Pharmaceutical Applications Notebook

DIONEX

Passion. Power. Productivity.

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Introduction to Pharmaceuticals

The pharmaceutical industry is the largest consumer of HPLC instrumentation. In drug discovery, HPLC and IC systems are used both as stand-alone tools and as front ends for mass spectrometers to screen drug candidates. In pre-clinical development, they are used for analyzing in-vitro and in-vivo samples. In clinical trials, they are used to gather data on a potential drug's safety and efficacy. They are used in manufacturing for many tasks including QA/QC, and the validation of cleaning procedures.

This applications notebook has been compiled to help the pharmaceutical scientist by providing a wide range of application examples relevant to the pharmaceutical market.

Dionex understands the demands of chemical analysis in the pharmaceutical industry. Our separation and detection technologies, combined with experience and applications competence, provide solutions for the analysis of inorganic ions, small drug molecules, and large components such as biologics and polysaccharides. Your laboratory now has a partner who can help you conduct reliable, accurate, and fast analyses. This notebook contains a wide range of pharmaceutical-related application notes and relevant information that will help address your challenges in drug discovery, development, and manufacturing.

Although, some of the applications published in this notebook were created some time ago, they are still relevant today. In the event that specific models of systems or modules used in these applications are no longer available, their methods may still be used on current instrumentation with similar performance.

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UltiMate 3000 LC Systems

Flexible, modular systems from nano to semiprep, for high-productivity LC solutions

The UltiMate® 3000 LC series delivers superior performance, while ensuring simple and reliable operation. The UltiMate 3000 LC solutions are geared to your application and operate over a wide dynamic flow rate range, from semipreparative to analytical to micro to nano/capillary scale, supporting applications such as discovery processes, quality control, and advanced research in proteomics and biomarker discovery.

The UltiMate 3000 LC Solutions are available for:

- Ultra High Performance LC (UHPLC)
- Proteomics MDLC
- LC Front-end for MS
- ×2 Dual LC
- Parallel and Tandem Analysis
- 2D-LC Analysis
- On-line SPE-LC Analysis
- Automated Application Switching
- Automated Method Scouting

The UltiMate 3000 LCi Series: intelligent solutions for performance, reliability, and ease-of-use.

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IC & RFIC Systems

A complete range of ion chromatography solutions for all performance and price requirements

Dionex has pioneered developments in Ion Chromatography (IC) for over 30 years with systems and solutions that represent state-of-the-art technological advancements and patented technologies, including:

- RFIC™ System technology
- Eluent generation
- Electrolytic suppression
- Continuously regenerated trap columns
- Three-dimensional electrochemical detection
- Dual capabilities to improve workflow, signal enhancement, and complex applications
- Award-winning systems

Dionex develops, manufactures, sells, and services IC systems to separate, isolate, and identify the components of chemical mixtures. Dionex products are used extensively in the environmental, food and beverage, pharmaceutical, life science, biotechnology, chemical, petrochemical, power generation, and electronics industries. Dionex customers include many of the world's largest companies, as well as government agencies, research institutions, and universities.

Technological developments such as eluent generation, continuously regenerated trap columns, and electrolytic suppression have changed the face of ion chromatography. Depending on your needs, a wide range of choices are available. For ion analysis, nothing compares to a Dionex IC system. Whether you have just a few samples or a heavy workload, whether your analytical task is simple or challenging, we have a solution to match your needs and budget.

Dionex has pioneered developments in ion chromatography for over 30 years with systems and solutions that represent state-of-the-art technological advancements.

MS Instruments

Single-point control and automation for improved ease-of-use in LC/MS and IC/MS

Dionex provides advanced integrated IC/MS and LC/MS solutions with superior ease-of-use and modest price and space requirements. The UltiMate 3000 System Wellness technology and automatic MS calibration allow continuous operation with minimal maintenance. The ICS-3000 and ICS-2100 RFIC systems automatically remove mobile phase ions for effort-free transition to MS detection. Other features include:

- MSQ® Plus, the smallest and most sensitive single quadrupole on the market for LC and IC
- Self-cleaning ion source for low-maintenance operation
- Chromeleon® software for single-point method setup, instrument control, and data management
- Compatible with existing IC and LC methods

The complete system includes the MSQ Plus, PC data system, ESI, and APCI probe inlets and vacuum system. You no longer need two software packages to operate your LC/MS system. The Chromeleon LC/MS software provides single-software method setup and instrument control, powerful UV, conductivity and MS data analysis, and fully integrated reporting.

MS Systems and Modules: MSQ Plus Mass Spectrometer; MSQ18LA nitrogen gas generator; AXP-MS digital auxiliary pump.

DIONEX

Chromatography Data Systems

The world's most complete chromatography software for HPLC, GC and IC

CHROMELEON 7 – THE NEW LOOK OF CHROMATOGRAPHY

Chromeleon 7 is the next-generation chromatography data system from Dionex. Using Operational Simplicity™ as its guiding design principle, Chromeleon 7 takes you from samples to results in the shortest time possible.

The modern, intuitive interface enables users to learn more quickly and reduce errors. Graphics and layout guide users to the correct action. Popular functions are visible and handy while specialized tools are easily discoverable. It also provides two new major processing features–the Cobra™ peak detection algorithm for fast accurate peak detection, and the SmartPeaks™ Integration Assistant for easy handling of unresolved peaks. It also offers eWorkflows, a new tool that automates all chromatography workflows via the simplest possible user interface.

- Find controls more easily with the fresh intuitive interface
- eWorkflows streamlines chromatography lab processes
- MiniPlots images of chromatograms provide extensive information at a glance
- Cobra Peak Detection Wizard walks users through all necessary steps
- SmartPeaks Integration Assistant shows users alternative integration treatments
- Create reports that fit your needs or choose a template from the library
- Comprehensive tool set enables CFR 21 Part 11 compliance

DCMS$^{Link™}$ provides direct control of Dionex instruments from Mass Spectrometry software including ABI/Sciex Analyst, Thermo Fisher Scientific Xcalibur, and Bruker Daltonics Hystar.

Combining Operational Simplicity and Intelligent Functionality...Chromeleon 7 is simply intelligent.

A Revolution in Chromatography

DIONEX

Process Analytical Systems and Software

Improve your process by improving your process monitoring

Dionex has nearly 25 years of experience installing on-line IC and HPLC capabilities in a wide range of industries to meet our customers' measurement requirements. Our Integral process analytical systems are used worldwide to provide timely analysis results by moving liquid chromatography-based measurements on-line.

Dionex Process Analytical (PA) systems provide more comprehensive, precise, and accurate information than can be provided by other process analyzers, and does so faster and at a higher frequency than is possible with laboratory-based results. Faster, more comprehensive information benefits process engineers responsible for plant performance in facilities both large and small, from the R&D lab bench to the factory floor.

Chromeleon PA 6.80 provides Integral analyzer control, data acquisition, and data reporting with a versatile software interface for viewing analyzer status, handling alarms, and reporting results.

Our Integral Migration Path™ approach enables you to use on-line IC/HPLC to generate timely, high resolution information when monitoring a small scale reactor in a process R&D lab, testing the production of tomorrow's new product in a pilot plant, or improving current processes in a manufacturing plant. No matter what the application, Integral has the versatility to place a solution using on-line IC/HPLC wherever and whenever it is needed.

Integral Process Analyzers are configured with various analytical modules to perform continuous on-line analysis. Chromeleon PA Software offers ex-panded capabilities for configuration, multi-system con-trol, conditional logic, and industrial connectivity for process systems.

DIONEX

Automated Sample Preparation

Better extractions in less time with less solvent

Solvent extractions that normally require labor-intensive steps are automated or performed in minutes, with reduced solvent consumption and reduced sample handling using Accelerated Solvent Extraction (ASE®) or AutoTrace®.

ASE is dramatically faster than Soxhlet, sonication and other extraction methods, and uses significantly less solvent and labor. ASE methods are accepted and established in the environmental, pharmaceutical, foods, polymers, and consumer product industries. ASE methods are accepted and used by government agencies:

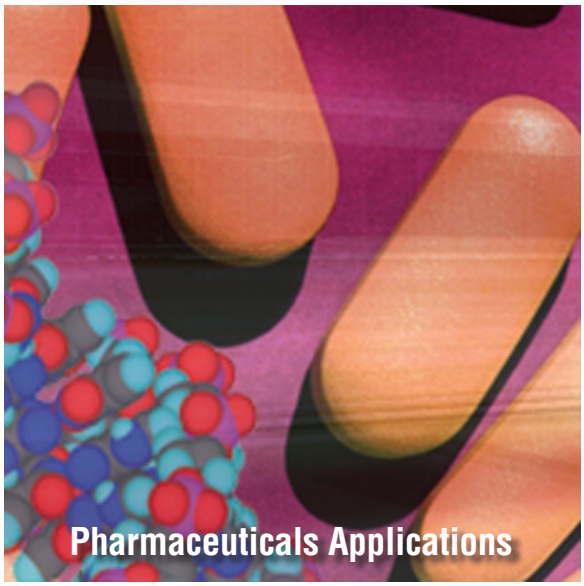
- US EPA Method 3545A
- CLP SOW OLM 0.42
- ASTM Standard practice D7210
- Chinese method GB/T 19649-2005
- German Method L00.00-34

AutoTrace is automated SPE instrument for extractions of large volume liquid sample matrixes. AutoTrace automates the standard SPE steps of condition, load, rinse and elute to reduce sample handling and improve productivity. AutoTrace systems are available in cartridge or disk formats.

ASE uses solvents at elevated tempratures and pressure to extract organic and ionic compounds from solid samples. AutoTrace automates the SPE process to provide high sample throughput combined with reliable analytical results

DIONEX

Part I: Analysis of Antibiotics



Analytes

Tobramycin
Neomycin B
Sulfur-Containing Antibiotics
Streptomycin
Paromomycin
Aminoglycosides
Cefepime
Sulfonamides

Determination of Tobramycin and Impurities Using HPAE-IPAD

INTRODUCTION

Tobramycin is a water-soluble aminoglycoside antibiotic used in a variety of pharmaceutical applications, including ophthalmic and intravenous administrations.[1] Tobramycin is purified from the fermentation of the actinomycete *Streptomyces tenebrarius*. Kanamycin B (also known as bekanamycin), nebramine, and neamine (also known as neomycin A) are three known impurities of tobramycin,[2] resulting from either incomplete purification of the drug or from degradation of tobramycin. Figure 1 shows the chemical structure of tobramycin and its major impurities. The amounts of these impurities must be determined and meet specified limit criteria before a manufactured lot of tobramycin may be used clinically. These aminoglycosides, like most carbohydrates, lack a good chromophore and therefore require high concentrations to be detected by UV absorbance. Many ingredients of manufacturing process intermediates and final pharmaceutical formulations are chromophoric and can interfere with the direct detection of tobramycin and its impurities by absorbance. Refractive index detection has similar limitations. Carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds can be oxidized and therefore directly detected by amperometry. This detection method is specific for those analytes that can be oxidized at a selected potential, leaving all other compounds undetected. Pulsed amperometric detection (PAD) is a powerful detection technique with a broad linear range and very low detection limits for aminoglycoside antibiotics.[3, 4]

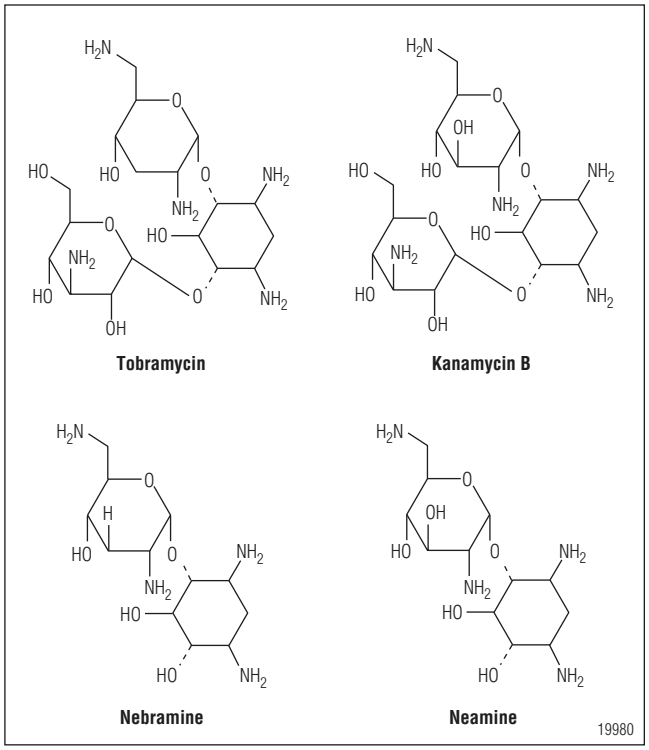


Figure 1. Chemical structures of tobramycin and known impurities (kanamycin, nebramine, and neamine).

High-performance anion-exchange chromatography (HPAE) is a technique capable of separating tobramycin and its impurities.[5,6] The CarboPac™ PA1 anion-exchange column retains tobramycin and its impurities, but requires a weak sodium hydroxide eluent (2 mM) that is difficult to prepare reproducibly without carbonate contamination. Varying amounts of carbonate contamination adversely affect retention time precision. This problem has limited the adoption of HPAE-PAD for tobramycin determinations.

In this application note, we show that an eluent generator solves the problem of consistent eluent preparation. An eluent generator can automatically prepare hydroxide eluents of precise concentrations that are essentially carbonate-free. The EG50 Eluent Generator automatically produces potassium hydroxide (KOH) eluent from water and a potassium electrolyte solution by means of electrolysis. The only carbonate in the mobile phase is that present in the water used to supply the eluent generator. The minor amounts of carbonate from the supply water, as well as borate and other contaminating anions, are removed by a Continuously Regenerated Anion Trap Column (CR-ATC) installed after the eluent generator. Consequently, the usual variability in hydroxide concentration associated with manual eluent preparation, and the variability of carbonate contamination due to adsorption of atmospheric carbon dioxide, are essentially eliminated, leading to highly reproducible retention times.

In addition to improving HPAE retention time reproducibility, we adopted disposable gold (Au) working electrodes to improve electrode-to-electrode (and system-to-system) reproducibility of tobramycin electrochemical response. Disposable Au working electrodes are manufactured in a manner that improves electrode-to-electrode reproducibility.[7,8] These electrodes are also easy to maintain (no polishing) and inexpensive to replace.

In this application note, we combine the CarboPac PA1, eluent generator with CR-ATC, and disposable Au working electrodes (Figure 2) to demonstrate an improved HPAE-PAD technology for tobramycin purity analysis. Key performance parameters are evaluated, including precision, limits of detection, linearity, and ruggedness, in a manner consistent with many requirements of normal method validation.[9–16] Overall, the described setup has improved sensitivity, good sample throughput (15 min per run), and improved retention time reproducibility. The automated production of KOH eluent improves reproducibility and eliminates eluent preparation errors.

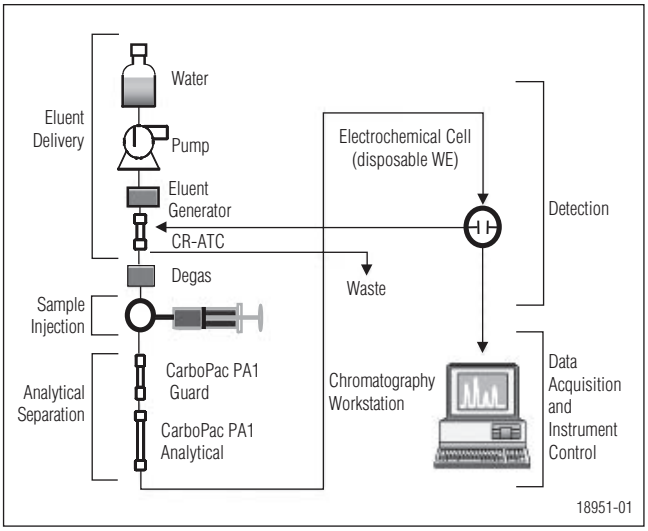


Figure 2. HPAE-PAD system for tobramycin determinations.

EQUIPMENT

Dionex BioLC® system consisting of:

- GP50 Gradient or IP25 Isocratic Pump, with vacuum degas option and GM-4 Gradient Mixer
- ED50 Electrochemical Detector and Combination pH/Ag/AgCl Reference Electrode (P/N 044198) with either:
  - Carbohydrate Certified (Au) Disposable Electrodes (P/N 060139, package of 6; or 060216, package of 24)
  - *AAA-Direct*™ Certified (Au) Disposable Electrodes (P/N 060082, package of 6; 060140, package of 24)
- EG50 Eluent Generator with EGC II KOH eluent generator cartridge (EluGen II Hydroxide; P/N 053921)
- EG40/50 Vacuum Degas Conversion Kit (P/N 055431)
- CR-ATC, Continuously Regenerated Anion Trap Column (P/N 060477)
- AS50 Autosampler with 20-µL injection loop
- AS50 Thermal Compartment
- EO1 Eluent Organizer, including four 2-L plastic bottles and pressure regulator

Chromeleon® Chromatography Workstation

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent nylon filter

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent)

0.3 mL Polypropylene Injection Vials with Caps (Vial Kit, Dionex P/N 055428)

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Standards

Tobramycin (Sigma-Aldrich Chemical Co, Cat. #T40014)

Kanamycin B (also known as bekanamycin sulfate; Sigma-Aldrich Chemical Co, Cat. #B5264)

Neamine hydrochloride (also known as Neomycin A hydrocloride; International Chemical Reference Substances; World Health Organization; Cat. #9930354)

CONDITIONS

Method

Columns: CarboPac PA1 Analytical, 4 × 250 mm (P/N 035391)
CarboPac PA1 Guard, 4 × 50 mm (P/N 043096)

Flow Rate: 0.5 mL/min

Injection Volume: 20 µL (full loop)

Temperature: 30 °C

Detection (ED50): Pulsed amperometry, Carbohydrate Certified disposable Au working electrodes (P/N 0600139), or *AAA-Direct* Certified disposable Au working electrodes (P/N 060082)

Background: 28–35 nC (using the Carbohydrate waveform)
33–96 nC (using the *AAA-Direct* waveform)

Typical System Operating Backpressure: 2460–2590 psi (with restrictor tubing installed between the degas apparatus and the injector)

Eluent Generation Method: 2 mM KOH; isocratic, 15-min run time

Carbohydrate Waveform for the ED50*

Time (s)	*Potential (V)*	*Integration*
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	–2.0	
0.42	–2.0	
0.43	+0.6	
0.44	–0.1	
0.50	–0.1	

Reference electrode in Ag/AgCl mode

* Waveform A in Technical Note 21.[17]

AAA-Direct* Waveform for the ED50 (Alternative, for increased sensitivity)*

Time (s)	*Potential (V)*	*Integration*
0.00	+0.13	
0.04	+0.13	
0.05	+0.33	
0.21	+0.33	Begin
0.22	+0.55	
0.46	+0.55	
0.47	+0.33	
0.56	+0.33	End
0.57	–1.67	
0.58	–1.67	
0.59	+0.93	
0.60	+0.13	

Reference electrode in pH mode

** Waveform used for this note. For the most current waveform, see the product manuals for the *AAA-Direct* Amino Acid Analysis System.[18]

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

It is essential to use high-quality water of high resistivity (18 MΩ-cm) that contains as little dissolved carbon dioxide as possible. Biological contamination should be absent. Source water must be obtained using a water purification system consisting of filters manufactured without electrochemically active substances (e.g., glycerol). Prior filtration through 0.2-µm porosity nylon under vacuum is recommended to remove particulates and reduce dissolved air. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of helium at all times to reduce diffusion of atmospheric carbon dioxide and opportunistic microorganisms.

STOCK STANDARDS

Solid tobramycin, kanamycin B, and neamine standards were placed in plastic vials and dissolved in deionized water to a 10-mg/mL concentration. The masses of moisture, salt, and impurities, as stated on the manufacturer's Certificate of Analysis, were subtracted from the measured mass to improve accuracy of the solutions. These solutions were further diluted with water to yield the desired stock mixture concentrations. For this note, all dilutions were made gravimetrically to ensure high accuracy. The solutions were maintained frozen at –40 °C until needed. Masses of 1, 2, 20, 100, 200, 300, 400, and 600 pmol tobramycin were injected for linearity studies.

Note: Tobramycin—and to a lesser extent kanamycin B—when dissolved in water, adsorbs to glass surfaces. Significant losses due to adsorption occur at dilute concentrations. Polypropylene injection vials and other labware must be used to ensure accurate results.

RESULTS AND DISCUSSION

Separation

Figure 3 shows the separation of tobramycin (peak 5) from five impurities (peaks 1, 2, 3, 4, and 6) using a CarboPac PA1 column set. Panel A shows the full display of the tobramycin peak, whereas panel B expands the baseline to view early-eluting impurity peaks. This isocratic method was optimized for throughput, for resolution of tobramycin (5.7 min) from impurities (3.0–3.1, 3.4–3.5, 4.1–4.2, and 4.6–4.7 min) and the void (2.7–2.8 min), and for noninterfering locations of baseline dips (6.0, 10.7, 15.5, and 31.0 min). Impurity peak 3 (Figure 3) was identified as kanamycin B, and peak 4 was identified as neamine (neomycin A) based on the retention time of standards. Impurity peak 1 was also observed to a lesser extent in the water blank injections, and it and other sporadically observed minor peaks were eliminated when injection vials were prerinsed three times with water before use.

Baseline dips associated with injections of water or samples are likely caused by trace organic impurities present in the sample or water separated on the CarboPac PA1 column by means of secondary interactions (e.g., hydrophobic interactions). When these compounds elute, they exclude electrochemically active ions in the eluent. The oxygen dip (~31-min retention time) is due

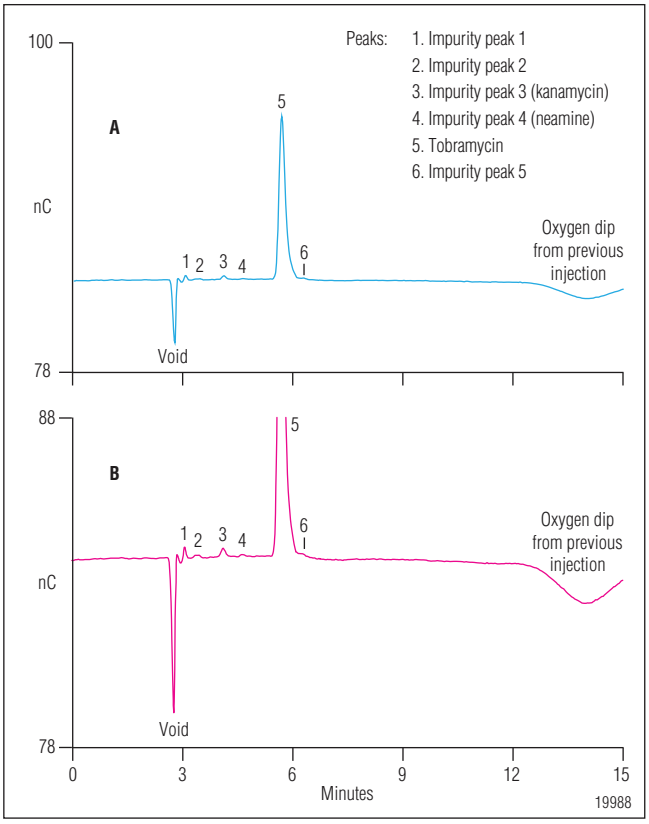


Figure 3. Determination of tobramycin (1.07 µM, 20-µL injection) using eluent generation (2 mM KOH) with 0.5 mL/min flow rate, 30 °C column temperature, and AAA-Direct *waveform. Full view (A) and expanded view of baseline (B).*

to oxygen present in the samples and appears as a function of the gas permeation volume of the column. Like some organic impurities, eluting oxygen produces less background than the eluent, so there is a dip in the baseline. The retention times of the oxygen dip and other baseline dips vary from column to column, and depend on the flow rate, not the eluent strength. Eluting the baseline dips just prior to the end of run, or timing their elution to occur at the end of the following injection, prevents the baseline dips from interfering with the peaks of interest.

We investigated tobramycin separations using the CarboPac PA10, PA20, and MA1, and the AminoPac® PA10 columns, but found inadequate retention of tobramycin and kanamycin B on these columns. Substitution of the CarboPac PA1 guard column with the AminoTrap™ column slightly increased retention times and broadened peaks.

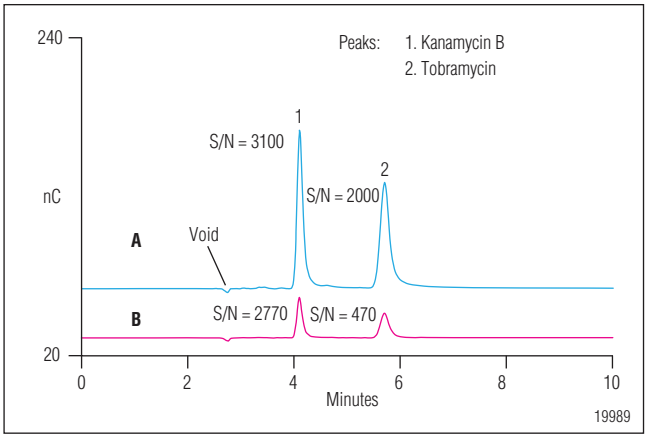


Figure 4. Comparison of 10 µM tobramycin and kanamycin B peaks (20-µL injection) using the AAA-Direct *waveform (A) and the carbohydrate waveform (B).*

The resolution (European Pharmacopoeia definition) between tobramycin and kanamycin B ranged from 5.80 and 6.16 over 7 days of consecutive analysis (mean ± SD; 6.00 ± 0.07, n = 572, 1.1% RSD). A European Pharmacopoeia method for tobramycin requires resolution to be greater than 3.0.[2] That method also allows adjustment of the mobile phase concentration to achieve this minimum resolution. The method presented in this application note easily achieves the resolution specification without mobile phase adjustment.

Detection

Figure 4 compares the peak heights for 10 µM tobramycin and kanamycin B (20-µL injection) detected using (A) the *AAA-Direct* waveform, and (B) the carbohydrate waveform. The use of the *AAA-Direct* waveform increased signal-to-noise (S/N) 2 to 4 times, depending on system noise. The *AAA-Direct* waveform improved tobramycin sensitivity, which is required to maximize the detection of tobramycin impurities. When high sensitivity is not required, the carbohydrate waveform is recommended because it allows longer use of each disposable Au working electrode and improves day-to-day peak area reproducibility. The *AAA-Direct* Certified disposable Au working electrode is guaranteed for 1 week when used with the *AAA-Direct* waveform, and the Carbohydrate Certified disposable Au working electrode is guaranteed for 2 weeks when used with the carbohydrate waveform.

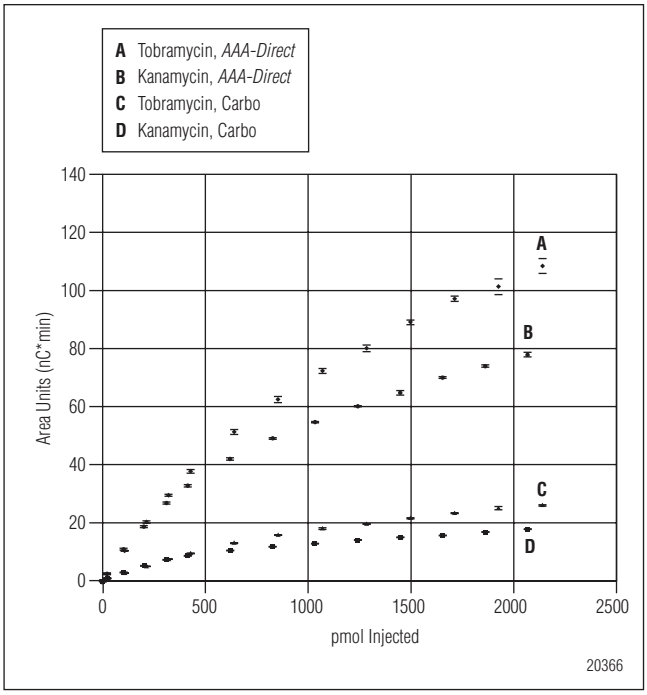


Figure 5. The relationship of peak area (mean ± SD, n = 4 injections each concentration) to mass of tobramycin and kanamycin B injected using the carbohydrate and AAA-Direct *waveforms for estimation of linear range.*

Linear Range

Figure 5 presents the relationship of tobramycin and kanamycin B peak area (pC*min) to pmole of the analyte injected (20 µL) using the carbohydrate and *AAA-Direct* waveforms over a broad range of injections, 1–2200 pmol. Figure 6 shows the same data over a narrower range, 1–650 pmol, where the relationship of response to mass injected is linear. Figure 5 shows the effect of column or detector overload where response becomes nonlinear. In this application note, we consider the linear concentration range to be where the response factor (ratio of peak area/mass injected) remains within a 20% variance from the mean of its optimum level. A plot that relates area response factor to the mass injected (data not presented) showed a typical plateau region that represented an optimal level for operation. The corresponding mean tobramycin area response factor for this region was 22.6 nC*min/pmol, whereas the mean kanamycin B response factor was 24.4 nC*min/pmol using the carbohydrate waveform.

Tobramycin injections having response factors below 18.1 nC*min/pmol (19.5 nC*min/pmol for kanamycin B) were considered outside the upper linear range. These results (Table 1) show tobramycin peak area linearity extends up to 700 pmol (35 µM for 20-µL injection), and kanamycin B linearity extends up to 500 pmol (25 µM for 20-µL injection) using the carbohydrate waveform. Using the same waveform, the tobramycin peak height was linear to 500 pmol, and kanamycin B peak height was linear to 400 pmol. The linear range typically extended over 3 orders of magnitude (0.7–700 pmol tobramycin, carbohydrate waveform; 0.3–750 pmol tobramycin, *AAA-Direct* waveform) using the estimated lower limit of detection (LOD) as the lower end of the range.

The mean peak area response factors for the plateau region using the *AAA-Direct* waveform was 95.6 nC*min/pmol for tobramycin and 98.1 nC*min/pmol for kanamycin B. Tobramycin injections having response factors below 76.5 nC*min/pmol (78.5 nC*min/pmol for kanamycin B) were considered outside the upper linear range. Tobramycin peak area linearity extended up to 750 pmol (38 µM for 20-µL injection), and kanamycin B linearity extended up to 425 pmol (21 µM for 20-µL injection) using the *AAA-Direct* waveform. The tobramycin peak height was linear to 525 pmol, and kanamycin B peak height was linear to 350 pmol using this waveform. The peak area linear range for tobramycin extended over 3 orders of magnitude, and was slightly larger for the carbohydrate waveform.

Linearity

Figure 6 shows the linear relationship of peak area response to mass of antibiotics injected for the concentrations ranging from near the lower limit of quantification to the upper limit of linearity. Masses ranging from 1 to 600 pmol produced a r^2 value of 0.9946 for tobramycin and 0.9874 for kanamycin B using the carbohydrate waveform, 0.9935 and 0.9917 for tobramycin and kanamycin B, respectively, using the *AAA-Direct* waveform. Table 1 summarizes the statistics for these four calibration curves. Slopes for tobramycin and kanamycin B were nearly identical for each waveform, however, slopes were 3–4 times greater using the *AAA-Direct* waveform (see Figure 6). The nearly identical slopes for tobramycin and kanamycin B indicate that accurate measure of kanamycin B impurity is expected using peak area percentages of tobramycin, reducing the need to run separate kanamycin B standards.

Table 1. Estimated Limits of Detection, Quantification, and Linearity for Tobramycin and Kanamycin B Using the Carbohydrate and *AAA-Direct* Waveforms

Carbohydrate Waveform		
	Tobramycin	**Kanamycin B**
Lower Limit Detection		
pmol	0.55–2.26	0.34–1.39
µM†	0.027–0.113	0.017–0.070
picogram	257–1055	164–673
µg/mL†	0.013 – 0.053	0.008–0.034
Lower Limit Quantitation		
pmol	1.83–7.52	1.13–4.64
µM†	0.091–0.376	0.056–0.232
picogram	855–3518	545–2243
µg/mL†	0.043–0.176	0.027–0.112
Upper Limit Linearity		
pmol	700	500
µM†	35	25
picogram	327000	242000
µg/mL†	16	12
Linearity Over Linear Range		
r^2	0.9946	0.9874
Slope (nC*min/pmol)	0.0206	0.0215
***AAA-Direct* Waveform**		
	Tobramycin	**Kanamycin B**
Lower Limit Detection		
pmol	0.22–0.36	0.12–0.20
µM†	0.011–0.018	0.006–0.010
picogram	102–167	59–97
µg/mL†	0.005–0.008	0.003–0.005
Lower Limit Quantitation		
pmol	0.72–1.19	0.41–0.67
µM†	0.036–0.060	0.020–0.034
picogram	339–558	197–325
µg/mL†	0.017–0.028	0.010–0.016
Upper Limit Linearity		
pmol	750	425
µM†	38	21
picogram	351000	206000
µg/mL†	18	10
Linearity Over Linear Range		
r^2	0.9935	0.9917
Slope (nC*min/pmol)	0.0821	0.0814

† 20-µL injections

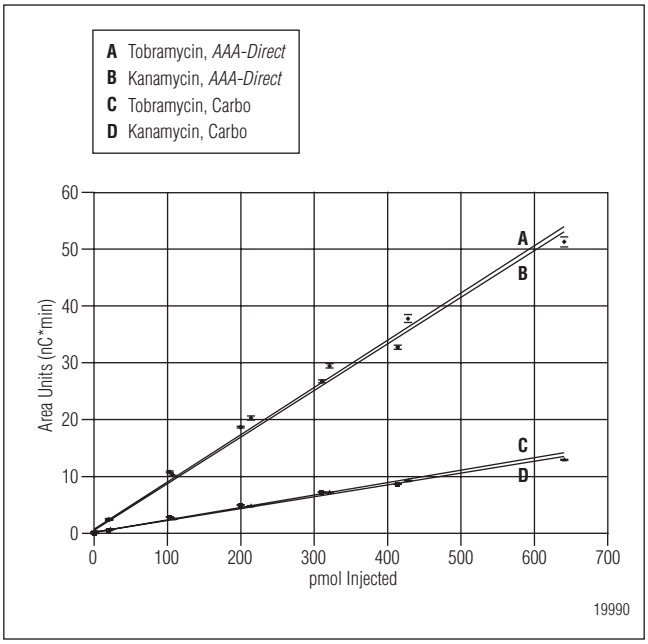


Figure 6. The linear relationship of tobramycin and kanamycin B peak area (mean ± SD, n = 4 injections each concentration) within their estimated linear range using the carbohydrate and AAA-Direct *waveforms.*

Lower Limits of Detection and Quantification

In this study, baseline (peak-to-peak) noise was determined from noise measured in 1-min intervals during blank runs. Noise is measured in peak height units, pC. Baseline noise for the carbohydrate waveform ranged from 12 to 91 pC (mean ± SD; 38 ± 21, n = 218 1-min intervals). Baseline noise for the *AAA-Direct* waveform ranged from 14 to 91 pC (mean ± SD; 37 ± 15, n = 308 1-min intervals). After installing new disposable electrodes, baseline noise tended to decrease over the several days that noise was monitored. This trend was observed for both waveforms. Noise stabilized to its lowest level (lower end of the range) between 1–2 days of electrode use. A range of lower limits of detection (LOD) were calculated from the minimum and maximum measured baseline noise collected periodically over 3 days, starting 100 min after installation of a new electrode. The concentration (or mass injected) of tobramycin at the lower limit of detection (LOD) was calculated from three times the average peak-to-peak noise (a height value), divided by the average peak height response factor for the antibiotic within its linear region. At this concentration, signal-to-noise ratio equals 3. The lower limit of quantification (LOQ) is the concentration (or mass injected) calculated from ten times the average peak-to-peak noise. The estimated LOD for tobramycin ranged from 0.55 to 2.3 pmol using the carbohydrate waveform, and ranged from 0.22 to 0.36 pmol using the *AAA-Direct* waveform using a 20-µL injection. The estimated LOD for kanamycin B ranged from 0.34 to 1.4 pmol, and the LOQ ranged from 1.1 to 4.6 pmol using the carbohydrate waveform. The estimated LOD for kanamycin B ranged from 0.12 to 0.20 pmol, and the LOQ ranged from 0.41 to 0.67 pmol using the *AAA-Direct* waveform. Table 1 summarizes these results. Figure 7 shows tobramycin and kanamycin B at their respective LODs using the *AAA-Direct* waveform.

When tobramycin is analyzed at the upper range of linearity (Figure 8; 750 pmol), this method can detect 0.048–0.20 and 0.016–0.027 mole percent kanamycin B impurity using the carbohydrate and *AAA-Direct* waveforms, respectively. In Figure 8, kanamycin B is a 0.11% impurity of tobramycin.

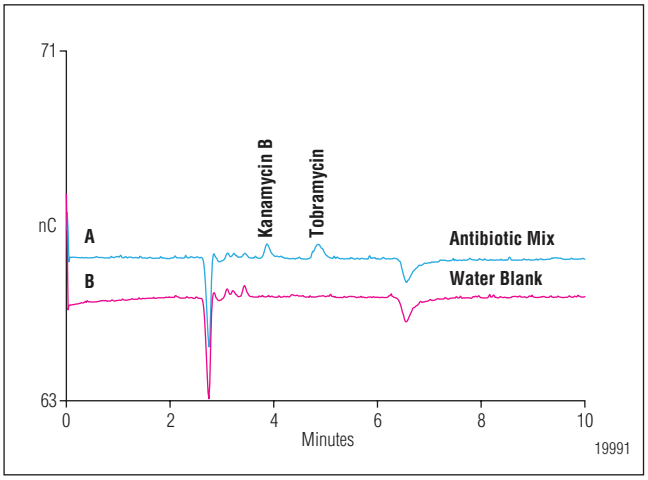


Figure 7. Determination of 0.22 pmol tobramycin (0.011 µM) and 0.20 pmol kanamycin B (0.010 µM) near their lower limits of detection (20-µL injection).

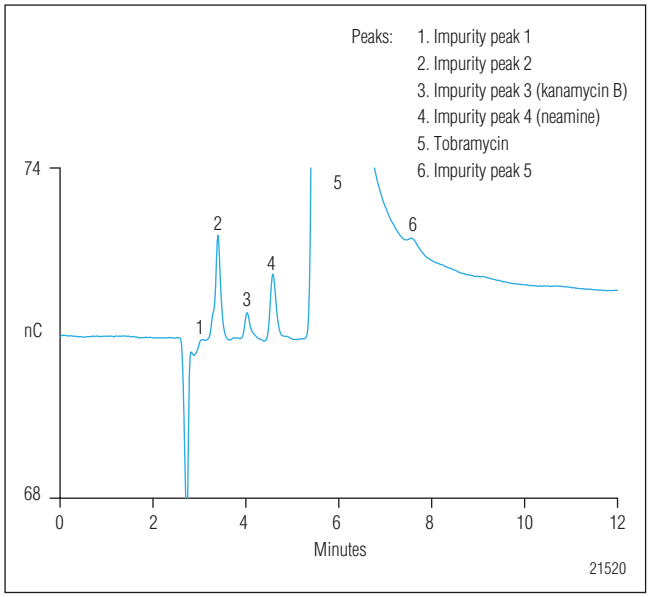


Figure 8. Determination of impurities when tobramycin is analyzed at the upper limit of linearity (0.038 mg/mL, 20-µL injection) using the AAA-Direct *waveform.*

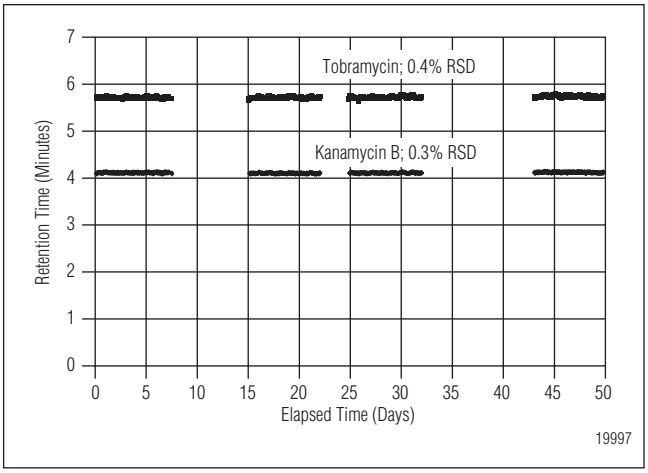


Figure 9. Reproducibility of tobramycin and kanamycin B retention time over 50 days using the eluent generator. Intervals without data represent periods when the system was either shut down, idle, or used for other tobramycin experiments.

Precision

The peak area and retention time RSDs were determined for replicate injections of a mixture of tobramycin and kanamycin B standards (10 µM for 20-µL injection) over 7 days (572 injections) for each waveform.

Retention Time

The mean (± SD) retention time for tobramycin was 5.74 ± 0.02 min over 7 days (572 injections), a 0.3% RSD. Kanamycin B retention time was 4.12 ± 0.01 min, a 0.2% RSD. The daily retention time RSDs (over a 24-h period) ranged from 0.2 to 0.4 % for tobramycin and 0.2 to 0.3% for kanamycin B. Figure 9 presents the long-term (50 days, 2368 injections) retention time data for tobramycin and kanamycin B using the eluent generator for four 7-day studies. The long-term tobramycin retention time RSD was 0.3%, and kanamycin B was 0.4%. The periods of time without data in Figure 10 reflect periods where the system was either shut down or used for other experiments. No upward or downward trend was observed, and the precision was the same for each 7-day study. The column was regenerated for 1 h at 100 mM KOH once per week. The method described in this application note is designed to analyze a relatively pure antibiotic and can be used without any column regeneration for at least 7 days.

Peak Area

The peak area for tobramycin in the study described above ranged from 3.71 to 4.43 nC*min (mean ± SD; 4.02 ± 0.16 nC*min) with a 4.0% RSD using the carbohydrate waveform. Peak area for kanamycin B injected for 7 days ranged from 3.92 to 4.45 nC*min (mean ± SD; 4.18 ± 0.11 nC*min) with 2.6% RSD. A slight increase in peak areas (8% for tobramycin, 4% kanamycin B) was observed over 7 days.

A similar study was performed using the *AAA-Direct* waveform. The peak area for tobramycin ranged from 17.1 to 20.1 nC*min (mean ± SD; 18.52 ± 0.42 nC*min) with a 2.3% RSD. Peak area for kanamycin B ranged from 16.8 to 18.5 nC*min (mean ± SD; 17.81 ± 0.33 nC) with 1.9 % RSD. An increasing trend in peak area was observed for both tobramycin and kanamycin B over the first 5 days, reaching a 4–5% change compared to day 1. Between 5 to 7 days, peak area trended back down to a 3% difference, compared to day 1.

Daily peak area RSDs ranged from 1.4 to 2.9% for the tobramycin and 1.2 to 1.8% for kanamycin B using the carbohydrate waveform. RSDs ranged 1.1–2.3% for the tobramycin and 0.8–1.7% for kanamycin B using the *AAA-Direct* waveform. The high retention time and response reproducibility indicate that this method is suitably rugged for this application.

Peak area precision is dependent on the concentration analyzed. As concentration approaches the LOQ and LOD, higher variance will be observed. This study used concentrations within the linear ranges for tobramycin and kanamycin B.

Robustness

Robustness was evaluated for influence of a 10% variance in eluent concentration, different disposable Au working electrodes, a 10% variance in flow rate, and a column change.

Eluent Concentration

The retention times of tobramycin and kanamycin B varied greatly with minor variations in mobile phase concentration. A 10% increase in KOH (2.2 mM) produced a retention time decrease to 4.7 min (–18 % change from 2.0 mM) for tobramycin, whereas a 10% decrease in KOH (1.8 mM) produced a retention time increase to 7.8 min (+36% change). Kanamycin B retention time decreased by 9.2% with 10% increase in eluent concentration, and increased 17% with a 10% increase in eluent concentration. The large percent change in retention time for a relatively small change in KOH eluent concentration demonstrates the importance of producing a consistent eluent concentration, which the eluent generator achieves.

Disposable Gold Working Electrode Response

Disposable electrodes were evaluated for their influence on response. Using the *AAA-Direct* waveform, with three *AAA-Direct* Certified electrodes from the same lot, tobramycin peak area response factors ranged from 83.6 to 94.8 pC*min/pmol (based on 20 µL of 10 µM), mean ± standard deviation of 90.1 ± 5.8 (6.5%). Kanamycin B peak area response factors ranged from 83.3 to 92.9 pC*min/pmol (based on 20 µL of 10 µM), mean ± standard deviation of 88.5 ± 4.8 (5.5%). Using the *AAA-Direct* waveform, with four *AAA-Direct* Certified electrodes from different lots, tobramycin response factors ranged from 73.7 to 90.1 pC*min/pmol, mean ± standard deviation of 83.8 ± 7.1 (8.5%). Kanamycin B response factors ranged from 75.5 to 88.5 pC*min/pmol, mean ± standard deviation of 83.6 ± 5.7 (6.8%).

Using the carbohydrate waveform, with four Carbohydrate Certified electrodes from the same lot, tobramycin peak area response factors ranged from 19.8 to 22.9 pC*min/pmol (based on 20 µL of 10 µM), mean ± standard deviation of 21.3 ± 1.7 (8.1%). Kanamycin B peak area response factors ranged (three different electrodes) from 21.0 to 25.4 pC*min/pmol (based on 20 µL of 10 µM), mean ± standard deviation of 23.0 ± 2.2 (9.6 %RSD). Using the carbohydrate waveform, with five Carbohydrate Certified electrodes from different lots, tobramycin response factors ranged from 21.3 to 25.6 pC*min/pmol, mean ± standard deviation of 23.6 ± 1.9 (8.1%). Kanamycin B response factors (5 electrodes) ranged from 21.5 to 24.8 pC*min/pmol, mean ± standard deviation of 23.1 ± 1.2 (5.1%).

Flow Rate

A 10% change in the operating column flow rate (0.50 mL/min) was evaluated for influence on tobramycin and kanamycin B retention time. At 10% higher flow rate, a 5–7% decrease in retention time was observed, and at 10% lower flow rate, a 13–14% increase in retention time was observed. At 10% higher flow rate, no significant change in peak area was observed, and at 10% lower flow rate, a 12–13% increase in peak area was observed using the *AAA-Direct* waveform. A 10% change in flow rate did not affect noise. The carbohydrate waveform was not investigated for these effects.

Column Reproducibility

The tobramycin retention time RSD for four separate columns was 7.0 %, whereas kanamycin B retention time RSD was 3.1% and neamine was 6.1%.

Retention times of baseline dips also vary slightly from column to column, and may change over the long-term (6–12 months) use of the column. Baseline dips do not interfere with determination of impurities. If the determination of a trace level of tobramycin is the analytical objective, and the tobramycin peak coincidently coelutes with the first baseline dip (at ~6 min) using 2.00 mM KOH, or the same retention times are desired from column to column, then KOH concentration may be adjusted as shown in Figure 10. Alternatively, replacement of the guard column, analytical column, or both can often correct this coelution. In this study, the peak area of the baseline dip 1 (a negative peak) at about 6 min was equivalent to 0.92 ± 0.42 pmol tobramycin (0.046 ± 0.021 µM, 20-µL injection, n = 28 measures over 287 days). If tobramycin coeluted with this dip, the error contribution of this dip was estimated to be insignificant at tobramycin concentrations above ~100 pmol (5 µM, 20-µL injection).

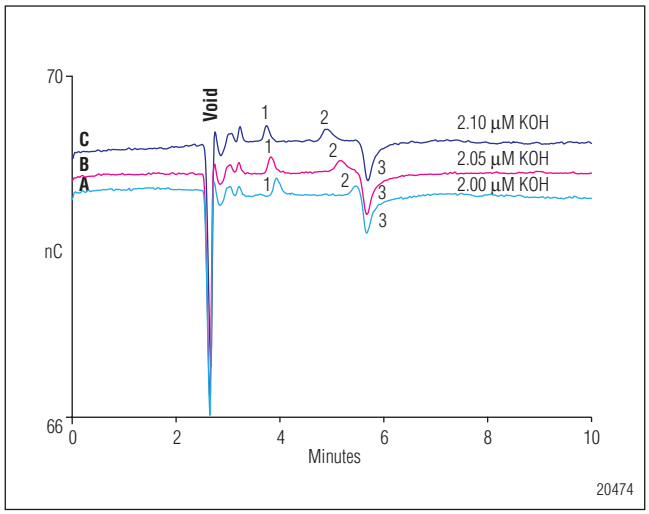


Figure 10. Effect of minor adjustments in KOH concentration on the separation of the tobramycin (peak 2) from the first baseline dip (peak 3). Mixture of tobramycin (0.011 µM) and kanamycin B (peak 1; 0.010 µM) near their lower limits of detection using (A) 2.00 mM KOH, (B) 2.05 mM KOH, and (C) 2.10 mM KOH (20-µL injection).

Sample Matrix

Salt exceeding 5 mM in the sample may cause retention time shifts in tobramycin and kanamycin and distort peaks. For some pharmaceutical formulations, a periodic column wash more frequent than every 7 days may be necessary, and will depend on the nature of the ingredients. At this time, we do not recommend this method for applications other than assessing the quality of pure tobramycin.

Instrument Operational Considerations

Weekly column washes at 100 mM KOH are recommended to restore retention times for tobramycin and kanamycin B when the system is used without column regeneration. The application of 100 mM KOH changes system equilibrium, and reequilibration at 2 mM KOH for 2 h is recommended to achieve high precision.

When the system is idle for short (1–2 week) periods, we recommend that the pump and eluent generator be left on at 0.5 mL/min or at a reduced flow rate to achieve rapid start-up. The cell should be turned off to extend disposable electrode life. The use of a lower flow rate, while maintaining the minimum backpressure of at least 2000 psi, extends the interval before water must be added to the reservoir. When the system must be shut down for a period of several weeks, the pump, eluent generator, and electrochemical cell may be turned off. For shutdown periods exceeding several weeks, all plumbing lines should be reconnected and the reference electrode should be removed from the electrochemical cell and stored in the original solution shipped with the reference electrode (saturated KCl). When the pump has been turned off for longer than

1 day, the column should be regenerated with 100 mM KOH for 1–2 h, and reequilibrated with 2 mM KOH for 2 h before analyzing samples.

CONCLUSION

HPAE-PAD with eluent generation can be used to determine tobramycin and its impurities. The linear range of electrochemical response extended over 3 orders of magnitude, from 0.03–0.11 µM (LOD) up to 35 µM (16 µg/mL; 20-µL injection) for the carbohydrate waveform, and from 0.01–0.02 µM (LOD) up to 38 µM (18 µg/mL; 20-µL injection) for the *AAA-Direct* waveform. Both the carbohydrate and *AAA-Direct* waveforms showed equivalent noise and linear range; however, the *AAA-Direct* waveform had 3–4 times greater response, and therefore had lower limits of detection. High-precision method ruggedness is possible for this antibiotic and impurities using either waveform, but the carbohydrate waveform—with its corresponding disposable electrode—provides longer guaranteed response stability. The recommended waveform choice (and corresponding disposable electrode) is based on the analytical requirements. The eluent generator makes this method reproducible and rugged with respect to retention time and peak separation. Because the pump is only required to pump water and no caustic eluent preparation is required, there is reduced pump seal wear and increased safety for the analyst. The disposable gold working electrodes provide consistently high detector response, assuring greater instrument-to-instrument and lab-to-lab reproducibility.

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LIST OF SUPPLIERS

J. T. Baker, 222 Red School Lane, Phillipsburg, NJ 08865 USA, Tel: 800-582-2537, www.jtbaker.com.

Fisher Scientific, 2000 Park Lane, Pittsburgh, PA 15275-1126 USA, Tel: 800-766-7000, www.fishersci.com.

Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 USA, Tel: 1-800-325-3010, www.sigma-aldrich.com.

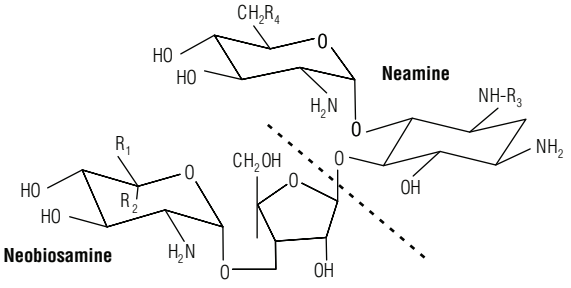
Praxair Specialty Gases and Equipment, 39 Old Ridgebury Road, Dansbury, CT 06810-5113 USA, Tel: 877-772-9247 and 716-879-4077, www.praxair.com/specialtygases.

World Health Organization (WHO) Collaborating Centre for Chemical Reference Substances; Apoteket AB; Produktion & Laboratories; Centrallaboratoriet, ACL; PrismavAgen 2; SE-141 75 Kungens Kurva, Sweden, Fax: + 46 8 740 60 40, who.apl@apoteket.se, www.who.int/medicines/strategy/quality_safety/trs917annl.pdf.

Determination of Neomycin B and Impurities Using HPAE-IPAD

INTRODUCTION

Neomycin is a complex of water-soluble aminoglycoside antibiotics purified from the fermentation of the actinomycete *Streptomyces fradiae* and used in a variety of pharmaceutical applications, including topical, ophthalmic, oral, and intravenous administrations (e.g., Neosporin®, NeoDecadron®, PediOtic® Suspension).[1] Neomycin B (also known as framycetin) is the main component of the complex and has the highest antibiotic activity. *S. fradiae* fermentation broth also contains less active forms of Neomycin: Neomycin A (also known as neamine), Neomycin C, Neomycin D (also known as paromamine), Neomycin E (paromomycin I), Neomycin F (paromomycin II). The acetylation of Neomycin A, B, and C also occurs during fermentation, lowering the antibiotic potency (LP = low potency), and has been described as Neomycin LP-A (mono-*N*-acetyl-neamine or 3-acetylneamine; low potency), Neomycin LP-B (mono-*N*-acetyl-Neomycin B, or LP-I in early publications), Neomycin LP-C (mono-*N*-acetyl-Neomycin C, or LP-II in early publications). Fradicin, an antifungal compound, and other antibiotic compounds have also been reported in *S. fradiae* fermentation broth.[2,3] Other impurities may result from chemical degradation during manufacture or storage.[4] For example, acid hydrolysis of Neomycin B yields Neomycin A and neobiosamine B; hydrolysis of Neomycin C yields Neomycin A and neobiosamine C. Neobiosamine B and C are composed of D-ribose and neosamine B and C, respectively. The current United States Pharmacopeia (USP 29, NF 24) compendial method for Neomycin sulfate measures Neomycin B as the primary antibiotic, with Neomycin A and B as impurities.[5] The current (5th Edition) monograph for the European Pharmacopoeia (EP) compendial method for Neomycin sulphate measures Neomycin B as the primary antibiotic, with Neomycin A, C, D, E, A-LP, and B-LP as impurities.[6] Figure 1 shows the chemical structure of Neomycin B and its major impurities. Generally, the amount of primary drug (Neomycin B) and all impurities must be determined and meet specified limit criteria before a manufactured lot may be used clinically. These aminoglycosides and their impurities, like most carbohydrates, lack a good chromophore and therefore require high concentrations to be detected by UV absorbance. Many ingredients of manufacturing process-intermediates and final pharmaceutical formulations are chromophoric and can interfere with the direct detection



Neomycin	R_1	R_2	R_3	R_4
A	NA	NA	–H	$–NH_2$
B	–H	$–CH_2NH_2$	–H	$–NH_2$
C	$–CH_2NH_2$	–H	–H	$–NH_2$
D	NA	NA	–H	–OH
E	–H	$–CH_2NH_2$	–H	–OH
F	$–CH_2NH_2$	–H	–H	–OH
3-Acetylneamine	NA	NA	$–COCH_3$	$–NH_2$

NA—not applicable, neobiosamine not part of the chemical structure.

23017

Figure 1. Chemical structures of neomycin and known impurities (neomycin A, B, C, and neobiosamine B and C).

of Neomycin B and its impurities by UV absorbance. Refractive index detection has similar limitations. Carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds can be oxidized and therefore directly detected by amperometry. This detection method is specific for those analytes that can be oxidized at a selected potential, leaving all other compounds undetected. Integrated pulsed amperometric detection (IPAD), a powerful detection technique with a broad linear range and very low detection limits, is ideally suited for aminoglycoside antibiotics and their impurities.[7–12]

High-performance anion-exchange chromatography (HPAE) is a technique capable of separating Neomycin B and its impurities.[7,10] The CarboPac® PA1 anion-exchange column retains Neomycin B and its impurities, but requires a weak sodium hydroxide eluent (2.40 mM) that is difficult to prepare reproducibly without carbonate contamination. Varying amounts of carbonate contamination adversely affect retention time precision. This problem has limited the adoption of HPAE-IPAD for Neomycin determinations.

In this application note, we show that an eluent generator solves the problem of consistent eluent preparation. An eluent generator automatically prepares hydroxide eluents of precise concentrations that are essentially carbonate-free. The EG50 Eluent Generator automatically produces potassium hydroxide (KOH) eluent from water and a potassium electrolyte solution by means of electrolysis. The only carbonate in the mobile phase is what exists in the water used to supply the eluent generator. The Continuously Regenerated Anion Trap Column (CR-ATC), installed after the eluent generator, removes the minor amounts of carbonate from the supply water, as well as borate and other contaminating anions. Consequently, the usual variability in hydroxide concentration associated with manual eluent preparation, and the variability of carbonate contamination due to adsorption of atmospheric carbon dioxide, are essentially eliminated, leading to highly reproducible retention times.

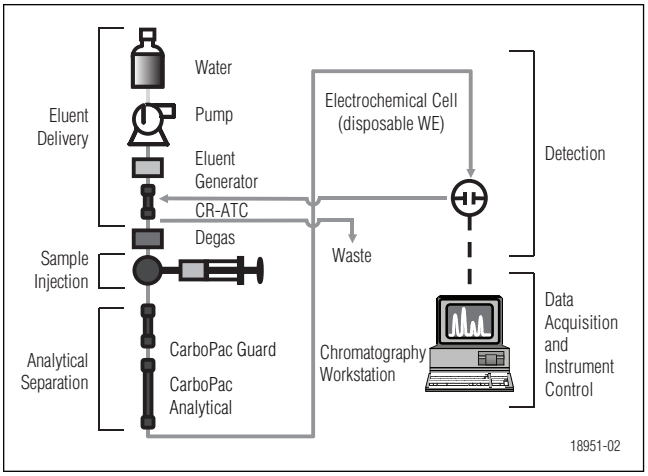


Figure 2. HPAE-PAD system for Neomycin determinations.

In addition to improving HPAE retention time reproducibility, we adopted disposable gold (Au) working electrodes to improve electrode-to-electrode (and system-to-system) reproducibility of Neomycin B electrochemical response. Disposable Au working electrodes are manufactured in a manner that improves electrode-to-electrode reproducibility.[11–14] These electrodes require no maintenance (e.g., polishing) and are economical to replace.

In this application note, we combine the CarboPac PA1, an eluent generator with CR-ATC, and disposable Au working electrodes (Figure 2) to demonstrate an improved HPAE-PAD technology for Neomycin B purity analysis and its determination in Neosporin topical ointment, a complex over-the-counter pharmaceutical formulation. Key performance parameters are evaluated including precision, limits of detection, linearity, and ruggedness in a manner consistent with many requirements of normal method validation.[15–22] Furthermore, Neomycin B purity is evaluated per the requirements of the International Conference on Harmonization.[23] Overall, the described setup has improved sensitivity, good sample throughput (15 min per run), and improved retention time reproducibility. The automated production of KOH eluent improves reproducibility and eliminates eluent preparation errors.

EQUIPMENT

Dionex BioLC system consisting of:

- GP50 Gradient or IP25 Isocratic Pump, with vacuum degas option and GM-4 Gradient Mixer
- ED50 Electrochemical Detector with:
  - Combination pH/Ag/AgCl Reference Electrode (P/N 044198)
  - *AAA-Direct*™ Certified (Au) Disposable Electrodes (P/N 060082, package of 6; P/N 060140, package of 24)
- EG50 Eluent Generator with EGC II KOH eluent generator cartridge (EluGen® II Hydroxide; P/N 053921)
- EG40/50 Vacuum Degas Conversion Kit (P/N 055431)
- CR-ATC, Continuously Regenerated Anion Trap Column (P/N 060477)
- AS50 Autosampler with 20-µL injection loop
- AS50 Thermal Compartment
- EO1 Eluent Organizer, including four 2-L plastic bottles and pressure regulator

Chromeleon® Chromatography Management Software

Helium, 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent nylon filter)

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent)

Polypropylene injection vials with caps, 0.3 mL (Vial Kit, Dionex P/N 055428)

Microcentrifuge tubes with detachable caps (plastic, 1.5 mL, Sarstedt, P/N 72.692.005, or equivalent)

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Standards

Neomycin B (Neomycin Sulfate; U.S. Pharmacopeia (USP) Reference Standard)

Neomycin A (Neamine hydrochloride, International Chemical Reference Substance, Control No. 193177, World Health Organization (WHO) Collaborating Centre for Chemical Reference Substances)

Samples

Neomycin Sulfate, commercial grade (Sigma-Aldrich, Cat. No. N-1876)

Neosporin (Original, Neomycin and Polymyxin Sulfates and Bacitracin Zinc First Aid Antibiotic Ointment, Pfizer Consumer Healthcare)

CONDITIONS

Method

Columns:	CarboPac PA1 Analytical, 4 x 250 mm (P/N 035391) CarboPac PA1 Guard, 4 x 50 mm (P/N 043096)
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL (full loop)
Temperature:	30 ˚C
Detection (ED50):	Pulsed amperometry, *AAA-Direct* Certified disposable Au working electrodes (P/N 060082)
Background:	11–89 nC
Backpressure:	2110–2840 psi (with restrictor tubing installed between the degas apparatus and the injector)
Eluent Generation Method:	2.40 mM KOH, isocratic, 15-min run time

AAA-Direct Waveform for the ED50*

Time (s)	Potential (V)	Integration
0.00	+0.13	
0.04	+0.13	
0.05	+0.33	
0.21	+0.33	Begin
0.22	+0.55	
0.46	+0.55	
0.47	+0.33	
0.56	+0.33	End
0.57	-1.67	
0.58	-1.67	
0.59	+0.93	
0.60	+0.13	

Reference electrode in pH mode.

* Waveform used for this note. For the most current waveform, see the product manuals for the *AAA-Direct* Amino Acid Analysis System.[24]

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

It is essential to use high-quality water of high resistivity (18 MΩ–cm) containing as little dissolved carbon dioxide as possible. Biological contamination should be absent. Source water must be obtained using a water purification system consisting of filters manufactured without electrochemically active surfactants (e.g., glycerol). Prior filtration through 0.2-µm porosity nylon under vacuum is recommended to remove particulates and reduce dissolved air. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of helium at all times to reduce diffusion of atmospheric carbon dioxide and opportunistic microorganisms.

Stock Standards and Drug Substance

Solid Neomycin A and Neomycin B standards and the Neomycin sulfate commercial material were placed in plastic vials and dissolved in deionized water to a 10 mg/mL concentration. The masses of moisture, salt, and impurities, as stated on the manufacturer's Certificate of Analysis, were subtracted from the measured mass to improve accuracy of the Neomycin free base solutions. These solutions were further diluted with water to yield the desired stock mixture concentrations. For this note, all dilutions were made gravimetrically to ensure high accuracy. The solutions were maintained frozen at -40 ˚C until needed. Masses of 1, 2, 20, 100, 200, 300, 400, and 600 pmol Neomycin B were injected for linearity studies.

Neosporin Extraction

Neosporin gel (14–32 mg) was placed in a 1.5-mL plastic microcentrifuge vial with a detachable screw cap, and combined with 1.0 mL water. The mass of the ointment and water were both weighed on an analytical balance during this process. The sealed vial was placed in an 80 ºC heating block for 5 min, with the tube vortexed (high setting) halfway through the heating (at 2.5 min). After 5 min, the melted ointment was vortexed (high setting) continuously for 5 min, and then placed in the refrigerator for >1 h. The chilled extract was centrifuged at 16,000 X g in a microcentrifuge for 10 min, and the supernatant was separated from an upper fat layer using a Pasteur pipette prerinsed with DI water, and transferred to another vial. This extract was then diluted 85.4-fold with water, using gravimetric techniques to accurately calculate the exact dilution. An aliquot of this diluted extract was injected for HPAE-IPAD analysis to determine the Neomycin B concentration. For spike recovery experiments, the 1.0 mL water used for extraction was replaced with 600 µM Neomycin B standard.

RESULTS AND DISCUSSION

Separation

Figure 3 shows the separation of 1 µM USP grade Neomycin B (peak 3) from the column void (peak 1) and 3 baseline dips (peaks 2, 4, 5) using a CarboPac PA1 column set with a 2.40 mM KOH eluent. Baseline dips associated with injections of water or samples are likely caused by trace organic impurities present in the sample or water separated on the CarboPac PA1 column by means of secondary interactions (e.g., hydrophobic interactions). When these compounds elute, they exclude electrochemically active ions in the eluent. The "oxygen dip" (~31-min retention time, peak 5) is due to oxygen present in the samples and appears as a function of the gas permeation volume of the column. Like some organic impurities, eluting oxygen produces less background than the eluent, and therefore causes a dip in the baseline. The retention times

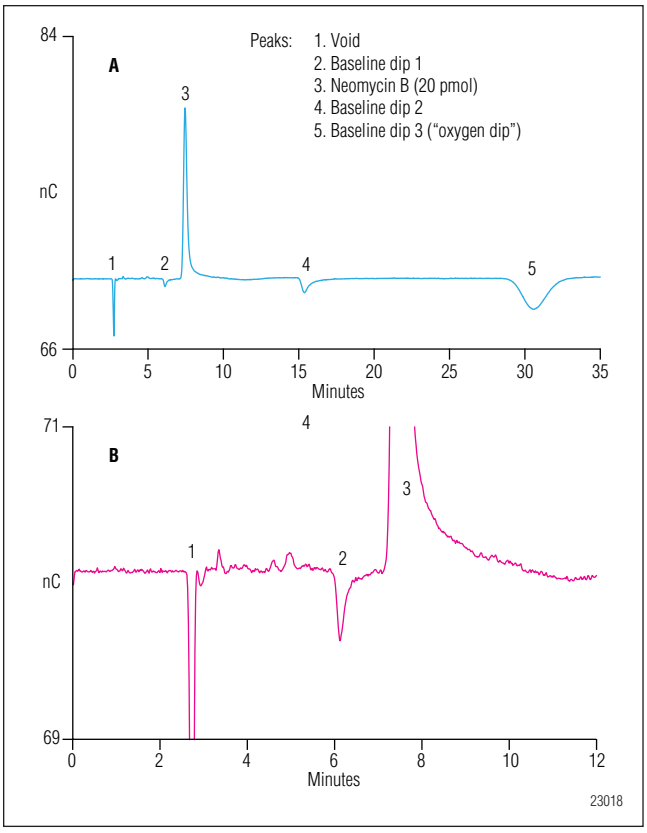


Figure 3. Determination of Neomycin B (1.0 µM, 20-µL injection) using eluent generation (2.40 mM KOH).

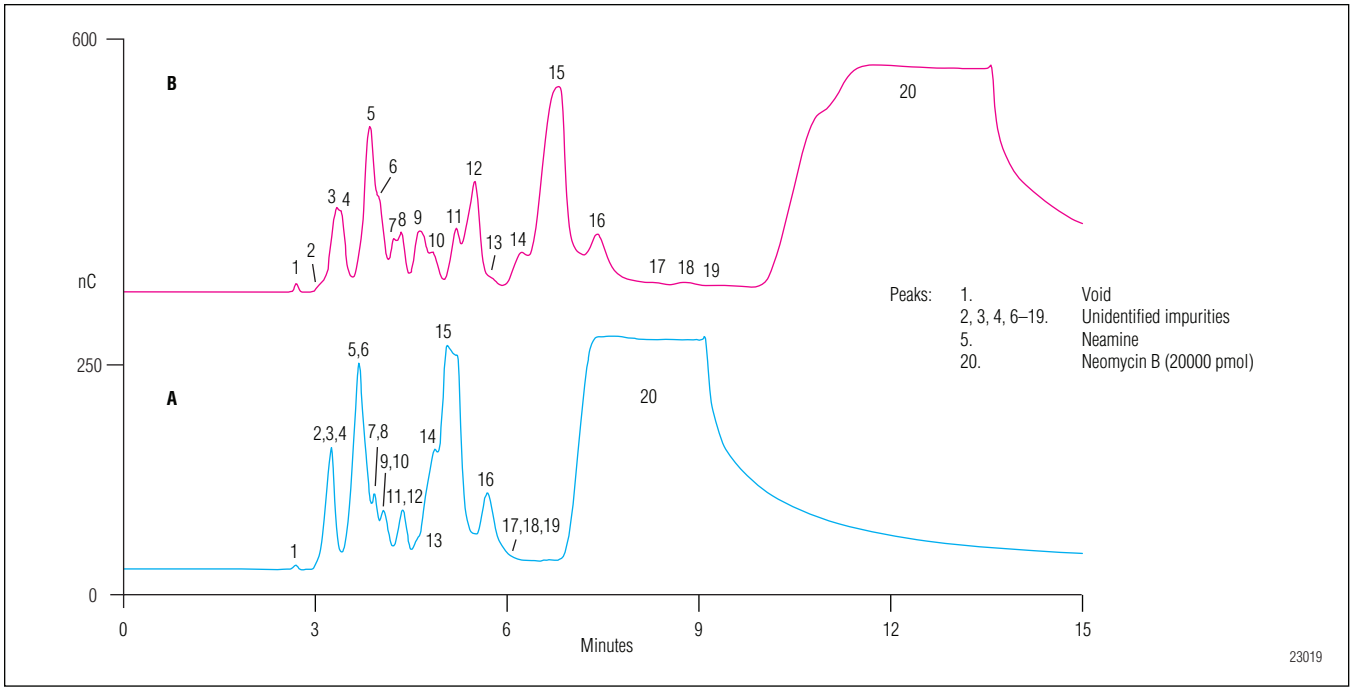


Figure 4. Separation of Neomycin B and impurities is highly dependent on eluent concentration. Comparison of the resolution of Neomycin B (1 mM, 20000 pmol) and impurities in commercial grade Neomycin sulfate separated using 2.40 mM KOH (chromatogram A) and 2.16 mM KOH (chromatogram B). Neomycin B (peak 20) is injected at a concentration outside its upper limit of detection.

of the "oxygen dip" and other baseline dips are constant for each column, but vary slightly from column to column; and depend on the flow rate, not the eluent strength. Eluting the baseline dips just prior to the end of run, or timing their elution to occur at the end of the following injection, prevents the baseline dips from interfering with the peaks of interest.

Separation of Neomycin B and its impurities is highly dependent on eluent concentration. Table 1 shows the effect of KOH eluent concentration on the retention times of Neomycin A and B. The greatest effect on retention of these two compounds was observed between 1 and 5 mM, where very minor changes in hydroxide concentration produced large changes in Neomycin A and B retention times. Figure 4 compares the resolution of impurity peaks for injections of 1 mM (0.5 mg/mL) commercial grade Neomycin B using 2.40 mM (chromatogram A) with 2.16 mM KOH (chromatogram B). The reduction in eluent concentration increases the retention time of Neomycin B, reducing throughput and increasing peak tailing; however, the separation of impurities is improved. The high concentration of Neomycin B used in Figure 4, compared to Figure 3, improves the detection of impurity peaks; however, the Neomycin

Table 1. Relationship of Neomycin B and Neomycin A Retention Time to Eluent Strength

	KOH Eluent Concentration (mM)							
	100	75	50	25	10	5	2	1
Peak Identity	**Retention Time (min)**							
Column Void	2.7	2.7	2.7	2.7	2.7	2.7	2.8	2.9
Neomycin A (Neamine)	2.8	2.8	2.8	2.8	2.9	3.0	4.6	51.6
Neomycin B	3.6	3.6	3.8	3.9	4.0	4.2	15.7	>60
Baseline Dip	15.6	15.6	15.6	15.4	15.4	15.4	15.4	15.4
Oxygen Dip	31.8	31.4	31.1	30.8	30.7	30.7	30.6	30.6

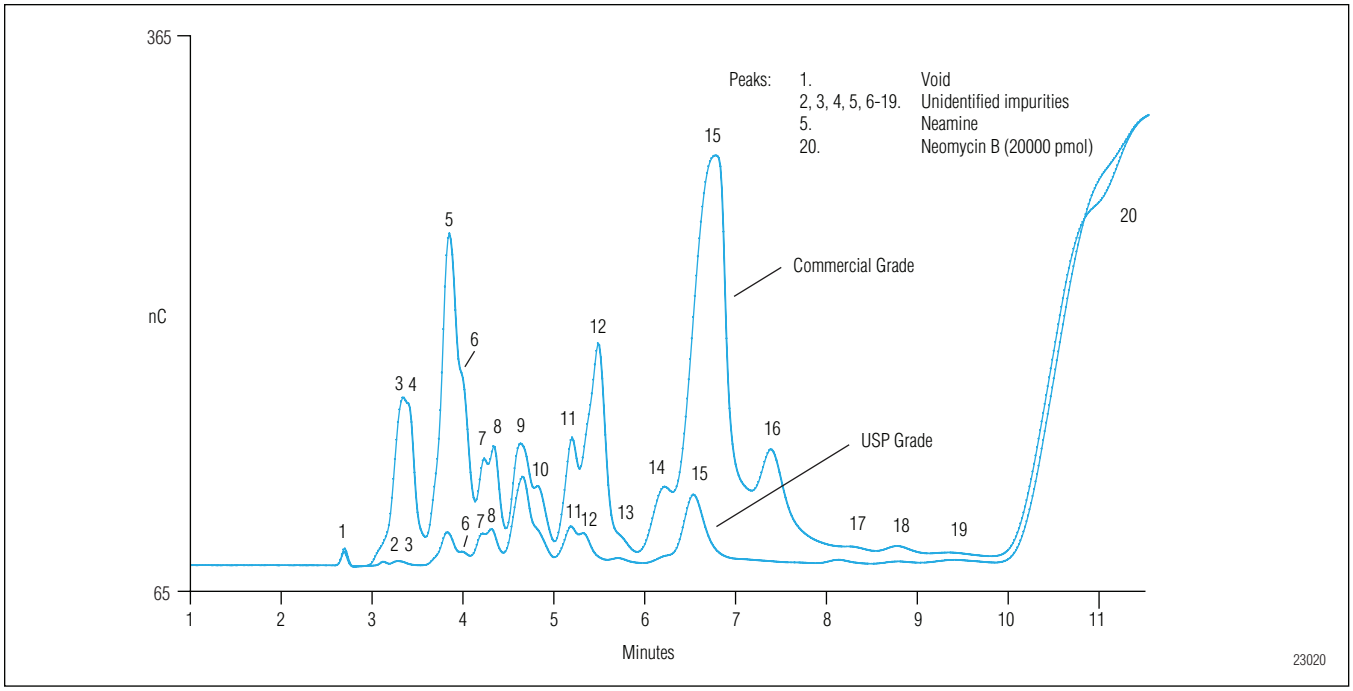


Figure 5. Comparison of the impurities found in USP and commercial grade Neomycin B (1 mM, 2 x 10^4 pmol) separated using 2.16 mM KOH. Neomycin B (peak 20) is injected at a concentration above its upper limit of detection.

B response is out of range and the peak appears as a plateau (peak 20). The response of impurities, if present in concentrations below their upper limit of linearity (see section "Detection: Linear Range" below), remains linear. Although decreasing the eluent strength to 2.16 mM KOH enables greater resolution of impurity peaks, the 2.40 mM KOH condition was optimized for throughput, for resolution of Neomycin B from impurities and the column void, and for noninterfering locations of baseline dips. For these reasons, the method evaluated in this note used the 2.40 mM KOH condition, unless otherwise specified. The impurity peak at 3.6 min (Figure 4, peak 5) was identified as Neomycin A based on the retention time of a standard. The major impurity peak 15 was presumed to be Neomycin C because it has been described as the most abundant impurity in commercial grade Neomycin sulfate.[4] Impurity peak 3 closely elutes with the column void and is probably a mixture of coeluting compounds. Also, this peak increases in the water blank injections when injection vials were not prerinsed three times with water before use. Figure 5 compares the separation of impurities in 1 mM USP grade Neomycin B from impurities in 1 mM commercial grade Neomycin B using 2.16 mM KOH. This figure shows the USP grade material has a significantly lower level of impurities compared to the same amount of commercial grade material injected.

The resolution (European Pharmacopoeia (EP) definition) between Neomycin B and its prior major eluting peak (peak 15, Figure 4, chromatogram A) presumed to be Neomycin C, ranged from 6.84 to 7.84 (mean ± SD; 7.35 ± 0.08, n = 845 injections, 1.2% RSD) over 10 days of consecutive injections without any column regeneration using 2.40 mM KOH. The EP method for Neomycin sulphate is a liquid chromatographic method that specifies a minimum resolution requirement between Neomycin B and C to be ≥2.0.[6] That method also allows adjustment of the mobile phase concentration to achieve this minimum resolution. The method presented in this application note easily achieves the resolution specification without mobile phase adjustment.

Detection

Linearity

Figure 6 presents the relationship of Neomycin B peak area (nC*min) to pmole of the analyte injected (20 µL) over a broad range of concentrations, 0 to 2 nmol. Figure 7 shows the same data over a narrower range, 1 to 400 pmol, where the relationship of response to mass injected is linear. In this application note, we consider the linear concentration range to be where the

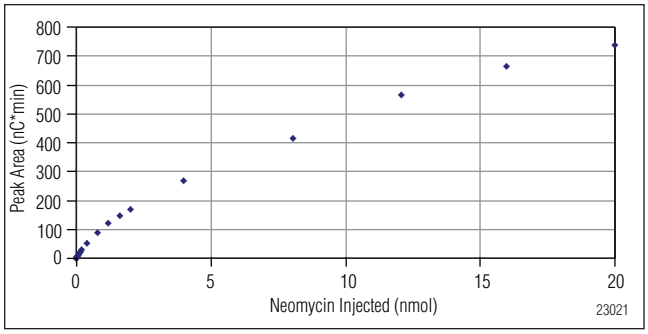


Figure 6. The relationship of peak area (mean) to nmol of Neomycin B injected for estimation of linear range.

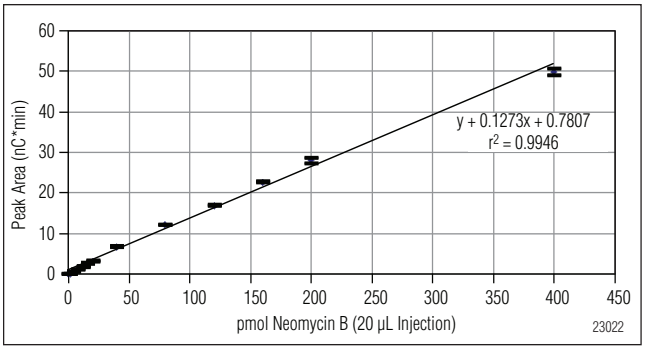


Figure 7. The linear relationship of Neomycin B peak area (mean ± SD) within its estimated linear range.

response factor (ratio of peak area/mass injected) remains within 20% from the mean. A plot that relates area response factor to the mass injected (data not presented) showed a typical plateau region that represented an optimal level for operation. These results (Table 2) show Neomycin B peak area linearity extends up to 400 pmol (20 µM for 20-µL injection). Neomycin B peak height linearity extends to only 160 pmol (7.8 µM for 20-µL injection). We therefore recommend peak area instead of peak height for quantification of Neomycin B. The linear range typically extended over 3 orders of magnitude (0.2 to 400 pmol Neomycin B) using the estimated lower limit of detection (LOD) as the lower end of the range.

Figure 7 shows the linear relationship of peak area response (mean ± standard deviation, n = 4 injections) to pmole of antibiotic injected for the concentrations ranging from near the lower limit of quantification to the upper limit of linearity. Quantities ranging from 0.2 to 400 pmol produced an r^2 value of 0.9946 for Neomycin B. Table 2 summarizes the statistics for this calibration curve. The slope for Neomycin B was 0.127 nC*min/pmol.

Lower Limits of Detection and Quantification

In this study, baseline (peak-to-peak) noise was determined from noise measured in 1-min intervals during blank runs. Noise is measured in peak height units, pC. Baseline noise ranged from 13 to 81 pC (mean ± SD; 34.7 ± 12.9, n = 186 1-min intervals) measured over an 11-day period. After installing new disposable electrodes, baseline noise tended to decrease over the first hour. Noise stabilized to its lowest level (lower end of the range) between 1–2 h of electrode use. The concentration (or mass injected) of Neomycin B at the lower limit of detection (LOD) was calculated from three times

Table 2. Estimated Limits of Detection, Quantitation, and Linearity for Neomycin B

Noise (pC)	
Mean ± SD; n = 186†	34.7 ± 12.9 pC
Range	13–81 pC
Lower Limit Detection	
pmol	0.21 ± 0.08
µM*	0.011 ± 0.004
picogram	130 ± 49
µg/mL*	0.0066 ± 0.0024
Lower Limit Quantitation	
pmol	0.72 ± 0.26
µM*	0.036 ± 0.013
picogram	440 ± 160
µg/mL*	0.022 ± 0.008
Upper Limit Linearity	
pmol	400
µM*	20
picogram	246,000
µg/mL*	12.3
Linearity Over Linear Range	
r^2	0.9946
Slope (nC*min/pmol)	0.127

* 20-µL injections

† Number of 1-min peak-to-peak readings over 11 days

the average peak-to-peak noise, divided by the average peak height response factor for the antibiotic within its linear region. At this concentration, the signal-to-noise ratio equals three. The lower limit of quantification (LOQ) is the concentration (or mass injected) calculated from 10 times the average peak-to-peak noise. The estimated LOD for Neomycin B was 0.21 ± 0.08 pmol (ranging from 0.004–0.02 µM for a 20-µL injection) over 11 days; and the LOQ was 0.72 ± 0.26 pmol (ranging from 0.01–0.08 µM). Table 2 summarizes these results. Figure 8 shows the Neomycin B peak at its LOD. The EP method specifies a minimum signal-to-noise ratio of ≥10 for an injection of 0.50 µg/mL (0.814 µM) Neomycin B. The signal-to-noise ratio determined for this method ranged from 101 to 616, the variance primarily a function of the range of the noise observed. This method easily exceeds this EP system suitability requirement.

When Neomycin B is analyzed at the upper range of linearity (400 pmol), a 0.20 pmol LOD is equivalent to a 0.05 mole percent impurity. This percent Neomycin B impurities can be determined from a single injection, where the Neomycin B peak area exists within its linear range and can be used for quantification. A lower percentage of impurities can be detected by injecting Neomycin B at concentrations outside its linear range. Injecting 20 µL of 0.50 mg/mL (1 mM) Neomycin B, equivalent to 20,000 pmol per injection (Figure 9), decreases lower detectable percentage of Neomycin B impurities to 0.001%, but requires a second injection of diluted Neomycin B (to within its linear range) to measure the amount of Neomycin B for percent impurity determination. No current USP specifications exist for the purity of Neomycin sulfate, while the EP require

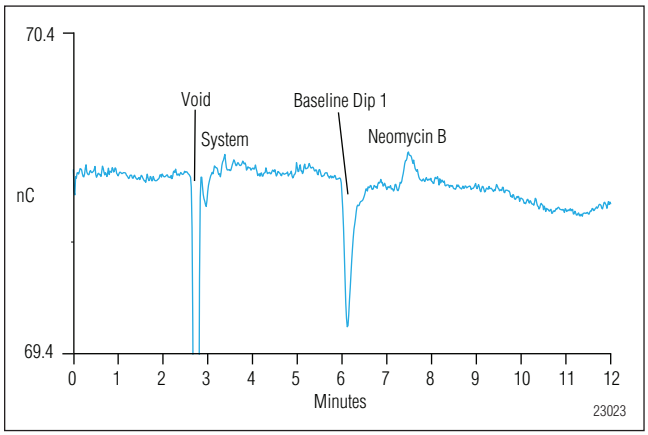


Figure 8. 0.20 pmol Neomycin B (0.010 µM, 20 µL) at its lower limit of detection.

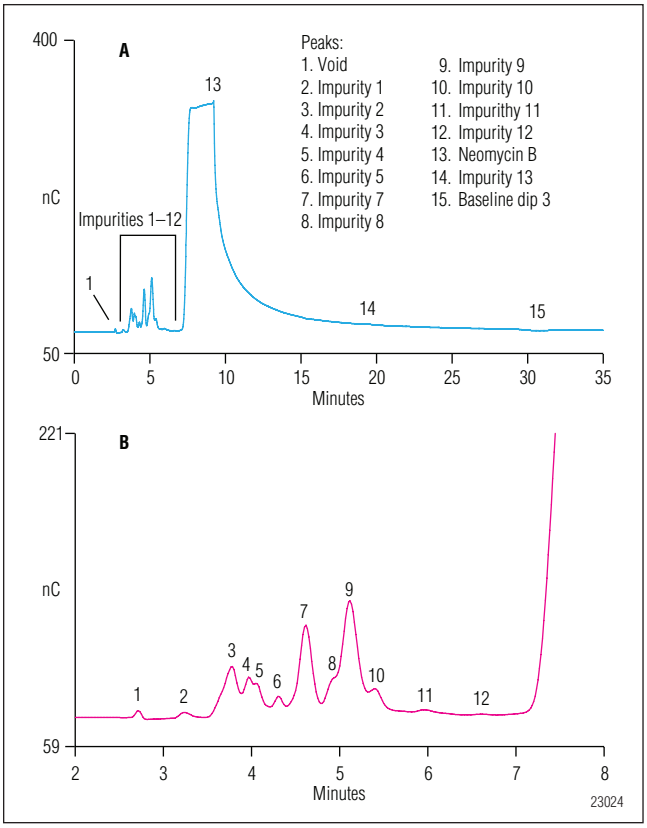


Figure 9. Determination of impurities when Neomycin B is analyzed outside the upper end of its linear range (0.50 mg/mL, 20-µL injection).

≤2% neamine (Neomycin A) and Neomycin C between 3 and 15%.[6] Injecting Neomycin B at the upper end of its linear range (400 pmol), this method can easily achieve the EP Neomycin B impurity levels.

The ICH Harmonized Tripartite Guideline for Impurities in New Drug Substances Q3A(R)[23] recommends 0.03% impurity (peak area) of the new drug as a reporting threshold for a >2 g/day daily dosage level. Neomycin B is not a new drug substance, and its impurities have been characterized for over 40 years since the drug was first discovered and developed. For this reason, the 0.03% level described in the ICH guidelines is strictly theoretical for Neomycin B. The usual oral dosage of Neomycin B in adults with normal renal function is 9 g/day, and maximum dosage for life-threatening infections (coma with hepatic disease) is 12 g/day.[25] If the targeted percent impurity level defined in the ICH Guideline (0.03%) was proportioned according to the maximum expected oral dosage (12 g/day), then the adjusted target would be 0.005%. If this drug were to be developed today, the measure of impurity levels required by ICH could be achieved using the method presented in this application

note, capable of detection to the 0.001% level. Note that all compendial purity methods for aminoglycoside antibiotics in the USP and EP assume the detection method can measure all unknown impurities or that the detection method responds the same as the parent compound. None of the existing methods can fulfill this requirement. The method presented in this application note is not an exception to this universal limitation.

Precision

The retention time and peak area RSDs were determined for replicate injections of a Neomycin B standard (10 µM for 20-µL injection) over 10 days (822 injections). Table 3 shows these results.

Retention Time

The mean (±SD) retention time for Neomycin B was 7.45 ± 0.05 min over 10 days (822 injections), with 0.64% RSD. The daily retention time RSDs (over a 24-h period) ranged from 0.2 to 0.4%, with the exception of the first day where column equilibration was needed following an initial 100 mM KOH column wash. The column was regenerated for 1 h at 100 mM KOH prior to this study. After the initial column equilibration was reached, no upward or downward trend was observed, and the precision was essentially the same for each 24-h period. The method described in this application note is designed to analyze a relatively pure antibiotic and can be used without any column regeneration for at least ten days.

Peak Area

The peak area for Neomycin B in this study ranged from 27.6 to 31.5 nC*min (mean ± SD; 29.92 ± 0.40 nC*min) with a 1.3% RSD. No statistically significant change in peak area (+0.2%) was observed over the 10-day period. Daily peak area RSDs ranged from 0.79 to 1.7%.

The high retention time and response reproducibility indicate that this method is suitably rugged for this application. Peak area precision is dependent on the concentration analyzed. As concentration approaches the LOQ and LOD, higher variance will be observed. This study used concentrations within the linear range for Neomycin B.

Table 3. Precision of Neomycin B Retention Time and Peak Area Over 10 Days Using the Eluent Generator

	Day										Over 10 Days (All Data)	Percent Change Over 10 Days
	1	2	3	4	5	6	7	8	9	10		
Retention Time (min)												
Mean	7.36	7.44	7.46	7.46	7.46	7.47	7.47	7.48	7.47	7.46	7.45	**1.37%**
SD	0.09	0.03	0.02	0.02	0.03	0.03	0.02	0.02	0.02	0.02	0.05	
N	72	84	85	85	83	85	82	82	80	84	822	
RSD	1.28%	0.39%	0.30%	0.33%	0.34%	0.43%	0.26%	0.32%	0.23%	0.30%	0.64%	
Peak Area (nC*min)												
Mean	29.92	29.65	29.96	29.88	29.86	29.88	29.92	30.05	30.10	29.98	29.92	**0.20%**
SD	0.50	0.50	0.34	0.33	0.41	0.30	0.42	0.42	0.24	0.31	0.40	
N	72	84	85	85	83	85	82	82	80	84	822	
RSD	1.66%	1.67%	1.14%	1.10%	1.39%	1.01%	1.41%	1.39%	0.79%	1.04%	1.33%	

Robustness

Robustness was evaluated for influence of a 10% variance in eluent concentration, a 10% variance in column temperature, a 10% variance in flow rate, a column change, and effect of sample salt concentration.

Eluent Concentration

The retention time of Neomycin B varied greatly with minor variations in mobile phase concentration. A 10% increase in KOH (2.64 mM) produced a retention time decrease from 7.50 min to 5.89 min (-21% change from 2.40 mM); while a 10% decrease in KOH (2.16 mM) produced a retention time increase to 10.90 min (+45% change). The 10% increase in eluent concentration increased peak area 2%, and the 10% decrease in eluent concentration decreased peak area 14%. Amperometric response is dependent on pH, and changes in eluent concentration changes peak area. The large percent change in retention time and peak response for a relatively small change in KOH eluent concentration demonstrates the importance of producing a consistent eluent concentration, which the eluent generator achieves.

Column Temperature

A 10% change in the operating column temperature (30 ºC) was evaluated for influence on Neomycin B retention times. At the recommended operating temperature of 30 ºC, the retention time for Neomycin B was 7.45 min. At 33 ºC, the retention time was 7.66 min, an increase of 2.7%. At 27 ºC, the retention time was 7.28 min, a decrease of 2.4%. The increase in retention times with an increase in column temperature may be due to increased ionization of functional groups. A 10% increase in temperature increased peak area by 5.4%, and a 10% decrease in temperature decreased peak area by 5.4%. At 10% higher temperatures, an 8% increase in background, and at a 10% lower temperature, a 5% decrease in background was observed. Noise was unaffected by 10% temperature changes. Although the electrochemical cell is not maintained at increased or decreased temperature under the conditions used in this study, the temperature of the eluent entering the cell is altered. Temperature-related changes in peak area and background may reflect the change in eluent/sample temperature.

Flow Rate

A 10% change in the operating column flow rate was evaluated for influence on Neomycin B retention time. At the recommended flow rate of 0.50 mL/min, the retention time for Neomycin B was 7.47 min. At 0.55 mL/min, the retention time was 6.76 min, a 9.4% decrease. At 0.45 mL/min, the retention time was 8.23 min, a 10.2% increase. At 10% higher flow rate, peak area decreased 4%, and at 10% lower flow rate, peak area increased 5%.

Sample Matrix

Salt exceeding 10 mM in the sample injected may cause a shift in Neomycin B retention time and distort peaks. Although slight peak distortions were observed at ≥5 mM NaCl, and progressed as concentrations increased, peak splitting occurred at ≥10 mM NaCl. Peak area tended to increase with increasing NaCl concentration. At 8 mM NaCl, peak area exceeded a 10% increase. Between 10 and 20 mM NaCl, a decreasing trend was observed for the combined peak area of the split peaks. The total sample salt concentration injected must be considered for applications other than assessing the quality of pure Neomycin. For some pharmaceutical formulations, a periodic column wash more frequent than 7–10 days may be necessary, and will depend on the nature of the ingredients.

Column Reproducibility

The Neomycin B retention time RSD for four columns was 5.6%, whereas Neomycin A retention time RSD was 3.3%. If the same retention times are desired from column-to-column, an adjustment of the KOH concentration may achieve that.

Retention time of baseline dips also vary slightly from column-to-column, and may change slightly over the long-term (6–12 months) use of the column. in this study baseline dips did not interfere with determination of Neomycin B or its impurities. If Neomycin B or its impurity peaks coelute with the first baseline dip (at ~6 min) using 2.40 mM KOH, or the same retention times are desired from column to column, then KOH concentration may be accurately and precisely adjusted using the eluent generator.

Table 4. Spike Recovery of Neomycin B from Neosporin Ointment						
			Percent Recovery of Neomycin B Extracted from Neosporin Ointment and from Water			
Sample	**Extract #**	**mg Neosporin Extracted**	**Mean ±SD (n = 4 injections of each Extract)**	**RSD**	**Mean ± SD (Within Each Sample)**	**RSD**
Water*	1	0	95.9 ± 0.73	0.76%		
	2	0	97.9 ± 1.3	1.3%		
	3	0	108 ± 1.3	1.2%		
	4	0	105 ± 1.3	1.2%	101.8 ± 5.8	5.7%
Neosporin	1	24.6	96.0 ± 1.2	1.2%		
	2	32.3	99.2 ± 1.8	1.8%		
	3	24.1	103 ± 1.2	1.2%		
	4	16.8	99.7 ± 2.3	2.3%		
	5	28.2	99.9 ± 1.6	1.6%	99.6 ± 2.5	2.5%

* Neomycin B in water, treated with the extraction procedure, is evaluated for recovery.

Table 5. Determination of Neomycin B in Neosporin Ointment						
			mg Neomycin B/gram Neosporin			
Trial Day	**Extract #**	**mg Neosporin Extracted**	**Mean ± SD (n = 4 injections of each Extract)**	**RSD**	**Mean ± SD (Within Each Day)**	**RSD**
1	1	29.7	4.08 ± 0.09	2.2%		
	2	14.0	3.97 ± 0.04	1.0%		
	3	19.3	4.31 ± 0.09	2.2%		
	4	15.0	3.94 ± 0.09	2.4%		
	5	25.6	4.28 ± 0.06	1.4%	4.12 ± 0.17	4.2%
2	1	21.4	4.17 ± 0.05	1.1%		
	2	20.1	4.16 ± 0.12	2.9%		
	3	31.6	4.19 ± 0.05	1.2%	4.17 ± 0.02	0.36%
3	1	19.0	4.17 ± 0.20	4.8%		
	2	13.7	4.07 ± 0.02	0.4%		
	3	29.6	4.35 ± 0.14	3.2%		
Between Days			4.15 ± 0.13	3.2%	4.20 ± 0.14	3.4%

Note: Days 2 and 3 used the same reference and disposable Au working electrode, different from day 1.
No significant difference in the Neomycin determination was observed with disposable electrode change.

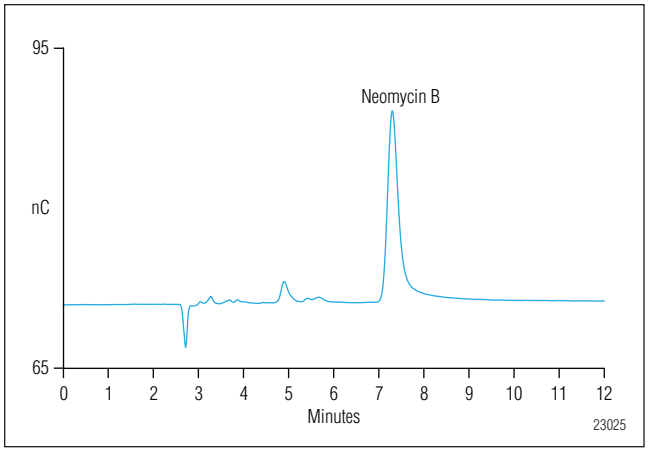


Figure 10. Determination of Neomycin B (2.12 µM, 25 µL injection) extracted from 25.6-mg Neosporin topical ointment with 1 mL water, and diluted 85-fold.

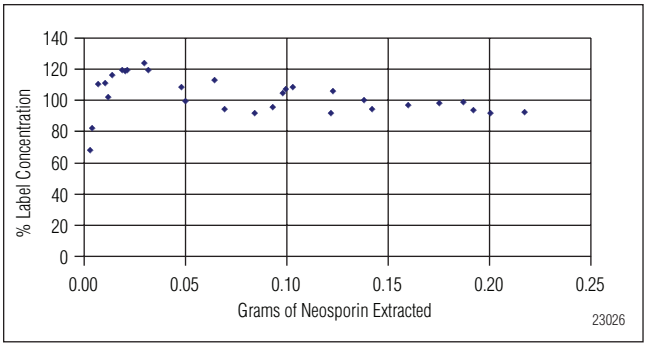


Figure 11. The relationship of Neomycin B yield and the mass of Neosporin ointment extracted.

Analysis of Formulations

Neosporin is a topical antibiotic ointment consisting of the active ingredients Neomycin (3.5 mg/g of ointment), bacitracin (400 units/g), polymyxin B (5000 units/g); and the inactive ingredients cocoa butter, cottonseed oil, olive oil, sodium pyruvate, vitamin E, and white petrolatum. This material was selected as a model pharmaceutical formulation because the largely water insoluble inactive ingredients and the presence of other antibiotics makes this a challenging mixture to analyze by liquid chromatography. The extraction and analysis of this ointment is relatively simple using HPAE-PAD for analysis. A known mass of ointment is melted and extracted in 1 mL of water at 80 ºC. The supernatant of the chilled extract is diluted and directly injected for HPAE-PAD. Figure 10 shows a chromatogram of Neomycin B recovered from the Neosporin extract. Neomycin B recovery is somewhat dependent on the mass of ointment extracted (Figure 11), and when the mass extracted was limited to the range of 14–32 mg, optimal recovery of 99.6 ± 2.5% was obtained for five separate extracts (Table 4). Table 5 shows Neomycin B was determined to be 4.15 ± 0.13 mg Neomycin B per g of Neosporin (3.2% RSD) over three trials conducted over three separate days, n = 11 extracts. The label of this product states a specified 3.5 mg/g concentration, and our measured level is 18.6% greater than expected. USP specifications allows ointments of this type to be not less than 90.0 and not more than 130.0% of the label value.[26] Our measurements show this product to be within these specifications. The slightly elevated concentration in this formulation is probably designed to ensure longer product shelf life.

Instrument Operational Considerations

Following an injection of 1 mM Neomycin B, useful for maximizing the LOD of this method (see section entitled "Lower Limits of Detection and Quantification"), the Neomycin B peak appears as a carryover peak in a subsequent injection of water or other blanks. In this study, we measured 0.0064% carryover (1.3 pmol) Neomycin B in the first injection of water. The carryover peak decreases, and falls below the detection limit after a total of four injections of water. Although the carryover is slight, its presence could affect the accuracy of Neomycin B quantification. The sequence of sample, standard, and blank injections should be designed to assure minimal artifacts due to carryover.

Weekly column washes at 100 mM KOH are recommended to restore retention times for Neomycin B when the system is used without column regeneration. The application of 100 mM KOH changes system equilibrium, and reequilibration at 2.40 mM KOH for >2 h is recommended to achieve high precision.

When the system is idle for short (1–2 week) periods, we recommend that the pump and eluent generator be left at a reduced flow rate to achieve rapid start-up, and the cell to be turned off to extend disposable electrode life. When the system must be shut down for a period of several weeks, the pump, eluent generator, and electrochemical cell may be simply turned off. For shutdown periods exceeding several weeks, all plumbing lines should be resealed, and the reference electrode should be removed from the electrochemical cell and stored in saturated KCl. When the pump has been turned off for longer than 1 day, the column should be regenerated with 100 mM KOH for 1–2 h, and reequilibrated with 2.40 mM KOH for 2 h before analyzing samples.

Unlike HPLC and TLC methods for aminoglycoside antibiotic determinations, where toxic reagents are required for separation and detection, this method produces dilute aqueous KOH as a waste stream. The container used for collecting KOH waste may be easily neutralized with hydrochloric acid to produce a nontoxic solution that may be disposed of without the added expense of hazardous waste disposal.

CONCLUSION

HPAE-PAD with eluent generation can be used to determine Neomycin B and its impurities. The linear range of electrochemical response extended over 3 orders of magnitude, from 0.011 ± 0.004 µM (LOD) up to 20 µM (12 µg/mL, 20-µL injection). The data in this application note suggests that this method is capable of meeting ICH guidelines for impurities in new drugs. Automated eluent generation makes this method reproducible and rugged with respect to retention time and peak separation. Because the pump is only required to pump water and no eluent preparation is required, pump seal wear is reduced and this increases efficiency and convenience for the analyst. The disposable gold working electrodes provided consistently high detector response, assuring greater instrument-to-instrument and lab-to-lab reproducibility. The practical application of this method was demonstrated from the chromatographic separation and measured high spike recovery of Neomycin B from other ingredients in a challenging over-the-counter topical ointment formulation.

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LIST OF SUPPLIERS

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Fisher Scientific, 2000 Park Lane, Pittsburgh, PA, 15275-1126, USA, Tel: 800-766-7000, www.fishersci.com.

Pfizer Consumer Healthcare, Morris Plains, NJ 07950, U.S.A. Tel: 1-800-223-0182 www.prodhelp.com

Praxair, 39 Old Ridgebury Road, Dansbury, CT 06810-5113, USA. Tel: 877-772-9247.

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A., 1-800-325-3010. www.sigma.sial.com

World Health Organization (WHO) Collaborating Centre for Chemical Reference Substances; Apoteket AB Produktion & Laboratorier Centrallaboratorier, ACL; Prismavägen 2; SE-141 75 Kungens, Kurva, Sweden. Tel: +46 8-466 1000. FAX: +46 8-740 6040. who.apl@apoteket.se http://www.who.int/medicines/library/pharmacopoeia/315to342.pdf

Determination of Sulfur-Containing Antibiotics Using Integrated Pulsed Amperometric Detection (IPAD)

INTRODUCTION

Antibiotics are often analyzed using high performance liquid chromatography (HPLC) with absorbance detection. Official methods to assess antibiotic identity, strength, quality, and purity are described in the Code of Federal Regulations (CFR Title 21) and in the United States Pharmacopeia National Formulary (USP NF). The HPLC methods described use absorbance detectors. Non-HPLC methods are required for some antibiotics with poor chromophoric properties. For example, lincomycin is certified for identity and potency[1,2] by a method that derivatizes this analyte and uses a gas chromatograph (GC) with flame ionization detection (FID)[3]. In this time-consuming method, lincomycin is dissolved in pyridine and then derivatized using a silylating reagent. An internal standard is added after derivatization. Identity is based on retention time, and potency is based on peak area relative to a lincomycin standard. Impurity is measured as lincomycin-B (4"-etillincomycin) content, the only measurement required to certify the purity of lincomycin. GC and HPLC methods using precolumn derivatization were developed to provide determinations of lincomycin and lincomycin-B, which lack strong chromophores. However, a derivatization reaction may not go to completion, so the accuracy of these methods can be questioned. Furthermore, use of derivatization makes it impossible to accurately assess the purity of the drug because silylation is a prerequisite for detection by GC-FID, and not all impurities can be derivatized. The CFR and USP contain other examples of antibiotics with poor chromophoric properties. Consequently, it is desirable to have methods that use a simple, direct, and sensitive detection method.

Sulfur-containing antibiotics that do not contain fully oxidized sulfur can be detected electrochemically. The electrochemical detection process for sulfur compounds on noble metal electrode surfaces has been described by LaCourse[5] and Johnson[20]. During the initial detection step, sulfur compounds are preadsorbed to the oxide-free noble metal (gold) surface by a nonbonded electron pair from the sulfur group. The adsorbed sulfur moiety is then oxidized concurrently with the gold surface. A detector signal results from analyte oxidation and gold oxide formation. The IPAD waveform removes the contribution of surface oxide formation from the detector signal.

Electrochemical detectors have been successfully used on other sulfur-containing substances, for example, sulfur-containing peptides[4–7] such as glutathione. This detection has also been used for the determination of sulfur-containing amino acids (e.g., cysteine[6–8], cystine[8], methionine[6, 9], homocysteine[9–10]), and amino acid derivatives such as S,S ‘-sulfonyldiethylenedicysteine, and S,S'-thiodiethylenedicysteine[11]. Simple inorganic compounds have also been determined by this detection, such as sulfur dioxide[12], sulfite[13–14], sulfide[8, 14, 15], disulfides[8], acid-volatile sulfur[15], and thiosulfate[14]. A broad assortment of organosulfur compounds such as thiourea[6], coenzyme A derivatives[5], bis-(2 hydroxyethyl) sulfoxide, thiodiethanol, mercaptoacetic acid, dithiodiacetic acid, thioxane, bis-(2-chloroethyl) sulfoxide, dithia-6-oxaundecane-1,11-diol, and dithiane also have been analyzed by electrochemical detection[11]. Recently, this detection has successfully been used on sulfur-containing antibiotics coupled to HPLC[5,17–19].

In this Application Note we present the determination of sulfur-containing antibiotics separated by reversed-phase HPLC and detected by integrated pulsed amperometric detection (IPAD). The HPLC eluent conditions described by LaCourse and Dasenbrock[18–19] (optimized for electrochemical detection) were used in conjunction with a modified version of their waveform to separate and detect a set of sulfur-containing antibiotics representing different structural classes (Figure 1). Absorbance detection with the same eluent system was also used and the results were compared to the IPAD results. Linear range, estimated limits of detection, and precision were determined for seven antibiotics (each representing a different structural class), including one non-sulfur-containing antibiotic. The recoveries of two antibiotics from a commercial tablet formulation were determined. The feasibility of performing a dissolution study with IPAD is also described. Chemical stability studies were performed on two antibiotics, monitoring the antibiotics' peak areas and the formation of decomposition products.

EQUIPMENT

Dionex DX-500 BioLC® system consisting of:

GP50 Gradient Pump with degas option

ED40 Electrochemical Detector

AD20 Absorbance Detector

LC30 or LC25 Chromatography Oven

AS3500 Autosampler

PeakNet™ Chromatography Workstation

For this Application Note, the AD20 cell preceded the ED40 cell.

REAGENTS AND STANDARDS

Reagents

Acetic acid, HPLC grade (J.T. Baker)

Acetonitrile, HPLC grade (Burdick & Jackson)

Deionized water, 18 MΩ-cm resistance or higher

Methanol, HPLC grade (Fisher Scientific)

Sodium hydroxide, 50% (w/w; Fisher Scientific)

Standards

Amoxicillin (Sigma)

Ampicillin, sodium salt (Fluka BioChemika)

Cefadroxil (Sigma)

Cefazolin, sodium salt (Fluka BioChemika)

Cefotaxim (Fluka BioChemika)

Cephalexin, hydrate (Sigma)

Cephaloridine, hydrate (Aldrich)

Cephalothin, sodium salt (Sigma)

Cephapirin, sodium salt (Sigma)

Cephradine (Sigma)

Cloxacillin, sodium salt, monohydrate (Sigma)

Lincomycin, hydrochloride (Sigma)

Penicillin G, potassium salt (benzylpenicillin; Fluka BioChemika)

Penicillin V (Sigma)

Sulfanilamide (Aldrich)

Sulfamethoxazole (Sigma)

Trimethoprim (Fluka BioChemika)

Samples

Sulfamethoxazole and trimethoprim tablets, USP (800 mg/160 mg; Sidmak Laboratories, Inc., East Hanover, NJ 07936)

CONDITIONS

Columns:	Vydac C8 Reversed-Phase Analytical (P/N 208TP5415)
Flow Rates:	1.0 mL/min
Injection Vol:	10 µL
Temperature:	30 °C
Eluents:	A: Water B: 500 mM sodium acetate, pH 3.75 C: 90% acetonitrile D: Methanol
On-line Degas:	30 s every 2 min
Program:	See table on next page
Detection:	AD20: Absorbance (200, 215, 254, or 275 nm depending on the antibiotic) ED40: Integrated pulsed amperometry, gold electrode, Ag/AgCl reference electrode
Typical system operating backpressure:	8.1–10.3 MPa (1170–1500 psi)

Waveform for the ED40:

Time (seconds)	*Potential (volts)*	*Integration*
0.00	0.24	
0.05	0.24	Begin
0.09	1.34	
0.13	0.24	
0.17	1.34	
0.21	0.24	
0.25	1.34	
0.29	0.24	
0.33	1.34	
0.37	0.24	
0.41	1.34	
0.45	0.24	
0.49	1.34	
0.53	0.24	
0.57	1.34	
0.61	0.24	
0.65	1.34	
0.69	0.24	
0.73	1.34	
0.77	0.24	
0.81	1.34	
0.85	0.24	End
0.86	-1.50	
0.87	-1.50	
0.88	1.34	
0.89	-0.21	
1.00	-0.21	

PREPARATION OF SOLUTIONS AND REAGENTS

On-line degassing is necessary because the amperometric detector is sensitive to oxygen in the eluent. Set the pump to degas for 30 s every 2 min.

Eluents

500 mM sodium acetate, pH 3.75 (Eluent B)

Combine 57 mL of glacial acetic acid with 1.8 L water; add 50% sodium hydroxide (50% w/w) until pH is increased to 3.75 (approximately 6.0–6.8 mL). Add water until the total volume is 2.0 L. Keep the eluents blanketed under 28–69 kPa (4–10 psi) of helium at all times.

90% (v/v) acetonitrile (Eluent C)

Combine 900 mL acetonitrile with 100 mL water.

SAMPLE PREPARATION

Stock Standards

Solid antibiotic standards were dissolved in purified water to 10 g/L concentrations, correcting for the percent weight of salt and water content as specified on the label. Sulfamethoxazole and trimethoprim were not readily soluble in water and were dissolved in 70% (v/v) methanol (MeOH) to water. For determinations of linear range and lower detection limits, 10 g/L solutions of cephradine, cephapirin, sulfamethoxazole, trimethoprim, sulfanilamide, lincomycin, and ampicillin were diluted with their respective solvents to concentrations of 0.01, 0.025, 0.05, 0.075, 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 250, 500, 750, 1000, 2500, 5000, 7500, and 10000 mg/L. The solutions were frozen at –20 °C until needed.

Program							
Eluent	**Analyte**	**Program**				**Background (IPAD)**	**Noise (Peak-to-Peak)***
100 mM Sodium Acetate (pH 3.75) with:	**Antibiotic**	**%A**	**%B**	**%C**	**%D**	**nC (Range)**	**pC (Range)**
9% Acetonitrile and 10% Methanol	Sulfamethoxazole, Trimethoprim	60	20	10	10	430–450	190–1590
9% Acetonitrile and 0% Methanol	Lincomycin	70	20	10	0	450–490	180–1300
6% Acetonitrile and 0% Methanol	Ampicillin	73.3	20	6.7	0	440–490	150–1570
4% Acetonitrile and 0% Methanol	Cephapirin, Cephradine	75.6	20	4.4	0	450–480	140–550
0% Acetonitrile and 0% Methanol	Sulfanilamide	80	20	0	0	410–460	100–340

*Measured peak-to-peak noise (IPAD) for 1-min intervals.

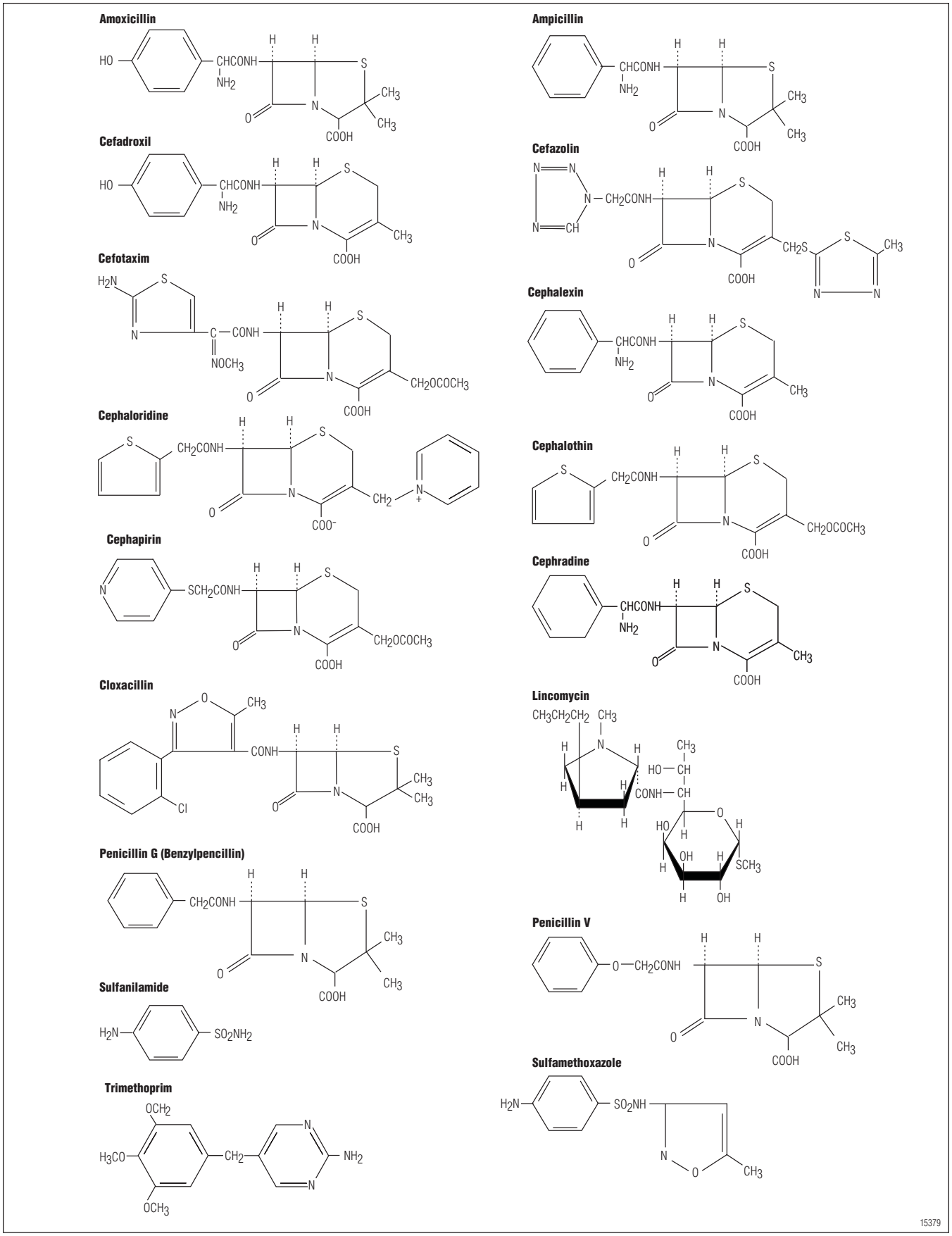

Figure 1. Chemical structures of antibiotics

Standard solutions of these antibiotics at concentrations ranging from 2.5 to 20000 times above the lower limit of detection and within the linear range were used to evaluate the precision of replicate injections.

Dissolution of Sulfamethoxazole and Trimethoprim Tablet in Water

One tablet containing 800 mg sulfamethoxazole and 160 mg trimethoprim (Sidmak Laboratories) was placed in a stainless steel mesh tea strainer and immersed in a clean, 1-L glass beaker containing 800 mL purified sterile water. The dissolution mixture was kept in constant motion using a magnetic stir bar with a rotation frequency of 70 rpm for 2 h, with 0.45 mL aliquots removed at frequent intervals between 0.5 and 10 min apart. Aliquots were diluted 3.3-fold with 1.05 mL MeOH, yielding an antibiotic sample in 70% MeOH. Insoluble particulates were removed by microcentrifugation (14000 x g, 10 min). Supernatants were directly analyzed (10-µl injection) by HPLC.

Complete Dissolution of Sulfamethoxazole and Trimethoprim Tablet in 70% Methanol

One tablet containing 800 mg sulfamethoxazole and 160 mg trimethoprim (Sidmak Laboratories) was placed in a 100-mL volumetric flask and brought to volume with 70% MeOH. The tablet in 70% MeOH was sonicated for 20 min. Some excipients listed on the product label (such as magnesium stearate, pregelatinized starch, and sodium starch glycolate) apparently did not dissolve under these conditions and were removed, with any insoluble drug, by centrifugation at 14,000 × g for 10 min. The supernatant was diluted 100-, 500-, and 1000-fold with 70% MeOH, and 10-µL aliquots were analyzed by HPLC.

RESULTS AND DISCUSSION

Selectivity

Sixteen sulfur-containing antibiotics and one non-sulfur-containing antibiotic were evaluated under different eluent conditions for their response, retention times, and detection of impurities. Appendix A shows the retention times of antibiotics with varying amounts of organic modifiers (acetonitrile and MeOH) in the mobile phase when using a Vydac C8 column flowing at 1 mL/min and a temperature of 30 °C. All sulfur-containing antibiotics tested in this Application Note are easily detected by IPAD after separation with this reversed-phase column. Sulfur-containing antibiotics that are poor chromophores (e.g., lincomycin and ampicillin) showed the most significant improvement in peak response by IPAD compared to absorbance detection in this eluent system. Figure 2A shows the chromatogram of a 1-µg injection of ampicillin (Peak 5) detected by absorbance at 215 nm. This peak is barely above the baseline noise. The ampicillin peak is large when it is detected by IPAD (Figure 2B). Furthermore, IPAD detects impurities in this antibiotic preparation that were not observed with absorbance detection (Peaks 2, 3, and 4). Similar results were obtained for lincomycin, another sulfur-containing antibiotic with poor chromophoric properties. Figure 3 shows a lincomycin chromatogram with (A) detection at 215 nm and (B) by IPAD. No peak

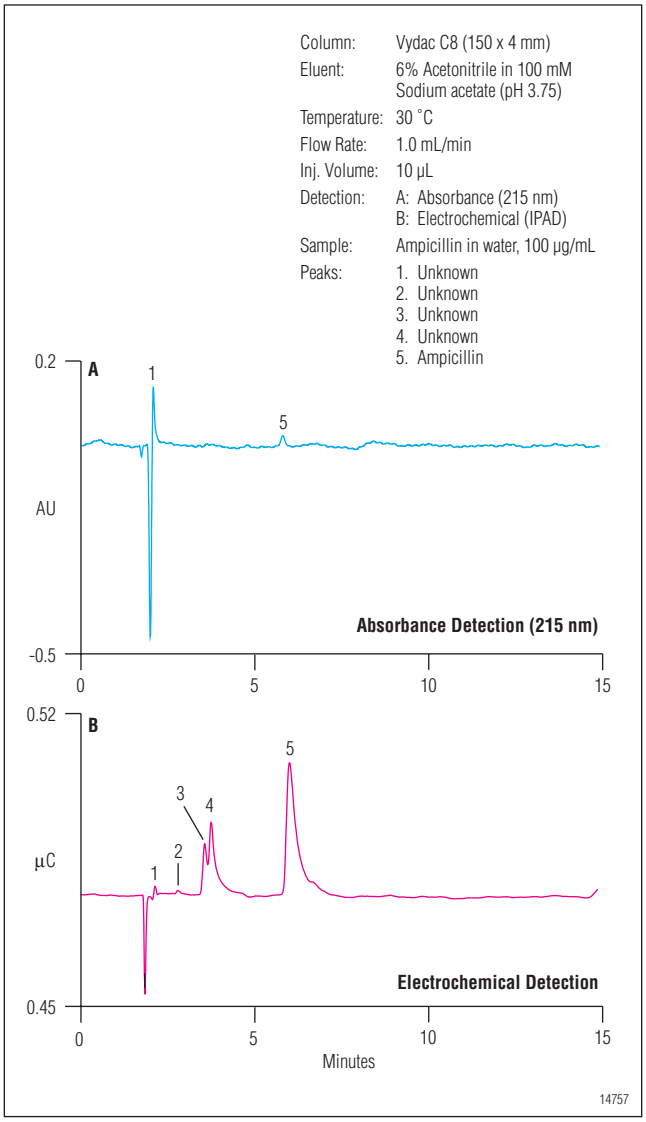

Figure 2. Ampicillin detected by (A) absorbance at 215 nm and (B) IPAD.

was observed in the absorbance trace, but a significant peak was observed in the IPAD trace. Similarly, impurities (Peaks 2, 3, and 5) were observed in the IPAD trace. These results show that a broad spectrum of sulfur-containing antibiotics can be detected using electrochemical detection. Furthermore, sulfur-containing impurities of antibiotics may respond poorly or be undetected by absorbance, but can be easily detected by electrochemical detection.

Stability of Detector Response

To test the long-term stability of the electrode response using the waveform described in this Application Note, 100-µg/mL solutions of cephradine and cephapirin were analyzed over 64 days. Analysis was performed using a Vydac C8 reversed-phase column with 100 mM sodium acetate and 4% acetonitrile as eluent at a flow rate of 1.0 mL/min. The average peak areas obtained for 10-µL injections of these antibiotic solutions were plotted over time; Figure 4 shows those results. Both antibiotics showed a stable response over at least two months.

When the organic solvent concentration of the eluent is lowered, retention time increases and IPAD peak area increases. Increased retention time has very little effect on absorbance detector response. We hypothesize that the lower organic solvent eluent content causes less suppression of the electrochemical response.

Linearity

Ampicillin, cephradine, cephapirin, lincomycin, sulfanilamide, sulfamethoxazole, and trimethoprim standards ranging from 0.01 to 10000 mg/L (0.10 to 100000 ng in 10 µL) were injected (two or three per concentration). The response factors (peak area per ng injected) for both detectors were tabulated for each concentration and the upper limits of linearity were calculated from the concentration points at which the response factors deviated more than 10% from the linear region. Table 1 shows that absorbance detection generally had a higher linear range than electrochemical detection. For example, lincomycin was linear by absorbance detection (215 nm) up to the highest concentration tested in the study (100-µg injection), but IPAD was linear to only 0.1–0.25-µg injection. The useful calculation range can be extended to higher concentrations by using nonlinear curve-fitting algorithms.

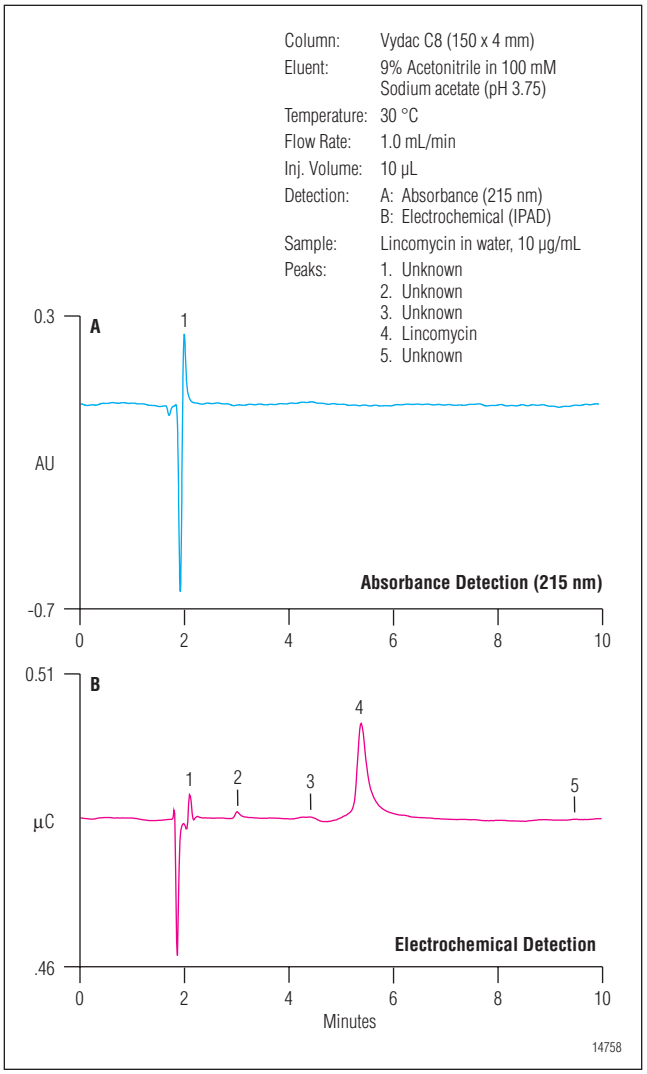

Figure 3. Lincomycin detected by (A) absorbance at 215 nm and (B) IPAD.

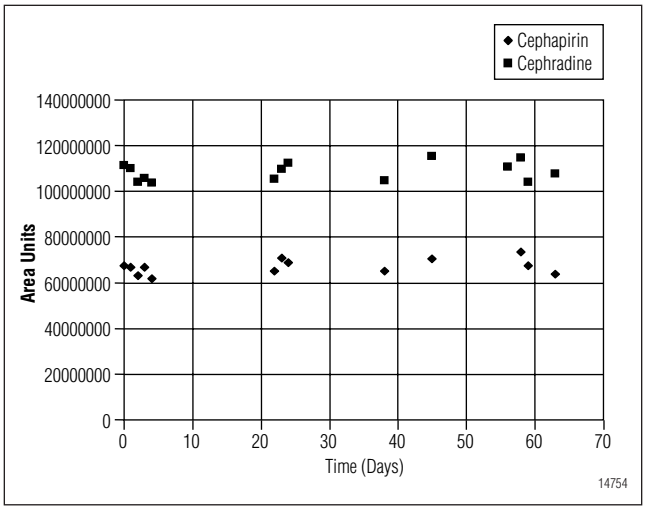

Figure 4. Stability of electrochemical response over 64 days.

Table 1 Upper Limit of Linearity

Antibiotic	Wavelength (nm)	UV Upper Limit*	IPAD Upper Limit*
Ampicillin	200	25–50 µg	1 µg
Ampicillin	254	>100 µg	1 µg
Cephapirin	254	5–7.5 µg	0.05–0.075 µg
Cephradine	254	>100 µg	0.1–0.25 µg
Lincomycin	215	>100 µg	0.1–0.25 µg
Sulfanilamide	254	1–2 µg	0.01–0.1 µg
Sulfamethoxazole	275	10–25 µg	0.025–0.05 µg
Trimethoprim	275	0.5 µg	0.05 µg

* Upper limit is defined here as the mass injected where response factor (area units/mass or slope) deviates from linearity by 10% or more.

Lower Limits of Detection

IPAD generally produced lower limits of detection (LODs) than absorbance detection in this eluent system. Estimated LOD values were calculated from the antibiotic concentrations yielding peak heights equivalent to 3 times the peak-to-peak noise. The noise was obtained from a 1-min interval of a solvent blank injection that included the retention time of the antibiotic peak. Table 2 presents the estimated LODs for detection of ampicillin, cephapirin, cephradine, lincomycin, sulfanilamide, sulfamethoxazole, and trimethoprim.

Table 2 Estimated Lower Limits of Detection

Antibiotic	Wavelength (nm)	Lower Limit of Detection*			
		UV** (µg/mL)	UV (ng Injected)	IPAD** (µg/mL)	IPAD (ng Injected)
Ampicillin	200	40	400	2	20
Ampicillin	254	10	100	2	20
Cephapirin	254	0.4	4	0.2	2
Cephradine	254	0.6	6	0.2	2
Lincomycin	215	520	5200	1	10
Sulfanilamide	254	0.04	0.4	0.01	0.1
Sulfamethoxazole	275	0.1	1	0.05	0.5
Trimethoprim	275	0.07	0.7	0.3	3

* Based on concentrations where peak heights are equal to 3 times the baseline noise.
** 10-µL injection

Nonchromophoric sulfur-containing antibiotics such as ampicillin and lincomycin showed the largest difference between the two detection methods. For example, lincomycin was detected by absorbance detection (215 nm) down to 5200 ng, and detected by IPAD down to 10 ng; hence IPAD was 520 times more sensitive under these conditions. Trimethoprim, a non-sulfur-containing antibiotic, was detected at greater sensitivity by absorbance than by IPAD. This is likely due to the absence of the sulfur atom and the presence of a chromophore. Ampicillin was evaluated at 200 and 254 nm. Although very little absorbance can be observed within the spectral region greater than about 220 nm, lower LODs were obtained at 254 nm than at 200 nm because the baseline (peak-to-peak) noise was significantly greater at lower wavelengths as a consequence of acetate absorbance at 200 nm. Neither wavelength yielded detection limits lower than those obtained by IPAD.

Because lower LODs depend on baseline noise levels and IPAD baseline noise levels in this method increase with the organic modifier content of the eluent, lower LODs are adversely affected by high levels of organic solvents in the mobile phase. To maximize detection limits, we recommend developing methods that minimize organic solvent in the eluent.

Peak Area Precision

The peak area RSDs were determined for replicate injections (n = 10) of ampicillin, cephapirin, cephradine, lincomycin, sulfanilamide, sulfamethoxazole, and trimethoprim. The results using both absorbance detection and IPAD are presented in Table 3. The precision was generally about the same for both methods. Except for ampicillin, the peak area RSD by absorbance detection ranged from 0.4 to 2%, and from 1 to 3% by IPAD. The peak area RSD for ampicillin by absorbance detection was 27% but only 3% by IPAD; this percentage is exceptionally high by absorbance detection because the concentration tested was only slightly greater than the lower limit of quantification. The results for ampicillin show the importance of high sensitivity to precision.

Table 3 Peak Area Precision (10 Injections)

Antibiotic	Conc. (µg/mL)	ng Injected*	Wavelength (nm)	% RSD UV	% RSD IPAD
Ampicillin	100	1000	200	27%	2.6%
Cephapirin	10	100	254	2.2%	2.4%
Cephradine	10	100	254	2.1%	1.5%
Lincomycin	100	1000	N/A	N/A	1.3%
Lincomycin	1000	10000	N/A	N/A	1.5%
Sulfanilamide	10	100	254	0.6%	2.2%
Sulfamethoxazole	10	100	275	1.7%	1.0%
Sulfamethoxazole	100	1000	275	0.6%	1.4%
Trimethoprim	10	100	275	2.2%	3.0%
Trimethoprim	100	1000	275	0.4%	2.6%

* 10-µL injection

Monitoring Antibiotic Stability

Some antibiotics maintained in aqueous conditions at ambient temperature (20–22 °C) chemically decompose over time. In this Application Note, the chemical stability of cephapirin and cephradine (10 µg/mL) were evaluated. Figure 5 presents cephradine peak area plotted against incubation time, detected by both absorbance detection and IPAD. Peak area loss was negligible over 69 h by both detectors. Figure 6 presents the same study conducted with cephapirin. This antibiotic showed a significant loss in peak area over time; the area units decreased at a rate of 10% per day for both detection methods. Chromatograms of fresh cephapirin (Figure 7) revealed a reasonably high level of purity based on the absence of spurious peaks. Some trace impurities were observed (Peaks 1 and 5) in both methods. After 69 h of incubation (Figure 8), two additional peaks were observed by absorbance detection (Peaks 2 and 3). Peak 4 was at or slightly above the baseline noise and could not be considered quantifiable. Four additional peaks were observed by IPAD (Peaks 2, 3, 4, and 6). Plotting the area of the extra peaks shows that both detectors can measure the same rate of change in peak areas of the new peaks, and that the higher sensitivity of IPAD for trace impurities can provide additional kinetic information not obtainable by absorbance detection (Figure 9).

Percent Recovery from Pharmaceutical Tablet Formulation

A tablet containing 800 mg sulfamethoxazole and 160 mg trimethoprim (according to the package's label) was dissolved in 100 mL of 70% MeOH:30% water. A slurry was produced that consisted of insoluble tablet excipients listed on the product label, such as magnesium stearate, pregelatinized starch, and sodium starch glycolate. Both sulfamethoxazole and trimethoprim were determined to be readily soluble in this solvent. The insoluble excipients were removed by centrifugation.

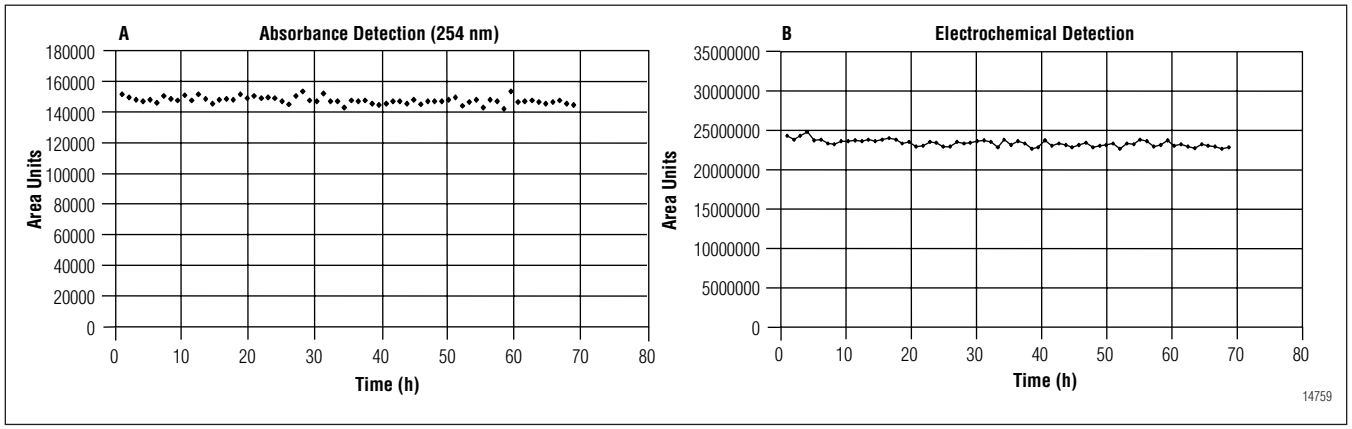

Figure 5. Monitoring cephradine (10 µg/mL) stability in water at ambient temperature by (A) absorbance detection and (B) IPAD.

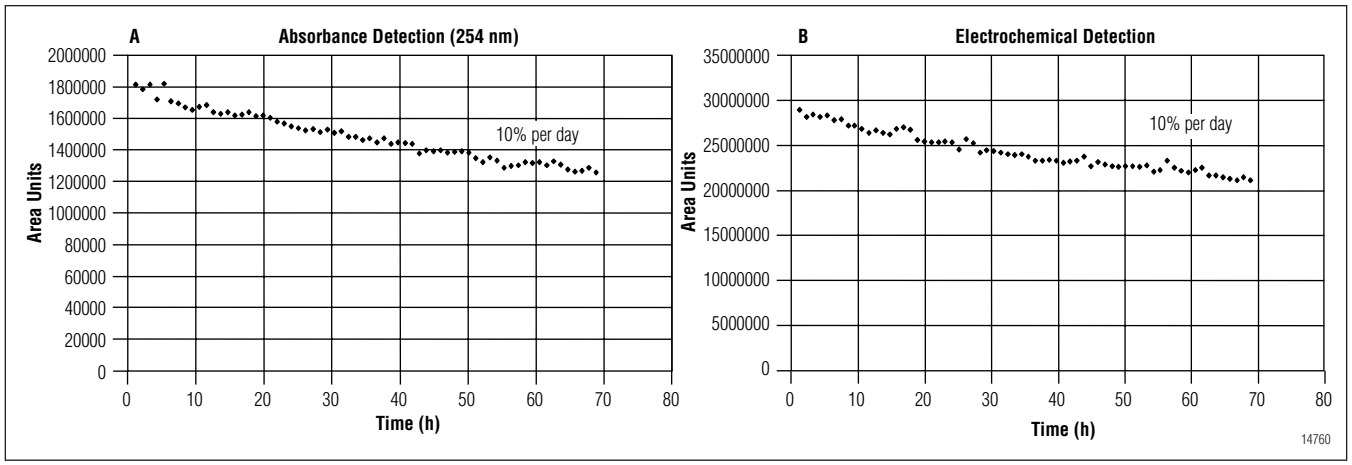

Figure 6. Monitoring cephapirin (10 µg/mL) stability in water at ambient temperature by (A) absorbance detection and (B) IPAD.

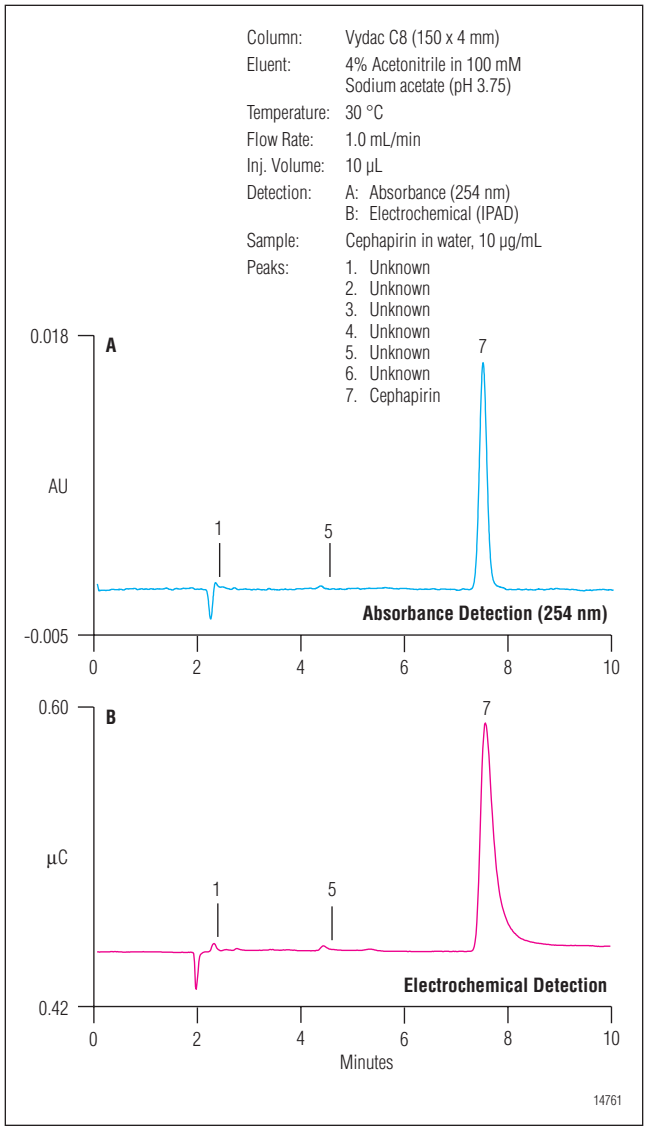

Figure 7. Chromatograms of cephapirin by (A) absorbance detection and (B) IPAD.

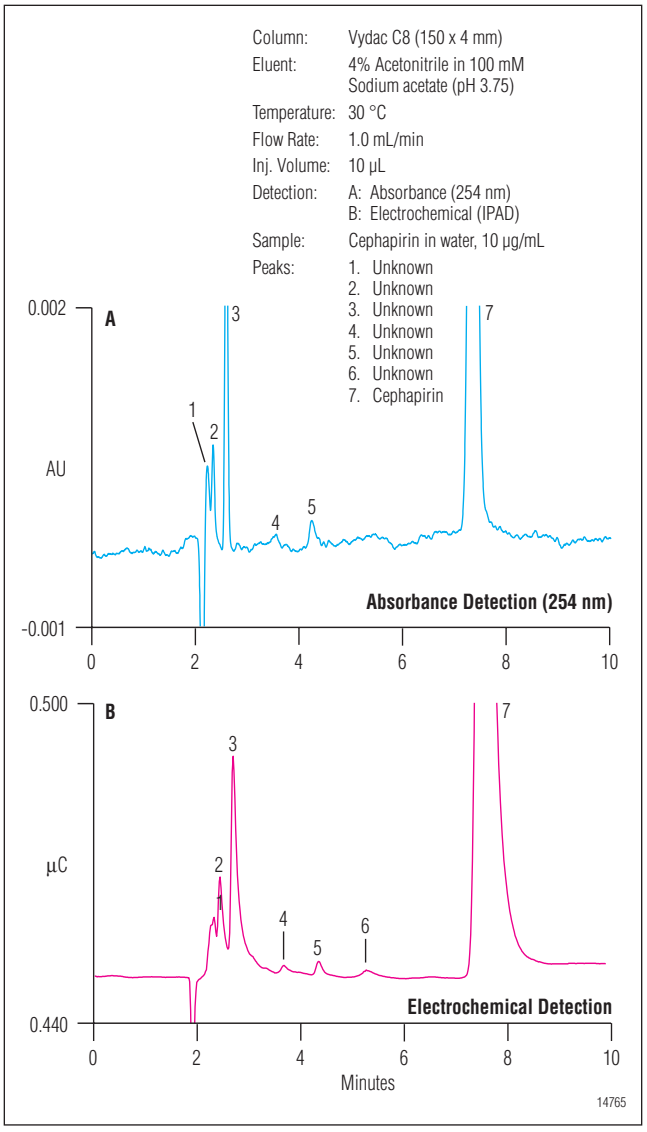

Figure 8. Chromatograms of cephapirin after 69-h incubation in water at ambient temperature by (A) absorbance detection and (B) IPAD.

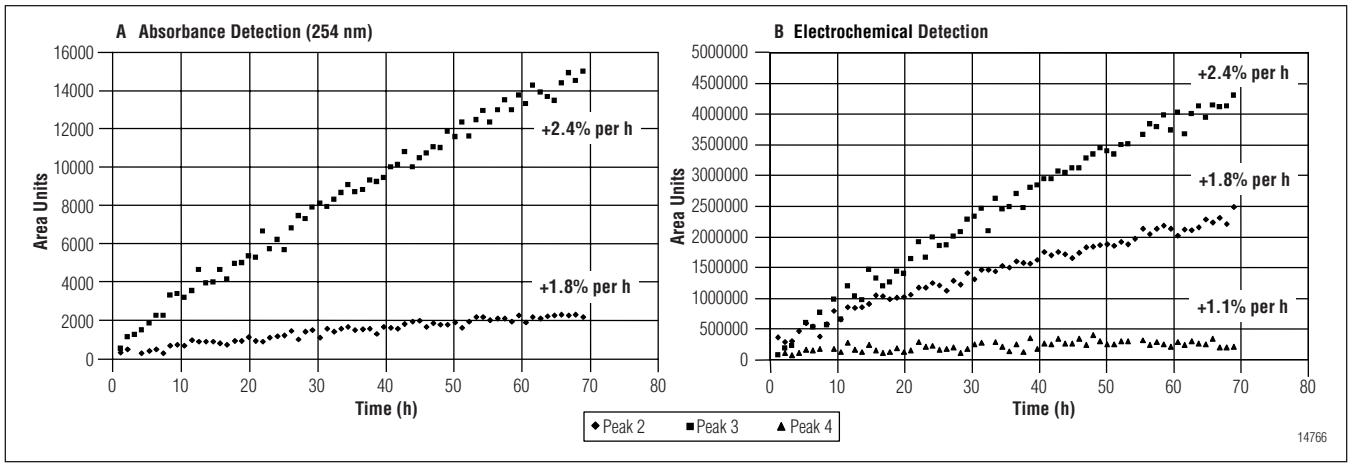

Figure 9. Monitoring cephapirin (10 µg/mL) decomposition products by (A) absorbance detection and (B) IPAD.

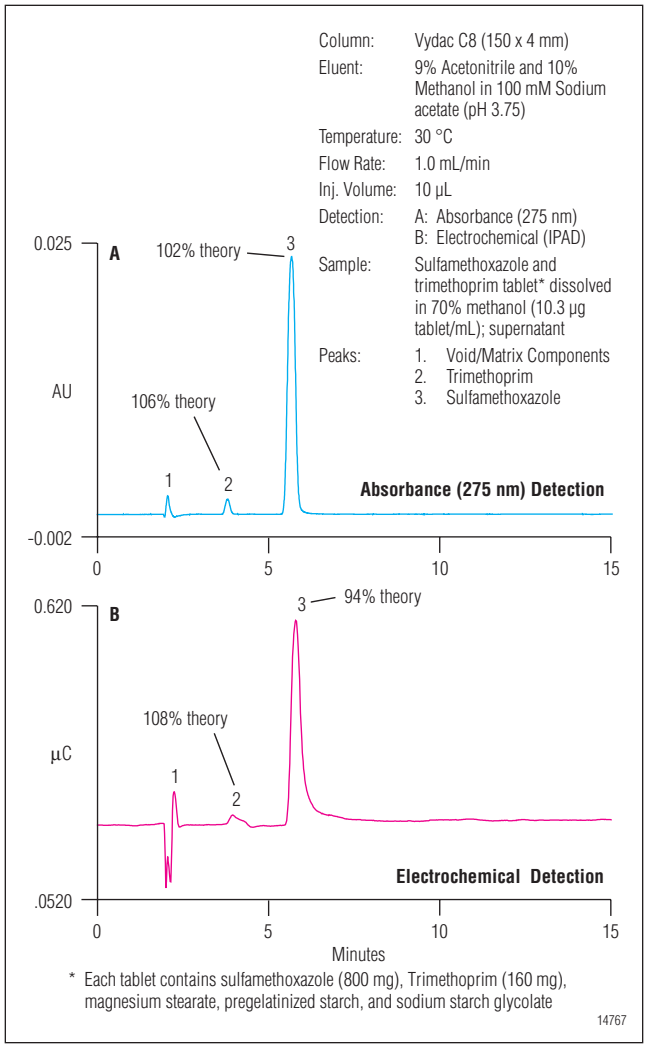

Figure 10. Recovery of sulfamethoxazole and trimethoprim from a tablet formulation by (A) absorbance detection and (B) IPAD.

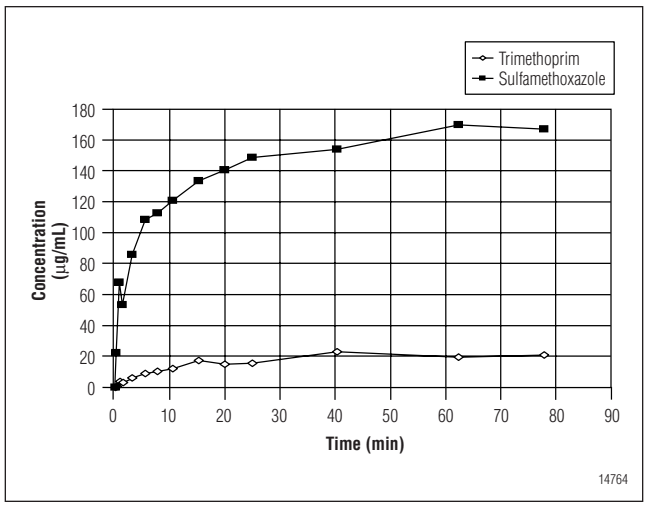

Figure 11. Determination of the dissolution of sulfamethoxazole and trimethoprim from a tablet formulation by IPAD.

The peak areas obtained for both antibiotics were related to standard calibration curves to determine their concentrations. Chromatograms produced by absorbance detection and IPAD are presented in Figure 10. The measured value of sulfamethoxazole recovered from the tablet by absorbance detection at 275 nm was 102% of the label value and 94% of the label value by IPAD. Trimethoprim measured by absorbance detection yielded 106% of the amount on the label, and 108% by IPAD. These recoveries demonstrate comparable accuracy for the two detection methods.

Dissolution of a Pharmaceutical Tablet Formulation in Water

We also investigated the feasibility of conducting a drug dissolution study using electrochemical detection. The sulfamethoxazole-trimethoprim tablet was used to study the kinetics of (1) dissolution in a nonoptimal solvent, (2) the drugs' release from insoluble excipients present in the tablet formulation, and (3) their release from the stainless steel wire mesh used to contain the tablet during dissolution. Neither sulfamethoxazole nor trimethoprim is readily soluble in water. Magnesium stearate, pregelatinized starch, and sodium starch glycolate are present in the tablet formulation as binders and, to some extent, facilitate the rate at which the drugs are released upon ingestion. In this study, a stainless steel mesh strainer was used to contain the tablet during dissolution. Solubility in water, release from an insoluble matrix, and release from the stainless steel container are all expected to participate in the measured release kinetics of the two drugs. To assure that all the released drugs were solubilized for analysis, aliquots of the suspension collected at designated time points were diluted in sufficient MeOH to produce a 70% MeOH solution and the insoluble particulates (excipient material or drugs) were then removed by centrifugation. Figure 11 presents the results of the dissolution study and shows that sulfamethoxazole reaches a steady state after about 50–60 min, and trimethoprim after 30–40 min. About 60% of the sulfamethoxazole was dissolved upon reaching its steady state, but only about 30% of the trimethoprim was dissolved after 30 min. These results were not collected by officially recognized dissolution procedures as described by the FDA or USP, and therefore should not be regarded as an accurate depiction of true kinetic behavior of this drug formulation. The purpose of the study was to show the feasibility of using IPAD for conducting drug

measurements in these types of studies. It is also expected that under circumstances where the excipients of a formulation are both chromophoric and soluble in the dissolution solvent, IPAD may be favorable in revealing the levels of either drug or drug-related impurities by reducing the level of interferences that are absorbing but electrochemically inactive.

CONCLUSION

IPAD is a good detection choice for nonchrom-ophoric sulfur-containing antibiotics. Some impurities resulting from the antibiotic manufacture or chemical decomposition may be detected better by IPAD than by absorbance. The specificity of IPAD for substances that can be oxidized using the electrode potentials selected for this study helps reduce interferences from chromophoric matrix ingredients or eluent components. IPAD may exhibit lower detection limits for sulfur-containing antibiotics and thus could be considered an alternative detection method for these compounds.

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LIST OF SUPPLIERS

Aldrich Chemical Company Inc., 1001 West St. Paul Avenue, P.O. Box 355, Milwaukee, Wisconsin 53233, U.S.A. Tel: 800-558-9160.

Burdick & Jackson, 1953 South Harvey Street, Muskegon, Michigan 49442, U.S.A. Tel: 800-368-0050.

Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219-4785, U.S.A. Tel: 800-766-7000.

Fluka BioChemika, Fluka Chemie AG, Industriestrasse 25, Buchs 9471, Switzerland. Tel: 081-755-25-11.

J.T. Baker Inc., 222 Red School Lane, Phillipsburg, New Jersey 08865, U.S.A. Tel: 800-582-2537.

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178, U.S.A. Tel: 800-325-3010.

100 mM Sodium acetate (pH 3.75) with:	Sulfanilamide	Amoxicillin	Cefadroxil	Cephapirin	Cephalexin	Ampicillin	Cephaloridine	Cefotaxim	Cefazolin	Cephradine	Lincomycin	Trimethoprim	Sulfamethoxazole	Cephalothin	Penicillin G (Benzylpenicillin)	Penicillin V	Cloxacillin
9% Acetonitrile and 40% MeOH	1.9			2.1		2.0	2.0	1.9	1.9	2.1	2.1	2.0	1.9	2.3	2.3	2.7	2.9
9% Acetonitrile and 30% MeOH	1.9			2.2		2.1	2.1		2.0	2.3	2.3	2.1	2.3	2.9	3.2	4.5	5.7
9% Acetonitrile and 20% MeOH	2.0	2.1		2.3	2.3	2.2	2.4	2.1	2.1	2.6	2.6	2.5	3.0	4.0	4.8	8.2	13.6
9% Acetonitrile and 10% MeOH	2.1	2.1		2.5	2.6	2.6	3.1	2.5	2.5	3.0	3.3	3.4	5.1	6.6	9.8	20.1	>30
9% Acetonitrile and 0% MeOH	2.3	2.2		3.3	3.6	3.5	5.2	4.0	4.2	4.7	5.3	6.6	11.5	18.3	25.1	>30	
6% Acetonitrile and 0% MeOH	2.4	2.4		5.1	6.0	6.2	8.3	7.5	8.4	8.5	9.5	12.3	18.4	>30	>60		
5% Acetonitrile and 0% MeOH	2.5	2.4	2.6	6.5	7.6	7.7	10.0	9.6	11.0	11.2	11.8	15.3	21.3				
4% Acetonitrile and 0% MeOH	2.5		2.9	7.5		10.5	12.9	13.9	16.2	14.6	16.0	21.3	26.2				
3% Acetonitrile and 0% MeOH	2.6	2.5	3.4	12.4	13.5	15.5	17.8	22.3	26.2	>30	22.6	31.9	33.6				
2% Acetonitrile and 0% MeOH	2.7	3.4	4.0	16.4	19.9	22.2	23.7	34.7	40.4		29.6	46.1	41.0				
1% Acetonitrile and 0% MeOH	2.9	3.5	5.3	33.6	35.6	39.1	38.5	>60	>60		48.5	>60	58.1				
0% Acetonitrile and 0% MeOH	3.0	4.0	6.3	>60	>60	54.1	52.0				>60		>60				

Identity based on major eluting peak

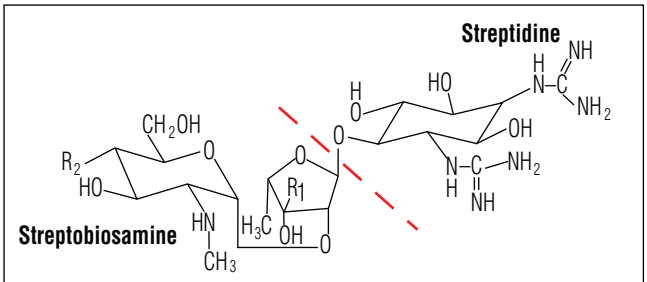
Appendix 1. Selectivity of sulfur-containing antibiotics on a Vydac C8 Column.

Determination of Streptomycin and Impurities Using HPAE-PAD

INTRODUCTION

Streptomycin is a water-soluble aminoglycoside antibiotic purified from the fermentation of the actinomycete *Streptomyces griseus* and used for intravenous administration[1] to treat infections. Streptomycin must be determined and all impurities must meet specified limits before a manufactured lot is used clinically. The current United States Pharmacopeia (USP 30, NF 25) compendial method for streptomycin sulfate measures streptomycin A as the primary antibiotic.[2,3] One of the thermal degradation products serves as a measure for system suitability. Streptomycin, also known as Streptomycin A, or O-2-Deoxy-2-(methylamino)-α-l-glucopyranosyl-(1→2)-O-5-deoxy-3-C-formyl-α-l-lyxofuranosyl-(1→2)-*N,N′*-bis(aminoiminomethyl)-d-streptamine, is the main antibiotic component of *S. griseus* fermentation broth, but also contains a less abundant form of streptomycin: mannosidostreptomycin, also known as streptomycin B. Unless otherwise noted, streptomycin in this note refers to streptomycin A. The precursors of streptomycin biosynthesis also occur during fermentation: streptidine and streptobiosamine (formed from streptose and *N*-methyl-l-glucosamine).[4–8] These and other compounds may result from chemical degradation during manufacture or storage.[9–12] Acid hydrolysis of streptomycin yields streptidine and streptobiosamine. Alkaline hydrolysis of streptomycin yields maltol. Thermal degradation of streptomycin, above 70 °C, produces streptidine and streptobiosamine, neither of which is commercially available. Figure 1 shows the chemical structure of streptomycin A and its major impurities. The system suitability peak used in the USP method is unidentified, but may be the less charged of the two thermal degradation products, streptobiosamine.

The aminoglycosides and their impurities, like most carbohydrates, lack a good chromophore and therefore require high concentrations to be detected by UV absorbance. Many ingredients of manufacturing process-intermediates and final pharmaceutical formulations are chromophoric and can interfere with the direct detection of streptomycin A and its impurities by absorbance. Refractive index detection has similar limitations. Carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds can be oxidized and therefore directly detected by amperometry. This detection method is specific for analytes that can be oxidized at a selected potential, leaving all other compounds undetected.



Streptomycin	R_1	R_2
A	–CHO	–OH
B	–CHO	–Mannose
Dihydro-	$–CH_2OH$	–OH

23932

Figure 1. Chemical structures of streptomycin A and some known impurities.

Pulsed amperometric detection (PAD), a powerful detection technique with a broad linear range and very low detection limits, is ideally suited for aminoglycoside antibiotics and their impurities.[13–17] High-performance anion-exchange chromatography (HPAE) is a technique capable of separating streptomycin A and its impurities.[13,16] The CarboPac® PA1 anion-exchange column retains streptomycin A and its impurities.

In this application note, we use an ICS-3000 system with PAD to run the USP Compendial Method for the assay of streptomycin sulfate. We show key performance parameters, including precision in determining streptomycin purity, limits of detection, linearity, and ruggedness, in a manner consistent with requirements of normal method validation.[18–24] We use disposable gold (Au) working electrodes to improve electrode-to-electrode (and system-to-system) reproducibility of streptomycin A electrochemical response. Disposable Au working electrodes are manufactured in a manner that improves electrode-to-electrode reproducibility.[17,25,26] We demonstrate HPAE-PAD technology for streptomycin A purity analysis and its feasibility for determinations in a fermentation broth. Finally, we evaluate streptomycin A purity per the requirements of the International Conference on Harmonization (for new drug substances).[27]

EQUIPMENT

Dionex ICS-3000 Ion Chromatography system with:

DP Dual Gradient or SP Gradient Pump, with vacuum degas option and GM-4 Gradient Mixer

DC Detector Chromatography Module equipped with dual temperature zones, 20-µL injection loop and an ECD Electrochemical Detector with Combination pH/Ag/AgCl Reference Electrode (P/N 061879)

Disposable Au Working Electrodes (P/N 060139, package of 6; 060216, package of 24)

AS Autosampler (with diverter valve for dual systems), and 2-mL vial tray

EO Eluent Organizer, including pressure regulator, and four 2-L plastic bottles for each system

Chromeleon® Chromatography Workstation

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent nylon filter)

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent; for degassing eluents)

Polypropylene Injection Vials (0.3 mL) with caps (Vial Kit, Dionex P/N 055428)

Microcentrifuge Tubes with detachable screw caps (polypropylene, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Standards

Streptomycin A (Streptomycin Sulfate; U.S. Pharmacopeia (USP) Reference Standard)

Samples

Streptomycin A (Streptomycin Sulfate; Sigma-Aldrich)

Bacto® YPD Broth (Pfizer Consumer Healthcare, BD Laboratories, Cat# 0428-17-5)

CONDITIONS

Method:

Columns:	CarboPac PA1 Analytical, 4 × 250 mm (P/N 035391)
	CarboPac PA1 Guard, 4 × 50 mm (P/N 043096)
Eluent Channel A:	Water
Eluent Channel B:	250 mM NaOH
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL (full loop)
Temperature:	30 °C column
	25 °C detector compartment
Detection:	Pulsed amperometry, carbohydrate certified disposable Au working electrodes (P/N 060139)

Isocratic Program:

Separating Eluent:	70 mM NaOH
Program:	72% A + 28% B
Run time:	35 min
Background:	7.9–32 nC
Typical System Operating Backpressure:	800–950 psi

Gradient Program:

Separating Eluent:	70 mM NaOH and 200 mM NaOH
Program:	72% A + 28% B for 22 min, then step to 20% A + 80% B for 18 min, then step to 60% A + 40% B for 20 min, for reequilibration to starting conditions
Run time:	60 min
Background:	7.9–36 nC
Typical System Operating Backpressure:	800–970 psi

Carbohydrate Waveform for the ED*

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	Off	Off
0.20	+0.1	On	On	On
0.40	+0.1	On	On	On
0.41	–2.0	Off	Off	Off
0.42	–2.0	Off	Off	Off
0.43	+0.6	Off	Off	Off
0.44	–0.1	Off	Off	Off
0.50	–0.1	Off	Off	Off

Reference electrode in Ag mode (Ag/AgCl reference).

*Settings required in the ICS-3000, but not used in older Dionex ECD systems.

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

Water (Eluent Channel A)

Use high-quality water of high resistivity (18 MΩ-cm) that contains minimal dissolved carbon dioxide and no biological contamination. Source water must be obtained using a water purification system consisting of filters manufactured without electrochemically active surfactants (e.g., glycerol). Filter through 0.2-µm porosity nylon under vacuum to remove particulates and reduce dissolved air. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of helium or nitrogen at all times to reduce contamination by carbon dioxide and microorganisms.

250 mM Sodium Hydroxide (Eluent Channel B)

Use high-quality water of high resistivity (18 MΩ-cm). Filter all water through a 0.2-µm nylon filter (Nalgene 90-mm Media-Plus, P/N 500-118; Nalge Nunc International) under vacuum to degas. Biological contamination should be absent. Minimize contamination by carbonate, a divalent anion at high pH that is a strong eluent and causes changes in carbohydrate retention times. Do not use commercially available NaOH pellets which are covered with a thin layer of sodium carbonate. Instead, use a 50% (w/w) NaOH solution that is much lower in carbonate (carbonate precipitates at this pH).

Dilute 26.2 mL of 50% (w/w) NaOH solution into 1974 g of thoroughly degassed water to yield 250 mM NaOH. Immediately blanket the NaOH eluents under 4–5 psi helium or nitrogen to reduce carbonate contamination. For more information on eluent preparation, please see Dionex Technical Note 71.

Stock Standards

Place solid streptomycin sulfate and dihydrostreptomycin sulfate in plastic microcentrifuge vials with screw caps (Sarstedt) and weigh them. The label for the USP material indicates that the material should be dried prior to use, using vacuum pressure not exceeding 5 mm (5 Torr) of mercury at 60 ºC for 3 h. Simultaneously centrifuge, heat, and dry the preweighed solid samples using a SpeedVac Evaporator at 0.35–0.60 Torr of vacuum for 20–24 h, set to 50 ºC. Within 1 min of completion of the drying, tightly seal and reweigh the vials to calculate the percent moisture content of the solid material. Dissolve the anhydrous solid in a weighed amount of deionized water (~1.0 mL) to obtain an accurate concentration of 100 mg dried solid/mL (assume density of H_2O = 1.000 g/mL). Adjust the 100 mg/mL streptomycin sulfate concentration to the streptomycin A base concentration using the reported percent sulfate stated on the manufacturer's Certificate of Analysis. Calculate the molar concentration using the molecular weight for the streptomycin base. An example of these calculations follows:

Certificate of Analysis information for streptomycin sulfate:

Sulfate:	18.7%
Potency:	758 IU/mg dried solid (based on the Third International Standard)
Loss On Drying:	2.9%
Molecular Weight (streptomycin sulfate):	728.69
Molecular Formula:	$C_{21}H_{39}N_7O_{12} - 1.5H_2SO_4$
Wet weight of solid (weight before SpeedVac drying):	115.34 mg
Dry weight of solid (weight after SpeedVac drying):	100.92 mg

(1) Calculation of percent moisture content:

$$\frac{(115.34 \text{ mg} - 100.92 \text{ mg})}{100.92 \text{ mg}} \times 100 = 14.29\%$$

(2) Calculation of exact concentration of dried streptomycin sulfate dissolved in 1.0023 g water:

$$\frac{100.92 \text{ mg dried streptomycin } SO_4}{1.0023 \text{ g } H_2O} \times 1.000 \text{ g/mL} = 100.69 \text{ mg/mL}$$

(3) Calculation of exact concentration of dried streptomycin base:

Bioassays base potency on relative biological response to an international standard reference material, and their units of measure are international units (IU). The Third International Standard used for determination of this material contains 785 units of streptomycin base per 1 mg dried streptomycin sulfate. For a 100% pure and active material, the theoretical mass of streptomycin base in anhydrous streptomycin sulfate is 798 µg. Unfortunately, the Third International Standard is neither pure nor completely anhydrous, and therefore it is necessary to assume that the standard is pure and anhydrous to convert from IU/mg units to µg/mg units of potency.[28,29] Another assumption is that the theoretical mass of streptomycin base is 798 µg, which relies on the calculation that 1.5 moles of sulfate exists as counter-ions per mole of streptomycin base, with sulfate equivalent to 20.19% by weight. Other counter-ions may exist in the true formula following manufacturing, which includes protonated forms, and therefore the exact distribution of sulfate ions may not be precisely 1.5:1. In spite of our significant assumptions, we use 785 IU = 798 µg to convert bioactivity units (IU) to mass units (µg). Fortunately, some manufacturers or distributors of streptomycin sulfate define potency in µg streptomycin base per mg anhydrous solid, in which case the conversion described above is not needed.

a) If needed, conversion of bioassay potency (IU/mg) to mass-based potency present in 1 mg (mg streptomycin base per mg dried solid):

$$758 \text{ IU} \times \frac{798 \text{ µg}}{785 \text{ IU}} = 770 \text{ µg streptomycin base in 1 mg}$$

b) Calculation of mg/mL streptomycin base concentration:

$$100.69 \text{ mg/mL} \times 0.770 \text{ mg/mg} = 77.5 \text{ mg/mL dried streptomycin base}$$

(4) Calculation of molar concentration of dried streptomycin base (optional):

Most formula weights reported by commercial vendors of streptomycin sulfate are erroneously referred to as molecular weights. Streptomycin A is ionic and may contain a counter-ion, typically sulfate, that varies depending on manufacturing. Therefore, it is important to differentiate between mass concentra-

tions (e.g., µg/mL) that include the anion and those that do not. In the example here, 100 mg/mL of streptomycin sulfate is equal to 77 mg/mL streptomycin base.

When calculating molar concentrations, it is essential to use the correct formula weight. In the example provided here, the formula weight provided by the vendor is for streptomycin sulfate, and the vendor defines the formula to contain 1.5 moles sulfate to 1 mole streptomycin base. Because 1.5 moles sulfate has a formula weight of 147.11, subtracting this from the molecular weight of the streptomycin base (728.6) equals 581.58. The 77.5 mg/mL mass concentration was calculated for the streptomycin base, and therefore the molecular weight of the streptomycin base must be used:

$$\frac{77.5\ \text{mg/mL}}{581.58} \times 1\ \text{M} = 0.133\ \text{M} = 133\text{-mM}_{\text{base}}^{\text{streptomycin}}$$

Also, the theoretical concentration of sulfate is then 1.5 × 133 mM = 200 mM sulfate. Another point of occasional confusion is the formula for streptomycin sesquisulfate, which actually is the same as streptomycin sulfate. This formula is occasionally expressed as $(C_{21}H_{39}N_7O_{12})\ 2 \bullet 3H_2SO_4$ with a formula weight of 1457.4, but the presence of two moles of streptomycin base may be overlooked, resulting in incorrect molarity calculations.[30]

Further dilute these solutions with water to yield the desired stock mixture concentrations. To ensure optimal accuracy, make all dilutions gravimetrically. Maintain solutions frozen at -40 °C until needed. For linearity studies, inject streptomycin masses of 0, 0.38, 0.78, 1.6, 2.4, 3.2, 3.6, 7.6, 15.5, 23.3, 31.1, 38.7, 80.2, 120, 160, 200, 240, 278, 319, 359, 400, 790, 1610, 2360, 3150, 3980, and 40,000 pmol. The USP compendial method uses a target concentration of 30 µg/mL (41 µM) for analysis. In this study, a 25% target level (10 µM) was also investigated.

System Suitability Sample Preparation

The thermal degradation of streptomycin A produces a number of products, but a single major product is used as part of a system suitability test to confirm satisfactory resolution of the chromatography system. The resolution of the major degradation peak and streptomycin A peak is required to be greater than three. To prepare this system suitability sample, place a 1-mL aliquot of the 30 µg/mL (41 µM) streptomycin B standard in sealed glass vials and heat at 75 ºC for 1 h. Do not use plastic vials.

Streptomycin and Dihydrostreptomycin Degradation Study

Evaluate streptomycin and dihydrostreptomycin for time-dependent changes in impurity content by exposure to elevated temperature. Incubate aliquots of 41 µM streptomycin and dihydrostreptomycin in water at 75 ºC for 0, 60 min, and 24 h. Evaluate the treated samples for changes in purity.

YPD Broth Media

Dissolve 1.0 g Bacto Yeast Extract-Peptone-Dextrose (YPD) Broth in 20.0 mL asceptically filtered (0.2 µm, nylon) water. Centrifuge an aliquot at 16,000 × g for 10 min and dilute 1000-fold in purified water. For spike recovery determinations, add concentrated streptomycin to the supernatant during dilution to final concentrations of 10 and 41 µM. Directly analyze the diluted supernatant.

INSTRUMENT OPERATION

Wash columns with 200–250 mM NaOH for 1 h to restore streptomycin A retention time after installing a column and for weekly column maintenance when analyses are made without column regeneration after each injection. The application of 200 mM NaOH changes system equilibrium, and reequilibration at 70 mM NaOH for >2 h is recommended to achieve high precision. For most work, however, commence injections after15 min. Retention time stability is observed 3 h from the start of column reequilibration, at which time retention is increased by 2.4%. Complete stability of retention time is observed after 10 h, at which time retention is increased by 3.0% from start of equilibration. When the pump has been turned off for longer than 1 day, regenerate the column with 200–250 mM NaOH for 1–2 h, and reequilibrate with 70 mM NaOH for 2 h before analyzing samples.

Peak area stability is observed 1 h after installation of a new disposable working electrode. Typically, at that time, no upward or downward trend is observed. Baseline noise stabilizes at low values after 1–2 h following installation of a new electrode. After this initial break-in, the electrode performs optimally within about 10 min of the cell being turned on.

When the system is idle for 1–2 week periods, we recommend that the pump be left on at a reduced flow rate of 0.05–0.10 mL/min to achieve rapid start-up, and the cell be turned off to extend disposable electrode life. When the system is shut down for up to several weeks, turn off the pump and electrochemical cell. For shutdown periods

exceeding several weeks, plug all plumbing lines leading to and from the cell, remove the reference electrode from the electrochemical cell, and store it in 3.5-M KCl solution.

RESULTS AND DISCUSSION

Separation

Figure 2 shows the separation of 10 µM USP grade streptomycin A (peak 8) from the column void (peak 1) and oxygen dip (peak 11) using a CarboPac PA1 column (70 mM NaOH eluent). The oxygen dip (~31–33-min retention time) is due to oxygen present in the samples and appears as a function of the gas permeation volume of the column. Like some organic impurities, eluting oxygen produces less background than the eluent, and therefore a dip in the baseline. The elution time of the "oxygen dip" varies slightly from column to column, depending on the flow rate, not the eluent strength. Eluting the oxygen dip just prior to the end of run, or timing its elution to occur at the end of the following injection, prevents the baseline dip from interfering with the peaks of interest.

Separation of streptomycin A and its impurities is highly dependent on eluent concentration. Table 1 shows the effect of NaOH eluent concentration on the retention time of streptomycin A. The greatest effect on retention was observed between 50 and 77 mM, where very minor changes in hydroxide concentration produced large changes in retention times. Figure 3 compares the resolution of impurity peaks for injections of 10 µM USP grade streptomycin A using 63 mM (chromatogram A) with 70 mM NaOH (chromatogram B). The 10% reduction in eluent concentration from the USP Monograph Method increases the retention time of streptomycin A, reducing throughput and increasing peak tailing; however, the separation of impurities is improved.

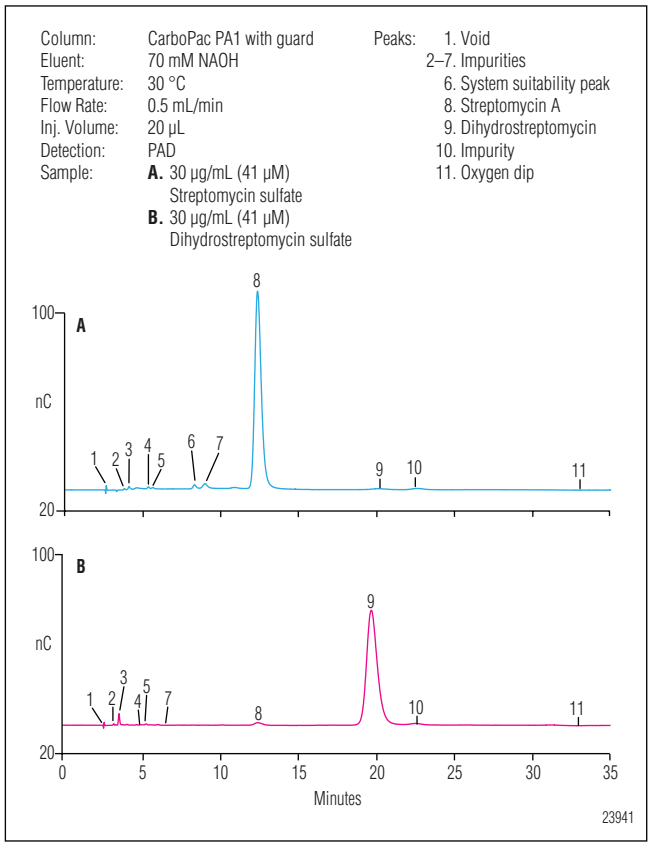


Figure 2. Determination of streptomycin A and dihydrostreptomycin.

Table 1. Effect of Eluent Concentration on Retention Time

	NaOH Eluent Concentration (mM)									
	100	**77**	**70**	**63**	**50**	**25**	**10**	**5**	**2**	**1**
	Retention Time (min)									
Column Void	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.8	2.9
Streptomycin A	4.9	9.3	12.0	15.8	21.3	>60	>60	>60	>60	>60

Although decreasing the eluent strength to 63 mM NaOH enables greater resolution of impurity peaks, the 70 mM NaOH concentration described in the compendial method appeared optimized for throughput, for resolution of streptomycin A from impurities and the column void, and for noninterfering location of the oxygen dip. For these reasons, the method evaluated in this note followed the USP method using the 70 mM NaOH condition, unless otherwise specified.

The impurity peak at 8 min (Figure 3, chromatogram A, peak 12) was identified as the USP system suitability peak based on the retention time of the major degradation peak produced using the heat-treatment procedure described in the USP method. This major impurity peak is presumed to be streptobiosamine because it has been described as the most abundant product of thermal degradation at neutral pH (in water).[7–10] Impurity peak 2 closely elutes with the column void and is probably a mixture of coeluting compounds. This peak increases in the water blank injections when injection vials were not prerinsed three times with water. Figure 4 compares the separation of impurities in 10 µM USP grade streptomycin sulfate (chromatogram A) with impurities in another commercial source (chromatogram B) using 70 mM NaOH. Chromatogram A shows a significantly different profile for the level of impurities than chromatogram B.

The resolution (USP definition) between streptomycin A and the system suitability peak (peak 10, Figure 4) ranged from 5.46 to 6.14 (mean ± SD; 5.83 ± 0.19, n = 23 injections, 3.3% RSD) over 1 day of consecutive injections. The mean resolution over four different days (interday) ranged from 4.08 to 5.76 (5.31± 0.83). The USP method for streptomycin specifies this resolution to be ≥3.0 for system suitability.[12] That method also allows adjustment of the mobile phase concentration to achieve this minimum resolution, but during this study no adjustment was required.

The production of the system suitability peak through thermal degradation of streptomycin A also produces other decomposition products. Most of these products elute near streptomycin A. One thermal decomposition product elutes at 160 min using 70 mM NaOH, and is shown in Figure 5. The identity of this late-eluting impurity peak is unknown, but its long retention time is of primary concern for this method because it will, if present, elute during subsequent injections and can cause either an extra unexpected peak or baseline disturbance leading to imperfect peak integrations.

If the peak is present, it will first elute during the tenth injection when the programmed run time is 15 min, with 2 min sample loading by the autosampler (with the Sample Overlap feature of Chromeleon disabled). It may also elute during the ninth injection when the Sample Overlap feature of Chromeleon is enabled, or during the fifth injection when run times are set to 35 min (Overlap disabled).

Unwanted elution can be avoided by using an eluent step change, where the streptomycin A and most impurity peaks are first allowed to elute at 70 mM NaOH, followed by a short elution of the late-eluting peak with 200 mM NaOH and reequilibration to 70 mM eluent concentration. This provides a method to rapidly determine all peaks, including the later impurity peak (Figure 6). The data presented in this note use the isocratic program.

The USP also specifies a tailing factor (asymmetry) value for the streptomycin A peak to be < 2, and peak efficiency to be >1000, to meet system suitability. We found peak asymmetry to range from 1.20 to 1.36 (1.25 ± 0.04) over one day of consecutive injections (intraday), and the mean asymmetry over four different days (interday) ranged from 1.23 to 1.25 (1.238 ± 0.006). The mean peak efficiencies ranged from 2209 to 2227 (2216 ± 8) for four separate days (interday).

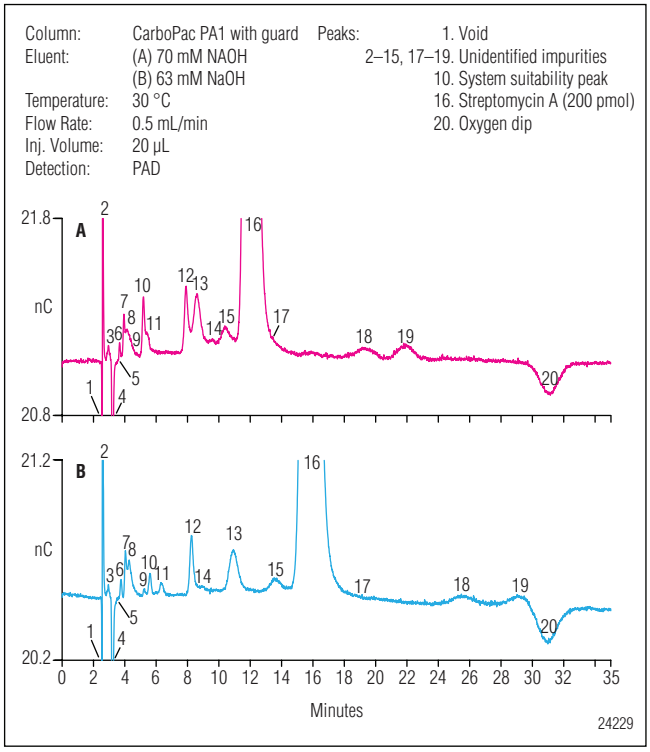


Figure 3. Comparison of USP streptomycin at 70- and 63-mM NaOH eluent concentrations.

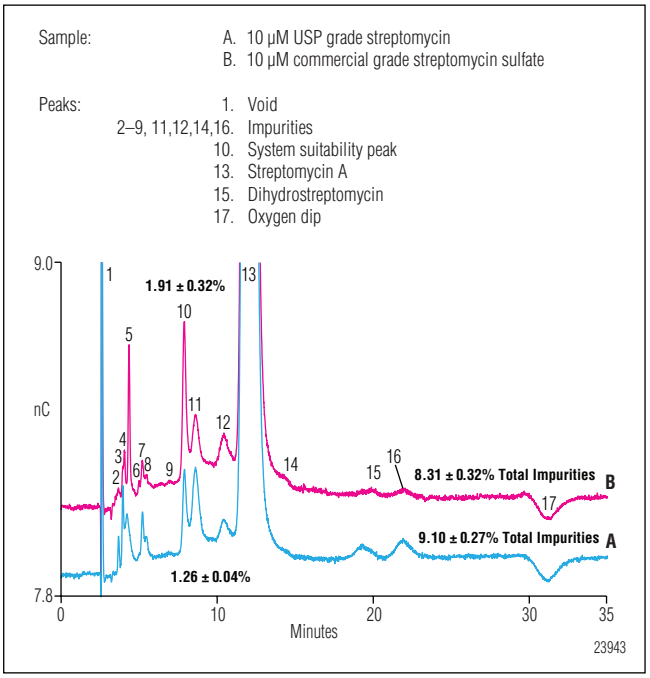


Figure 4. Comparison of USP and a second commercial source of streptomycin sulfate at a 70-mM NaOH eluent concentration.

Detection

Linear Range

The linear concentration range is characterized by the response factor (ratio of peak area/mass injected) remaining within 20% of the mean optimal level. In an evaluation between 0.4 pmol and 40 nmol injected, we found the optimal response between 120 and 400 pmol. The corresponding mean streptomycin A area response factor was 0.0447 ± 0.0006 nC • min/pmol (n = 34 injections, concentrations between 6–20 µM). We considered streptomycin A injections having response factors below 0.03576 nC • min/pmol outside the linear range (2.9–211 µM), which we calculated to be below 58 pmol and above 4.2 nmol. This range extended over nearly two orders of magnitude. We arbitrarily choose a 20% threshold to define the upper and lower limit of linearity. At this upper or lower concentration, the error in the calibration curve for accurately calculating concentration is approximately 20%, using the slope and y-intercept calculated by first order linear regression. For the concentration range of 4–200 µM (80–4000 pmol per 20 µL injection), we obtained an r^2 value of 0.9976 (see Table 2). Streptomycin A peak height linearity extends to only 2990 pmol (150 µM for 20-µL injection). We therefore recommend peak area for quantification of streptomycin A.

Linearity

Figure 7 shows a narrower concentration range of 4–80 µM (80 to 1600 pmol, 20-µL), where the linear relationship of response to mass is improved (r^2 = 0.9990). The narrower range produces a slope (0.0407) closer to the mean optimal response factor of 0.0447 nC • min/pmol. Generally, the narrower the range centered around 260 pmol (13 µM), the higher the linearity and the lower the possible error in calibration. Although the target concentration specified in the USP compendial method, 41 µM (30 µg/mL), is near the upper end of the linear range, it is at an appropriate concentration for this method to accommodate the typical 90–130% target concentrations described for most aminoglycoside antibiotic drug products defined by USP and EP Formulatory Monographs.

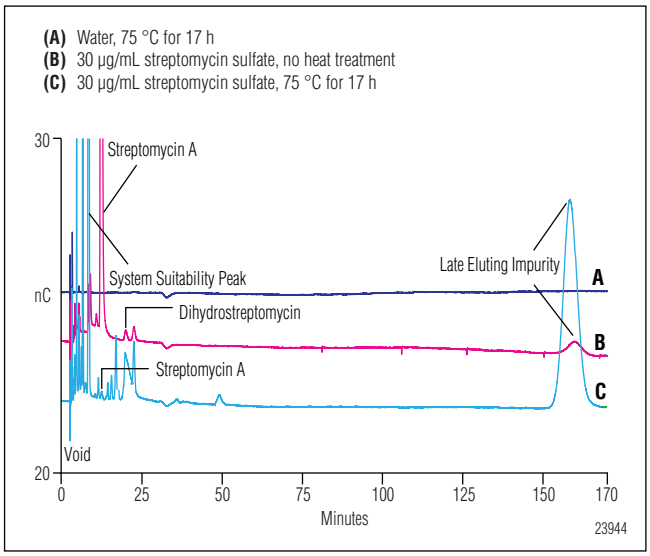


Figure 5. Late eluting thermal degradation peak.

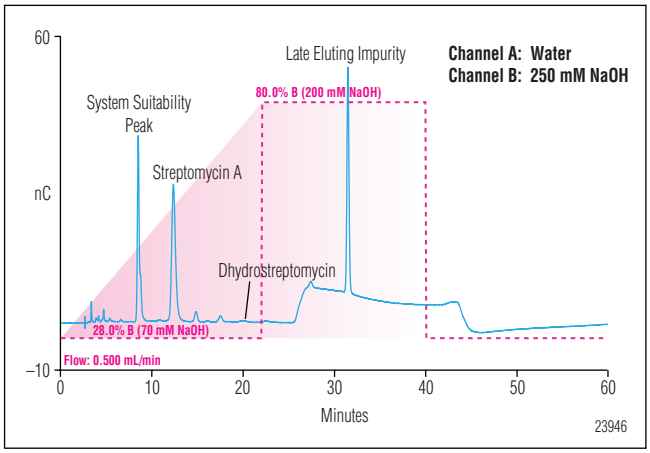


Figure 6. Use of a different elution program to more quickly elute the thermal degradation peak.

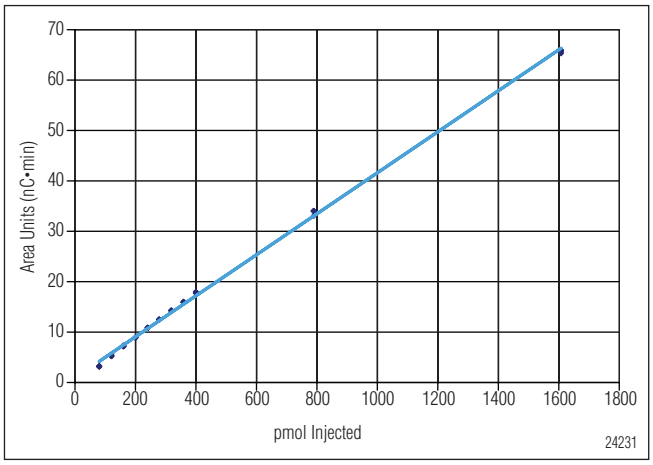


Figure 7. Linearity of streptomycin within the range of 80–1600 pmol (4–80 µM, 20 µL injection).

Lower Limits of Detection and Quantification

Baseline, peak-to-peak noise was determined from noise measured in 1-min intervals during blank runs. Baseline noise ranged from 9.8 to 194 pC (mean ± SD; 31.4 ± 25.3, n = 510 1-min intervals) measured over a 73-day period. After installing new disposable electrodes, baseline noise tended to decrease over the first two hours. After two hours, the concentration (or mass injected) of streptomycin A at the lower limit of detection (LOD) was calculated from three times the average peak-to-peak noise (a height value), divided by the average peak height response factor for the antibiotic within its linear region. The lower limit of quantification (LOQ) is the concentration (or mass injected) calculated from 10 times the average peak-to-peak noise. The estimated LOD for streptomycin A was 1.7 ± 1.4 pmol; and the LOQ was 5.6 ± 4.5 pmol. Table 2 summarizes these results.

Precision

The retention time and peak area RSDs were determined for replicate injections of a streptomycin A standard (10 µM for 20 µL injection) over one day (intraday, n = 34 injections). Precision was also determined on two separate additional days (interday variance). Table 3 shows these results.

Table 2. Estimated Limits of Detection, Quantification, and Linearity for Streptomycin A

Noise (pC)	Mean ± SD	31.4 ± 25.3 n = 510†
	range	98.4 – 194
Lower Limit Detection	pmol	1.2
	µM*	0.06
	nanogram	0.70
	µg/mL*	0.035
Lower Limit Quantification	pmol	4.0
	µM*	0.20
	nanogram	2.3
	µg/mL*	0.12
Upper Limit of Linearity**	pmol	4200
	µM*	211
	nanogram	2500
	µg/mL*	120
Linearity Over Linear Range	r^2	0.9976
	Y-intercept (nC•min)	2.54
	slope (nC•min/pmol)	0.03674

* 20-µL injections

** Linear range is defined as the corresponding concentrations having 20% deviation from mean optimal peak area.

† Number of 1-min peak-to-peak reading over 73 days

Table 3. Precision of Steptomycin A Retention Time and Peak Area

	Retention time				Peak area (nC•min)*			
INTRA-DAY	**MEAN**	**SD**	**N**	**RSD**	**MEAN**	**SD**	**N**	**RSD**
Chemist 1	11.99	0.01	5	0.08%	7.498	0.097	5	1.30%
Chemist 2	11.45	0.04	8	0.37%	7.816	0.144	8	1.84%
Chemist 3	11.92	0.11	8	0.91%	8.745	0.168	8	1.92%
Chemist 4	12.01	0.07	34	0.60%	8.895	0.152	34	1.71%
Intraday	**11.84**	**0.26**	**4**	**2.24%**	**8.24**	**0.69**	**4**	**8.34%**

* 20-µL injections of 10 µM streptomycin A

Intraday results for eluent prepared by separate chemists on separate days

ACCURACY

We evaluate three different sources of error in this method: sample preparation, calibration, and spike recovery.

Sample Preparation Error

The preparation of standards and samples normally involves weighing a solid streptomycin sulfate material, followed by dissolving in water, and then calculating the resulting concentration. These steps are subject to error from pipetting, moisture content of the material, and salt content. Pipetting errors were eliminated using gravimetric techniques for standard and sample preparation. Recording the weights of the liquids transferred using the pipettors enables review of actual volumes used in calculations.

Table 4. Effect of Moisture Content on Accuracy

Sample Preparation	Replicate Injection Number	% Moisture Content Reported in the C of A	Measured Moisture Content After Speed Vac Drying	Percent Error of Measured Drug Substance (Commercial Grade Material) from Expected Concentrated	
				With Pre-Drying (using SpeedVac)	Without Pre-Drying
Chemist 1	1 2	2.9%	10.49%	0.39% –0.32%	9.56% 8.79%
Chemist 2	1 2	2.9%	10.29%	0.66% 0.34%	9.86% 9.50%
Chemist 3	1 2	2.9%	10.36%	0.79% –0.95%	10.00% 8.10%
Chemist 4	1 2	2.9%	10.36%	–1.27% 0.41%	7.75% 9.58%
Mean				**0.01%**	**9.14%**
SD				**0.77%**	**0.84%**

The second source of error is the moisture content. The manufacturers and distributors of streptomycin provide data for the percent moisture content of each lot. Depending on the storage container, age, humidity of the different storage locations, and the initial drying method used by the manufacturer, we find moisture content changes from the time it was first assayed. This change is of particular concern for streptomycin sulfate and other aminoglycoside antibiotics because they are hygroscopic. Any increase in moisture content of the solid streptomycin sulfate from the amount stated for the material in its Certificate of Analysis reduces the accuracy of the concentration by that same percentage. Table 4 shows the results for moisture content of the same streptomycin sulfate material, preweighed and redried by four different chemists using a previously unopened bottle. The moisture content for these four preparations ranged from 10.3% to 10.5%—7.4% to 7.6% greater than the moisture content of 2.9% provided by the Certificate of Analysis (C of A). In addition, the USP streptomycin sulfate had a measured moisture content of 9.1%; a difference in 4.1% from the 5% stated on its C of A. When the commercial material is analyzed using this HPAE-PAD method both with and without predrying, the error in accuracy of the dried material averaged 0.01%, while the undried material had an average error of 9.1% (see Table 4). Because the USP glass vials appear sufficiently sealed, either the moisture content changed prior to their sealing, or the drying method used for its manufacture was not as effective as the SpeedVac method (using 0.5 torr of vacuum, 20–24 h, 50 °C).

The third source of error, salt content, was previously discussed in the section titled *Preparation of Solutions and Reagents, Stock Standards*. The percent of salt present in the streptomycin is an important factor used in the calculation of the streptomycin base concentration. The mass percentage of sulfate is theoretically 20.19% of streptomycin sulfate, assuming exactly 1.5 moles of sulfate per mole of streptomycin base. The presence of different types of salts can alter this percentage. For this reason, an accurate measure of the anionic salts presence in the anhydrous streptomycin sulfate material can assure an accurate potency factor of the material is used during sample preparation. Application Note 190[33] shows how ion chromatography with suppressed conductivity can be used to obtain a profile of the different major salts present in aminoglycoside antibiotics and help make accurate determinations of their potency. Using the ICS-3000 system with dual pump and dual detector, both the aminoglycoside base (using HPAE-PAD) and the salt composition (using IC) of the material are determined simultaneously.

Calibration Error

Calibration errors are associated with deviation from linearity. The percent error in the measured concentration for standards at 30 µg/mL, using the calibration curve from 80 to 4000 pmol per injection, ranged from 5.3 to 8.7%, while the percent error, using the calibration curve for 80 to 1500 pmol per injection, ranged from 0.0 to 3.2%. For this reason, to achieve the highest accuracy, it is recommended to select a target concentration of the standard, drug substance, and of diluted drug product that is within the center of the highest linear range of 6–20 µM (3–15 µg/mL) and then to extend the range of the calibration curve for routine use to match the requirements of the drug formulation limits (e.g., 90–115% of the target concentration).[31,32]

Spike Recovery

A third challenge to analytical accuracy is interference from sample matrices, often associated with sample preparation techniques. Errors of this type are ordinarily not a concern for analysis of pure drug substances using the same diluting solvents. When measuring drug substances in complex matrices such as fermentation broths, the recovery of the analytes may not be complete due to adsorption to matrix, or other reasons. In this note, streptomycin A was spiked at 10 and 41 µM concentrations into 1000-fold diluted YPD broth (Figure 8), a very complex and undefined medium closely resembling that used for the fermentation of Streptomyces for the production of streptomycin A. The spike recoveries were 82.6 ± 0.6% (n=4) and 92.9 ± 0.6% (n=4) for the 10 and 41 µM concentrations, respectively.

Purity

The USP Monographs describe eight categories of impurities in official chemical material: foreign substances, residual solvents, toxic impurities, concomitant components, signal impurities, ordinary impurities, related substances, and process contaminants.[33] This method is useful for many toxic impurities, concomitant components, ordinary impurities, related substances, and some process contaminants. For determination of process contaminants such as chloride, sulfate, bromate, and other inorganic and some organic anions, Application Note 190[30] may be useful. Streptomycin A purity was determined by comparing two different commercial sources of streptomycin sulfate, and evaluating its thermal and

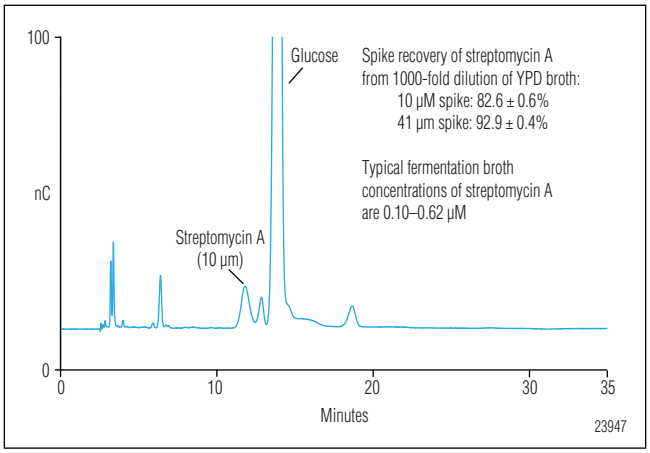


Figure 8. Determination of streptomycin A in YPD fermentation broth.

chemical degradation. The percent impurities may be presented in two ways: the percentage of non-streptomycin A peak area relative to the total peak area for all detected peaks (also known as chromatographic purity), or non-streptomycin A peak area relative to the streptomycin A peak area (ideally, relative to a highly purified standard streptomycin A peak area). In this note, we used the later definition, as recommended in the Chromatography section of the USP Monograph for Physical Tests.[24] Due to the lack of a highly purified standard, the impurity peak area was related to the streptomycin A peak area within the same chromatogram for the untreated USP standard and Sigma-Aldrich drug substance, but impurity peak area in thermally or chemically treated streptomycin sulfate was related to untreated streptomycin A peak area.

A comparison of impurities present in two dried commercial sources of streptomycin sulfate is shown in Figure 4. The endogenous system suitability peak area in streptomycin sulfate obtained from Sigma-Aldrich was determined to be 1.91 ± 0.02%, and 1.26 ± 0.04% obtained from USP. Total peak impurities, not including the late eluting impurity found at 160 min, was 8.31 ± 0.05% for Sigma-Aldrich and 9.10 ± 0.27% for the USP material. With the 70 mM NaOH eluent, peaks for ≥20 impurities were observed. The late-eluting impurity peak was 6.2% in the USP streptomycin sulfate, therefore the total impurity content was calculated to be 15.3%. When the USP streptomycin sulfate was heated at 75 °C for 60 min, as required for production of the USP system suitability material, the percent total impurity peak area for 26 peaks rose to 85%, where the system suitability peak amounted to 33% and the late eluting peak 39%.

Total peak impurities for dihydrostreptomycin, not including the late eluting impurity found at 160 min, was 8.55%, and of this percentage, streptomycin A as an impurity in dihydrostreptomycin was 1.6%, and the system suitability peak was 0.046%. The late eluting impurity was 1.5%. Combined, the total calculated impurity content was 10.1%. A similar heat-treatment of dihydrostreptomycin sulfate, but for 24 h, yielded 24% total impurity peak area for 29 peaks, and of this percentage, the system suitability peak amounted to 0.013% and the late eluting peak was 0.44%. The major impurity peak after heat-treatment eluted at 3.4 min (16%). The higher level of impurities generated for streptomycin sulfate than for dihydrostreptomycin upon heat-treatment is consistent with the higher stability known for dihydrostreptomycin, and these results help support the validity of this technique for purity analysis.

Ruggedness

Ruggedness was evaluated for influence of a 10% variances in eluent concentration, column temperature, detector temperature, and flow rate. The variance due to different columns manufactured over several years was also studied.

Eluent Concentration

The retention time of streptomycin A and the system suitability peak varied greatly with minor variations in mobile phase concentration. A 10% increase in NaOH (77 mM) decreased streptomycin A retention time from 12.0 min to 9.3 min (-22% change from 70 mM), while a 10% decrease in NaOH (63 mM) increased retention time to 15.8 min (+32% change). A 10% increase in NaOH decreased system suitability peak retention time from 7.9 min to 7.0 min (-11% change from 70 mM), while a 10% decrease in NaOH increased retention time to 8.3 min (+4% change). A 10% increase in NaOH decreased the resolution of the streptomycin and system suitability peaks by 37%, while a 10% decrease in NaOH increased this resolution by 50%. The 10% increase or decrease in eluent concentration did not produce any significant change in peak area, baseline noise, or peak asymmetry. The measured theoretical plates increased 7 and 4% for 10% increases and decreases, respectively.

Column Temperature

A 10% change in the operating column temperature was evaluated for influence on performance of this method. At the recommended operating temperature of 30 ºC, the retention time for streptomycin A was 11.6 min. At either 27 or 33 ºC, the retention time, baseline noise, peak area, peak height, were not significantly different from 30 ºC. In spite of the lack of statistical difference in retention time for the system suitability peak comparing 27 with 30 ºC, or 33 and 30 ºC, a trend was observed where this peak eluted later with decreasing column temperature. The retention time for this peak at +10% was significantly different from the -10% level. The streptomycin A peak did not show this effect. For this reason, the resolution of streptomycin A and the system suitability peak was significantly affected by column temperature; -38% change for 10% decrease in temperature, and +14% change for 10% increase. The effect of temperature on both peak asymmetry and efficiency was statistically significantly due to the high precision of these values. Asymmetry decreased with increased temperature (by 1–2% per 10% temperature change), while theoretical plates decreased (by 6–7% per 10% change).

Detector Compartment Temperature

A 10% change in the operating detector temperature (25 ºC) was evaluated for influence on streptomycin A peak area. A 10% increase in temperature increased peak area by 8.7%, and a 10% decrease in temperature decreased peak area by 6.3%. A similar percent change was observed for peak height. Baseline noise, background response, peak asymmetry and efficiency, retention time and resolution were unaffected by 10% temperature changes.

Flow Rate

A 10% change in the eluent flow rate was also evaluated for influence on method performance. At the recommended flow rate of 0.50 mL/min, the retention times were 8.0 and 11.6 min respectively for the system suitability and streptomycin A peaks. At 0.55 mL/min, their retention times were 7.2 (-11%) and 10.6 min (-9%), respectively. At 0.45 mL/min, their retention times were 8.7 (+11%) and 12.9 min (11%), respectively. At 10% higher flow rate, peak area decreased 3.5%, and at 10% lower flow rate, peak area increased 9.7%. Peak efficiency decreased with increasing flow rate (by 5% per 0.05 mL/min change), while the efficiency increased (-2% for -10% change, +7% for +10% change). Background response, baseline noise, and asymmetry were unaffected.

Column Reproducibility

Upon initial installation of a new column, or after storage of a previously used column, the column was washed for 1 h with 200 mM NaOH and then reequilibrated with 70 mM. The mean system suitability and streptomycin A peak retention times for four different CarboPac PA1 analytical columns manufactured over two years were 8.16 ± 0.34 and 12.15 ± 0.51 min. The mean resolution between these peaks was 6.89 ± 0.70.

CONCLUSION

HPAE-PAD is useful in assaying streptomycin A and its impurities. This method is accurate, reproducible, and rugged with respect to all the system suitability criteria defined in the USP compendial method for streptomycin sulfate. With HPAE-PAD, analysts can assay and determine the purity of streptomycin without costly and time-consuming sample derivatization. Overall, the described approach has good sensitivity, good peak area, retention time reproducibility, and high sample throughput.

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LIST OF SUPPLIERS

Gast Manufacturing Corp., 2550 Meadowbrook Road, Benton Harbor, MI 49022, U.S.A. Tel: 1- 269-926-6171, http://www.gastmfg.com.

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625, U.S.A. Tel: 1-800-625-4327, http://www.nalgenunc.com.

Pfizer Consumer Healthcare, Morris Plains, NJ 07950, U.S.A. Tel: 1-800-223-0182, www.prodhelp.com.

Praxair, 39 Old Ridgebury Road, Dansbury, CT 06810-5113, U.S.A. Tel: 877-772-9247, http://www.praxair.com.

Sarstedt AG & Co., Rommelsdorfer Straße, Postfach 1220, 51582 Nümbrecht, Germany Tel.: +49-2293-305-0, http://www.sarstedt.com.

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A., Tel: 1-800-325-3010, www.sigma.sial.com.

U.S. Pharmacopeia (USP), 12601 Twinbrook Parkway, Rockville, MD 20852-1790 U.S.A. Tel: 1-800-227-8772, http://www.usp.org.

Analysis of Paromomycin by HPAE-IPAD

INTRODUCTION

Paromomycin (Figure 1) is an aminoglycoside antibiotic produced by *Streptomyces rimosus var. paromomycinus.*[1] The antibacterial spectrum of paromomycin is similar to other aminoglycosides that demonstrate broad spectrum activity against some gram-positive and many gram-negative bacteria.[2] Paromomycin has been widely used in human and veterinary medicine for the treatment of various bacterial infections. In humans, paromomycin has been used to treat leishmaniasis, cryptosporidiosis, and amebiasis.[3-6] Leishmaniasis is a parasitic disease that is transmitted from the bite of a sandfly, and is primarily concentrated in India, Bangladesh, Sudan, and Brazil.[3] Previous treatments for this parasitic disease used antimony, which can be toxic to the heart, liver, kidneys, and pancreas.[7] More recently, paromomycin has resurfaced as treatment for leishmaniasis, due to its effectiveness against the disease, low toxicity, and low cost relative to other available antibiotics.[8] Due to the work of the Institute for OneWorld Health, paromomycin was granted orphan drug status for the treatment of leishmaniasis. The Orphan Drug Act of 1983 encourages the development of drugs that are necessary but would be unprofitable to produce under normal circumstances.[9]

Determination of the active component(s) of a drug is critical to ensure a safe and effective formulation before release to the market. The current U.S. Pharmacopeia (USP) compendial method for assaying paromomycin uses a microbial assay.[10] This assay is qualitative and it lacks specificity, accuracy, and is time-consuming. In addition, microbial assays neither identify active ingredient(s) nor yield information on the total composition of the antibiotic formulation.

HPLC with UV or fluorescence detection has also been used for the determination of paromomycin.[8,12,13]

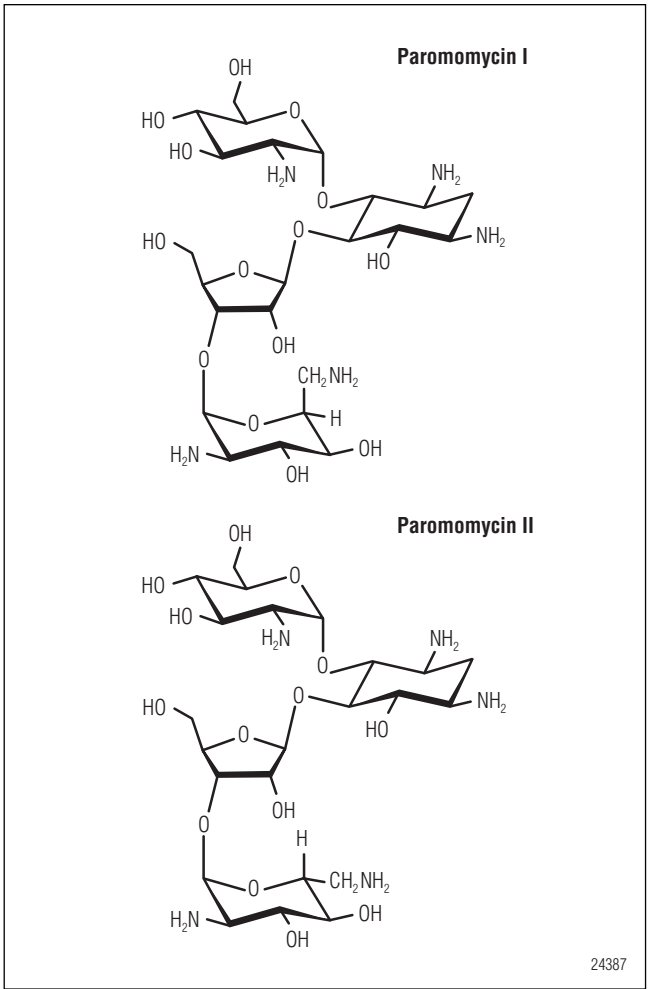

Figure 1. Paromomycin I and II.

Although these methods permit determination of the paromomycin isomers, pre- or postcolumn derivatization is required to achieve adequate sensitivity due to the lack of a suitable chromophore. Aminoglycoside compounds contain oxidizable groups (e.g., amines and hydroxyls) and can therefore be detected electrochemically.

Electrochemical detection has advantages relative to other techniques in that an oxidation potential can be selected for specific analytes while other compounds remain undetected, and derivatization is not required for detection, which simplifies the analysis. Integrated pulsed amperometric detection (IPAD) and PAD have been used succesfully to determine aminoglycosides, such as neomycin and tobramycin.[13,14] However, as previously reported, a six-potential IPAD waveform provides better sensitivity for aminoglycosides than a three or four-potential PAD waveform.[14,15] Therefore, the work presented in this application used a *AAA-Direct*™ waveform for the detection of paromomycin.

This Application Note demonstrates the use of an electrolytically generated potassium hydroxide eluent combined with the CarboPac® PA1 anion-exchange column and IPAD using a disposable *AAA* Au working electrode for the determination of paromomycin in a bulk pharmaceutical formulation. Similar to neomycin, separation of paromomycin with the CarboPac PA1 requires a weak hydroxide eluent (1.80 mM) making it difficult to use manually prepared eluents. Manually prepared NaOH may contain elevated concentrations of carbonate, which can impact retention time precision and chromatographic efficiency. An eluent generator (EG) prepares KOH eluent that is essentially carbonate-free, at accurate, precise concentrations. Carbonate that is present in the deionized water source used to supply the EG is removed from the system using a Continuously Regenerated Anion Trap Column (CR-ATC), which is installed after the eluent generator cartridge. This method accurately determines paromomycin without the need for pre- or postcolumn derivatization and meets the current USP performance requirements.

EQUIPMENT

Dionex ICS-3000 system consisting of:
- SP Single Pump or DP Gradient Pump with in-line degas option
- DC Detector Compartment (single or dual temperature zones) with electrochemical cell consisting of a pH/Ag/AgCl reference electrode (P/N 061879) and *AAA-Direct*™ Certified Au disposable working electrode (P/N 060082, package of 6; P/N 060140,package of 24)
- EG Eluent Generator module
- EluGen EGC II KOH cartridge (P/N 058900)
- EG Vacuum Degas Conversion Kit (P/N 063353)
- Continuously Regenerated Anion Trap Column, CR-ATC (P/N 060477)
- AS Autosampler with 20 µL injection loop
- Chromeleon® Chromatography Workstation
- Polypropylene injection vials with caps, 0.3 mL (Vial Kit, Dionex P/N 055428)
- Microcentrifuge tubes with detachable caps (plastic, 1.5 mL, Sarstedt, P/N 72.692.005, or equivalent)

REAGENTS AND STANDARDS

Deionized water, Type I reagent grade, 18 MΩ-cm resistivity or better

Paromomycin sulfate (USP, Catalog # 1500003 Lot G was used in this study)

Paromomycin sulfate (Sigma-Aldrich, P9297)

Sample

Humatin® (Paromomycin sulfate capsules, USP)

CONDITIONS

Columns:	CarboPac PA1 Analytical, 4 × 250 mm (P/N 035391) CarboPac PA1 Guard, 4 × 50 mm (P/N 043096)
Eluent:	1.8 mM KOH
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	0.50 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C (lower compartment) 30 °C (upper compartment)
Detection:	Integrated pulsed amperometry, *AAA-Direct* Certified Disposable Electrodes (P/N 060082)
Background:	40-55 nC
System Backpressure:	~2600 psi

Waveform:

Time (s)	Potential (V vs. pH)	Gain Region	Ramp	Integration
0.00	+0.13	Off	On	Off
0.04	+0.13	Off	On	Off
0.05	+0.33	Off	On	Off
0.21	+0.33	On	On	On
0.22	+0.55	On	On	On
0.46	+0.55	On	On	On
0.47	+0.33	On	On	On
0.56	+0.33	Off	On	Off
0.57	-1.67	Off	On	Off
0.58	-1.67	Off	On	Off
0.59	+0.93	Off	On	Off
0.60	+0.13	Off	On	Off

PREPARATION OF SOLUTIONS AND REAGENTS

The use of electrolytically generated potassium hydroxide eluent is critical in order to maintain the retention time and peak area precision of paromomycin as described here; conditions in this method cannot be successfully duplicated using manually prepared hydroxide eluents. It is essential to use high quality deionized water with a resistivity of 18 MΩ-cm or better, with a low concentration of dissolved carbon dioxide. Eluents should be kept under a blanket of helium (~5-8 psi) at all times to minimize the introduction of atmospheric carbon dioxide.

USP Reference Standard Solutions

An official USP paromomycin sulfate reference standard (~120 mg) was placed in a pre-weighed 1.5 mL polypropylene microcentrifuge tube with screw cap, and the exact weight of the undried solid was determined. The vial (without cap) containing solid paromomycin sulfate was placed in a SpeedVac® Evaporator heated to 50 °C for 24 h at <0.7 mm Hg. The vial, cap, and dried paromomycin sulfate were reweighed together to determine the dried weight and the percent moisture content (for information only). The dried solid was dissolved in a volume of deionized water to make a 100 mg/mL concentration. The assay results stated by the USP were used to calculate the concentration for the paromomycin free base, which subtracts the mass of sulfate. USP lot G of paromomycin sulfate standard used for this application contained 730 µg paromomycin per mg solid (free base, dry basis). The calculated mg/mL concentration of paromomycin was converted to mM concentration using the paromomycin free base molecular weight of 615.6 daltons (Da) and was labeled as the "Stock Standard Concentrate Solution."

A 1 mM stock standard intermediate solution was prepared by adding 123.1 µL of the 100 mg/mL stock concentrate solution to a 20 mL scintillation vial with deionized water added to bring the total volume to 20 mL. The 1 mM paromomycin stock intermediate solution was diluted to 100 µM paromomycin (2 mL of 1 mM solution in 18 mL of deionized water) and labeled "Stock Standard Solution." Stock standard intermediate solution and stock standard solutions were prepared fresh daily. All solutions were stored at -40 °C until needed.

Stock Drug Substance Solutions

The drug substance was obtained from Sigma-Aldrich. The same procedure described above for the preparation of the USP reference standard solutions was used to prepare the Sigma drug solutions. The stock drug concentrate solution, the stock drug intermediate solution, and the stock standard solution were prepared fresh daily.

Working Standard Solutions

Prepare working standard solutions at lower concentrations by adding the appropriate amount of the 100 µM stock standard solutions and diluting with deionized water. For this Application Note, USP paromomycin reference standards were prepared at 1.25, 2.50, 3.50, 4.50, 5.00, 5.50, 6.00, 6.50, 7.50, 8.50 and 10.00 µM paromomycin as calibration standards. Once linearity was established, working drug substance solutions and working product solutions were prepared at the same concentrations as the USP reference standard solutions from their respective 100 µM stock standard.

SAMPLE PREPARATION

A paromomycin sulfate capsule (containing the equivalent of 250 mg paromomycin) was weighed on an analytical balance and the mass recorded. The capsule was carefully dissembled to expose the solid material and the entire capsule with solid was placed in a pre-weighed 120 mL HDPE bottle containing 100.0 ± 0.1 g of deionized water to dissolve the solid material. Duplicate 1.0 mL volumes of the dissolved solution

were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 10 min at 16,000 rpm, after which 0.50 mL of supernatant from each microcentrifuge tube was transferred to separate microcentrifuge tubes. Based on the label concentration, the mg/mL concentration of the solution was calculated using the following equation:

$$\text{mg/mL paromomycin free base} = \frac{250\text{ mg}}{\text{tablet}} \times \frac{\text{tablet}}{100.0\text{ mL}} = 2.50\text{ mg/mL}$$

This solution was diluted to 100 µM paromomycin base by adding 0.493 mL of 2.50 mg/mL stock product solution to a 20 mL volumetric flask and bringing to volume. The 100 µM paromomycin sample was labeled as the stock product solution. The sample was prepared fresh daily.

SYSTEM PREPARATION AND SETUP

Determination of paromomycin using eluent generation (EG) requires installation of the ICS-3000 EG Vacuum Degas Conversion Kit (P/N 063353) to allow sufficient removal of the hydrogen gas formed with the potassium hydroxide eluent. Because installation of the kit requires access to the DP-3000 electronics compartment, the degas conversion kit must be installed by a Dionex Support Technical Representative or other authorized person. After installation of the degas conversion kit is complete, install an EGC II KOH cartridge in the EG-3000 and configure the setup of the cartridge with the Chromeleon server configuration. Connect the cartridge to the EG degas assembly and install backpressure tubing (~91.4 cm of 0.003" i.d.) in place of the column set to produce a system pressure of ~2000 psi at 1 mL/min. Condition the cartridge with 50 mM KOH for 30 min at 1 mL/min. After completing the conditioning process, disconnect the backpressure tubing temporarily installed in place of the column set. Install a CR-ATC between the EGC II KOH cartridge and the EGC degas. Hydrate the CR-ATC prior to use by following the instructions outlined in the EluGen Cartridge Quickstart Guide (Document No. 065037-02).

Install a 4 × 50 mm CarboPac PA1 guard and 4 × 250 mm CarboPac PA1 analytical column set. Ensure system backpressure is at an optimal pressure of 2400 ± 200 psi when 1.8 mM KOH is delivered at 0.5 mL/min. Install additional backpressure tubing between the EG degas and injection valve as necessary to achieve an optimal pressure reading. Calibrate the pH electrode according to the instructions provided in the Chromeleon software. Install a disposable *AAA* Au working electrode in the electrochemical cell, then install a short piece (~25 cm) of black tubing (0.010" i.d.) on the cell outlet.

The CarboPac PA1 column is stored in 200 mM NaOH. Upon installation, rinse the column set with 100 mM KOH for at least 1 h prior to connecting to the cell inlet. After completing the rinse step, equilibrate the column with 1.8 mM KOH for 24 h to obtain optimum retention time precision. Select the "Amino Acids (pH, Ag, AgCl reference)" waveform in Chromeleon. Set the waveform mode and reference electrode to "IntAmp" and "pH" respectively. Note: While the use of the carbohydrate waveform promotes a longer lifetime of the disposable Au electrode, the *AAA* waveform provides better sensitivity and was therefore used in this study.[15] Dionex specifies a lifetime of one week for the disposable Au electrode when the *AAA* waveform is used. However, actual lifetime may vary, depending on conditions. For more information, refer to the product manual for Disposable Gold Electrodes (Document No. 065040-03). After selecting the waveform, confirm flow is passing through the cell and turn the cell voltage to the ON position. The pH recorded by the reference electrode in the electrochemical cell should be between 11.2-11.5 once the column has been equilibrated with 1.8 mM KOH at 0.5 mL/min. Significant deviation from this range may be an indication of an excessive potential shift, and may require replacement of the reference electrode (typically every 6-12 months for the ICS-3000 cell). The electrochemical background recorded in this series of experiments was 44.5 ± 1.0 nC over a three week period. Generally, the background should be within 40-50 nC when operating under the specified method parameters. A significantly higher or lower background may be an indication of electrode malfunction or contamination within the system.

RESULTS AND DISCUSSION

Separation

Figure 2 shows separation of 5 µM USP grade paromomycin on the CarboPac PA1 column. The paromomycin isomers (paromomycin I and II) are represented by the two largest peaks eluting at approximately 5 and 8 min, respectively. Two baseline dips are observed in

the chromatogram using 1.80 mM KOH eluent. The first baseline dip (~15 min) may be caused by the presence of trace organic impurities present in the standard or sample injected, resulting in a negative response due to the exclusion of electrochemically active ions in the eluent. The second baseline dip (~30 min) is due to the presence of oxygen (also called the "oxygen dip") in the standard or sample and appears as a function of the gas permeation volume of the column. The retention times of the baseline dips vary slightly from column to column, but are affected by flow rate, not eluent strength. The elution of the oxygen dip can be timed to occur at the end of the following injection to avoid interference with the target analytes. In this Application Note, the run time was reduced from 32 to 16 min to increase sample throughput.

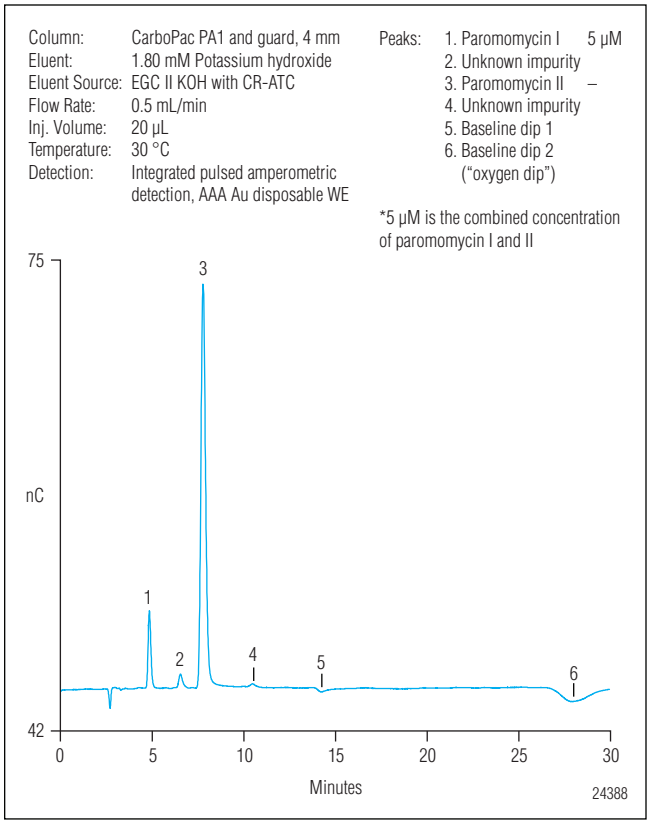

Figure 2. Determination of paromomycin (5 µM) using the CarboPac PA1 column with eluent generation.

Minor changes in the hydroxide eluent concentration can produce significant changes in the retention times of paromomycin I and II. For example, a concentration of 1.6 mM KOH eluent produced retention times of 6.2 min and 11.1 min for paromomycin I and II, respectively, compared to retention times of 5.1 min and 8.3 min using 1.8 mM KOH eluent. Reducing the eluent concentration increases the retention time of paromomycin, reduces sample throughput, and increases peak tailing. However, the resolution of some sample impurities will improve at lower hydroxide concentrations. A concentration of 1.8 mM KOH eluent was shown to provide good resolution between paromomycin II and an unknown impurity (Figure 2, peak 4) with a run time that allows for optimum sample thoughput.

LINEARITY, LIMIT OF QUANTITATION, AND LIMIT OF DETECTION

Linear range was determined by injecting paromomycin over a broad concentration range (0.005 to 20 µM) and plotting the sum of the peak areas of the two largest peaks (paromomycin I and II) against the injected concentration. Optimum linear range was considered to be where the response factor (ratio of paromomycin I + II peak areas/concentration injected) remained within 10% of the mean (average response factor for 2, 4, 5, and 6 µM paromomycin). The plot of the response factor versus the injected concentration showed a typical plateau region that represents an optimum level for operation (data not shown). The results demonstrated the optimum linearity for paromomycin was between 1.25 µM and 10 µM. The USP reference standard, Sigma drug substance, and Humatin sample were each prepared within the specified calibration range on three separate days. A summary of the calibration data is shown in Table 1. Each calibration was found to be linear using a least squares regression curve with correlation coefficients (r^2) of 0.9991 or better.

The USP method for validation specifies a signal-to-noise (S/N) ratio of 10 for the determination of the limit of quantitation (LOQ). Baseline noise was determined over a one minute time period during an analysis of a blank. The baseline noise ranged from 19 to 90 pC with an average noise of 46 ± 17 pC (n = 71 one-minute segments) measured over three weeks using three different *AAA* Au disposable electrodes. The LOQ for paromomycin based on the ratio of the sum of the peak heights to the average baseline noise was determined to be 0.10 µM (S/N = 10). The limit of detection (LOD) was estimated to be 0.030 µM (S/N = 3) for paromomycin (by extrapolation).

Table 1. Summary of Calibration Data for Paromomycin (Three Day Study)							
Day	**Analyte**	**Source**	**Range (µM)**	**Linearity (r^2)**	**RSD**	**Intercept**	**Slope**
1	Paromomycin	USP RS	1.25-10	0.9991	1.88	0.341	1.918
	Paromomycin	Sigma	1.25-10	0.9994	1.95	0.287	1.759
	Paromomycin	Humatin	1.25-10	0.9994	1.57	0.242	2.091
2	Paromomycin	USP RS	1.25-10	0.9993	2.08	0.203	1.895
	Paromomycin	Sigma	1.25-10	0.9992	2.41	0.069	1.886
	Paromomycin	Humatin	1.25-10	0.9995	1.41	0.347	2.173
3	Paromomycin	USP RS	1.25-10	0.9995	2.01	0.008	1.969
	Paromomycin	Sigma	1.25-10	0.9995	1.74	0.197	1.873
	Paromomycin	Humatin	1.25-10	0.9992	1.34	0.495	2.121

METHOD PERFORMANCE

Method performance was measured in terms of precision of replicate injections of paromomycin and recovery of spiked samples. The relative standard deviations (RSDs) were calculated for the sum of the paromomycin peak areas from a 5 µM standard. The intraday precision (i.e., a sequence of consecutive injections, n = 3) was <2% for USP grade paromomycin, ≤1% for paromomycin prepared from the Sigma drug substance, and <1% for paromomycin prepared from the Humatin sample based on independently prepared solutions analyzed on three separate days. The between-day precision for a three day period (i.e., day-to-day, n = 9) was <2% for paromomycin prepared from the USP reference standard, the Sigma drug substance, and the Humatin sample.

Ruggedness of an analytical method is defined by the USP as a measure of the degree of reproducibility for the same samples under a variety of conditions.[16] This is typically expressed as the lack of influence on the assay results under different conditions that would normally be expected from laboratory to laboratory and from analyst to analyst when operating under the defined method parameters. The ruggedness of the paromomycin assay was evaluated based on results from different analysts, instruments, lots of the column, and eluent generator cartridges. Each analyst used a USP reference standard solution containing 5 µM paromomycin, and the Sigma drug substance and Humatin product prepared at 100% of the target concentration (5 µM paromomycin) using different instruments, two different lots of the CarboPac PA1 column, and two different KOH eluent generator cartridges. Table 2 shows the overall procedure RSD and the RSD from two different eluent generator cartridges. Evaluation by single factor analysis of variance (ANOVA) test demonstrated that results obtained using different eluent generator cartridges or different CarboPac PA1 columns were not significantly different with a 95% confidence interval. The method was found to be rugged with respect to the variables evaluated in this study.

Humatin is a broad spectrum antibiotic that is supplied as a water-soluble capsule containing the equivalent of 250 mg paromomycin. This sample was analyzed over three days using independently prepared standards and diluted dosage solutions. The average paromomycin concentration was determined to be 279 ± 10 mg (3.7% RSD) over three trials performed on three separate days. The label states the product contains 250 mg paromomycin, however, our results indicate an average measured concentration that is 11.6% above the expected value. The USP specifies that the paromomycin sulfate capsules can contain the equivalent of not less than 90% and not more than 125% of the labeled amount of paromomycin. Our results demonstrate that this product concentration falls within these specifications. The higher measurement values found in this formulation may be designed to ensure longer shelf life. The accuracy of the procedure was evaluated by spiking the samples with known amounts of paromomycin. For samples spiked with 0.5, 1.0, and 2.5 µM paromomycin, recoveries were in the range of 96-106%, 98-107%, and 95-103%, respectively.

Table 2. Results of Ruggedness Study[a]						
Analyte	**Source**	**Eluent Generator Cartridge C**		**Eluent Generator Cartridge D**		**Overall Precision**
		Average (µM)	**RSD**	**Average (µM)**	**RSD**	**RSD**
Paromomycin	Sigma	4.80	2.03	4.84	1.00	1.12
		Average (mg/capsule)	**RSD**	**Average (mg/capsule)**	**RSD**	**RSD**
Paromomycin	Humatin	265	3.56	268	3.42	3.29

[a]*Average concentrations based on combined data from chemists A and B and columns E and F, n = 12.*

Table 3. Average Recoveries of Paromomycin Spiked Into a Bulk Pharmaceutical Formulation						
Sample	**Analyte**	**Amount Added (µM)**	**Day 1 Average Recovery (%)**	**Day 2 Average Recovery (%)**	**Day 3 Average Recovery (%)**	**Overall Recovery[b] (%)**
Humatin	Paromomycin	0.5	102.0 ± 5.2	100.2 ± 1.3	99.4 ± 3.3	100.5 ± 2.2
Humatin	Paromomycin	1.0	102.2 ± 4.0	100.2 ± 0.5	101.2 ± 3.8	101.2 ± 2.8
Humatin	Paromomycin	2.5	97.7 ± 1.7	99.4 ± 0.3	98.6 ± 4.0	98.6 ± 1.2

[a]*Average recoveries based on three independently prepared solutions, n = 3.*
[b]*Combined average recoveries over three days from independently prepared solutions, n = 9.*

Figure 3 compares a chromatogram of paromomycin detected in the Humatin sample to the same sample spiked with 1.0 µM paromomycin. Table 3 summarizes the average recoveries of known concentrations of paromomycin spiked into the Humatin sample over three days.

CONCLUSION

This Application Note demonstrates the use of HPAE-IPAD combined with electrolytic generation of potassium hydroxide eluent for the determination of paromomycin in a bulk pharmaceutical product. The data suggests that the method is linear, precise, and accurate for determintion of paromomycin and therefore meets current USP performance requirements. The use of automated eluent generation improves the consistency in producing a low potassium hydroxide concentration, making the method reproducible and rugged with respect to retention time and peak area precision. The use of disposable *AAA* Au electrodes further simplifies the method, providing good electrode-to-electrode reproducibility and assuring greater accuracy between instruments as well as between laboratories.

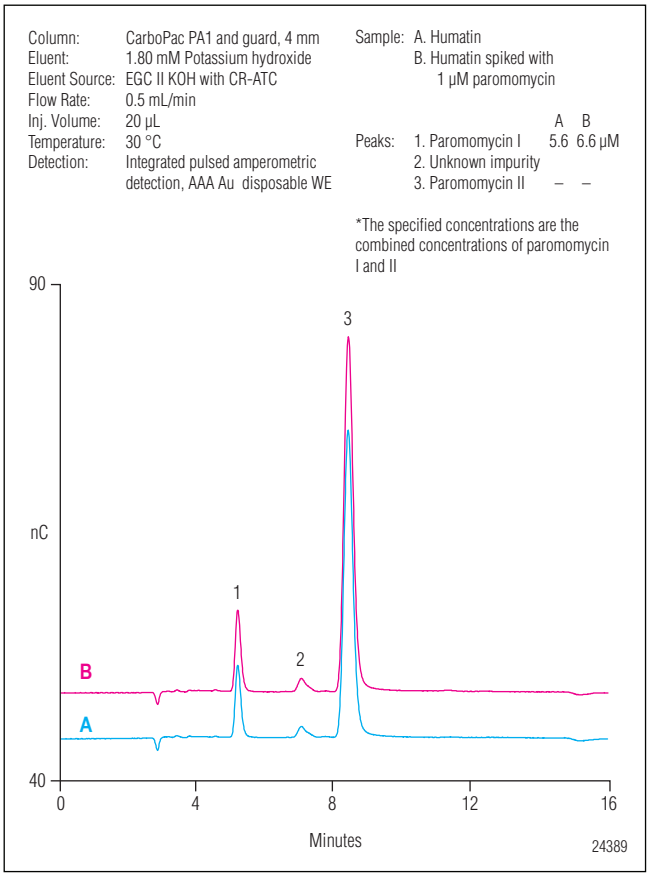

Figure 3. Comparison of A) unspiked and B) spiked Humatin sample containing paromomycin.

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Determination of Sulfate Counter Ion and Aionic Impurities in Aminoglycoside Drug Substances by Ion Chromatography with Suppressed Conductivity Detection

INTRODUCTION

Most drug substances are produced synthetically in bulk and formulated into convenient dosage forms, such as tablets, capsules, suspensions, ointments, and injectables.[1] Many of these substances are manufactured in specific salt forms to promote solubility, stability, and bioavailability.[2,3] The most common pharmaceutical counter ions used in the development of basic drugs include chloride (~50%) and sulfate (5-10%).[4] It is important to accurately determine the concentration of these counter ions to establish the correct molecular mass of the drug, the stoichiometric relationship between the drug and counter ion, and the completeness of salt formation.

During the early stages of drug product development, it is also critical to determine the concentrations of unknown ionic impurities. Impurities can originate from a variety of sources, such as raw materials, intermediates, byproducts, degradation products, and contaminants in the synthetic pathway.[5] The International Conference on Harmonization (ICH) has developed a guideline for the control of impurities in the pharmaceutical industry. In general, the ICH guidelines propose a qualification threshold of 0.1% if the maximum daily does is ≤2 g/day and 0.05% if the maximum daily dose exceeds 2 g/day. However, higher or lower limits may be implemented based on scientific rationale with respect to safety considerations.[6] In all cases, all impurities should be identified and quantified.

Ion chromatography (IC) with suppressed conductivity detection is well established and the most common technique for determining inorganic and organic ions in a wide range of matrices, including those of pharmaceutical origin. A suppressor significantly reduces background conductivity and effectively increases the analyte signal, thereby providing very low detection limits. Previous reports have successfully demonstrated the use of IC to determine counter ions and impurities in a variety of pharmaceutical products.[7–13] This application note describes the use of two hydroxide-selective anion-exchange columns with suppressed conductivity detection to determine sulfate counter ion and anionic impurities in aminoglycoside drug products.

Aminoglycosides are a large and diverse class of antibiotics that are active against aerobic, gram-negative bacteria and some gram-positive organisms.[14,15] These antibiotics are typically used in the treatment of severe infections of the abdomen and urinary tract, but they have also been used to treat bacteremia and endocarditis.[15] Some of the most common aminoglycosides include gentamicin, tobramycin, amikacin, and streptomycin. Approximately 20–30% (w/w) of the total molecular mass of many aminoglycoside compounds is sulfate. Figure 1 shows the chemical structures of three of the aminoglycosides investigated in this study.

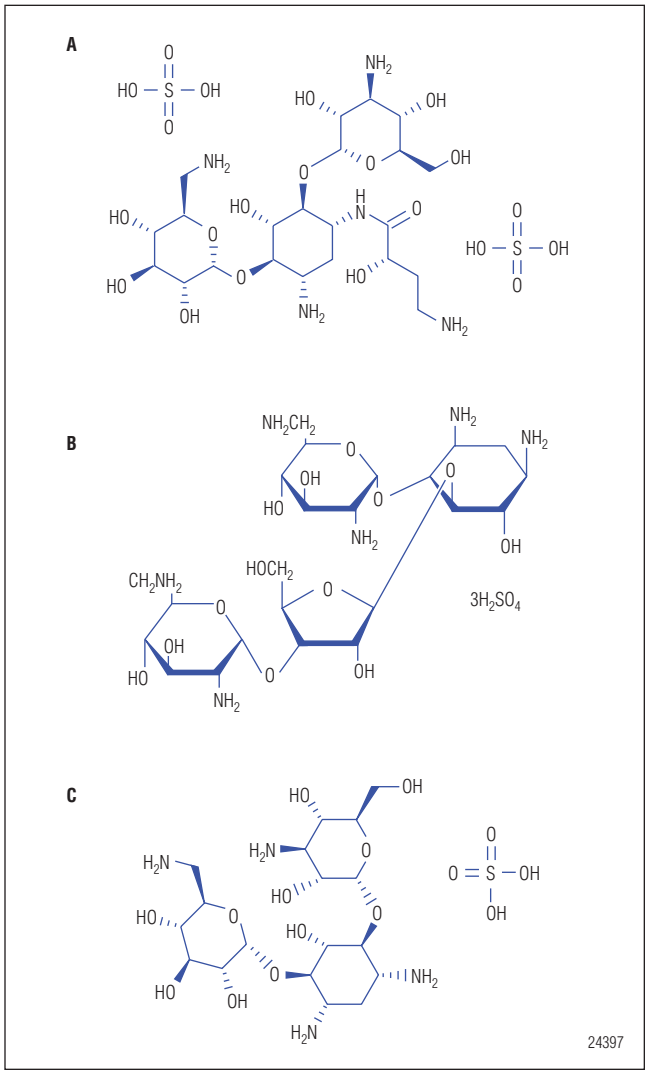

Figure 1. Chemical structures of some aminoglycoside sulfate compounds: A) amikacin disulfate, B) neomycin B trisulfate, C) kanamycin A sulfate.

The methods described herein use either an IonPac® AS18 or IonPac AS11-HC anion-exchange column to determine sulfate and anionic impurities in aminoglycoside drug substances. The AS18 packing consists of a highly cross-linked core with a latex anion-exchange layer that is functionalized with very hydrophilic quaternary ammonium groups. The selectivity of the AS18 is optimized for the separation of common inorganic anions and small organic acids in a variety of sample matrices. This column is ideal for determining the major anionic counter ions of a pharmaceutical. The AS11-HC packing consists of a 9-µm macroporous resin bead with an anion-exchange layer that is functionalized with quaternary ammonium groups. The selectivity of the AS11-HC is optimized for the separation of a large number of organic acids and inorganic anions in complex matrices. This column is ideal for the determination of trace components and for separating organic acids in uncharacterized samples.

Both columns are designed for use with hydroxide eluents, which can be generated electrolytically on-line using deionized water and an eluent generator. This application note describes the linearity, detection limits, precisions, and recoveries using anion-exchange chromatography with an electrolytically-generated potassium hydroxide eluent for the determination of sulfate counter ions and impurities in aminoglycosides. The combination of a Reagent-Free™ IC (RFIC™) system and hydroxide-selective column meets the needs of the pharmaceutical industry for counter ion analysis by providing accurate, precise, and robust methods that are easily transferred between laboratories.

EQUIPMENT

Dionex ICS-3000 RFIC system consisting of:

- DP Dual Pump (an SP Single Pump can be used if determining only the ions in this application)
- EG Eluent Generator
- DC Detector/Chromatography module (single or dual temperature zone configuration)
- AS Autosampler

EluGen® EGC II KOH cartridge (P/N 058900)

Continuously-Regenerated Anion Trap Column, CR-ATC (P/N 060477)

Chromeleon® 6.8 Chromatography Workstation

REAGENTS AND STANDARDS

Deionized water, Type I reagent grade, 18 MΩ-cm resistivity or better

Sodium acetate ($C_2H_3O_2Na$) (Sigma-Aldrich, P/N 71179)

Sodium chloride (NaCl) (J.T. Baker; VWR P/N JT3625-1)

Sodium sulfate (Na_2SO_4) (Aldrich 29,931-3)

Sodium phosphate, dibasic, anhydrous (Na_2HPO_4) (J.T. Baker; VWR P/N JT4062-1)

Sodium pyrophosphate, tetrabasic decahydrate ($Na_4P_2O_7•10H_2O$) (Sigma-Aldrich, P/N 71515)

Samples

Amikacin disulfate ($C_{22}H_{43}N_5O_{13}•2H_2SO_4$, Sigma-Aldrich A1774)

Dihydrostreptomycin sesquisulfate ($C_{21}H_{41}N_7O_{12}•3/2H_2SO_4$, Sigma-Aldrich D7253)

Kanamycin sulfate, kanamycin A ($C_{18}H_{36}N_4O_{11}•H_2SO_4$, Sigma-Aldrich K4000)

Kanamycin B sulfate, bekanamycin ($C_{18}H_{37}N_5O_{10}•xH_2SO_4$, Sigma-Aldrich B5264)

Neomycin trisulfate hydrate ($C_{23}H_{46}N_6O_{13}•3H_2SO_4•xH_2O$, Sigma-Aldrich N5285)

Paromomycin sulfate ($C_{23}H_{45}N_5O_{14}•H_2SO_4$, Sigma-Aldrich P9297)

Paromomycin sulfate ($C_{23}H_{45}N_5O_{14}•H_2SO_4$, USP Catalog # 1500003)

Sisomicin sulfate ($2C_{19}H_{37}N_5O_7•5H_2SO_4$, Sigma-Aldrich S7796)

Streptomycin sulfate ($C_{21}H_{39}N_7O_{12}•1.5H_2SO_4$ Sigma-Aldrich S6501)

Humatin® (paromomycin sulfate capsules, USP, Monarch Pharmaceuticals, Bristol, TN)

CONDITIONS

Method 1

Columns:	IonPac AG18 Guard, 2 × 50 mm (P/N 060555) IonPac AS18 Analytical, 2 × 250 mm (P/N 060553)
Eluent:	22 mM potassium hydroxide 0–7 min, 22–40 mM from 7–8 min, 40 mM from 8–20 min*
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	0.25 mL/min
Temperature:	30 °C (lower compartment) 30 °C (upper compartment)
Inj. Volume:	5 µL (full-loop injection)
Detection:	Suppressed conductivity, ASRS® ULTRA II (2 mm), Recycle mode Power setting: 25 mA
System Backpressure:	~2600 psi
Background Conductance:	~0.6–0.7 µS
Noise:	~2–3 nS/min peak-to-peak
Run Time:	30 min

*The column equilibrates at 22 mM KOH for 5 min prior to the next injection.

METHOD 2

Columns:	IonPac AG11-HC Guard, 2 × 50 mm (P/N 052963) IonPac AS11-HC Analytical, 2 × 250 mm (P/N 052961)
Eluent:	1 mM potassium hydroxide 0–5 min, 1–5 mM from 5–9 min, 5–38 mM from 9–20 min, 38–60 mM from 20–25 min, 60 mM from 25–30*
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	0.38 mL/min
Temperature:	30 °C (lower compartment) 30 °C (upper compartment)
Injection Vol:	5 µL (full-loop injection)
Detection:	Suppressed conductivity, ASRS ULTRA II (2 mm), Recycle mode Power setting: 62 mA
System Backpressure:	~2600 psi
Background Conductance:	~0.6–0.8 µS
Noise:	~2–3 nS/min peak-to-peak
Run Time:	30 min

*The column equilibrates at 1 mM KOH for 5 min prior to the next injection.

PREPARATION OF SOLUTIONS AND REAGENTS

Stock Standard Solutions

To prepare individual 1000 mg/L stock standards of acetate, chloride, sulfate, phosphate, and pyrophosphate, add 0.1389 g NaOAc, 0.1649 g NaCl, 0.1479 g Na_2SO_4, 0.1479 g anhydrous Na_2HPO_4, and 0.2564 g $Na_4P_2O_7$• 10 H_2O, respectively to separate 100 mL volumetric flasks. Dilute each to volume with deionized water, and mix thoroughly.

Primary Dilution Standards

Method 1: Prepare 10 mg/L chloride and 10 mg/L phosphate by adding 1 mL from their respective 1000 mg/L stock standard solutions to separate 100 mL volumetric flasks, and diluting to volume with deionized water.

Method 2: Prepare 50 mg/L each of chloride and pyrophosphate and 100 mg/L each of acetate and phosphate. To prepare chloride and pyrophosphate, add 5 mL from each respective 1000 mg/L stock standard solution to a separate 100 mL volumetric flask and dilute to volume with deionized water. To prepare acetate and phosphate, add 10 mL from each respective 1000 mg/L stock standard solution to a separate 100 mL volumetric flask and dilute to volume with deionized water.

Calibration Standards

For Method 1, prepare the calibration standards for chloride and phosphate from their respective 10 mg/L primary dilution standards using an appropriate dilution from each standard. Prepare sulfate standard from the 1000 mg/L stock standard solution using an appropriate dilution. For Method 2, prepare the calibration standards for acetate, chloride, phosphate, and pyrophosphate from their respective primary dilution standards. Prepare the sulfate standard from the 1000 mg/L stock standard solution using the appropriate dilution.

SAMPLE PREPARATION

In the present analyses, for each aminoglycoside sulfate compound, approximately 120 mg of solid was placed in a separate pre-weighed 1.5 mL polypropylene microcentrifuge tube with a screw cap, and the exact weight of the undried solid was determined. The vials (without caps) containing the solid aminoglycoside compounds were placed in a SpeedVac® Evaporator heated to 50 °C for 24 h at <0.7 mm Hg. The vials, caps, and dried solids were reweighed to determine the dry weights and percent moisture content (0.7–11.4% in this study). The dried solids were dissolved in the appropriate weight of deionized water to make a 100 mg/mL concentration. A primary sample dilution containing 1.0 mg/mL for each compound was prepared by adding 0.20 mL of the respective 100 mg/mL stock solution to a separate 20 mL scintillation vial, and adding deionized water to a total volume of 20 mL. A final sample dilution containing 0.05 mg/mL for each compound was prepared by adding 1 mL of the respective 1.0 mg/mL primary sample dilution to a 20 mL scintillation vial, and adding deionized water to a total volume of 20 mL. The 0.05 mg/mL solutions of individual anhydrous aminoglycoside sulfate compounds were used to determine the sulfate counter ion and anionic impurities using the IonPac AS18 column.

Humatin (paromomycin sulfate capsule containing the equivalent of 250 mg paromomycin) was weighed on an analytical balance and the mass recorded. The capsule was carefully moved apart to expose the solid material, and the entire capsule with solid was placed in a pre-weighed 120-mL HDPE bottle containing 100.0±0.1 g of deionized water to dissolve the solid material. Eight 1.0-mL volumes of the dissolved solutions were transferred to separate 1.5-mL microcentrifuge tubes and centrifuged for 10 min at 16,000 rpm. The solutions were combined in a 20-mL scintillation vial. Based on the label concentration, the final concentration was equivalent to 2.50 mg/mL paromomycin free base. This solution was injected directly on an IonPac AS11-HC column to determine the concentration of impurities in the sample and diluted 1:10 to determine the sulfate concentration.

RESULTS AND DISCUSSION

Many aminoglycosides occur naturally as products of various Actinobacteria, particularly from the genera *Streptomyces* and *Micromonospora*. Although the chemical synthesis of many aminoglycosides has been achieved, the production of these compounds by fermentation remains the most economical route.[14] Aminoglycosides are commonly purified with adsorbents or ion exchange materials with an acid, such as sulfuric acid. Therefore, these antibiotics should contain only the aminoglycoside free base and sulfuric acid. However,

the compounds also typically contain some water due to their hygroscopic nature. In addition, small amounts of ionic impurities may be present as byproducts from the fermentation process or synthetic and purification pathways. Fermentation broths are complex media containing a wide range of inorganic and organic anions[16] that can be carried over from the isolation and purification of the aminoglycoside antibiotic compounds.

The methods reported in this application note compare two hydroxide-selective anion exchange columns, the IonPac AS18 and AS11-HC, for the determination of sulfate counter ion and ionic impurities in aminoglycoside compounds. A hydroxide-selective column combined with a potassium hydroxide eluent gradient permit the separation of a wide variety of inorganic and organic anions, from single to polyvalent charged ionic species.

Low analyte concentrations can be detected using suppressed conductivity detection. Although good sensitivity is not required to detect the sulfate counter ion, the detection must be sufficiently sensitive to determine impurities at concentrations less than 0.1%. To further simplify method development and avoid the difficulties often encountered when preparing hydroxide eluents, the RFIC system produces a high-purity carbonate-free potassium hydroxide eluent automatically. The EG essentially eliminates the hydroxide eluent absorption of carbon dioxide that can cause undesirable baseline shifts, irreproducible retention times, and therefore compromise the integrity of the analytical results.

Method 1

The IonPac AS18 column was used to determine the concentration of sulfate and impurities in eight different aminoglycoside sulfate compounds. Using the conditions described for Method 1, common inorganic anions were separated by the IonPac AS18 column in about 16 min. Therefore, this column and method are recommended for high-throughput analysis of samples that do not contain a wide variety of inorganic and organic anions. An initial screening for inorganic impurities in each aminoglycoside compound prepared at a concentration of 0.05 mg/mL revealed the presence of chloride in all samples and phosphate in neomycin sulfate and paromomycin sulfate. The IonPac AS18 column was calibrated for chloride, sulfate, and phosphate by performing duplicate injections of the target anions in the range of 0.025–0.15 mg/L (0.05–0.3 wt. %), 5–25 mg/L (10–50 wt. %), and 0.02–0.15 mg/L (0.04–0.3 wt %), respectively.

Table 1. Calibration Data and Detection Limits Using Method 1

Analyte	Range (mg/L)	Linearity (r^2)	Estimated Limit of Detection[a] (g/L)
Chloride	0.025–0.15	0.9998	3.0
Sulfate	5.0–25	0.9994	7.7
Phosphate	0.020–0.15	0.9994	9.3

[a]LODs estimated from 3 x S/N

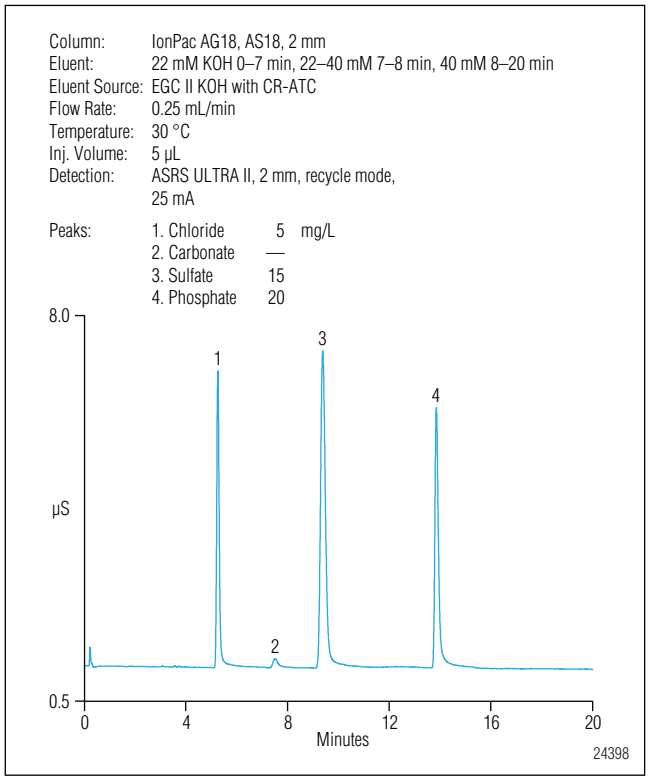

Figure 2. Separation of target anions on the IonPac AS18 column.

Table 1 summarizes the calibration data and limits of detections (LODs) for the target anions. Figure 2 shows a standard separation of chloride, sulfate, and phosphate on the IonPac AS18 column using an electrolytically-generated potassium hydroxide eluent. For samples that do not contain phosphate, the run time can be reduced to about 12 min to increase sample throughput.

Table 2. Percentages of Sulfate Counter Ion and Anionic Impurities Determined in Anhydrous Aminoglycoside Sulfate Compounds Using Method 1				
Aminoglycoside Sample	**Theoretical Sulfate (%)**	**Experimental Sulfate (%)**	**Chloride (%)**	**Phosphate (%)**
Amikacin	24.6	22.3	0.110	—
Dihydrostreptomycin	19.7	16.8	0.052	—
Kanamycin A	16.5	13.7	0.057	—
Kanamycin B	28.2	24.8	0.065	—
Neomycin	29.1	25.0	0.090	0.042
Paromomycin, Sigma Lot 1	23.7	22.5	0.021	0.097
Paromomycin, Sigma Lot 2	23.7	24.2	0.036	0.058
Paromomycin, USP	23.7	23.6	0.016	0.040
Sisomicin	34.6	30.2	0.056	—
Streptomycin	18.7	17.3	0.098	—

Table 2 summarizes the average percentages (n = 3) of sulfate and impurities (chloride and phosphate) determined in the aminoglycoside sulfate compounds. The percentage of sulfate varied from 13.6 to 30.2%. The total impurities from chloride and phosphate (if present) were in the range of 0.056–0.13%. Sigma-Aldrich does provide the stoichimoetry of the aminoglycoside freebase to sulfate for most samples analyzed in this study, with the exception of kanamycin B sulfate and paromomycin sulfate. We verified the accuracy of the moles of sulfate provided by Sigma-Aldrich based on the determinations shown in Table 2 using the IonPac AS18 column. The stoichiometry of paromomycin free base to sulfate is 1:2, which is in agreement with a previous study.[17] Kanamycin B (bekanamycin) sulfate was also found to contain two moles of sulfate per mole of the aminoglycoside free base. Determination of the correct stoichiometry is important in the pharmaceutical industry to establish an accurate molecular mass of the compound being investigated.

In this study, we also investigated two different paromomycin sulfate lots from Sigma-Aldrich and one lot from the U.S. Pharmacopeia (USP). The sulfate percentages from the separate lots varied slightly from 22.5 to 24.2% with a maximum relative difference of 1.2% between the experimental and theoretical sulfate percentages. The USP paromomycin sulfate contained the least amount of impurities (0.056%), and was within 0.1% of the theoretical sulfate value.

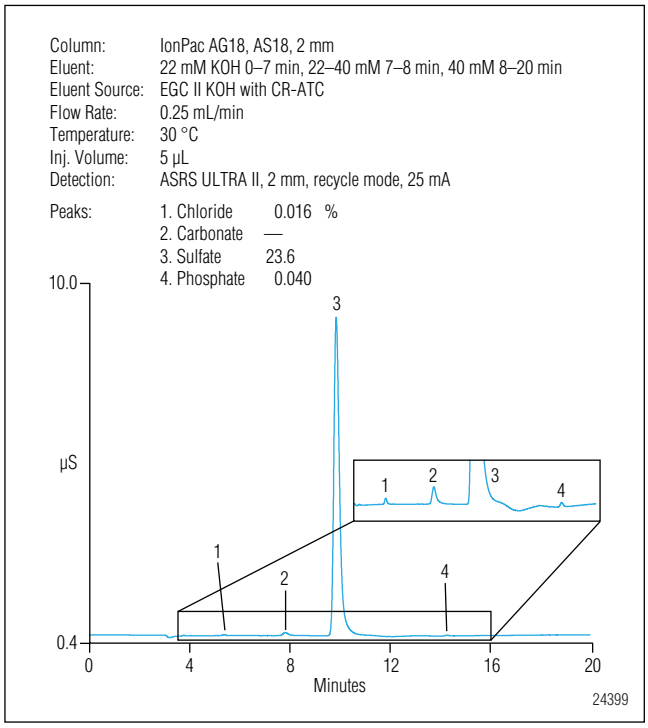

Figure 3. Separation of sulfate counter ion and anionic impurities in USP-grade paromomycin sulfate.

Figure 3 demonstrates the separation of sulfate and trace concentrations of chloride and phosphate in USP-grade paromomycin sulfate. The absolute difference between the theoretical sulfate concentration and the experimentally-determined values ranged from -4.4% to +0.5% for all aminoglycoside sulfate compounds

Table 3. Analyte Recoveries for Sulfate Counter Ion and Impurities Detected in Anhydrous Aminoglycoside Sulfate Compounds Using Method 1

Aminoglycoside Sample	Chloride Recovery (%)	Sulfate Recovery (%)	Phosphate Recovery (%)
Amikacin	107.2	98.8	—
Dihydrostreptomycin	102.2	99.5	—
Kanamycin A	108.7	99.1	—
Kanamycin B	106.1	99.7	—
Neomycin	105.0	97.6	95.0
Paromomycin, Sigma Lot 1	102.1	98.5	102.9
Paromomycin, Sigma Lot 2	92.0	98.9	91.2
Paromomycin, USP	108.3	97.1	95.3
Sisomicin	112.0	100.7	—
Streptomycin	103.5	97.8	—

(Table 2); however, most percent differences were <3% from the theoretical values. The larger theoretical percent error may be attributed to the presence of impurities that are not detected by suppressed conductivity, and are possibly due to the presence of some solvent that is not completely removed upon drying. In a previous study, Olsen et al. demonstrated that methanol present in a paromomycin sulfate compound was quantitatively equivalent before and after repeated drying under reduced pressure (<5 mm Hg) at 60 °C, indicating that the methanol is trapped in the sample matrix under these drying conditions and is not available until the matrix is dissolved.[17]

The peak area RSDs from the triplicate sample injections were <3% for chloride and phosphate and <2% for sulfate. To verify the accuracy of the method for determining chloride, sulfate, and phosphate in the aminolycoside sulfate compounds, each sample was spiked with known concentrations of the target anions. The average recoveries were in the range of 92–112%, 97–101%, and 91–103% for chloride, sulfate, and phosphate, respectively (Table 3).

Table 4. Calibration Data and Detection Limits Using Method 2

Analyte	Range (mg/L)	Linearity (r^2)	Estimated Limit of Detection[a] (g/L)
Acetate	1.0–10	0.9998	50
Chloride	0.50–5.0	0.9998	12
Sulfate	50–150	0.9998	25
Phosphate	5.0–15	0.9996	53
Pyrophosphate	0.50–5.0	0.9999	150

[a]LODs estimated from 3 x S/N

Method 2

For the most accurate determination of the molecular mass and stoichiometry of a drug product, a total assay that measures the aminoglycoside free base and sulfate counter ion should be performed. The aminoglycoside free base can be determined using a CarboPac® PA1 column with integrated pulsed amperometric detection (IPAD), while the sulfate composition is determined by IC with suppressed conductivity detection. Neomycin B, tobramycin, and paromomycin have been determined previously with the CarboPac PA1 column and IPAD using a disposable *AAA* Au working electrode.[18-20]

The ICS-3000 instrument is equipped with dual channels that can be used to determine the aminoglycoside free base on one channel while determining sulfate and anionic impurities on the second channel. This configuration simplifies determination of the free base and salt counter ion while reducing the time normally required to change system configurations for the separate assays.

Humatin is a broad-spectrum antibiotic that is supplied as a water-soluble paromomycin sulfate capsule containing the equivalent of 250 mg paromomycin. Humatin was previously analyzed to determine the concentration of paromomycin free base.[20] The present study determined the sulfate counter ion and impurities in Humatin using the IonPac AS11-HC, a column recommended for the determination of a wide variety of inorganic and organic anions in uncharacterized samples.[16] After screening the sample for ionic impurities, we calibrated the system for acetate, chloride, sulfate,

Table 5. Percentages of Sulfate Counter Ion and Anionic Impurities Detected in Humatin Using Method 2						
Sample	**Theoretical Sulfate (%)**	**Experimental Sulfate (%)**	**Acetate (%)**	**Chloride (%)**	**Phosphate (%)**	**Pyrophosphate (%)**
Humatin	23.7	24.7	0.080	0.025	0.23	0.035

phosphate, and pyrophosphate. Table 4 summarizes the calibration data and detection limits determined with the IonPac AS11-HC column and an electrolytically generated potassium hydroxide eluent.

In the previous study, the Humatin capsules were found to contain 274 mg paromomycin free base.[20] Combining those results with the sulfate counter ion concentration in this study indicates that the Humatin capsules contain the equivalent of 364 mg paromomycin sulfate. The sulfate percentage in each capsule is 24.7%, which is 1.0% higher than the theoretical value. The inorganic impurities (acetate, chloride, phosphate, pyrophosphate) in the sample ranged from 0.025–0.23% with total impurities of 0.37%, which is nearly three times the impurity levels found in the Sigma-Aldrich samples.

Table 5 summarizes the data for the sulfate percentages and anionic impurities found in Humatin using the IonPac AS11-HC column. Figure 4A shows the determination of impurities in Humatin (prepared as 2.50 mg/mL paromomycin); Figure 4B shows the same sample diluted 1:10 to determine the percentage of sulfate counter ion. As shown, all target anions are well-resolved on the IonPac AS11-HC column. The peak area RSDs for triplicate injections were <3% for the target anions. The accuracy of the analysis was verified by spiking known concentrations of acetate, chloride, sulfate, phosphate, and pyrophosphate in the sample. The average recovery for the sulfate counter ion was 97.5%. The recoveries for impurities found in the sample were in the range of 93–111%, based on triplicate sample injections.

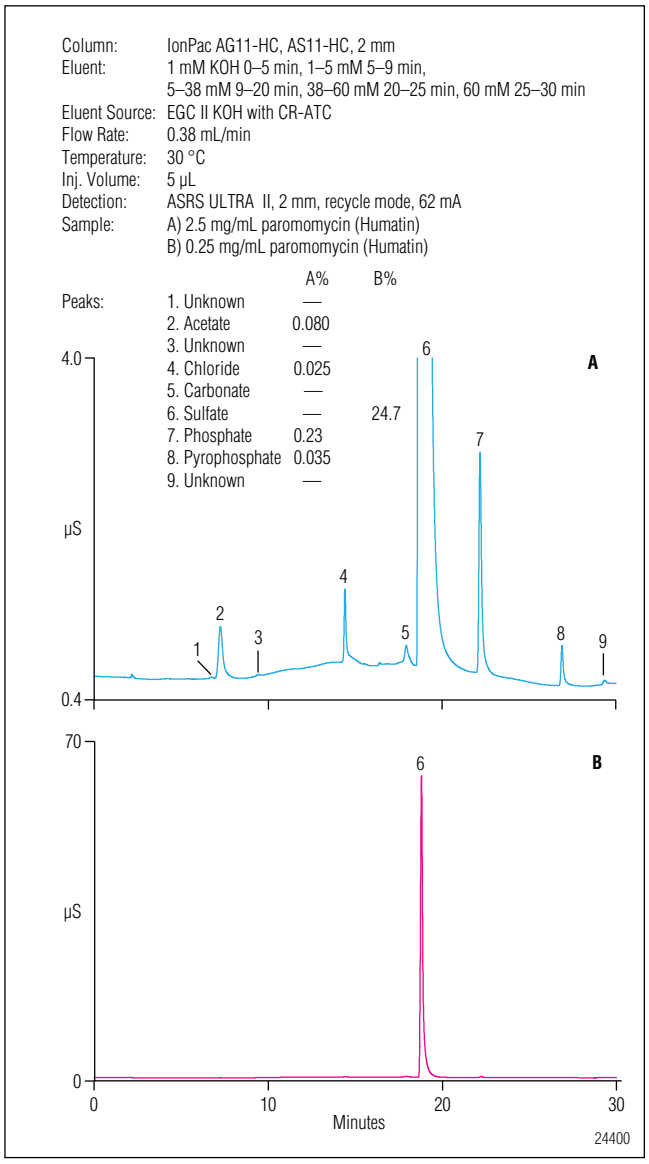

Figure 4. Separation of sulfate counter ion and anionic impurities in Humatin using the IonPac AS11-HC.

CONCLUSION

This application note demonstrates the determination of sulfate counter ion and anionic impurities in aminoglycoside sulfate compounds using either the IonPac AS18 or AS11-HC column. The IonPac AS18 column resolves common inorganic anions in about 16 min and is therefore recommended for high-throughput analysis of well-characterized pharmaceutical matrices. The IonPac AS11-HC column can separate a wide variety of inorganic and organic anions in uncharacterized pharmaceutical formulations, enabling the analysts to obtain more information on the content of a sample.

An RFIC system eliminates the need to manually prepare eluents and increases the level of automation and ease-of-use of the IC system, improving data reproducibility between analysts and laboratories. The excellent sensitivity of the RFIC system reliably detects anionic impurities well below 0.1%. In addition, an ICS-3000 instrument with a dual pump and dual eluent generator enables the analyst to accomplish a total assay of the aminoglycoside at once in a single system. One channel of the dual system can determine the aminoglycoside free base, while the second channel determines the salt counter ion. Overall, an RFIC system is the ideal chromatography system for pharmaceutical companies required to perform counter-ion analyses.

LIST OF SUPPLIERS

VWR Scientific, P.O. Box 7900, San Francisco, CA 94120, USA. Tel: 1-800-252-4752. www.vwr.com

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178 USA. Tel: 800-325-3010.www.sigma-aldrich.com

U.S. Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD 20852, USA. Tel: 1-800-227-8772 www.usp.org

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Determination of *N*-Methylpyrrolidine in Cefepime Using a Reagent-Free Ion Chromatography System

INTRODUCTION

Cephalosporins are currently the most prescribed class of antibiotics worldwide for the treatment of bacterial infections.[1] Their low toxicity and broad range antimicrobial activity against Gram-negative and Gram-positive bacteria have contributed to their widespread use.[1,2] Third generation cephalosporins were developed with enhanced activity against Gram-negative bacilli, but are less active against Gram-positive bacilli. Therefore, further synthetic modifications were incorporated to achieve a more balanced antimicrobial spectrum, which resulted in fourth-generation cephalosporins.[3] Cefepime (Figure 1A) is a semi-synthetic, fourth generation cephalosporin that is commonly prescribed for the treatment of pneumonia, febrile neutropenia, urinary tract infections, skin or soft-tissue infections, and abdominal infections.[4]

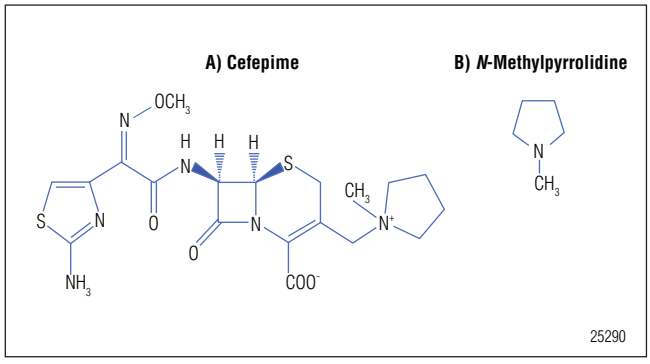

Figure 1. Chemical structures of A) cefepime and B) N*-methyl-pyrrolidine.*

Cefepime is unstable and will degrade slowly even during storage at 4 °C. Degradation is more rapid at higher temperatures, with cefepime content decreasing by 10% at 37 °C in approximately 13 h.[5] This can be problematic if cefepime is kept at room temperature or body temperature during infusion over extended periods of time. Degradation of cefepime includes cleavage of the R2 side chain and opening of the β-lactam ring to yield 2-[[(2-amino-4-thiazolyl)((Z)-methoxyimino) acetyl]amino]acetoaldehyde and *N*-methylpyrrolidine (NMP, Figure 1B).[5] The accumulation of alkaline degradation products increases the pH and therefore increases the rate of cefepime degradation. The degradation of cefepime is also associated with colorimetric changes from a colorless solution (no degradation) to a characteristic orange/brown appearance (complete degradation).[6]

The primary concerns with degradation are loss of potency and the potential toxicity of degradation products to patients. According to one study, an administration of 50 mg/kg NMP in monkeys for 28-30 consecutive days caused ataxia and esotropia ("cross-eyes") during or shortly after treatment.[7] Although this dose was approximately 25-fold higher than the maximum NMP likely to contaminate a daily 6 g dose of cefepime, and no significant effects were observed with lower doses, the potential for side effects is still of concern. Therefore, the determination of NMP in cefepime is critical to assess the purity of the pharmaceutical product due to the potential toxicity of NMP to patients.

The current U.S. Pharmacopeia (USP) compendial method for determining the limit of NMP in cefepime describes the use of cation-exchange chromatography with a 10 mM nitric acid/10% acetonitrile eluent followed by direct conductivity detection. This yields a typical background conductance of approximately 3500 µS.[8,9] The significantly higher background conductance generated by direct conductivity detection (i.e., non-suppressed conductivity detection) relative to suppressed conductivity detection produces higher baseline noise and therefore higher detection limits. Larger injection volumes are required to achieve adequate sensitivity. However, non-suppressed conductivity detection requires low-capacity resins with dilute eluents to achieve a reasonably low background signal. The conflicting requirements of low column capacity and high injection volume make method optimization difficult. The dilute eluents result in long retention times and low sample throughput. A more detailed comparison between non-suppressed and suppressed conductivity detection can be found in Dionex Application Note 157.[10]

The current USP method has additional disadvantages. The USP methods for cefepime hydrochloride and cefepime for injection require nitric acid as a diluent (0.01 N and 0.05 N, respectively). These dilutions yield a sample pH ≤ 2, even though cefepime is most stable at pH values between 4 and 6.[5] Degradation of the sample during testing can lead to artificially high results. Other disadvantages include the 3–4 h time required per injection and a retention time difference of 10–15% between NMP in the test (sample) and standard solutions.[11,12]

This application note describes a cation-exchange chromatography method that significantly reduces the time between injections relative to the current USP method (by approximately 3 h) due to the very low hydrophobic character of the IonPac® CS17 column, enabling a faster elution of strongly retained compounds (e.g., cefepime). The proposed method is also simplified by using an electrolytically generated methanesulfonic acid (MSA) eluent and requires only a deionized water source for operation. The method uses a Reagent-Free™ ion chromatography system with the IonPac CS17 column and suppressed conductivity detection for the determination of NMP in cefepime hydrochloride. The IonPac CS17 is a hydrophilic, moderate capacity (363 µeq/column, 2 × 250 mm), carboxylate-functionalized cation exchanger that was specifically developed for the separation of hydrophobic and polyvalent amines. This stationary phase can also successfully separate hydrophilic amines from common cations. The linearity, detection limits, precision, and recovery of NMP in cefepime hydrochloride are determined. The limit of quantitation for this method is approximately 0.001% NMP, well within the limit of 0.3% set in the USP method.[8]

EQUIPMENT

Dionex ICS-3000 Reagent-Free Ion Chromatography system with Eluent Generation (RFIC-EG™ system) consisting of:

- SP Single Pump or DP Dual Pump module
- EG Eluent Generator module with EGC II MSA eluent generator cartridge (EluGen® II MSA; P/N 058902) and Continuously Regenerated Anion Trap Column (CR-CTC II; P/N 066262)
- DC Detector/Chromatography module (dual temperature zone configuration)
- AS Autosampler with sample tray temperature control

Chromeleon® Chromatography Management Software

1.5 mL glass injection vials with caps and septa (Dionex P/N 058902)

REAGENTS AND STANDARDS

Deionized water, Type 1 reagent grade, 18 MΩ-cm resistivity or higher

Methanesulfonic acid, (Dionex, P/N 033478)

DL-Arginine (Sigma-Aldrich; P/N A4881)

N-Methylpyrrolidine (Sigma-Aldrich, P/N M79204)

SAMPLES

Cefepime hydrochloride (USP, Catalog # 1097636), Lot G0D116 was used in this study.

Cefepime Hydrochloride System Suitability RS (USP, Catalog # 1097647), Lot F0C095 was used in this study.

CONDITIONS

Column:	IonPac CS17 Analytical, 2 × 250 mm (P/N 060561) IonPac CG17 Guard, 2 × 50 mm (P/N 060563)
Eluent:	6 mM MSA from 0–7.5 min, step change to 85 mM at 7.5 min, 85 mM from 7.5–20 min, step change back to 6 mM at 20 min, 6 mM from 20–30 min[a]
Eluent Source:	EGC II MSA with CR-CTC II
Flow Rate:[b]	0.4 mL/min
Injection Volume:	5 µL (full loop)
Temperature:	40 °C (column compartment) (50 °C was used for the system suitability test) 30 °C (detector compartment)
Detection:	Suppressed conductivity, CSRS® 300 (2 mm), AutoSuppression® recycle mode, 100 mA
Background Conductance:	0.5–0.7 µS
Noise:	0.2–0.4 nS
System Backpressure:	~2300 psi

[a]The column was equilibrated an additional 5 min at 6 mM MSA prior to injection.

[b]The equivalent flow rate for this application using a 4 mm CS17 column would be 1.6 mL/min. At this flow rate, the maximum MSA concentration is 62.5 mM due to the suppressor current limitations. Therefore, we strongly recommend using a 2 mm column for this application, which requires relatively low flow rates. This enables the use of a higher MSA concentration to remove cefepime from the column, and reduces eluent consumption and waste production.

PREPARATION OF REAGENTS AND STANDARDS

Eluent Solution

Generate the MSA eluent online by pumping high quality deionized water (18 MΩ-cm resistivity or better) through the EGC II MSA cartridge. Chromeleon software will track the amount of MSA used and calculate the remaining lifetime.

Alternatively, prepare 100 mM MSA by carefully adding 9.61 g of concentrated MSA to a 1 L volumetric flask containing about 500 mL of deionized water. Bring to volume and mix thoroughly. Degas the eluents and store in plastic labware. Proportion this MSA solution with degassed deionized water to generate the appropriate eluent concentrations listed in the method conditions.

Standard Solutions

Accurately dispense and weigh 0.16 mL NMP (d = 0.819 g/mL at 25 °C) beneath a well ventilated fume hood in a 100 mL volumetric flask, bring to volume with deionized water, and mix to prepare a final NMP concentration of 1.31 mg/mL. Store the stock solution at 4 °C when not in use. Prepare working standards for generating the calibration curve with an appropriate dilution of the stock standard in deionized water. Store at 4 °C.

SAMPLE PREPARATION

Accurately weigh 100 mg of cefepime hydrochloride into a 20 mL scintillation vial, dissolve in 10 mL of deionized water, and mix. Prepare the simulated Cefepime for Injection solution by combining 100 mg of cefepime hydrochloride with 72.5 mg of arginine in a 20 mL scintillation vial. Dissolve in 10 mL of deionized water and mix. Prepare the cefepime system suitability sample by weighing 10 mg of the sample into a 1.5 mL glass AS vial. Dissolve the solution in 1 mL of deionized water and mix. Further dilute the sample to a final concentration of approximately 1.4 mg/mL prior to analysis. Note: These solutions should be analyzed within an hour if stored at 25 °C or within 10 h if stored between 4–6 °C. We strongly recommend that the AS sample tray temperature control be set to at least 6 °C for the duration of this method.

RESULTS AND DISCUSSION

Previous studies have demonstrated with mass spectrometry data that degradation of cefepime includes cleavage of NMP and opening of the cephem (β-lactam ring). Similar degradation pathways have been observed with other cefepime related compounds.[7] An increase in the percentage of NMP in the drug would be indicative of a decrease in the potency of the active component. Therefore, it is critical to determine the amount of NMP in cefepime to assess the purity and stability under different storage conditions over time. The USP monograph specifies a limit of <0.3% NMP in cefepime hydrochloride and <1% in Cefepime for Injection.[8,9] The latter is a dry mixture of cefepime hydrochloride and L-arginine. The L-arginine is added at an approximate concentration of 725 mg/g of cefepime to maintain the pH of the constituted solution between 4 and 6.[4]

In an acidic media, cefepime is positively charged and therefore is expected to be retained on a cation-exchange column. This can be problematic due to the large size of the molecule, which can produce a longer retention time, a broader peak shape, and therefore a lower sample throughput. The use of organic solvent, as described in the USP monograph, can decrease the retention time and improve the peak shape of cefepime by reducing the hydrophobic interaction with the stationary phase. However, cefepime is still reported to elute as a broad peak at approximately 55 min. In addition, the monograph recommends that the column be flushed with a column rinse solution, which is a more concentrated solution than the eluent, for 30 min at 1 mL/min after each injection of 10 mg/mL of cefepime hydrochloride. This significantly increases the time required for each sample injection and can cause a lack of retention time stability. There is significant opportunity to improve the current method.

Separation

The IonPac CS18 was initially investigated for the determination of NMP in cefepime, but preliminary experiments showed that the IonPac CS17 was superior for this application. Its low hydrophobic character produced a more efficient cefepime peak, shorter retention times, and higher sample throughput. The separation of NMP in cefepime hydrochloride was optimized on the CS17 by using an initial concentration of 6 mM MSA to elute NMP and then a step change to 85 mM at 7.5 min to remove the cefepime from the column. An increase in column temperature from 30 to 40 °C and flow rate from 0.25 to 0.40 mL/min improved sample throughput by reducing the cefepime retention time from 20 to 12 min.

The IonPac CS17 provides several advantages over the cation-exchange column described in the current USP monograph by 1) allowing the use of a simple acidic eluent with no organic solvent, 2) reducing the cefepime retention time to 12 min relative to the 55 min described in the USP monograph, 3) increasing the sample throughput from approximately 3–4 h to 35 min, and 4) by not requiring a separate "column rinse solution" to remove cefepime from the column.

Figure 2 compares the separation of a 25 µg/mL NMP standard to the cefepime hydrochloride solution prepared in deionized water, using the IonPac CS17 column with an electrolytically generated MSA eluent. The retention time of NMP was approximately 5.3 min in both the standard and sample solutions. An additional 5 min column equilibration was added before each analysis, resulting in a total analysis time of 35 min. It was also determined that common cations, which may appear in the blank or other sources, did not interfere with the determination of NMP.

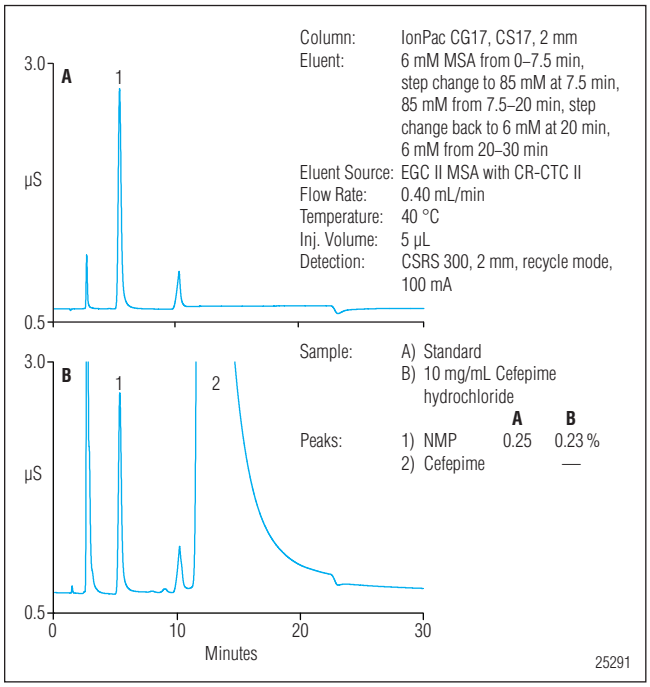

Figure 2. Comparison of the separation of NMP in A) a standard solution and B) cefepime hydrochloride.

Linearity, Limit of Quantitation, Limit of Detection

To determine the linearity of the method, calibration standards were injected in duplicate at eight concentration levels in the range of 0.45–200 µg/mL of NMP. A plot of peak area versus concentration produced a correlation coefficient (r^2) value of 0.9999 using a least squares regression fit. The USP compendial method for validation <1225> specifies a signal-to-noise (S/N) ratio of 10 for the determination of the limit of quantitation (LOQ).[13] The baseline noise was determined by measuring the peak-to-peak noise in a representative one-minute segment of the baseline where no peaks elute. Typical baseline noise for this method using the CSRS 300 suppressor in the recycle mode is 0.2–0.4 nS/min. The LOQ for NMP was determined to be 0.10 µg/mL (S/N = 10), which represents 0.001% NMP in a 10 mg/mL cefepime hydrochloride solution. The limit of detection (LOD) for NMP was estimated to be 0.03 µg/mL (S/N = 3).

Accuracy and Precision

The performance of the method was evaluated with replicate injections of standard and sample solutions, and the recovery of known concentrations of NMP added to cefepime hydrochloride samples. The relative standard deviations (RSDs) of the retention times and measured peak areas were calculated from 10 replicate injections of standard solutions prepared at concentrations of 25 and 50 µg/mL NMP. The calculated peak area precisions for replicate injections of these NMP standards were 1.2% and 0.3%, respectively. The average NMP retention time was 5.3 min and the retention time precision was <0.1% for the standard solutions.

The method was used to assay three independently prepared sample solutions prepared at 10 mg/mL cefepime hydrochloride from a single USP lot over three consecutive days. The average NMP concentration detected in cefepime was 0.236 ± 0.003%. This value meets the <0.3% NMP specification for cefepime hydrochloride according to the USP 31-NF 26 monograph. The intraday retention time and peak area precisions (i.e., a sequence of consecutive injections, n = 10) were ≤0.8% and ≤1.3%, respectively. The between-day retention time and peak area precisions over three consecutive days (i.e., day-to-day, n = 30) were 0.5% and 1.5%, respectively. Table 1 summarizes the amount of NMP determined in the independently prepared cefepime sample solutions and the retention time and peak area precisions.

Table 1. Summary of NMP Determined in Independently Prepared Solutions of 10 mg/mL Cefepime Hydrochloride over Three Consecutive Days

Day	n	Average NMP (%)	Average Retention Time (min)	Retention Time RSD	Peak Area RSD
1	10	0.232	5.3	0.3	1.36
2	10	0.239	5.3	0.8	0.99
3	10	0.236	5.3	0.1	1.01

The accuracy of the method was evaluated by spiking three different concentrations of NMP in the sample and calculating the recoveries based on the difference in response between the unspiked and spiked sample. For the samples spiked with 0.26, 0.52, and 1.0% NMP, the average recoveries for triplicate injections were 102.0 ± 3.2%, 100.6 ± 0.8%, and 97.8 ± 0.4%, respectively, suggesting that the method is accurate.

Cefepime for Injection contains a mixture of the hydrochloride salt and a sufficient amount of L-arginine to provide a reconstituted solution pH between 4 and 6. Arginine is reported to be strongly retained on the cation-exchange column described in the current USP method. The combination of arginine and cefepime, which is also strongly retained, can significantly increase the analysis time required for each sample as has been reported for the USP method.[12] Based on data from previous experiments, arginine is not expected to be problematic for this assay. In addition, the signal response from arginine after suppression should be significantly less than a direct conductivity system. This was confirmed by preparing a solution containing cefepime and arginine. Figure 3 shows an example chromatogram of 10 mg/mL cefepime containing approximately 7.25 mg/mL arginine. As shown, NMP was well-resolved from arginine and no significant difference in the amount of NMP, relative to previous sample preparations, was observed. However, the resolution between NMP and arginine can be improved further by reducing the starting MSA concentration from 6 to 5 mM. The retention time and peak area precisions for triplicate injections of the simulated Cefepime for Injection sample were 0.6% and 1.1%, respectively using the conditions described in Figure 3. (Note: arginine can not be quantified using this method.)

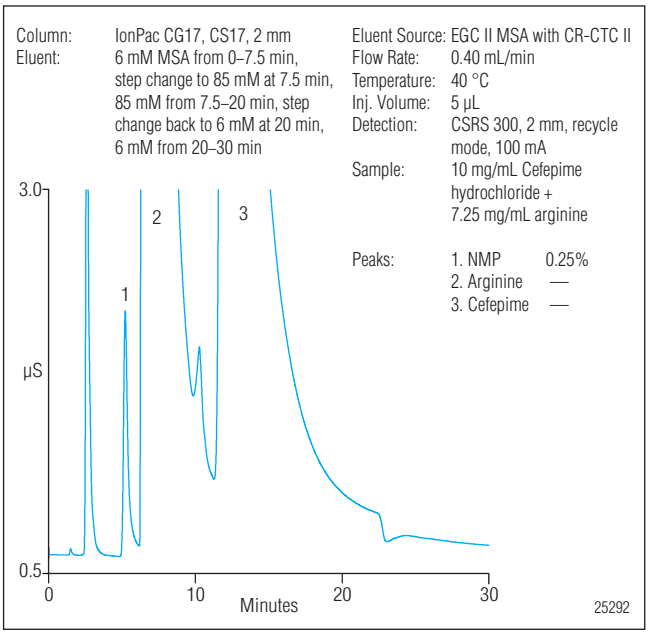

Figure 3. Determination of NMP in a simulated Cefepime for Injection sample.

The method described in this application note was also used to determine the concentration of NMP in the USP cefepime system suitability sample. This material consists of a mixture of 93.8% cefepime hydrochloride, 0.9% cefepime related compound A, and 1.4% cefepime related compound B. The USP requires the analysis of this sample to assess the resolution between cefepime and its related compounds, but does not require the determination of the limit of NMP. However, the analysis of the system suitability sample with the IonPac CS17 column further demonstrates a significant lack of influence of the active pharmaceutical ingredient (i.e., cefepime) on the determination of NMP relative to the current USP method. In addition, the presence of three high molecular weight compounds could have increased the complexity of the analysis due to their strong hydrophobic characteristics.

An initial analysis of this sample using the method conditions previously described produced low resolution between NMP and an unidentified peak, which is most likely derived from the cefepime related compounds, with $R_S = 0.82$. Therefore, further optimization was required to improve the resolution of NMP and remove cefepime and its related compounds from the column within a reasonable time. Reducing the initial MSA concentration from 6 to 2 mM provided an R_S value of 1.39, but increased the NMP retention time from 5.3 to ~12.5 min and total analysis time from 35 to 45 min. In addition, an increase in the column temperature from 40 to 50 °C was determined to further improve the separation by decreasing the NMP retention time to 11.5 min, slightly decreasing the retention of cefepime, and improving the resolution between NMP and the unidentified peak with an R_S value of 1.67. The process of modifying the method conditions to produce an optimum separation for NMP in the USP cefepime system suitability sample was simplified by altering the electrolytically generated MSA eluent concentration using the Chromeleon workstation.

Figure 4 demonstrates the separation of NMP in the cefepime system suitability sample using the optimized conditions shown in the chromatogram. The modified method was used to assay three independently prepared dilutions of approximately 1.4 mg/mL each on three different days. The average NMP concentration in the cefepime system suitability sample was 0.80 ± 0.02%. The detection of a significantly higher NMP concentration, relative to the USP cefepime hydrochloride sample, was expected due to the presence of other cephalosporins related to cefepime, which contain NMP as part of their chemical structure. The intraday retention time and peak area precisions from six replicate injections were <0.1% and ≤0.7%, respectively. The between-day retention time and peak area precisions for replicate injections over three different non-consecutive days (n = 18) were 0.3% and 2.7%, respectively. Table 2 summarizes the data for NMP in three independently prepared cefepime system suitability samples.

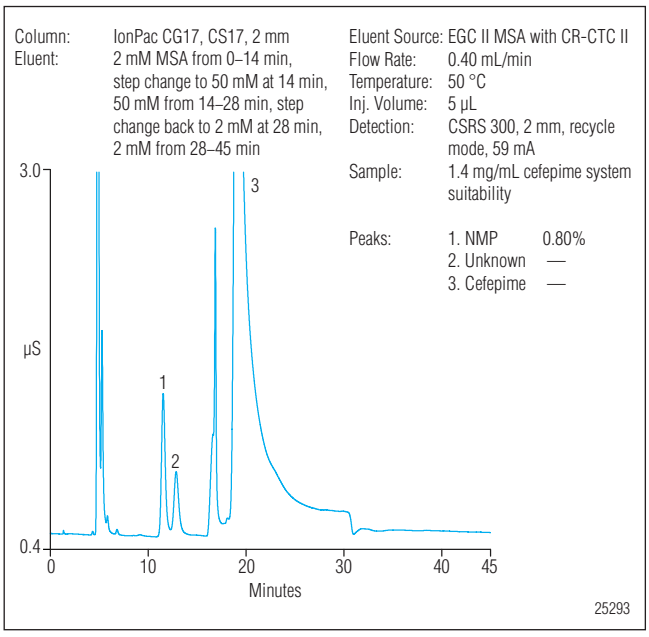

Figure 4. Determination of NMP in USP Cefepime Hydrochloride System Suitability RS sample.

Table 2. Summary of NMP Determined in Independently Prepared Solutions of 1.4 mg/mL Cefepime Suitability Sample over Three Days					
Day	**n**	**Average NMP (%)**	**Average Retention Time (min)**	**Retention Time RSD**	**Peak Area RSD**
1	6	0.81	11.5	0.03	0.39
2	6	0.82	11.5	0.02	0.74
5	6	0.77	11.5	0.04	0.35

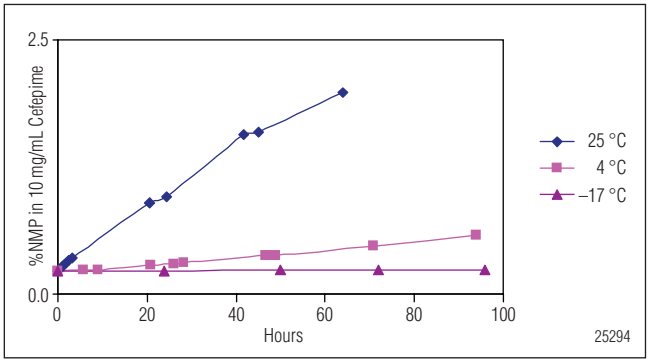

Figure 5. Stability of NMP in USP Cefepime Hydrochloride RS sample.

Sample Stability

An earlier study demonstrated that the percentage of cefepime remaining after 24 h was 90% when stored at 25 °C. This is currently the USP limit for some cefepime related compounds; however, neither the US nor the European Pharmacopoeia has set a limit of degradation of cefepime in solution.[6] A decrease in the cefepime concentration should correspond to an increase in the NMP concentration. Previous research has shown that NMP in cefepime does increase if stored at 25 °C or at elevated temperatures (40 and 60 °C).[11,12] In this study, we examined the stability of NMP in cefepime when stored at room temperature (25 °C), in a cooled AS sample tray (4 °C), and in the freezer (-17 °C) up to four consecutive days. Three independently prepared solutions containing 10 mg/mL cefepime hydrochloride each were subjected to the different temperature environments and analyzed in duplicate. Figure 5 shows the results from this study. As illustrated in this graph, the most significant increase in NMP was observed when the solution was stored at 25 °C. In approximately one hour, the NMP concentration increased from 0.23 to 0.27% and continued to increase to nearly 2% over the next three days. No further studies were attempted for NMP stored at this temperature due to the formation of a precipitate and a change in solution color, which is in agreement with previous observations.[14] For cefepime stored at 4 °C, the NMP concentration did not significantly change within 6 h. However, within approximately 24 h the percent NMP increased from 0.22 to 0.29%. For cefepime stored at -17 °C, no significant increase in NMP concentration was observed after 96 h of storage.

CONCLUSION

The IonPac CS17, a hydrophilic weak-acid cation-exchange column, combined with suppressed conductivity detection, was successfully used for the determination of NMP in cefepime hydrochloride, cefepime for injection, and cefepime system suitability samples. This method enabled the separation of NMP in less than 10 min. It also provided efficient removal of strongly retained compounds that allowed significantly lower analysis times and good retention time stability relative to the current method for the limit of NMP in cefepime hydrochloride described in USP monograph USP 31-NF 26. In addition, the described method used a simple electrolytically generated MSA eluent, without the organic solvent required for the method in the USP monograph. This also reduced the time required to optimize the separation of NMP from an unidentified peak in the system suitability sample, because the eluent concentration could be controlled simply by changing the current, instead of reformulating eluents. The exceptionally low baseline background and noise using suppressed conductivity detection enabled the quantification of 0.001% NMP in cefepime hydrochloride, which is significantly better than would be anticipated using a non-suppressed conductivity system.

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Determination of Cefepime and Cefepime-Related Substances Using HPLC with UV Detection

INTRODUCTION

Cephalosporins are currently among the most widely prescribed antibiotics in hospitals.[1] Development of these antibiotics has led to compounds with a broad spectrum of activity against both Gram-positive and Gram-negative bacteria and with low toxicity profiles. Derivatives of penicillins, these drugs universally contain a β-lactam ring (Figure 1). This four member ring is inherently strained and prone to hydrolysis and photolysis, limiting its stability.[2] In addition to degradation products, isomers and dimers of the synthesis reagents are produced during manufacture of the compound. These impurities can persist in the drug product and many are of unknown toxicity.

Despite extensive research on this class of drugs, quantitative analysis and purity assays remain problematic.[3] The chemical instability of the strained β-lactam ring system and the variable stability of different substituted groups (R1 and R2 in Figure 1) require that analysis of these compounds be rapid. In addition to the need for fast analysis times, superior resolution is necessary to separate synthetic byproducts. Both the chemical instability and the structural similarities of the impurities to the desired product make analysis of these antibiotics difficult.

Cefepime, a fourth generation cephalosporin, is a broad spectrum antibiotic with improved activity against Gram-negative bacteria over other commercially available cephalosporin drugs.[4] Analysis of cefepime purity is particularly challenging due to isomeric and other impurities with similar structures (Figure 2). Additionally, cefepime is particularly labile and its stability is highly

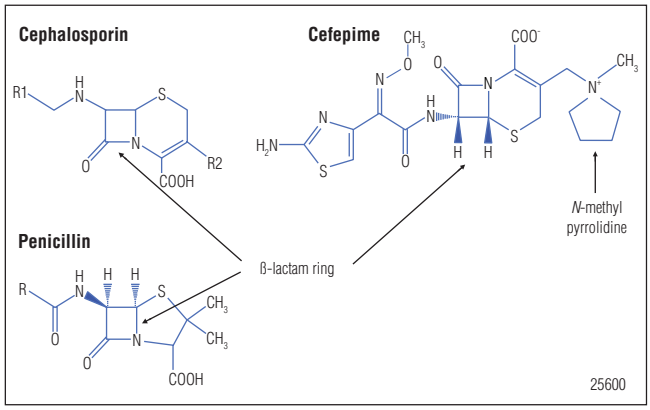

Figure 1. Penicillin, cephalosporin, and cefepime.

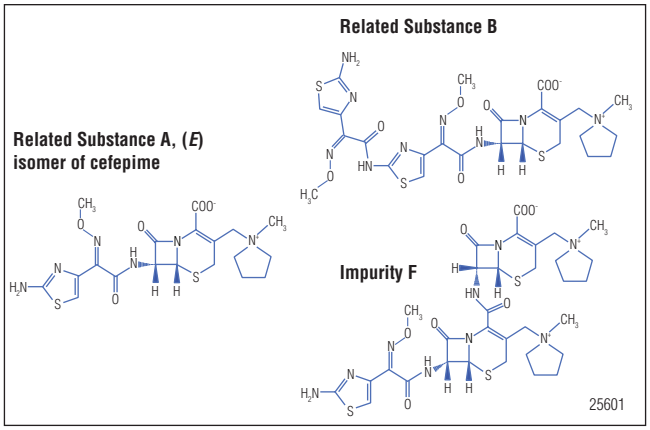

Figure 2. Cefepime-related substances.

pH dependent, in part due to rapid N-methylpyrrolidine (NMP) cleavage at room temperature.[5] An IC method for the determination of NMP in cefepime preparations is described in Dionex Application Note 199.[6]

Both the United States Pharmacopeia (USP) and the European Pharmacopeia (EP) publish monographs for determining the concentration and purity of cefepime.[7-8] The related substances methods for purity analysis that are provided by the EP and USP are chromatographically similar, requiring a type L1 column and chromatographic conditions consisting of a short isocratic elution using 10/90 acetonitrile/5 mM potassium phosphate followed by a linear gradient of acetonitrile in 5 mM aqueous monobasic potassium phosphate. Mobile phase preparation details and subsequent calculations to evaluate the purity of the cefepime differ, but the analytical methods are the same. In addition to the purity methods published by these two organizations, faster concentration assays are also available. The EP uses a modified version of the purity method to determine the cefepime concentration. This is convenient in that it does not require additional mobile phase preparation, column equilibration, or system set up. Rather than use the isocratic portion of the purity method, the USP method uses a pentane sulfonate/acetonitrile based mobile phase for the assay method.

This application note describes modifications to the related substances method to maximize either the speed or the resolution. The Acclaim® 120 C18, 3 µm column is used, an L1 column as defined by the USP. It is manufactured using high purity silica with a 120 Å pore diameter, with very high surface coverage and extremely low metal content. This C18 phase exhibits low polarity, high hydrophobicity, and good steric selectivity, which results in a high-capacity column with unique selectivity. This steric selectivity makes it an excellent choice for resolving structurally similar compounds. The performance of this column is compared to data from the same method using the Acclaim PolarAdvantage (PA), a sulfonamide-embedded polar stationary phase column. Finally, a modified version of this method for use as a concentration assay for cefepime is discussed. Linearity, precision, the limit of detection (LOD), and the limit of quantification (LOQ) are demonstrated for the concentration assay.

The Acclaim 120 C18, 3 µm can be used to meet and exceed the criteria set by the USP for determining related substances and assaying the purity of cefepime. The improved efficiency of the column allows for shorter run times without sacrificing resolution, leading to fast, high-resolution methods. The lower flow rate used with this column saves resources and produces less waste than the original assay.

EQUIPMENT

Dionex UltiMate® 3000 Intelligent LC system:

- SRD-3600 Solvent Rack (Dionex P/N 5035.9230)
- DGP-3600M pump (Dionex P/N 5035.0050)
- WPS-3000T autosampler (Dionex P/N 5820.0020)
- FLM-3100 flow manager (Dionex P/N 5720.0010)* or TCC-3200 column compartment (Dionex P/N 7522.0025)
- VWD-3400 detector (Dionex P/N 5074.0010)
- Semi-Micro Peek flow cell, 2.5 µL (Dionex P/N 6074.0300)

Chromeleon® 6.8 Chromatography Workstation

Glass injection vials with caps and septa, 1.5 mL (Dionex P/N 055427)

Nalgene® Filter Unit, 0.2 µm nylon membrane, 1 L capacity (Nalgene P/N 164-0020)

**An FLM-3100 flow manager was used as a temperature controlled column compartment with the flow controller disabled. The FLM is not necessary and a thermostatted column compartment (TCC) can be used for this application.*

REAGENTS AND STANDARDS

Deionized water, Type 1 reagent grade, 18 MΩ-cm resistivity.

Acetonitrile, HPLC Grade or better (B&J P/N 015-4)

Potassium phosphate, monobasic, HPLC grade (Fisher P/N P286-1)

Potassium hydroxide concentrate, 45% (J T Baker P/N 314301)

ortho-Phosphoric acid, HPLC grade, (Fisher P/N A260-500)

pH buffers, 4.00 (VWR P/N 34170-127) and 7.00 (VWR P/N BDH5046-500mL)

SAMPLES

Cefepime Hydrochloride (USP, Catalog # 1097636), Lot G0D116

Cefepime Hydrochloride System Suitability RS (USP, Catalog # 1097647), Lot F0C095

CONDITIONS

Column:	Acclaim 120 C18, 3 µm Analytical, 2.1 × 150 mm, (Dionex P/N 059130)
Mobile Phases:	**USP Method** A: 90/10 5 mM potassium phosphate/ acetonitrile B: 50/50 5 mM potassium phosphate/ acetonitrile -or- **Increased Resolution Method** A: 94/6 5 mM potassium phosphate/ acetonitrile B: 50/50 5 mM potassium phosphate/ acetonitrile
Gradient:	**USP Method** 100% A for 10 min, 0–50% B in 20 min, 50% B for 5 min, 9 min of equilibration prior to injection -or- **Shortened Runtime Method** 100% A for 8 min, 0–50% B in 10 min, 50% B for 3 min, 5 min of equilibration prior to injection
Flow Rate:	0.20 mL/min
Temperature:	30 °C (column compartment)
Inj. Volume:	1 µL
Detection:	Variable Wavelength UV-Vis detector, 254 nm
Noise:	~12-18 µAU
System Backpressure:	~110 bar (~1600 psi)

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phases

Mobile Phase A:

Dissolve 0.68 g of HPLC grade monobasic potassium phosphate in 1000 mL of DI water. Remove 100 mL of the solution and add 100 mL of HPLC grade acetonitrile. Adjust the pH to 5.00 ± 0.05 with 100 fold diluted 45% KOH. Filter the mobile phase through a 0.2 µm nylon filter unit and degas. Transfer the solution to a glass eluent bottle.

Optional: To prepare 94/6 5 mM monobasic potassium phosphate/acetonitrile, dissolve 0.68 g of HPLC grade monobasic potassium phosphate in 1000 mL of DI water. Remove 60 mL of potassium phosphate solution and add 60 mL of HPLC grade acetonitrile. Continue the preparation as described above.

Mobile Phase B:

Dissolve 0.34 g of monobasic potassium phosphate in 500 mL of DI water. Add 500 mL of acetonitrile. Adjust the pH to 5.0 ± 0.05 with 100-fold diluted 45% KOH or 100-fold diluted HPLC grade phosphoric acid. Filter the mobile phase through a 0.2 µm nylon filter unit and degas. Transfer the solution to a glass eluent bottle.

Consistency in the amount of acetonitrile in the mobile phase is critical to reproducible chromatography between mobile phase preparations. Care should be taken to ensure that the amounts of acetonitrile added are reproducible and that degassing does not remove the solvent from the aqueous phosphate solution. It is recommended that the mobile phase be used as soon as practical after filtration. Additionally, cefepime acts as a zwitterion over a broad pH range.[9] With zwitterionic compounds, the mobile phase pH can strongly affect both retention time and peak shape. Care must be taken during preparation of the mobile phase to adjust the pH properly in order to avoid peak shifting and broadening.[10]

Autosampler Syringe Wash Solution

In order to prevent carryover from the autosampler, a wash solution of 10% acetonitrile in DI water was used. Carryover from injections of 1.4 mg/mL cefepime solutions was not observed when this wash solution was used.

Sample Solutions

Prepare stock solutions of cefepime hydrochloride gravimetrically by accurately weighing 10.0 mg of powder in a 1.5 mL vial, dissolving the powder in 1 mL (1 g) of deionized water, and mixing thoroughly. Prepare the cefepime system suitability standard (SSS) similarly by weighing 10 mg of the sample in a 1.5 mL glass AS vial, adding 1 mL of deionized water, and mixing. Store these stock solutions at -19 ºC or below. Prepare samples volumetrically by diluting an aliquot of stock solution in Mobile Phase A to produce a final concentration of 1.4 mg/mL prior to analysis. Prepare standards for testing linearity of the assay method by volumetric dilution of the cefepime hydrochloride stock standard with Mobile Phase A to produce the desired concentration. Note: Samples should be analyzed within 24 h if stored in the dark at 4 ºC. We strongly recommend that the WPS autosampler sample compartment temperature control be set to 4 ºC for the duration of this method.

RESULTS AND DISCUSSION

Separation

The SSS was used to test the separation on the Acclaim 120 C18 column. Figure 3 shows the separation of both the SSS and cefepime using the USP conditions. The separation meets the USP requirements for the method. The asymmetry of the cefepime peak in the SSS sample is 1.4, meeting the requirement of ≤1.5. The resolution between cefepime and cefepime-related substance A (RSA) is 23, and the resolution between cefepime and cefepime-related substance B (RSB) is 120, exceeding the requirements of 5 and 10 for RSA and RSB respectively. The capacity factor, k', is 1.0, greater than the requirement of 0.6, and 9600 theoretical plates are calculated for cefepime, more than double the 4000 specified. The relative retention times are 2.5 for RSA and 6.6 for RSB. In addition to the related substances specified by the USP, another peak is visible just past cefepime. This peak has been assigned as impurity F as described in EP method 2126.

Improved Resolution

In order to improve the resolution between cefepime and impurity F, Mobile Phase A was modified by reducing the amount of acetonitrile from 10% to 6%. Figure 4 shows the separation of the SSS sample on an Acclaim 120 C18 3 µm under these conditions. In this case, the impurity F peak is baseline resolved from cefepime. Another option for improving the resolution that does not require changing the mobile phases, is to use an Acclaim PA 3 µm, 2.1 × 150 mm column. This column contains a phase with an embedded polar group that is compatible with 100% aqueous mobile phases, is well suited for samples containing polar and nonpolar analytes, and delivers excellent peak shapes for acidic and basic compounds. Using this column and the same mobile phase conditions shown in Figure 3, the resolution between cefepime and impurity F is significantly improved compared to the Acclaim 120 C18, increasing from 2.5 to 4.9 (Figure 5). The relative retention times for RSA and RSB are 2.1 and 6.2, respectively. In addition to the enhanced resolution, the asymmetry of the cefepime peak is also improved on this column (1.1 vs 1.4) and the overall peak shapes are excellent.

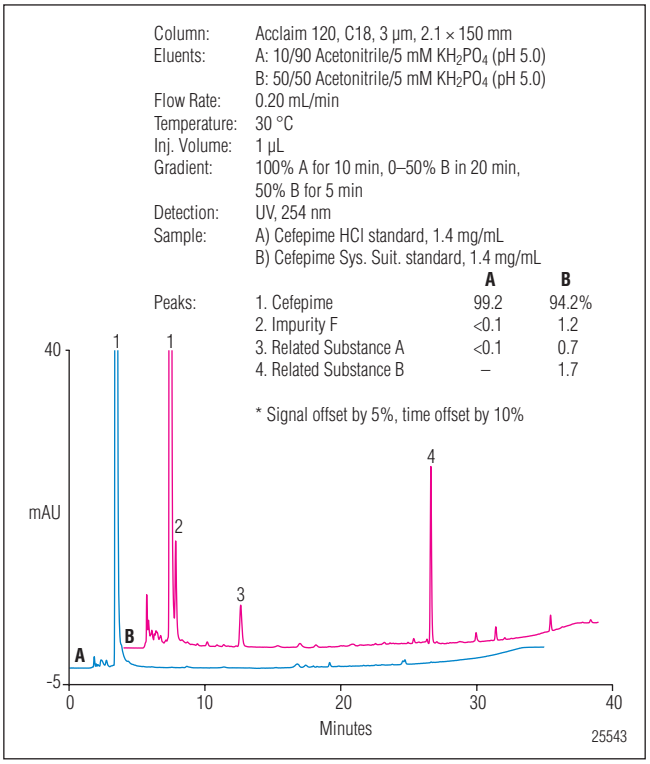

Figure 3. Separation of cefepime and Cefepime System Suitibility Standard.

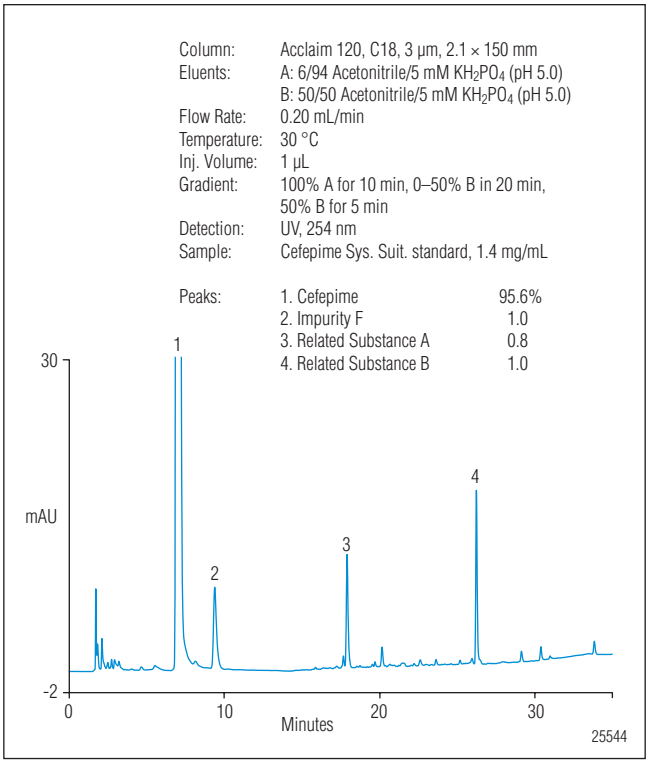

Figure 4. Resolution improvement with 6% acetonitrile in mobile phase A.

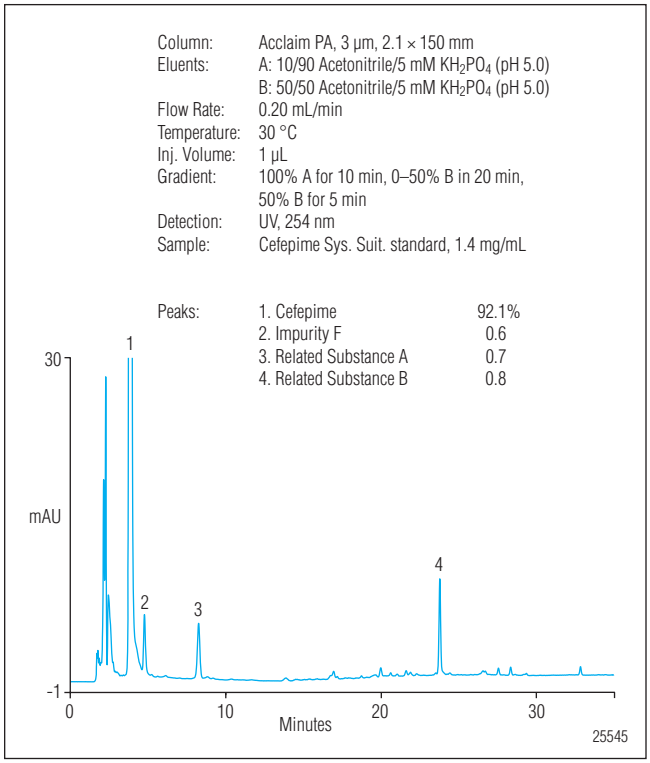

Figure 5. Improved resolution using the Acclaim PA column.

Faster Analysis

Due to the smaller column size and the 3 µm particle size of the Acclaim 120, C18 column, the gradient can be considerably shortened and still meet the USP conditions. Figure 6 illustrates the separation possible with a gradient method that removes 20 min from the run time for each injection. This shortened gradient meets the USP criteria. The only differences are slight changes in the resolution between cefepime and RSA (25) and RSB (110). The precision of this shortened gradient was tested, and retention times and peak areas were reproducible (Table 1).

Table 1. Reproducibility of Rapid Gradient, 6% Acetonitrile Mobile Phase A, n=10

Analyte	Retention Time (min)	Area (mAU*min)	Relative Area (%)	Retention Time Precision (RSD)	Peak Area Precision (RSD)
Cefepime	6.95	168.5	95.6	0.05	0.10
Related Substance A	9.39	1.73	1.0	0.06	0.22
Related Substance B	15.0	1.40	0.8	0.03	0.82
Impurity F	19.0	1.76	1.0	0.03	0.52

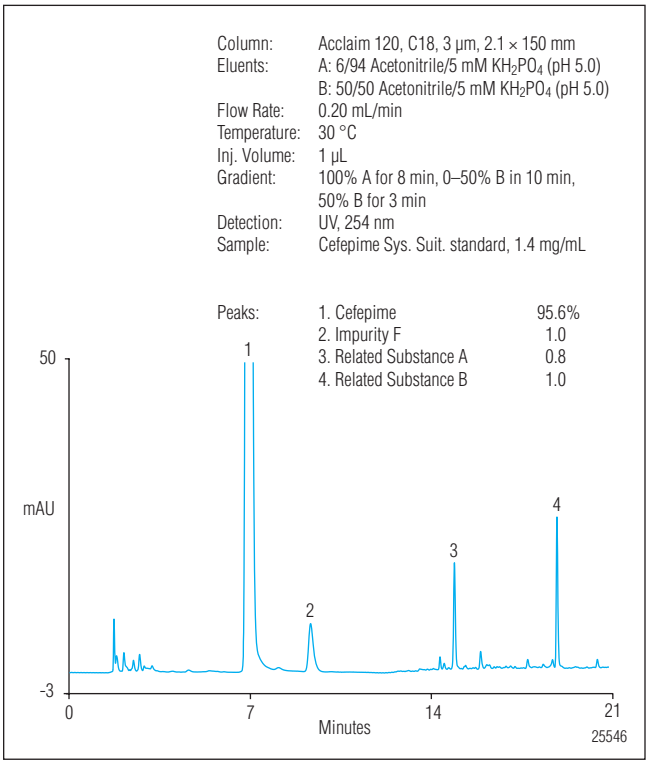

Figure 6. Separation with a rapid gradient using the Acclaim 120 C18 column

Table 2. Linearity, Precision, LOD, and LOQ for Isocratic Cefepime Assay Methods							
Mobile Phase Composition	**Range (µg/mL)**	**Coor. Coeff. (r^2)**	**LOD (µg/mL)**	**LOQ (µg/mL)**	**Retention Time (min)**	**Retention Time Precision (RSD)**	**Peak Area Precision (RSD)**
6/94 Acetonitrile/ 5mM KH_2PO_4 (pH 5.0)	1.0–1400	0.99995	0.062	0.20	6.95	0.02[a]	0.08[a]
10/90 Acetonitrile/ 5mM KH_2PO_4 (pH 5.0)	1.0–1400	0.99989	0.032	0.12	3.62	<0.01[b]	0.04[b]

[a]n=10
[b]n=15

Quantification Assay Linearity, Limit of Quantitation, Limit of Detection, and Precision

Isocratic methods for assaying the concentration of cefepime using mobile phase A were tested for linearity, LOQ, LOD, and precision. Both 10% acetonitrile and 6% acetonitrile in 5 mM monobasic potassium phosphate mobile phases were tested. The 10% acetonitrile mobile phase A run time is very fast (6 min), but the 6% acetonitrile mobile phase provides better resolution with a 10 min run time. The EP recommends a run time of 1.4 times the retention time of cefepime. However, if the samples contain significant amounts of RSA, the time should be extended to 12 min for mobile phase A containing 10% acetonitrile and 25 min for mobile phase A containing 6% acetonitrile, in order to avoid quantification interference from RSA in subsequent injections. The linearity, LOQ, LOD, and precision data for both mobile phases using the isocratic assay method are listed in Table 2. In both cases, the linearity and precision are excellent. The LOD is improved for 10% acetonitrile mobile phase due to the shorter retention time leading to narrower peak widths. In this method, RSB and other less polar compounds are retained on the column. For this reason a periodic 30 min wash of 50:50 mobile phase A/mobile phase B is recommended to preserve column life.

Sample Stability

Cefepime solutions are sensitive to hydrolytic and photolytic decompositions. To determine the stability of the cefepime solutions in the mobile phase, samples were studied at ambient room temperature (average of 25 ºC), 4 ºC (WPS autosampler), and -19 ºC (freezer). All samples were stored in the dark, with the exception of the room temperature samples that were stored in a covered HDPE container. A single solution of 1.4 mg/mL cefepime was prepared in a glass 10 mL volumetric flask. The solution was transferred to individual vials for storage under the three conditions. Samples were injected for 5 consecutive days at 24, 48, 72, and 96 h from the initial injections. From these data, storage at both 4 ºC and -19 ºC conditions were nearly equivalent, with no change in the peak area of cefepime over 96 h (4 days). Storage at room temperature resulted in a 2.9% loss of peak area after 24 h. After 96 h of storage at room temperature there was a 12% loss of peak area (Figure 7A). Storage at room temperature prior to analysis for any extended length of time is not recommended. Comparison of the purity of the cefepime by relative peak area revealed more subtle effects of thermal instability (Figure 7B). Over 96 h there was no change in the absolute peak area or the relative peak area of cefepime in samples stored at -19 ºC. However, storage at 4 ºC did result in a slight decrease in the purity of cefepime from 99.4% to 99.0%. The absolute peak area data for samples stored at 4 ºC does not show this change. Similar to the results from measuring the absolute peak area, the purity data for samples stored at room temperature showed dramatic loss of cefepime from the solution. During the 96 h of the study, the purity by relative peak area of the remaining cefepime dropped from 99.4% to 93.3%. It is strongly recommended that the samples are stored in the autosampler at 4 ºC and that analysis for related substances be completed within 24 h of sample preparation.

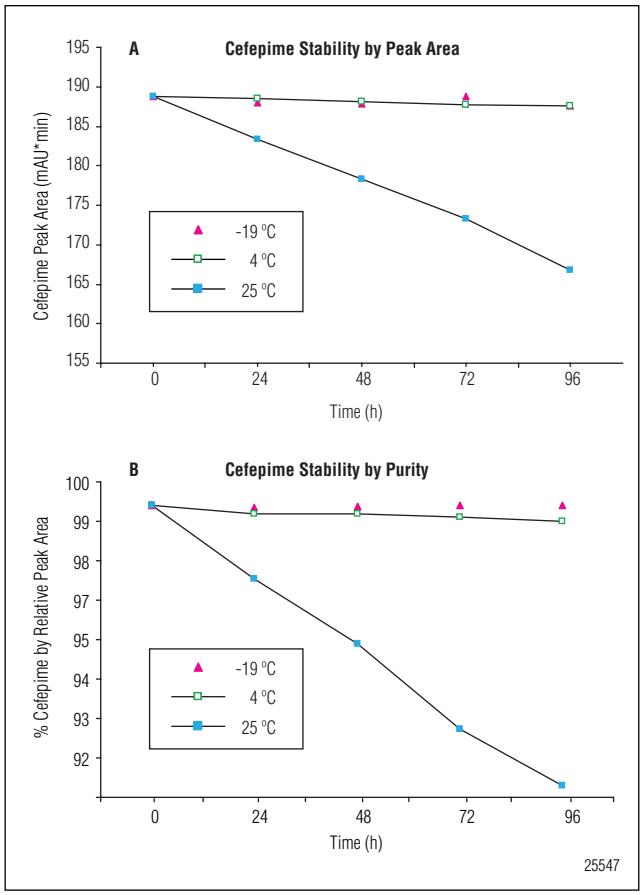

Figure 7. Stability of cefepime over 96 h. A) Absolute cefepime peak area. B) Relative cefepime peak area.

CONCLUSION

In this application note, the Acclaim 120 C18 column, an L1 column with good steric selectivity, combined with UV detection was successfully used for the determination of cefepime and cefepime-related substances, and for assaying cefepime hydrochloride. The methods were modified to decrease the time needed for analysis and improve the resolution as compared to the current methods described in USP monograph USP 30-NF 25-Supplement 1. In addition, the described method uses the same mobile phase preparation for both methods, rather than two separate types, adding convenience and time savings to the method. Finally, the low flow rates used in this method save time and resources spent on mobile phase preparation and reduce waste production.

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Sulfonamide Antibiotics on the Acclaim 120 C18 RSLC Column

INTRODUCTION

During the first half of the 20th century, sulfonamide antibiotics played a major role in the fight against bacterial infections. Sulfanilamide, also called sulfa, traces its beginnings back to the early 1930's, predating penicillin. The accidental poisoning of a large number of people who ingested an untested formulation of sulfanilamide played a major role in bringing about the passage of the Federal Food, Drug and Cosmetic act of 1938 and the modern era of federal drug regulation. There are many sulfanilamide containing drugs in use today and are prescribed for the treatment of acne and urinary tract infections.

METHOD

This application demonstrates how a fast gradient employed on a 2 µm particle UHPLC column can be used to separate eight sulfonamide antibiotics in a single run of only three minutes. The column selected is an Acclaim 120 C18 with dimensions of 2.1 × 100 mm. The Ultimate 3000 RS system provides UHPLC separations with the ease of conventional HPLC. Detection is accomplished using UV absorbance at 265 nm.

RESULTS

As shown in the figure below, the method provides baseline resolution of all 8 sulfonamides in a short period of time. This separation is clearly suitable for assaying any of the compounds listed. Additionally, because of the short analysis time, relatively low flow rate (0.75 mL/min) and 2.1 mm diameter column, consumption of mobile phase is quite low. This reduces cost of solvent, cost of waste disposal, and cost of operation.

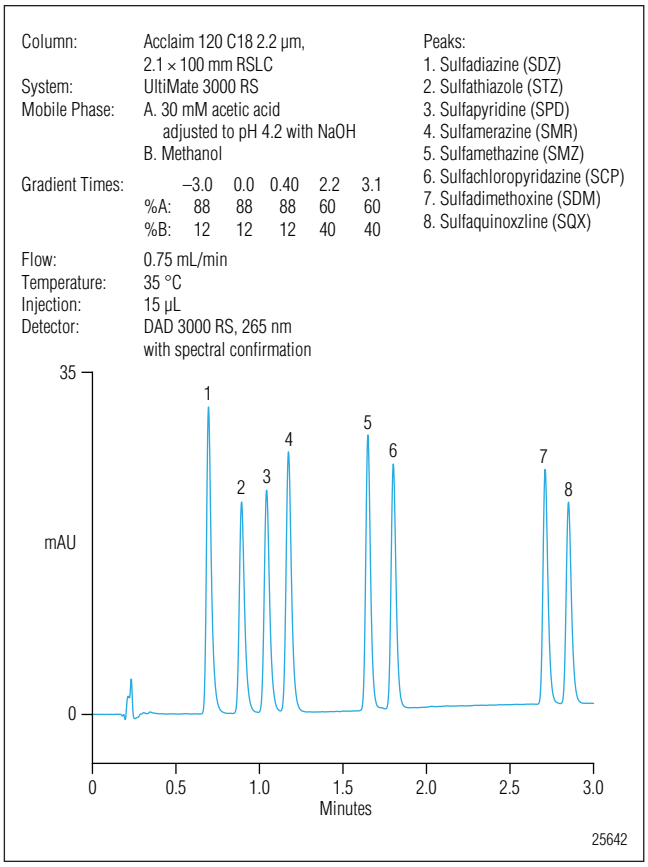
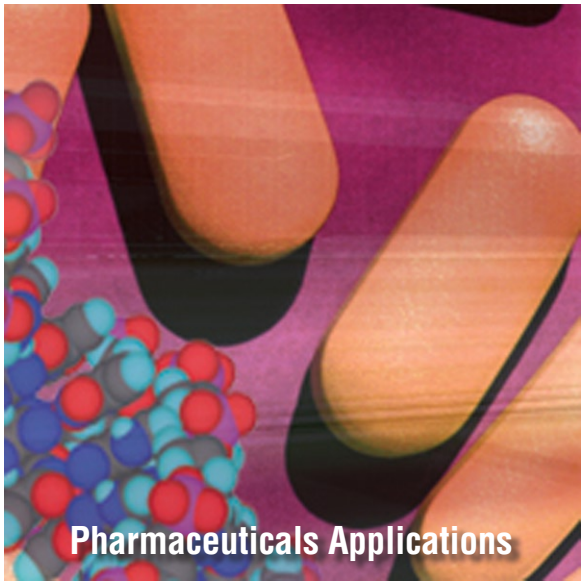


Figure 1. Sulfonamide antibiotics on Acclaim 120 C18 RSLC.

DIONEX

Part II: Analysis of Controlled Drugs



Analytes

Bethanechol
Nevirapine
Carbachol
Verapamil
Glucosamine
Urea and Allantoin
Sumatriptan
Budesonide and Related Substances
Glucocorticosteroids
Propafenone
Loperamide
Trimipramine

Determination of Bethanechol by Ion Chromatography

INTRODUCTION

Amines are widely used in various industries, such as the chemical, manufacturing, power, and pharmaceutical industries. In pharmaceuticals, amines may be used in the production of emulsifying agents and medications. Bethanechol chloride, 2-[(aminocarbonyl)oxy]-*N,N,N*-trimethylpropanaminium chloride, is a quaternary ammonium compound that is structurally and pharmacologically related to acetylcholine. It is administered either as an injection or tablet for the treatment of urinary retention. A method in the *U.S. Pharmacopeia* (USP) 24 NF 19 (page 230) recently proposed that the gravimetric assay for bethanechol chloride be replaced with a more specific and rugged ion chromatography assay that also measures stability.[1] The proposed method specifies the use of a Dionex IonPac® CS14 separator column using a manually prepared methanesulfonic acid (MSA) eluent and suppressed conductivity detection. In this application note, we applied electrolytic on-line generation of MSA, using the EG40 eluent generator to optimize reproducibility, convenience, and method transfer between laboratories. We describe the linearity, method detection limits (MDLs), and potential interferences during the determination of bethanechol and its degradation product, 2-hydroxypropyltrimethylammonium chloride.

EQUIPMENT

Dionex DX-600 ion chromatography system consisting of:
- GP50 gradient pump with vacuum degas option
- ED50A Electrochemical Detector
- EG40 Eluent Generator
- EluGen® EGC-MSA cartridge (Dionex P/N 053922)
- AS50 Autosampler
- AS50 Thermal Compartment with conductivity cell
- Chromeleon® 6.4 Chromatography Workstation

REAGENTS AND STANDARDS

Deionized water, Type I reagent-grade, 17.8 MΩ-cm resistivity or better

Combined Six Cation Standard-II (Dionex P/N 046070)

Bethanechol chloride (U.S. Pharmacopeia)

CONDITIONS

Columns:	IonPac CS14 Analytical, 4 × 250 mm (Dionex P/N 044123) IonPac CG14 Guard, 4 × 50 mm (Dionex P/N 044124)
Eluent:	20 mM MSA
Eluent Source:	EG40
Flow Rate:	1.0 mL/min
Temperature:	30 °C
Injection:	25 µL
Detection:	Suppressed conductivity, CAES™ (Dionex P/N 056118) Power setting 67 mA
Expected Background:	~0.2 µS
Expected Backpressure:	~2100 psi
Run Time:	15 min

PREPARATION OF SOLUTIONS AND REAGENTS

Reagent Water

Use Type I reagent-grade distilled or deionized water with a specific resistance of 17.8 MΩ-cm or greater, filtered through a 0.2-µm filter immediately before use.

Eluent Solution

Generate 20 mM MSA eluent on-line by pumping deionized water through the EG40 with an EGC-MSA cartridge. Chromeleon software tracks the amount of MSA used and calculates the remaining lifetime. Replace the EGC-MSA cartridge when the remaining lifetime drops below 10%.

Alternatively, prepare 20 mM MSA by diluting 50 mL of 0.4 N methanesulfonic acid eluent concentrate (Dionex P/N 057562) to 1.0 L with deionized water. Degas the eluent by sonicating under vacuum for 10 min or by sparging with helium. Store the eluent in plastic labware.

As another alternative, prepare a 1.0 N methanesulfonic acid stock solution. Carefully add 96.10 g of methanesulfonic acid (MSA, >99%, Dionex P/N 033478) to a 1-L volumetric flask containing about 500 mL of deionized water. Dilute to the mark and mix thoroughly. Prepare 20 mM MSA by diluting 20 mL of the 1.0 N MSA stock solution to 1.0 L with deionized water. Degas the eluent and store in plastic labware.

Stock Standard Solutions

1000 mg/L Bethanechol Standard Solution

To prepare the stock standard, weigh 0.050 g bethanechol chloride into a 125 mL plastic bottle, add 50.0 g deionized water, sonicate to dissolve, and mix.

1000 mg/L 2-Hydroxypropyltrimethylammonium (2-HPTA) Standard Solution

To prepare the stock standard solution, weigh 0.050 g bethanechol chloride into a 125 mL plastic bottle, add 50 mL of 0.1 N NaOH, sonicate to dissolve, and mix. Allow five days for bethanechol to completely hydrolyze to 2-HPTA chloride.

Working Standard Solutions

Prepare composite working standards at lower concentrations by diluting appropriate volumes of the stock standards with deionized water. For the calibration shown here, the following standards for bethanechol and 2-HPTA were prepared: 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 25, 50, 100, 200, 500, and 1000 mg/L. The only exception was that the maximum 2-HPTA was 500 mg/L.

SYSTEM PREPARATION AND SETUP

Prepare the CAES for use by hydrating the eluent chamber. Use a disposable plastic syringe to slowly push approximately 3 mL of deionized water through both the "Eluent-In" port and "Regen-In" port of the suppressor. Allow the suppressor to sit for approximately 20 min to fully hydrate the suppressor. For more information on CAES operation, consult the *Installation and Troubleshooting Instructions for the CAES* (Document No. 031770-02).

Install the EG40, connect it to the network, and configure it with the Chromeleon chromatography data system. Condition the EluGen MSA cartridge, as directed in the EG40 manual, by running a gradient from 1 to 60 mN MSA in 20 min, then 60 mN for 40 min at 1.0 mL/min. For instructions on EG40 installation and use, see the *Operator's Manual* for the EG40 eluent generator system (Document No. 031373).

Remove the 0.005 in. PEEK backpressure tubing temporarily installed during conditioning of the EluGen cartridge. Install a 4 × 50 mm IonPac CG14 guard column and a 4 × 250 mm IonPac CS14 column. Make sure the system pressure displayed by the pump is at least 2000 psi when 20 mM MSA is delivered at 1.0 mL/min, because the EG40 high-pressure degas tubing assembly requires at least 2000 psi (14 MPa) backpressure to efficiently remove hydrolysis gas from the eluent. If necessary, install backpressure coils supplied with the EG40 ship kit to bring the system pressure between 2000 and 2800 psi. Because the system pressure can rise over time, occasional trimming of the backpressure coil may be necessary to maintain system pressure under 3000 psi. Do not exceed 3000 psi.

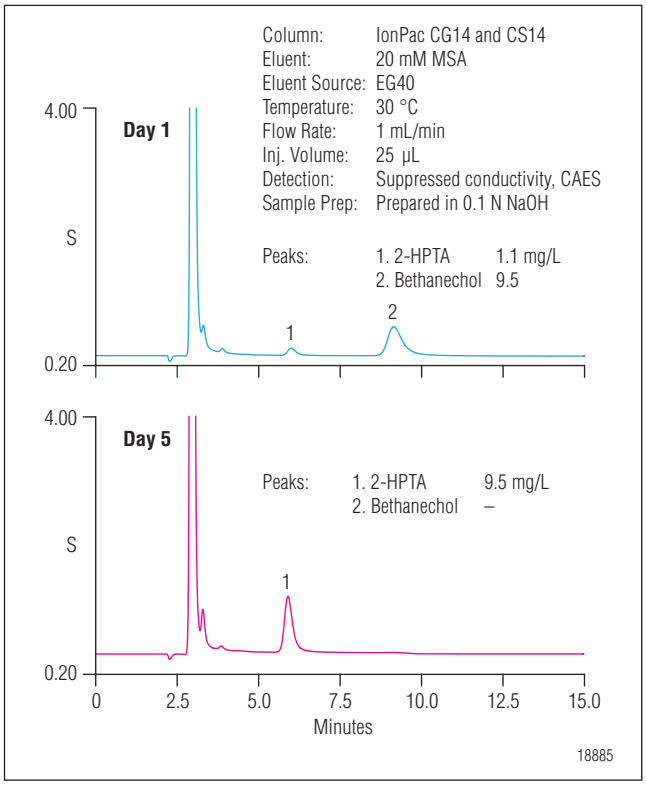


Figure 1. Conversion of Bethanechol to 2-HPTA in the presence of NaOH.

Allow the CS14 to properly equilibrate by pumping 20 mM MSA at 1.0 mL/min for approximately 60 min. Prior to sample analysis, analyze a system blank of deionized water. Prepare a 500× dilution of the Six Cation Standard (Dionex P/N 046070) and a make a 25-µL full-loop injection. Subsequently, prepare a 5 mg/L combined standard of bethanechol and 2-HPTA, and make a 25- µL full-loop injection. No peaks should be eluting at the same retention times as the analytes of interest. When duplicate injections of the bethanechol and 2-HPTA standard produce identical retention times, the system is equilibrated.

Peak area precision and accuracy depend on autosampler performance. Replace the water in the flush reservoir daily with freshly filtered and degassed deionized water. Inspect the AS50 daily for bubbles in the sample syringe or its tubing. Purge to remove any bubbles by following the instructions in the AS50 manual.

The precision and accuracy of the AS50 will vary depending on the mode of injection. The most accurate and precise injections can be made with a calibrated sample loop in the full-loop injection mode. To conserve sample, use a partial-loop injection mode. Refer to the AS50 reference manual for a complete discussion of the different injection modes.

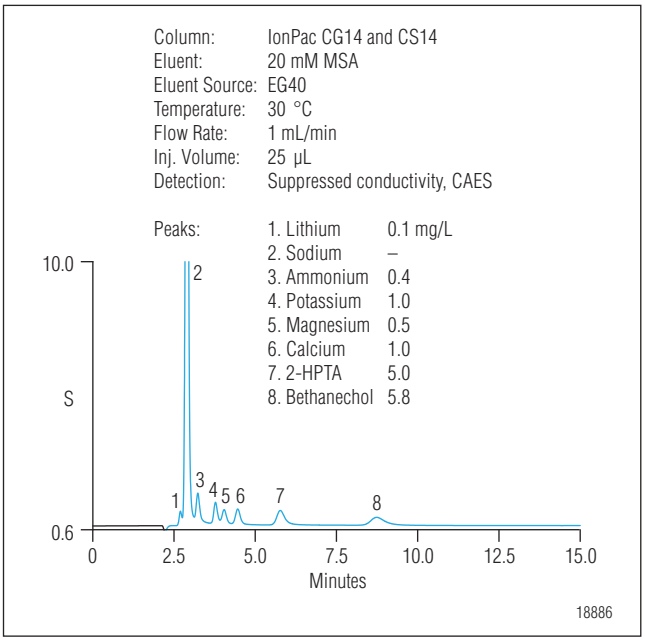


Figure 2. Separation of Bethanechol and 2-HPTA from common inorganic cations using the IonPac CS14.

Make sure the correct sample "Loop Size" and "Sample Syringe Volume" are entered in the AS50 Plumbing Configuration Screen.

RESULTS AND DISCUSSION

In the presence of an alkaline solution, bethanechol undergoes hydrolysis to 2-HPTA. In this application note, NaOH was used to prepare the hydrolysis product by combining 0.1 N NaOH with bethanechol and allowing the solution to stand for five days. Figure 1 illustrates the conversion of 9.5 mg/L bethanechol to 2-HPTA from day one to day five.

To determine the system suitability for the analysis of bethanechol and its degradation product, 2-HPTA, the analytes were analyzed in the presence of six common cations (Figure 2). The relative retention values reported by the U.S. Pharmacopeia[1] for Na^+, Mg^{2+}, Ca^{2+}, 2-HPTA, and bethanechol were 1.0, 1.4, 1.6, 2.0, and 2.8, respectively. We found the relative retention values were 1.0, 1.3, 1.5, 2.0, and 2.9, respectively. According to reference 1, the resolution between calcium and 2-HPTA should be greater than 2, with peak efficiency for bethanechol greater than 350 theoretical plates, and a peak tailing factor less than 4.5. The corresponding values from the separation in Figure 2 were 4.66, 2189, and 1.29, respectively.

Table 1. Linear Range and MDLs for Bethanechol and 2-HPTA

Cation	Range (mg/L)	Linearity (r^2)	Calculated MDL* (mg/L)	MDL Standard (mg/L)
Bethanechol	0.02–1000	0.9999	0.01	0.05
2-HPTA	0.02–500	1.0000	0.006	0.02

* The MDLs were calculated as MDL = *(t)* x (S) Where *t* = Student's *t* value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom (*t* = 3.14 for seven replicates of the MDL Standard), and S = standard deviation of the replicate analysis.

Table 1 summarizes the calibration data and MDLs for bethanechol and 2-HPTA. Calibration was linear over four orders of magnitude with correlation coefficients for 2-HPTA and bethanechol of 1.0000 and 0.9999, respectively (see "Appendix"). The intraday precision based on the retention time RSD of 7 replicate injections was 0.31%, and 88 injections of varying analyte amounts during 4 days of a 14-day period yielded a retention time RSD of 1.6%. The high retention time reproducibility is a result of a continuous generation of an exact high-purity MSA concentration by the EG40. The EG40 provides an increased level of automation, decrease in operator error, and greater precision in comparison to a manually prepared eluent.

SUMMARY

This application note discussed the separation and detection of bethanechol and 2-HPTA using the IonPac CS14 column with 20 mM MSA and suppressed conductivity detection. These pharmaceutically important amines were shown to be well resolved from the common inorganic cations that may be present as inactive ingredients. The good day-to-day reproducibility of the retention times of these analytes was possible with the continuous on-line generation of MSA using the EG40.

APPENDIX

The proposed USP method specifies a 50- µL sample injection with a concentration range up to 1000 mg/L bethanechol. It is our experience that injecting concentrations at this level with a 50- µL sample volume will overload the column, resulting in a nonlinear calibration curve. However, we believe a change in the sample loop injection volume is considered a minor modification of the USP method according to the system suitability specifications.[2] Therefore, in this application note, we described the determination of bethanechol using a 25- µL injection. The decrease in injection volume allowed bethanechol to be measured up to 1000 mg/L without overloading the column, resulting in good linearity ($r^2 = 0.9999$). However, any degradation of bethanechol at this concentration will compromise the linearity.

REFERENCE

1. *Pharmacopeial Forum* **2001** *27(1)*, 155–157.
2. U.S. Pharmacopeia. 24 NF19, **2000,** 24(1), 1923.

SUPPLIER

U.S. Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD 20852 USA, Tel: 800-277-8772, www.usp.org.

Determination of Nevirapine Using HPLC with UV Detection

INTRODUCTION

Combination therapy has proven to be one of the most effective approaches to treat HIV infection.[1–3] Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) with activity against human immunodeficiency virus type 1 (HIV-1) that is already marketed for the treatment of HIV-1 infected adults. Nevirapine is recommended for treating HIV infections in combination with other reverse transcriptase inhibitors such as stavudine and lamivudine.[4]

The method in the United States Pharmacopeia (USP)—monograph for determining nevirapine and its related compounds, A and B—uses a reversed-phase separation with UV detection.[5] The method calls for a 4.6 × 150 mm column packed with L60 (spherical, porous silica gel, 10 µm or less in diameter, the surface of which has been covalently modified with alkyl amide groups and endcapped). Due to the strong retention of impurity C, the separation requires about 30 min. Here, we report an optimized HPLC-UV method that requires less time per analysis and satisfies the chromatographic parameters of the USP method.

EQUIPMENT

Dionex UltiMate® 3000 Intelligent LC system:

- LPG-3000 pump
- WPS-3000 autosampler
- TCC 3200 column compartment
- VWD-3400 detector

Chromeleon® 6.70 chromatography management system

REAGENTS AND STANDARDS

Acetonitrile, Fisher HPLC Grade or equivalent

Water, Milli-Q water from Milli-Q Gradient A10

Ammonium phosphate monobasic ($NH_4H_2PO_4$), Fluka ACS reagent, ≥99%, or equivalent

Nevirapine (99.99%), stavudine (98.19%), and lamivudine (99.43%) standards, generous gifts from a customer

Thymine, 99% from Sigma

PREPARATION OF MOBILE PHASE AND STANDARDS

To prepare the mobile phase, weigh 2.882 g $NH_4H_2PO_4$ into a 200-mL beaker. After dissolving with water, move the solution to a 1000-mL volumetric flask and dilute to 1000 mL. Filter through a 0.45-µm PVDF Millicup-HV filter.

Prepare the stock standard solution by weighing 100 mg of nevirapine into a 250-mL volumetric flask together with 50 mL of MeCN and 50 mL of 25 mM $NH_4H_2PO_4$ buffer. After sonication for 5 min, add 90 mL water and continue sonication for 10 min. After cooling, bring the solution to volume with water and filter an aliquot through a 0.2-µm filter. The concentration of nevirapine was 0.4 mg/mL.

Prepare serial standard solutions with concentrations of 0.01, 0.05, 0.10, and 0.30 mg/mL nevirapine by taking the proper amount of stock standard solution and diluting with a mixture of 25 mM ammonium phosphate and acetonitrile that equal the initial eluent concentration. To prepare 100 mL of this mixture, add 18 mL of acetonitrile to 82 mL of the 25 mM ammonium phosphate solution.

PREPARATION OF SAMPLES

A nevirapine sample solution was a generous gift from a customer with a labeled concentration of 0.24 mg/mL nevirapine, 0.00012 mg/mL nevirapine-related compound A, and 0.00012 mg/mL nevirapine-related compound B.

Dilute the sample 1:4 and 1:9 with mobile phase at its initial concentration (see Preparation of Standards for preparation of this mobile phase concentration).

CHROMATOGRAPHIC CONDITIONS

Column:	Acclaim® PA, 4.6 × 150 mm, 5 µm (P/N 061320)
Mobile Phase:	25 mM $NH_4H_2PO_4$ and MeCN (see gradient table)
Flow Rate:	1.5 mL/min
Inj. Volume:	20 µL
Detection:	Absorbance at 220 nm
Column Temperature:	35 ºC

Gradient Table

Time (min)	25 mM $NH_4H_2PO_4$ (%)	MeCN (%)	Curve
0.0	82	18	
8.0	70	30	5
10.0	70	30	5
10.5	82	18	5
15.0	82	18	5

RESULTS AND DISCUSSION

Separation of Nevirapine and Its Related Compounds A and B on the Acclaim Polar Advantage (PA) Column

The chemical structure of nevirapine is shown in Figure 1. Using isocratic conditions, nevirapine and its related compounds A and B could be baseline resolved, but the chromatographic resolution between nevirapine and its related compounds were not as high as the values prescribed in the USP method. Therefore, we developed a gradient method.

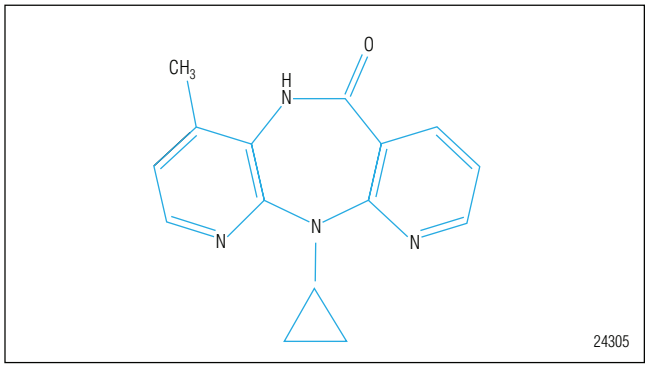


Figure 1. Chemical structure of nevirapine.

The Acclaim PA column contains a sulfonamide-embedded reversed-phase silica-based stationary phase[6] ideal for separating nevirapine and its related compounds, A and B. This stationary phase shares some chemical properties with L60. It also has selectivity similar to an ordinary C18 column for many analytes of low polarity, and is compatible with aqueous-only mobile phases for analytes of high polarity. Using the Acclaim PA under the chromatographic conditions (eluents, flow rate, detection wavelength, column dimensions, and column temperature) described in the USP monograph method for nevirapine, we developed a gradient separation of nevirapine and its related compounds A, B, and C. This separation meets the chromatographic requirements of the USP method. Using the Acclaim PA, related compound C is eluted within 11 min, allowing a total analysis time about half that of the USP method.

Resolution

Figure 2 shows a chromatogram of the undiluted nevirapine sample. The calculated resolution between nevirapine-related compound B and nevirapine was 6.5, and that between nevirapine and nevirapine-related compound A was 10.9, exceeding the values in the USP method. The USP values are ≥ 5.0 and ≥ 7.4, respectively.

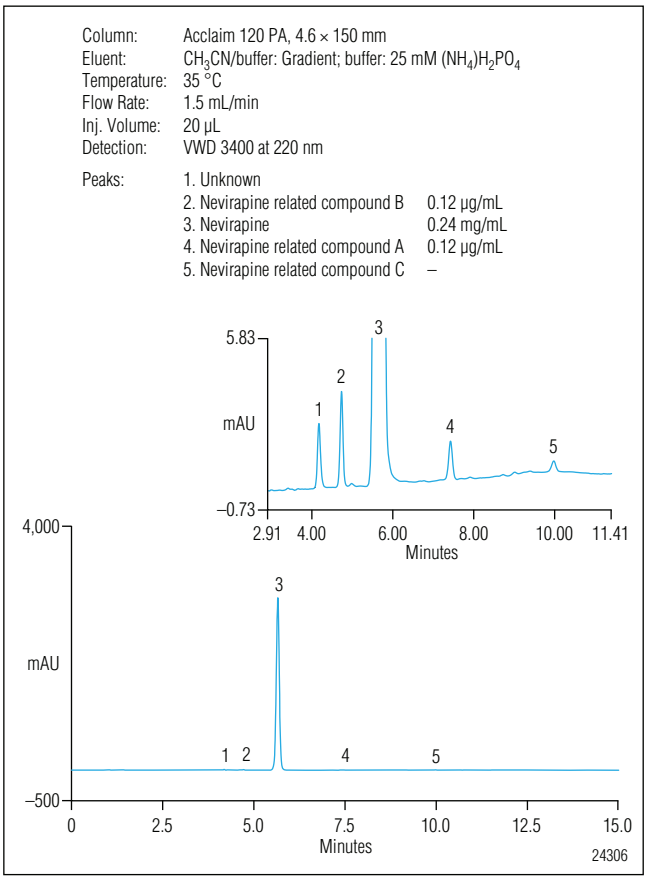


Figure 2. Chromatogram of nevirapine and its related compounds.

Reproducibility

The reproducibility was estimated by making replicate injections (n = 6) of a nevirapine standard solution (0.05 mg/mL). The relative standard deviation (RSD) was 0.030% for retention time, 0.284% for peak area, and 0.366% for peak height.

Linearity

Calibration linearity for UV detection of nevirapine was found to extend over the range from 5.0 mg/mL to 300 mg/mL based on making replicate injections (n = 6) of serial standard solutions of nevirapine at four concentrations. The linear regression equation was:

$$y = 1095.6x$$

where y is peak area (mAU·min), x is sample concentration (mg/mL), and the origin was used as the first point. Figure 3 shows the linearity of nevirapine (correlation coefficient, R^2, of 0.9999). Table 1 summarizes the related data. The detection limit of nevirapine, calculated by using S/N = 3, was 3.18 ng/mL.

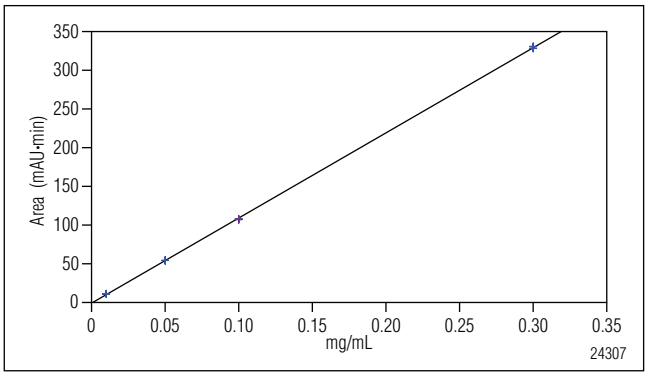


Figure 3. Linearity of nevirapine (n = 6).

Table 1. Data of Nevirapine Standards for Calibration

	Peak Areas (mAU·min)			
Injection Number	**0.01 (mg/mL)**	**0.05 (mg/mL)**	**0.1 (mg/mL)**	**0.3 (mg/mL)**
1	11.234	54.198	107.71	330.69
2	11.152	54.457	108.16	330.68
3	11.129	54.001	107.39	328.70
4	11.108	54.333	107.25	328.05
5	10.991	54.251	106.96	330.72
6	10.957	54.179	106.92	328.13
Average	11.095	54.237	107.40	329.49
RSD	0.935	0.284	0.442	0.405

Sample Analysis

Figure 4 shows the chromatograms of the 1:10 diluted nevirapine sample and that sample spiked with nevirapine. The recovery of nevirapine (n = 5) ranged from 102% to 102.9%. The average concentration of nevirapine determined in the undiluted sample solution was 0.25 mg/mL, consistent with the labeled value, 0.24 mg/mL.

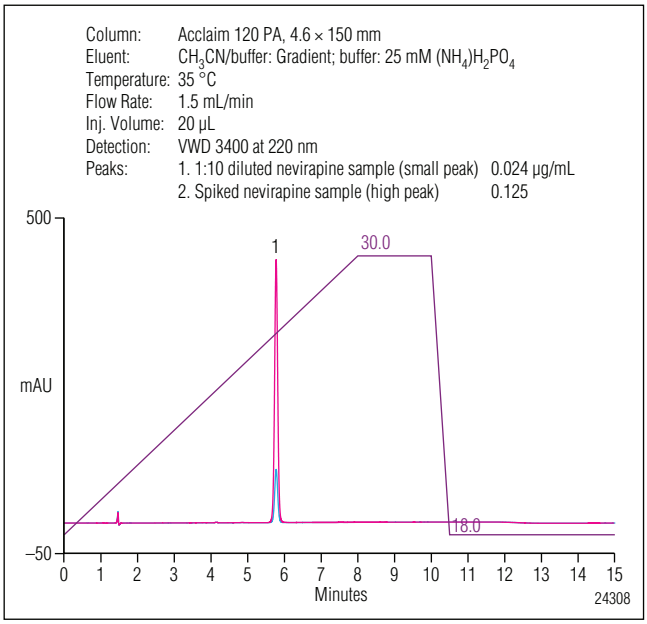


Figure 4. Overlay of chromatograms of 1:10 diluted nevirapine sample with and without a nevirapine spike.

Application to the Analysis of Other NNRTIs

This method can be used to analyze other non-nucleoside reverse transcriptase inhibitors (NNRTIs) with activity against HIV-1. The commonly used NNRTIs are zidovudine, lamivudine, stavudine, nevirapine, and indinavir. Figure 5 shows the separation of thymine, lamivudine, stavudine, and nevirapine. Indinavir was not analyzed.

Faster Analysis of Nevirapine

As shown in Figure 6, using a 100 mm narrow bore column can shorten the analysis of the nevirapine sample to < 10 min. This analysis should be performed using a high pressure mixing gradient pump to minimize delay volumes and requires a change to a 2.5-µL flow cell.

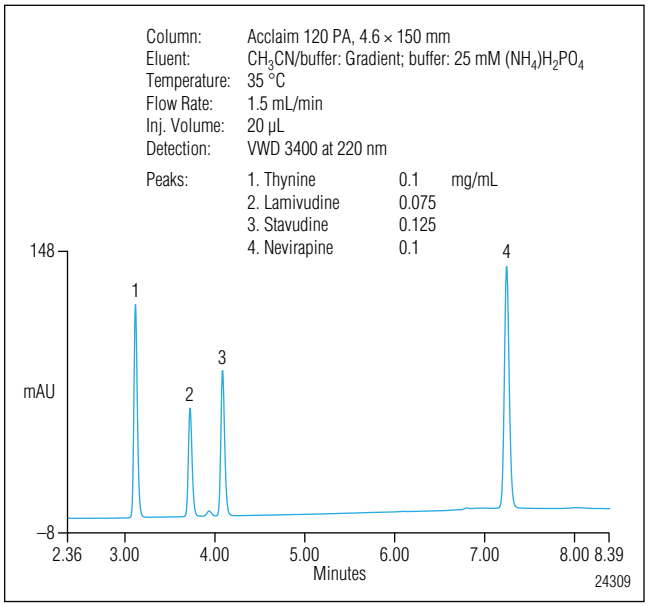


Figure 5. Separation of thymine, lamivudine, stavudine, and nevirapine.

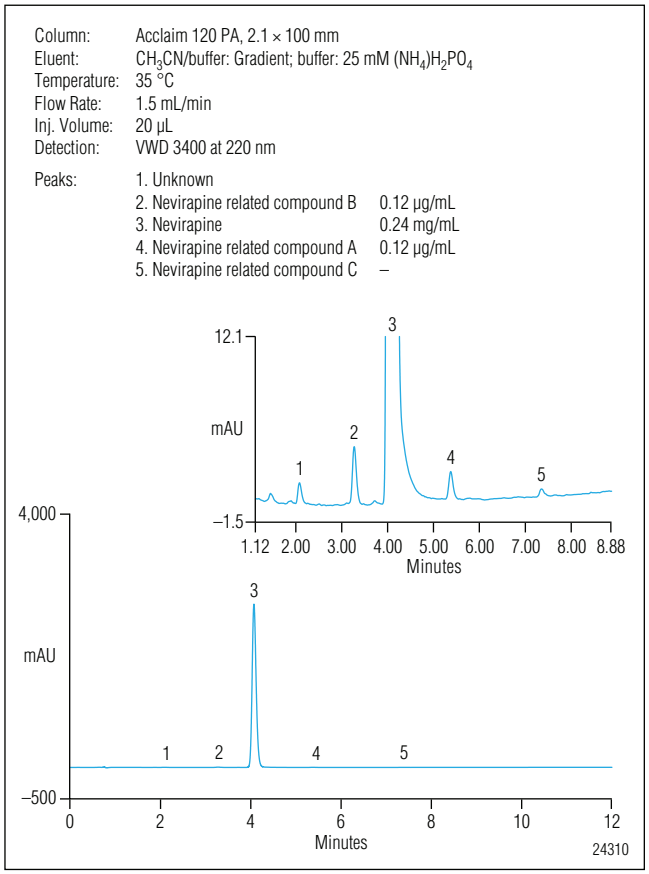


Figure 6. Fast separation of nevirapine and its related compounds, A and B, on a Summit® HPG HPLC system (modified for narrow bore analysis: using microflow kit and 2.5-µL flow cell) using an Acclaim PA column (2.1 × 100 mm). The inset is an enlarged view of the main chromatogram.

Performance of the UltiMate 3000 Intelligent LC System

Simultaneous Determination of Nevirapine and Related Compounds with Different Concentrations

As shown in Figure 2, the peak height of the main peak, nevirapine, was 2800 mAU, and the peak heights of the impurities including the related compounds A and B were between 0.3–4 mAU, which demonstrates the exceptional performance of the VWD-3400 detector for simultaneously determining a main constituent and its trace level impurities. This was verified by measuring the linearity of the response of the impurities, related compounds A and B, and nevirapine from replicate injections of the nevirapine sample and 1:4 and 1:9 diluted nevirapine samples, respectively (n = 6). Figure 7 shows an overlay of chromatograms of nevirapine-related compound B at different concentrations, and Figure 8 shows the graphs of peak area versus amount for nevirapine and its related compounds A and B. The correlation coefficients were 0.9999 for both nevirapine and nevirapine-related compound B, and 0.9992 for nevirapine-related compound A, demonstrating the excellent performance of the VWD-3400 over this broad concentration range difference between analytes. Table 2 summarizes related data from this experiment, from which we can conclude that the VWD-3400 detector provides accurate analysis in applications with varying analyte concentrations.

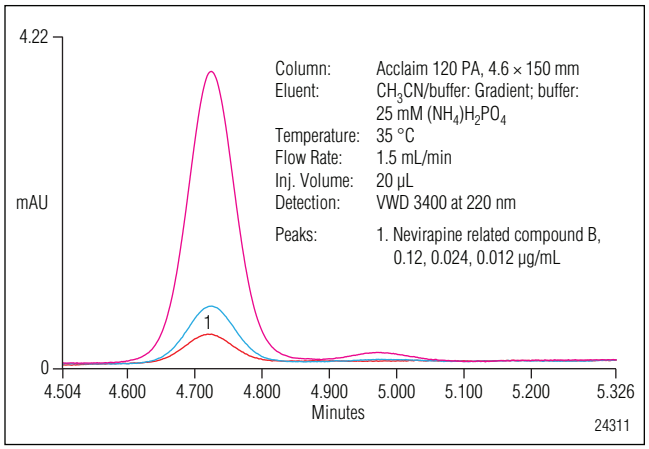


Figure 7. Overlay chromatograms demonstrating trace-level detection of nevirapine-related compound B.

Table 2. Related Data of Impurities Performance Test*

	Related compound B					Related compound A				
H_{peak} of Nevirapine (mAU), 2820	H_{peak} (mAU)	Level (%)	S/N	RT RSD (%)	H peak RSD (%)	H_{peak} (mAU)	Level (%)	S/N	RT RSD (%)	H peak RSD (%)
Original sample	3.6	0.13	279	0.101	0.19	1.5	0.053	113	0.080	0.44
1:4 diluted sample	0.7	0.025	55	0.027	1.06	0.3	0.011	22	0.039	2.67
1:9 diluted sample	0.35	0.012	22	0.122	2.44	0.14	0.005	12	0.168	4.29

* Mean value of six determinations

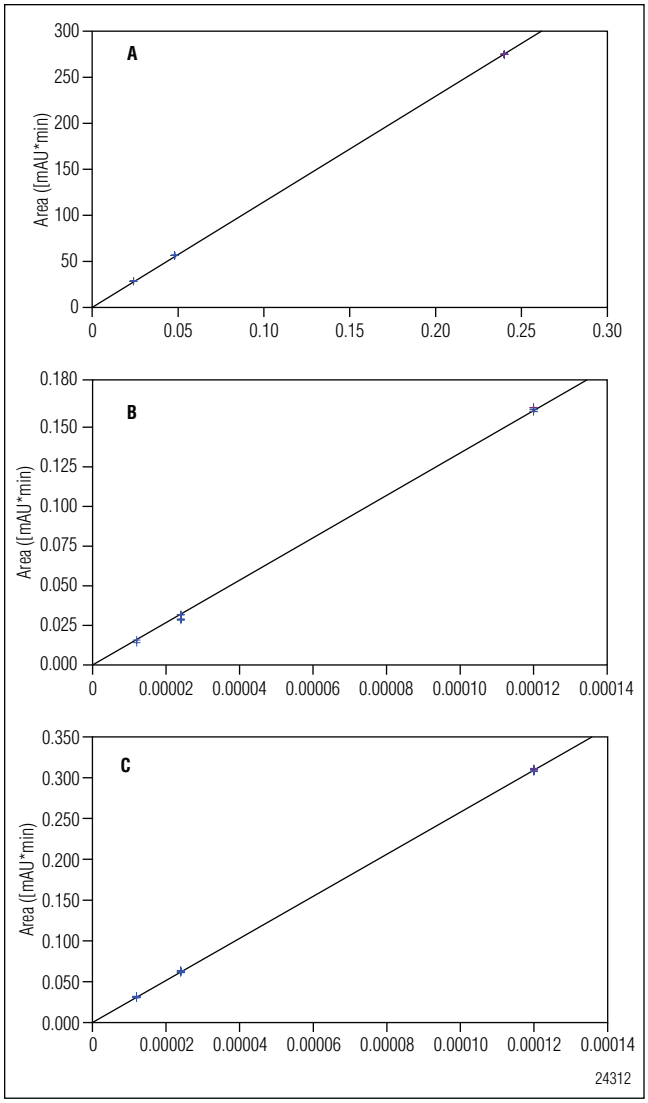


Figure 8. Linearity of (A) nevirapine, (B) related compound B, and (C) related compound A (n = 6).

Carry-Over Performance of the WPS-3000

Figure 9 shows exceptional carry-over performance for the WPS-3000 autosampler without the need for an external needle wash. There was no cross contamination observed when using WPS-3000 autosampler for this application.

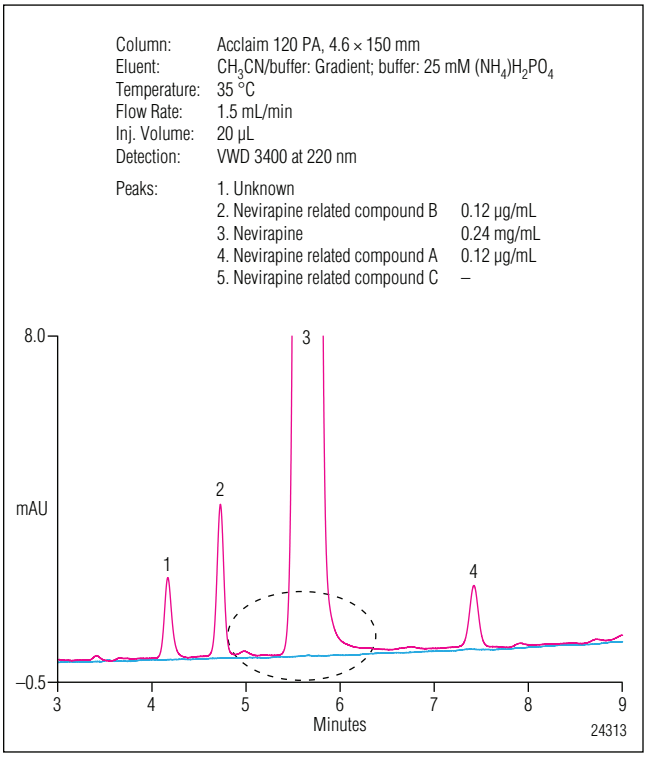


Figure 9. Carry-over test on theWPS-3000 autosampler. Original nevirapine sample solution and blank solution (25 mM $NH_4H_2PO_4$ buffer) were injected in series.

CONCLUSION

This application note describes an optimized method for determining nevirapine on an UltiMate 3000 Intelligent LC system with an Acclaim PA column. This method meets or exceeds the chromatographic requirements of the USP monograph method for nevirapine while requiring about half the analysis time per sample. This method is optimized on the UltiMate 3000 due to the system's elimination of cross contamination from the WPS-3000 autosampler, low noise from the VWD-3400 detector, and other benefits. The Acclaim PA and UltiMate 3000 are ideally suited for determining both polar and nonpolar pharmaceutical compounds and their impurities.

PRECAUTIONS

Exercise care when handling acetonitrile, ideally filling the eluent bottle in a fume hood. Use proper methods for disposal of waste.

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Determination of Carbachol In Ophthalmic Solutions Using a Reagent-Free Ion Chromatography System

INTRODUCTION

Carbachol is a choline ester and a positively charged quaternary ammonium compound used primarily for ophthalmic applications, such as solutions used for glaucoma treatment or ophthalmic surgery. Carbachol is a potent cholinergic agent which constricts the iris and ciliary body resulting in reduction of intraocular pressure in patients with glaucoma.[1] The exact mechanism by which carbachol lowers intraocular pressure is not precisely known; however it is believed to increase the amount of fluid drained from the eye.

Diminished concentrations of carbachol in an ophthalmic formulation may prevent effective reduction of intraocular pressure, which may have deleterious effects such as iris prolapse. Analytical methods are therefore needed to ensure that concentrations in these solutions remain at therapeutically active levels. The current USP monograph (USP 29-NF 24) describes a colorimetric method for the determination of carbachol in ophthalmic solutions.[2] Colorimetric methods can be both time- and labor-intensive, and yield significant measurement errors.

Choline is a member of the B vitamin group and the parent member of a class of drugs referred to as cholinergic. Carbachol and bethanechol are two clinically useful choline derivatives.[3] In alkaline solutions, carbachol degrades to choline. Therefore, a method to selectively detect carbachol and choline is required.

Prior Applications describe methods for detection of choline and other analytes related to carbachol. Dionex Application Note 124 (AN 124) describes the use of an IonPac® CS12A column for the determination of free and bound choline from dried milk in infant formula.[4] In the method described here, optimized conditions for carbachol analysis were used to determine choline linearity, method detection limits (MDL), and separation from carbachol in lens and saline solutions.

Bethanechol chloride is a quaternary ammonium compound that is structurally and pharmacologically related to carbachol.[5] AN 148 reports a Reagent-Free™ Ion Chromatography (RFIC™) method for the determination of bethanechol chloride. Method parameters used for determining carbachol can also be used for bethanechol analysis, as shown in this Application Note. Linearity, MDL, and potential interferences with the breakdown product of bethanechol, 2-hydroxypropyltrimethylammonium chloride (2-HPTA) were also determined.

Here, a simple RFIC method is described for determination of carbachol, bethanechol, and choline in 25 min with a Dionex IonPac CS17 column. Methanesulfonic acid (MSA) eluent is delivered isocratically by an Eluent Generator (EG). Use of an EG eliminates eluent preparation errors and helps ensure retention time reproducibility. The sensitivity of suppressed conductivity detection allows carbachol determination in ophthalmic solutions with only a simple sample dilution.

EQUIPMENT

ICS-2000 (Dionex P/N 061098)

AS Autosampler

Chromeleon® 6.8 SP2 Chromatography Workstation

CONSUMABLES AND REAGENTS

CR-CTC II (Dionex P/N 066202)

CSRS® ULTRA II 4 mm (Dionex P/N 061563)

EluGen® II MSA Cartridge (Dionex P/N 058902)

Carbachol Chloride (USP reference standard P/N 1092009)

Bethanechol Chloride (USP reference standard P/N 1071009)

Six Cation Standard II (Dionex P/N 046070)

Dimethylamine (Fluka P/N 38960)

Alcon OPTI-FREE® RepleniSH™ Multipurpose Disinfecting Lens Solution

Bausch & Lomb Gentle Sensitive Eyes® Plus Saline Solution

Type I reagent-grade distilled water or deionized water with a specific resistance of 17.8 MΩ-cm or greater, filtered through a 0.2 µm filter immediately before use.

CONDITIONS

Columns:	IonPac CG17 4 mm 4 × 50 mm (Dionex PN 060560) IonPac CS17 4 mm 4 × 250 mm (Dionex PN 060557)
Eluent:	5 mM Methanesulfonic Acid
Flow Rate:	1.0 mL/min
Temperature:	30 °C
Injection Volume:	25 µL
Detection:	Suppressed conductivity, CSRS ULTRA 4mm (P/N 053948) recycle mode
Power setting:	20 mA
Background Conductivity:	< 1 µS
Noise:	< 0.5 nS/min
Backpressure:	2300 psi
Run Time:	25 min

ELUENT SOLUTION

5 mM MSA eluent is generated on-line using an EG Eluent Generator with an MSA EluGen cartridge. Fill the eluent reservoir with reagent water and maintain an inert helium atmosphere of 3-5 psi in the eluent reservoir. Chromeleon software tracks the amount of MSA used and calculates the remaining lifetime. Replace the MSA cartridge when the remaining lifetime drops below 10%.

Alternately, manually prepared MSA may be used. First prepare a 1.0 N stock solution by adding 96.10 g of MSA to a 1 L volumetric flask containing approximately 500 mL of deionized water. Bring to volume with deionized water, and mix thoroughly. Prepare 5 mM MSA by diluting 5 mL of the 1 N MSA stock solution to 1 L with deionized water. Degas the eluent and store in a plastic container.

STOCK STANDARD SOLUTIONS

1000 mg/L Carbachol Solution

Dissolve 0.1762 g of carbachol chloride in approximately 75 mL of reagent water and dilute to 100 mL in a volumetric flask. Store the stock solution in a high-density polyethylene or polypropylene bottle at 4 °C.

1000 mg/L Choline Solution

Weigh 0.0881g of carbachol chloride into a 125 mL plastic bottle, add 50 mL of 0.1N NaOH, sonicate to dissolve, and mix. Allow five days for the carbachol to completely hydrolyze to choline.

1000 mg/L Bethanechol Solution

Dissolve 0.1 g of bethanechol chloride in approximately 75 mL of reagent water and dilute to 100 mL in a volumetric flask. Store the stock solution in high-density polyethylene or polypropylene bottle at 4 °C.

1000 mg/L 2-Hydroxypropyltrimethylammonium (2-HPTA) Solution

HPTA was prepared as directed in AN 148. Weigh 0.050g of bethanechol chloride into a 125 ml plastic bottle, add 50 mL 0.1N NaOH, sonicate to dissolve, and mix. Allow five days for the bethanechol to completely hydrolyze to 2-HPTA chloride.

1000 mg/L Dimethylamine Solution

Dissolve 0.1 g of dimethylamine in approximately 75 mL of reagent water and bring to volume in a 100 mL volumetric flask. Store the stock solution in a high-density polyethylene or polypropylene bottle at 4 °C.

WORKING STANDARD SOLUTIONS

To prepare working standards, use a calibrated pipet to deliver the appropriate volume of the 1000 mg/L stock standard into a volumetric flask and bring to volume with reagent grade water. For method linearity studies, the following standards of bethanechol, choline, carbachol,

and 2-HPTA were used: 1000, 500, 200, 100, 50, 25, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 mg/L. The exceptions were the linearity studies on 2-HPTA and choline, with maximum concentrations of 500 mg/L.

To prepare mixed standards containing carbachol and other compounds, combine appropriate volumes of the carbachol stock standard with the Cation Standard II, or single-component standards, in a volumetric flask and bring to volume with reagent water.

INTERFERENCE STUDIES

To confirm no other compounds interfere with carbachol determinations using this method, a mixed standard was injected containing carbachol (1 mg/L) along with lithium (0.1 mg/L), sodium (0.4 mg/L), ammonium (0.5 mg/L), potassium (1 mg/L), magnesium (0.5 mg/L), calcium (1 mg/L), choline (1 mg/L), bethanechol (1 mg/L), and dimethylamine (1 mg/L).

SAMPLES

Alcon OPTI-FREE RepleniSH Multipurpose Disinfecting Lens Solution and Bausch & Lomb Gentle Sensitive Eyes Plus Saline Solution were each diluted 1:1000 with reagent water and spiked with the desired amount of carbachol for linearity and (MDL) determinations as well as recovery and precision studies.

SYSTEM PREPARATION AND SETUP

Verify that the pump flow rate is within specifications and recalibrate if necessary. (The pump should deliver liquid at ± 0.5% of the specified volume against a constant backpressure of 2300 psi.) Verify that the conductivity cell constant is within specifications and recalibrate if necessary. Consult the pump or detector manuals for procedural details.

Install the EG, and condition the EluGen II MSA Cartridge as directed in the manual by running a gradient from 1 to 60 mM MSA in 20 min, then 60 mM for 40 min at 1 mL/min. (For instructions on installation and use, see the ICS-2000 IC system installation instructions, Document No. 031857.)

Install and configure the autosampler. Use a calibrated sample loop in "full loop" mode to obtain the best accuracy and precision. Note: If making partial loop injections, program a sample volume that is less than half the volume of the installed sample loop, with a cut volume of 8 µL. This injection procedure should provide peak area precision of < 1% RSD.

Install a 1-mL sample syringe and set the syringe speed to 3. Enter the correct sample loop size and sample syringe volume in the AS plumbing configuration screen. Refer to the ICS-2000 Ion Chromatography System Installation Instructions, (Document No. 031857) for details.

Install an IonPac CG17 4 × 50 mm guard column and an IonPac CS17 4 × 250 mm analytical column. Confirm that the system pressure displayed by the pump is at least 2300 psi when 5 mM MSA is delivered at 1.0 mL/min. This allows the degas assembly to effectively remove electrolysis gas from the eluent. If necessary, install backpressure coils supplied with the EG ship kit to adjust the system pressure to between 2300 and 2800 psi. Because system pressure can rise over time, it may be necessary to trim the backpressure coil to maintain system pressure under 3000 psi. Do not exceed 3000 psi or the degas assembly tubing may rupture.

Prepare the CSRS-ULTRA 4 mm suppressor for use by hydrating the eluent chamber. Pump approximately 5 mL reagent water through the Regen In port. Next, pump approximately 5 mL reagent water through the Eluent In port. Allow approximately 20 min to fully hydrate the suppressor screens and membranes. Install the CSRS-ULTRA in Recycle Mode, following the installation and troubleshooting instructions for the CSRS-ULTRA, (Document No. 031370).

Equilibrate the column with 6 mM MSA eluent for 60 min, and analyze a system blank of reagent water. A well-equilibrated system should have a background conductivity of approximately 1 µS, and peak-to-peak noise should be < 0.5 nS/min.

Make a 25 µL full injection of a 1:1000 dilution of the six cation standard along with 1 mg/L carbachol. None of the peaks in the standard should coelute with carbachol. Once the column is equilibrated, duplicate injections of the standard should produce identical or nearly identical retention times for all analytes.

Peak area precision and accuracy depend on the performance of the autosampler. The water in the flush reservoir should be replaced daily, and the sample syringe and tubing should be regularly inspected for bubbles. If bubbles are observed, they should be removed by purging as outlined in the autosampler manual. The injection mode used also affects precision and accuracy; the most accurate way to make an injection is by using a calibrated sample loop, in "full loop" injection mode.

RESULTS AND DISCUSSION

Chromatography and Interference Studies

In order to determine the system suitability for the analysis of carbachol in the presence of commonly occurring cations, the compound was analyzed in the presence of lithium, sodium, ammonium, potassium, magnesium, calcium, choline, bethanechol, and dimethylamine. Figure 1 shows a chromatogram of a 1 mg/L carbachol standard along with several commonly occurring cations, and compounds that may potentially interfere with the analysis of carbachol. The retention times of lithium, sodium, ammonium, potassium, dimethylamine, choline, carbachol, bethanechol, magnesium, and calcium were 4.01, 4.50, 4.87, 5.51, 6.10, 8.81, 10.53, 11.66, 19.35 and 22.13, minutes, respectively. Thus, all the compounds are well separated from carbachol, and do not interfere with its determination. Figure 2 shows the conversion of carbachol to choline in the presence of NaOH, on Day 1 and Day 5 of exposure to 0.1N NaOH.

Determination of Linearity for Carbachol and Choline

Prior to evaluation of carbachol in samples, a calibration using different concentrations of carbachol was performed with the standards prepared in reagent grade water. Table 1 summarizes the data for a typical calibration curve obtained by injecting calibration standards at 1000, 500, 200, 100, 50, 25, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 mg/L of carbachol. Table 1 also summarizes the calibration data for choline using the same calibration standards with the exception of the 1000 mg/L standard. Calibration for both compounds was linear over four orders of magnitude, with a correlation coefficient of 0.9999 for carbachol and choline.

Table 1. Linear Range for Carbachol and Choline

Analyte	Range (mg/L)	r^2	Offset	Slope
Carbachol	0.02 – 1000	0.99998	-0.036	0.085
Choline	0.02 – 500	0.99999	-0.011	0.060

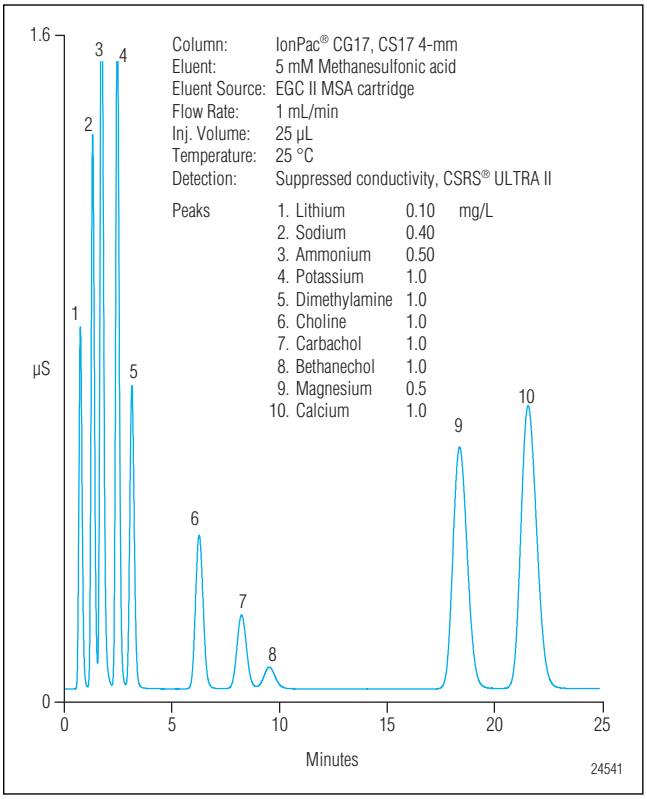

Figure 1. Separation of 1 mg/L carbachol, choline, and bethanechol with a mixed cation standard.

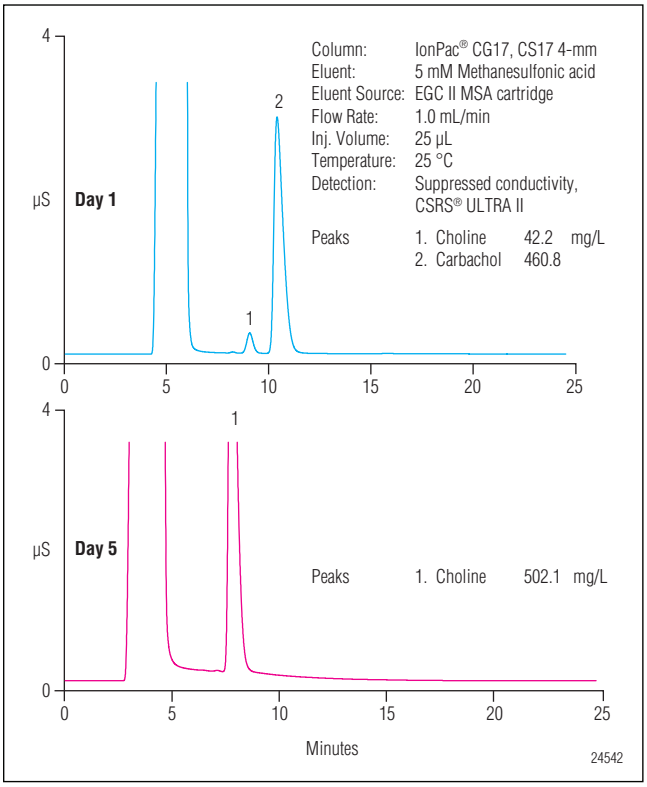

Figure 2. Conversion of carbachol to choline in the presence of 0.1 N NaOH.

Minimum Detection Limit (MDL) for Carbachol and Choline

The MDL is defined as the minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero. It is a measure of the precision of preparing and analyzing low-level samples according to the method parameters. The MDL of carbachol was determined by making seven injections of a low-level solution fortified with carbachol at a level yielding a signal/noise ratio of approximately 3–5 µS. The amount was determined using the calibration curve, and the MDL was calculated.

The MDL for carbachol in water was determined by making seven replicate injections of reagent water fortified with carbachol at 0.02 mg/L. Using this method, the calculated MDL for carbachol in water is 5 µg/L. The calculated MDL for choline obtained by the same method is 1 µg/L. Table 2 summarizes the data for the determination of the MDLs for carbachol and choline.

Table 2. Determination of Carbachol and Choline MDLs

Analyte	Range (mg/L)	MDL Standard (mg/L)	RSD	S/N	Calculated MDL (µg/L)
Carbachol	0.02 – 1000	0.02	0.12	5.61	5
Choline	0.02 – 500	0.01	0.04	2.96	1

** The MDLs were calculated as MDL = (t) x (SD), where t = Student's t value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom [t = 3.14] for seven replicates of the MDL Standard, and SD = standard deviation of the replicate analysis.*[5]

SAMPLE ANALYSIS

Carbachol is used in wash solutions for surgical procedures. As these solutions are not commercially available, this method was developed and tested using over-the-counter eyecare solutions, including Alcon Optifree RepleniSH Multi-Purpose Disinfecting Lens Solution and Bausch & Lomb Gentle Sensitive Eyes Plus Saline Solution spiked with carbachol. These solutions share similar properties to the wash solutions used during surgical procedures. Because they contain large amounts of sodium, sample dilution is necessary to prevent overloading of the column with the matrix ions. Overloading may cause the carbachol peak to appear shorter and broader, may reduce carbachol recovery, and may compromise integration reliability.

Figure 3 shows determination of 1 mg/L carbachol spiked into Alcon Lens Solution diluted 1:1000. Sodium was observed along with carbachol. The Bausch & Lomb saline solution was also diluted 1:1000 and spiked with carbachol. Figure 4 shows determination of 1 mg/L carbachol spiked into diluted Bausch & Lomb saline solution using optimized conditions. The solution contains sodium and potassium. Neither cation interferes with carbachol in either matrix.

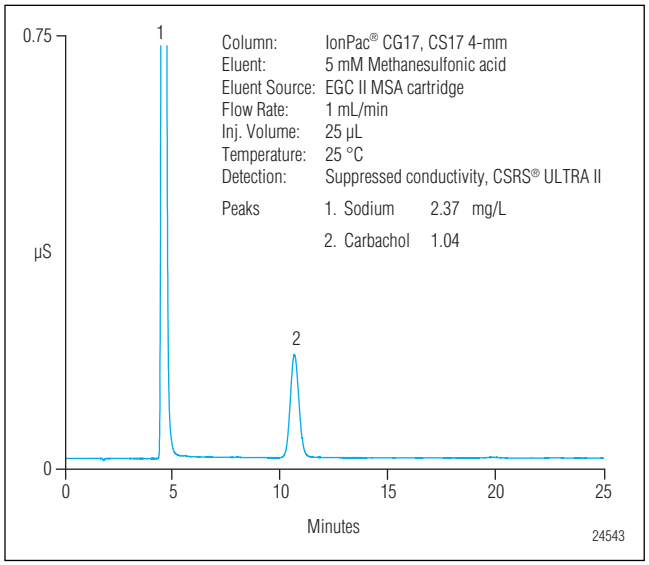

Figure 3. Alcon Lens Solution (diluted 1:1000) spiked with 1 mg/L of carbachol.

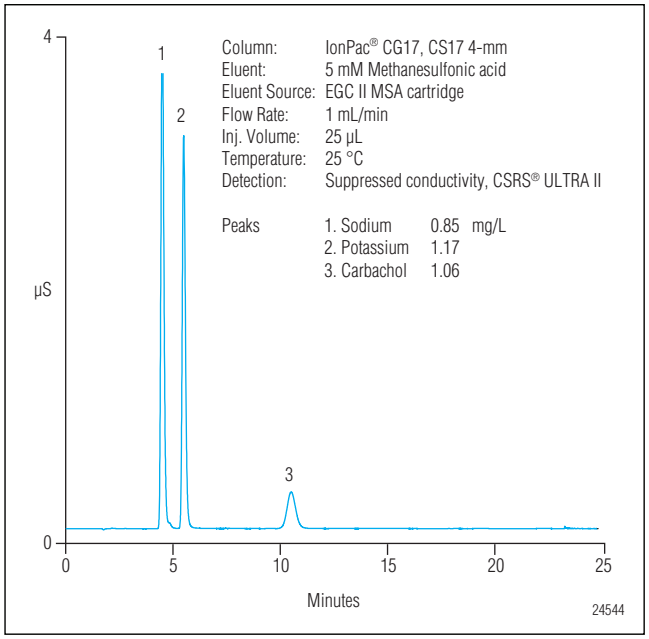

Figure 4. Saline solution (diluted 1:1000) spiked with 1 mg/L of carbachol.

Carbachol was spiked into the two matrices mentioned above, and precision, recovery, linearity, and MDL were evaluated. The results from the linearity and MDL studies for both matrices are summarized in Table 3. Precision and recovery data are shown in Table 4.

Table 3. Linear Range and Detection Limits of Carbachol in Two Over-the-Counter Ophthalmic Solutions

Matrix	Range (mg/L)	r2	MDL Standard (mg/L)	RSD	S/N	Calculated MDL (µg/L)
Alcon Lens Solution	0.01 – 500	0.99999	0.02	0.035	4.12	4
Bausch & Lomb Saline Solution	0.01 – 500	0.99995	0.01	0.11	13.5	3

** MDLs were calculated as MDL = (t) x (SD) where t = Student's t value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom [t = 3.14] for seven replicates of the MDL Standard, and SD = standard deviation of the replicate analysis.*[5]

Table 4. Recovery of Carbachol from Ophthalmic Samples

Matrix	Amount Added (mg/L)	Recovery	Precision (RSD)
Alcon Lens Solution	0.5	96	0.77
Bausch & Lomb Saline Solution	0.5	98	0.67

Short-term, between-day reproducibility was measured by injecting five replicates of a 5 mg/L standard each day for 6 days. The between-day the precision based on the retention time RSD was 0.043% with saline and 0.101% with lens solution. The high retention time reproducibility is a result of a continuous generation of high-purity eluent by the eluent generator, which provides an increased level of automation, decreased operator error, and greater precision as compared to manual preparation of mobile phases.

Determination of Bethanechol

As shown in Dionex AN 148, bethanechol undergoes hydrolysis to 2-HPTA in the presence of an alkaline solution, therefore 2-HPTA was also evaluated in this study. Different concentrations of bethanechol standards were prepared in reagent grade water and a calibration procedure was performed. Table 6 shows a typical calibration curve obtained by injecting standards at 1000, 500, 200, 100, 50, 25, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 mg/L of bethanechol. Table 6 also summarizes the calibration data for 2-HPTA using the same concentrations, with the exception of the 1000 mg/L standard. Calibration for both compounds was linear over four orders of magnitude, with a correlation coefficient of 0.9999 for bethanechol and 2-HPTA. Table 7 summarizes the data for the determination of MDL for those two compounds. Figure 5 shows the conversion of bethanechol to 2-HPTA in an alkaline solution.

Separation of choline from carbachol and bethanechol was also evaluated in two over-the-counter eye care products. Figure 6 shows separation of choline from carbachol and bethanechol when using lens solution as a matrix. The chromatogram demonstrates reliable separation, even in the presence of sample matrix cations.

Table 5. Reproducibility

Matrix	Concentration (mg/L)	RSD		
		Retention Time	Height	Area
Alcon Lens Solution	5	0.10	0.78	0.88
Bausch & Lomb Saline Solution	5	0.43	0.74	0.89

Table 6. Linear Range for Bethanechol and 2-HPTA

Analyte	Range (mg/L)	r2	Offset	Slope
Bethanechol	0.02 – 1000	0.99928	-0.007	0.036
2-HPTA	0.02 – 500	0.99997	-0.011	0.028

Table 7. Determination of MDL for Bethanechol and 2-HPTA

Analyte	Range (mg/L)	MDL Standard (mg/L)	RSD	S/N	Calculated MDL (µg/L)
Bethanechol	0.02 – 1000	0.05	0.05	2.8	2
2-HPTA	0.02 – 500	0.05	0.12	3.3	5

** The MDLs were calculated as MDL = (t) x (SD) where t = Student's t value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom [t = 3.14] for seven replicates of the MDL Standard, and SD = standard deviation of the replicate analysis.*[5]

PRECAUTIONS

Carbachol is hazardous to humans and to the environment. It can be toxic if swallowed and harmful if inhaled. It may cause skin irritation, and may be harmful if absorbed through the skin. It may cause irritation of the eyes as well as the upper respiratory tract and mucous membranes. To dispose of this material, contact a licensed waste disposal service.

Bethanechol chloride and choline are harmful if inhaled, swallowed, or absorbed through the skin. These materials may cause serious damage to the eyes; wear protective gloves and clean body-covering clothing, chemical safety goggles, and work in a well-ventilated area. These materials should be disposed of in accordance with the appropriate federal, state and local regulations.

Strongly retained compounds can accumulate on the column and degrade its performance. Signs of a fouled column include loss of capacity, loss of resolution, shortened retention times, higher noise and background, spurious peaks, and peak tailing. When cleaning an analytical and guard column in series, ensure that the guard column is placed after the analytical column in the flow path. Flush the columns for 15 min with 10 mM HCl at a flow rate of 1.0 mL/min, followed by a 1M HCl flush for 60 min to help remove contaminants. (For more information on column troubleshooting and cleanup, see the installation instructions and troubleshooting guide for the IonPac CS17 analytical Column, Document No. 031877.)

Some samples contain particulates that may plug the column and increase backpressure. Use a guard column to protect the analytical column. Inspect the column bed supports for discoloration and change if discolored. Replace the guard column if a sample causes a sudden increase in total backpressure greater than 3000 psi.

SUMMARY

The method outlined in this Application Note quantifies mg/L or lower concentrations of carbachol-fortified eye care products. Separation and detection of carbachol, choline, bethanechol and 2-HPTA using an IonPac CS17 column with 5 mM MSA and suppressed conductivity detection are also examined. Using the method described here, these cholinergic agents are well resolved from commonly occurring inorganic cations. The method demonstrates high precision, high recovery and excellent day-to-day reproducibility for analysis of carbachol.

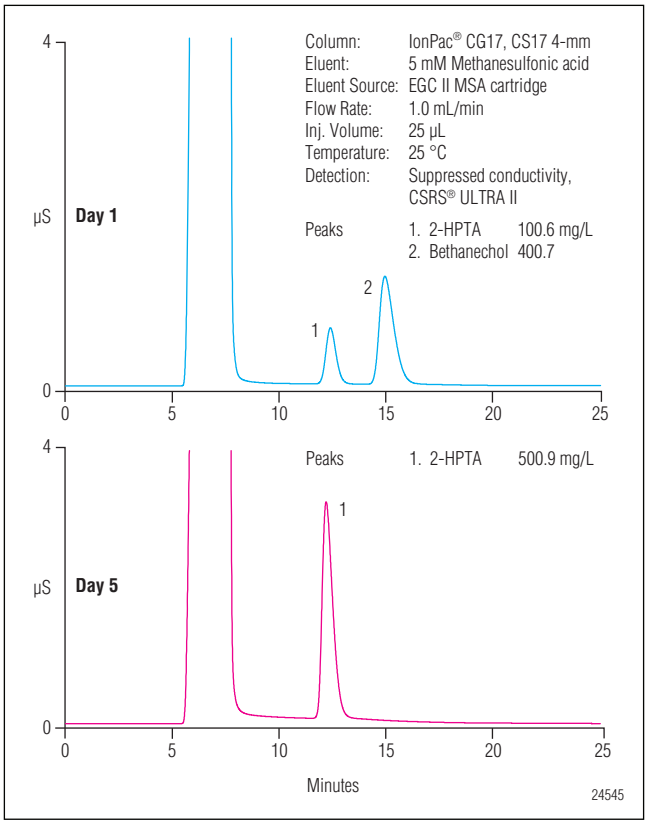

Figure 5. Conversion of bethanechol to 2-HPTA in the presence of 0.1 N NaOH.

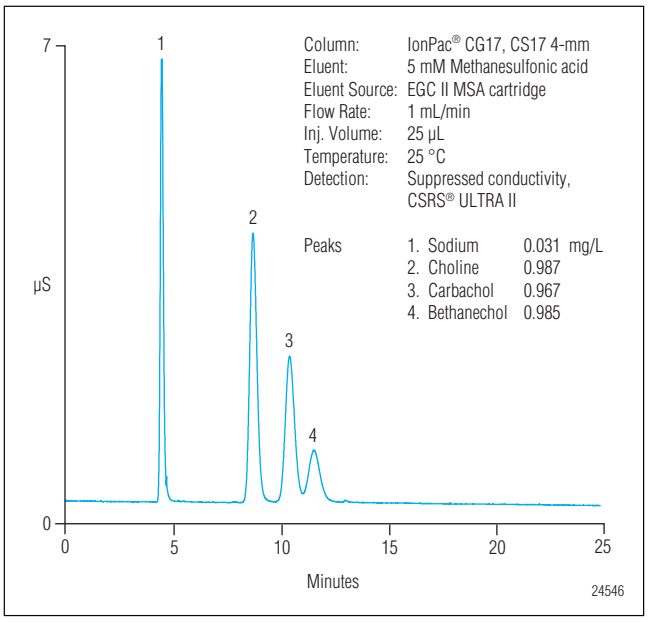

Figure 6. Separation of choline from carbachol and bethanechol in lens solution.

SUPPLIER

U.S. Pharmacopeia, 12701 Twin Brook Parkway, Rockville, MD 20852 USA, (800) 277-8772, www.usp.org

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3. Application Note 70, Choline and Acetylcholine, Dionex Corporation. LPN 034516.
4. Application Note 124, Determination of Choline in Dry Milk and Infant Formula, Dionex Corporation. LPN 1054.
5. Application Note 148, Determination of Bethanechol by Ion Chromatography Dionex Corporation. LPN 1510.

Determination of Verapamil Hydrochloride Purity Using the Acclaim PA Column

INTRODUCTION

Verapamil-based medications are prescribed for several heart and blood pressure indications. The fast-acting formulations (verapamil hydrochloride and Isoptin®) are taken for angina, as well as irregular heartbeat and high blood pressure. The United States Pharmacopeia (USP®) has a monograph method to determine verapamil hydrochloride purity. This method separates verapamil hydrochloride and verapamil-related compound B using HPLC with a C18 (USP designation L1) column.[1] There is a recent proposal to revise the method by using a L60 column to determine verapamil hydrochloride and verapamil-related compounds B and D. The existing method can not determine compound D under the prescribed eluent. The proposed method is time consuming (61 min), and requires a special column.[2]

In this application note, we describe a new method for the fast determination of verapamil hydrochloride and verapamil-related compounds A, B, and D, using a polar-embedded reversed-phase column, the Acclaim® PolarAdvantage (PA). The new method requires only about half the time of the proposed USP monograph method, provides significant eluent and therefore cost savings, and meets the resolution requirement.

EQUIPMENT

UltiMate® 3000 HPLC

HPG 3400 pump with SRD 3400 degasser

WPS 3000 TSL autosampler

TCC 3000 thermostatted column compartment

VWD-3400 UV-Vis detector

Chromeleon® 6.80 SP1 Chromatography Workstation

REAGENTS AND STANDARDS

Water, Milli-Q water from Milli-Q Gradient A10

Acetonitrile (CH_3CN), Fisher, HPLC grade

KH_2PO_4, reagent grade, (AR, analytical pure, grade in China)

H_3PO_4, reagent grade, (AR, analytical pure, grade in China)

Verapamil HCl (Sigma, CAS: 152-11-4), purity > 99.0%

Verapamil-related compound A (USP, P/N: 71130)

Verapamil-related compound B (USP, P/N: 71140)

Verapamil-related compound D (LGC Promochem GmbH, P/N: USP1711428)

PREPARATION OF REAGENTS AND STANDARDS

Prepare two solutions for testing, consistent with the requirements of the USP monograph method.

System suitability solution:

Prepare a mixture of verapamil hydrochloride, verapamil-related compound B, and verapamil-related compound D where each component has a concentration of 25 µg/mL. Add an additional component not described in the USP method, verapamil-related compound A, also at 25 µg/mL. Use this solution for method development.

Standard solution and test solution:

Prepare a 2.5 mg/mL standard solution of verapamil hydrochloride. To this solution add the three related compounds to achieve a final concentration of 2.5 µg/mL each, which serves as the test solution to simulate real samples.

CHROMATOGRAPHIC CONDITIONS

Column: Acclaim PA, 5 µm, 4.6 × 250 mm (P/N 061321)

Temperature: 35 °C

Inj. Volume: 5 µL

Mobile Phase: A: 20 mM KH_2PO_4 adjust pH to 3.0 with H_3PO_4

B: CH_3CN

Detection: Absorbance at 278 nm

Gradient Table:

Time	Flow Rate	Buffer (%)	CH_3CN (%)	Curve
0.0	1.0 mL/min	70	30	
4.0	1.0 mL/min	70	30	5
29.0	1.0 mL/min	45	55	5
29.5	1.0 mL/min	45	55	5
30.0	1.0 mL/min	70	30	5
35.0	1.0 mL/min	70	30	5

RESULTS AND DISCUSSION

Verapamil (Figure 1) with two aromatic rings is ideally suited for analysis by reversed-phase HPLC. Using the proposed USP monograph method as a starting point, we developed a much faster method that requires significantly less eluent. Figure 2 shows a separation of the system suitability solution described in the proposed revision to the USP monograph method with an addition of verapamil-related compound A. All four compounds are well resolved. The resolution of verapamil and verapamil-related compound B is >5.0 as required by the proposed method and >1.5 as required by the current USP monograph method.

Verapamil-related compound D is eluted, as required by the proposed USP method. Table 1 shows the repeatability for retention time and peak areas for five injections of the system suitability standard. The method described here requires only 35 min compared to the 61 min required by the proposed USP method. In addition to the 26 min savings, there is 56.5 mL of eluent savings per injection because the method described here is run at 1.0 mL/min rather than 1.5 mL/min. Chromatography of the test solution (Figure 3) shows that this method easily detects 0.1% quantities of verapamil-related compounds A, B, and D relative to verapamil at 2.5 mg/mL, making it ideal for verapamil purity analysis.

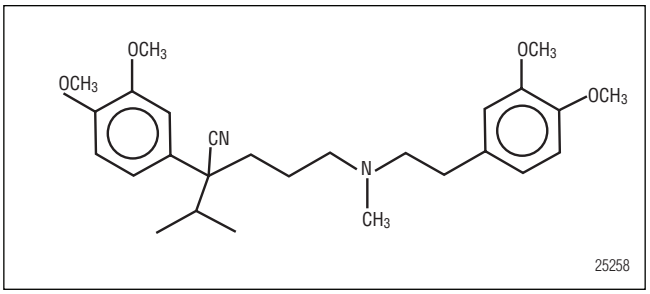

Figure 1. Structure of verapamil.

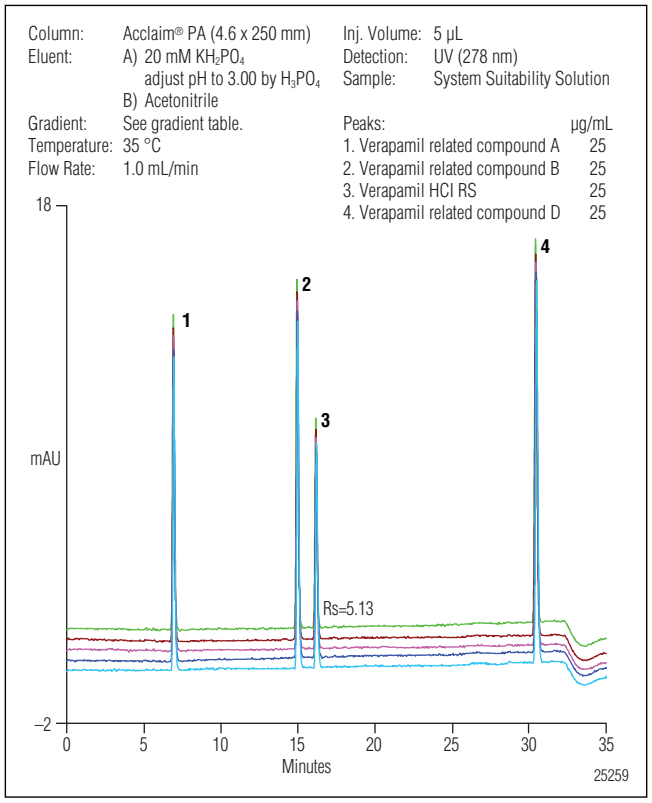

Figure 2. Overlay of five chromatograms of the system suitability solution.

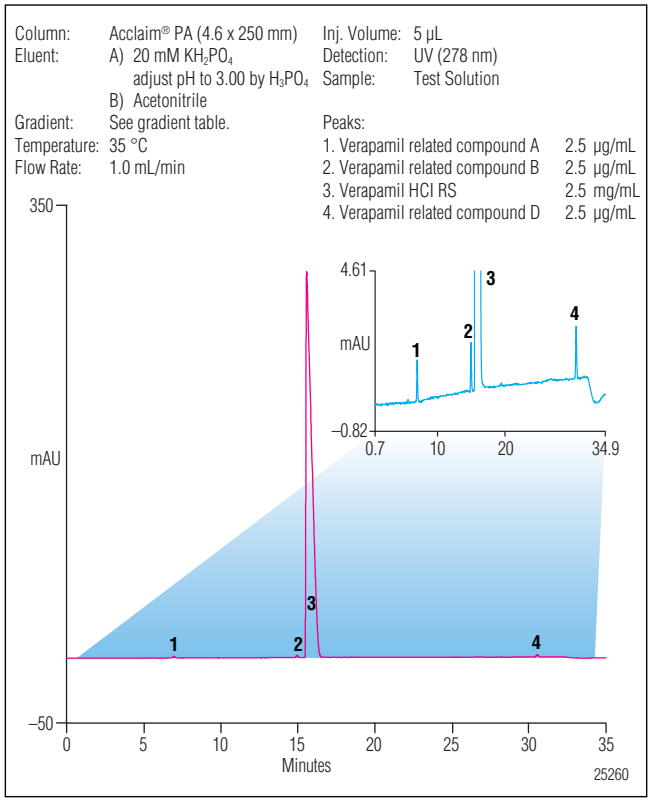

Figure 3. Chromatogram of the test solution.

Table 1. Repeatability of Retention Time and Peak Areas of Five Injections of the System Suitability Solution

RT (min)/ Area (mAU•min)	Verapamil Related Compound A		Verapamil Related Compound B		Verapamil HCl RS		Verapamil Related Compound D	
Injection 1	6.997	1.7798	15.084	15.084	2.0539	16.037	1.2225	2.553
Injection 2	6.991	1.7572	15.083	15.084	2.0654	16.305	1.2338	2.5308
Injection 3	6.99	1.7789	15.082	15.084	2.0771	16.301	1.2186	2.5278
Injection 4	6.989	1.7884	15.075	15.084	2.082	16.295	1.2391	2.54
Injection 5	6.984	1.7802	15.068	15.084	2.0538	16.29	1.228	2.5497
RSD	0.07	0.66	0.04	0.63	0.04	0.67	0.04	0.44

To achieve these improvements over the proposed USP monograph method, our method uses a polar-embedded reversed-phase column, the Acclaim PA, rather than an L60. No current L description suitably describes the resin in the PA column. It previously proved successful in improving the USP monograph method for nevirapine, which also prescribes a L60 column.[3] To shorten run time, achieve the best resolution, and work at a pH that will ensure the longest possible column life, the pH of the mobile phase is adjusted to 3.0 rather than the 7.2 pH recommended for the L60 column mobile phase.

Effect of Mobile Phase pH

During method development, testing the effect of pH 2.4 to 7.0 showed that lower pH provides higher resolution of verapamil-related compound B and verapamil. However, pH values lower than 3.1 did not significantly improve resolution. To meet the USP resolution requirement (> 5.0), a 250 mm column was required (same length as the L60). If a 150-mm column is preferred and a resolution of 5.0 between verapamil and verapamil-related compound B is not required, the 150-mm column will yield a resolution of about 4.0.

Figures 4 and 5 show the system suitability and test solutions analyzed at pH 2.9, 3, and 3.1. The chromatography shows that the method is rugged when challenged by small changes in pH.

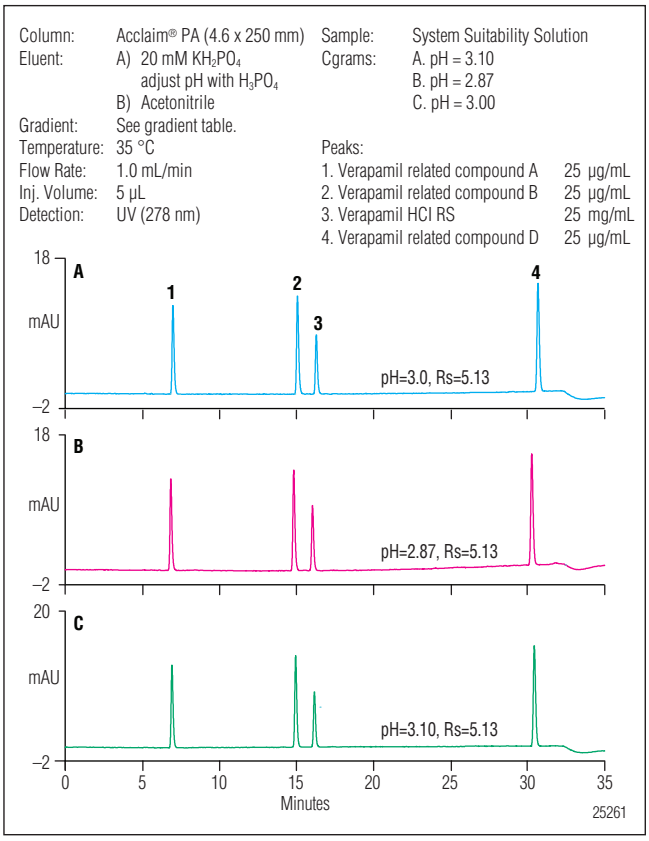

Figure 4. Overlay of chromatograms of the system suitability solution analyzed with different pH mobile phases. Peaks: 1) verapamil-related compound A; 2) verapamil-related compound B; 3) verapamil HCl RS, 4) verapamil-related compound D.

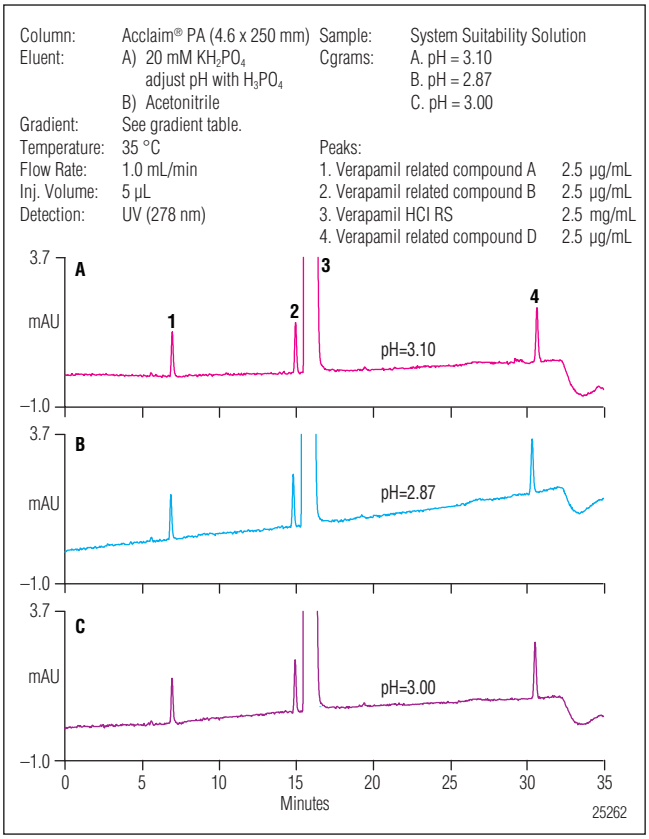

Figure 5. Analysis of the test solution with different pH mobile phases. Peaks: 1) verapamil-related compound A (2.5 µg/mL); 2) verapamil-related compound B (2.5 µg/mL), 3) verapamil HCl RS (2.5 mg/mL); 4) verapamil-related compound D (2.5 µg/mL).

REFERENCES

1. United States Pharmacopeia National Formulary USP30/NF25 (2007), 3454.
2. Pharmacopeial Forum (2006) 32(2) 389.
3. Dionex Application Note 180.

Determination of Glucosamine in Dietary Supplements Using HPAE-PAD

INTRODUCTION

Glucosamine (GlcN), an amino sugar, occurs naturally in the human body. It is a major structural component in the biosynthesis of glycosaminoglycans, compounds involved in normal joint function. Use of GlcN as a dietary supplement in the management of osteoarthritis has attracted considerable attention.[1] Results of the 2002 National Health Interview Survey showed that GlcN was one of the five nonvitamin, nonmineral herbal products/dietary supplements most frequently used by adults in the U.S.A.[2] Increased use in Canada was also noted.[3] While the principal use for GlcN dietary supplements is for arthritis management, especially in older adults, its use as a preventive measure to maintain health[4] and in veterinary medicine[5] also has been reported.

The 1994 Dietary Supplement Health and Education Act granted the United States FDA authority to prescribe good manufacturing practices for dietary supplements.[6] The final rule, published in June, 2007, established regulations requiring current good manufacturing practices (cGMP) for dietary supplements.[7] Using the cGMP regulation model for foods, the rule ensures that dietary supplements are produced in a quality manner, do not contain contaminants or impurities, and are accurately labeled.

Previously-reported methods for the determination of glucosamine in dietary supplements have used HPLC with UV or fluorescence detection.[8,9] As GlcN lacks a chromophore, these methods require either pre- or postcolumn derivatization and are often limited to determining only the glucosamine. However, carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds can be oxidized and therefore detected directly without derivatization using amperometry. Pulsed amperometric detection (PAD), a powerful detection technique with a broad linear range and very low detection limits, is ideally suited for determination of GlcN and related substances. This detection method is specific for those analytes such as GlcN that can be oxidized at a selected potential, leaving all other compounds undetected.

High-performance anion-exchange with pulsed amperometric detection (HPAE-PAD) chromatography is a sensitive, direct-detection technique capable of separating mono- and disaccharides rapidly and efficiently.[10,11] At approximately pH 12, the CarboPac® PA20 anion-exchange column will separate and elute neutral monosaccharides, aminosaccharides, and disaccharides while retaining oligosaccharides. The use of HPAE-PAD has been reported for the determination of saccharides in dietary glyconutritional products.[12]

Generating highly reproducible retention times for HPAE chromatographic systems relies on the use of a high purity hydroxide eluent mobile phase prepared with an accurate and precise concentration. An eluent generator (EG) produces such an eluent. The usual variability in hydroxide concentration associated

with manual eluent preparation, and the variability of carbonate contamination due to absorption of atmospheric carbon dioxide, are essentially eliminated by the EG, leading to highly reproducible retention times.

In this application note, a rapid, rugged HPAE-PAD method for determining GlcN in dietary supplement tablets, gelatin capsules, and fortified liquids is described. Key performance parameters are evaluated including accuracy, precision, and limits of detection/quantification, linearity, and ruggedness. The system setup (Figure 1) provides good sample throughput (7.5 min run time) while retaining the selectivity to resolve many other mono- and disaccharides that may be present in the supplement formulation.

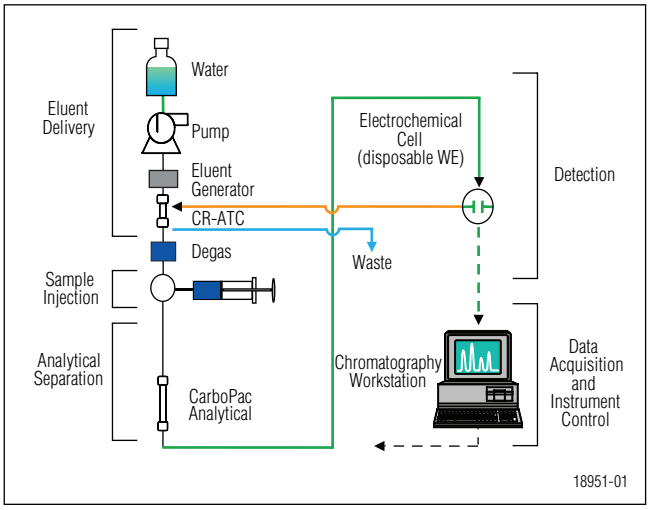

Figure 1. HPAE-PAD system for glucosamine determinations.

EQUIPMENT

Dionex ICS-3000 Reagent-Free™ Ion Chromatography system with Eluent Generation (RFIC-EG™ system) consisting of:

- DP Dual Gradient or SP Single Gradient Pump, with the EG/DP/SP Vacuum Degas Conversion Kit (P/N 063353) and GM-4 Gradient Mixer (P/N 049135)
- Eluent Generator with EGC II KOH eluent generator cartridge (EluGen® II Hydroxide; P/N 058900) and Continuously Regenerated Anion Trap Column (CR-ATC; P/N 060477)
- DC Detector/Chromatography module equipped with single or dual temperature zones, injection valve(s) and 10 µL injection loop, ED Electrochemical Detector (P/N 061718), ED cell and spacer block (P/N 061756) with combination pH/Ag/AgCl Reference Electrode (P/N 061879) and Carbohydrate Disposable Au Working Electrodes (P/N 060139, package of 6; 060216, package of 24)
- AS Autosampler (with diverter valve for dual systems), and 2 mL vial tray
- EO Eluent Organizer, including pressure regulator, and four 2 L plastic bottles for each system

Chromeleon® Chromatography Management Software

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm nylon (Nalgene® 90 mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent nylon filter)

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent; for degassing eluents)

1.5 mL glass injection vials with caps (Vial Kit, Dionex P/N 055427)

Microcentrifuge tubes with detachable screw caps (polypropylene, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher

D(+)Glucosamine (Sigma-Aldrich; P/N G4875)

Sucrose (Thermo Fisher Scientific; P/N S5500)

Glucose (Sigma-Aldrich; P/N G5250)

D-Sorbitol (Sigma-Aldrich; P/N S1876)

myo-Inositol (Sigma-Aldrich; P/N I5125)

N-Acetyl-D-glucosamine (Sigma-Aldrich; P/N A8625)

D(-)Fructose (Mallinckrodt Baker; P/N M55605)

Mannitol (Sigma-Aldrich; P/N M9546)

Glycerol (EMD Chemicals; formerly EM Science; P/N GX0190-6)

Propylene glycol (1,2-propanediol; Sigma-Aldrich; P/N P6209)

SAMPLES

Samples of GlcN-containing tablets, capsules, and beverages were purchased from retail groceries or drugstores. Table 1 lists the expected amount per serving size, source, the salt form of GlcN in each sample, other ingredients listed on the label, and the amount used to prepare the sample.

Table 1. Description of Glucosamine–Containing Samples					
Sample	**mg GlcN (Serving Size)**	**Size Used for Analysis**	**GlcN Salt Form**	**GlcN Source**	**Other Ingredients**
Supplement A	1500 (2 tablets)	1 tablet	HCl	Shellfish	MSM*, cellulose, hypromellose, croscarmellose sodium, stearic acid, silicon dioxide, magnesium stearate, corn starch, povidone, polyethylene glycol
Supplement B	1500 (1 tablet)	1 tablet	HCl	Shellfish	Cellulose, hydroxypropyl cellulose, stearic acid, coating (titanium dioxide, polydextrose, hydroxypropyl methylcellulose, triacetin, polyethylene glycol, magnesium trisilicate), copolyvidone, croscarmellose sodium, silicon dioxide
Supplement C	750 (1 tablet)	1 tablet	HCl	Vegetarian	Sorbitol, dibasic calcium phosphate, stearic acid, modified cellulose gum, colloidal silicon doixide, wheat/gluten
Supplement D	1000 (1 tablet)	1 tablet	HCl	Vegetarian	Cellulose, modified cellulose gum, stearic acid, magnesium stearate
Supplement E	1000 (1 tablet)	1 tablet	H_2SO_4	Not disclosed	Potassium chloride, cellulose, modified cellulose gum, stearic acid, magnesium stearate
Supplement F	1500 (2 capsules)	1 capsule	H_2SO_4	Shellfish	Potassium chloride, gelatin, magnesium stearate
Supplement G	1500 (1 can**)	1-237 mL can	HCl	Not disclosed	Sparkling water, orange juice concentrate, citric acid, mango juice concentrate, passionfruit juice, sodium hexametaphosphate, sucralose, potassium sorbate, coloring extracts

*MSM - Methylsulfonylmethane (dimethylsulfone) present at 1500 mg/serving

**One can contains 237 mL of liquid

CONDITIONS

Column:	CarboPac PA20 Analytical, 3 × 150 mm (P/N 060142)
Eluent:	20 mM KOH, isocratic, 7.5 or 15 min run time
Eluent Source	EGC II KOH
Flow Rate:	0.5 mL/min
Injection Volume:	10 µL (full loop)
Temperature:	30 °C
Detection:	Pulsed amperometry, using Carbohydrate Disposable Au Working Electrodes (P/N 060139, package of 6; P/N 060216, package of 24)
Background:	40–65 nC
Typical System Backpressure:	2580–2730 psi

Carbohydrate 4-Potential Waveform for the ED

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the ICS-3000, but not used in older Dionex systems.

Reference electrode in Ag mode (Ag/AgCl reference).

Instrument Operational Considerations

Analyze a GlcN check standard at regular intervals to assess both retention time (RT) and peak area precision. When required, a column wash at 100 mM KOH will restore RT for GlcN. The column requires at least 2 h after the column wash to reequilibrate to 20 mM KOH and achieve the highest RT precision. Shorter reequilibrations may yield acceptable precision.

When the system is idle for short (1–2 week) periods, we recommend that the pump and eluent generator be left on at 0.5 mL/min and 20 mM KOH or at a reduced flow rate to allow rapid start-up, and the cell to be turned off to extend disposable electrode life. The use of a lower flow rate, while maintaining the minimum backpressure of at least 200 psi, can extend the interval before water must be added to the eluent reservoir. When the system must be shut down for a period of several weeks, the pump, eluent generator, and electrochemical cell may be turned off. For shutdown periods exceeding several weeks, all plumbing lines should be resealed, and the reference electrode should be removed from the electrochemical cell and stored in the original solution in which it was shipped by Dionex (3.5 M KCl). When the pump has been turned off for longer than 1 day, the column should be washed with 100 mM KOH for 1–2 h, and reequilibrated with 20 mM KOH for 2 h or less (see above) before analyzing samples.

PREPARATION OF REAGENTS AND STANDARDS

Eluents

It is essential to use high-quality water of high resistivity (18 MΩ-cm) containing as little dissolved carbon dioxide as possible. Biological contamination should be absent. Source water must be obtained using a water purification system consisting of filters manufactured without electrochemically active surfactants or other leachable substances (e.g., glycerol). Prior filtration through 0.2 µm porosity nylon under vacuum is recommended to remove particulates and reduce dissolved air. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of helium at all times to reduce carbonate contamination and opportunistic microorganisms.

Although not used to produce the data in this application note, a manually prepared NaOH eluent can be used. Follow the instructions in Dionex Technical Note 71 to prepare 100 or 200 mM NaOH and allow the pump to proportion the 20 mM eluent. Results obtained using manually prepared eluent may not be equivalent to the results reported here.

Stock Standards

Prepare stock solutions of GlcN and other ingredients in the dietary supplements by accurately weighing standards into tared plastic vials. Add filtered and degassed DI water and weigh the resulting solution. Prepare stock standard solutions at concentrations of approximately 1.0 mM. Store stock standards at –15 °C. Dilute stock standards with filtered, degassed water to yield the desired working mixture concentrations. For this application note, all dilutions were made gravimetrically to ensure high accuracy and concentrations reported as GlcN free base.

SAMPLE PREPARATION

Place tablet or capsule sample in a 1.0 L volumetric flask and add approximately 500 mL of filtered DI water. Place the flask into an ultrasonic bath until the sample is fully dispersed (20–30 min) and then bring to volume with filtered DI water. Pour liquid dietary supplement sample into a 1.0 L volumetric flask, carefully degas under vacuum, and bring to volume with filtered, degassed DI water. Make further dilutions by placing 1 mL aliquots in 1.5 mL plastic microcentrifuge vials with detachable screw caps and centrifuge at 16,000 × g in a microcentrifuge for 20 min. Dilute the supernatant gravimetrically to produce sample stock solutions expected to have 1.0 mM (180 µg/mL) GlcN free base concentrations based on product label information. Further dilute aliquots from the 1.0 mM solutions gravimetrically to produce solutions for injection into the HPAE-PAD system.

Quantitative results for GlcN concentration and for concentrations of other putatively identified ingredients were converted to the masses of these compounds in the original sample (one tablet or capsule or one 237 mL can of liquid). Two factors, the dilution factor (DF) and the molar conversion factor (CF) were needed for this calculation. The DF represents the factor required to dilute product solutions from their concentration in the 1.0 L volumetric flask to their injected target concentrations. Dilutions used for this application note are listed in Table 3. The CF represents the factor that converts concentrations found for GlcN and other

putatively identified ingredients to mass of the analyte in the original sample. For supplements containing GlcN as the sulfate salt, CF was 228 (half the FW of 2GlcN·H_2SO_4). Supplements E and F contained GlcN as its H_2SO_4 salt. For Supplements A, B, C, D and G, which contained GlcN as its chloride salt, the CF was 216 (the FW of GlcN·HCl). For other substances, CF was the compound's MW. To convert the measured GlcN free base concentration (expressed as µM, µmoles/L) to mg of GlcN as its appropriate salt form per unit dissolved in the original 1.0 L of water, the following equation was used:

$$\frac{\text{mg GlcN (salt form)}}{\text{unit}} =$$

$$\frac{\mu\text{mol GlcN}}{\text{L}} \times \text{DF} \times \text{CF} \times 1.0 \frac{\text{L}}{\text{unit}} \times 0.001 \frac{\text{mg}}{\mu\text{g}}$$

A unit of supplement is a tablet, capsule, can, packet, or any other amount of product dissolved or diluted in 1.0 L of water to prepare the sample concentrate. For example, if the GlcN concentration in the diluted sample of Supplement A is determined to be 10.0 µM, the amount of GlcN·HCl in the tablet dissolved in 1.0 L water is:

$$\frac{\text{mg GlcN·HCl}}{\text{unit}} =$$

$$\frac{10\ \mu\text{mol}}{\text{L}} \times 350 \times 216 \frac{\mu\text{g GlcN·HCl}}{(\mu\text{mol GlcN free base})} \times 1.0 \frac{\text{L}}{\text{unit}}$$

$$\times 0.001 \frac{\text{mg}}{\mu\text{g}} = 756 \frac{\text{mg}}{\text{unit}}$$

Method accuracy was assessed from recovery of known amounts of GlcN spiked into either DI water or Supplement B previously diluted to an expected GlcN concentration of 9.9 µM (1.8 µg/mL). A 1.00 mM (179 µg/mL) GlcN standard was used to accurately spike the Supplement B sample at 50% and 100% of the expected GlcN concentration in the supplement.

RESULTS AND DISCUSSION

Separation

Figure 2A shows chromatograms for the seven GlcN dietary supplements diluted to the target 10 µM (1.8 µg/mL) GlcN concentration. The CarboPac PA20, combined with PAD, yielded simple chromatograms for most of the supplements tested. In Supplement A, the high concentration of methylsulfonylmethane (MSM), another active ingredient in this product, was not detected and did not interfere with the GlcN determination. Sorbitol in Supplement C, an inactive ingredient (preservative), was detected but did not interfere. In liquid Supplement G, glucose, fructose, sucrose, and *myo*-inositol were also observed and sufficiently separated from GlcN. The

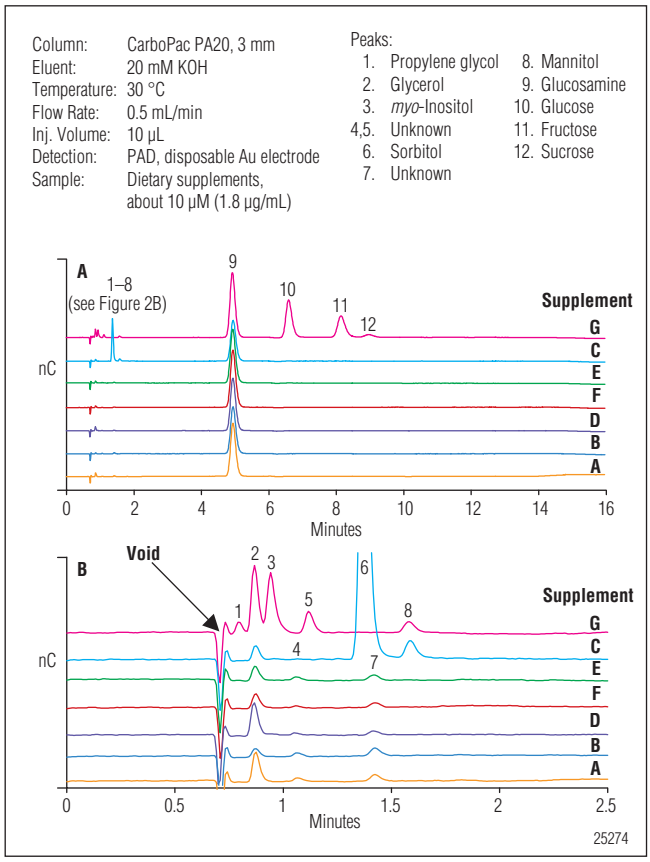

Figure 2. HPAE-PAD analysis of GlcN-containing dietary supplements. Seven dietary supplement samples diluted to approximately 10 µM (1.8 µg/mL) GlcN, 10-µL injection. A) Full chromatogram. B) Expanded early RT region of the chromatogram.

added non-nutritive sweetener, sucralose, was retained on the CarboPac PA20 column and was not eluted using this method. Sucralose can be determined using similar methods.[13,14] Although we expected the possibility that *N*-acetyl-glucosamine might be present in some of the dietary supplements from shellfish sources, it was not detected.

Trace amounts of other, unidentified ingredients can be seen in Figure 2B. Peaks 4 and 7 are detected in all tablet and capsule samples analyzed, except in Supplement C, where peak 7 may be masked by sorbitol, peak 6. Neither peak was detected in the beverage (Supplement G). Table 1 lists the other ingredients present in the seven dietary supplements evaluated in this note. The combined use of HPAE and the specificity of PAD yields an uncomplicated chromatogram for determination of GlcN.

Eluent concentrations of 10–15 mM KOH caused the GlcN peak to coelute with a baseline dip, typically having a retention time of 6 min. Baseline dips associated with injections of water or samples are caused by the elution of non-electrochemically active trace organic impurities present in the sample. When these compounds elute, they exclude electrochemically active ions present in the eluent and appear as negative peaks. The "oxygen dip" (~16 min retention time for the column used in this study) is due to oxygen present in the samples and appears as a function of the gas permeation volume of the column. The retention times of the "oxygen dip" and other baseline dips are constant for each column, but vary slightly from column to column; and many depend on the flow rate, not the eluent strength. Increasing the eluent strength to 20 mM KOH decreased the GlcN retention time to 5.0 min and thus removed any effect of the dip at 6 min on GlcN peak integration.

Eluting the baseline dips just prior to the end of the run, or timing their elution to occur at the end of the following injection, prevents the baseline dips from interfering with the peaks of interest. Using the overlapping sample preparation configuration (flushing the injection port, needle, and autosampler tubing for the next sample during the separation of the current sample), a run time of 7.5 min (total time between injections of 8.6 min) will produce a relatively flat baseline for integration of peaks having retention times between 1–6 min. For samples with compounds eluting later than GlcN, the run time can be set to 16 min without significant baseline interfere from the oxygen dip

.

Detection

Linearity

Figure 3A presents the relationship of GlcN peak area (nC*min) to concentration of the GlcN injected (10 µL) over a broad range of concentrations, 0 to 1000 µM (0–179 µg/mL). In this study the lower limit of detection was estimated to be 0.09 µM (0.02 µg/mL). The full linear range in this study covered more than 3 orders of magnitude, 0.30–340 µM, 0.06–61 µg/mL, for a 10 µL injection. For routine GlcN determination we recommend a dietary supplement dilution scheme that targets a 10 µM (1.8 µg/mL) GlcN concentration. Figure 3B presents a plot covering a narrower concentration range of 1.8–36 µM (0.32–6.4 µg/mL) where the target concentration is near the middle of this range. The r^2 value in this range is >0.9998.

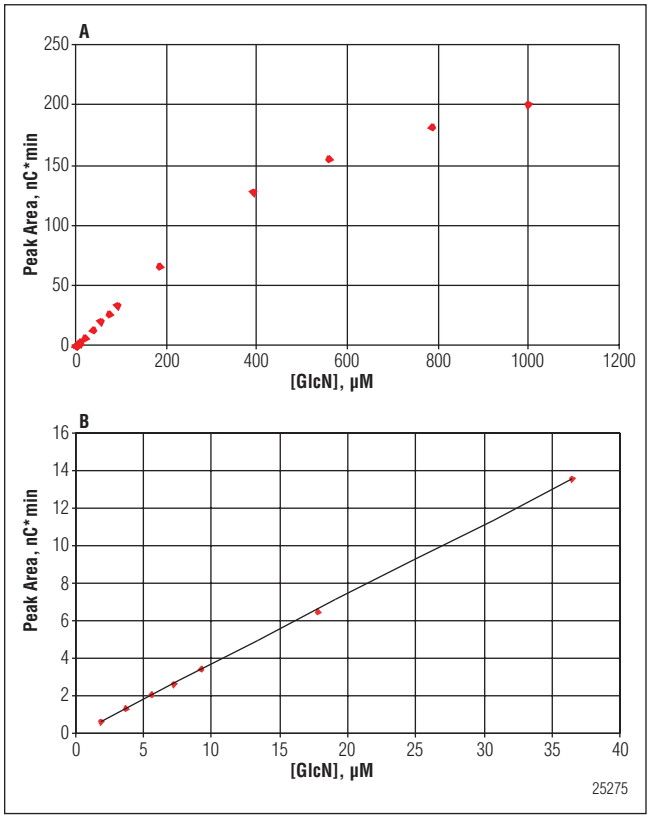

Figure 3. The relationship of peak area (mean) to glucosamine concentration injected for estimation of linear range (n = 8). A) Wide range curve. B) Narrower range used for GlcN quantification.

Table 2. Precision of Glucosamine Retention Time and Peak Area for Supplement B Injected Consecutively Over 5 Days

	Day					All 5 Days	% Change over 5 Days
	1	2	3	4	5		
Retention Time (min)							
Mean	4.972	4.937	4.908	4.899	4.896	4.922	–1.53
SD	0.011	0.011	0.007	0.007	0.009	0.03	
N	144	146	145	141	142	718	
RSD	0.22	0.22	0.14	0.14	0.18	0.61	
Peak Area (nC*min)							
Mean	4.096	4.073	4.049	4.029	4.034	4.057	–1.51
SD	0.034	0.027	0.034	0.045	0.035	0.043	
N	144	146	145	141	142	718	
RSD	0.83	0.66	0.84	1.12	0.87	1.06	

Precision

GlcN retention time and peak area RSDs were determined for replicate injections of Supplement B supernatant (GlcN concentration targeted to 10 µM [1.8 µg/mL] for 10 µL injection) over 5 days (718 injections). Supplement B was chosen for this study because the label lists several cellulosic compounds as part of this dietary supplement tablet and was considered among the more challenging matrices of the products investigated in this note. Run times were 7.5 min (injections made every 8.6 min). Table 2 shows these results on a daily basis and for the 5-day period. The column was washed for 1 h at 100 mM KOH prior to this study, but no wash was performed during this 5-day period.

Retention Time

Buildup on the stationary phase of non-eluting sample ingredients and carbonate contaminants from the eluent can result in decreasing capacity and eventually can decrease the retention time for GlcN. An EG essentially eliminates carbonate contamination; therefore, the only remaining concern is loss of column capacity due to sample ingredients. The data in Table 2 shows high retention time precision and little loss of retention time over the 5 days, despite injecting a challenging sample with no column washes during the 5-day period.

Peak Area

Peak area precision is a measure of the ECD response stability and the variance in response for replicate injections. Table 2 shows there was good GlcN peak area reproducibility during the 5-day study.

Accuracy

GlcN recovery from DI water and a diluted aqueous extract of a dietary supplement was evaluated in this application note. Percent recovery (mean ± SD) from DI water at 5.1 µM (0.91 µg/mL) and 10.1 µM (1.81 µg/mL) was 101 ± 1.3 and 102 ± 0.3 %, respectively. Recoveries from Supplement B supernatant spiked at 5.1 µM (0.91 µg/mL) and 9.9 µM (1.77 µg/mL) were 93.4 ± 3.0 and 99.0 ± 2.5 %, respectively, indicating that the method was accurate.

Table 3. Determination of Glucosamine in Dietary Supplement Samples

Sample	Dilution Factor (DF)	Measured Amount, mg/unit[a]	Expected Amount, mg/unit[b]	% GlcN Found ± SD
Supplement A	350	959 ± 9.5	750	128 ± 1.3
Supplement B	659	1650 ± 2.5	1500	110 ± 1.7
Supplement C	455	966 ± 3.5	750	129 ± 0.5
Supplement D	413	1130 ± 4.1	1000	113 ± 0.4
Supplement E	467	1370 ± 2.5	1000	137 ± 0.3
Supplement F	315	991 ± 5.2	750	132 ± 0.7
Supplement G	680	2270 ± 11	1500	152 ± 0.7

[a]Calculated amount = [GlcN] found × DF × CF, converted to mg
[b]Expected amount derived from Supplement Facts on label

Table 4. Determination of Other Substances Detected in Dietary Supplements

Sample	Analyte[a]	Calculated amount/unit (mg/unit)[b,c] ± SD	% Relative to Measured [GlcN] ± SD
Supplement A	Glycerol	28.1 ± 0.6	2.9 ± 0.1
Supplement B	Glycerol	17.6 ± 1.1	1.1 ± 0.1
Supplement C	Glycerol	16.8 ± 0.4	1.74 ± 0.04
	myo-Inositol	0.7 ± 0.4	0.07 ± 0.04
	Sorbitol	307 ± 1.5	31.8 ± 0.2
	Mannitol	43.9 ± 1.4	4.5 ± 0.1
Supplement D	Glycerol	37.3 ± 1.8	3.3 ± 0.2
Supplement E	Glycerol	18.5 ± 0.5	1.35 ± 0.04
Supplement F	Glycerol	12.7 ± 0.3	1.28 ± 0.03
Supplement G	Propylene glycol	5.44 ± 0.08	0.24 ±0.01
	Glycerol	125 ± 2.7	5.5 ± 0.1
	myo-Inositol	61.7 ± 0.8	2.72 ± 0.04
	Mannitol	43 ± 2.2	1.9 ± 0.1
	Glucose	1380 ± 11	60.8 ± 0.6
	Fructose	1939 ± 4.5	85.4 ± 0.5
	Sucrose	300 ± 11	13.2 ± 0.5

n = 5 injections per sample
[a]Putative identification based on retention time matches with standards
[b]A unit is 1 tablet, 1 capsule, or 1 237-mL can of liquid
[c]Calculated amount = [substance] found × DF × MW, converted to mg

Application

Figure 2 presents chromatograms for the seven GlcN-containing dietary supplements studied. No other peaks were observed when run times were extended to 30 min. Table 3 shows the measured amounts of GlcN in the seven dietary supplements analyzed for this note, derived from a 7-point calibration over the 1.8–36 µM (0.32–6.4 µg/mL) range. The determined amounts of GlcN for all seven supplement samples were above the stated label amounts, ranging from 110%–152% of the GlcN label value.

Some dietary supplements showed significant amounts of PAD-responsive related substances using this method (Figure 2). The peaks for these related substances were putatively identified by matching their retention times with those of carbohydrate and glycol standards. Single-level calibrations were used to estimate the amount of these ingredients in the supplements. Table 4 shows the amounts of these related substances, expressed as mg/unit. Unknown ingredient peaks 4 and 7 (Figure 2B), present in all products except Supplement G, showed peak areas relative to GlcN ranging from 0.08–0.27% and 0.29–0.63%, respectively. This method can also be used to determine other carbohydrates or glycols present in dietary supplements. Higher concentration GlcN solutions can be injected for determination of trace mono- and disaccharide concentrations, if desired, for evaluation of GlcN quality.

CONCLUSION

HPAE-PAD with eluent generation can be used to determine glucosamine in dietary supplements without the pre- or postcolumn derivatization required when using UV or fluorescence detection. Sample preparation consists of simply dissolving samples in DI water and diluting the resulting solution to a target concentration within the linear range. The high capacity of the CarboPac PA20 and the use of eluent generation enable the isocratic analysis of over 100 samples per day for 5 days with the analyst required to add only water and samples to the system. This method works for a variety of sample matrices, as demonstrated by the practical application of this method to the accurate determination of GlcN in seven dietary supplements.

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SUPPLIERS

EMD Chemicals Inc., 480 South Democrat Road, Gibbstown, NJ 08027, U.S.A. Tel: 1-800-222-0342 http://www.emdchemicals.com.

Gast Manufacturing Corp., 2550 Meadowbrook Road, Benton Harbor, MI 49022, U.S.A. Tel: 1- 269-926-6171, http://www.gastmfg.com.

Mallinckrodt Baker, 222 Red School Lane, Phillipsburg NJ 08865, U.S.A. 1-800-582-2537 http://www.mallbaker.com.

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625, U.S.A. Tel: 1-800-625-4327, http://www.nalgenunc.com.

Praxair, 39 Old Ridgebury Road, Danbury, CT 06810-5113, U.S.A. Tel: 877-772-9247, http://www.praxair.com.

Sarstedt AG & Co., Rommelsdorfer Straße, Postfach 1220, 51582 Nümbrecht, Germany Tel.: +49-2293-305-0, http://www.sarstedt.com.

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A., Tel: 1-800-325-3010, www.sigma.sial.com.

Thermo Fisher Scientific, 4500 Turnberry Drive, Hanover Park, IL 60133, U.S.A. Tel: 1-800-766-7000 www.fishersci.com.

Determination of Urea and Allantoin in Cosmetics Using the Acclaim Mixed-Mode HILIC Column

INTRODUCTION

Reversed-phase (RP) silica columns (e.g., C18 and C8) are widely used for separating small molecules. However, these columns are unsuitable for retaining and separating highly polar compounds. Some modified RP columns, such as the Acclaim® PolarAdvantage (PA) column, a type of polar-embedded column, retain some polar compounds. However, chromatographers often need to use a buffer, which usually impairs MS detection and results in insufficient retention of some highly polar compounds. Hydrophilic interaction chromatography (HILIC), termed by Alpert in 1990, is a technique capable of retaining these highly polar compounds with additional benefits, including complementary selectivity compared to RP columns, enhanced sensitivity for MS detection, and simplified sample preparation.[1] However, due to the low-hydrophobicity surfaces associated with traditional HILIC columns (i.e., silica-, cyano-, amino-, and diol- phases), small molecules cannot be separated via hydrophobic interaction. Mixed-mode hydrophilic interaction-cation-exchange chromatography (HILIC-CEX) promotes hydrophilic interactions overlaid on ionic interactions with a cation-exchange matrix and this high-performance technique has the potential for peptide separations.[2–5]

The Acclaim Mixed-Mode HILIC column is a new stationary phase that combines both HILIC and RP characteristics.[6] The new phase is based on high purity and spherical silica functionalized with a silyl ligand containing both hydrophilic and hydrophobic functionalities. This packing material can be used in either HILIC mode (in high organic conditions) or RP mode (in high aqueous conditions). The optimal balance between the hydrophilic and hydrophobic moieties on the silica surface provides unique chromatographic properties that make this new phase useful for many applications, including determination of hydrophobe distribution and degree of ethoxylation (EO number) in a broad variety of ethoxylated surfactants.[6]

In this application note, we investigate the chromatographic behavior of highly polar compounds on the Acclaim Mixed-Mode HILIC column using allantoin and urea as test compounds. We discuss the influence of different sample diluents, and different concentrations and pH values of the buffer in the mobile phases on HILIC. After method optimization, we determined the concentrations of allantoin and urea in cosmetic products.

EQUIPMENT

UltiMate® 3000 HPLC system:

HPG 3400A pump with SRD 3400 Solvent Rack with degasser

TCC-3000 thermostatted column compartment

WPS-3000TSL autosampler

VWD-3400 UV-Vis detector

Chromeleon® 6.80 SP2b Chromatography Workstation

Anke TDL80-2B centrifuge, Anting Scientific Instrumental Factory, Shanghai, China

85-2 magnetic stirrer, Hongpu Instrumental Factory, Minhang, Shanghai, China

PH 140A constant temperature oven, Yiheng Science and Technique Ltd., Shanghai, China

REAGENTS AND STANDARDS

Deionized (DI) water from a Milli-Q® Gradient A10 (Millipore)

Acetonitrile (CH_3CN), HPLC grade, Fisher

Allantoin, purum, ≥ 98%, Fluka

Urea, analytical grade, SCRC, China

n-Heptane, analytical grade, SCRC, China

Acetic acid (HAc), analytical grade, SCRC, China

Ammonium acetate, analytical grade, SCRC, China

Diatomaceous earth, Hyflo Super Cel®, Sigma-Aldrich

CHROMATOGRAPHIC CONDITIONS

Column:	Acclaim Mixed-Mode HILIC column, 5 µm, 4.6 × 150 mm (P/N: 066843)
Mobile Phase:	Premix of 97% CH_3CN : 3% H_2O (v/v, using premixed mobile phase yields a more stable base-line at the 200 nm detection wavelength)
Flow Rate:	1.0 mL/min
Temperature:	30 °C
Inj. Volume:	5 µL
Detection:	Absorbance at 200 nm

PREPARATION OF STANDARDS

Stock Standard Solutions

The concentrations of stock standard solutions were 1000 mg/L for allantoin and 10,000 mg/L for urea. They were prepared with DI water.

Mixed Working Standard Solutions

The mixed stock standard solution was diluted with a solution of 90% acetonitrile, 10% DI H_2O (v/v) to prepare the mixed working standard solutions used for calibration. The concentrations of each analyte in the mixed working standard solutions are shown in Table 1.

Table 1. Concentration of the Mixed Working Standard Solutions

Analyte	Concentration (mg/L)					
	# 1	# 2	# 3	# 4	# 5	# 6
Allantoin	3.125	6.25	12.5	25.00	50.00	125.0
Urea	25.00	50.00	100.0	200.0	400.0	1000

SAMPLE PREPARATION

Two cosmetic products (samples 1 and 2) were purchased from a local market. About 0.5 g of sample and 0.5 g diatomaceous earth were placed into a 50 mL beaker. 10 mL n-heptane was added, and the mixture was stirred for 5 min using a magnetic stirrer. The organic phase was discarded, and the inorganic phase was extracted two more times. The residue was dried completely at 60 ºC in a constant temperature oven. Three milliliters of water and 7 mL acetonitrile were added to the dried residue, stirred for 3 min, allowed to stand for 10 min, and then the solution layer was moved to a 10 mL centrifuge tube and centrifuged at 3000 rpm for 10 min. The same sample was extracted two more times with water and acetonitrile. The solution layer of all three extracts was moved to a 100 mL glass flask and diluted to the mark with acetonitrile. Prior to injection, the extract was filtered though a 0.45 µm filter.

RESULTS AND DISCUSSION

Comparison of the Retention of Highly Polar Compounds on Acclaim Mixed-Mode HILIC, Acclaim 120 C18, and Acclaim PA Columns

Allantoin and urea (structures shown in Figure 1) are compounds with high polarity and therefore are good candidates for HILIC. Typical RP silica columns such as the Acclaim 120 C18 are unable to retain these compounds, as shown by chromatogram B in Figure 2. Reducing the CH_3CN to 30% does not result in retention of either compound. These compounds are retained on the Acclaim PA column (a polar-embedded phase, chromatogram C in Figure 2) under conditions that should yield the maximum retention of polar compounds (no CH_3CN), but resolution is poor. Chromatogram A of Figure 2 shows that allantoin and urea are well retained and resolved on the Acclaim Mixed-Mode HILIC column.

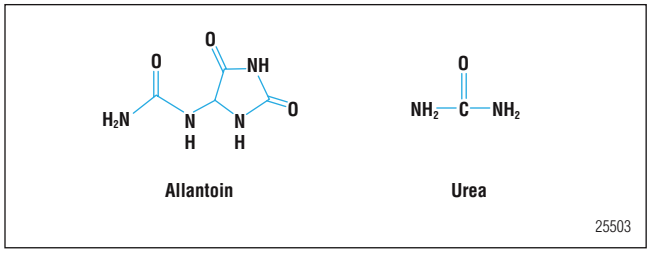

Figure 1. Structures of allantoin and urea.

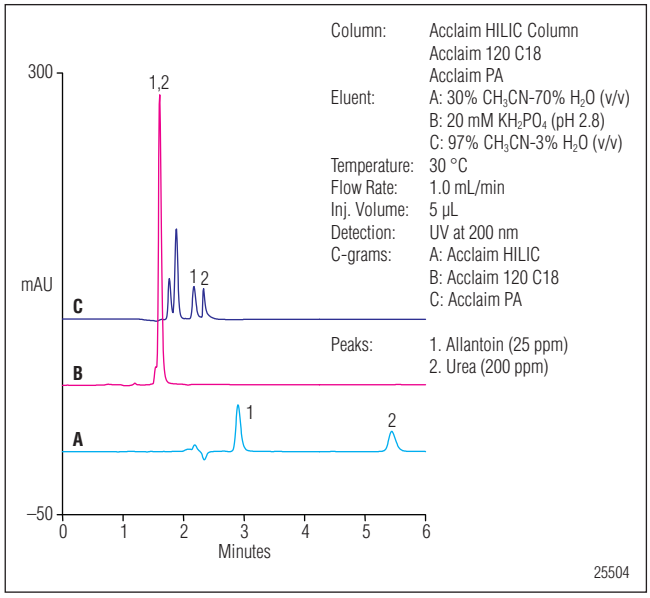

Figure 2. Chromatograms of allantoin and urea on A) Acclaim Mixed-Mode HILIC, B) 120 C18, and C) PA columns.

Influence of Sample Diluent on Acclaim Mixed-Mode HILIC Column Chromatography

As in RP-HPLC, sample diluent can strongly influence peak shape and sample solubility during HILIC chromatography. Here, three solvents commonly used in HPLC, differing in solvent strength in the order CH_3CN> CH_3OH >water, were used to prepare standard solutions of the polar compounds allantoin and urea. The ideal sample diluent should be 100% CH_3CN or as close to initial mobile phase conditions as possible. As shown in Figure 3, the best peak shapes for allantoin and urea are obtained using 100% CH_3CN as the sample solvent. However, highly polar analytes often have low solubilities in organic solvents, making some samples difficult to run on a HILIC column. Although 100% water can dissolve polar samples better, it is not suitable for injecting on a HILIC column because of the resulting poor peak shape (chromatogram A, Figure 3).

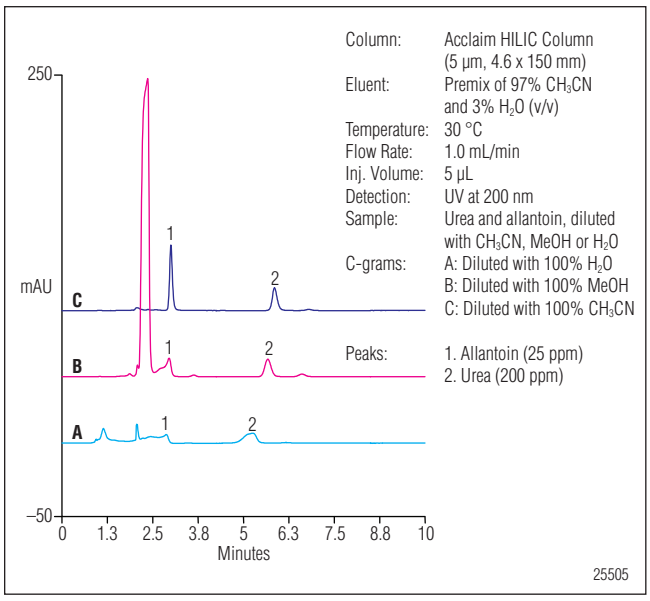

Figure 3. Chromatograms of allantoin and urea diluted with A) water, B) methanol, and C) acetonitrile.

Therefore, the influence of using methanol/water and CH_3CN/water diluents with different proportions on peak shapes of allantoin and urea were investigated. Figure 4 shows chromatograms of allantoin and urea using three different methanol/water mixtures as diluents, and none yielded acceptable peak shapes. Using CH_3CN/water mixtures as diluents, peak shapes are acceptable at 75% CH_3CN and higher (Figure 5). When the proportion of CH_3CN increases to 90%, sharp and symmetric peak shapes are observed. Most polar analytes are soluble in 90% CH_3CN/10% water.

Influence of Mobile Phase Buffer on HILIC Chromatography

Phosphate buffers are not recommended due to precipitation in the highly organic mobile phases commonly used in HILIC. The buffers usually used with HILIC columns are ammonium formate, ammonium acetate, formic acid, and phosphoric acid. We evaluated the influences of the pH value and concentration of an acetic acid-ammonium acetate buffer on HILIC chromatography. The pH value of the acetic acid-ammonium acetate buffer was adjusted by changing the proportion of acetic acid and ammonium acetate. As shown in Figure 6, although the retention time of allantoin

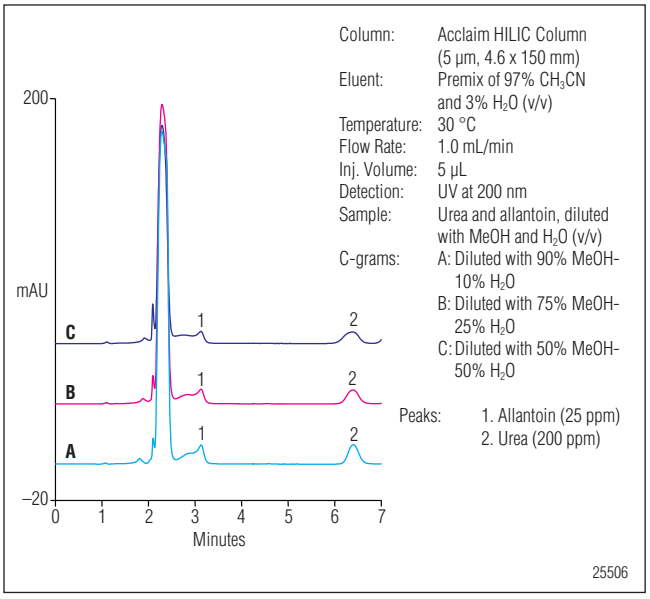

Figure 4. Chromatograms of allantoin and urea diluted with methanol/water with differing proportions.

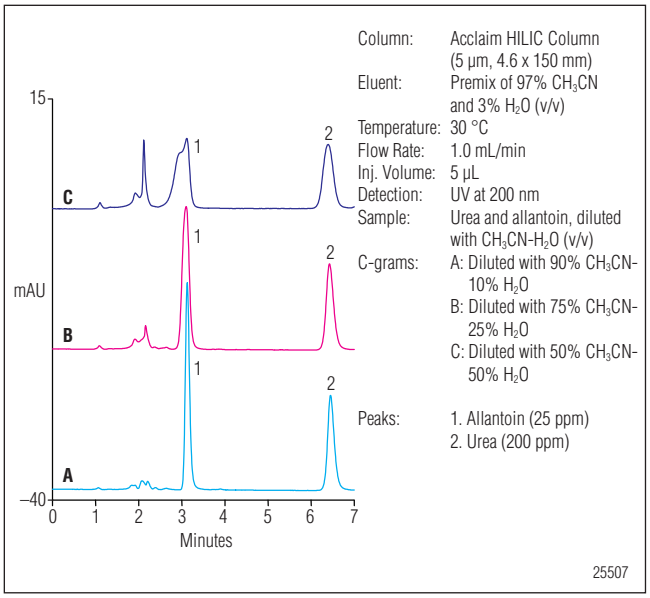

Figure 5. Chromatograms of allantoin and urea diluted with acetonitrile/water with differening proportions.

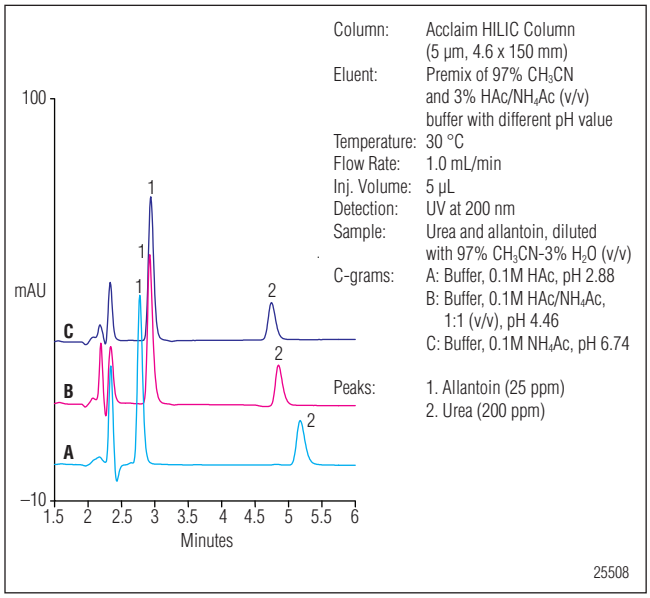

Figure 6. Chromatograms of allantoin and urea when the buffers in mobile phase are at different pH values.

increases and that of urea decreases with increasing buffer pH, their peak shapes are maintained. Figure 7 shows chromatograms of allantoin and urea when the ammonium acetate concentration in the mobile phase changes from 100 to 0 mM (100% H_2O). Peak shapes are also maintained with changes in the buffer concentration, even in the absence of buffer. However, for some ionic compounds, e.g., benzoate, a buffer in the mobile phase is needed for good peak shape and retention (Figure 8).

Reproducibility, Linearity and Detection Limits

Prior to sample analysis, we estimated the reproducibility by making seven replicate injections of a mixed standard solution with concentrations of 25 mg/L for allantoin and 200 mg/L for urea. The RSDs for retention time were both 0.000, and the RSDs for peak area were 0.178 for allantoin and 0.379 for urea.

Calibration linearity for allantoin and urea was investigated by making replicate injections of a mixed standard prepared at six different concentrations. The external standard method was used to calculate the calibration curve and to quantify these compounds in samples. Table 2 shows the data from the calibration as calculated in Chromeleon. The single-sided Student's test method was used for estimating method detection limits (MDL). These data are also reported in Table 2.

Table 2. Calibration Data as Reported by Chromeleon and MDLs for the Two Analytes

Analyte	Equation	r	RSD	MDL (mg/L)
Allantoin	A = 0.1362 c + 0.0163	0.9998	1.6408	0.66
Urea	A = 0.0111 c + 0.0039	0.9990	2.9740	11.6

Note: The single-sided Student's test method (at the 99% confidence limit) was used for determining MDL, where the standard deviation (SD) of the peak area of seven injections is multiplied by 3.14 to yield the MDL.

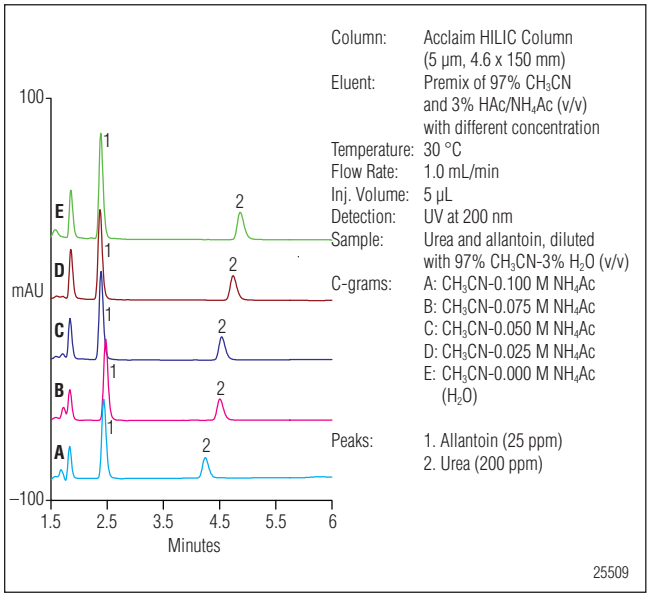

Figure 7. Chromatograms of allantoin and urea diluted when changing buffer (NH_4Ac) concentration in mobile phase

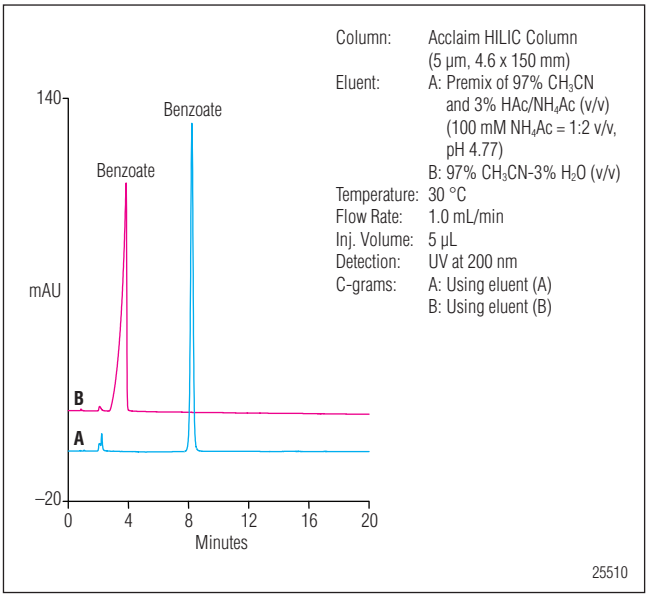

Figure 8. Chromatograms of benzoate on the Acclaim Mixed-Mode HILIC column using different mobile phases.

Sample Analysis

Allantoin and urea are added to cosmetic products for skin protection and regeneration, especially for the treatment of dry skin. We analyzed two different cream samples. Figure 9 shows chromatograms of a blank, sample 2, and the same sample spiked with standards. The amounts of allantoin and urea in each sample and the spike recovery from sample 2 are summarized in Table 3. Urea was found in both of the samples, and allantoin was found in sample 2.

CONCLUSION

For HILIC, sharp symmetric peaks for polar compounds are obtained using 90% acetonitrile, 10% water as the sample diluent. Our recommended buffer for HILIC mobile phases is ammonium acetate. For the determination of urea and allantoin, the pH value of the buffer does not have a significant influence on peak shape. However, keeping a certain buffer concentration in the mobile phase can yield better peak shape for some ionic polar compounds (e.g., for benzoate). Our experiments demonstrate that the Acclaim Mixed-Mode HILIC column is suitable for separation of highly polar compounds such as allantoin and urea and their determination in cosmetic products.

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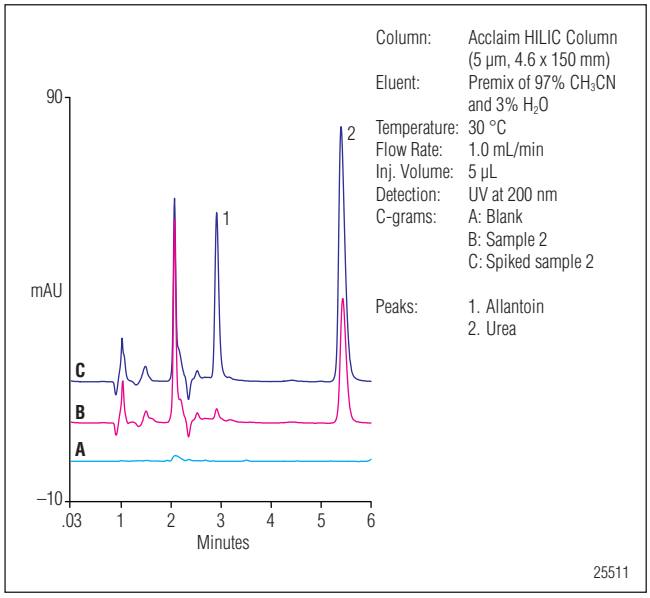

Figure 9. Overlay of chromatograms of the blank, sample 2 and spiked sample 2.

Table 3. Analysis Results for Cosmetic Products

Analyte	Sample 1	Sample 2			
	Detected (g/Kg)	Detected (g/Kg)	Added (g/Kg)	Found (g/Kg)	Recovery (g/Kg)
Allantoin	ND	0.37	5.00	4.92	98.4
Urea	53.5	76.6	90.0	87.6	97.3

Note: 1. One sample and spiked sample were prepared, respectively, and 3 injections were made for each.
2. Detected = Measured Value of sample x Diluted fold
3. Found = Measured value of spiked sample — Measure value of sample
4. ND = "not detected"

Analysis of Sumatriptan in Cerebro-spinal Fluid Using Capillary LC/MS/MS

INTRODUCTION

Sumatriptan helps to relieve headache pain and associated symptoms of migraine (nausea, vomiting, sensitivity to light and sound). It also helps to constrict dilated blood vessels that may contribute to development of migraines. Most analysis techniques for the separation, detection and quantification of sumatriptan are based on conventional high-performance liquid-chromatography techniques. Here the gain in sensitivity is demonstrated by using capillary LC/MS/MS.

RESULTS AND DISCUSSION

The sample preparation steps for sumatriptan in cerebro spinal fluid (CSF) prior to LC/MS analysis is straight forward and demonstrated in the Figure 1. The UltiMate®/FAMOS™ Capillary LC System was equipped with a 300 µm I.D. x 15 cm column packed with C18, 3 µm stationary phase at flow rate of 5 µL/min. Mobile phase A: 0.1% aqueous formic acid, B: acetonitrile. Gradient: 5 to 60% B in 8 min. MS/MS analysis was conducted with a triple quadrupole MS equipped with an electrospray interface.

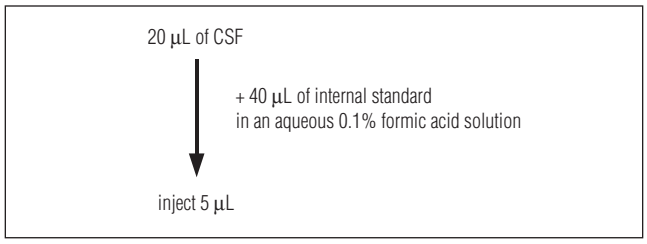

Figure 1. Schematics of sample handling prior to capillary LC/MS/MS.

Figure 2 shows multiple reaction monitoring (MRM) chromatograms of sumatriptan analysis by means of Capillary and conventional HPLC. The upper trace corresponds to a chromatogram taken when 1.25 pg of sumatriptan was injected on column and the lower trace shows the limits of detection achieved with conventional HPLC, which is approximately equal to 300 pg. The overall gain in sensitivity is approximately a 700 fold. For spiked CSF samples, the peak area ratios of analyte to internal standard were linear over the calibration range 0.5–15 ng/µL. A typical calibration curve is shown in the Figure 3. The limit of quantitation was found to be equal to 300 pg/µL.

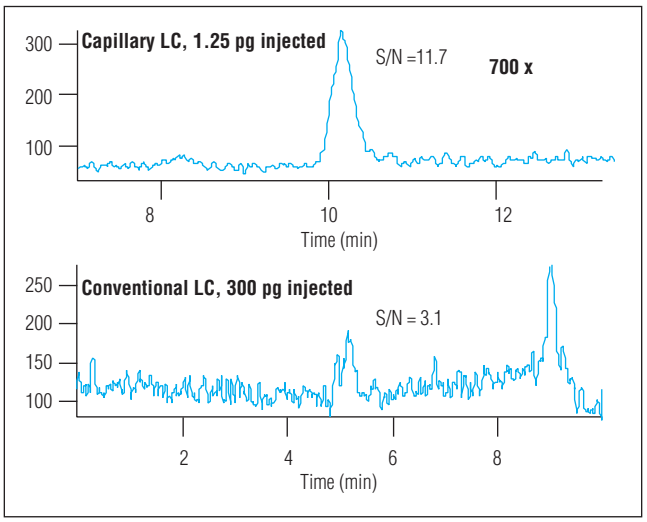

Figure 2. Capillary (top) and conventional (bottom) LC/MRM chromatograms of sumatriptan. Gradient conditions are given in the text.

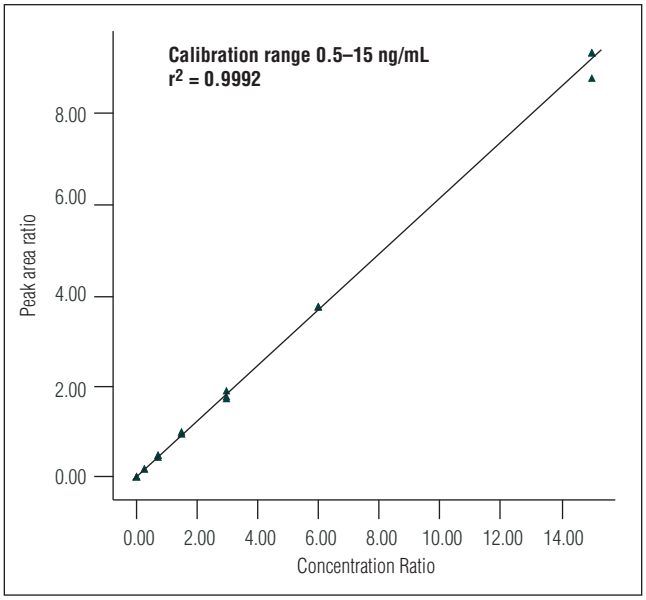

Figure 3. Calibration curve for the analysis of sumatriptan in CSF.[1]

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1. Courtesy Prof. G. Moneti, CIMS, University of Florence, Italy

Budesonide and Related Substances on the Acclaim 300 C18 Column

INTRODUCTION

Budesonide is a highly effective steroidal antihistamine decongestant, and is available as a mixture of two epimers. The safety of budenoside-based pharmaceuticals depends on careful monitoring of impurities associated with the epimers. The related substances are decomposition products and synthetic byproducts. The Acclaim 300 C18 column is a direct replacement for the column in the referenced method.

METHOD

In this application, 6 µL of a degraded budesonide sample (500 µg/mL) is injected onto a 3 µm Acclaim 300 C18 column with the dimensions of 4.6 × 150 mm. The isocratic separation of the budesonide epimers from the decomposition products and synthetic byproducts is accomplished using a mobile phase of 66% aqueous and 34% organic. The aqueous portion is 0.1% phosphoric acid and the organic portion is a mixture of acetonitrile: ethanol (15:1). The total run time is roughly 16 min. Detection is accomplished using UV absorbance at 240 nm.

RESULTS

As shown in Figure 1, the method provides baseline resolution of the two major components as well as the eight impurities. This separation is clearly suitable for assaying budesonide.

REFERENCE

1. Hou S, Hindle M, Byron PR; *J. Pharm. Biomed. Analy.* **2001** *24:* 371–80.

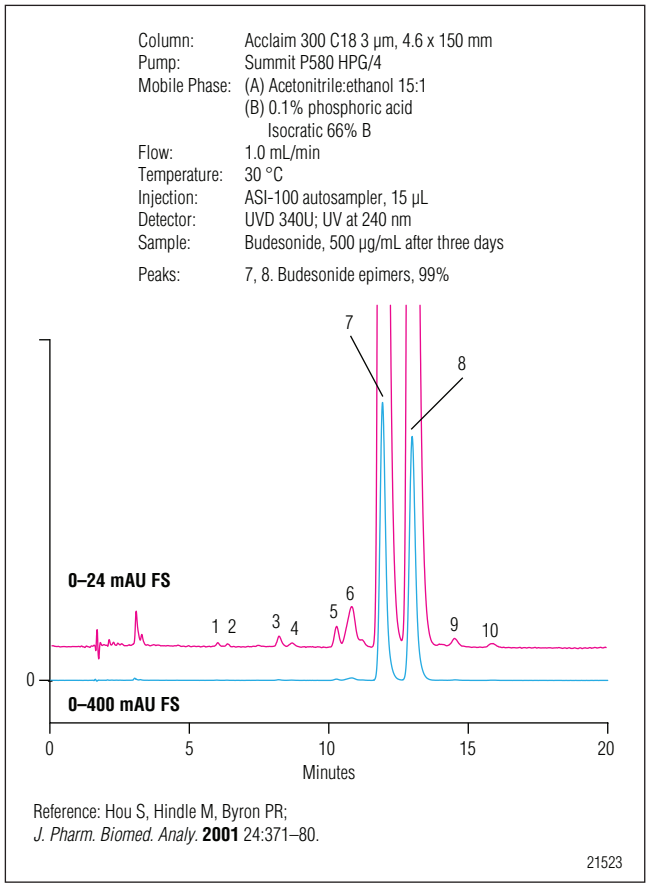


Figure 1. Budesonide and related substances.

Glucocorticosteroids in Serum on the Acclaim 120 C18 Column

INTRODUCTION

Accurate analysis of glucocorticosteroids, both natural and synthetic, is necessary for monitoring the health of patients with a number of medical conditions. The referenced method is widely used in clinical laboratories. The Acclaim 120 C18 column is a direct substitute for the referenced column.

SAMPLE PREPARATION

The sample is prepared by extracting bovine serum (alkalized with sodium hydroxide) with ethyl acetate and injecting 60 µL.

METHOD

A 5 µm Acclaim 120 C18 column with the dimensions of 4.6 × 250 mm is used to separate 6 glucocorticosteroids from the internal standard, Fludrodcortisone. The separation is performed under isocratic conditions using methanol:THF:water (3:25:72) at a flow rate of 1.0 mL/min.The total run time for the analysis is under 25 min. Detection is accomplished using UV absorbance at 240 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of the six glucocorticosteroids with a wide window for the internal standard. This separation is clearly suitable for quantitatively assaying a number of glucocorticosteroids.

REFERENCE

1. McWhinney B C, Ward G, Hickman P E; *Clin. Chem,* **1996,** *42:* 979–81.

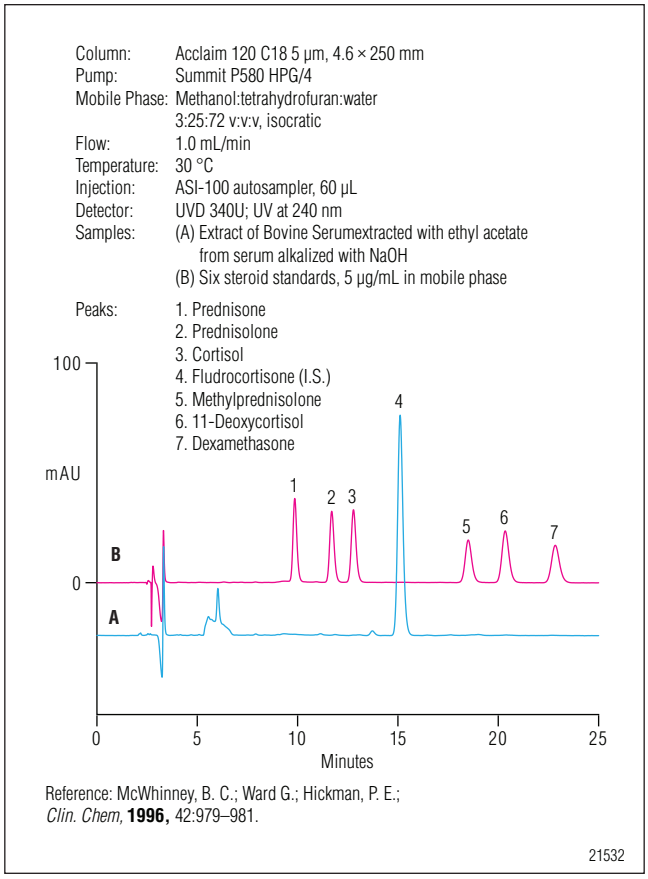


Figure 1. Glucocorticosteroids in serum on Acclaim 120 C18.

Propafenone and Related Substances Using the Acclaim PA Column

INTRODUCTION

Propafenone is a sodium channel blocker used to treat irregular heart rhythm. This analysis of the formulated drug resolves the known impurities and degradation products.

SAMPLE PREPARATION

In this application brief, sample preparation is accomplished by initially dissolving the sample in methanol and then further diluting 1 mL of the sample in 40% mobile phase A and 60% mobile phase B.

METHOD

20 µL of sample is injected onto a 5 µm Acclaim PA column with the dimensions of 4.6 × 150 mm. A gradient solvent system at a flow rate of 1.0 mL/min is used to separate the early eluting propafenone from five related substances in 30 min. Mobile phase A consists of 10 mM ammonium acetate at pH 2.4. Mobile phase B is methanol. The gradient starts out with a 5 min ramp from 20% to 50% B followed with a 5 min ramp to 60% B. Mobile phase B is increased to 70% at 15 min and 95% at 30 min before returning to 20% for column re-equilibration. Detection is accomplished using UV absorbance at 249 nm.

RESULTS

As shown in Figure 1, the method provides more than adequate resolution of propafenone and the five related substances. This separation is clearly suitable for assaying propafenone.

ACKNOWLEDGEMENTS

Data courtesy of V. Bhate of Analytical Solutions

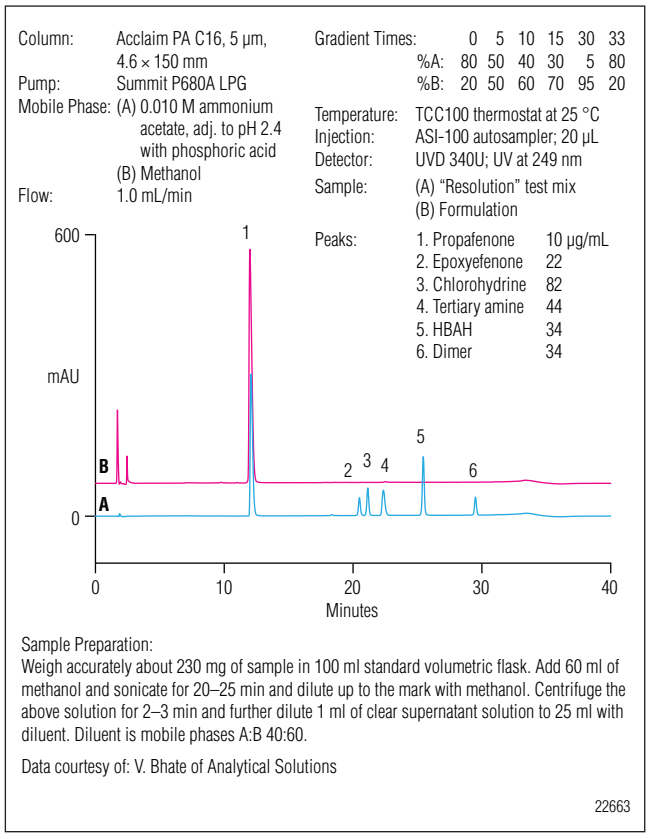


Figure 1. Propafenone and related substances using Acclaim PA.

Loperamide in Anti-diarrhea Tablets on the Acclaim 120 C8 Column

INTRODUCTION

Loperamide is the active ingredient in many formulations used to treat diarrhea. The U.S. Pharmacopeia assay specifies an L7 column be used for this analysis. Here the Acclaim 120 C8 column is used for determination of this compound.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving ground up time-release tablets in a solution of 5% H_3PO_4, methanol and water. After sonication, followed by filtration, 20 µL of the sample is injected.

METHOD

In this application, a 3 µm Acclaim 120 C8 column with the dimensions of 4.6 × 150 mm is used to separate loperamide hydrochloride. The column was maintained at 30 °C. The separation is performed under isocratic conditions at a flow rate of 1.2 mL/min. The mobile phase consisted of 45% acetonitrile and 55% buffer. The buffer was composed of 3.0 g of Et_3N HCl plus 1.0 mL of H_3PO_4 in 550 mL water. The method yielded a total run time of just over 5 min with the detection of the compound using UV absorbance at 214 nm.

RESULTS

As shown in Figure 1, this method provides excellent retention of the loperamide peak. Clearly this separation is suitable for the quantitative assaying of loperamide in anti-diarrheal tablets.

REFERENCE

1 USP-31 NF-26, pg. 2545 (2008).

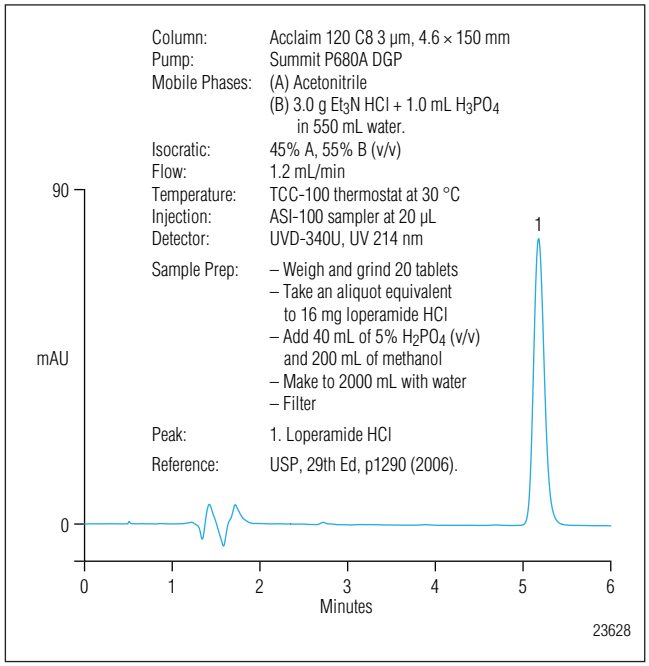


Figure 1. Loperamide in anti-diarrhea tablets on Acclaim 120 C8.

Simultaneous Analysis of a Basic Drug and Its Counterion (Trimipramine)

INTRODUCTION

Retaining a hydrophobic base and its hydrophilic counterion can prove very difficult using conventional reversed-phase columns. The Acclaim Mixed-Mode WAX-1 retains the hydrophobic base by reversed-phase interaction, and the hydrophilic counterion by anion-exchange interaction. In this way, it can eliminate a separate test for the two components.

SAMPLE PREPARATION

In this application, the sample is prepared by dissolving trimipramine maleate in mobile phase at a concentration of approximately 0.5 mg/mL. 2.5 µL of sample is injected on column.

METHOD

For this analysis, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate trimipramine from its counterion, maleate. The separation is performed under isocratic conditions using an acetonitrile/buffer (30:70) mobile phase at a flow rate of 1.0 mL/min.The buffer was 50 mM phosphate at pH 6.0. The total run time for the analysis is under 7 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown in Figure 1, this HPLC method produces a chromatogram with the trimipramine peak well-resolved from its maleate counterion peak. This separation is clearly suitable for quantitatively assaying both trimipramine and its counterion in a single analysis.

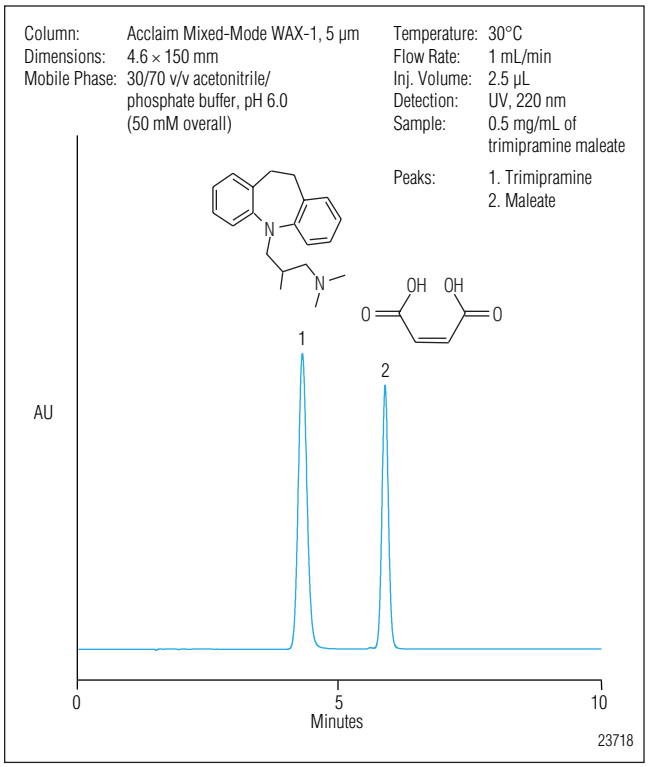
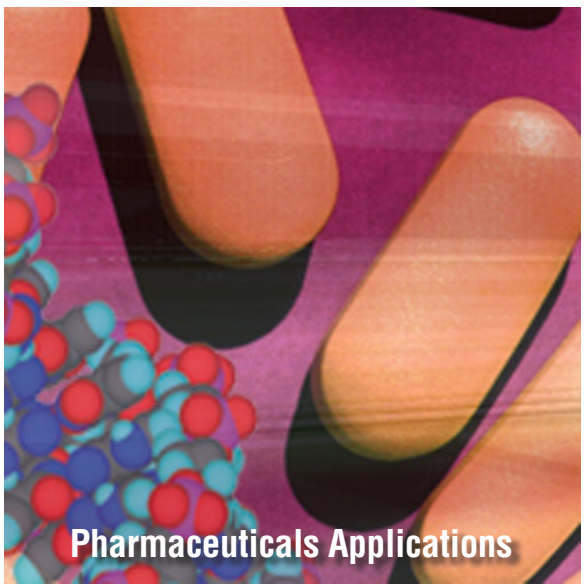


Figure 1. Simultaneous analysis of a basic drug and its counterion (trimipramine).

DIONEX

Part III: Analysis of Over-the-Counter Drugs



Analytes

Aspirin
Multi-symptom Cold Remedy
Loratidine
Miconazole
Ketoconazole
Water-Soluble Vitamins
Fat-Soluble Vitamins and Carotenoids
APIs
Nonsteroidal Anti-inflammatory Drugs
Hydrocortisone
Omeprazole
Alkaloids in Bitter Orange

Accelerated USP Assay of Aspirin on the Acclaim 120 C18 RSLC Column

INTRODUCTION

USP and EP monographs are available to pharmaceutical manufacturers as records of standard procedures for the analysis of drug substances and drug products. The current USP monograph given for both coated and uncoated aspirin tablets[1] is based on conventional HPLC instrumentation and indicates a 4 × 300 mm column operated at a flow rate of 2.0 mL/min. Although the monograph's system suitability test calls for a resolution between the salicylic acid and aspiring peaks of no less than 2.0, when used with the Acclaim 120 C18 column it delivers a resolution of 5.7. However the analysis time is 15 min and thus is an excellent candidate for acceleration using a UHPLC method. Simply recomputing the operating parameters for a 2.1 × 50 mm column leads to a 10-fold increase in throughput which translates into a 96% savings in mobile phase. The accelerated method still produces baseline resolution of the two components that is greater than the required 2.0 stated in the monograph.

RESULTS

As shown in Figure 1, the original HPLC method produces a chromatogram with a salicylic acid/aspirin peak resolution of 5.7, far more than the monograph's stated specification of 2.0. By transferring the method to a UHPLC method using a shorter, narrower column with smaller particle diameter the analysis time is reduced to less than 2 min. The resulting resolution is still sufficient at a value of 2.9.

Reference

1. USP-31 NF-26, page 1450.

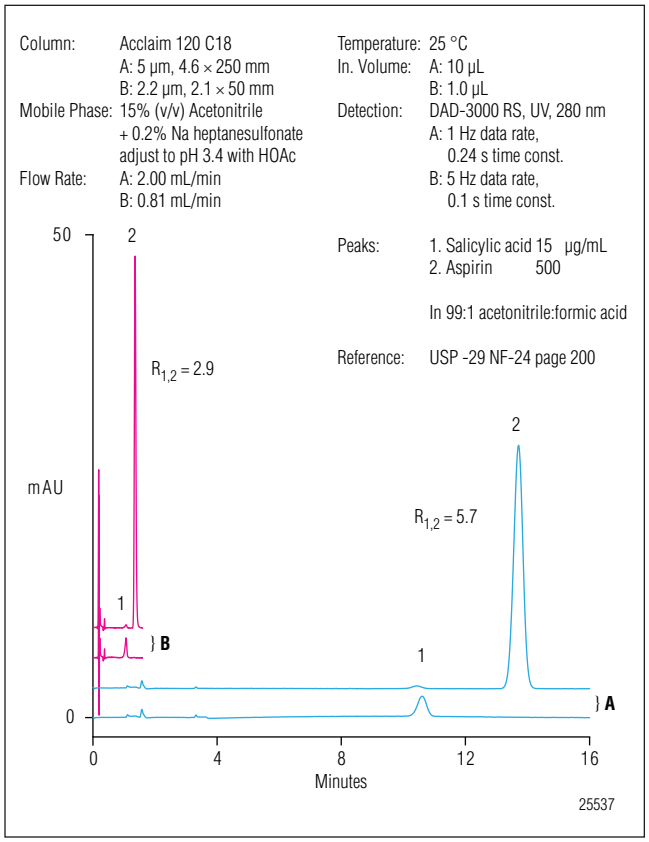


Figure 1. Injections of salicylic acid (peak 1) and aspirin (peak 2) under HPLC conditions (lower chromatograms) and UHPLC conditions (upper chromatograms).

Determination of Four Active Ingredients in a Multi-symptom Cold Remedy Using the Acclaim PA Column

INTRODUCTION

This simple application of the Acclaim PA resolves all the active ingredients and most of the inactive ones in cough syrup. At pH 3, three of the active ingredients are only weakly hydrophobic and one is moderately hydrophobic. The Acclaim PA column is well suited to the low-organic conditions needed for this separation. Note that the Acclaim PA column delivers a sharp peak for pseudoephedrine, a hydrophilic, basic drug that is difficult for columns with high silanol activity.

SAMPLE PREPARATION

For the two chromatograms in Figure 1, the sample is prepared by a simple dilution of the cold remedy with mobile phase B at a rate of 20:1 and 400:1, respectively.

METHOD

A 3 µm Acclaim PA column with the dimensions of 4.6 × 150 mm is used to separate a 10 µL injection of the diluted sample into the 4 major components of the multi-symptom cold remedy. The gradient separation starts with a 2 min composition hold (8% mobile phase A) followed by an 8 min ramp from 8% to 20% A. The gradient then ramps up to 50% A over the next 7 min. The flow rate is 1.0 mL/min. yielding a run time of 17 min. Detection is accomplished using UV absorbance at 210 nm.

RESULTS

Trace A shows the acetaminophen peak at a 400:1 dilution. Trace B is a chromatogram of the sample diluted 20:1. At this concentration you can clearly see the other three active ingredients as well as the non-active components. As shown in the figure, the Acclaim PA column provides sufficient separation efficiency to baseline resolve the four active ingredients. It also provides baseline resolution of five other non-active ingredients.

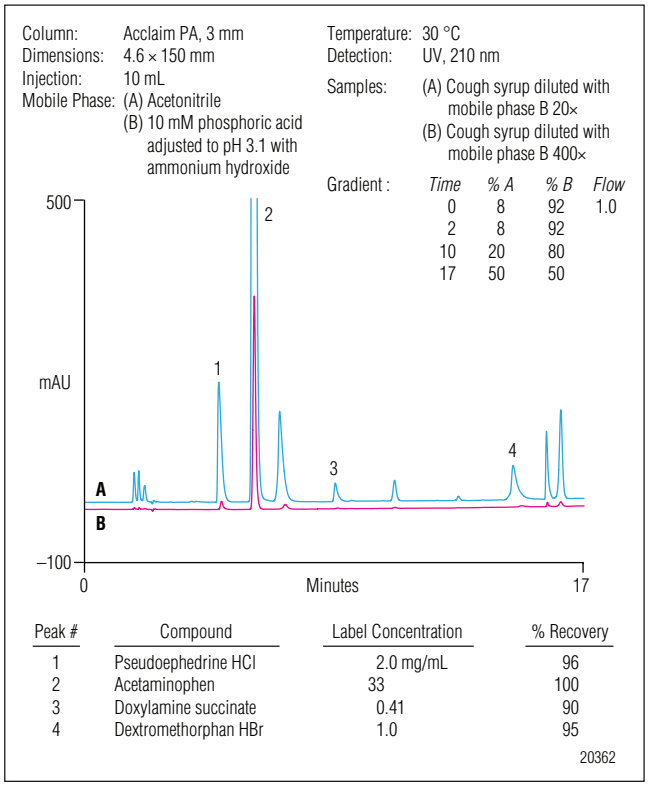


Figure 1. Determination of four active ingredients in a multisymptom cold remedy using Acclaim PA.

Fast Analysis for Loratidine and Pseudoephedrine in a Time-Release Tablet on the Acclaim PA Column

INTRODUCTION

Loratidine is a second-generation, nonsedating antihistamine. Pseudoephedrine is a decongestant. This combination is a popular treatment for relief of allergy symptoms. The analysis is difficult because the two drugs have very different hydrophobicities. Also, both are strong basic amines with a tendency to tail. The Acclaim PolarAdvantage (PA) column is well suited to this challenge as it delivers good base symmetry over a wide range of mobile phase compositions. The short, 50 mm column enables this analysis to be completed in less than 4 min.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving a time-release tablet in a solution of roughly 10% methanol, 3% 1N HCl and 87% water. After filtration, the 10 µL of the sample is injected.

METHOD

A 5 µm Acclaim PA column with the dimensions of 4.6 × 50 mm is used to separate the two active ingredients. The column was thermostatically maintained at 35 °C. The separation starts with a 0.41 min composition hold at 0% mobile phase B. Next there is essentially a gradient step up to a final composition of 100% B which is then held to the end of the run. Mobile phase A is an acetonitrile/water (15:85) solution containing 0.05% TFA. Mobile phase B is also an acetonitrile/water solution but at a ratio of 40:60 and it also contains 0.05% TFA. The flow rate is 1.25 mL/min. Detection is accomplished using UV absorbance at 257 nm.

RESULTS

As shown in Figure 1, this HPLC method using the Acclaim PA column produces a chromatogram showing more than adequate resolution of pseudoephedrine and loratidine. Furthermore, the separation is accomplished in under 4 min. At a flow rate of 1.25 mL/min. the analysis consumes less than 5 mL of mobile phase.

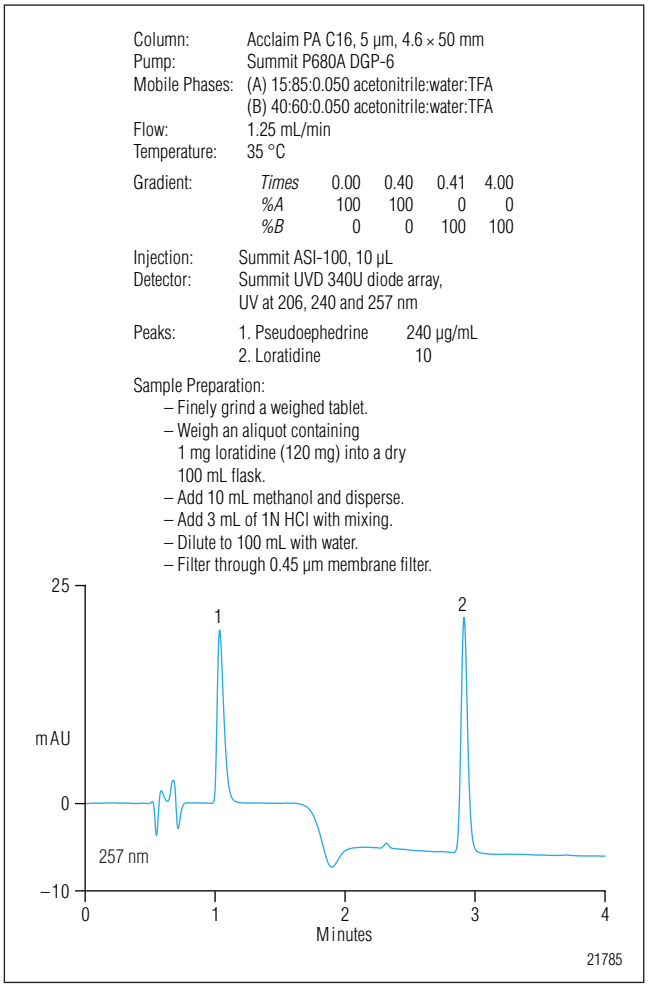


Figure 1. Fast analysis for loratidine and pseudoephedrine in a time-release tablet on the Acclaim PA column.

Miconazole in Athlete's Foot Ointment on the Acclaim 120 C18 Column

INTRODUCTION

Miconazole is a popular antifungal agent used in various forms to treat athlete's foot and related conditions. Here, an Acclaim C18 column is used to determine miconazole content in a topical ointment. The label listed the active ingredient concentration as 2.0%. The analysis measured it at 2.1%.

SAMPLE PREPARATION

In this application, the sample is prepared using liquid-liquid extraction to remove the miconazole from the ointment. The ointment is first dissolved in a solution of ethanol and heptane. Water is then added to the ethanol/heptane solution, the mixture is centrifuged and the miconazole is extracted in the aqueous layer. 4 µL of the aqueous extract is injected on column.

METHOD

A 3 µm Acclaim 120 C18 column with the dimensions of 4.6 × 150 mm is used to separate miconazole from the other aqueous-soluble ingredients. The separation is performed under isocratic conditions using 80% methanol and 20% buffer. The buffer is composed of 10 mM TEA adjusted to pH 2.7 with phosphoric acid. The flow rate is 1.0 mL/min. which produces a run time of less than 6 min. Detection is accomplished using UV absorbance at 225 nm.

RESULTS

As shown in Figure 1, the miconazole is very well-retained and easily distinguished from the other components. This separation is clearly suitable for quantitatively assaying of miconazole in difficult samples, such as creams and ointments.

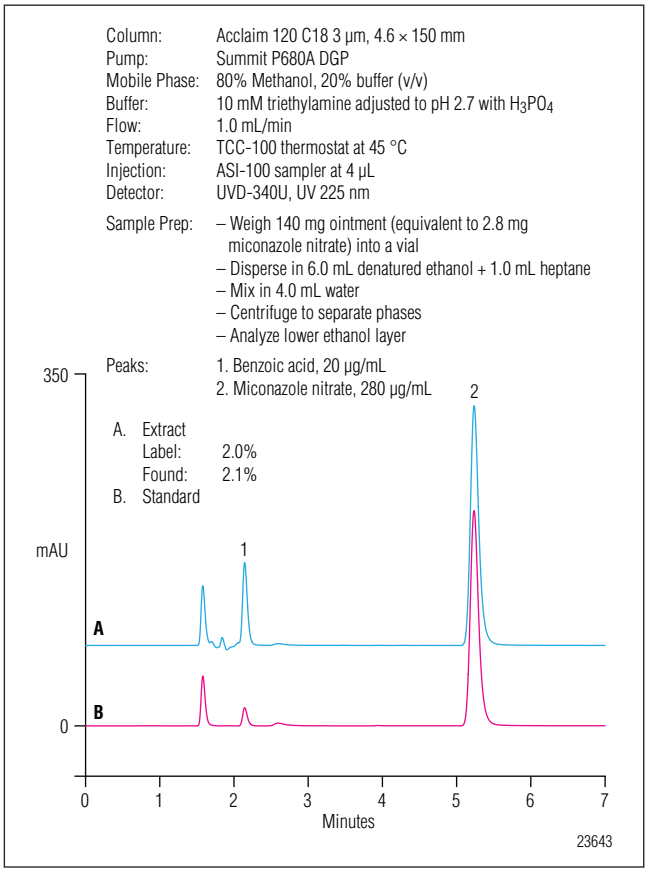


Figure 1. Miconazole in athlete's foot ointment on Acclaim 120 C18.

Ketoconazole in Anti-Dandruff Shampoo on the Acclaim 120 C18 Column

INTRODUCTION

Ketoconazole is an antifungal agent used both internally and as a topical treatment for dandruff. Here an Acclaim C18 column easily resolves this active ingredient from shampoo, a matrix that is complex and can be difficult to analyze.

The current USP monograph given for ketoconazole[1] is based on a 4.6 × 250 mm column packed with 5 µm C18 stationary phase. However the analysis time can be easily shortened by substituting a shorter (150 mm) column packed with smaller (3 mm) particles to maintain adequate resolution.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving 400 mg of shampoo in 3 mL of water to which is added 10 mL of methanol. After filtration, 4 µL of the sample solution is injected.

METHOD

A 3µm Acclaim 120 C18 column, with the dimensions of 4.6 × 150 mm and maintained at 30 °C, is used to resolve ketoconazole from other formulation ingredients. The separation is performed under isocratic conditions using a mobile phase consisting of 2.55 g tetrabutylammonium hydrogen sulfate dissolved in 750 mL of water and 250 mL of acetonitrile. A flow rate of 1.0 mL/min. produces a run time of less than 6 min. Detection is accomplished using UV absorbance at 223nm.

RESULTS

As shown in Figure 1, the ketoconazole is very well retained and easily distinguished from the other components. This separation is clearly suitable for quantitatively assaying of ketoconazole in formulations such as creams and shampoos.

Column:	Acclaim 120 C18 3 µm, 4.6 × 150 mm
Pump:	Summit P680A DGP
Mobile Phase:	2.55 g $Bu_4N^+HSO_4^-$, 750 mL water, 250 mL acetonitrile
Flow:	1.0 mL/min
Temperature:	TCC-100 thermostat at 30°C
Injection:	ASI-100 sampler at 4 µL
Detector:	UVD-340U, UV 223 nm
Sample Prep:	– Weigh 400 mg shampoo (equivalent to 4 mg ketoconazole) into a flask – Disperse in 3.0 mL water – Dilute to 10.0 mL with MeOH – Filter
Peaks:	1. Ketoconazole, approx. 400 µg/mL
Reference:	U.S. Pharmacopeia 29th Ed., p.1216 (2006).

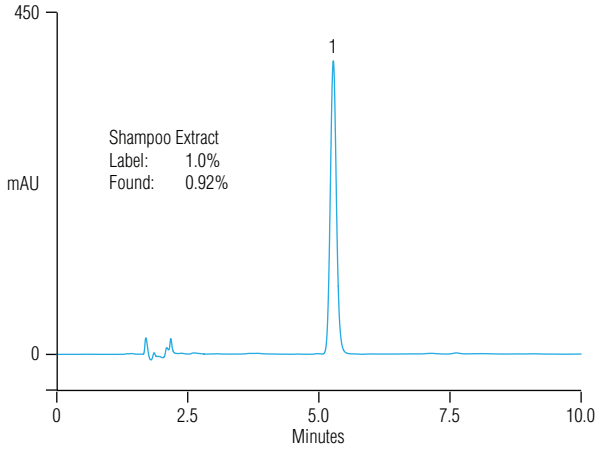


Figure 1.Ketoconazole in anti-dandruff shampoo on Acclaim 120 C18.

REFERENCE

1. USP-31 NF 36, page 2488 (2008).

Assay for Water-Soluble Vitamins

INTRODUCTION

Vitamin supplement tablets are complex formulations with many ingredients. Some vitamins are strongly hydrophilic, so the column needs to operate in 100% aqueous buffer to gain sufficient retention to resolve them from the matrix. The Acclaim PA column can do this reliably whereas a hydrophobic C18 column would likely suffer dewetting. The multiple wavelength capability of the diode array detector provides primary wavelengths for quantitation and alternate wavelengths for confirmation.

SAMPLE PREPARATION

For this analysis, the vitamin tablet was crushed and dissolved in 100 mL of mobile phase buffer. After sonication followed by filtration, 20 µL of the sample solution was injected.

METHOD

A 3 µm Acclaim PA column, with the dimensions of 4.6 × 150 mm is used to resolve 8 vitamins. The gradient separation ramps from 0% to 20% mobile phase B in 15 min. Mobile phase A is phosphate buffer at pH 3.4. Mobile phase B is acetonitrile. A flow rate of 1.0 mL/min. produces a run time of less than 17 min. Detection is accomplished using UV absorbance at 210, 246, 265 and 285 nm.

RESULTS

As shown in Figure 1, the eight vitamins contained in the vitamin tablet are all well resolved using the Acclaim PA column. By selectively monitoring multiple wavelengths, sensitivity can be maximized while avoiding potential interferences. This separation is clearly suitable for quantitatively assaying water soluble vitamin tablets.

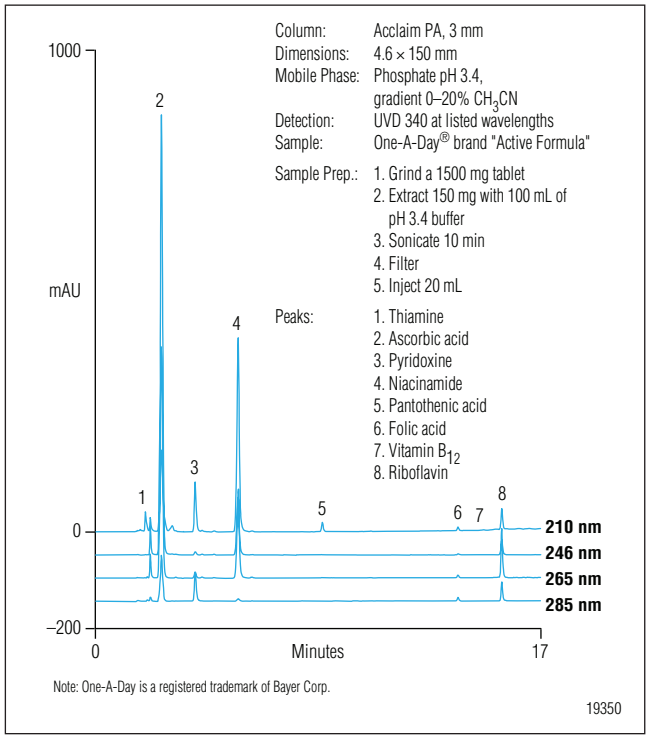


Figure 1. Assay for water-soluble vitamins in vitamin tablets using the Acclaim PA column.

Fat-Soluble Vitamins and Carotenoids in a Vitamin Tablet on the Acclaim PA Column

INTRODUCTION

Vitamin supplements often include carotenoids together with fat-soluble vitamins in their formulations. This convenient analysis measures vitamins A and E and a complex of carotenoids in a single run. The Acclaim PA column shows a different selectivity under low-aqueous conditions than conventional reversed-phase columns. The conditions above have been used with a variety of sample types.

SAMPLE PREPARATION

In this application, the sample is prepared using liquid-liquid extraction to remove extract the vitamins from the tablet. A portion of the crushed tablet is taken up in a mixture of water and ethyl acetate. The mixture is sonicated and 2 mL of the ethyl acetate layer is removed and evaporated to dryness. The residue is then reconstituted in 1mL of mobile phase B. 10 µL of the reconstituted sample is injected on column.

METHOD

A 3 µm Acclaim PA column, with the dimensions of 4.6 × 150 mm and maintained at 30 °C, is used to resolve 7 fat-soluble vitamins and carotenoids. The separation starts with a 8 min composition hold at 95% mobile phase B. Next, there is essentially a gradient step up to a final composition of 100% B which is then held to the end of the run. Mobile phase A is water and mobile phase B is a solution of methanol, acetonitrile and isopropanol at a ratio of 54:44:2. A flow rate of 1.25 mL/min. produces a run time of less than 18 min. Detection is accomplished using multiple wavelength UV absorbance at 285 and 450 nm.

RESULTS

As seen in Figure 1, the vitamins and carotenoids contained in the vitamin tablet are all resolved using the Acclaim PA column. By selectively monitoring two wavelengths, sensitivity can be maximized while avoiding potential interferences. This separation is clearly suitable for quantitatively assaying fat-soluble vitamin tablets.

Column:	Acclaim® PA C16, 3 µm, 4.6 × 150 mm
Pump:	Summit P580 HPG/4
Mobile Phase:	(A) Water (B) 54:44:2 methanol:acetonitrile:isopropanol

Gradient:	Time	0	8.0	8.1	25
	%A	5	5	0	0
	%B	95	95	100	100

Flow:	1.25 mL/min
Temperature:	30 °C
Injection:	Summit ASI-100, 10 µL
Detector:	Summit UVD 340U diode array, 450 and 285 nm, and spectra 200–595 nm

Sample Prep.:
- Finely grind a weighed tablet
- Take an aliquot of about 1/10 tablet (150 mg)
- Partition between 2.0 mL water and 6.0 mL ethyl acetate with sonication
- Evaporate 2.0 mL of ethyl acetate phase and reconstitute in 1.0 mL mobile phase B

Peaks:
1. *trans*-Retinol acetate
2. *trans*-Lutein
3. α-Tocopherol acetate
4. *trans*-Lycopene
5. *cis*-Lycopene
6. *trans*-β-Carotene
7. *cis*-β-Carotene

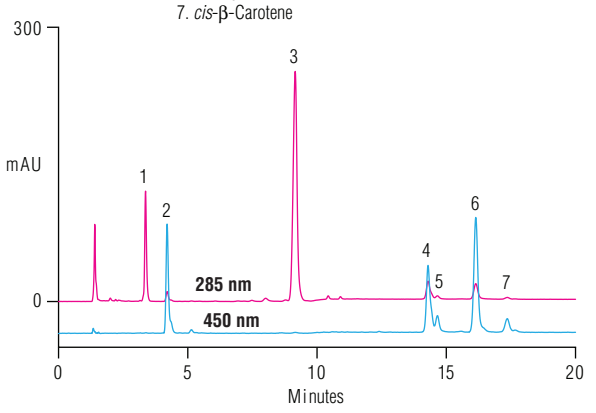


Figure 1. Fat-soluble vitamins and carotenoids in a vitamin tablet.

Analysis of APIs in a Pain-Relief Medicine on the Acclaim Mixed-Mode WAX-1 Column

INTRODUCTION

Pain relief medications often contain a combination of ingredients that can be challenging to separate. The Acclaim Mixed-Mode WAX-1 column resolves all the active pharmaceutical ingredients (APIs) in this product.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving a ground up time-release tablet in water. After sonication, followed by filtration, 1 µL of the sample is injected.

METHOD

In this application, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate five active pharmaceutical ingredients found in a pain-relief medication. The column was maintained at 30 °C. The separation is performed under isocratic conditions at a flow rate of 1.0 mL/min. The mobile phase consisted of 40% acetonitrile and 60% buffer. The buffer was composed of 6.8 g KH_2PO_4 and 0.5 g NaP_2O_7*10 H_2O in 1000 mL water. The pH of the buffer was adjusted to 6.0 with NaOH. The method yielded a total run time under 15 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of all five components in the mixture. Clearly this separation is suitable for the quantitative assaying of a multi-component pain-relief medication.

Note: Pyrophosphate in the buffer suppresses interference from metal ions.

Column:	Acclaim Mixed-Mode WAX-1, 5 µm, 4.6 × 150 mm
Mobile Phase:	40/60 v/v Acetonitrile/buffer (6.8 g KH_2PO_4 and 0.5 g $NaP_2O_7 \bullet 10H_2O$ in 1000 g D.I. H_2O, pH is adjusted to 6.0 with NaOH)
Temperature:	30 °C
Flow Rate:	1 mL/min
Inj. Volume:	1 µL
Detection:	UV @ 220 nm
Note:	Pyrophosphate in the buffer suppresses interference from metal ions
Peaks:	1. Caffeine 2. Acetaminophen 3. Salicylamide 4. Acetyl salicylic acid (Aspirin) 5. Salicylic acid

Sample Preparation:
1. A 500 mg tablet is ground into fine powder.
2. Weigh 400 mg of the powder and dissolve with 40 g of D.I. water.
3. After sonicating for 5 min, the resulting suspension was filtered with 0.45 µm membrane filter.
4. Dilute the filtrate 4 times with D.I. water

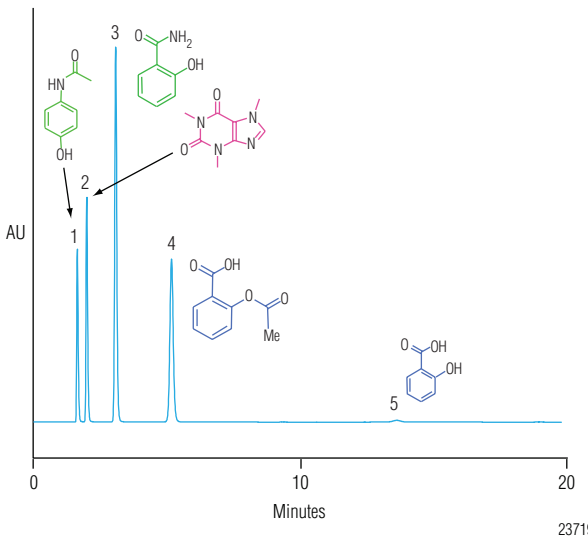


Figure 1. Analysis of APIs in a pain-relief medicine on the Acclaim Mixed-Mode WAX-1 column.

Nonsteroidal Anti-inflammatory Drugs on the Acclaim PA2 C18 column

INTRODUCTION

Nonsteroidal Anti-inflammatory Drugs (SAIDs) are a widely-used class of drugs for pain relief in joints and muscles. These acidic drugs may be separated with excellent performance using the Acclaim PA2 column at low pH.

METHOD

In this application, a 5 µm Acclaim PA2 column with the dimensions of 4.6 × 150 mm is used to separate three active pharmaceutical ingredients found in an anti-inflammatory medication. During the analysis, the column was maintained at 30 °C. The separation is performed under isocratic conditions at a flow rate of 1.0 mL/min. The mobile phase consisted of 55% acetonitrile and 45% phosphoric acid (0.1%). The method yielded a total run time of approximately 10 min and the compounds were detected using UV absorbance at 265 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of ketoprofen and naproxen and both elute much earlier than ibuprofen. Clearly this separation is suitable for the quantitative assaying of a multi-component nonsteroidal anti-inflammatory medication.

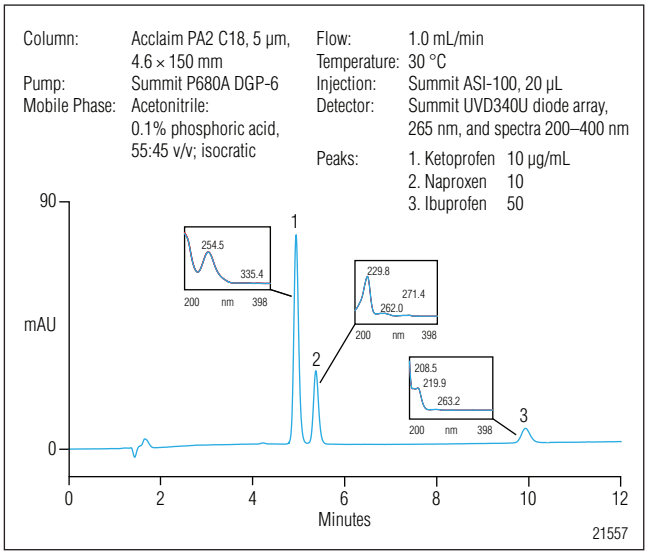


Figure 1. Nonsteroidal anti-inflammatory drugs on the Acclaim PA2 C18 column.

Hydrocortisone in Skin Ointment

INTRODUCTION

Hydrocortisone is the active ingredient in a number of topical formulations used to treat many types of skin rashes. The product formulations often have an assortment of other ingredients making the analysis complex and demanding. Here, we have employed a straightforward liquid-liquid extraction and simple isocratic analysis for quick, clean, and accurate results.

SAMPLE PREPARATION

In this application, sample preparation is accomplished by dissolving the sample ointment in a mixture of ethanol and heptane followed by an aqueous extraction of interfering compounds. The ethanol layer is then filtered and 6 µL injected.

METHOD

A 3 µm Acclaim C18 column with the dimensions of 3.0 × 75 mm is used to separate hydrocortisone from two preservatives in a topical ointment. The total run time is only four min. The Ultimate 3000 system provides high resolution and fast analysis without producing UHPLC pressures. Detection is accomplished using UV absorbance at 245 nm.

RESULTS

As shown in Figure 1, the method provides more than adequate resolution of all three components in a short period of time. This separation is clearly suitable for assaying this formulation. Additionally, because of the short analysis time and 3.0 mm diameter column, consumption of mobile phase is only 4 mL per analysis. This reduces cost of solvent, cost of waste disposal, and cost of operation.

Column:	Acclaim® 120 C18 3 µm, 3.0 × 75 mm
Pump:	UltiMate® LPG-3600A
Mobile Phase:	Acetonitrile:methanol:water 15:25:60 v/v/v
Flow:	1.0 mL/min
Temperature:	TCC-3000 thermostat at 50 °C
Injection:	WPS-3000 TSL sampler at 6 µL
Detector:	VWD-3000 UV at 245 nm

Sample Prep:
- Disperse 100 mg of ointment in 4.0 mL denatured ethanol + 1.0 mL heptane.
- Mix in 6.0 mL water.
- Centrifuge to separate emulsion.
- Filter ethanol layer.

Peaks: 1. Methyl paraben
2. Hydrocortisone
3. Propyl paraben

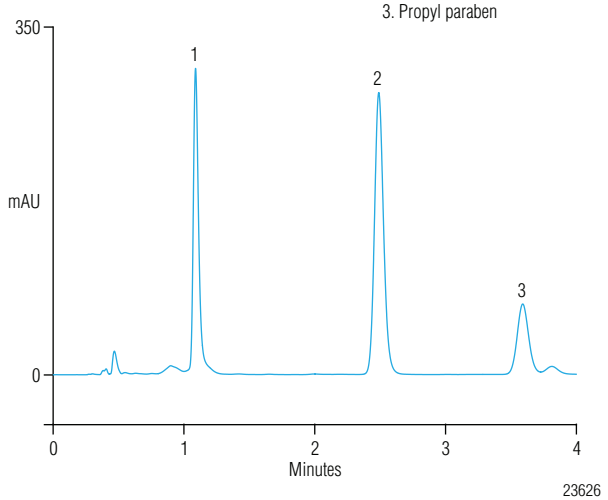


Figure 1. Hydrocortisone in skin ointment on Acclaim 120 C18.

Omeprazole in Anti-Heartburn Tablet on the Acclaim PA2 Column

INTRODUCTION

Omeprazole is an inhibitor of gastric acid secretion and is widely used to treat chronic heartburn. It is commonly available under its original brand names as well as a number of generic medicines. This application brief demonstrates the use of the Acclaim PA2 column which gives excellent performance for this assay. The method described in this applications brief is suitable for performing the USP assay[1] and chromatographic purity.

SAMPLE PREPARATION

The sample is prepared by dissolving an omeprazole tablet in a solution of 20% ethanol and 80% buffer (10 mM borate, 1.5 mM EDTA, pH 11.0). the sample is filtered prior to injecting 10 µL on column.

METHOD

This method uses a 4.5 × 150 mm column packed with 5 µm Acclaim PA2 stationary phase maintained thermostatically at 30 °C to separate omeprazole from its related substances.

The separation is achieved using a 20 min binary gradient going from 12% to 60% mobile phase B at a flow rate of 1.2 mL/min. Mobile phase A is also a solution of 85% acetonitrile and 15% methanol and mobile phase B is a 40 mM glycine solution adjusted to pH 9.0. Detection is by UV absorbance at 224 nm.

RESULTS

As shown in Figure 1, the HPLC method produces a chromatogram showing good retention of the omeprazole peak (trace A). The recovery of omeprazole was calculated as 19.5 mg or 97.5% of the labeled amount.

REFERENCE

1. USP-31 NF-26, page 2850, (2008).

Column:	Acclaim PA2, 5 µm, 4.6 × 150 mm
Pump:	UltiMate® LPG-3400
Mobile Phases:	(A) Acetonitrile:methanol 85:15 (v/v) (B) 40 mM glycine, pH 9.0
Gradient Time:	–7 0 20 %A 12 12 60 %B 88 88 40
Flow:	1.2 mL/min
Temperature:	TCC-3100 thermostat at 30 °C
Injection:	WPS-3000 SL sampler at 10 µL
Detector:	VWD-3400, UV 305 nm
Sample Prep:	– Weight and finely grind tablets. – Extraction solvent: 20% ethanol, 80% (10 mM borate, 1.5 mM EDTA, pH 11.0) (v/v) – Extract with sonication an aliquot equiv. to 20 mg omeprazole in 100 mL solvent. – Filter
Peak:	1. Omeprazole, approx. 225 µg/mL
Reference:	U.S. Pharmacopeia, 29th Ed., p.1578, (2006).

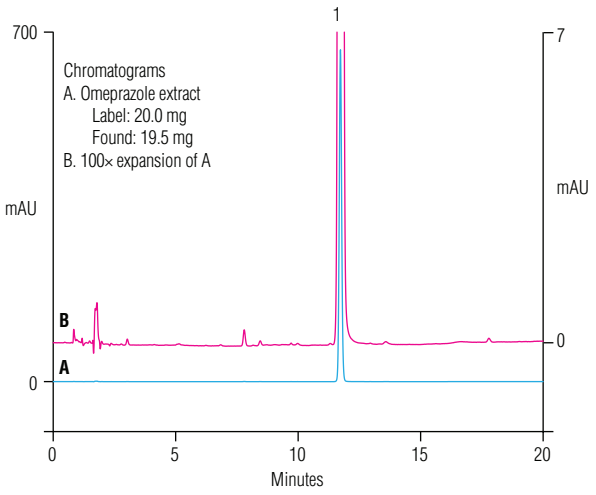


Figure 1. Omeprazole in anti-heartburn tablet on Acclaim PA2 column

Alkaloids in Bitter Orange on the Acclaim 120 C18 Column

INTRODUCTION

Combinatorial libraries are not the only source for pharmacologically active compounds. For thousands of years, civilizations around the world have employed naturally occurring ingredients to treat human maladies. Currently there is much research taking place to study areas such as Traditional Chinese Medicines (TCM). Bitter orange, the immature fruit of Citrus Aurantium, is an ingredient in traditional Chinese medicines. It contains tyramine alkaloids, with synephrine as a major component.

METHOD

The method described in this application is suitable for assaying ingredients and formulations for bitter orange and its active constituents. The method uses a 3 × 150 mm column packed with 3 µm Acclaim 120 C18 stationary phase. The column is maintained thermostatically at 35 °C.

A buffer composed of 20 mM boric acid adjusted to pH 8.2 with KOH was first created for use in sample dilution and mobile phase modification. Mobile phase A is 10 mM sodium hexanesulfonate solution made up with buffer. Mobile phase B is also a 10 mM sodium hexanesulfonate solution but made up with a mixture of 20% acetonitrile and 80% buffer. The separation is achieved using a 30 min binary gradient going from 100% mobile phase A to 100% mobile phase B. Detection is by UV absorbance at 224 nm.

RESULTS

As shown in Figure 1, the HPLC method produces a chromatogram showing good separation of six potential components of Bitter Orange (trace A). The separation of the bitter orange extract (trace B) clearly indicates the presence of synephrine demonstrating its usefulness in assaying components of bitter orange extracts.

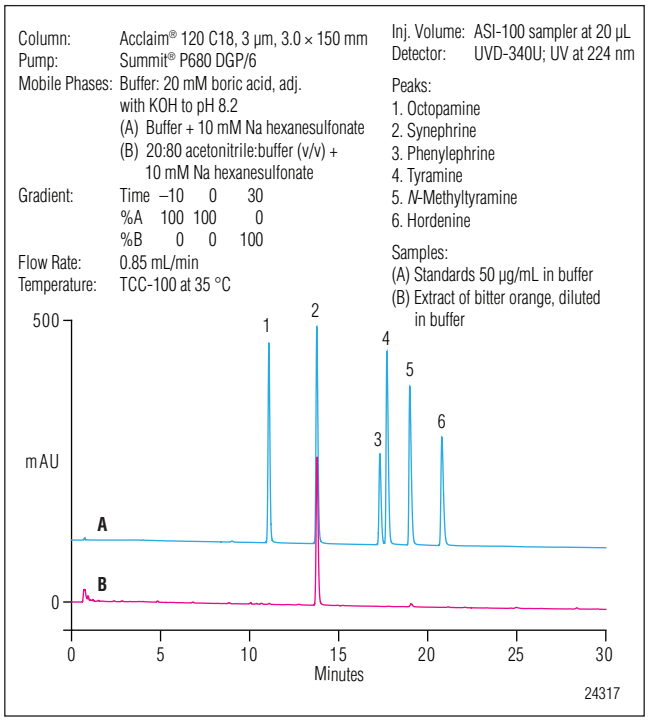
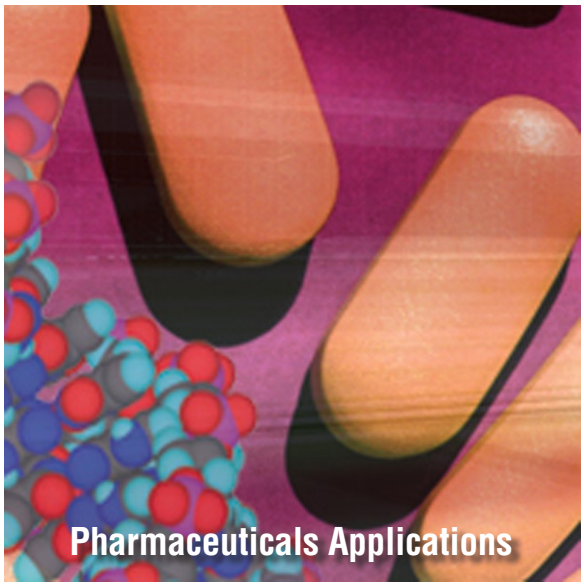


Figure 1. Alkaloids in bitter orange extract on Acclaim 120 C18 column.

DIONEX

Part IV: Analysis of Anions, Cations, and Organic Acids



Analytes

Transition Metals
Trifluoroacetic Acid
Anions in Pharmaceuticals
Fermentation Broths
Trifluoroacetate
Citrate and Phosphate
Cyanate
Water-Insoluble Pharmaceuticals

Determination of Transition Metals in Serum and Whole Blood by Ion Chromatography

INTRODUCTION

The determination of transition metals in physiological fluids is of considerable interest in clinical chemistry. In recent years several studies have linked the concentrations of specific transition metals to various diseases. Low serum copper level is used as a marker for Wilson's disease. Serum copper levels are elevated in a large number of chronic and acute illnesses such as Hodgkin's disease, leukemia, and many other malignancies.[1] Zinc is an important nutritive factor as well as a cofactor for many metalloenzymes. Zinc is necessary for the growth and division of cells, especially during the stages of life when growth rates are high. Zinc deficiency is associated with syndromes that cause short stature and dwarfism.[2] There is also interest in the biochemical relationship of copper and zinc.[3] Studies have linked an increase in plasma copper level with decreasing plasma zinc concentration in childhood lymphatic leukemia. Determination of iron in whole blood is used to monitor anemia.

Traditionally, atomic absorption spectrophotometric (AAS) techniques have been used by most clinical chemistry laboratories to determine transition metals in physio-logical fluids. These techniques have their limitations. Flame AAS has limited sensitivity for copper, and graphite atomic absorption spectrophotometry is susceptible to solute vaporization interferences such as depression of element signal, especially in physiological samples.[4–6] The protein content of the physiological fluid samples can cause absorption abnormalities. High sodium chloride content can hamper sensitivity, linearity, and cause burner clogging.

This application note describes an attractive alternative to traditional spectroscopic methods by using the principles of ion exchange. As a sample moves through the ion exchange column, bands of transition metals migrate through at differential rates determined largely by the relative affinities of the different metal-ligand complexes for the stationary ion exchange sites. A strong metal complex-ing colorimetric reagent is supplied pneumatically and mixed with the column effluent. The bands of transition metals are then determined at a visible wavelength using an absorbance detector.[7] Separation between individual metals can be enhanced or altered simply by changing eluents. Figure 1 illustrates the selectivity differences observed on an IonPac® CS5A column when using A) a pyridine-2,6-dicarboxylic acid eluent, and B) an oxalic acid eluent. This method is precise, sensitive, and requires minimum sample preparation.

RECOMMENDED EQUIPMENT

Dionex DX-500 system consisting of:
- GP40 Gradient Pump
- AD20 Absorbance Detector
- LC20 Chromatography Module

PC10 Postcolumn Pneumatic Controller

PC10 Automation Kit (optional)

PeakNet® Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Deionized water, 17.8 MΩ-cm resistance or higher

MetPac™ PDCA Eluent Concentrate and/or MetPac Oxalic Acid Eluent Concentrate

MetPac Postcolumn Diluent

4-(2-Pyridylazo)resorcinol, monosodium, monohydrate (P/N 039672)

Nitric acid, trace-metal grade (Fisher Scientific)

Sulfuric acid (Fisher Scientific)

Hydrogen peroxide, 30% (Fisher Scientific)

Trichloroacetic acid (Fluka Chemika-BioChemika)

Standards

Transition metal standards of 1000 mg/L are available from chemical supply companies (e.g. Aldrich, Sigma) for use with atomic absorption spectrometry. These are always dissolved in dilute acid solutions and can be used as IC standards.

CONDITIONS

Either of two analytical systems may be used for the chromatographic determination of transition metals. The most current method employs the IonPac CS5A, a highly efficient, solvent-compatible, mixed anion/cation exchange column. An older column, the IonPac CS5, may be used for these analyses, but efficiencies are superior and cadmium is better resolved on the CS5A. Refer to Table 1 for a complete listing of experimental conditions for both columns.

PREPARATION OF SOLUTIONS AND REAGENTS

Two eluent systems can be used for transition metal separations with the IonPac CS5A or CS5 column. The PDCA eluent is used for iron, copper, nickel, zinc, cobalt, cadmium, and manganese. The oxalic acid eluent is used for lead, copper, cobalt, zinc, and nickel. Cadmium and manganese coelute using the oxalic acid eluent.

MetPac PDCA Eluent

Dilute 200 mL of the MetPac PDCA Eluent Concentrate to 1.0 L with deionized water.

Table 1. Conditions for Two Analytical Systems Used for the Chromatographic Determination of Transition Metals

Columns	IonPac CS5A analytical and IonPac CG5A guard	IonPac CS5 analytical and IonPac CG5 guard
Eluents	A) MetPac PDCA Eluent (Alternatively, 8.0 mM PDCA, 66 mM potassium hydroxide, 74 mM formic acid, and 5.6 mM potassium sulfate may be used.) B) MetPac Oxalic Acid Eluent (Alternatively, 8 mM oxalic acid, 50 mM potassium hydroxide, and 100 mM tetramethylammonium hydroxide may be used.)	A) 6.0 mM PDCA 40 mM Sodium hydroxide 90 mM Acetic acid B) 50 mM Oxalic acid 95 mM Lithium hydroxide
Flow Rate	1.2 mL/min	1.0 mL/min
Detection	Absorbance, 530 nm	Absorbance, 520 nm
Postcolumn Reagent	0.5 mM PAR, dissolved in MetPac Postcolumn Diluent. (Alternatively, 1.0 M 2-dimethylaminoethanol, 0.50 M ammonium hydroxide, and 0.30 M sodium bicarbonate may be used.)	0.4 mM PAR 1.0 M 2-dimethylaminoethanol 0.50 M Ammonium hydroxide 0.30 M Sodium bicarbonate
Postcolumn Reagent Flow Rate	0.7 mL/min	0.5 mL/min

MetPac Oxalic Acid Eluent

Dilute 100 mL of the MetPac Oxalic Acid Eluent Concentrate to 1.0 L with deionized water.

PAR [4-(2-Pyridylazo)resorcinol] Postcolumn Reagent

Prepare the postcolumn reagent directly in the 1-L plastic reagent reservoir container. Add 0.15 g of 4-(2-pyridylazo)resorcinol, monosodium, monohydrate, to 1.0 L of the MetPac Postcolumn Diluent and ultrasonicate for five minutes. Add a stir bar and stir for several minutes to ensure that the PAR has completely dissolved. The color of the final solution should be yellow to yellow-orange. Place the reagent container in the reagent reservoir.

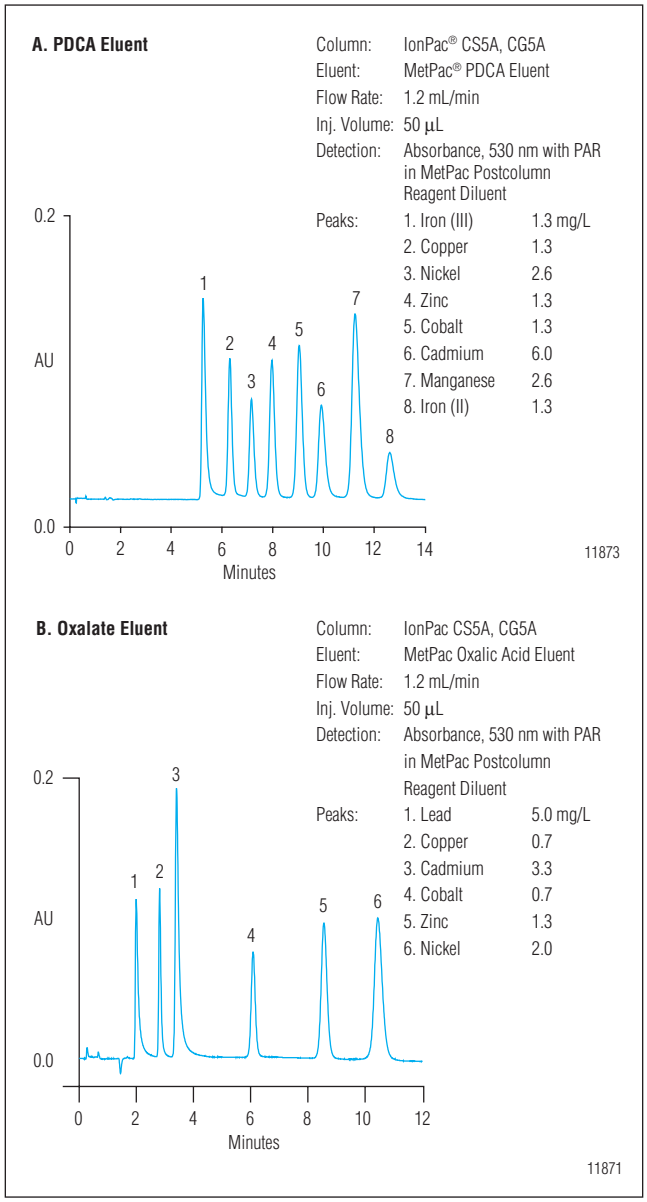


Figure 1. Separation of transition metals using A) PDCA Eluent and B) Oxalate Eluent (IonPac CS5A).

SAMPLE PREPARATION

Biological matrices contain higher concentrations of alkali and alkaline earth metals than transition metals. In such instances, a chelation concentration step can be used where the alkali and alkaline earth metals are removed from the matrix and the transition metals are selectively concentrated. For the determination of trace metals in physiological fluids or tissues, the sample must first be acid-digested to a single phase. In this application, the serum and whole blood samples were digested using the following procedure: to a 250-mL evaporation dish add 10–100 mL of sample. Next, add 5 mL of concentrated HNO_3 and 2 mL of 30% H_2O_2. Evaporate on a hot plate at medium heat to a volume of 15 to 20 mL. Cover with a watch glass to avoid sample loss by spattering. Transfer the concentrate and any precipitate to a 125 mL conical flask using 5 mL of concentrated HNO_3. Add 10 mL of concentrated H_2SO_4 and a few boiling chips or glass beads. Evaporate on a hot plate in a hood until dense white fumes of SO_3 appear. If the solution does not clear, add 10 mL of concentrated HNO_3 and repeat evaporation. Remove all HNO_3 before continuing treatment. (All HNO_3 is removed when the solution is clear and no brownish fumes are evident.[8]) Cool and dilute to about 50 mL with eluent.

Alternatively, samples can be digested using nitric acid. Add 40 g of concentrated nitric acid to approximately 75 g of sample. Add a 10-mL aliquot of the digested sample to 20 mL of 2 M ammonium acetate. The final pH of the sample should be 5.5.

A trichloroacetic acid deproteinization procedure is sometimes used for serum and plasma samples. Add 0.2 mL of 50% trichloroacetic acid to 0.4 mL of serum or plasma sample. Centrifuge the mixture for 5 minutes at 1500 x g. Inject an appropriate volume of the supernatant (e.g. 25–50 µL).

DISCUSSION AND RESULTS

The method outlined in this application note permits rapid separation of various transition metals. The separations are based on one of two different eluent systems. The first is a pyridine-2,6-dicarboxylic acid (PDCA) eluent, which is a strong complexing agent that separates the metal ion complexes by anion exchange. PDCA is best suited for iron(II) and iron(III), copper, nickel, zinc, cobalt, cadmium, and manganese (see Figure 2). This method allows one to speciate the oxidation states of iron, Fe(II) and Fe(III). However, since ferrous ion is easily oxidized to ferric, oxygen must be removed from the eluent by degassing. Oxygen should also be purged from the analytical column by pumping 0.1 M sodium sulfite (12.6 g/L Na_2SO_3) through the column for 2 hours.

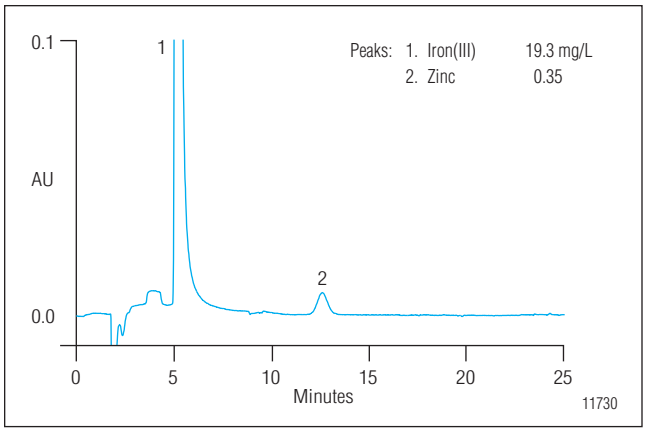


Figure 2. Determination of iron(III) and zinc in whole blood (IonPac CS5, PDCA eluent).

An alternative eluent system uses an oxalic acid-based eluent, which is a moderate strength complexing agent that separates the metals by a mixed mode mechanism. The oxalate eluent separates lead, copper, cobalt, zinc, and nickel (see Figure 3). Cadmium and manganese coelute with this eluent.

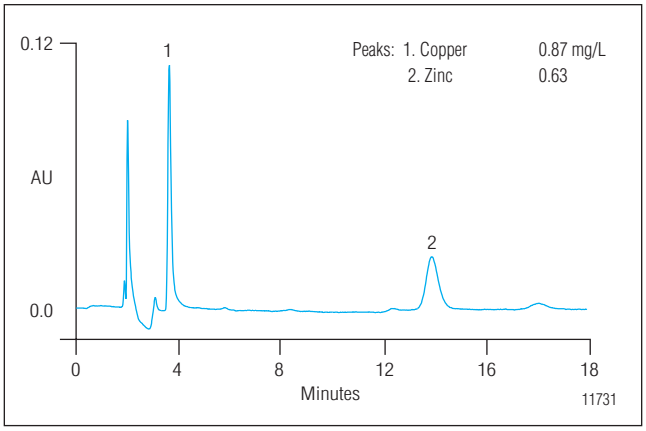


Figure 3. Determination of copper and zinc in serum (IonPac CS5, Oxalic Acid Eluent).

The separated metals from the analytical column enter a postcolumn reaction system where they are derivatized with 4-(2-pyridylazo)resorcinol and then detected at 520–530 nm using a UV/visible absorbance detector. This method is ideal for complex matrices such as physiological fluids. This method is highly sensitive, specific, and precise. Analyte recoveries for various physiological fluid matrices are listed in Tables 2 and 3.

Table 2. Precision and Recovery Data for Transition Metals in Whole Blood[a]

Analyte	Amount Found (mg/L)	Amount Spiked (mg/L)	Mean Recovery (%)	RSD (%)
Iron(III)[b]	420	—	—	2.7
Zinc	1.2	3.0	95	1.3

[a]n = 7 replicates, 50 µL injected.
[b]Iron(III) was not spiked.

Table 3. Precision and Accuracy Data for Transition Metals in Serum[a]

Analyte	Amount Found (mg/L)	Amount Spiked (mg/L)	Mean Recovery (%)	RSD (%)	MDL[b] µg/L
Copper	0.57	1.0	98	3.8	45
Zinc	0.85	2.0	95	2.4	70

[a]n = 7 replicates, 50 µL injected.
[b]MDL = SD x $(t_s)_{99\%}$.

PRECAUTIONS

The analytical flow path must have no metal com-ponents. This includes tubing end fittings, stainless steel washers, omni-fittings, etc., as well as columns and valves that contain stainless steel. Use caution in preparing and transferring reagents to minimize contamination. The prepared PAR is easily oxidized. If at anytime the PAR takes on a red color, it has been contaminated and should be discarded. Prepared reagents should be stored under an inert gas, such as nitrogen or helium, and used within two weeks of preparation. Be sure that the eluent is being pumped through the columns when the postcolumn pneumatic controller is turned on.
Failure to do so may cause the PAR reagent to back up through the analytical column.

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8. Franson, M. H. (Ed.) "Standard Methods for the Examination of Water and Wastewater," 15th Edition, Amer. Public Health Assoc., Washington, D. C., **1985**.

SUPPLIERS

Aldrich Chemical Company
1001 West Saint Paul Avenue
P.O. Box 355
Milwaukee, WI 53233, USA
Tel.: (800) 558-9160

Fisher Scientific, 711 Forbes Avenue
Pittsburgh, Pennsylvania 15219-4785, USA
Tel.: (800) 766-7000

Fluka Chemika-BioChemika, Fluka Chemie AG
Industriestrasse 25, CH-9471
Buchs, Switzerland
Tel.: +81 755 25 11

Sigma Chemical Company
P.O. Box 14508
St. Louis, MO 63178, USA
Tel.: (800) 325-3010

Determination of Trifluoroacetic Acid (TFA) in Peptides

INTRODUCTION

Trifluoroacetic acid (TFA) is commonly used in the manufacturing process to release synthesized peptides from solid-phase resins. TFA or acetate is also used during reversed-phase HPLC purification of peptides. TFA is manufactured using acetate and fluoride as precursors, and residual levels of these compounds may be present whenever TFA is used. Residual TFA, fluoride, and, to a much lesser extent, acetate are toxic and undesirable in peptides intended for preclinical and clinical studies. A method for the determination of TFA, acetate, and fluoride must be suitable for peptide formulations and be capable of verifying the removal of these anions during the production process.

TFA has been assayed by gas chromatography[1–5], GC mass spectroscopy[6], reversed-phase HPLC[7], isotachophoresis[8–10], infrared spectrometry[11], titration[12–13], spectrophotometry[14], and ion-exchange chromatography[15–18]. Ion chromatography (IC) is advantageous because it is sensitive, simple, and can be automated.

The separation mechanism of IC is based on an anion-exchange displacement process occurring between the sample ions and eluent ions with the anion-exchange functional groups bonded to the stationary phase. A typical stationary phase consists of a grafted, solvent-compatible, alkyl-based ion-exchange resin. The separation of TFA, acetate, and fluoride illustrated in this application note uses a stationary phase functionalized with alkyl quaternary ammonium groups. Effluent from the analytical column is passed through a suppressor that reduces the total background conductance of the eluent and increases the electrical conductance of the analyte ions. With suppressed conductivity, signal-to-noise ratios are improved approximately 50-fold compared to nonsuppressed conductivity.

This application note describes the analysis of commercially prepared, water-soluble peptides using ion chromato-graphy. This method requires minimal sample preparation, and the analytes, fluoride, acetate, and TFA, are easily separated without significant peptide interference.

EQUIPMENT

Dionex DX 500 system consisting of:

- GP40 Gradient Pump
- CD20 Conductivity Detector or
  ED40 Electrochemical Detector
- LC30 Oven or LC20 Chromatography Module
- AS3500 Autosampler

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Sodium carbonate, 0.5 M (Dionex P/N 37162)

Sodium bicarbonate, 0.5 M (Dionex P/N 37163)

Deionized water, 18 MΩ-cm resistance or higher

Standards

Sodium fluoride, ACS grade (Fisher Scientific, Cat. No. S-299)

Sodium acetate, trihydrate (Sigma Chemical Co., Cat. No. S-8625)

Trifluoroacetic acid, anhydrous, protein sequencing grade (Sigma Chemical Co., Cat. No. T-1647)

Five Anion Standard (fluoride, chloride, nitrate, phosphate, sulfate) (Dionex, P/N 37157)

CONDITIONS

Columns:	IonPac® AS14, Analytical (4 mm) IonPac AG14, Guard (4 mm)
Eluent:	3.5 mM Sodium carbonate/ 0.8 mM Sodium bicarbonate
Flow Rate:	1.2 mL/minute
Inj. Volume:	10 µL
Detection:	Suppressed conductivity, ASRS™ AutoSuppression™ recycle and external water modes
Expected Background Conductivity:	15 µS
Expected System Operating Backpressure:	12.4 MPa (1800 psi)

PREPARATION OF SOLUTIONS AND REAGENTS

3.5 mM Sodium carbonate / 0.8 mM Sodium bicarbonate

Combine 1980 mL of deionized water with 14.0 mL of 0.5 M sodium carbonate and 3.2 mL of 0.5 M sodium bicarbonate. Degas for 20 minutes. Connect the eluent reservoir to the instrument and pressurize with helium.

STOCK STANDARDS

Prepare a 4.5-mg/mL stock analyte standard of fluoride by combining 10.0 mg of sodium fluoride with 1.00 mL of water. Prepare a 7.2-mg/mL acetate standard by combining 10.0 mg of sodium acetate trihydrate with 1.00 mL of water. For a 9.9-mg/mL trifluoroacetate stock solution, mix 10.0 mg of trifluoroacetic acid with 1.00 mL of water. Combine and dilute standard solutions to desired concentrations using the mobile phase eluent as the diluent. Standard solutions should be frozen until needed.

SAMPLE PREPARATION

Commercial Peptides

Tyr-[Trp^2]-MSH Release Inhibiting Factor;
Tyr-Pro-Trp-Gly-NH_2
Trifluoroacetate salt, abbreviated here as MSH-RIF.

[Sar^1, Thr^8]-Angiotensin II;
Sar-Arg-Val-Tyr-Ile-His-His-Pro-Thr
Acetate salt, abbreviated here as AT-II.

Ala-D-Isoglutaminyl-Lys-D-Ala-D-Lys
Acetate salt, abbreviated here as IGA.

[Gln^4]-Neurotensin; pGlu-Leu-Tyr-Gln-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
No counterion specified, abbreviated here as NT.

FMRF Amide Related Peptide;
Asn-Arg-Asn-Phe-Leu-Arg-Phe amide
Trifluoroacetate salt, abbreviated here as FMRF.

Peptide Samples from In-Process Manufacturing

Eleven Amino Acid Crude Peptide; Ac-D-Ala-Gly-Arg-His-Tyr-Ala-Arg-Val-Ala-Leu-Arg-amide
No purification, abbreviated as "Crude Peptide".

Eleven Amino Acid >70% Pure Peptide; Ac-D-Ala-Gly-Arg-His-Tyr-Ala-Arg-Val-Ala-Leu-Arg-amide
Purified by gel permeation chromatography, abbreviated as "GPC Pure Peptide".

Commercial peptide samples are dissolved in eluent to peptide concentrations of 1 mg/mL. Peptide samples from in-process manufacturing are dissolved in eluent to dry weight concentrations of 1 mg/mL. Both the commercial and in-process peptide solutions are further diluted with eluent to concentrations of 40 and 400 µg/mL. Peptide solutions are also diluted with standard solutions to evaluate the spike recovery by the method of standard addition.

DISCUSSION AND RESULTS

Figure 1 shows the separation of fluoride, acetate, chloride, nitrate, phosphate, sulfate, and TFA using the 3.5 mM sodium carbonate/0.8 mM sodium bicarbonate eluent.

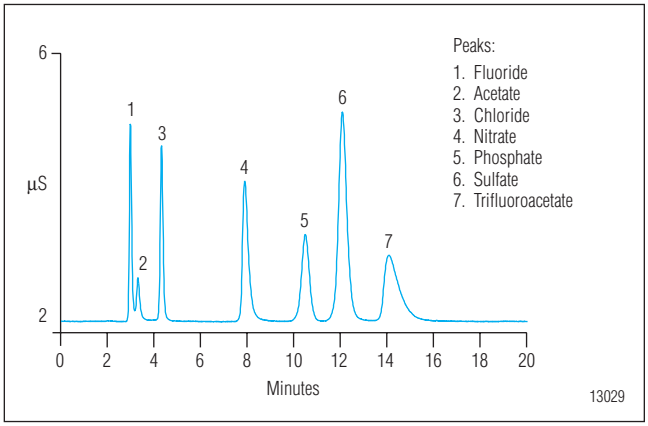

Figure 1. Seven common anions found in peptide samples.

Method Detection Limits

The method detection limits (MDL) for a 10-µL injection of fluoride, acetate, and trifluoroacetate are given in Table 1. The MDL is defined as the minimum concentration required to produce a signal-to-noise ratio of 3. Two modes of suppression were compared for their effect on MDL. The recycle mode, which used the postdetector eluent as feed through the regenerant chamber to achieve suppression, was compared to the external water mode, which used deionized water from a separate, pressurized bottle as regenerant feed. Suppression in the external water mode compared to the recycle mode produces lower background noise, thus a lower MDL. The MDL can be further decreased by increasing the injection volume above the 10 µL used in this Application Note.

Table 1. Detection Limits

Sample Mode	Recycle Mode		External Water	
	(ng)	(ng/mL)	(ng)	(ng/mL)
Fluoride	0.3	30	0.1	10
Acetate	2	200	1	100
Trifluoroacetate	6	600	3	300

Linearity

Fluoride standards of 0.023, 0.045, 0.23, 0.45, 2.3, 4.5, 23, 45, 230, 450 µg/mL were injected (n = 6 per concentration) for this study. The method was found to be linear for fluoride over the range tested (r^2 = 1.000). Acetate and TFA were also linear (r^2 = 0.999 in each case), using 0.36, 0.72, 3.6, 7.2, 36, 72, 360 µg/mL acetate standards and 0.50, 1.0, 5.0, 100, 50, 100, 500 µg/mL trifluoroacetate standards (n = 6). For all three analytes, linearity was demonstrated over at least three orders of magnitude.

Stability

Standards of fluoride (4.5 µg/mL), acetate (7.2 µg/mL), and TFA (10 µg/mL) were injected over 48 hours using an equilibrated system. Sample vials were at ambient temperature. Peak areas (Figure 2) and retention times (Figure 3) were reasonably stable throughout this period.

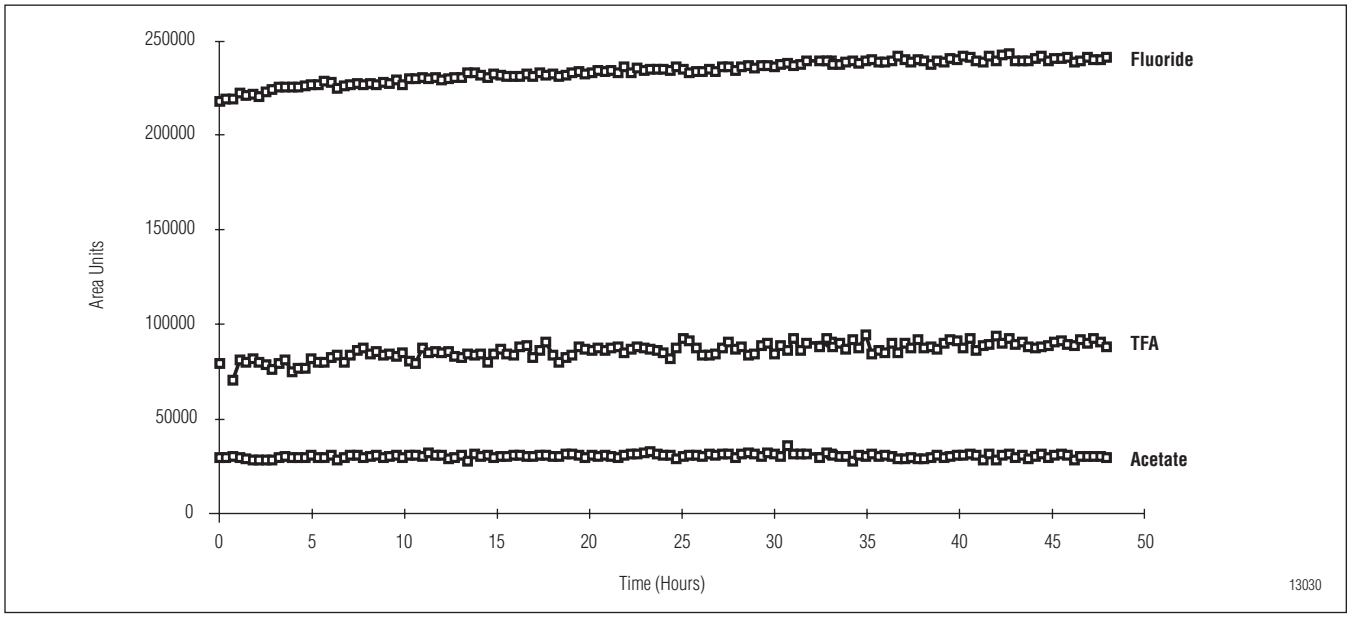

Figure 2. Peak area stability over 48 hours.

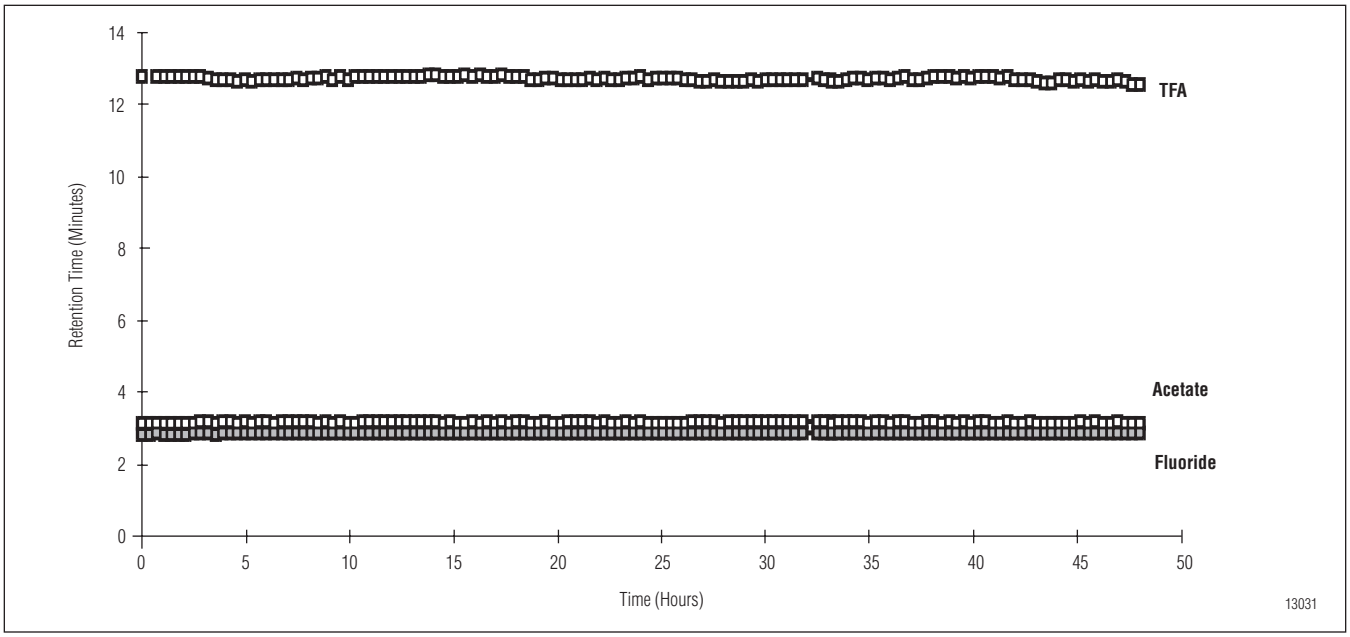

Figure 3. Retention time stability over 48 hours.

Table 2. Recovery of Fluoride, Acetate, and Trifluoroacetate from Peptides

Anion	Percent Recovery (Mean)					
	Spiked (µg/mL)	MSH-RIF	AT-II	IGA	NT	FMRF
Fluoride	1.8	93.3	93.7	96.7	95.4	93.6
	3.6	94.8	95.2	98.4	96.9	96.4
	5.4	96.4	97.4	99.4	98.0	99.9
Acetate	2.8	97.9	79.2	96.4	95.2	101
	5.7	102	99.6	99.9	98.2	101
	8.6	98.3	99.4	97.7	100	106
TFA	4.0	105	98.1	91.1	104	101
	8.0	105	102	94.4	103	101
	12.0	98.3	100	99.7	106	103

Precision

Precision is affected by concentration; RSD values increase as the concentrations approach the MDL. The peak area RSD values for fluoride (45 ng/injection), acetate (72 ng/injection), and TFA (99 ng/injection) were 0.5, 2.3, and 3.8% respectively for 12 injections.

Retention time precision (RSD) values were 0.4, 0.4, and 0.3% for fluoride, acetate, and TFA, respectively.

Table 3. Effect of Peptide (FMRF) Concentration on Recovery of Fluoride, Acetate, and Trifluoroacetate

Peptide Inj. (ng)	Percent Recovery (Mean)		
	Fluoride	Acetate	TFA
140	97.1	115	99.3
420	96.4	101	101
1010	97.7	102	120

Recovery from Peptide Matrix

Table 2 shows the recovery of fluoride, acetate, and TFA from commercial peptides. Anions were spiked into the peptide solutions by the method of standard addition. Recovery for fluoride ranged from 93 to 100%, acetate from 79 to 106%, and TFA from 91 to 106%. Only fluoride showed a slightly higher recovery with increasing levels of standard. The amount of peptide (FMRF) injected had no effect on recovery (see Table 3).

Table 4. Fluoride, Acetate, and Trifluoroacetate in Commercial Peptides

	mg anion/g peptide		
Peptide	**Fluoride**	**Acetate**	**Trifluoroacetate**
MSH-RIF	0.26	0.90	202
AT-II	0.18	93	<0.3
IGA	24	80	<0.3
NT	0.02	0.13	184
FMRF	0.02	0.15	193

Residual Anions in Commercial Peptides

Commercially available peptides contained counterions that were quantifiable by ion chromatography. Table 4 lists the fluoride, acetate, and trifluoroacetate measured in the commercial peptides. All peptides labeled by the manufacturer as trifluoroacetate salts (MSH-RIF and FMRF) contained TFA at approximately the same concentrations (193–202 mg of TFA per gram of peptide). All peptides labeled as acetate salts (AT-II and IGA) contained acetate at measurable levels. The peptide NT was labeled as containing salts, but did not indicate which salts. By this method, NT was determined to contain TFA at levels similar to those found in MSH-RIF and FMRF. Furthermore, IGA was labeled as containing acetate as the counterion, but additionally contained fluoride. All the peptides investigated in this Application Note also contained other anions, such as chloride, sulfate, phosphate, and nitrate, which can be effectively resolved by this method. Other anions were detected but not identified.

Residual Anions During In-Process Peptide Manufacturing

This IC method can be used to evaluate the effectiveness of purification during manufacturing. A crude synthetic peptide sample (prior to purification) was determined to contain 19.6% TFA by dry weight. Gel permeation chromatography (GPC) was used as a primary purification step after synthesis, producing a peptide sample containing 16.7% TFA. Figures 4A and 4B show chromatograms of these peptide solutions. These results suggest that TFA was not effectively removed by GPC. Appreciable levels of chloride and sulfate were measured in the crude peptide (0.02 and 1.0%, respectively). After GPC, much higher levels were observed (0.83% chloride and 2.6% sulfate). The GPC-purified peptide sample also contained fluoride and nitrate at 0.026% and 0.014% respectively. Knowing the amount and type of these counterions in peptide preparations is important to the ultimate safety and effectiveness of the product. Ion chromatography is therefore an effective in-process quality control method. This ion chromatography method can also assist with defining the mass balance of the peptide preparations.

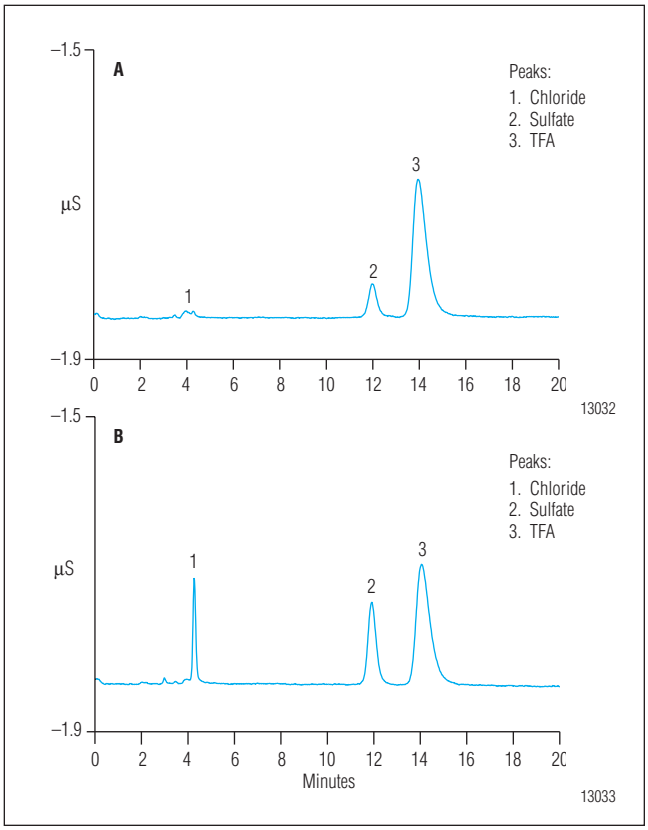

Figure 4. (A) Crude in-process peptide (10 µL of 40 µg/mL). (B) GPC-purified in-process peptide (10 µL of 40 µg/mL).

CONCLUSION

This isocratic IC method using the IonPac AS14 column with suppressed conductivity detection can be used to evaluate peptides for residual TFA, fluoride, and acetate. The method also resolves other common anions such as chloride, sulfate, nitrate, and phosphate. TFA, fluoride, and acetate can be detected at the mg/L level. The recovery of fluoride, acetate, and TFA from peptide matrices is normally greater than 90%.

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LIST OF SUPPLIERS

Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennsylvania, 15219-4785, U.S.A., 800-766-7000.

Sigma Chemical Corporation, P.O. Box 14508, St. Louis, Missouri, 63178, U.S.A., 800-325-3010.

Quantification of Anions in Pharmaceuticals

INTRODUCTION

The United States Food and Drug Administration (U.S. FDA)[1–3] and regulatory agencies in other countries require that pharmaceutical products be tested for composition to verify their identity, strength, quality, and purity, with increased attention to inactive as well as active ingredients. Analytical techniques that can adequately test complex formulations composed of chromophoric and nonchromophoric ingredients are desirable. Nonchromophoric ingredients, many of which are ionic, cannot be detected by absorbance, but can be detected using suppressed conductivity. Suppressed conductivity is a powerful detection technique with a broad linear range and very low detection limits. Suppression lowers the background conductivity caused by the eluent and effectively increases the conductivity of the analyte.[4–5]

This Application Note describes the use of two anion-exchange columns with suppressed conductivity detection to analyze common anions in pharmaceutical formulations. Two oral, over-the-counter medications were selected as representative pharmaceutical products: a cough suppressant and a multisymptom cold/flu medication. These formulations contain complex mixtures of ingredients that are commonly found in other medications, many of which are ionic and nonchromophoric. Furthermore, these formulations also contain sugar alcohol, glycol, and carbohydrate ingredients that can be analyzed using the IonPac® ICE-AS1, CarboPac™ MA1, and CarboPac PA10 columns with electrochemical detection.[6]

In the methods outlined in this Note, the selectivities of the IonPac AS14 and AS11 columns for the analysis of anionic ingredients in pharmaceutical formulations were compared. The AS14 packing has a highly crosslinked core with an anion-exchange layer grafted to the surface. The anion-exchange layer is functionalized with alkyl quaternary ammonium functional groups and is grafted to crosslinked ethylvinylbenzene. This anion-exchange resin is selective for the more hydrophobic anions. The AS11 column packing has a pellicular structure consisting of an alkyl quaternary ammonium latex bonded to a crosslinked ethylvinylbenzene core. Another important difference between the two columns is that the AS14 column is designed for isocratic conditions, while the AS11 can be used with hydroxide gradients. Both columns are compatible with eluents containing organic solvents, which can be used to reduce undesirable secondary interactions some organic anions have with stationary phases. Expected detection limits, linearity, selectivity, accuracy, and precision are reported for the AS11 column.

EQUIPMENT

Dionex DX-500 system consisting of:

- GP40 Gradient Pump, with degas option
- CD20 Conductivity Detector or
  ED40 Electrochemical Detector
- LC30 or LC25 Chromatography Ovens or
  LC20 Chromatography Module
- AS3500 Autosampler

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Sodium carbonate, 0.5 M (Dionex P/N 37162)

Sodium bicarbonate, 0.5 M (Dionex P/N 37163)

Sodium hydroxide, 50% (w/w) (Fisher Scientific, P/N SS254-500)

Deionized water, 18 MΩ-cm resistance or higher (Prior to use, Dionex recommends testing the water for trace anions using the intended ion chromatography method.)

Standards

Five Anion Standard (fluoride, chloride, nitrate, phosphate, sulfate; Dionex P/N 37157)

Sodium acetate, anhydrous (Fluka Biochemika, P/N 71179)

Sodium bromide (Aldrich Chemical Co., P/N 31,050-6)

Sodium chloride (Fisher Scientific, P/N S271-500)

Sodium citrate, monohydrate (Fisher Scientific, P/N A104-500)

Sodium benzoate (Sigma Chemical Co., P/N B-3375)

Saccharin (Sodium salt; Sigma Chemical Co., P/N S-1002)

CONDITIONS

System 1

Column:	IonPac AS14 Analytical (P/N 46124), IonPac AG14 Guard (P/N 46134)
Eluent:	3.5 mM Sodium carbonate 0.8 mM Sodium bicarbonate
Flow Rate:	1.2 mL/min
Injection Vol.:	10 µL
Detection mode:	Suppressed conductivity, ASRS™, AutoSuppression™ recycle mode
Expected Background Conductivity:	15 µS
Expected System Operating Backpressure:	12.4 MPa (1800 psi)

System 2

Column:	IonPac AS11 Analytical (P/N 44076), IonPac AG11 Guard (P/N 44078), ATC-1 Anion Trap Column (P/N 37151)
Eluent:	Linear sodium hydroxide gradients: 0.5 mM Sodium hydroxide for 2.5 min, then 0.5 to 5 mM sodium hydroxide for 3.5 min, then 5 to 38 mM sodium hydroxide for 12 min, then 0.5 mM sodium hydroxide for 7 min.
Flow Rate:	2.0 mL/min
Injection Vol.:	10 µL
Detection mode:	Suppressed conductivity, ASRS, AutoSuppression recycle mode
Expected Background Conductivity:	0.5 µS
Expected System Operating Backpressure:	12.4 MPa (1800 psi)

PREPARATION OF SOLUTIONS AND REAGENTS

Sodium carbonate/bicarbonate eluent

3.5 mM Sodium carbonate/0.8 mM Sodium bicarbonate

Combine 1980 mL of deionized water with 14.0 mL of 0.5 M sodium carbonate and 3.2 mL of 0.5 M sodium bicarbonate. Degas for 20 minutes. Connect the eluent reservoir to the instrument and pressurize with helium.

Sodium hydroxide eluents

5 mM Sodium hydroxide

Degas 2000 mL of deionized water for 20 min and combine with 520 µL of 50% (w/w) sodium hydroxide.

100 mM Sodium hydroxide

Degas 1990 mL of deionized water for 20 min and combine with 10.4 mL of 50% (w/w) sodium hydroxide.

STOCK STANDARDS

Combine the Five Anion Standard (fluoride, chloride, nitrate, phosphate, sulfate; Dionex P/N 37157) with acetate, bromide, citrate, benzoate, and saccharin standards and purified water to yield stock concentrations of:

Fluoride	1.6 mg/L	Phosphate	12 mg/L
Acetate	9.8 mg/L	Chloride	2.4 mg/L
Nitrate	8.0 mg/L	Citrate	10 mg/L
Bromide	9.8 mg/L	Benzoate	10 mg/L
Sulfate	12.0 mg/L	Saccharin	100 mg/L

Dilute the stock solution with water to the desired concentrations.

For determinations of linear range, combine 10-g/L solutions of chloride, bromide, benzoate, citrate, and saccharin to make a 1.0-g/L solution of standard mix. Dilute with water to concentrations of 0.8, 0.6, 0.4, 0.2, 0.1, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, and 0.001 g/L. Dilute saccharin and citrate separately to evaluate these analytes without interference using concentrations of 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1 g/L.

SAMPLE PREPARATION

Dilute viscous products with water on a weight per weight basis. A 10-fold (w/w) dilution was obtained by combining 1 gram of medication with 9 grams of water. The multisymptom cold/flu medication was further diluted to a 100-fold (w/w) final concentration. Determine the densities of the products by measuring the weights of known volumes. Calculate the final concentrations of the ingredients based on the densities of these medications. The ingredients of each medication are presented in Tables 1 and 2. The ingredients noted in bold-face type can be analyzed by anion-exchange columns with suppressed conductivity; other ingredients listed can be analyzed using CarboPac columns with electrochemical detection.[6]

Table 1. Cough Suppressant

Dextromethorphan Hydrobromide	Active
Citric Acid	Inactive
FD&C Red 40	Inactive
Flavors	Inactive
Glycerin (glycerol)	Inactive
Propylene Glycol	Inactive
Saccharin Sodium	Inactive
Sodium Benzoate	Inactive
Sorbitol	Inactive
Water	Inactive

Table 2. Multisymptom Cold/Flu Medication

Pseudoephedrine Hydrochloride	Active
Acetaminophen	Active
Dextromethorphan Hydrobromide	Active
Citric Acid	Inactive
FD&C Yellow #6	Inactive
Flavor	Inactive
Glycerin (glycerol)	Inactive
Polyethylene Glycol	Inactive
Propylene Glycol	Inactive
Purified Water	Inactive
Saccharin Sodium	Inactive
Sodium Citrate	Inactive
Sucrose	Inactive

Any purified water used for dilutions should be tested for trace anions by ion chromatography prior to use. Sample containers should be tested for residual anions prior to use by adding pure water, shaking or vortexing, and then testing the liquid. Plastic vials are usually lower in residual anions than glass. Prerinsing the vials with purified water can reduce artifacts.

DISCUSSION AND RESULTS

Selectivity

Figure 1 shows the separation of fluoride, acetate, chloride, nitrate, phosphate, and sulfate standards using a 3.5 mM sodium carbonate/0.8 mM sodium bicarbonate eluent with the IonPac AS14 column. The isocratic conditions eliminate any need to reequilibrate the column, thereby decreasing run times and increasing throughput. These anions can be analyzed within 14 minutes. Citrate, benzoate, and saccharin are not eluted by this method.

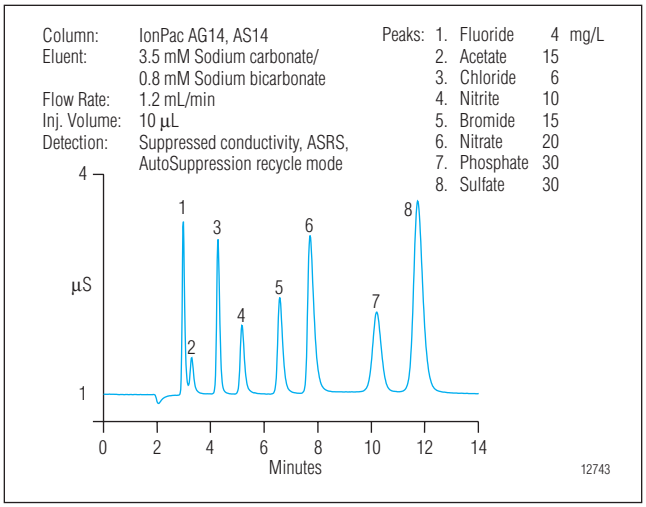

Figure 1. Rapid separation of inorganic anions.

With the IonPac AS11 column, inorganic anions, acetate, citrate, benzoate, and saccharin are eluted using a sodium hydroxide gradient (see Figure 2). Although the gradient results in slightly longer run times, both organic and inorganic anions are effectively separated. Shorter run times are possible for either method by adjusting the eluent strength, but some resolution may be lost for fluoride and acetate.

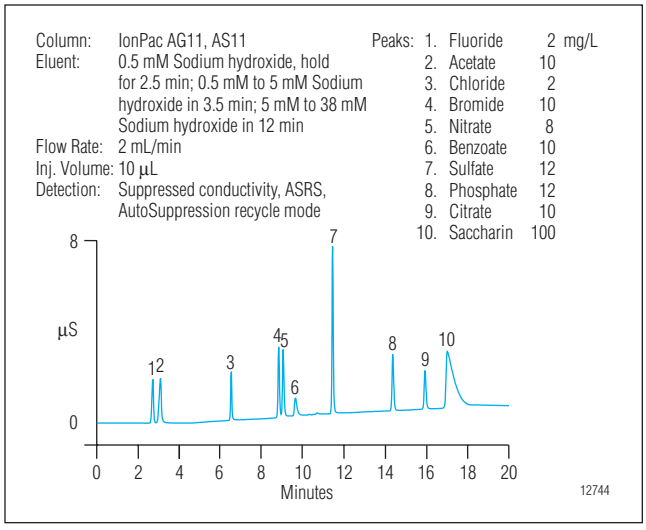

Figure 2. Gradient separation of both inorganic and organic anions.

Saccharin retention times significantly shorten above 1000 ng per injection, causing coelution with citrate. Saccharin retention depends on secondary interactions with the column. At high concentrations, saccharin exceeds the capacity of the column and may elute earlier than at lower concentrations. When both saccharin and citrate coexist in a formulation, they both should be adjusted to below 100 mg/L for a 10-µL injection. Saccharin peaks tail, but the addition of organic solvents in the eluent (e.g., 10% acetonitrile) reduces tailing. Addition of acetonitrile alters the column selectivity, decreases peak area response, and increases background noise.

Although not presented here, nearly a dozen over-the-counter medications have been analyzed using the IonPac AS14 and AS11 columns with suppressed conductivity. These medications include both solid and liquid formulations such as nasal and oral decongestants, astringents, antacids, enemas, sleep aids, analgesics, cleaning and disinfecting solutions, antihistamines, and allergy syrups. The known anionic ingredients in each formulation were separated from each other without any apparent interference. Trace levels of unlabeled ingredients were also detected as minor peaks and, when identified, measured at low concentrations.

Method Detection Limits

The method detection limits (MDL) for a 10-µL injection of common pharmaceutical anions with the AS11 column are described in Table 3. The MDL is defined as the minimum concentration required to produce a signal-to-noise ratio of 3. The MDL can be further decreased by increasing the injection volume above the 10 µL used in this Application Note or by using external water mode suppression.[7]

Table 3 . Estimated Lower Detection Limits

	System 2 (IonPac AS11 column)	
	ng	µg/L
Fluoride	0.3	30
Acetate	2	200
Chloride	0.5	50
Bromide	4	400
Nitrate	3	300
Sulfate	1	100
Phosphate	4	400
Citrate	4	400
Benzoate	7	700
Saccharin	20	2000

Linearity

Chloride, bromide, benzoate, citrate, and saccharin standards ranging from 1 to 1000 mg/L (10 ng to 10,000 ng) were injected (in triplicate) on the AS11 column (data not shown). The peak area response was found to be linear for chloride, bromide, citrate, and saccharin over this range ($r^2 \geq 0.999$). Benzoate was linear over the range of 1 to 200 mg/L (10 ng to 2,000 ng per injection; $r^2 = 0.999$). For the range of 1 to 1000 mg/L (10 ng to 10,000 ng), the correlation coefficient for benzoate was 0.995. Although linearity is good for citrate and saccharin when run independently, the two peaks coelute at concentrations above 100 mg/L (1000 ng). Therefore, linearity cannot be evaluated when both compounds are present at this concentration. For all analytes, linearity was demonstrated over at least two orders of magnitude.

Representative calibration curves for both the AS14 and the AS11 columns are presented in Figures 3 and 4.

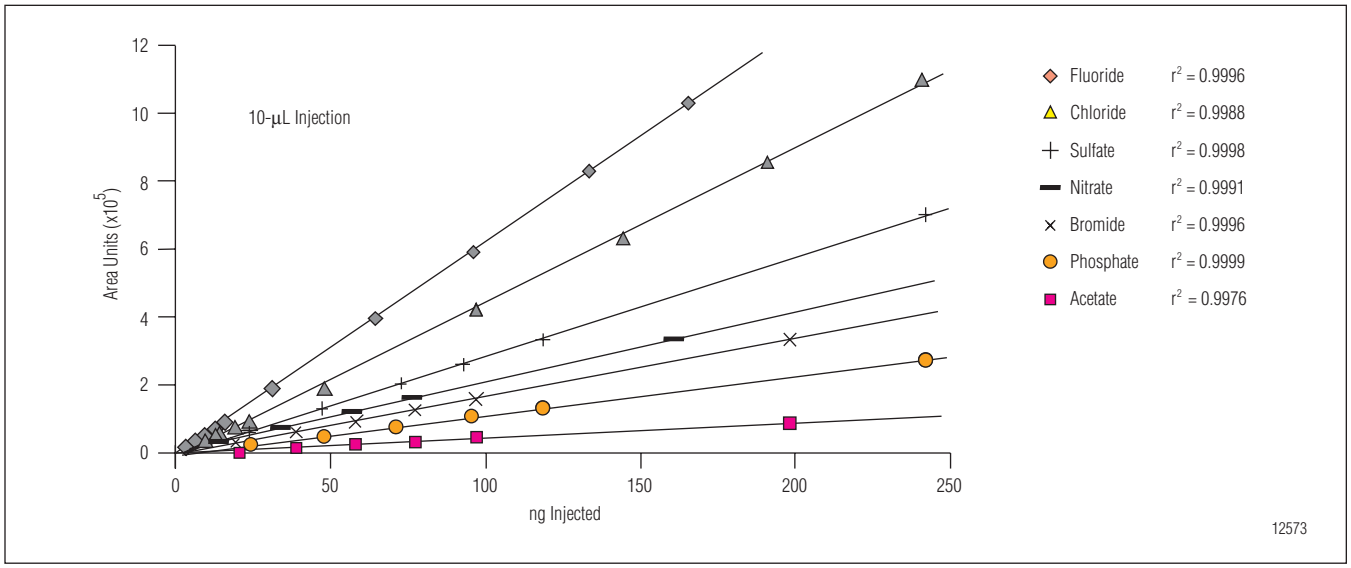

Figure 3. Method linearity for the IonPac AS14 column (isocratic method).

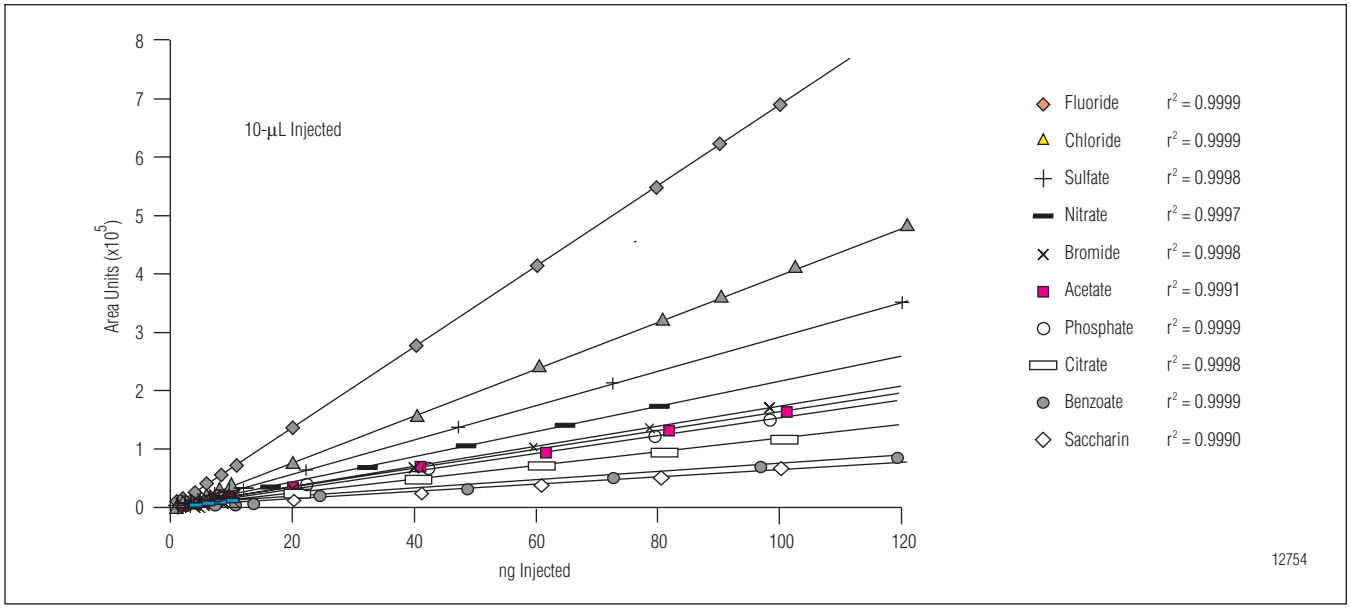

Figure 4. Method linearity for the IonPac AS11 column (gradient method).

Table 4. Peak Area and Retention Time Precision*

	RSD (%)	
	Area	Retention Time
Chloride	0.1	0.2
Bromide	0.1	0.1
Benzoate	0.2	0.1
Citrate	0.3	0.4
Saccharin	0.4	0.3

* 100-mg/L standards, 10 µL per injection (n = 15).

Table 5. Peak Area and Precision of Pharmaceutical Products*

Analyte	Measured Conc. (mg/L)	RSD (%)	
		Cough Suppressant	Cold/Flu Medication
Bromide	30	0.5	–
Bromide	1	–	1.0
Chloride	3	–	0.3

* 10-fold (w/w) dilutions of cough suppressant, 100-fold (w/w) dilutions of cold/flu medication, 10-µL injection (n = 10).

Table 6. Recovery of Active Ingredient Counterions in Medications

	Dextromethorphan hydrobromide	Pseudoephedrine hydrochloride
Cough suppressant	98.9%	N/A
Multisymptom cold/flu formulation	97.3%	109%

Precision

The peak area and retention time RSDs using the AS11 column are presented in Table 4. Area precision is affected by concentration; RSD values increase as the concentrations approach the MDL.

Replicate, 10-µL injections (n = 10) of a 10-fold (w/w) dilution of cough suppressant yielded a bromide peak area RSD of 0.5% when measured at 30 µg/mL. A 100-fold (w/w) dilution of the multisymptom cold/flu medication resulted in a bromide RSD value of 1.0% when measured at 1 µg/mL for 10-µL injections; chloride was 0.3% at 3 µg/mL for 10-µL injections. The retention time RSD values ranged from 0.1% to 0.2% for these anions. These sample RSDs are summarized in Table 5 and are consistent with those found for standards analyzed at equivalent concentrations.

Recovery from Sample Matrix

Figures 5 and 6 show the separation of bromide from other ingredients in cough suppressant using the AS14 and AS11 columns, respectively. Bromide is the counterion in the active ingredient dextromethorphan hydrobromide. Figures 7 and 8 show the separation of both bromide and chloride from other ingredients in a multisymptom cold/flu formulation. Chloride is the counterion in pseudoephedrine hydrochloride. These anions are unique to these active ingredients in both medications; therefore, their quantification is an orthogonal method for the determinations of dextromethorphan and pseudoephedrine in these formulations.

The measured levels of these anions using the AS11 column are compared to the labeled values of these active ingredients in each formulation and expressed as a percent recovery (see Table 6). In all cases, the levels of bromide and chloride were within 10% of the label value. This orthogonal method for quantification of an active ingredient in a pharmaceutical can be a valuable troubleshooting tool. For example, during stability studies, samples can either change in concentration or components can decompose. Analysis of both the organic component and the inorganic counterion can serve to differentiate between these symptoms.

The dosage of inactive ingredients is not specified on the label of most medications. Therefore, it was not possible to evaluate the accuracy of the formulation for benzoate, citrate, and saccharin. However, these ingredients were quantified (see Figures 6 and 8).

Other Anions in Pharmaceutical Products

Besides the labeled content of the pharmaceutical products, other anionic ingredients may also be present. These may arise from trace levels of ingredients found in the raw materials used in manufacturing. Expanding the baseline of the above chromatograms (Figures 6B and 8B) reveals the presence of minor peaks. Some can be identified based on retention time, while others cannot. Sulfate (2 µg/mL) and phosphate (1 µg/mL) were measured in the cough suppressant. Five other minor peaks were observed but were not identified. Sulfate (13 µg/mL) was measured in the multisymptom cold/flu formulation, while three minor peaks were unidentified.

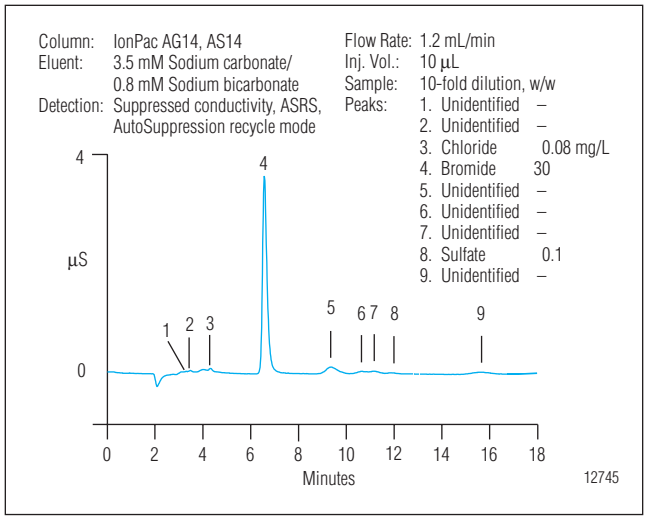

Figure 5. Counterions in cough suppressant.

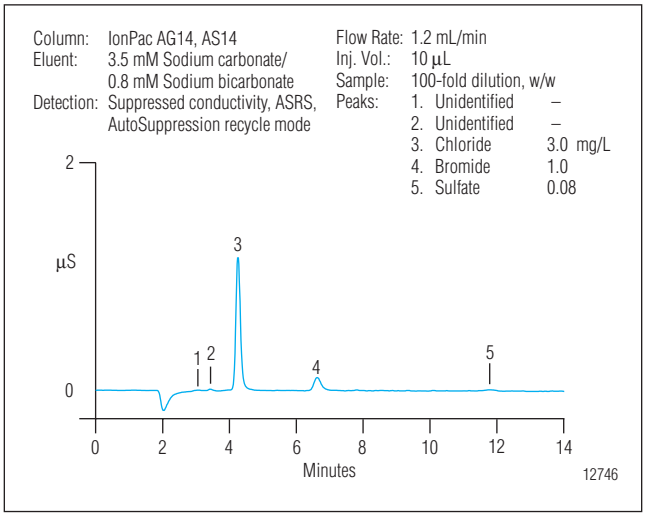

Figure 7. Counterions in multisymptom cold/flu medication.

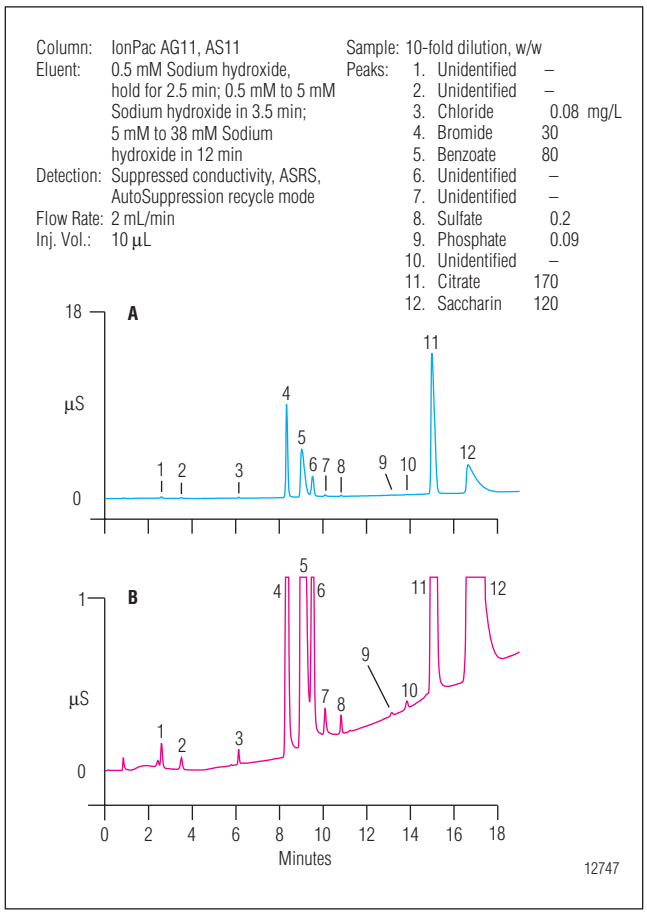

Figure 6. Anionic ingredients in cough suppressant.

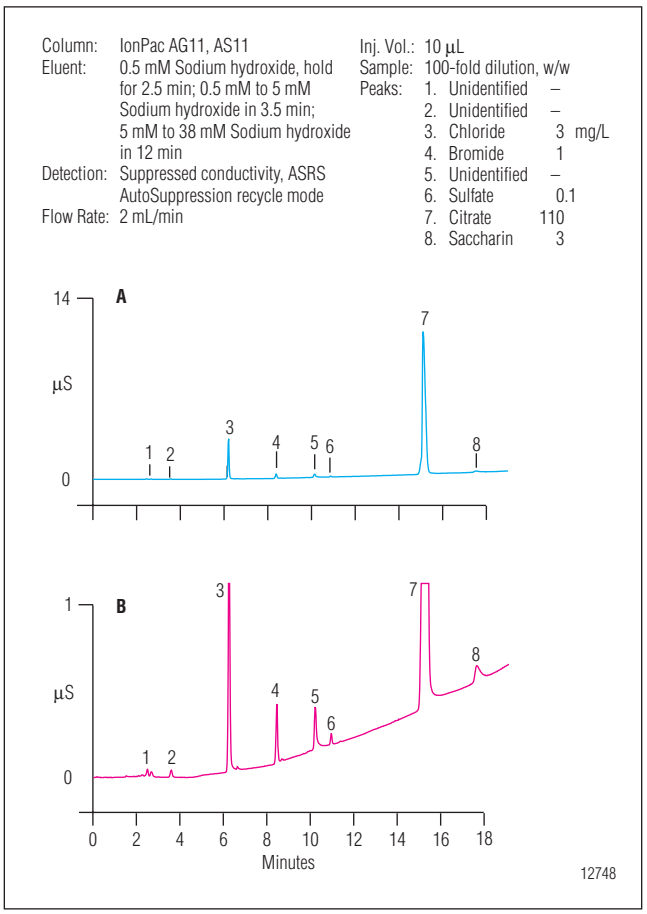

Figure 8. Anionic ingredients in multisymptom cold/flu medication.

CONCLUSION

Using the IonPac AS11 column with suppressed conductivity detection, pharmaceutical formulations can be analyzed for both organic and inorganic anions. In the same injection, the IonPac AS11 resolves common inorganic anions such as fluoride, chloride, bromide, sulfate, nitrate, and phosphate, as well as common organic anions such as benzoate, sorbate, citrate, and saccharin. The IonPac AS14 resolves common inorganic anions, but is not suitable for common organic anions (except acetate). The AS14 column uses an isocratic eluent and is therefore faster for this analysis. Suppressed conductivity eliminates potential interferences from the nonionic ingredients in the formulation and provides a sensitive means to detect nonchromophoric analytes. Inorganic anions can be detected at the 30 to 400-µg/L levels, while organic anions can be detected at the 200 to 2000-µg/L levels. Both organic and inorganic anions were linear over more than two orders of magnitude. The recovery of chloride and bromide from pharmaceutical formulations was greater than 97% of the labeled concentrations for the active ingredients. Both methods are also well suited for evaluating trace levels of anionic contaminants.

REFERENCES

1. CFR Title 21, Foods and Drugs, Chapter 1, FDA, B Part 211.22, "Responsibilities of quality control unit."
2. CFR Title 21, Foods and Drugs, Chapter 1, FDA, I Part 211.160, "General requirements."
3. CFR Title 21, Foods and Drugs, Chapter 1, FDA, I Part 211.165, "Testing and release for distribution."
4. Dionex Corporation, "Ion Chromatography in the Pharmaceutical Industry", Application Note 106.
5. Rabin, S.; Stillian, J.R.; Barreto, V.; Friedman, K.; and Toofan, M. *J. Chromatogr.* **1993**, *640,* 97–109.
6. Dionex Corporation, "Quantification of Carbohydrates and Glycols in Pharmaceuticals", Application Note 117.
7. Dionex Corporation, "Determination of Trifluoroacetic Acid (TFA) in Peptides", Application Note 115.

LIST OF SUPPLIERS

Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennsylvania, 15219-4785, USA. Tel: 1-800-766-7000.

Fluka Chemika-BioChemika, Fluka Chemie AG, Industriestrasse 25, CH-9471 Buchs, Switzerland. Tel: 081 755 25 11.

Aldrich Chemical Company, Inc., 1001 West Saint Paul Avenue, P.O. Box 355, Milwaukee, Wisconsin, 53233, USA. Tel: 1-800-558-9160.

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178, USA. Tel: 1-800-325-3010.

Determination of Inorganic Anions and Organic Acids in Fermentation Broths

INTRODUCTION

Fermentation broths are used in the manufacture of biotherapeutics and many other biologically derived products using recombinant genetic technology. Broths are also used for the production of methanol and ethanol as alternative energy sources to fossil fuels. In addition, many food and beverage products such as alcoholic beverages, vinegars, fermented vegetables, sauces, and dairy products are all prepared by controlled fermentation processes. Fermentation monitoring is also important in detection of spoilage of fruit juices and food products. Recently, attention has been given to characterizing the ingredients of fermentation broths because carbon sources and metabolic by-products can impact the yield of the desired products. Carbohydrates (glucose, lactose, sucrose, maltose, etc.) are carbon sources essential for cell growth and product synthesis, while alcohols (ethanol, methanol, sugar alcohols, etc.), glycols (glycerol), and organic anions (acetate, lactate, formate, etc.) are metabolic by-products, many of which reduce desired yields. Fermentation broths are complex mixtures of nutrients, waste products, cells and cell debris, and desired products, such as antibiotics. Many of these ingredients are nonchromophoric and cannot be detected by absorbance.

Organic and inorganic anions are ionic and therefore can be determined by ion chromatography using suppressed conductivity detection. Suppressed conductivity is a powerful detection technique with a broad linear range and very low detection limits. Nonionic compounds are not detected. Suppression lowers the background conductivity caused by the eluent and effectively increases the conductivity of the analyte.[1, 2] Anion-exchange chromatography is a technique capable of separating complex mixtures of organic acids and inorganic anions. For complex samples like fermentation broths, the high resolving power of ion-exchange chromatography and the specificity of suppressed conductivity allow the determination of ionic fermentation broth ingredients, with little interference from other broth ingredients.[3-5] Although biosensor and flow-injection analysis methods are commonly used to evaluate fermentation broths,[6, 7] these techniques cannot simultaneously determine multiple compounds. Gel permeation chromatography with refractive index detection, and anion-exchange chromatography with UV-VIS detection, have been used for analysis of fermentation broths, but both are limited by poor selectivity and sensitivity.[8, 9] Anion-exchange chromatography with suppressed conductivity monitors, by direct injection, a large number of different compounds simultaneously, using a single instrument and chromatographic method.[10]

This application note describes the use of two different anion-exchange columns, with suppressed conductivity detection, to analyze common organic and inorganic anions in yeast and bacterial fermentation broths. The yeast Saccharomyces cerevisiae in yeast extract-peptone-dextrose (YPD) broth and the bacteria Escherichia coli in Luria-Bertani (LB) broth are common fermentation broth cultures and represent eukaryotic and prokaryotic systems. Both fermentation broth cultures are complex and contain undefined media ingredients, and thus are a great challenge for most separation and detection technologies. These formulations also contain carbohydrates, sugar alcohols, alcohols, and glycols that have been analyzed using the CarboPac™ PA1, PA10, and MA1 anion-exchange columns with pulsed amperometric detection.[11]

In the methods outlined in this application note, the selectivities of the IonPac® AS11 and IonPac AS11-HC anion-exchange columns are compared for the determination of anionic analytes in fermentation broths. The IonPac AS11 column packing consists of an alkanol quaternary ammonium latex bonded to a microporous crosslinked ethylvinylbenzene core. The AS11-HC (high capacity) latex is bonded to a macroporous crosslinked ethylvinylbenzene core. Due to the greater surface area of its core, the AS11-HC has six times more anion-exchange capacity than the AS11. Both columns are designed for separation of organic and inorganic anions using sodium hydroxide gradients. Organic solvents can be added to eluents to modify the selectivity of these columns.

Expected detection limits, linearity, selectivity, stability, and precision for organic and inorganic anions in fermentation broths are reported for the IonPac AS11 and AS11-HC columns using the Dionex DX-500 BioLC® system with suppressed conductivity detection.

EQUIPMENT

Dionex DX-500 BioLC system consisting of:
- GP40 Gradient Pump with degas option
- ED40 Electrochemical Detector
- LC30 or LC25 Chromatography Oven
- AS3500 Autosampler

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Sodium hydroxide, 50% (w/w) (Fisher Scientific and J. T. Baker)

Deionized water, 18 MΩ-cm resistance or higher was used for preparing all standards and eluents. Water that was used to prepare YPD broth was filter sterilized by passage through a 0.2-µm filter.

Standards

Lactic acid (Fisher Scientific)
Succinic acid (Aldrich Chemical Co.)
Pyruvic acid, sodium salt (Fisher Scientific)
dl-Isocitric acid, trisodium salt (Sigma Chemical Co.)
n-Butyric acid, sodium salt (Sigma Chemical Co.)
Sodium formate (Fisher Scientific)
Phenylacetic acid (Sigma Chemical Co.)
Propionic acid, sodium salt (Sigma Chemical Co.)
Maleic acid, disodium salt (Sigma Chemical Co.)
Oxalic acid, sodium salt (Fluka Chemika)
l-Malic acid (Eastman Chemical Co.)
Pyrophosphoric acid (Fluka Chemika)
Trichloroacetic acid (Fluka Chemika)
Chloroacetic acid (Aldrich Chemical Co.)
Glycolic acid (Sigma Chemical Co.)
l-Glutamic acid (Sigma Chemical Co.)
Fumaric acid (Fluka Chemika)
d-Gluconic acid, sodium salt (Sigma Chemical Co.)
Oxalacetic acid (Sigma Chemical Co., and Fluka Chemika)
Methylmalonic acid (Sigma Chemical Co.)
5-Keto-d-Gluconic acid, potassium salt (Sigma Chemical Co.)
2-Keto-d-Gluconic acid, hemicalcium salt (Sigma Chemical Co.)
Valeric acid (Aldrich Chemical Co.)
Isovaleric acid (Sigma Chemical Co.)
Isobutyric acid (Sigma Chemical Co.)
Sodium bromate (Fluka Chemika)
Sodium arsenate, dibasic, 7-hydrate (J.T. Baker Chemical Co.)
Sodium acetate, anhydrous (Fluka Chemika)
Sodium fluoride (Fisher Scientific)
Sodium nitrate (Fisher Scientific)
Sodium chloride (Fisher Scientific)
Potassium phosphate, dibasic, anhydrous (Fisher Scientific)
Citric acid, monohydrate (Fisher Scientific)
Sodium bromide (Aldrich Chemical Co.)
Sodium sulfate, anhydrous (EM Science)
Sodium carbonate, monohydrate (Fisher Scientific)

Culture and Media

Bacto YPD Broth (DIFCO Laboratories, Cat# 0428-17-5)
Bacto Yeast Extract (DIFCO Laboratories, Cat# 0127-15-1)
Bacto Peptone (DIFCO Laboratories, Cat# 0118-15-2)
LB Broth (DIFCO Laboratories, Cat# 0446-17-3)
Yeast, S. cerevisiae; Bakers Yeast type II (Sigma Chemical Co., Cat# 45C-2)
Bacteria, E. coli (donated by SRI International)

Table 1. Chromatographic Conditions

	Conditions	
	System 1	**System 2**
Column:	IonPac AS11 Analytical (P/N 44076) IonPac AG11 Guard (P/N 44078) ATC-1 Anion Trap Column (P/N 37151)	IonPac AS11-HC Analytical (P/N 52960) IonPac AG11-HC Guard (P/N 52962) ATC-1 Anion Trap Column (P/N 37151)
Flow Rate:	2.0 mL/min	1.5 mL/min
Injection Volume:	10 µL	10 µL
Oven Temperature:	Ambient	30 °C
Detection (ED40):	Suppressed conductivity, ASRS®, AutoSuppression® recycle mode, 300 mA	Suppressed conductivity, ASRS, AutoSuppression recycle mode, 300 mA
Eluents:	A: Water B: 5 mM sodium hydroxide C: 100 mM sodium hydroxide	A: Water B: 5 mM sodium hydroxide C: 100 mM sodium hydroxide
Gradient:	0.5–38 mM sodium hydroxide: 0.5 mM sodium hydroxide, hold for 2.5 min; 0.5–5 mM sodium hydroxide in 3.5 min; 5–38 mM sodium hydroxide in 12 min.	1–60 mM sodium hydroxide: 1 mM sodium hydroxide, hold for 8 min; 1–15 mM sodium hydroxide in 10 min; 15–30 mM sodium hydroxide in 10 min. 30–60 mM sodium hydroxide in 10 min; 60 mM sodium hydroxide, hold for 2 min.
Method:	Time (min) / A (%) / B (%) / C (%) 0.0 / 90 / 10 / 0 2.5 / 90 / 10 / 0 6.0 / 0 / 100 / 0 18.0 / 0 / 62 / 38 18.1 / 90 / 10 / 0 25.0 / 90 / 10 / 0	Time (min) / A (%) / B (%) / C (%) 0.0 / 80 / 20 / 0 8.0 / 80 / 20 / 0 18.0 / 85 / 0 / 15 28.0 / 70 / 0 / 30 38.0 / 40 / 0 / 60 40.0 / 40 / 0 / 60 40.1 / 80 / 20 / 0 50.0 / 80 / 20 / 0
Typical Background Conductivity:	0.5 mM sodium hydroxide: 0.5–1 µS 38 mM sodium hydroxide: 2–3 µS	1 mM sodium hydroxide: 0.5–1 µS 60 mM sodium hydroxide: 2–3 µS
Typical System Operating Backpressure:	12.4 Mpa (1800 psi)	15.2 Mpa (2200 psi)

CONDITIONS

See "Conditions" (Table 1).

PREPARATION OF SOLUTIONS AND REAGENTS

Sodium Hydroxide Eluents

5 mM Sodium Hydroxide

It is essential to use deionized water of high resistance (18 MΩ-cm) that is as free of dissolved carbon dioxide as possible. Carbonate is formed in alkaline eluents from carbon dioxide. Carbonate, a divalent anion at high pH, binds strongly to the columns and causes a loss of chromatographic resolution and efficiency. Carbonate can be removed by placing an anion trap column (ATC-1, P/N 37151) between the pump and the injection valve. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide.

Dilute 0.524 mL of 50% (w/w) sodium hydroxide solution into 2000 mL of thoroughly degassed water to yield 5 mM sodium hydroxide. Keep the eluents blanketed under 5–8 psi (34–55 kPa) of helium at all times.

100 mM Sodium Hydroxide

Follow the same precautions described above for the 5 mM sodium hydroxide eluent. Dilute 10.4 mL of 50% (w/w) sodium hydroxide solution into 1990 mL of thoroughly degassed water to yield 100 mM sodium hydroxide. Keep the eluents blanketed under 5–8 psi (34–55 kPa) of helium at all times.

Stock Standards

Solid standards were dissolved in water to 10 g/L anionic concentrations. These were combined and further diluted with water to yield the desired stock mixture concentrations.

The solutions were kept frozen at –20 °C until needed. For determinations of linear range, combine 10-g/L solutions of chloride, bromide, and citrate to make a 1-mg/L standard mix solution. Dilute with water to concentrations of 800, 600, 400, 200, 100, 80, 60, 40, 20, 10, 4, and 1 µg/L. Standard solutions of acetate, bromide, nitrate, sulfate, phosphate, and citrate were also prepared for estimating lower detection limits and linearity at concentrations of 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.08, 0.06, 0.04, 0.02, and 0.01 mg/L. Chloride was prepared at concentrations of 2.4, 1.9, 1.5, 1.0, 0.49, 0.24, 0.19, 0.15, 0.098, 0.048, 0.024, 0.019, 0.014, 0.0097, 0.0048, and 0.0024 mg/L.

SAMPLE PREPARATION

Yeast Fermentation Broth Culture—Standard Media

In a sterile 500-mL Erlenmeyer flask, dissolve 10 g of Bacto YPD Broth (DIFCO Laboratories, Cat# 0428-17-5) in 200 mL filter-sterilized water. Bacto YPD Broth contains 2 g Bacto Yeast Extract, 4 g Bacto Peptone, and 4 g dextrose (glucose) per 10 g. Dissolve 1.0 g yeast (S. cerevisiae; Bakers Yeast type II; Sigma Chemical Co., Cat# 45C-2) in the YPD broth. Cap the flask with a vented rubber stopper. Incubate the culture in a 37 °C shaking water bath (500–600 rpm) for 24 h, removing aliquots at designated time points and placing them on ice. For this study, samples were taken after the addition of yeast at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 24-h intervals. The incubation starts when yeast is added to the medium. Aliquots are centrifuged at 14,000 × g for 10 min and diluted 10- and 100-fold in purified water. Diluted supernatant (10 µL) was analyzed directly.

Heat-inactivated yeast fermentation broth supernatant was spiked with anions for the recovery and stability study. To inactivate the culture, broth supernatant was diluted 10-fold, and heated in boiling water for 10 min. An aliquot of heat-inactivated supernatant was then diluted another 10-fold using 100 µg/mL lactate, acetate, formate, pyruvate, sulfate, oxalate, phosphate, and citrate. The final concentration of each anion was 10 µg/mL. Another aliquot of heat-inactivated yeast culture supernatant was diluted 100-fold in water, serving as an unspiked "blank".

E. Coli Fermentation Broth Culture—Standard Media

LB Broth is dissolved to a concentration of 25 g/L with water, heated to a boil, and autoclaved for 15 minutes at 121 psi. A liter of LB broth contains 10 g of tryptone, 5 g yeast extract, and 10 g of sodium chloride per 25 g. The culture was incubated and sampled as described for the yeast standard media.

RESULTS AND DISCUSSION

Selectivity

IonPac AS11

Figure 1A shows the separation of the common fermentation broth anions using an IonPac AS11 column set with a 0.5–38 mM NaOH gradient (Table 1, System 1) flowing at 2.0 mL/min. The organic and inorganic anions were well-resolved. The analytes were eluted from the column in less than 20 min. The retention times of the anions in Figure 1A are listed in Table 2. In general, monovalent anions eluted first, followed by di- and trivalent anions.

IonPac AS11-HC

Figure 1B shows the analysis of common fermentation broth anions using the IonPac AS11-HC column. Analytes were eluted using a 1–60 mM sodium hydroxide gradient (Table 1, System 2) flowing at 1.5 mL/min. A stronger eluent was needed to elute anions from this column due to its higher capacity. The higher capacity improves resolution of early eluting peaks. For example, lactate and acetate are better resolved on the AS11-HC than the AS11. The elution order of the AS11-HC is similar to the AS11. Table 2 also summarizes the retention times of different anions on the AS11-HC column. These results demonstrate that the AS11-HC column has

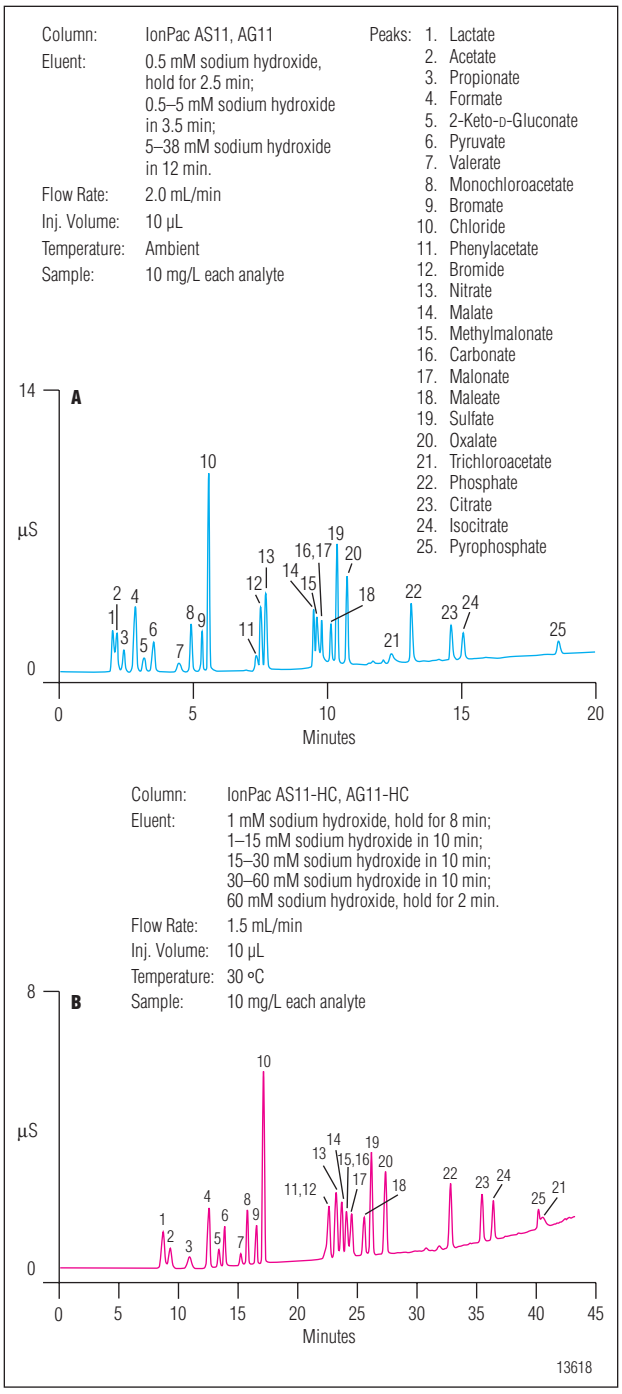

Figure 1. Common organic and inorganic anions found in fermentation broths analyzed on the IonPac AS11 and AS11-HC columns with suppressed conductivity.

Table 2. Retention Times for Common Organic and Inorganic Anions

	Retention Times (Minutes)	
Analyte	**IonPac AS11/AG11**	**IonPac AS11-HC/AG11-HC**
Fluoride	2.3	8.7
Gluconate	2.3	8.2
Lactate	2.5	8.8
Acetate	2.6	9.5
Glycolate	2.6	9.4
Propionate	2.9	11.0
Isobutyrate	3.2	12.3
Formate	3.4	12.4
Butyrate	3.6	12.8
2-Keto-D-Gluconate	4.0	13.1
Pyruvate	4.3	13.5
Isovalerate	4.3	13.7
Valerate	5.1	14.8
Monochloroacetate	5.5	15.3
Bromate	5.8	16.1
Chloride	6.1	16.7
Phenylacetate	8.0	21.7
Bromide	8.2	21.9
5-Keto-D-Gluconate	8.3	20.1
Nitrate	8.4	22.4
Glutarate	N/A	22.5
Succinate	10.1	22.9
Malate	10.1	23.0
Carbonate	N/A	23.5
Methylmalonate	10.2	23.4
Malonate	10.4	23.8
Maleate	10.7	24.9
Sulfate	11.0	25.4
Oxalate	11.4	26.6
Fumarate	11.4	26.8
Oxalacetate	12.2	29.2
Trichloroacetate	13.5	39.0
Phosphate	13.9	31.8
Arsenate	15.1	33.9
Citrate	15.5	34.4
Isocitrate	16.0	35.3
Pyrophosphate	19.7	39.1

N/A - Not available

slightly different selectivity than the AS11. For example, the AS11 column elutes phenylacetate, bromide, and nitrate several minutes before malate; the AS11-HC elutes these compounds much closer to malate. Also, trichloroacetate elutes before phosphate on the AS11, but elutes after pyrophosphate on the AS11-HC. The high capacity of the AS11-HC permits larger sample loads.

Table 3. Estimated Lower Detection Limits (10-µL Injection)

	System 1 AS11	
	ng	µg/L
Acetate	2	200
Chloride	0.5	50
Bromide	4	400
Nitrate	3	300
Sulfate	1	100
Phosphate	4	400
Citrate	4	400

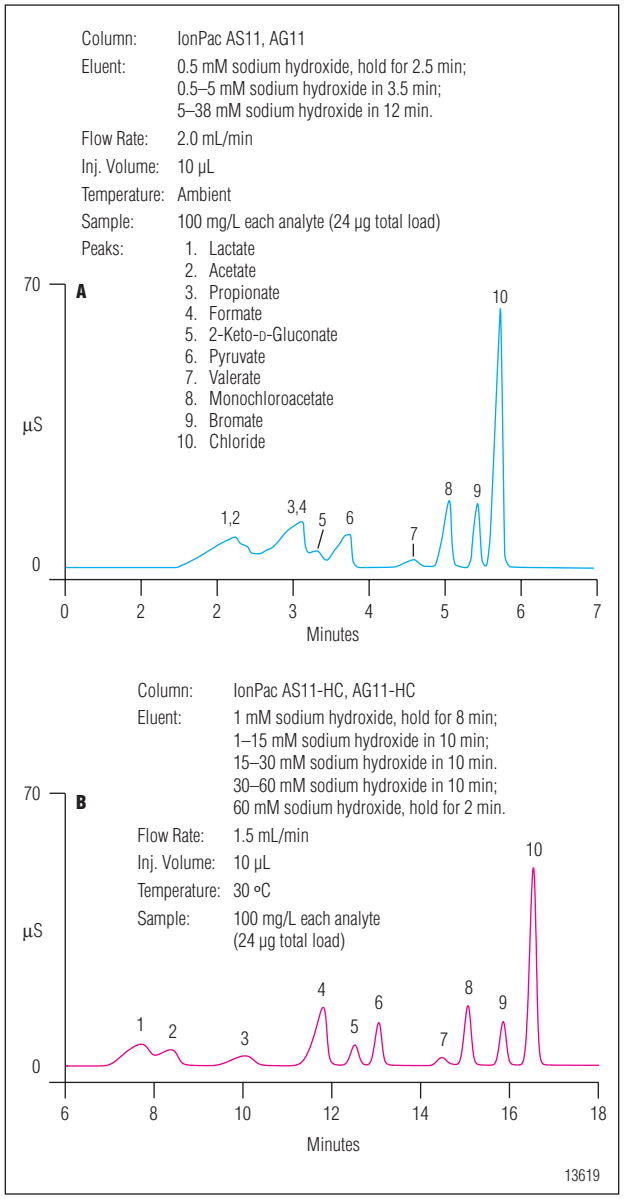

Figure 2. Separation of early eluting organic and inorganic anions at high levels (24 µg total load) using the IonPac AS11 and AS11-HC.

Figure 2A shows early-eluting peaks from the analysis of 24-µg sample of fermentation broth anions analyzed on the AS11, and Figure 2B shows the same analysis on the AS11-HC. At this sample load, the AS11 is overloaded.

Detection Limits

The detection limits for a 10-µL injection of representative fermentation broth anions, in the absence of broth matrix, using the AS11 column, are shown in Table 3. The detection limit is defined as the minimum concentration required to produce a peak height signal-to-noise ratio of 3. The detection limit can be further decreased by increasing the injection volume above the 10-µL injection volume used for this application note. If increasing injection volume also increases sample load beyond the AS11 column capacity, the higher capacity AS11-HC can overcome this limitation. The detection limit can be further decreased by using smoothing algorithms available in PeakNet software and by using external water mode.

Table 4. Peak Area Precision (RSD, %)

Analyte	Last 8 Hours	First 48 Hours	Second 48 Hours	96 Hour Period
Lactate	0.2	0.4	0.3	0.5
Acetate	0.2	0.7	0.4	0.6
Formate	0.1	0.3	0.2	0.4
Pyruvate	0.5	0.5	0.7	0.8
Chloride	0.6	0.5	0.4	0.5
Sulfate	0.3	1.2	1.3	1.3
Oxalate	0.4	0.8	0.5	1.2
Phosphate	0.7	1.6	0.5	1.9
Citrate	0.3	1.8	0.6	2.1

Retention Time Precision (RSD, %)

Analyte	Last 8 Hours	First 48 Hours	Second 48 Hours	96 Hour Period
Lactate	0.1	0.2	0.2	0.3
Acetate	0.1	0.2	0.1	0.3
Formate	0.1	0.2	0.1	0.3
Pyruvate	0.0	0.2	0.1	0.3
Chloride	0.0	0.2	0.1	0.4
Sulfate	0.0	0.3	0.1	0.7
Oxalate	0.0	0.3	0.1	0.7
Phosphate	0.0	0.3	0.1	0.7
Citrate	0.0	0.3	0.0	0.7

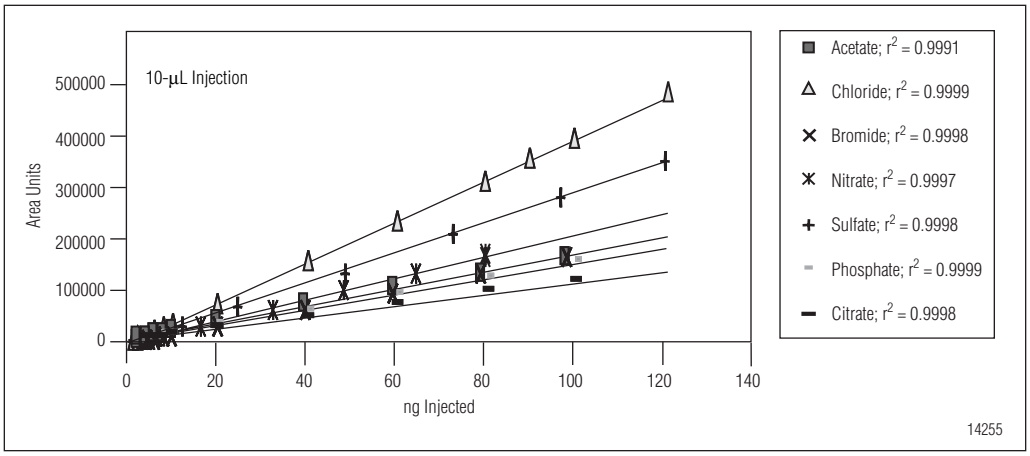

Figure 3. Method linearity for IonPac AS11 with suppressed conductivity detection.

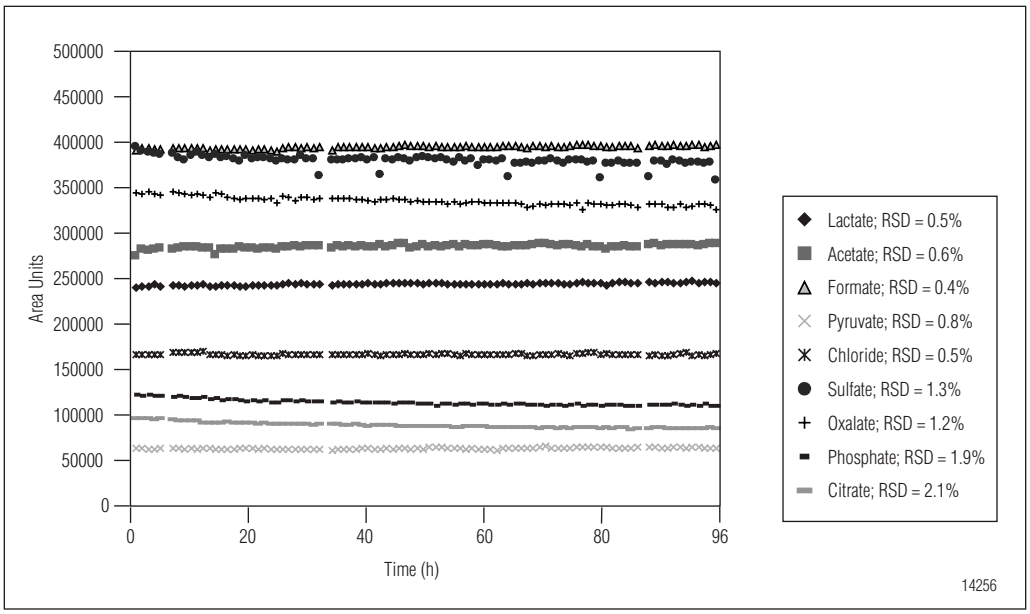

Figure 4. Peak Areas during 4 day repetitive analysis of heat-inactivated yeast fermentation broth.

Table 5. Recovery of Anions in the Yeast Fermentation Broth

Analyte	Percent Recovery
Lactate	100
Acetate	88
Formate	101
Pyruvate	99
Sulfate	101
Phosphate	100
Citrate	84

Linearity

Chloride, bromide, and citrate standards ranging from 1–1000 mg/L (10–10,000 ng) were injected (in triplicate) on the AS11 column. For these analytes, the peak area response was found to be linear over this range ($r^2 \geq 0.999$). Acetate, nitrate, sulfate, and phosphate were investigated over the concentration range of 0.1–12 mg/L (1–120 ng) and showed high linearity ($r^2 \geq 0.999$). Broad linear ranges help reduce the need to repeat sample analyses when components vary greatly in concentration. Representative calibration curves for the AS11 column is presented in Figure 3.

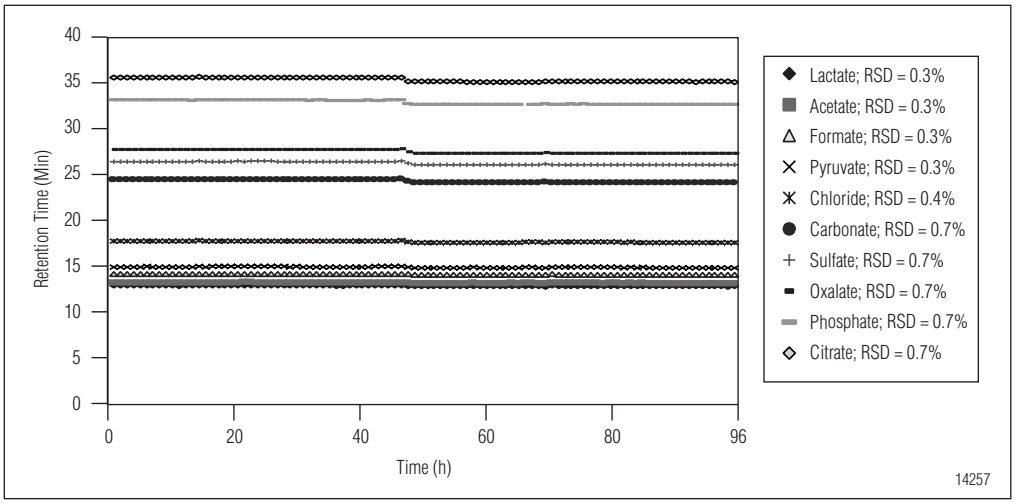

Figure 5. Retention times during 4 day repetitive analysis of heat-inactivated yeast fermentation broth.

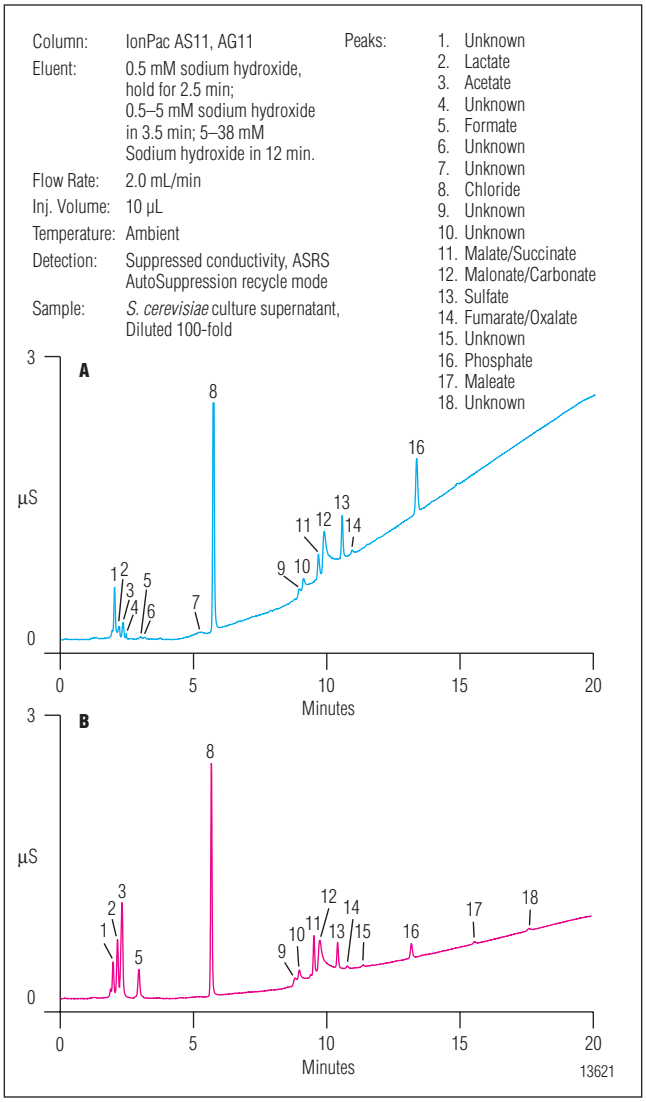

Figure 6. S. cerevisiae fermentation broth culture (100-fold dilution) using the IonPac AS11 column at 0 h (A) and 24 h (B) of incubation.

Precision and Stability

The peak area and retention time RSDs were determined for replicate injections of common anions spiked into yeast fermentation broth. Anion standards were added to heat-inactivated S. cerevisiae fermentation broth culture supernatant to yield 10 mg/L spike concentrations and then analyzed repeatedly for 96 h (10-µL injections) on the AS11-HC column. Statistics for this experiment are presented in Table 4. Figures 4 and 5 show peak areas and retention times for every injection of this experiment. Peak area RSDs were 0.4–2.1% over 96 h. Retention time RSDs ranged from 0.3–0.7%. Retention times shifted slightly at 45 h (Figure 5) when the 100 mM sodium hydroxide eluent was replenished during the study. These results demonstrate that changing eluents can affect retention time precision.

Recovery from Sample Matrix

After correction for endogenous amounts, the measured levels of selected anions spiked into a heat-inactivated yeast fermentation broth culture were compared to their expected levels. These results are presented in Table 5, and show good recovery of anions from the yeast fermentation broth.

Yeast (*S. cerevisiae*) Culture

Yeast were grown in Bacto YPD broth at 37 °C for up to 24 h. Figure 6 shows the separation of fermentation broth ingredients in a yeast culture at the beginning (Figure 6A) and after 24 h (Figure 6B) of incubation. Lactate, acetate/glycolate, formate, valerate, methylmalonate, and citrate increased during the 24-h

Table 6. Anions in Yeast Fermentation Broth During in 24 h Incubation

		Broth Concentration (µg/mL)									
	Incubation Time (h)	0	0.5	1	2	3	4	5	6	7	24
Anions	**Lactate**	59	67	66	70	88	85	84	89	90	338
	Acetate	72	122	153	187	199	222	227	247	235	704
	Propionate	11	10	4	9	4	6	4	7	4	11
	Formate	7	10	11	13	11	14	6	7	6	21
	2-Keto-D-Gluconate	4	9	2	10	0	0	0	11	4	2
	Pyruvate	10	14	19	24	14	16	17	17	17	6
	Valerate	0	0	0	0	0	5	5	4	11	24
	Chloride	348	345	353	320	355	356	357	354	371	347
	Malate	0	7	13	9	8	15	15	12	15	11
	Methylmalonate	100	125	169	180	224	248	247	237	253	229
	Malonate	428	452	569	476	474	547	563	531	577	563
	Sulfate	68	68	79	63	67	65	64	63	61	57
	Oxalate	12	14	14	10	12	13	12	16	15	11
	Phosphate	165	124	92	62	55	57	58	58	59	62
	Citrate	0	0	0	13	15	12	13	13	10	0

incubation. Table 6 lists the measured concentrations of these and other analytes during the 24-h incubation. Between 7 and 24-h, no additional time points were taken; however, substantial increases in the levels of lactate, acetate/glycolate, formate, and valerate occur. Some anions remained constant throughout the 24-h incubation, including chloride, malonate, sulfate, and oxalate/fumarate. Phosphate concentration decreased, presumably due to incorporation into the biomass (e.g., DNA, RNA, membrane phospholipids, etc.). At least 10 unidentified peaks were observed. The area units for eight of these peaks changed over the course of the incubation period. Changes in lactate, acetate, and formate concentrations are expected as a result of normal metabolic processes. Trending can be used to track culture status.

Bacteria (*E. coli*) Culture

Bacteria (*E. coli*) was grown on LB broth for 24 h at 37 ºC. Figure 8A shows the anions present in this broth at the beginning of the culture, and Figure 8B shows anions after 24 h.

To examine anions at lower concentrations, injections of a more concentrated culture are needed and the AS11-HC column is the best choice. Yeast fermentation broth (diluted only 10-fold) was analyzed by both the AS11 and AS11-HC columns, and is presented in Figures 7A and 7B, respectively.
The AS11 column did not resolve the first unknown peak from lactate, while the AS11-HC did. Lactate and acetate were better resolved on the AS11-HC. Butyrate was resolved from formate on the

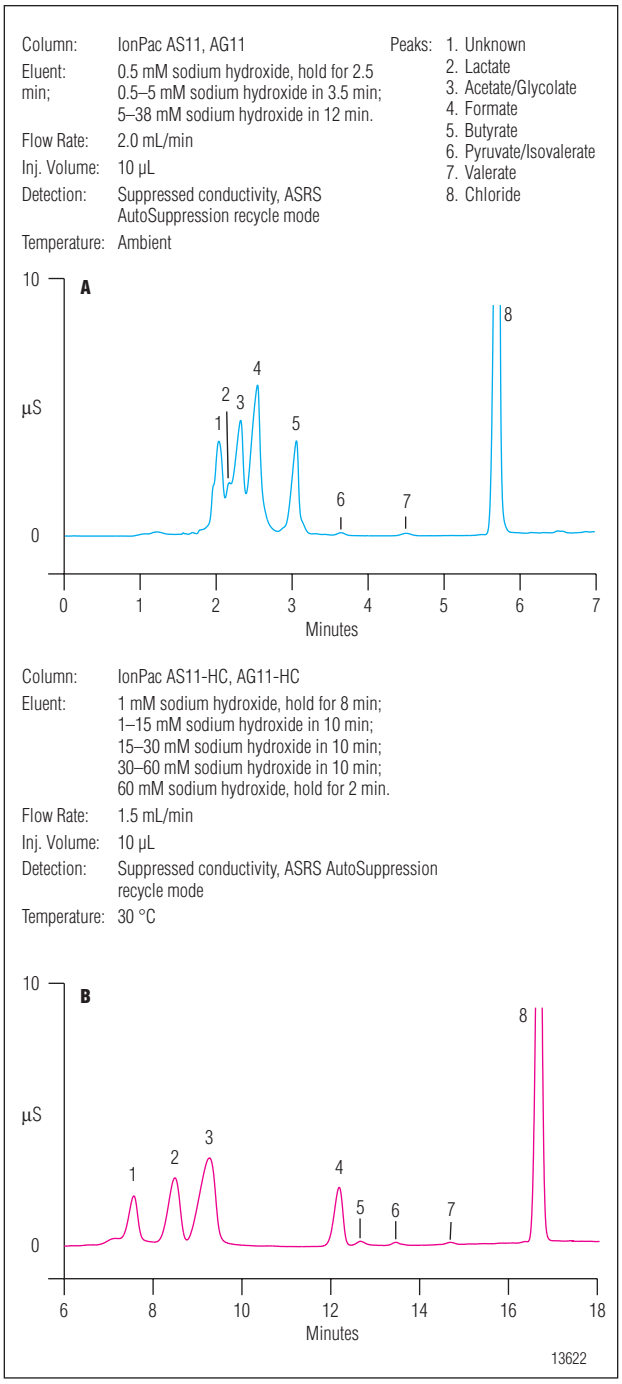

Figure 7. S. cerevisiae *fermentation broth culture (10-fold dilution) using the IonPac AS11 and AS11-HC column at 24 h of incubation.*

AS11-HC column. Furthermore, many of the trace components that could not be measured using a 100-fold dilution could be measured with a 10-fold diluted

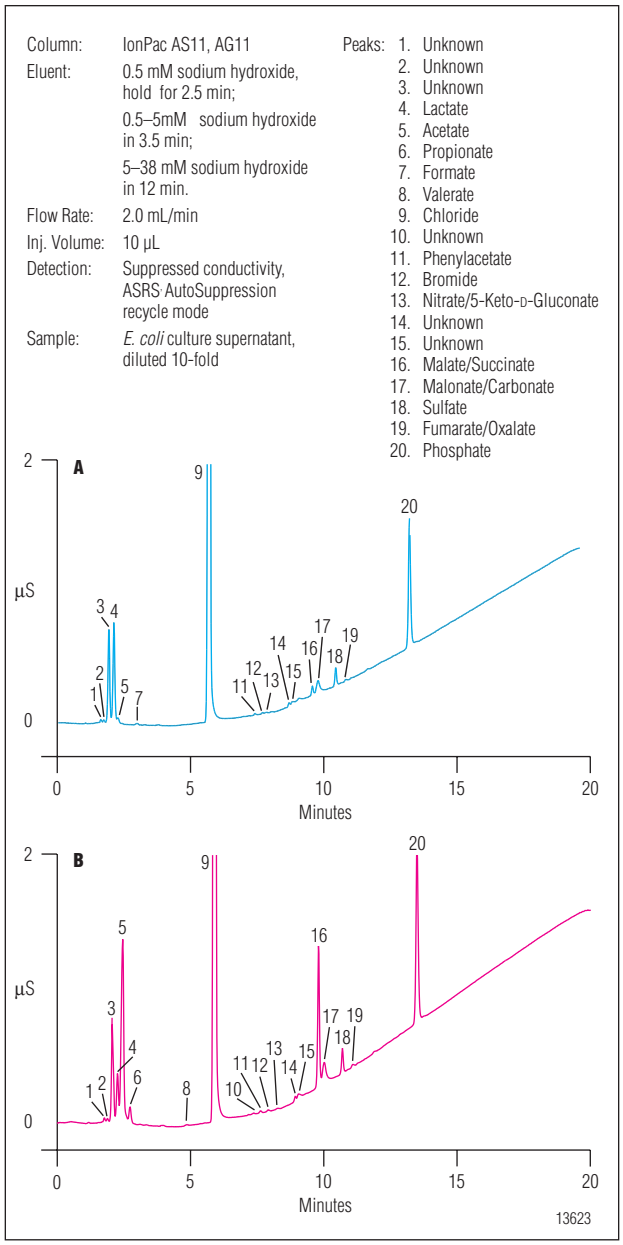

Figure 8. E. coli *fermentation broth culture using the IonPac AS11 column at 0 h (A) of incubation and the AS11 column at 24 h (B) of incubation.*

sample using the AS11-HC column. Concentrations of anions were determined at different time points during incubation. After 24 h, lactate decreased, while acetate increased in concentration. Malate/succinate increased over this period. Chloride remained unchanged. Peaks having retention times equal to propionate and valerate were present after 24 h of incubation.

CONCLUSION

These results show that both yeast and bacterial culture fermentation broths can be analyzed for anion composition using ion chromatography and suppressed conductivity. Two columns (IonPac AS11 and AS11-HC) are available for fermentation broth analysis of organic acids and inorganic anions. The AS11-HC permits higher sample loading due to higher capacity. The high capacity of the column is able to resolve lactate, acetate, and formate. Complex mixtures of organic and inorganic anions can be monitored simultaneously during fermentation, providing the analyst with some of the information needed to optimize the fermentation.

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LIST OF SUPPLIERS

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Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178 USA, Tel.: 1-800-325-3010, www.sigmaaldrich.com

Determination of Residual Trifluoroacetate in Protein Purification Buffers and Peptide Preparations by Reagent-Free Ion Chromatography

INTRODUCTION

A Reagent-Free ion chromatography (RFIC) system allows the determination of anions or cations with only the addition of deionized water. For anion analysis, the RFIC system prepares high-purity, carbonate-free potassium hydroxide eluents. After separation on the anion-exchange column, an anion self-regenerating suppressor automatically suppresses the eluent and the sample anions are detected by suppressed conductivity. RFIC allows rapid method development and easy transfer of methods to other labs. In this application note, we used RFIC to determine the concentration of residual trifluoroacetate (TFA) in samples of interest to the pharmaceutical and biotechnology industries.

TFA is commonly used during the purification of pharmaceutical and biotechnology products. For example, TFA is used with an acetonitrile gradient on a preparative reversed-phase HPLC column to purify synthetic peptides. Because TFA is toxic, its removal must be reliably measured in products intended for preclinical or clinical applications. The high-capacity IonPac® AS18 anion-exchange column was used to separate trace TFA from an excess of chloride, phosphate, and other anions in three different pharmaceutical buffers. The method presented in this application note expands on the work presented in Application Note 115, "Determination of Trifluoroacetate (TFA) in Peptides", that described the use of a carbonate/bicarbonate eluent with the IonPac AS14. This new method, based on the use of RFIC with an IonPac AS18 column, improves the sensitivity of TFA determinations and allows more samples to be analyzed directly.

EQUIPMENT

Dionex Ion Chromatography system (ICS-2000 or ICS-2500) consisting of:

- GP50 Gradient Pump
- CD25A Conductivity Detector
- EG50 Eluent Generator with EluGen® EGC II KOH cartridge (P/N 060585)

AS50 Autosampler with thermal compartment

Columns: IonPac AS18 analytical, 4 × 250 mm (P/N 060549)
IonPac AG18 guard, 4 × 50 mm (P/N 060551)

50-µL sample loop (P/N 42950) or 100-µL sample loop (P/N 42951)

Suppressor: ASRS® ULTRA II, 4 mm (P/N 61561)

CR-ATC (Continuously Regenerated Anion Trap Column) (P/N 060477)

Chromeleon® Chromatography Workstation (Release 6.5 and higher)

REAGENTS AND STANDARDS

Deionized water, Type I reagent-grade, 18 MΩ-cm resistance

Sodium trifluoroacetate (trifluoroacetic acid, sodium salt) (Aldrich P/N 13,210-1)

SAMPLES

Phosphate-Buffered Saline, pH 7.4 (Invitrogen Life Technologies catalog number 10010)

- 1.06 mM potassium phosphate, monobasic (KH_2PO_4)
- 155.17 mM sodium chloride
- 2.96 mM sodium phosphate, dibasic 7-hydrate ($Na_2HPO_4 \cdot 7H_2O$)

Protein Purification Buffer #1[4]

- 0.1 M acetic acid, pH 3
- 0.25 M sodium chloride
- 0.01% Tween® 20
- 1 mg/mL bovine serum albumin (Fluka P/N 05468)

Protein Purification Buffer #2[4]

- 0.1 M Tris, pH 7.4
- 0.14 M sodium chloride
- 0.01 % Tween 20
- 1 mg/mL bovine serum albumin (Fluka P/N 05468)

Commercial Peptide – Human Angiotensin II (Sigma-Aldrich P/N A9525)

- Asp – Arg – Val – Tyr – Ile – His – Pro – Phe, Acetate salt

CONDITIONS

Eluent:	Potassium hydroxide (EG50 as the source)
Temperature:	30 °C
Eluent Flow Rate:	1.0 mL/min
Detection:	Suppressed conductivity, ASRS ULTRA II, recycle mode
ASRS Current Setting:	See method
Expected Background Conductivity:	<1 µS (22 mM KOH)
Typical System Backpressure:	14 MPa (2000 psi) to 17.2 MPa (2500 psi)
Sample Volume:	5–100 µL

SEPARATION METHOD

Time (min)	EG50 Conc (mM)	SRS Current (mA)
0.00	22.0	80
Comments: Load sample loop, 80 mA SRS current setting, Acquisition ON		
6.00	22.0	80
6.01	28.0	80
Comment: Step to 28 mM KOH		
12.00	28.0	80
12.01	50.0	80
Comment: Step to 50 mM KOH for cleanup		
14.00	50.0	124
Comment: Step to 124 mA SRS current		
15.00	50.0	124
15.01	22.0	124
Comment: Step back to 22 mM KOH		
17.00	22.0	80
Comment: Step back to 80 mA SRS current		
20.00	22.0	80
Comment: Acquisition OFF		

PREPARATION OF SOLUTIONS AND REAGENTS

TFA Stock Standard Solution 1000 µg/mL

Dissolve 0.1203 g of sodium trifluoroacetate in deionized water and dilute to 100 mL in a volumetric flask. Dilute this stock standard solution to the desired concentrations.

SYSTEM PREPARATION AND SETUP

This section describes the procedures for the initial installation and start-up of the ASRS ULTRA II, EGC II KOH EluGen cartridge, and CR-ATC. Prepare the ASRS according to the *Quickstart Instructions for the ASRS ULTRA II* (Document No. 031951). Install the EGC II OH EluGen cartridge according to the instructions in the *Operator's Manual for the EG50 Eluent Generator System* (Document Number 031908). Install the CR-ATC between the EGC II KOH cartridge and the degas module in the EG50 according to the *Operator's Manual for the Continuously Regenerated Anion Trap Column* (Document Number 031910).

Connect the columns and suppressor in the IC system by using the black PEEK 0.010-in. (0.25-mm) tubing. Keep the lengths of connecting PEEK tubing as short as possible to minimize the system void volume and thus ensure efficient chromatographic performance. Carefully use a plastic tubing cutter to ensure the tubing cuts have straight, smooth surfaces. Irregularity on the surface of a tubing end can result in unwanted additional dead volume.

SYSTEM OPERATION

Turn on the gradient pump to begin the flow of eluent through the system. If the system backpressure is below 14 MPa (2000 psi), a length of yellow PEEK 0.003-in. (0.075-mm) tubing should be added between the outlet of the degas assembly in the EG50 and the inlet of the injection valve. A system backpressure of 15.9 MPa (2300 psi) is ideal. Confirm that the chromatographic pathway has no leaks. For more information, see the *Operator's Manual for the EG50 Eluent Generator System* (Document Number 031908).

Using the Chromeleon workstation, turn on the EG50 to deliver the highest eluent concentration required by the method. Allow the AS50 thermal compartment to stabilize at 30 °C. Determine the status of the system by measuring the short-term noise. Baseline noise should be less than 5 nS over a period of 5–10 min when measured in 1-min segments. It may take 12 h or more for the system to equilibrate to a stable background conductivity for trace analysis. When performing trace analysis, we recommend running the system overnight to equilibrate for use the following day.

RESULTS AND DISCUSSION

An anion-exchange column for monitoring residual TFA in high-ionic-strength pharmaceutical buffers should ideally have two characteristics. The column should have a sufficient ion-exchange capacity for the high ionic matrix and should separate TFA from the matrix anions that are present at high concentrations. The IonPac AS18 column has both of these characteristics. The 4-mm AS18 set column has an anion-exchange capacity of 285 µeq and is an excellent match for the target application. TFA is well resolved from the early-eluting anions under optimized conditions. Figure 1 shows the AS18 separation of an anion standard that includes TFA.

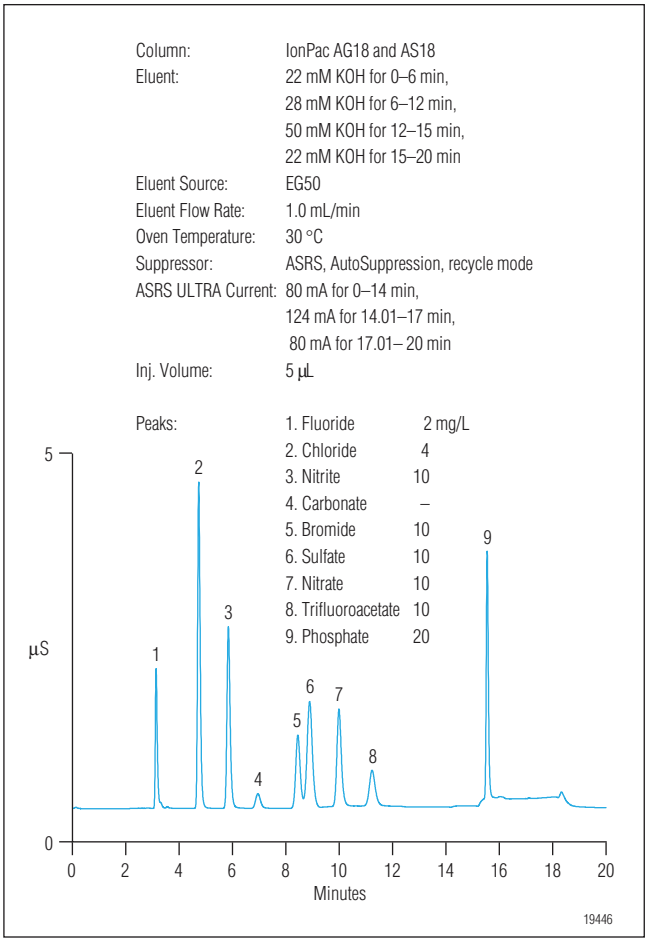

Figure 1. Analysis of anion standard with TFA.

The separation of TFA from the anions in the samples was achieved with a series of eluent concentration step changes. The method begins with an initial eluent concentration of 22 mM KOH to elute weakly retained ions such as fluoride, acetate, and formate. An eluent step change to 28 mM KOH at 6 min separates trifluoroacetate from the other matrix anions such as sulfate and nitrate. The SRS current setting at the time of separation of TFA is set at 80 mA. This current provides the optimum suppression with the least baseline noise. After TFA has eluted, the eluent is step changed to 50 mM KOH at 12 min to clean the column of any highly retained matrix anions such as phosphate. Afterward, the eluent is stepped back to 22 mM KOH to reequilibrate the column for the next injection.

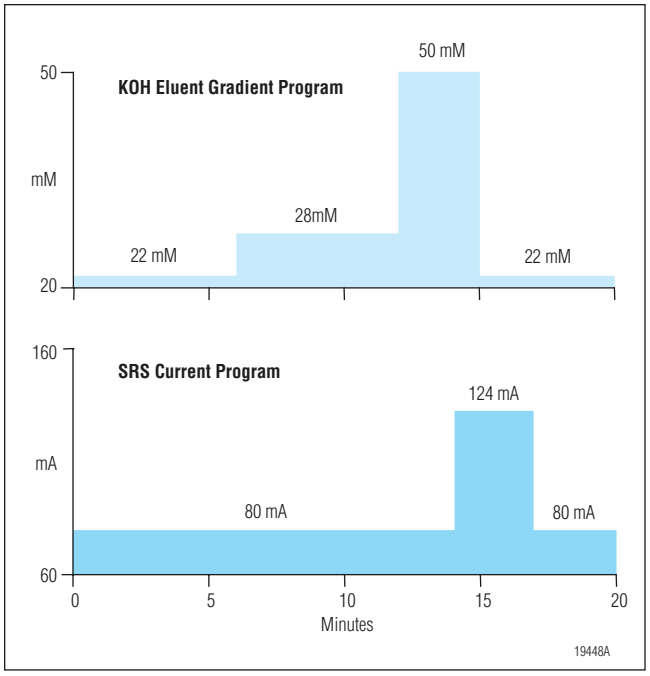

Figure 2. IC method for determination of TFA using the IonPac AS18 and RFIC.

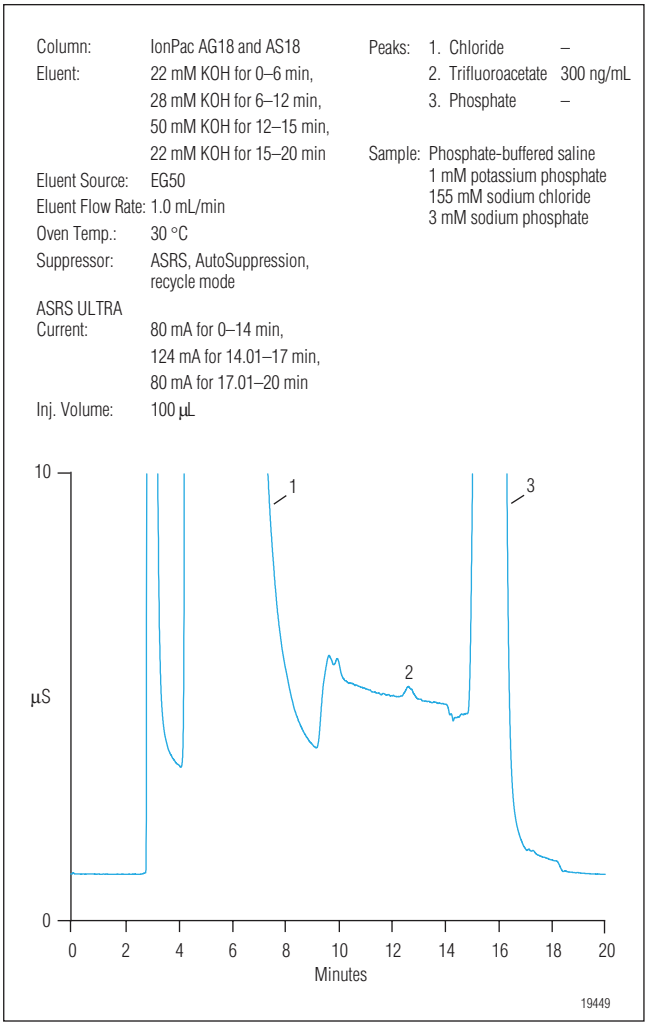

Figure 3. Determination of trace TFA in phosphate-buffered saline using the IonPac AS18 and RFIC.

This optimized eluent step change method was quickly developed using the RFIC system. By simply programming the EG50 Eluent Generator, a number of isocratic methods and a variety of step-gradient eluent methods were quickly evaluated. This evaluation did not require the preparation of different eluents to achieve the ideal proportioning for proper method evaluation. A higher ASRS current setting of 125 mA is applied at 14 min to account for the higher eluent concentration used. A delay of approximately 2 min occurs for the higher eluent concentration to reach the suppressor after the eluent concentration change command is given to the EG50. This delay is mainly due to the column void volume. In contrast, the change in current setting to the ASRS shows an immediate response. By applying the optimal current to the suppressor, it is possible to achieve low noise at every point of the separation. Separations are performed at 30 °C to provide the best retention time reproducibility. Figure 2 illustrates the eluent gradient program and ASRS current program for the method.

This method was applied to the determination of TFA in the following samples: a phosphate-buffered saline (PBS),[1] two buffers used in a recombinant protein recovery process,[4] and a commercial peptide.[2,3] Figure 3 shows the determination of 300 ng/mL of TFA in PBS using a 100-µL injection. This separation is challenging because of the excess presence of matrix components that elute before and after the detection of a trace amount of TFA. We recommend using the detection parameter in Chromeleon software called "Inhibit Integration" to ensure the accurate determination of TFA. Setting the "Inhibit Integration" command to "On" at 0.00 min stops the integration from the beginning of the run. It remains off until the command to turn the "Inhibit Integration" command "Off" is set at 11.0 min. This setting starts integration until "Inhibit Integration" is turned "On" again at 13.5 min. Integration will be inhibited from this point until the end of the run. Because TFA is the only analyte of interest, this detection parameter greatly simplifies detection and data processing. The integration window for stopping and starting detection should be modified according to the specific matrix of interest.

A calibration curve was obtained using TFA standards at 100, 300, and 1000 ng/mL prepared in PBS. Three replicate injections were performed at each concentration level. Results showed that TFA yielded a linear response with a coefficient of determination (r^2) of 0.9979. The method detection limit (MDL) was estimated to be 100 ng/mL TFA in PBS by measuring a TFA peak three times higher than the background noise (S/N = 3). An MDL was calculated using the standard deviation for seven replicate injections of 100 ng/mL TFA in the PBS.[5] TFA was spiked at 100 ng/mL to be in the same concentration range as the estimated MDL and multiplied by the Student's *t* value for the 99.5% confidence limit. The standard deviation was multiplied by the Student's *t* value for the 99.5% confidence limit. A method detection limit for TFA was calculated to be 86 ng/mL in the PBS matrix under these conditions. Recovery of TFA for a 300-ng/mL spike in PBS was 98.7% for (6) replicate injections (293 ± 2.7 ng/mL). The retention time of TFA was 11.7 ± 0.011 min with an RSD of 0.09%. These results compare favorably with the work by Fernando and coworkers using the IonPac AS11-HC column.[1] They reported an MDL of 10 ng/mL for TFA in PBS after reduction of the matrix chloride concentration using an OnGuard® Ag pretreatment cartridge. We report an MDL of 86 ng/mL for TFA in PBS without a sample preparation step.

This method is also applicable to monitoring TFA in the buffers used in a recombinant protein recovery process. Buffers were prepared according to the specification of the manufacturer (see the "Samples" section) with the addition of 1 mg/mL bovine serum albumin to simulate the presence of protein. The method was optimized using a 50-µL injection to give the best sensitivity for the determination of TFA in these two buffers. Recovery of TFA for a 300-ng/mL spike in this buffer was 98% for five replicate injections (296 ± 19 ng/mL) based on a calibration curve prepared in the matrix. The retention time of TFA was 12.7 ± 0.021 min with an RSD of 0.17%. Kabakoff and coworkers reported an MDL of 300 ng/mL for TFA in this sample with a 10-µL injection using a 4 × 250 mm IonPac AS14 column (65 µeq/column capacity) with a carbonate-based eluent.[4] By using a higher injection volume (50 µL) with the higher-capacity IonPac AS18 column, we were able to achieve an MDL of 36 ng/mL for TFA. Figures 4 and 5 show chromatograms for a 300 ng/mL TFA spike in both of these buffers. Linearity for TFA in these two buffers yielded coefficients of determination (r^2) greater than 0.999. Recovery of TFA for a 50 ppb spike in Protein Buffer #2 was 115% for seven replicate injections.

Column:	IonPac AG18 and AS18
Eluent:	22 mM KOH for 0–6 min, 28 mM KOH for 6–12 min, 50 mM KOH for 12–15 min, 22 mM KOH for 15–20 min
Eluent Source:	EG50
Eluent Flow Rate:	1.0 mL/min
Oven Temperature:	30 °C
Suppressor:	ASRS, AutoSuppression, recycle mode
ASRS ULTRA Current:	80 mA for 0–14 min, 124 mA from 14.01–17 min, 80 mA for 17.01–20 min
Inj. Volume:	50 µL
Peaks:	1. Acetate/chloride 2. Trifluoroacetate 300 ng/mL
Sample:	Protein Buffer #1 0.1 M acetic acid 0.25 M sodium chloride 0.01% Tween 20, pH3 1 mg/mL bovine serum albumin

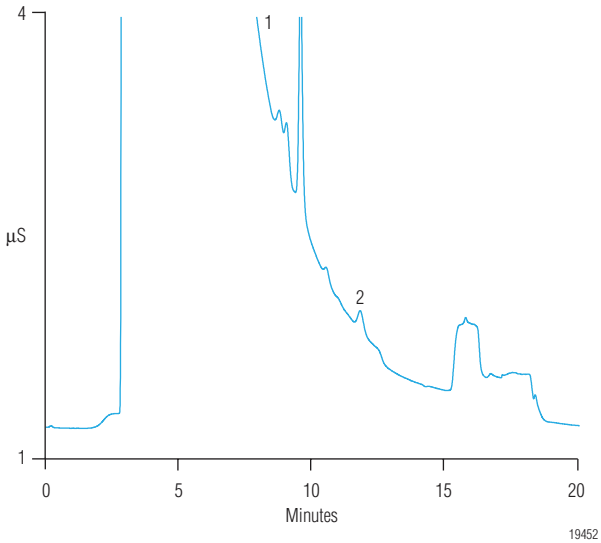

Figure 4. Determination of trace TFA in protein buffer #1.

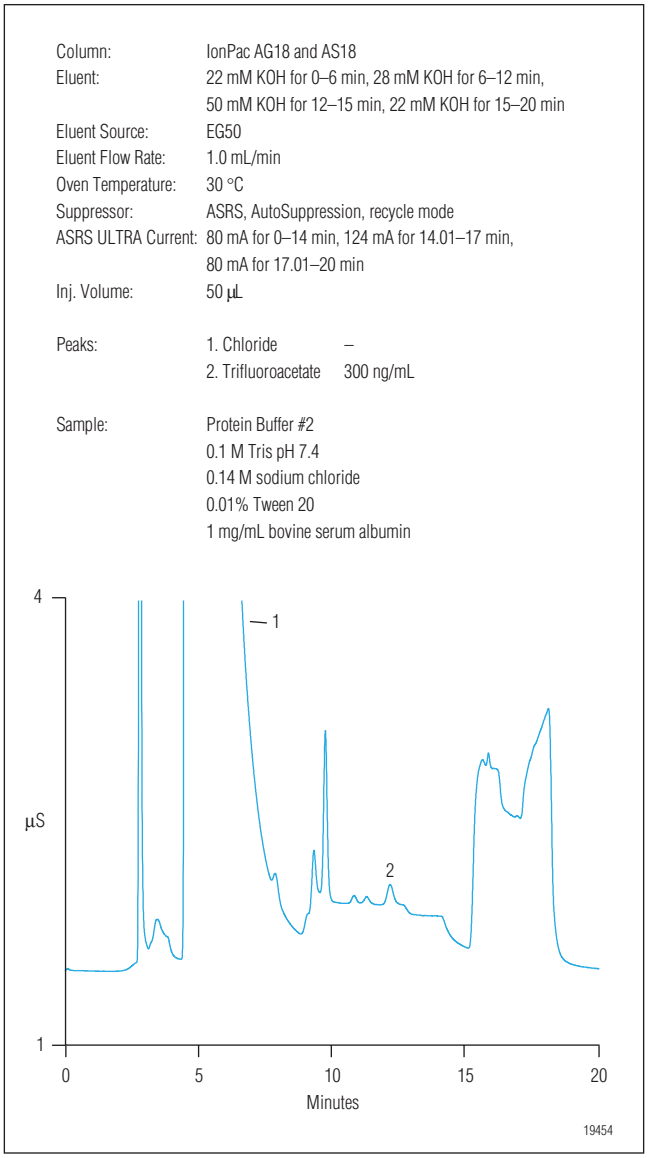

Figure 5. Determination of trace TFA in protein buffer #2.

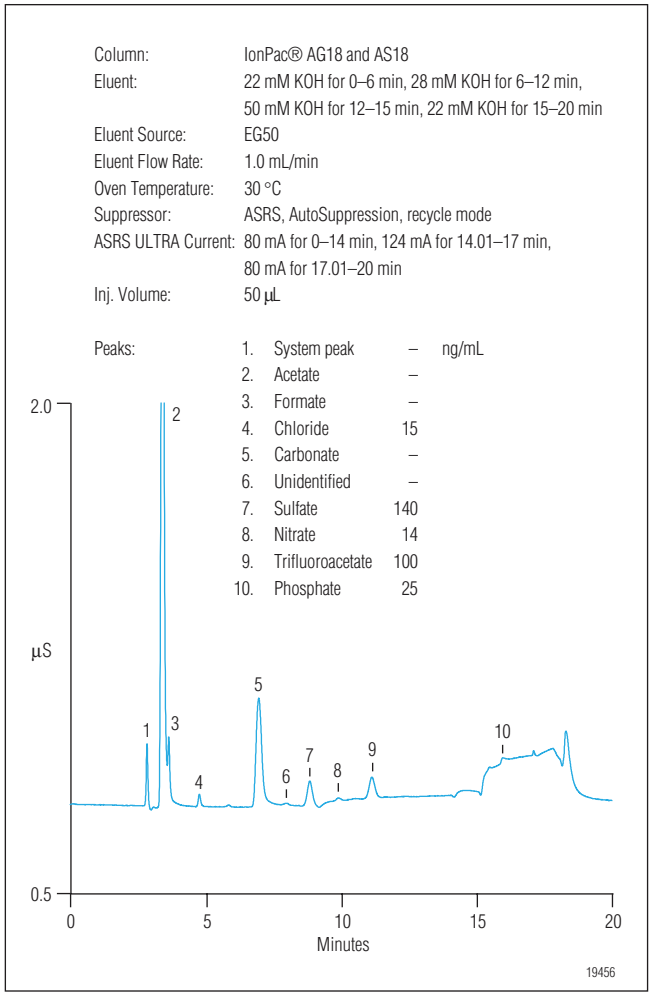

Figure 6. Determination of trace TFA in a commercial peptide: 40 µg/mL angiotensin II spiked with 100 ng/mL TFA.

This method was also applied to the determination of TFA in a commercial peptide. A solution of human angiotensin II protein was prepared at 40 µg/mL in deionized water with and without a spike of 100 ng/mL of TFA. Both solutions were analyzed using the method developed in this study. No TFA was detected in the peptide preparation. Figure 6 shows the peptide solution spiked with 100 ng/mL of TFA. Because this peptide was prepared as an acetate salt, a large acetate peak appears. Trace amounts of chloride and sulfate were also detected. A 50 ng/mL spike of TFA was completely recovered (101% for n = 7), demonstrating that the method is valid for determining TFA in this sample. Table 1 summarizes the calibration results and calculated method detection limits in the human angiotensin II protein solution, including the three different buffers.

Table 1. Calibration Results and Calculated MDLs in the Human Angiostensin II Protein Solution Including the Three Different Buffers					
Matrix	**Data points****	**r^2**	**Dynamic Range (ng/mL)**	**Method Detection Limit (MDL)* (ng/mL)**	**Standard Used to Calculate MDL (ng/mL)**
Phosphate Buffered Saline	9	0.9979	100–1000	86	100
Protein Buffer #1	9	0.9997	100–1000	36	100
Protein Buffer #2	9	0.9986	100–1000	15	30
Commercial Peptide	9	0.9997	30–300	4	10

* MDL = (S.D.) × (t_s) 99.5%, where (t_s) is for a 99.5% single-sided Student's *t* test distribution for n = 7

** Three concentrations injected in triplicate

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SUPPLIERS

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VWR Scientific Products, 1310 Goshen Parkway, West Chester, PA 19380 USA, Tel: 800-932-5000, www.vwr.com.

Assay for Citrate and Phosphate in Pharmaceutical Formulations Using Ion Chromatography

INTRODUCTION

Citric acid is a common ingredient found in many pharmaceutical formulations. The most common use of citric acid in pharmaceuticals is the effervescent effect it produces when combined with carbonates or bicarbonates in antacids and dentifrices. Citrate is also widely used as a flavoring and stabilizing agent in pharmaceutical preparations to mask the taste of medicinal flavors. Citric acid can act as a buffering agent and assist in the dispersion of suspensions to help maintain stability of the active ingredients[1] and improve the effectiveness of antioxidants.[2] It may also be used as an anticoagulant to preserve blood for transfusion and as an ingredient of rectal enemas.[2]

The United States Pharmacopeia (USP) has adopted several different assays for citrate in various pharmaceutical dosage forms. These analytical techniques include calorimetry, gravimetry, ion-exclusion chromatography, and reversed-phase liquid chromatography.[3] Method variation is usually required for many of these techniques to assay a specific dosage form. This method variation results in the use of different color-forming reagents, mobile phases, columns, and detectors. For instance, a dosage form containing citrate and phosphate requires the use of pyridine and acetic anhydride for the determination of citrate, and ammonium molybdate, hydroquinone, and sodium sulfite for a separate determination of phosphate. The prescribed assays are time consuming, labor intensive, require extensive analyst training, and may yield significant measurement errors.

Citrate has been successfully separated by ion-exchange,[4,5] ion-exclusion,[6,7] and reversed-phase[8] liquid chromatography in a wide range of sample matrices, including those of pharmaceutical and biological origin. The most common reported detection of these separations is indirect UV absorbance; however, conductivity and refractive index detection have also been used. Separation of citric acid by reversed-phase liquid chromatography requires a low mobile phase pH to inhibit the ionization of citric acid.[8] Furthermore, ion-exclusion separations generally have long retention times for citric acid unless an organic modifier is used.[7] Because citrate is a very poor absorbing analyte, a mobile phase with a strong UV-absorbing chromophore is required for indirect UV detection.[9] Chalgari and Tan described a citrate assay for some pharmaceutical dosage forms with USP monographs that uses ion chromatography (IC) with indirect photometric detection.[10] This method used trimesic acid, a UV-absorbing eluent, as the mobile phase to detect citrate as a negative peak at a wavelength of 280 nm. However, the method required proper pH adjustment of the mobile phase with NaOH to produce consistent retention times. The retention time of citric acid decreases as its ionization decreases at low pH values (pH 3.2–4.5) and increase at higher pH values (pH 4.5–6.0) as ionization increases.[11]

IC with suppressed conductivity detection is the chromatographic technique of choice for citrate determinations.[12] In addition, IC can simultaneously determine phosphate and other anions that are present in some pharmaceutical formulations and uses eluents that do not require expensive reagents or pH adjustments. Aliphatic carboxylic acids, such as citric acid, generally exhibit high affinities for anion-exchange stationary phases. Thus, low-ionic-strength carbonate/bicarbonate buffer solutions are typically not suitable as eluents. However, when hydroxide eluents are used, citric acid can be easily eluted from the column.[13]

In this application note, we report on the validation of an IC method for the determination of phosphate and total citric acid in pharmaceutical formulations with a hydroxide-selective, anion-exchange column and suppressed conductivity detection. The method incorporates an electrolytic eluent generator to automatically produce a simple isocratic potassium hydroxide eluent, allowing the separation of phosphate and citrate on an IonPac® AS11 column in less than 10 min. The results indicate that this method can replace 18 USP monographs for the assay of citric acid or phosphate in *USP 27-NF 22*. The method was evaluated in terms of linearity, precision, accuracy, ruggedness, and limit of quantitation for phosphate and citrate.

EQUIPMENT

A Dionex ICS-2000 Reagent-Free™ Ion Chromatography (RFIC) System was used in this work. The ICS-2000 is an integrated ion chromatograph and consists of:

- Eluent generator
- Column heater
- Pump with degas
- EluGen® EGC II KOH Cartridge (Dionex P/N 058900)
- CR-ATC (Dionex P/N 060477)

AS50 Autosampler

Chromeleon® 6.5 Chromatography Workstation

This application note is also applicable to other RFIC systems.

REAGENTS AND STANDARDS

Deionized water, Type I reagent-grade, 18 MΩ-cm resistivity or better

Citric acid (USP, Catalog #1134368)

Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4 \cdot H_2O$ (EM Science)

Calcium chloride dihydrate, $CaCl \cdot 2H_2O$ (Fisher Scientific)

Sodium acetate anhydrous (Fluka Chemical Co.)

Sodium chloride (J. T. Baker)

Magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$ (Sigma-Aldrich)

Sodium citrate dihydrate (Sigma-Aldrich)

Potassium chloride (Sigma-Aldrich)

Sodium hydroxide, 50% (J. T. Baker)

CONDITIONS

Columns:	IonPac AS11 Analytical, 4 × 250 mm (Dionex P/N 044076) IonPac AG11 Guard, 4 × 50 mm (Dionex P/N 044078)
Eluent:	20 mM potassium hydroxide
Eluent Source:	ICS-2000 EG with CR-ATC
Flow Rate:	2.0 mL/min
Temperature:	30 °C
Injection:	10 µL
Detection:	Suppressed conductivity, ASRS® ULTRA II, 4 mm (Dionex P/N 061561) AutoSuppression® recycle mode 100 mA current
System Backpressure:	~2300 psi
Run Time:	10 min

PREPARATION OF SOLUTIONS AND STANDARDS

Eluent Solution

Generate 20 mM KOH eluent on-line by pumping deionized (DI) water through the ICS-2000 EG device. Set the eluent concentration using Chromeleon software or from the front LCD panel of the ICS-2000. Chromeleon Chromatography Management Software tracks the amount of KOH used and calculates the remaining lifetime of the EGC II KOH cartridge.

Alternatively, prepare 20 mM NaOH by pipetting 1.05 mL of 50% (w/w) aqueous NaOH from the reagent bottle into a 1.00-L volumetric flask containing about 500 mL of degassed DI water. Bring to volume with degassed DI water, mix, and degas by sparging with helium or sonicating under vacuum for 10 min. Atmospheric carbon dioxide readily dissolves in dilute basic solutions, forming carbonate. Carbonate contamination of eluents can affect analyte retention times, resulting in performance that is not equivalent to electrolytically producing the hydroxide eluent on-line using an eluent generator. Store the eluent in plastic labware. Maintain an inert helium atmosphere of 3–5 psi in the eluent reservoir to minimize carbonate contamination.

Stock Standard Solutions

An official USP citric acid reference standard was dried in an oven at 105 °C for 2 h immediately before use. To prepare a 500-mg/L citric acid stock standard, weigh exactly 250 mg of the dried citric acid, add to a 500-mL volumetric flask, and dilute to volume with DI water. To prepare a mixed citrate/phosphate stock standard with 300 mg/L phosphate (as $NaH_2PO_4 \cdot H_2O$), weigh 150 mg $NaH_2PO_4 \cdot H_2O$, add to a 500-mL volumetric flask containing 250 mg citric acid, and dilute to the mark with DI water. Store in polypropylene bottles at 4 °C.

Working Standard Solutions

Prepare working standard solutions at lower concentrations by adding an appropriate amount from the stock standard solutions and 5 mL of 20 mM NaOH to a 100-mL volumetric flask. Dilute to the mark with DI water. The 20 mM NaOH solution used for standard and sample preparation should be prepared fresh daily.

SAMPLE PREPARATION

All liquid samples should be appropriately diluted with DI water so that the concentration of citrate and phosphate fit within the calibration range. For solid citrate samples, such as potassium citrate extended-release tablets that contain insoluble components, a portion equivalent to ~100 mg citric acid (powdered form) was added to 300 mL of hot DI water (~80 °C) and magnetically stirred for ~30 min while maintaining the temperature between 70–80 °C. The solution was allowed to cool and then transferred to a 500-mL volumetric flask and diluted to volume with DI water to prepare the sample stock solution. For completely soluble solid samples containing citrate (e.g., effervescent tablets), a finely ground portion equivalent to ~100 mg citric acid should be added to 300 mL of DI water in a 500-mL volumetric flask and diluted to the mark to prepare the sample stock solution. In this study, most samples were diluted 1000-fold for citrate determinations and approximately 200-fold for phosphate determinations.

SYSTEM PREPARATION AND SETUP

Prepare the ASRS ULTRA II for use by hydrating the suppressor. Use a disposable plastic syringe and push approximately 3 mL of degassed DI water through the "Eluent Out" port and 5 mL of degassed DI water through the "Regen In" port. Allow the suppressor to sit for approximately 20 min to fully hydrate the suppressor screens and membranes. Install the ASRS ULTRA II for use in the recycle mode according to the *Installation and Troubleshooting Instructions for the ASRS ULTRA II* (Document No. 031956).

Install the EGC II KOH cartridge in the ICS-2000 and configure it with Chromeleon chromatography software. Condition the cartridge as directed by the *EGC II Quickstart* (Document No. 031909) for 30 min with 50 mM KOH at 1 mL/min. Install a 4 × 50 mm IonPac AG11 and 4 × 250 mm IonPac AS11 column. Make sure that the system pressure displayed by the pump is at an optimal pressure of ~2300 psi when 20 mM KOH is delivered at 2.0 mL/min so the degas assembly can effectively remove hydrolysis gas from the eluent. If necessary, install additional backpressure tubing supplied with the ICS-2000 shipping kit to adjust the pressure to 2300 ± 200 psi. Because the system pressure can rise over time, trim the backpressure coil as necessary to maintain a system pressure between 2100–2500 psi.

RESULTS AND DISCUSSION

In general, highly charged analytes, such as citrate, are difficult to elute from most anion-exchange columns without using a concentrated eluent. To reduce the elution time, the eluent anion should have a high selectivity for the resin. Therefore, an anion-exchange column with a high selectivity toward hydroxide eluent, in combination with a low anion-exchange capacity of 45 µeq/column, was chosen to assay for citric acid. This column allows the separation of a wide range of inorganic and organic anions, including polyvalent ions, such as citrate, using a low to moderate eluent concentration. In addition, a hydroxide eluent has the following advantages: (1) it is readily available, (2) it can be electrolytically generated at an appropriate concentration, and (3) it is suppressed to water to yield an exceptionally low background conductance and lower noise, therefore improving the limits of detection and quantitation. An electrolytically generated potassium hydroxide eluent was used for the separation of phosphate and citrate in different pharmaceutical formulations to increase method automation and allow easy method transfer between laboratories. The assay was evaluated in terms of linearity, limit of quantitation, specificity, precision, accuracy, and ruggedness.

All calibration standards used in this assay were prepared in 1 mM NaOH. A total of 12 calibration data sets were acquired using either combined citric acid and phosphate standards or standards containing only citric acid. A calibration curve was generated with citrate in the range of 2–100 mg/L using seven concentration levels for the combined standard to assay formulations containing citrate and phosphate, and in the range of 5–70 mg/L using five concentration levels for assay of dosage forms containing only citrate. A calibration curve was generated with phosphate in the range of 1.2–60 mg/L. Each calibration was linear over the specified range using a least-squares regression curve with correlation coefficient (r^2) of 0.9990 or better. Figure 1A shows a chromatogram of a combined phosphate and citrate standard separated on an IonPac AS11 and Figure 1B shows the same analytes analyzed in an anticoagulant citrate, phosphate, dextrose, and adenine dosage form.

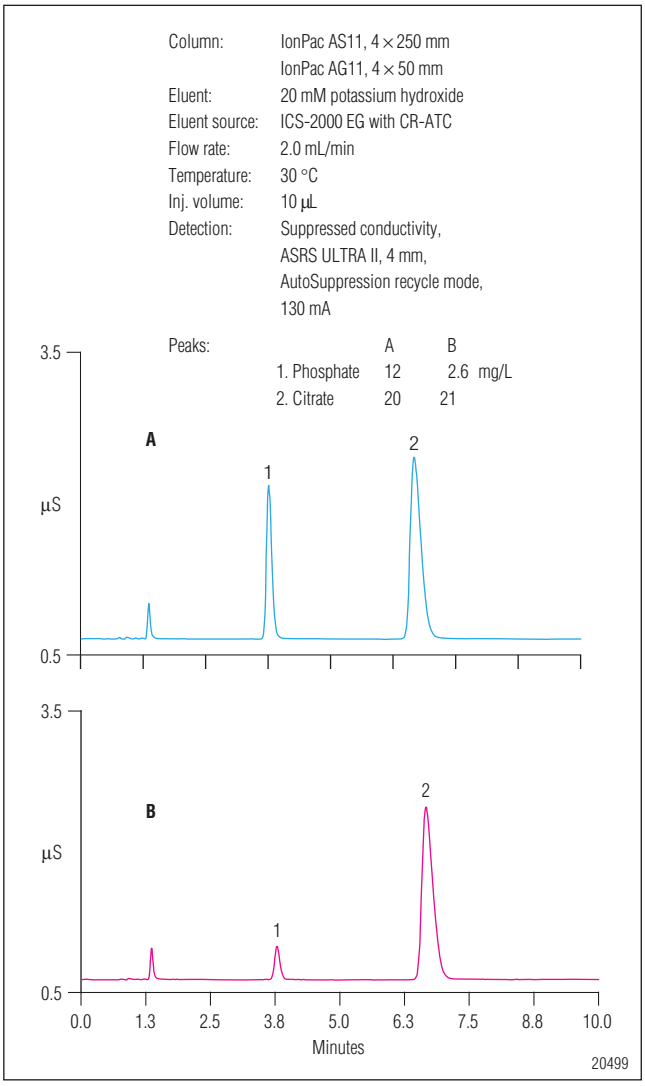

Figure 1. Separation of phosphate and citrate on the IonPac AS11 with (A) standard and (B) assay for citrate in an anticoagulant citrate, phosphate, dextrose, and adenine dosage form.

The USP compendial method for validation <1225> specifies a signal-to-noise (S/N) ratio of 10 for the determination of the limit of quantitation (LOQ).[14] The baseline noise was determined by measuring the peak-to-peak noise in a representative 1-min segment of the baseline where no peaks elute. Typical baseline noise for this method using the ASRS ULTRA II suppressor in the recycle mode is ~2 nS/min. The determined LOQ for phosphate and citric acid over three consecutive days was approximately 0.2 mg/L (S/N = 10). The method validation did not require a determination of the detection limit. Table 1 summarizes the calibration and LOQ data.

Table 1. Summary of Calibration and Limit of Quantitation Data for Citrate and Phosphate

Analyte	Concentration Range (mg/L)	Coefficient of Determination Range[a] (r^2)	LOQ[a] (mg/L)
Citrate	2–100	0.9993–0.9994	0.20
Citrate	5–70	0.9990–0.9998	0.20
Phosphate	1.2–60	0.9999	0.20

[a] For three independent calibrations (3 days)

Method performance was measured in terms of the precision of replicate sample injections and recovery of spiked samples. The relative standard deviations (RSDs) of the measured peak areas were calculated for the target analytes prepared at a concentration of ~20 mg/L citrate and 12 mg/L phosphate. The intraday precision (i.e., a sequence of consecutive injections) for citrate and phosphate from independently prepared solutions analyzed on three separate days was <1% for citrate and <0.5% for phosphate on each day. The interday (i.e., day to day) precision for a three-day period (i.e., three independent sample preparations) for citrate was <2% and for phosphate <1%. Recoveries were determined by adding known amounts of analyte to the sample solutions. The calculated recoveries were within 95–105% for all samples. Table 2 summarizes the precision and recovery data for citrate and phosphate.

Table 2. Accuracy and Precision for Citrate and Phosphate in Pharmaceutical Formulations

Analyte	Intraday Precision Range (% RSD)	Interday Precision Range (% RSD)	Range of Recoveries (%)
Citrate	0.16–0.91[a]	0.49–1.94[a]	95.3–105.1[c]
Phosphate	0.19–0.49[b]	0.41–0.51[b]	94.8–104.8[d]

[a] Precision range for eight samples analyzed on three separate days from independently prepared solutions containing citric acid

[b] Precision range for two samples analyzed on three separate days from independently prepared solutions containing phosphate

[c] Added 1–2.5 mg/L citric acid to nine samples prepared at 100% of the target concentration

[d] Added 0.6–1.5 mg/L phosphate to two samples prepared at 100% of the target concentration

The USP defines ruggedness as a measure of the reproducibility of the method obtained by the analysis of the same samples under a variety of conditions. Ruggedness is typically expressed as the lack of influence on the assay results under different conditions that would normally be expected from laboratory to laboratory and from analyst to analyst when operating under the specified method parameters. We evaluated ruggedness of the method by using analysts, instruments, batch lots of the same column, and eluent preparation procedures as variables. The precision was determined by using the average measured concentrations (based on duplicate injections) and calculating the RSDs for the separate assays. Table 3 shows the precisions for each variable tested for both an individual analyst and for both analysts. For analyst A, the RSDs ranged from 0.17–2.00% compared to 0.31–2.60% for analyst B. The greatest total RSD for both analysts was ~2.40%. Based on these data, the method was found to be rugged in terms of the variables investigated.

Table 4 summarizes the results from the assay of nine different pharmaceutical formulations for citric acid and phosphate (if present). The same samples were analyzed on three consecutive days using independently prepared standards and diluted dosage solutions. The calculated concentrations of these samples measured on separate days was consistent with a maximum difference of ~2% from day to day. In most cases, the measured values were very close to the label amounts and within the specifications according to their respective *USP 27-NF 22* monographs. However, the assay for phosphate in the anticoagulant solution and the assay for citrate in the oral rehydration solution were outside the specifications established by the USP. The amounts stated on the labels are based on the average from 20 to 25 sample containers. In this applications note, the values are based on the assay of one or two bottles. Also, the methods used to determine the label values are based on current USP procedures that are significantly different than the method presented in this application note. Therefore, the difference in the formulation label values and the experimental values may be due to method variation, variation in the precision of the current USP methods, and the differences in sample size used to establish the label and experimental values.

Table 3. Summary of Results from the Ruggedness Study				
Sample	**Analyte**	**Analyst A (% RSD)**	**Analyst B (% RSD)**	**Total (% RSD)**
Anticoagulant citrate phosphate dextrose adenine solution for citrate assay	Citrate	0.17	0.31	1.51
Anticoagulant citrate phosphate dextrose adenine solution for phosphate assay	Phosphate	2.00	2.60	2.17
Citric acid, magnesium oxide, sodium carbonate irrigation solution	Citrate	0.93	1.60	2.39
Potassium citrate extended release tablets	Citrate	1.65	0.64	1.72

Table 4. Comparison of the Citrate and Phosphate Concentrations Obtained by IC with Suppressed Conductivity Detection to the Label Amounts			
Sample	**Analyte**	**Label Amount (mg/mL)[a]**	**Experimental Average[c] ± SD (mg/mL)[a]**
Anticoagulant citrate phosphate dextrose adenine solution for citrate assay	Citrate	20.17	21.18 ± 0.10
Anticoagulant citrate phosphate dextrose adenine solution for phosphate assay	Phosphate	2.22	2.81 ± 0.010
Citric acid, magnesium oxide, sodium carbonate irrigation solution	Citrate	29.6	29.9 ± 0.4
Potassium citrate extended release tablets	Citrate	10 meq	10.3 ± 0.2 meq
Anticoagulant citrate phosphate dextrose solution for citrate assay	Citrate	20.17	20.79 ± 0.23
Anticoagulant citrate phosphate dextrose adenine solution for phosphate assay	Phosphate	2.22	2.20 ± 0.02
Magnesium citrate oral solution	Citrate	NLT[b] 75.9	86.9 ± 1.8
Sodium citrate and citric acid oral solution	Citrate	126.4	128.3 ± 1.6
Sodium bicarbonate and citric acid effervescent tablets	Citrate	1000 mg/tab	1044.7 ± 21.5 mg/tab
Multiple electrolytes injection type 2	Citrate	0.513	0.517 ± 0.003
Oral rehydration solution	Citrate	1.92	2.55 ± 0.05

[a] Amounts expressed as mg/mL citric acid or $NaH_2PO_4 \cdot H_2O$ unless otherwise noted

[b] NLT = Not less than

[c] Average and standard deviation of three independent determinations, two injections per day

CONCLUSION

An IC method using a low-capacity, hydroxide-selective, anion-exchange column with suppressed conductivity detection provides an efficient and rapid separation of phosphate and citrate in different pharmaceutical formulations. This method meets USP performance requirements in terms of specificity, linearity, precision, and recovery of samples spiked with phosphate and citrate. There are currently nine different USP assays for 18 pharmaceutical formulations for citrate or citrate/phosphate in USP monographs. Laboratories that currently support multiple USP citrate assays for different pharmaceutical formulations may be able to standardize on this single IC assay. This assay can incorporate electrolytic generation of the potassium hydroxide eluent to enhance the consistency of the results between analysts and laboratories.

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SUPPLIERS

U.S. Pharmacopeia, 3601 Twinbrook Parkway, Rockville, MD 20852 USA, Tel.: 800-227-8772, www.usp.org.

VWR Scientific Products, 1310 Goshen Parkway, West Chester, PA 19380 USA, Tel: 800-932-5000, www.vwr.com.

Sigma-Aldrich Chemical Co., P.O. Box 14508, St. Louis, MO 63178 USA, Tel: 800-325-3010, www.sigma-aldrich.com.

Fisher Scientific, 2000 Park Lane, Pittsburgh, PA 15275-1126 USA, Tel: 800-766-7000, www.fishersci.com.

Direct Determination of Cyanate in a Urea Solution and a Urea-Containing Protein Buffer

INTRODUCTION

Urea is commonly used in protein purification, including large scale purification of recombinant proteins for commercial purposes, and in recombinant protein manufacturing to denature and solubilize proteins.[1–3] Some proteins are readily soluble and denature at moderate urea concentrations (4–6 M), however, most solubilize and denature at higher concentrations (8–10 M).[4–6] Urea degrades to cyanate and ammonium in aqueous solutions. The maximum rate of cyanate production occurs near neutral pH, the typical pH range of biological buffers.[7]

Cyanate can carbamylate proteins through a reaction with free amino, carboxyl, sulfhydryl, imidazole, phenolic hydroxyl, and phosphate groups.[8] These are unwanted modifications that can alter the protein's stability, function, and efficiency. While some of these reactions can be reversed by altering the pH of the solution, cyanate-induced carbamylation reactions to N-terminal amino acids, however–such as arginine and lysine–are irreversible.[8] Urea solutions are commonly deionized to remove cyanate for this reason. Unfortunately, high cyanate concentrations can accumulate in urea solutions regardless of prior deionization, with some urea buffers reporting cyanate concentrations as high as 20 mM.[7] An accurate, sensitive method for measuring cyanate in high ionic strength matrices is needed to help monitor and control quality in these buffers.

Ion chromatography (IC) is an ideal method for cyanate determination. A 2004 publication showed determination of cyanate in urea solutions by IC using a Dionex IonPac® AS14 column with 3.5 mM Na_2CO_3/ 1.0 mM $NaHCO_3$ eluent, and suppressed conductivity detection.[9] This method was used to evaluate the efficiency of cyanate scavengers and make recommendations for protein buffers that reduced cyanate accumulation and subsequent carbamylation reactions. The authors evaluated separate 0.1 M citrate, phosphate, and borate buffers in 8 M urea across a pH range of 5–9. They reported that the citrate (pH = 6) buffered urea solution demonstrated the best suppression of cyanate accumulation (<0.2 mM cyanate). However, phosphate buffers at pH 6 and 7 (<0.5 mM cyanate) were preferred over the citrate buffers because citrate actively carboxylates proteins. (It is sometimes used as a carboxylating agent.) While this analytical method was effective, the authors believed it could be improved by using a high-capacity hydroxide-selective anion-exchange column with better chloride-cyanate resolution. Hydroxide eluent delivers better sensitivity than carbonate/bicarbonate. Improved resolution between chloride and cyanate, combined with the capability to inject more concentrated samples due to the higher column capacity also provides increased method sensitivity.

This application note shows determination of cyanate in samples of 8 M urea, 8 M urea with 1 M chloride, and 8 M urea with 1 M chloride and 50 mM phosphate buffer (pH = 8.4) using a Reagent-Free™ Ion Chromatography (RFIC™) system. This method provides improved sensitivity, allows smaller volume sample injections, lowers the required dilution, and demonstrates higher resolution of cyanate from chloride compared to the previously published method. Cyanate is separated using

a hydroxide-selective IonPac AS15 (5-µm) column with electrolytically generated 25 mM potassium hydroxide eluent and suppressed conductivity detection. The IonPac AS15 column is a 3 × 150 mm high-capacity (60 µEq/column) column, with a smaller particle size, diameter, and length than the IonPac AS14 column described in a previous method. These changes improve sensitivity, reduce eluent consumption, and allow higher sample throughput. The mobile phase is electrolytically generated, which reduces labor, improves consistency, and provides the reproducibility of an RFIC system. Linearity, limit of detection (LOD), precision, recoveries, and stability of cyanate in urea as a function of temperature are discussed.

EXPERIMENTAL

Equipment

Dionex ICS-3000 RFIC-EG™ system consisting of:

SP Single gradient pump

DC Detector and Chromatography module, single or dual temperature zone configuration

CD Conductivity Detector

EG Eluent Generator

AS Autosampler with Sample Tray Temperature Controlling option and 1.5 mL sample tray

EluGen® EGC II KOH cartridge (P/N 058900)

Continuously Regenerated Anion Trap Column (CR-ATC, P/N 060477)

Chromeleon® 6.8 Chromatography Workstation

Virtual Column™ Separation Simulator (optional)

Sample Vial kit, 0.3-mL polypropylene with caps and septa (P/N 055428)

[a] This application can be performed on other Dionex RFIC-EG systems.

REAGENTS AND STANDARDS

Reagents

Deionized water, Type 1 reagent-grade, 18.2 MΩ-cm resistivity, freshly degassed by ultrasonic agitation and applied vacuum.

Use only ACS reagent grade chemicals for all reagents and standards.

Chloride standard (1000 mg/L; Dionex, P/N 037159)

Sodium carbonate, anhydrous (Na_2CO_3) (VWR, JT3602)

Sodium chloride (NaCl) (VWR, JT3624)

Sodium cyanate (NaOCN) (Aldrich, 185086)

Sodium phosphate, dibasic anhydrous (Na_2HPO_4) (VWR, JT3828)

Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) (VWR, JT3818)

Urea (H_2NCONH_2) (VWR, JT4204-1)

Urea Matrix Sample Solutions

Urea solutions were prepared from solid compounds in 18.2 MΩ-cm deionized water and diluted 50× prior to cyanate determinations:

8 M urea

8 M urea with 1 M chloride

8 M urea with 1 M chloride and 50 mM phosphate (pH = 8.4)

All solutions containing urea were prepared the same day of the experiments unless otherwise stated.

CONDITIONS

Columns:	IonPac AS15 5-µm Analytical (3 × 150 mm, P/N 057594) IonPac AG15 5-µm Guard, (3 × 30 mm, P/N 057597)
Eluent[a]:	25 mM KOH
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	0.5 mL/min
Column Temperature:	30 ºC
Tray Temperature:	4 ºC[b]
Injection Volume:	5 µL
Detection:	Suppressed conductivity, ASRS® 300 (2 mm, P/N 064555), recycle mode, 31 mA
Background Conductivity:	<1 µS
Baseline Noise:	<2 nS
System Backpressure:	~2200 psi
Run Time:	22 min

[a] Add a step change at 12 min to 65 mM KOH when determining cyanate in samples containing phosphate. The eluent conditions are: 25 mM KOH for 0 to 12 min, and 65 mM KOH from 12 to 20 min, recycle mode, 81 mA.

[b] Temperature was maintained at 4 °C using the temperature-controlled autosampler tray to minimize changes in cyanate concentration.

PREPARATION OF SOLUTIONS AND REAGENTS

It is essential to use high quality, Type 1 water, with a resistivity of 18.2 MΩ-cm or greater, and it must be relatively free of dissolved carbon dioxide. Degas the deionized water using ultrasonic agitation with applied vacuum.

1 M Stock Cyanate Standard Solution

To prepare a 1 M stock cyanate standard solution, dissolve 6.50 g of sodium cyanate (NaOCN, FW 65.01 g/mol) with deionized water in a 100 mL volumetric flask and bring to volume. Gently shake the flask to thoroughly mix the solution.

Primary and Working Cyanate Standard Solutions

To prepare a 1.00 mM cyanate primary standard, pipette 100 µL of the 1 M cyanate stock standard solution into a 100-mL volumetric flask, bring to volume with deionized water, and shake the flask gently to mix.

To prepare 1, 2, 4, 10, 20, 50, and 100 µM cyanate individual working standards, pipette 50, 100, 200, 500, 1000, 2500, and 5000 µL, respectively of the 1.00 mM cyanate primary standard solution into separate 50 mL volumetric flasks. Bring to volume with deionized water and shake gently to mix.

Prepare the 0.13, 0.25, and 0.50 µM cyanate detection limit standards by diluting the 1.0 µM cyanate working standard using serial dilutions. For example, a portion of the 1.0 µM cyanate standard is diluted 50% to 0.50 µM cyanate. The 0.13 and 0.25 µM cyanate standards are prepared in a similar way from the 0.25 and 0.50 µM cyanate standards, respectively.

Store all standards at 4 °C. Prepare the 1–20 µM standard solutions weekly and the primary and stock standard solutions monthly.

Urea Matrix Sample Solutions

To prepare 8 M urea (H_2NCONH_2, FW 60.06 g/mole) matrix sample solution, dissolve 48.05 g urea with deionized water in a 100 mL volumetric flask, dilute to volume, and mix thoroughly.

Prepare the combined 8 M urea, 1 M sodium chloride (NaCl, FW 58.44 g/mole) matrix sample solution in a similar manner using 48.05 g urea, 5.84 g sodium chloride, and deionized water in a 100 mL volumetric flask.

Prepare the combined 8 M urea, 1 M sodium chloride, and 50 mM phosphate (49.5 mM sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$), 0.5 mM sodium phosphate dibasic (Na_2HPO_4) matrix solution using 48.05 g urea, 5.84 g sodium chloride, 0.683 g sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$, FW 58.44 g/mole), 0.007 g sodium phosphate dibasic (Na_2HPO_4, FW 141.96 g/mole), and deionized water in a 100 mL volumetric flask. Bring to volume, and mix thoroughly (pH = 8.4).

To prepare 100-, 50-, and 10-fold dilutions of the matrix sample solutions for the dilution experiments, pipette 200, 400, and 2000 µL, respectively, of the matrix sample solution into a (tared) 20 mL polypropylene scintillation vial and add deionized water until a total weight of 20.00 g is reached.

PRECAUTIONS

The urea solutions must be stored frozen at –20 or -40 °C and defrosted before use or prepared fresh daily as the cyanate concentrations increase over time and with increased temperature.

SYSTEM SETUP

Refer to the instructions in the ICS-3000 Installation[10] and Operator's[11] manuals, AS Autosampler Operator's manual,[12] and column,[13] and suppressor[14] product manuals for system setup and configuration.

RESULTS AND DISCUSSION

Method Development and Optimization

The challenge in this application is to accurately determine low concentrations of cyanate in a matrix with high concentrations of salts (up to 20,000× higher) and to resolve a cyanate peak eluting near a significantly larger chloride peak. In a previous study, cyanate was separated on a Dionex IonPac AS14 column using carbonate/bicarbonate eluent at a flow rate of 1.2 mL/min, with suppressed conductivity detection.[9] The authors reported a 1.3 min retention time difference between chloride and cyanate. However, cyanate is not fully resolved from chloride in urea solutions containing 1 M chloride. The authors also reported that the chromatogram's baseline was affected when 8 M urea solutions were injected. As urea eluted through the column, the baseline of the chromatogram increased >2 µS at approximately 3 min, then slowly drifted downward to the original baseline. The cyanate detection limit corresponding to the undiluted urea solution is high (200 µM). An RFIC system with a hydroxide-selective column and electrolytically generated hydroxide eluent can be easily optimized to resolve low concentrations of cyanate from high concentrations of chloride in urea samples. Electrolytically generated hydroxide eluent improves sensitivity and reduces baseline noise.

In order to optimize conditions and to minimize the time required to select the hydroxide-selective, high-capacity column with the highest resolution for cyanate, the authors used the Chromeleon Virtual Column simulator to model separation conditions. Nitrite was used to model cyanate because it is not currently part of the Virtual Column database and was chosen after reviewing chromatograms in column manuals for various high-capacity hydroxide-selective columns. (Only these types of columns were considered because some urea-containing solutions contain molar concentrations of chloride and other anions that may overload low-capacity columns). Virtual Column simulator was used to evaluate the separation of chloride, nitrite, carbonate, and phosphate with isocratic hydroxide eluents at 30 °C. The simulator results demonstrated that the IonPac AS15 column using 20 mM KOH provided the optimum chloride/nitrite and nitrite/carbonate resolution with $R_s > 3$. The Virtual Column simulator proved to be a valuable tool in helping accelerate method development by eliminating the time required to select a column and eluent suitable for this application.

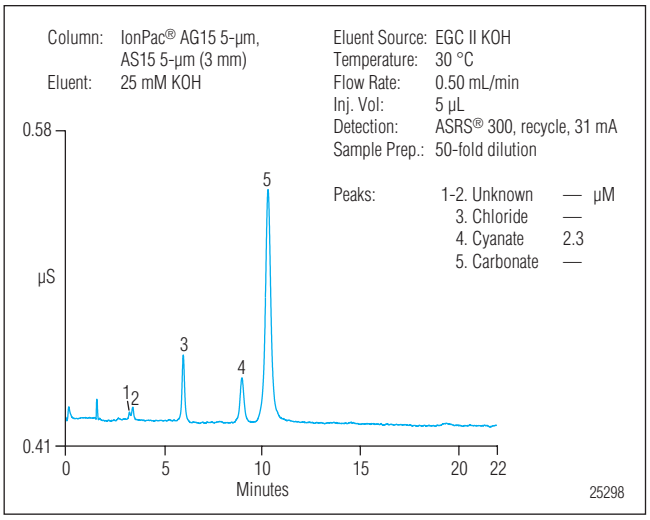

Figure 1. Determination of 2 µM cyanate by ion chromatography.

To refine the results obtained by Virtual Column simulator, the retention times of a 5 µL injection of 1 mM cyanate, chloride, and carbonate were determined on an IonPac AS15 5-µm, 3 x 150 mm column, using 20, 25, and 30 mM potassium hydroxide at 0.5 mL/min and 30 °C. The experiments showed cyanate well resolved from chloride and carbonate by 3.2 min ($R_s > 3$) using either 20 or 25 mM potassium hydroxide, and, as expected, retention times decreased with increasing eluent strength. 25 mM potassium hydroxide eluent was selected for this assay. Figure 1 demonstrates good peak response and peak asymmetry for 2 µM cyanate separated on the IonPac AS15 5-µm column using electrolytically generated 25 mM potassium hydroxide at 0.5 mL/min.

During the initial method evaluation, the effects of column overload using 8 M urea samples with and without spiked concentrations of 1 M sodium chloride were tested. The chromatograms of undiluted 8 M urea showed a similar baseline disturbance (3 µS) from urea as reported in the literature[9] (not shown). As urea elutes from the column, the baseline shifts upward at approximately 3 min then slowly back to the original baseline at 15 min. All tested urea-containing solutions demonstrated similar results. The magnitude of the baseline shifts reduced with dilution (Figures 2–4). Urea contains minor unidentified ions that do not interfere with the cyanate peak and elute from the column in less than 20 min. 8 M urea samples did not overload the column. To determine whether the molar concentrations of chloride expected in the samples would cause column overloading, 20-, 50-, and 100-fold dilutions of 8 M urea solutions containing 1 M chloride

were injected. The chromatography of the 20-fold dilution samples (50 mM chloride) showed distorted chloride peaks typical of column overloading. At least 50× dilutions of 8 M urea with 1 M chloride are required to avoid this phenomenon and to obtain adequate resolution of chloride and cyanate.

Method Qualification

To qualify the cyanate method, linearity, system noise, limit of quantification, and LOD were evaluated. The peak area response of cyanate was determined from 1 to 100 µM, using triplicate injections of the calibration standards. The linearity of cyanate peak area responses was determined with Chromeleon software using a least squares regression fit. The resulting correlation coefficient (r^2) was 0.9993.

The peak-to-peak baseline noise was measured in 1 min segments from 20 to 60 min without injecting a sample. The noise was acceptably low (0.95 ± 0.13 nS (n = 3)). To determine the limit of detection, seven replicates of 0.13, 0.25, and 0.50 µM cyanate were injected. The peak responses of cyanate were compared against the baseline noise using 3× S/N. The LOD was 0.25 µM cyanate (S/N 3.01). These detection limits are significantly lower than previously reported (2 µM) using an IonPac AS14 column with bicarbonate/carbonate eluents.[9] This improvement is likely due to the advantages of using electrolytically generated hydroxide eluent with suppressed conductivity detection. The IonPac AS15 column has similar capacity per column but 30% less volume than the AS14 column. These differences provide improvement in peak response and sensitivity. This allows for a smaller sample (5 µL) to be injected, resulting in less column overload and longer column life. The limit of quantification, using 10× S/N, is 0.8 µM (1.0 µM, S/N 11.6).

Determination of Cyanate in Urea and Urea-Containing Solutions

The method was applied to 50-fold dilutions of 8 M urea, 8 M urea with 1 M chloride, and 8 M urea with 1 M chloride and 50 mM phosphate buffer (pH 8.4). The authors determined the urea and urea-containing solutions had similar cyanate concentrations of 1.1 ± 0.1 µM in 50× diluted solutions (Table 1). The cyanate peak was well resolved from chloride and carbonate, and had good peak shape (Figure 2A). An acceptably small baseline drift (<0.15 µS) from urea was observed starting at 2.4 min and ending at about 15 min. In urea-containing solutions with molar concentrations of chloride, cyanate elutes on the base of the large chloride peak and is not fully resolved from chloride (Figure 3A). The chromatography is similar for urea-containing solutions with chloride and phosphate (Figure 4A).

To determine the method accuracy, multiple additions of cyanate (1.2, 2.2, and 3.6 µM) were added to 50× dilutions of 8 M urea samples. In addition, 50× dilutions of 8 M urea with 1M chloride and 8 M urea with 1 M

Table 1. Recoveries of Cyanate in Urea Solutions (Dilution Factor: 50×)

Matrix[a]	Amount Present (µM)	RSD	Amount Added (µM)	Amount Measured (µM)	RSD	Recovery (%)	Amount Added (µM)	Amount Measured (µM)	RSD	Recovery (%)	Amount Added (µM)	Amount Measured (µM)	RSD	Recovery (%)
A	1.13	0.50	1.20	2.26	0.64	96.7	2.20	3.32	1.38	99.7	3.62	4.92	0.87	103.6
B	1.11	1.07	0.91	1.88	1.03	93.1	1.31	2.43	1.44	100.4	2.22	3.48	0.55	104.4
C	1.00	1.41	0.81	1.60	1.10	89.5	1.24	2.27	0.69	101.1	1.70	2.66	1.16	98.0

A) 8 M Urea
B) 8 M Urea, 1 M Chloride
C) 8 M Urea, 1 M Chloride, 50 mM Phosphate Buffer pH = 8.4

chloride, and 50 mM phosphate buffer (pH = 8.4) were similarly spiked with 0.9–2.2 µM, and 0.8–1.7 µM cyanate, respectively. The calculated recoveries were >89% for all solutions (Table 1). The chromatograms of the 50× dilution of 8 M urea and the same solution spiked with 3.6 µM cyanate are shown in Figure 2. Peaks 6–8 are unidentified ions from the urea matrix that elute from the column within 20 min, while run time was extended to 22 min to ensure that these ions were fully eluted before the next injection. The chromatograms of unspiked and spiked with 2.2 µM cyanate in 50-fold dilution of 8 M urea 1 M chloride are shown in Figure 3. The chromatograms of
8 M urea with 1 M chloride and 50 mM phosphate buffer (pH=8.4), both unspiked and spiked with 1.7 µM cyanate and diluted 50× are shown in Figure 4.

To determine the retention time and peak area precisions seven replicate injections of 2 µM cyanate were spiked into deionized water, 50× dilutions of 8M urea, 8 M urea with 1M chloride, and 8 M urea with 1M chloride and 50 mM phosphate buffer (pH 8.4). Cyanate had similar retention times for all samples—8.99 to 9.07 min (Table 2). The retention time and peak area precisions were <0.1 and <2 % for all three samples.

Table 2. Retention Time and Peak Area Precisions of 2 µM Cyanate Spiked Into 50-fold Dilution of Urea Solution

Matrix[a]	Retention Time (min)	RSD	Peak Area RSD
Deionized Water	9.07	0.02	1.06
A	9.07	0.02	1.65
B	9.03	0.04	0.65
C	8.99	0.06	1.69

A) 8 M Urea
B) 8 M Urea, 1 M Chloride
C) 8 M Urea, 1 M Chloride, 50 mM Phosphate Buffer pH = 8.4

n = 7
[a] Freshly prepared solutions

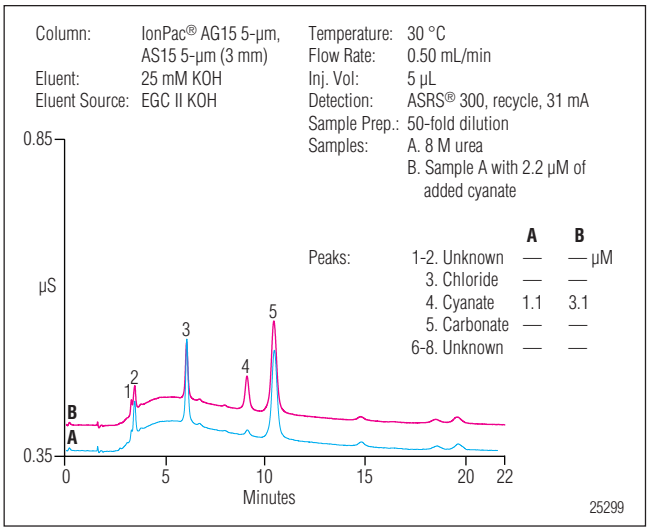

Figure 2. Comparison of A) 8 M urea B) 8 M urea with 2.2 µM cyanate.

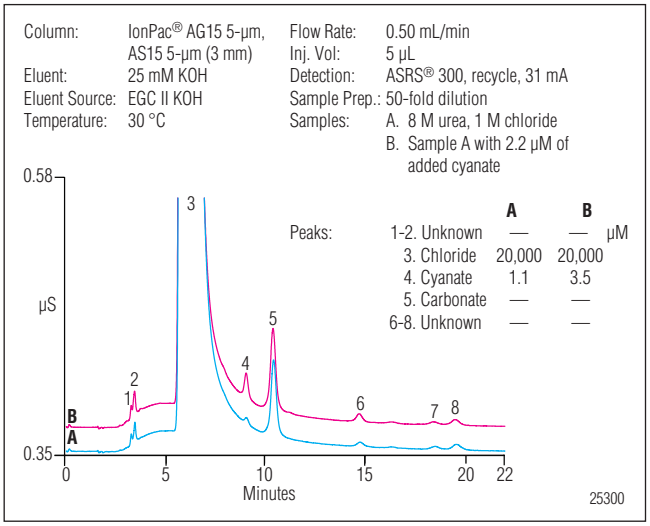

Figure 3. Comparison of A) 8 M urea 1 M chloride B) 8 M urea 1 M chloride with 2.2 µM cyanate.

Sample Stability

The accumulation of cyanate in urea as a function of temperature is frequently discussed in the literature.[1,4–9] To determine the stability of cyanate in urea over 4 days, cyanate concentrations were determined from 50-fold dilutions of 8 M urea, 8 M urea with 1 M chloride, and 8 M urea with 1 M chloride and 50 mM phosphate buffer solutions (pH = 8.4). The solutions were stored at -40 °C, 4 °C (AS autosampler tray), and 25 °C during the four-day experiment. To elute phosphate in the phosphate buffered urea solution, the method was modified with a step change to 65 mM KOH after the carbonate peak at 12 min.

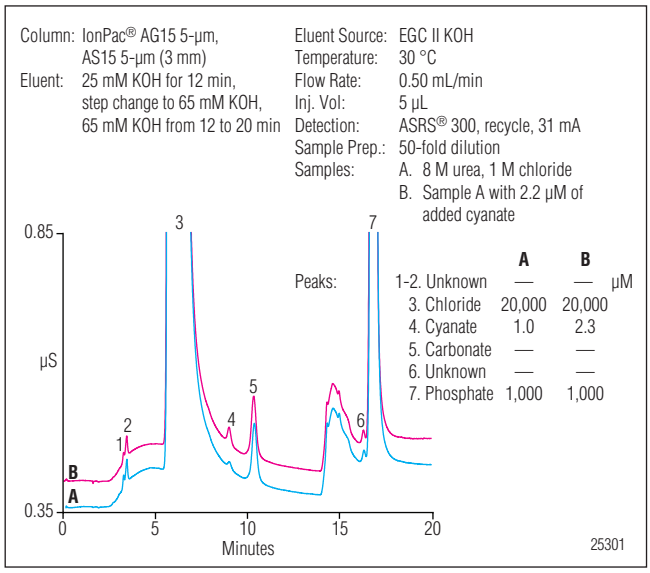

Figure 4. Comparison of A) 8 M urea 1 M chloride and 50 mM phosphate buffer (pH = 8.4) to B) Sample A spiked with 1.2 µM cyanate.

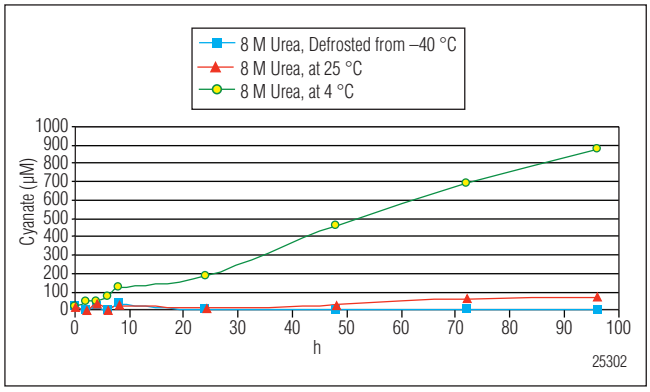

Figure 5. Effect of temperature on cyanate from urea solutions.

The experiments showed the total cyanate concentrations were stable in 8 M urea when stored at –40 °C. However, cyanate concentration increased more than 10-fold over four days when stored at 4 °C (from 6 to 75 µM) and increased significantly when stored at 25 °C (from 24 to 886 µM) (Figure 5). Cyanate had similar stability in 8 M urea with 1 M chloride and 8 M urea with 1 M chloride and 50 mM phosphate buffer as in the 8 M urea solutions. Cyanate accumulation in urea was not inhibited by the phosphate buffer. In the previous study, the authors reported that scavengers–in addition to phosphate and other buffers–were needed to effectively suppress the accumulation of cyanate in urea.[9] These results agree with the previous study.

CONCLUSION

Urea degrades to cyanate, an unwanted contaminant in urea-containing buffers used for protein purification. Using a high-capacity anion-exchange column with suppressed conductivity detection, the authors accurately determined low (µM) concentrations of cyanate in 50-fold dilutions of 8 M urea and urea solutions containing molar concentrations of chloride and mM concentrations of phosphate. This method allows fast, accurate determination of cyanate in urea-containing solutions. A Reagent-Free IC system ensures the highest precision, eliminates the need to prepare eluents, and eliminates possible eluent preparation errors.

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Determination of Inorganic Anion Impurities in a Water-Insoluble Pharmaceutical by Ion Chromatography with Suppressed Conductivity Detection

INTRODUCTION

The U.S. Food and Drug Administration (FDA) is responsible for protecting consumers by ensuring that pharmaceuticals are safe by requiring the manufacturers to verify their identity, strength, quality, and purity characteristics. Impurities that are present even in small amounts may influence the safety and efficacy of the pharmaceutical product. According to the International Conference on Harmonization, impurities are defined as any component of the active pharmaceutical ingredient (API) that is not the chemical entity defined as the API.[1]

Pharmaceutical impurities are categorized as organic, inorganic, or residual solvents. Inorganic impurities that may be derived from the manufacturing process of bulk drugs include reagents, catalysts, ligands, heavy metals, and other materials (e.g., filter aids, charcoal).[2] For example, inorganic impurities may be present in the raw materials or may be derived from reagents, such as phosphate buffers, used during the production of the pharmaceutical. While the presence of many inorganic impurities at low concentrations have few toxicological consequences, significant variation in the impurity levels from batch-to-batch can indicate that the manufacturing process of the drug product is not adequately controlled.[3,4] In most cases, these impurities should be removed or at least minimized in the final product. Therefore, the identification, quantification, and control of impurities are important during drug development in the pharmaceutical industry.

Ion chromatography (IC) with suppressed conductivity detection is a well-established technique for the determination of inorganic and organic ions in pharmaceuticals.[5–7] For the determination of anions, a hydroxide eluent is commonly used. Hydroxide is suppressed to water, which provides exceptionally low background conductivity and baseline noise and, therefore, very low detection limits. In Application Note 190 (AN190), we demonstrated the determination of sulfate counter ion and anionic impurities in several water-soluble aminoglycoside antibiotics.[8] Most of the samples described in AN190 could be analyzed by direct injection after dilution with deionized water. In this Application Note (AN), we demonstrate the development of an IC method for the determination of anionic impurities in a proprietary water-insoluble pharmaceutical. A 2-mm IonPac® AS15 column with an electrolytically generated potassium hydroxide eluent was used for the determination of sub-mg/L concentrations of inorganic anion impurities in a proprietary pharmaceutical dissolved in 100% MeOH. A 100-µg/L sample was concentrated on an IonPac UTAC-ULP1 concentrator followed by elimination of the MeOH matrix and pharmaceutical with 1 mL of deionized water to permit the determination of the target inorganic anions without matrix interferences. The linearity, detection limits, precision, and accuracy of the method are described.

EQUIPMENT

Dionex ICS-3000 Reagent-Free™ Ion Chromatography (RFIC) system consisting of:

DP Dual Pump module (an SP Single Pump module can also be used)

EG Eluent Generator module

DC Detector/Chromatography module (single or dual temperature zone configuration)

AS Autosampler with a 1-mL syringe (P/N 055066)

EluGen EGC II KOH cartridge (P/N 058900)

Continuously-Regenerated Anion Trap Column, CR-ATC (P/N 060477)

Chromeleon® 6.8 Chromatography Data System

REAGENTS AND STANDARDS

Deionized water, Type I reagent grade, 18 MΩ-cm resistivity or better

Combined Seven Anion Standard, 100 mL (Dionex P/N 056933)

Fluoride Standard 1000 mg/L, 100 mL

(Dionex P/N 037158 or Ultra Scientific, VWR P/N ULICC-003)

Chloride Standard 1000 mg/L, 100 mL

(Dionex P/N 037159 or Ultra Scientific, VWR P/N ULICC-002)

Sulfate Standard 1000 mg/L, 100 mL

(Dionex P/N 037160 or Ultra Scientifc, VWR P/N ULICC-006)

Nitrate Standard 1000 mg/L, 100 mL (Ultra Scientific, VWR P/N ULICC-004)

Phosphate Standard 1000 mg/L, 100 mL (Ultra Scientifc, VWR P/N ULICC-005)

Methanol, ACS grade (99.8% min), BDH (VWR P/N BDH1135-4LG)

CONDITIONS

Columns:	IonPac AG15 Guard, 2 × 50 mm (P/N 053943) IonPac AS15 Analytical, 2 × 250 mm (P/N 053941)
Eluent:	10 mM potassium hydroxide 0–8 min, 10 – 40 mM from 8 – 14 min, 40 – 60 mM from 14 – 20 min, 60 mM from 20 – 30 min*
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	0.40 mL/min
Temperature:	30 °C (lower compartment) 30 °C (upper compartment)
Inj. Voume:	100 µL
Matrix Elim. Vol.:	1000 µL (DI water)
Concentrator:	IonPac UTAC-ULP1, 5 × 23 mm (P/N 063475)
CRD:	CRD 200, 2-mm (P/N 062986)
Detection:	Suppressed conductivity, ASRS® 300 (2 mm), Recycle mode, 60 mA current
System Backpressure:	~2400 psi
Background Conductance:	~0.5-0.7 µS
Noise:	~1-2 nS/min peak-to-peak
Run Time:	30 min

*The column equilibrates at 10 mM KOH for 5 min prior to the next injection

PREPARATION OF SOLUTIONS AND REAGENTS

Mixed Inorganic Anion Stock Solution

To estimate the concentration of the target anions in the sample, prepare a 1000-fold dilution of the Combined Seven Anion Standard. Inject 100 µL of this standard followed by 1000 µL of deionized water. The separation should be similar to that shown in Figure 1. For this application, nitrite and bromide were excluded from the calibration standards because these anions were not detected in the sample or matrix blank.

Stock Standard Solutions for Target Anions (1000 mg/L)

For several of the analytes of interest, 1000 mg/L standard solutions are available from Dionex or other commercial sources. When commercial standards are not available, 1000 mg/L standards can be prepared by dissolving the appropriate amounts of the required analytes from ACS reagent grade salts (or better) in 100 mL of deionized water. Standards are stable for at least one month when stored at 4 °C.

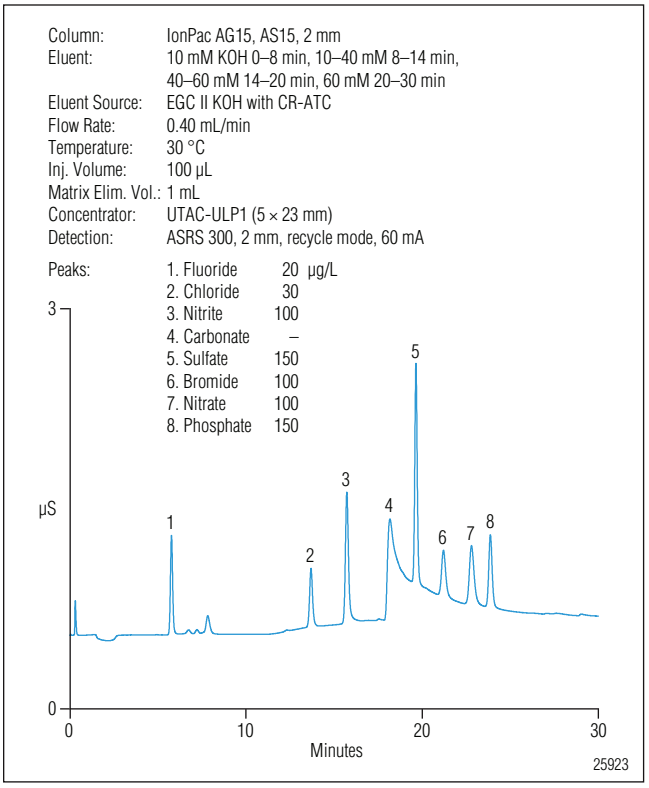

Figure 1. Separation of common inorganic anions in a 1000-fold dilution of a mixed common anion standard on the IonPac AS15 column.

Primary Dilution Standards

Prepare 100 mg/L each of fluoride and phosphate standards in separate 20 mL scintillation vials by combining 2 mL of the respective 1000 mg/L stock solutions with 18 mL of deionized water. Prepare 1 mg/L each of chloride, sulfate, and nitrate standards in separate 125 mL HDPE bottles by combining 100 µL of the respective 1000 mg/L stock solutions with 99.9 mL of deionized water.

Calibration Standards

Prepare calibration standards in the low-µg/L to mg/L range by adding the appropriate volumes from the target anions primary dilution standards to separate 125 mL HDPE bottles and dilute to 100 mL with deionized water. Four levels of calibration standards were used in this study to cover the expected concentrations found in the pharmaceutical sample.

SAMPLE PREPARATION

Weigh approximately 30 ± 2 mg of sample on an analytical balance and then transfer to a previously weighed 100 mL polypropylene volumetric flask. Dissolve the solid in 100 mL of ACS grade MeOH (d = 0.7918 g/mL) to prepare a final sample concentration of 0.30 mg/mL (w/v). C*aution: MeOH is flammable. Work under a hood.* Record the weight of this solution in the volumetric flask and subtract from the weight of the empty volumetric flask and solid to obtain the weight of MeOH used to prepare the sample. To completely dissolve the solid material, sonicate the solution for approximately 15 min.

SYSTEM PREPARATION AND SETUP

1. Install an EGC II KOH cartridge in the EG-3000 module.
2. Install backpressure tubing in place of the columns to produce a total backpressure of ~2000-2500 psi at a flow rate of 1 mL/min.
3. Condition the cartridge by setting the KOH concentration to 50 mM at 1 mL/min for 30 min.
4. Disconnect the backpressure tubing installed in place of the column set.
5. Install a CR-ATC between the EGC II KOH cartridge and the EGC degas.
6. Hydrate the CR-ATC prior to use by following the instructions outlined in the EluGen Cartridge Quickstart Guide.
7. Install 2 × 50 mM AG15 and 2 × 250 mm AS15 columns in the lower compartment of the DC using red PEEKTM tubing (0.005” i.d.) between connections.
8. Install a 5 × 23 mm UTAC-ULP1 concentrator in place of the sample loop on valve #1 using black PEEK (0.010” i.d.) tubing. Direction of sample loading should be opposite of analytical flow.
9. Make sure the pressure is ~2200-2500 psi using the operating conditions described earlier to allow the degas assembly to effectively remove electrolysis gases. If necessary, install additional backpressure tubing or trim tubing between degas assembly and the injection valve to achieve the recommended pressure.
10. Hydrate and install ASRS 300 suppressor and Carbonate Removal Device (CRD 200) according to the instructions in the product manuals. Install both in recycle mode using red PEEK tubing for all connections.

The AS autosampler was used in this AN to concentrate the sample and eliminate the matrix from the UTAC-ULP1 concentrator column. To install and configure the AS autosampler:

1. Install a 1-mL sample syringe (P/N 055066).
2. From the front panel (and under System Parameters), configure the AS autosampler sample mode to "Concentrate."
3. Connect the AS injection port tubing directly to the injection valve. Be sure the tubing is properly calibrated before operating the autosampler.
4. The AS autosampler Concentrate option allows the AS to deliver sample to a low pressure concentrator at a maximum pressure of 100 psi. Therefore, the sample syringe dispense speed should be no greater than 2 in the Chromeleon program.

This application requires a matrix elimination step using deionized water to remove MeOH from the concentrator column. There are two possible procedures to accomplish this task:

1. Rinse a 10 mL AS sample vial several times with deionized water and then fill the vial with deionized water. When performing the matrix elimination step in the program, direct the AS autosampler to aspirate 1 mL from the vial. Separate vials are strongly recommended for different calibration standards and samples to minimize cross contamination. The deionized water in the vial should be changed frequently. For ease-of-use, this option for performing the matrix elimination step was used.
2. Alternatively, the matrix elimination step can be performed by using the sample prep syringe of the AS autosampler with a 5 mL syringe installed. However, this setup requires an 8.2-mL sampling needle assembly (P/N 061267) to accommodate the larger volume. The use of the sample prep syringe for eliminating the matrix from the concentrator will require more time per injection.
3. To setup the concentrate and matrix elimination steps in Chromeleon, use the program wizard and go to the Sampler Options section. By default, the first line of the Sampler Options steps should appear. The first line should read:

   1 Concentrate Loadposition Aspirate = 3 Dispense = 1

   Change the dispense speed from 1 to 2 and click Enter.

   Click the mouse pointer on the next line and then select Reagent Flush from the drop down menu. To use a vial as the source of the matrix elimination solution, as described in #1 above, enter the appropriate vial # in the box. To use the second option, described in #2 above, choose the appropriate Reagent Reservoir that contains the solution used to eliminate the matrix. For the volume, enter 1000 µL and Valve Position should equal No Change. Click insert to insert the line in the sampler options steps. This completes the steps required to concentrate the sample and eliminate the matrix from the concentrator column.

RESULTS AND DISCUSSION

A primary consideration in the development of a suitable IC method for pharmaceuticals is the solubility of the API in water. Many drugs and intermediates are insoluble in water and other aqueous solutions that are typically used in IC systems. This poses a potential analytical challenge as it could lead to precipitation of the API in the chromatography system and therefore cause excess backpressure and column contamination.[9] To overcome this challenge, a sufficient amount of organic solvent can be added to the eluent to maintain the solubility of the API or the API can be precipitated and the resulting solution filtered prior to analysis.[9] The former approach requires a manually prepared eluent and therefore precludes the use of a Reagent-Free ion chromatography (RFIC) system, while the latter increases analysis complexity that can lead to potential contamination and measurement errors.

This AN describes the development of an IC method for the determination of monovalent to polyvalent inorganic anions commonly found in pharmaceuticals. The method combines preconcentration with matrix elimination to detect trace concentrations of inorganic impurities in a proprietary water-insoluble drug. A 100 µL of the pharmaceutical dissolved in 100% MeOH is concentrated on a UTAC-ULP1 concentrator column to trap the inorganic anion impurities, while the MeOH matrix is eliminated with 1 mL of deionized water before analysis. This approach eliminates the need for organic solvent in the eluent or the offline precipitation of the API and therefore improves the methods ease-of-use.

The IonPac AS15 column was chosen as the separation column because it is a high-capacity, hydroxide-selective column specifically developed for the rapid and efficient separation of trace concentrations of inorganic anions in matrices with varying ionic strength. The use of an electrolytically generated hydroxide eluent for this application produces an exceptionally low background and baseline noise and therefore lower detection limits, which enables the detection of inorganic impurities that are less than 0.001% (w/w) in the 0.30 mg/mL pharmaceutical sample analyzed in this study.

It is important to establish a matrix blank and ensure its stability before proceeding to analyze the sample. In this AN, MeOH was required to dissolve the pharmaceutical sample. In general, organic solvents are known to contain trace concentrations of inorganic anions and low molecular weight organic acids as discussed in AU163.[10] However, trace anions in solvents can also be derived from sample handling procedures and contaminated materials used to transport the solution for analysis. Therefore, it is critical to use the same set of containers and other components used to prepare the samples to obtain a representative blank. As shown in Figure 2, trace concentrations of fluoride, chloride, sulfate, and nitrate were detected in 100% MeOH.

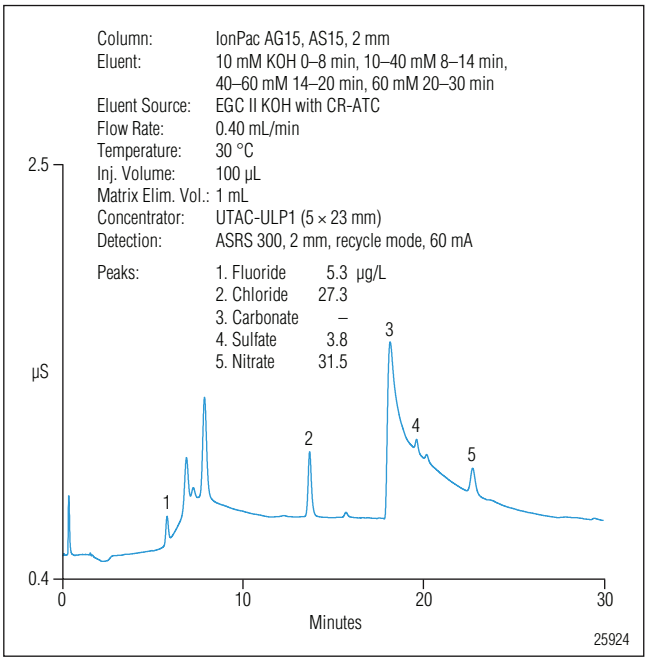

Figure 2. Target anions detected in a representative MeOH blank.

To establish a suitable concentration range for the target anions, the anions detected in the pharmaceutical sample (0.30 mg/mL) and MeOH matrix blank were compared against a 1000-fold dilution of a mixed common anion standard (Figure 1). Table 1 summarizes the range of the calibration curves and the linearity for each target anion. The results demonstrate that the calibration curves for the target anions were linear with correlation coefficients (r^2) greater than 0.997. Table 1 also summarizes the estimated limits of detections (LODs) for the target analytes, calculated based on three times the signal-to-noise ratio (S/N).

Table 1. Calibration Data and Detection Limits

Analyte	Range (µg/L)	Linearity (r^2)	Estimated Limits of Detection[a] (µg/L)
Fluoride	500–2000	0.9996	0.16
Chloride	10–100	0.9989	0.39
Sulfate	5.0–50	0.9974	0.46
Nitrate	10–100	0.9997	1.3
Phosphate	250–1000	0.9997	1.7

[a]LODs estimated from 3 × S/N

The method performance was evaluated by analyzing three different preparations of the pharmaceutical sample over three days. Figure 3 demonstrates the applicability of the method for determining trace anions in a 0.30 mg/mL proprietary pharmaceutical product. As shown, the pharmaceutical sample primarily consists of fluoride and phosphate with only trace concentrations of chloride, sulfate, and nitrate. When the sample is corrected for the MeOH blank, the concentrations of the trace anions (chloride, sulfate, nitrate) were determined to be significantly less than the background concentrations. Therefore, this AN focuses on the primary anion constituents, fluoride and phosphate, in the pharmaceutical sample. The average concentrations for fluoride and phosphate detected in the sample over three days were 967 ± 12 µg/L and 339 ± 10 µg/L, respectively. The presence of fluoride in some inorganic raw materials used for the preparation of pharmaceuticals is well-known. Calcium salts are the most contaminated with fluoride due to their manufacturing process. The determination

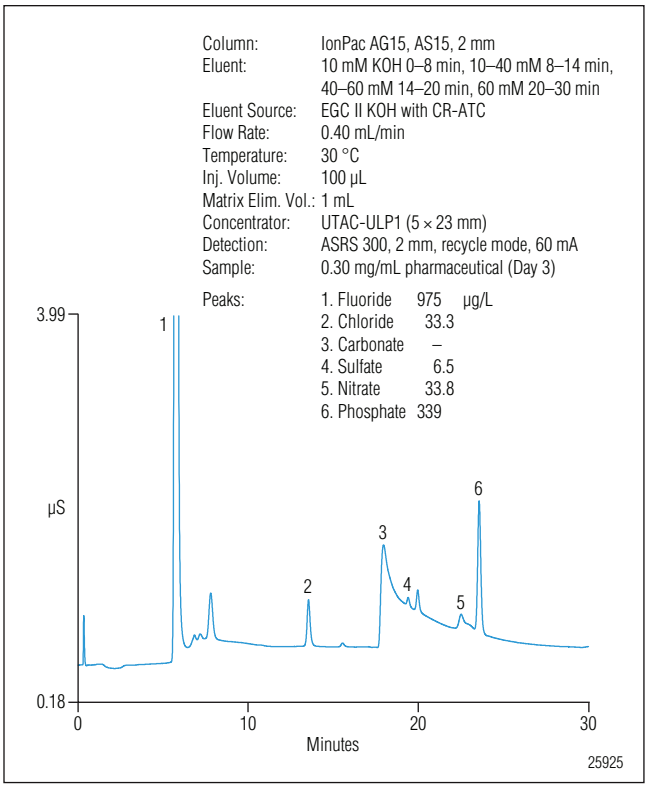

Figure 3. Determination of inorganic anion impurities in a proprietary water-insoluble pharmaceutical compound

of fluoride in pharmaceuticals is critical because excess fluoride is toxic and can cause bone diseases, such as fluorosis, osteoporosis, and skeletal fragility.[11] The presence of phosphate is also not uncommon in pharmaceuticals as phosphate buffers are commonly used during the preparation of the final formulation. Table 2 summarizes the results for the determination of fluoride and phosphate in the pharmaceutical sample. For the three day study, the retention time and peak area RSDs were <0.1% and <1.2%, respectively, for the target anions. The method accuracy was also evaluated by determining the recoveries of fluoride and phosphate spiked into the sample at concentrations that were nearly equivalent to the unspiked sample. The calculated recoveries for fluoride and phosphate were 102.6% and 107.7%, respectively. The good recoveries obtained in this study indicate that the method performed well for the determination of the target anions in a proprietary water-insoluble pharmaceutical compound.

Table 2. Summary of Data Obtained for Target Anions in a Water-Insoluble Pharmaceutical Product

Day	Analyte	Amount Found (µg/L)	% (w/w) in a 0.30 mg/mL Pharmaceutical	Retention Time RSD[a]	Peak Area RSD[a]
1	Fluoride	973.5	0.25	0.06	0.12
	Phosphate	328.9	0.08	0.02	1.1
2	Fluoride	953.9	0.24	0.12	0.41
	Phosphate	349.0	0.09	0.01	0.76
3	Fluoride	974.6	0.25	0.04	0.38
	Phosphate	339.0	0.09	0.01	0.40

[a]n = 6

CONCLUSION

In this AN, we demonstrated the ability to determine trace anions in a proprietary water-insoluble pharmaceutical using preconcentration with matrix elimination. This method was designed to provide a simpler approach that avoids the potential complications of column contamination and excess column backpressure that can occur when analyzing water-insoluble samples. The use of a hydroxide-selective AS15 column provided an efficient separation of common anions from low to high µg/L concentrations that are typically found in pharmaceuticals. In addition, the combination of a hydroxide-selective column with an electrolytically generated potassium hydroxide eluent eliminates the problems associated with the manual preparation of hydroxide eluents and therefore further increases the ease-of-use and method automation. This method demonstrated good linearity, sensitivity, precision, and accuracy for determining inorganic anion impurities in a water-insoluble pharmaceutical compound.

LIST OF SUPPLIERS

VWR Scientific, P.O. Box 7900, San Francisco, CA 94120, USA. Tel: 1-800-252-4752. www.vwr.com

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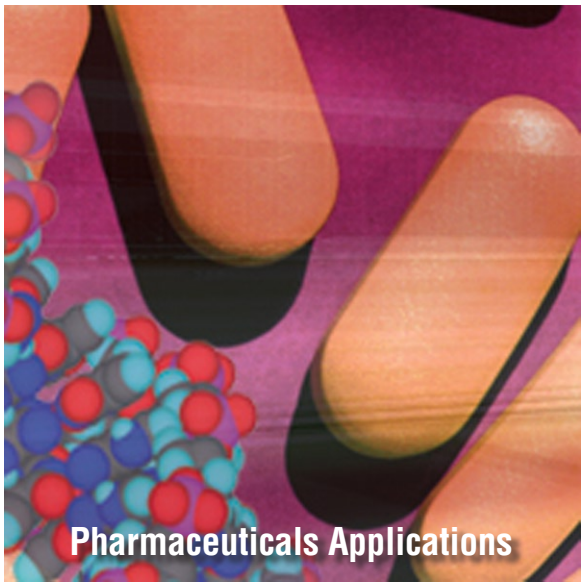
DIONEX

Part V: Analysis of Other Compounds



Analytes

Carbohydrates
Glycols
Alcohols
Active Ingredients in Sunscreen
Triton X-100

Quantification of Carbohydrates and Glycols in Pharmaceuticals

INTRODUCTION

The United States Food and Drug Administration (U.S. FDA)[1–3] and the regulatory agencies in other countries require that pharmaceutical products be tested for composition to verify their identity, strength, quality, and purity. Recently, attention has been given to inactive ingredients as well as active ingredients. Some of these ingredients are nonchromophoric and cannot be detected by absorbance. Some nonchromophoric ingredients, such as carbohydrates, glycols, sugar alcohols, amines, and sulfur-containing compounds, can be oxidized, and therefore can be detected using amperometric detection. This detection method is specific for those analytes that can be oxidized at the selected potential, leaving all other nonoxidizable compounds transparent.[4–5] Amperometric detection is a powerful detection technique with a broad linear range and very low detection limits.

This Application Note describes the use of three different anion-exchange columns with amperometric detection to analyze common simple sugars, sugar alcohols, and glycols in pharmaceutical formulations. Two oral, over-the-counter medications were selected as representative pharmaceutical products. A cough suppressant and a multisymptom cold/flu medication were chosen because they contain a complex mixture of simple sugars, glycols, and sugar alcohols. These carbohydrates and glycols are also commonly found in other medications. Furthermore, these formulations contain inorganic and organic anionic ingredients that have been analyzed using the IonPac® AS14 and AS11 anion-exchange columns with suppressed conductivity detection.[6]

In the methods outlined in this Note, the selectivities of the IonPac ICE-AS1 ion exclusion, CarboPac™ PA10, and CarboPac MA1 anion-exchange columns for the analysis of carbohydrate and glycol ingredients in pharmaceutical formulations are compared. The ICE-AS1 resin bead is a completely sulfonated polystyrene/divinylbenzene polymer with a capacity of about 27 meq/column and with moderate hydrophilic characteristics. The retention mechanisms possible in this column include ion exclusion, steric exclusion, and adsorption. Weakly-ionized acids are separated by pKa differences, size, and hydrophobicity. This column is ideal for the determination of aliphatic organic acids and alcohols in complex or high-ionic strength samples.

The CarboPac PA10 column packing consists of a nonporous, highly crosslinked polystyrene/divinylbenzene substrate agglomerated with 460-nm diameter latex. The MicroBead™ latex is functionalized with quaternary ammonium ions, which create a thin surface rich in anion-exchange sites. The packing is specifically designed to have a high selectivity for monosaccharides. The PA10 has an anion-exchange capacity of approximately 100 µeq/column.

The CarboPac MA1 resin is composed of a polystyrene/divinylbenzene polymeric core. The surface is grafted with quaternary ammonium anion-exchange functional groups. Its macroporous structure provides an extremely high anion-exchange capacity of 1450 µeq/column. The CarboPac MA1 column is designed specifically for sugar alcohol and glycol separations. The ICE-AS1 and PA10 columns, but not the MA1 column, are compatible with eluents containing organic solvents, which can be used to clean these columns.

Expected detection limits, linearity, selectivity, accuracy, and precision are reported for the CarboPac MA1 column. The performance of the CarboPac PA10 column for monosaccharide analysis is presented in Technical Note 40.[7]

EQUIPMENT

Dionex DX-500 system consisting of:

GP40 Gradient Pump, with degas option

ED40 Electrochemical Detector

LC30 or LC25 Chromatography Oven

AS3500 Autosampler

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Sodium hydroxide, 50% (w/w) (Fisher Scientific)

Perchloric acid, 70% (w/w) (Fisher Scientific)

Deionized water, 18 MΩ-cm resistance or higher

Standards

Propylene glycol, anhydrous (Sigma Chemical Co.)

Glycerol (EM Science)

Sorbitol (Eastman Chemical Company)

Mannitol, ACS grade (J. T. Baker Incorporated)

Maltitol (Aldrich Chemical Co.)

Glucose, reference grade (Pfanstiehl Laboratories)

Sucrose, ACS certified (Fisher Scientific)

On-line degassing is necessary because the amperometric detector is sensitive to oxygen in the eluent. High flow rates of 2 mL/minute, such as those used in the ICE-AS1 system, require more frequent on-line degassing intervals (every 5 minutes) to eliminate cyclic baseline drift.

PREPARATION OF SOLUTIONS AND REAGENTS

Perchloric Acid Eluent

100 mM Perchloric acid

Degas 1983 mL of deionized water for 20 minutes and mix with 17.1 mL 70% (w/w) perchloric acid. Connect the eluent reservoir to the instrument and pressurize with helium.

Conditions

	System 1	System 2	System 3
Columns:	IonPac ICE-AS1 Analytical (P/N 43197) NG1 Neutral Guard (P/N 39567)	CarboPac PA10 Analytical (P/N 46110) CarboPac PA10 Guard (P/N 46115)	CarboPac MA1 Analytical (P/N 44066) CarboPac MA1 Guard (P/N 44067)
Flow Rate:	2.0 mL/min	1.5 mL/min	0.4 mL/min
Injection Volume:	10 µL	10 µL	10 µL
Oven Temperature:	30 °C	30 °C	30 °C
Detection (ED40):	Integrated amperometry, platinum electrode	Integrated amperometry, gold electrode	Integrated amperometry, gold electrode
Waveform for ED40:	Time (s) / Potential (V) / Integration (Begin/End) 0.00 +0.30 0.05 +0.30 Begin 0.25 +0.30 End 0.26 +1.40 0.60 +1.40 0.61 +0.10 1.00 +0.10	Time (s) / Potential (V) / Integration (Begin/End) 0.00 +0.05 0.20 +0.05 Begin 0.40 +0.05 End 0.41 +0.75 0.60 +0.75 0.61 -0.15 1.00 -0.15	Time (s) / Potential (V) / Integration (Begin/End) 0.00 +0.05 0.20 +0.05 Begin 0.40 +0.05 End 0.41 +0.75 0.60 +0.75 0.61 -0.15 1.00 -0.15
Eluent Components:	A: 100 mM Perchloric acid	A: Water B: 200 mM Sodium hydroxide	A: Water B: 1.0 M Sodium hydroxide
Eluent Concentration:	100 mM Perchloric Acid	18 mM Sodium hydroxide	480 mM Sodium hydroxide
Method:	Time (min) / A (%) 0.0 100 End 100	Time (min) / A (%) / B (%) 0.0 91 9 11.0 91 9 11.1 0 100 17.6 0 100 17.7 91 9 40.0 91 9	Time (min) / A (%) / B (%) 0.0 52 48 60.0 52 48

Sodium Hydroxide Eluents

200 mM Sodium hydroxide

It is essential to use high-quality water of high resistivity (18 MΩ-cm) as free of dissolved carbon dioxide as possible. Biological contamination should be absent. Additionally, borate, a water contaminant that can break through water purification cartridges (prior to any other indication of cartridge depletion), can be removed by placing a BorateTrap column (P/N 47078) between the pump and the injection valve. It is extremely important to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the columns, causing a loss of chromatographic resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide.

Dilute 20.8 mL of a 50% (w/w) sodium hydroxide solution into 1980 mL of thoroughly degassed water to yield a 200 mM sodium hydroxide solution. Keep the eluents blanketed under 5–8 psi (34–55 kPa) of helium at all times.

1.0 M Sodium hydroxide

Follow the same precautions described above for the 200 mM sodium hydroxide eluent. Dilute 104 mL of a 50% (w/w) sodium hydroxide solution into 1896 mL of thoroughly degassed water to yield 1.0 M sodium hydroxide. Keep the eluents blanketed under 5–8 psi (34–55 kPa) of helium at all times.

STOCK STANDARDS

Solid standards were dissolved in purified water to 10 g/L concentrations. These were combined and further diluted with purified water to yield the desired stock mixture concentrations.

For determinations of linear range, combine 10 g/L solutions of propylene glycol, glycerol, sorbitol, mannitol, glucose, maltitol, and sucrose to make a 1.0 g/L standard mix solution. Dilute with water to concentrations of 800, 600, 400, 200, 100, 80, 60, 40, 20, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 mg/L. Maintain the solutions in a frozen state at –20 °C until needed.

SAMPLE PREPARATION

Dilute viscous products with water on a weight per weight (w/w) basis. Combine 1 gram of medication with 9 grams of water to obtain a 10-fold dilution. Further dilute the medication to yield 100 and 1000-fold dilutions on a weight per weight (w/w) basis. Determine product densities by measuring the weights of known volumes. Calculate the final concentrations based on the densities of these medications. The ingredients of each medication are presented in Tables 1 and 2. The ingredients noted in bold-face type can be analyzed by anion-exchange chromatography with amperometric detection. Many of the other ingredients listed below can be analyzed using the IonPac AS14 and AS11 columns with suppressed conductivity detection.[6]

Any purified water used for dilutions should be tested for trace carbohydrates prior to its use. Test the sample containers for residual carbohydrates prior to use by adding pure water, shaking or vortexing, and then testing the liquid. Prerinsing the vials with purified water can eliminate artifacts and erroneous results.

DISCUSSION AND RESULTS

Selectivity

Figure 1 shows the separation of sorbitol, glycerol, and propylene glycol standards using a 100 mM perchloric acid eluent with the IonPac ICE-AS1 analytical column and the NG1 guard column. The separation was isocratic, which decreases injection-to-injection run times and increases sample throughput. Glycerol, propylene glycol, and sugar alcohols were determined within 10 minutes. Sucrose, maltitol, and mannitol were not well resolved by this method (results not shown). Oxygen is reduced using the same waveform used to

Table 1. Cough suppressant ingredients

	Type
Dextromethorphan Hydrobromide	Active
Citric Acid	Inactive
FD&C Red 40	Inactive
Flavors	Inactive
Glycerin (glycerol)	Inactive
Propylene Glycol	Inactive
Saccharin Sodium	Inactive
Sodium Benzoate	Inactive
Sorbitol	Inactive
Water	Inactive

Table 2. Multisymptom Cold/Flu Ingredients	
	Type
Pseudoephedrine Hydrochloride	Active
Acetaminophen	Active
Dextromethorphan Hydrobromide	Active
Citric Acid	Inactive
FD&C Yellow #6	Inactive
Flavor	Inactive
Glycerin (glycerol)	Inactive
Polyethylene Glycol	Inactive
Propylene Glycol	Inactive
Purified Water	Inactive
Saccharin Sodium	Inactive
Sodium Citrate	Inactive
Sucrose	Inactive

detect sugar alcohols. Oxygen dissolved in the sample eluted at the total permeation volume, and appeared as a dip in the baseline at just before 8 minutes. Use of the NG1 neutral guard column increased the total permeation volume and moved the oxygen dip away from the analytes. Without the NG1, sucrose, sorbitol, maltitol, and mannitol were slightly better resolved, but the oxygen dip encroached on the propylene glycol peak. An alternative way to reduce or eliminate the oxygen dip is to degas the sample prior to injection.

The CarboPac PA10 column separated propylene glycol, glycerol, sorbitol, mannitol, maltitol, glucose, and sucrose using an isocratic sodium hydroxide eluent (Figure 2). Propylene glycol and glycerol eluted near the void and were not baseline-resolved.

Propylene glycol, glycerol, sorbitol, mannitol, maltitol, and sucrose were completely resolved when analyzed on the CarboPac MA1 (Figure 3). Shorter run times are possible for both the PA10 and MA1 methods by adjusting the eluent strength, but resolution may be lost.

Although not presented here, nearly a dozen over-the-counter medications have been analyzed using the ICE-AS1 and MA1 columns with amperometric detection. These medications include both solid and liquid formulations such as nasal and oral decongestants, astringents, antacids, enemas, sleep aids, analgesics, cleaning and disinfecting solutions, antihistamines, and allergy syrups. In most cases, the known carbohydrate ingredients in each formulation were separated from each other using the MA1 column without any apparent interference.

Figures 4 and 5 compare the selectivity of the ICE-AS1 and MA1 columns for the analysis of a cough suppressant. Figure 5B is an expanded view of Figure 5A to better reveal the minor peaks. In general, peak elution order from the ICE-AS1 column is the reverse of that of the MA1 column. The early eluting peaks for sugar alcohols and carbohydrates were not resolved on the ICE-AS1 column. Propylene glycol and glycerol eluted later and were completely resolved on this column. Figure 6 shows the analysis of a multisymptom cold/flu medication using the MA1 column.

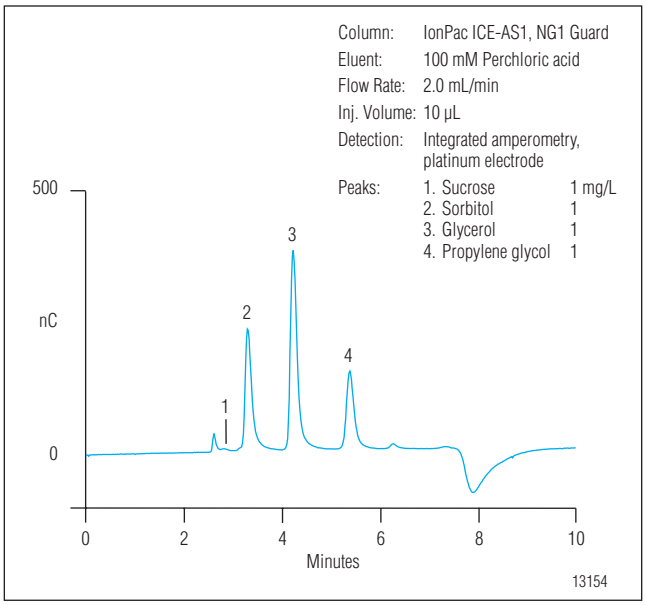


Figure 1. Separation of common glycols, sugar alcohols, and carbohydrates in pharmaceutical formulations on an IonPac ICE-AS1 column.

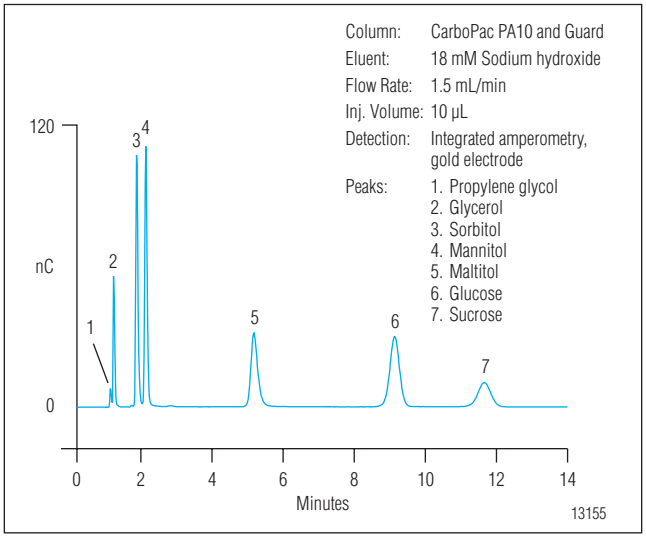


Figure 2. Separation of common glycols, sugar alcohols, and carbohydrates in pharmaceutical formulations on a CarboPac PA10 column.

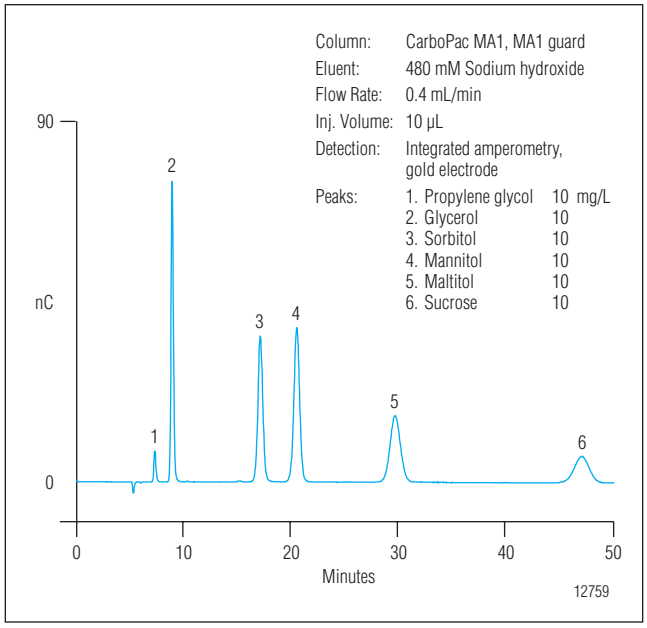


Figure 3. Separation of glycols, sugar alcohols, and carbohydrates using the CarboPac MA1 column.

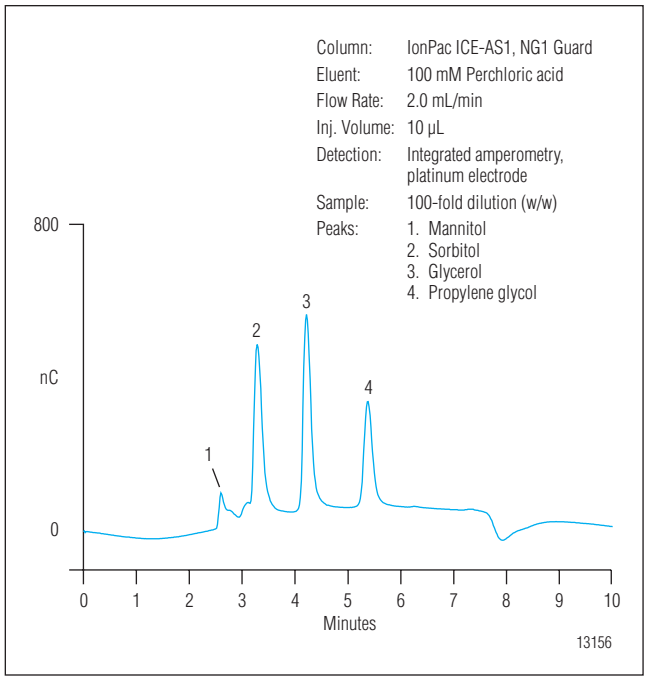


Figure 4. Separation of sugar alcohols and glycols in cough suppressant using the IonPac ICE-AS1 column.

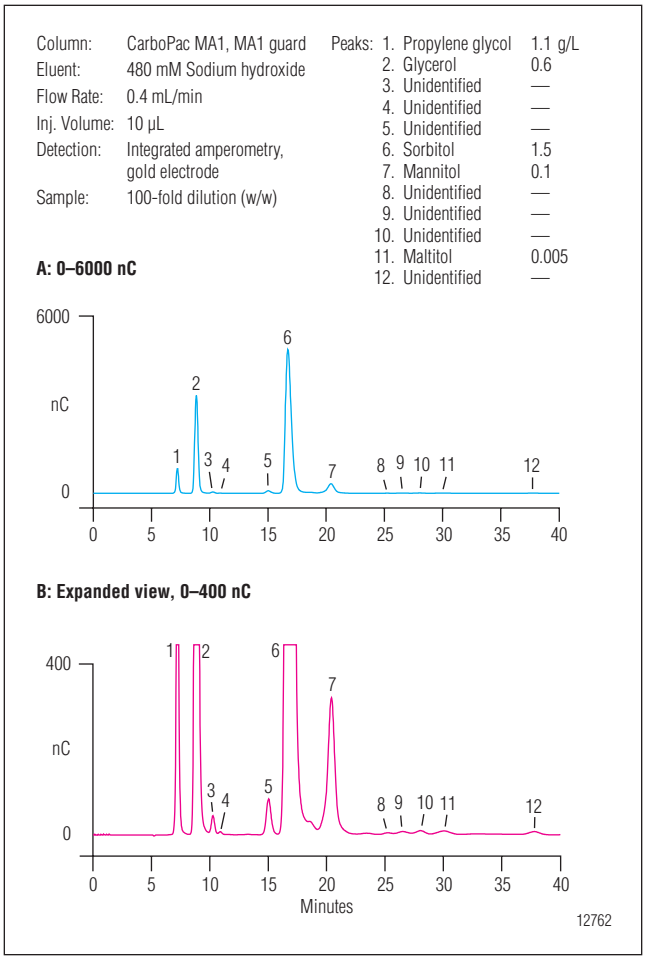


Figure 5. Separation of sugar alcohols and glycols in cough suppressant using the CarboPac MA1 column.

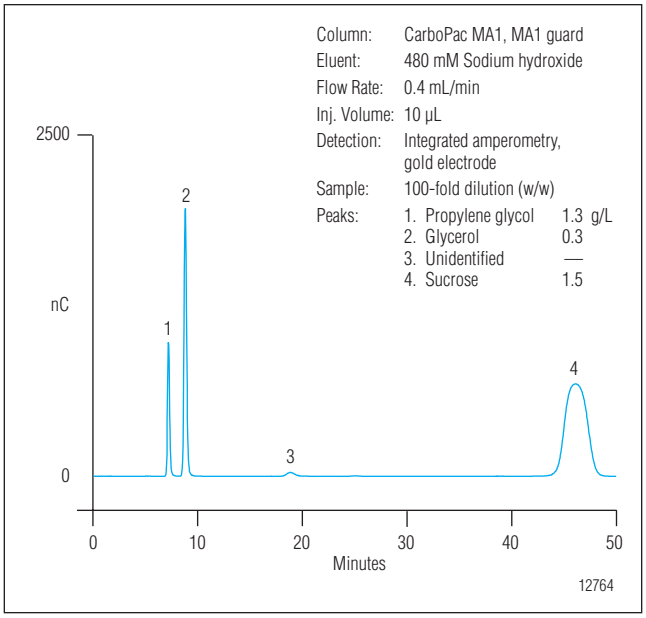


Figure 6. Sugar alcohols, glycols, and carbohydrates in multi-symptom cold/flu medication.

The selection of the best method depends on the analytes tested. If a rapid analysis of sugar alcohols and simple sugars is needed, with little interest in propylene glycol or glycerol, the CarboPac PA10 column is the best choice. If a rapid analysis of glycerol and propylene glycol is desired, with little interest in carbohydrates, the IonPac ICE-AS1 is the best choice. If the analysis of all of these compounds is required, a CarboPac MA1 is the best choice.

Method Detection Limits

The method detection limits (MDL) for a 10-µL injection of common pharmaceutical constituents using the MA1 column are shown in Table 3. The MDL is defined as the minimum concentration required to produce a signal-to-noise ratio of 3. The MDL can be further decreased by increasing the injection volume above the 10-µL injection volume used in this Application Note, and by using smoothing algorithms available in PeakNet software.[7]

Linearity

Propylene glycol, glycerol, sorbitol, mannitol, glucose, maltitol, and sucrose standards ranging from 0.1 to 1000 mg/L (1 ng to 10,000 ng) were injected (n = 2 to 3 per concentration) onto a CarboPac MA1 column. The method was found to be linear for propylene glycol, sorbitol, mannitol, glucose, maltitol, and sucrose over this range ($r^2 \geq 0.999$). Glycerol was linear over the range of 0.1 to 200 mg/L (1 ng to 2,000 ng per injection; $r^2 = 0.999$). For the range of 0.1 to 1000 mg/L (1 ng to 10,000 ng), glycerol deviated from linearity ($r^2 = 0.995$). Using a second order polynomial regression, the r^2 for glycerol was 0.9998 over the range of 0.1 to 1000 mg/L (1 ng to 10,000 ng). For all analytes, linearity was demonstrated over at least three orders of magnitude. Broad linear ranges help eliminate the need to repeat sample analyses when components vary greatly in concentration.

Calibration curves for the MA1 column are presented in Figure 7.

Precision

The peak area and retention time RSDs for 10-mg/L injections of standards (10 µL per injection, 12 injections) run on the MA1 are presented in Table 4. RSDs varied from 2–4% at this concentration. Precision is affected by concentration; RSD values increase as the concentrations approach the MDL. RSDs increase near the MDL because peak integration becomes less precise from the contribution of variation in baseline noise from run-to-run.

Table 3. Estimated Detection Limits Using the CarboPac MA1

	ng	µg/L
Propylene glycol	4	400
Glycerol	0.7	70
Sorbitol	1	100
Mannitol	1	100
Glucose	1	100
Maltitol	2	200
Sucrose	7	700

Table 4. Peak Area and Retention Time Precision

	RSD (%)	
	Area	Retention Time (min)
Propylene glycol	2.4	0.0
Glycerol	3.0	0.2
Sorbitol	2.9	0.1
Mannitol	2.9	0.1
Glucose	2.7	0.1
Maltitol	2.6	0.1
Sucrose	3.7	0.1

Table 5. Recovery of Carbohydrates in Medications

	Percent Recovery	
Analyte	Cough Suppressant	Multisymptom Cold/Flu Medication
Propylene glycol	96	111
Glycerol	98	109
Sorbitol	87	105
Mannitol	99	105
Glucose	101	104
Maltitol	103	106
Sucrose	103	114

Recovery from Sample Matrix

To assess the accuracy of this method, evaluate both medications by the method of standard addition. Combine each 1000-fold (w/w) diluted formulation with an equal weight of a 100 mg/L mixture of propylene glycol, glycerol, sorbitol, mannitol, maltitol, sucrose, and glucose to yield a 50 mg/L spiked solution [2000-fold dilution (w/w)]. Subtract the amount of each analyte measured in the sample before it was spiked from the total amount of each analyte measured in the spiked

sample to yield the amount of spiked analyte recovered. The amount of spiked analyte recovered relative to the known amount added then yields the percent recovery.

Figure 5 shows the separation of carbohydrate and glycol ingredients in cough suppressant using the MA1 column. Figure 6 shows the separation of carbohydrates and glycols in a multisymptom cold/flu formulation. The percent recovery after standard addition [50 mg/L spike, 2000-fold dilution, (w/w)] is presented in Table 5. Percent recovery ranged from 87 to 114% for the glycols, sugar alcohols, and carbohydrates tested.

Table 6 Concentration of carbohydrates in medications

Analyte	Concentration (g/L)	
	Cough Suppressant	Multisymptom Cold/Flu Medication
Propylene glycol	184*	215*
Glycerol	144*	56*
Sorbitol	288*	0.1
Mannitol	13	1.5
Glucose	0.2	0.2
Maltitol	0.2	Not Detected
Sucrose	Not Detected	459*

* Ingredients that are listed on the product containers.

Concentration of Known Ingredients in Pharmaceutical Products

Ingredients in pharmaceutical products that could be identified by retention times are listed in Table 6. Their respective concentrations were determined and also presented in Table 6. The ingredients listed on the product container are marked with an asterisk (*). It is not the normal practice of drug manufacturers to state the concentrations of inactive ingredients on their product labels; therefore, the accuracy of these formulations against the stated label concentrations could not be evaluated.

In addition to the labeled content of the pharmaceutical products, other carbohydrate or glycol ingredients may be present. Unlabeled ingredients are not marked with an asterisk. Trace levels of unlabeled carbohydrates were determined by injecting less dilute samples [1000 and 100-fold dilutions (w/w)]. Expanding the chromatogram for the cough suppressant (Figure 5B) reveals the presence of minor peaks. Besides sorbitol, which is a labeled ingredient of the cough suppressant, the unlabeled ingredients, mannitol and maltitol, were also identified based on their retention time. Mannitol and maltitol are probably trace impurities of sorbitol. Seven other peaks were detected in the cough suppressant (Figure 5B), but were not identified. The multisymptom cold/flu medication contained one unidentified minor peak (Figure 6).

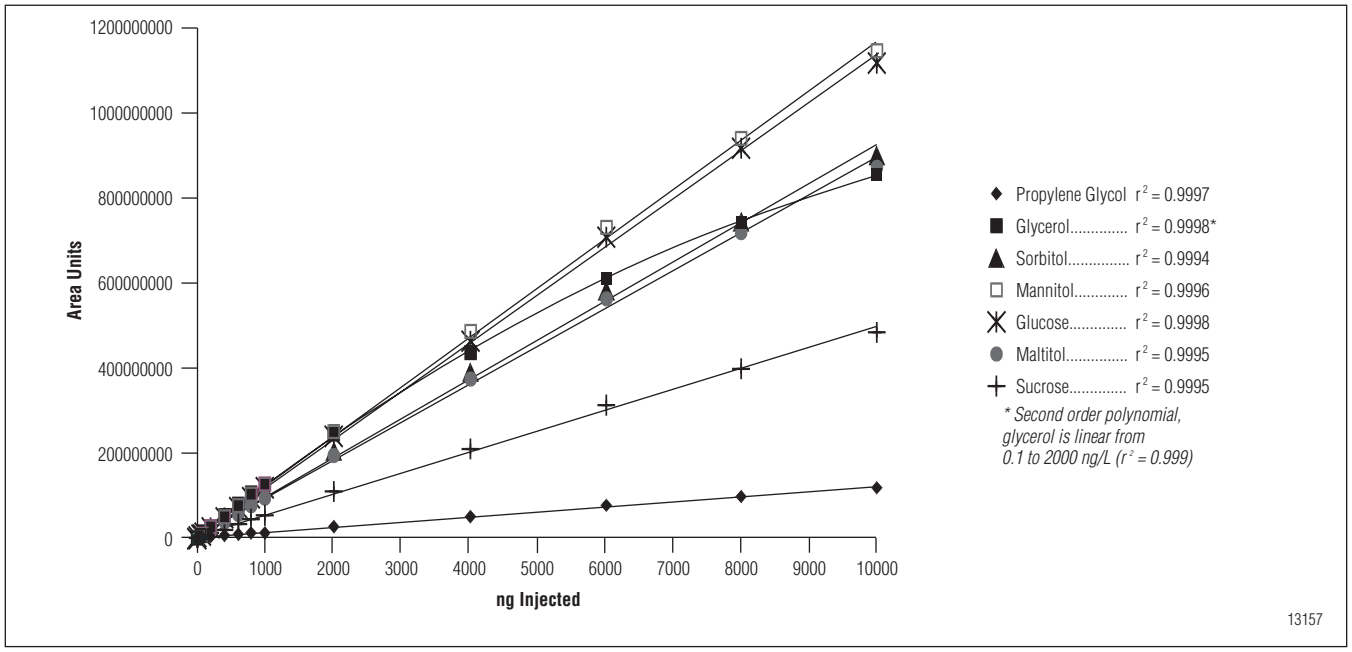


Figure 7. Method linearity for CarboPac MA1 with amperometric detection.

CONCLUSION

Pharmaceutical formulations can be analyzed for both glycols and carbohydrates using the CarboPac MA1 column with amperometric detection. The MA1 resolves glycols such as propylene glycol and glycerol. It also resolves sugar alcohols such as sorbitol, mannitol, maltitol, and carbohydrates such as sucrose and glucose in the same injection. The CarboPac PA10 also resolves sugar alcohols and carbohydrates, but is not suitable for glycols such as propylene glycol and glycerol. The IonPac ICE-AS1 with the NG1 can be used to rapidly separate propylene glycol and glycerol, but is less effective for many carbohydrates and some sugar alcohols. The ICE-AS1 reverses the elution order of glycols and carbohydrates when compared to the CarboPac columns.

All of these columns use isocratic eluents, which simplify analysis. The MA1 is a high-capacity column and generally has longer run times than the PA10 and the ICE-AS1. Amperometric detection eliminates potential interferences from the nonoxidizable ingredients in the formulation and provides a sensitive means to detect nonchromophoric analytes. Carbohydrates, glycols, and sugar alcohols can be detected at the 70–700 µg/L levels using the CarboPac MA1 column with amperometric detection. The three classes of compounds tested were linear over more than three orders of magnitude using this system. The recoveries from pharmaceutical formulations were greater than 87% based on the method of standard addition. This method can also be used to evaluate trace levels of carbohydrate, glycol, and sugar alcohol contaminants.

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7. Dionex Corporation, "Glycoprotein Monosaccharide Analysis Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)", Technical Note 40.

LIST OF SUPPLIERS

Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennsylvania, 15219-4785, U.S.A., 1-800-766-7000.

Aldrich Chemical Company, Inc., 1001 West Saint Paul Avenue, P.O. Box 355, Milwaukee, Wisconsin, 53233, U.S.A., 1-800-558-9160.

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178, U.S.A., 1-800-325-3010.

EM Science, P.O. Box 70, 480 Democrat Road, Gibbstown, New Jersey, 08027, U.S.A., 1-800-222-0342.

Eastman Chemical Company, 1001 Lee Road, Rochester, New York, 14652-3512, U.S.A., 1-800-225-5352.

J. T. Baker Incorporated, 222 Red School Lane, Phillipsburg, New Jersey, 08865, U.S.A., 1-800-582-2537.

Pfanstiehl Laboratories, Inc., 1219 Glen Rock Avenue, Waukegan, Illinois, 60085-0439, U.S.A. 1-800-383-0126.

The Determination of Carbohydrates, Alcohols, and Glycols in Fermentation Broths

INTRODUCTION

Fermentation broths are used in the manufacture of biotherapeutics and many other biological materials produced using recombinant genetic technology, as well as for the production of methanol and ethanol as alternative energy sources to fossil fuels.

Recently, attention has been given to characterizing the ingredients of fermentation broths because carbon sources and metabolic by-products have been found to impact the yield of the desired products. Carbohydrates (glucose, lactose, sucrose, maltose, etc.) are carbon sources essential for cell growth and product synthesis, while alcohols (ethanol, methanol, sugar alcohols, etc.), glycols (glycerol), and organic anions (acetate, lactate, formate, etc.) are metabolic by-products that often reduce yields.

Fermentation broths are complex mixtures of nutrients, waste products, cells, cell debris, and desired products. Many of these ingredients are nonchromophoric and cannot be detected by absorbance. Carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds can be oxidized and detected by amperometry. This detection method is specific for analytes that can be oxidized at a selected potential, leaving all other compounds undetected.

Pulsed amperometric detection (PAD) is a powerful detection technique with a broad linear range and very low detection limits. High-performance anion-exchange chromatography (HPAE) is capable of separating complex mixtures of carbohydrates. For complex samples such as fermentation broths, the high resolving power of HPAE and the specificity of PAD allow the determination of carbohydrates, glycols, sugar alcohols (alditols), and other alcohols such as ethanol and methanol, with little interference from other broth ingredients.[1–3] Although biosensor and flow-injection analyzer-based methods are commonly used to evaluate fermentation broths, these techniques cannot simultaneously determine multiple compounds.[4,5] Refractive index detection has been used for the analysis of fermentation broths, but is limited by poor sensitivity and selectivity.[6,7] Postcolumn derivatization with UV/Vis detection has also been used, but is complicated by the additional reaction chemistry and poor sensitivity.[8,9] HPAE-PAD provides the analytical capability to monitor, without derivatization, a large number of different compounds simultaneously using a single instrument and chromatographic method.

This Application Note describes the use of two different anion-exchange columns with amperometric detection to analyze simple sugars, sugar alcohols, alcohols, and glycols in yeast and bacterial fermentation broths. The yeast *Saccharomyces cerevisiae* in Yeast Extract-Peptone-Dextrose (YPD) broth and the bacteria *Escherichia coli* (*E. coli*) in Luria-Bertani (LB) broth are common eukaryotic and prokaryotic fermentation systems, respectively. Both fermentation broth cultures are complex and contain undefined media ingredients, and thus are a great challenge for most separation and detection technologies. These formulations contain inorganic and organic anionic ingredients that have been analyzed using the IonPac® AS11 and AS11-HC anion-exchange columns with suppressed conductivity detection.[10]

In the methods outlined in this Note, the selectivities of the CarboPac™ PA1 and CarboPac MA1 anion-exchange columns are compared for the analysis of carbohydrate, alcohol, and glycol ingredients in fermentation broths.

The CarboPac PA1 column packing consists of a 10-µm nonporous, highly crosslinked polystyrene/divinylbenzene substrate agglomerated with 350-nm diameter latex. The MicroBead™ latex is functionalized with quaternary ammonium ions, which create a thin surface rich in anion-exchange sites. The PA1 has a unique MicroBead pellicular resin structure that gives it stability from pH 0–14 at all concentrations of buffer salts, and enables excellent mass transfer, resulting in rapid gradient equilibration. The PA1 has an anion-exchange capacity of approximately 100 µeq/column and is specifically designed as a general purpose carbohydrate column.

The CarboPac MA1 resin is composed of a polystyrene/divinylbenzene polymeric core. The surface is grafted with quaternary ammonium anion-exchange functional groups. Its macroporous structure provides an extremely high anion-exchange capacity of 1450 µeq/column. The CarboPac MA1 column is designed specifically for sugar alcohol and glycol separations.

Expected detection limits, linearity, selectivity, and precision are reported for the CarboPac MA1 column using a Dionex DX-500 BioLC® system with pulsed amperometric detection.

EQUIPMENT

Dionex DX-500 BioLC system consisting of:
- GP40 Gradient Pump with degas option
- ED40 Electrochemical Detector
- LC30 or LC25 Chromatography Oven
- AS3500 Autosampler

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Sodium hydroxide, 50% (w/w) (Fisher Scientific and J.T. Baker)

Deionized water, 18 MΩ-cm resistance or higher

Standards

D-Arabinose, anhydrous (Sigma Chemical Co.)
L-Arabitol (Aldrich Chemical Co.)
2,3-Butanediol (Sigma Chemical Co.)
D-Cellobiose, anhydrous (Sigma Chemical Co.)
2-Deoxy-D-glucose, reference grade (Pfanstiehl Laboratories)
Erythritol (Pfanstiehl Laboratories)
Ethanol (EM Science)
D-Fructose, reference grade (Pfanstiehl Laboratories)
Fucose, reference grade (Pfanstiehl Laboratories)
Galactitol, reference grade (Pfanstiehl Laboratories)
D-Galactose, reference grade (Pfanstiehl Laboratories)
Galactosamine, reference grade (Pfanstiehl Laboratories)
D-Glucosamine, reference grade (Pfanstiehl Laboratories)
β-D-Glucose, reference grade (Pfanstiehl Laboratories)
Glycerol (EM Science)
α-Lactose, monohydrate (Sigma Chemical Co.)
Maltose, monohydrate, reference grade (Pfanstiehl Laboratories)
Maltitol (Aldrich Chemical Co.)
Maltotriose, hydrate (Aldrich Chemical Co.)
Mannitol, ACS grade (J.T. Baker Inc.)
Methanol (EM Science)
Raffinose, pentahydrate, reference grade (Pfanstiehl Laboratories)
L-Rhamnose, monohydrate (Pfanstiehl Laboratories)
D-Ribose, reference grade (Pfanstiehl Laboratories)
Ribitol, reference grade (Pfanstiehl Laboratories)
Sorbitol (Eastman Chemical Co.)
Sucrose (Fisher Scientific)
α-α-Trehalose, dihydrate, reference grade (Pfanstiehl Laboratories)
D-Xylose, anhydrous (Sigma Chemical Co.)

Culture and Media

Bacto YPD Broth (DIFCO Laboratories, Cat. No. 0428-17-5)
Bacto Yeast Extract (DIFCO Laboratories, Cat. No. 0127-15-1)
Bacto Peptone (DIFCO Laboratories, Cat. No. 0118-15-2)
LB Broth (DIFCO Laboratories, Cat. No. 0446-17-3)
Yeast, *Saccharomyces cerevisiae*, Bakers Yeast type II (Sigma Chemical Co., Cat. No. 45C-2)
Bacteria, *Escherichia coli* (donated by SRI International)

CONDITIONS

CarboPac MA1 Method:

Columns: CarboPac MA1 Analytical (P/N 44066)
CarboPac MA1 Guard (P/N 44067)

Flow Rates: 0.4 mL/min

Eluent: A: 480 mM Sodium hydroxide

Program:

Time (min)	A (%)
0.0	100
70.0 (End)	100

CarboPac PA1 Method:

Columns: CarboPac PA1 Analytical (P/N 35391)
CarboPac PA1 Guard (P/N 43096)

Flow Rate: 1.0 mL/min

Eluents: A: Water
B: 100 mM Sodium hydroxide
C: 250 mM Sodium hydroxide

Program:

Time (min)	A (%)	B (%)	C (%)
0.00	84	16	0
60.0	84	16	0
60.1	0	0	100
70.0	0	0	100
70.1	84	16	0
90.0 (End)	84	16	0

Common to Both Methods:

Injection Volume: 10 µL

Temperature: 30 ºC

Detection (ED40): Pulsed amperometry, gold electrode

Waveform for the ED40:

Time (s	Potential (V)	Integration
0.00	+0.05	
0.20	+0.05	Begin
0.40	+0.05	End
0.41	+0.75	
0.60	+0.75	
0.61	-0.15	
1.00	-0.15	

PREPARATION OF SOLUTIONS AND REAGENTS

Sodium Hydroxide Eluents

100 mM Sodium hydroxide

It is essential to use high-quality water of high resistivity (18 MΩ-cm) as free of dissolved carbon dioxide as possible. Biological contamination should be absent. Additionally, borate, a water contaminant that can break through water purification cartridges (prior to any other indication of cartridge depletion), can be removed by placing the BorateTrap™ column (P/N 47078) between the pump and the injection valve. It is extremely important to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the columns, causing a loss of chromatographic resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide.

Dilute 10.4 mL of 50% (w/w) sodium hydroxide solution into 1990 mL of water to yield 100 mM sodium hydroxide. Keep the eluents blanketed under 34–55 kPa (5–8 psi) of helium at all times.

250 and 480 mM Sodium hydroxide

When preparing these eluents, follow the same precautions described above for the 100 mM sodium hydroxide eluent.

Sodium Hydroxide (mM)	50% Sodium Hydroxide (mL)	Water (mL)
250	26	1974
480	50	1950

Keep the eluents blanketed under 34–55 kPa (5–8 psi) of helium at all times. On-line degassing is necessary because the amperometric detector is sensitive to oxygen in the eluent. Set the pump to degas for 30 seconds every 4 minutes.

STOCK STANDARDS

Keep solid standards desiccated and under vacuum prior to use. Dissolve in purified water to 10 g/L concentrations. Combine and further dilute with purified water to yield the desired stock mixture concentrations. Maintain the solutions frozen at –20 °C until needed.

For determinations of linear range and lower detection limits, combine 10 g/L solutions of 2,3-butanediol, glycerol, erythritol, rhamnose, arabitol, sorbitol, galactitol, mannitol, arabinose, glucose, galactose, lactose, ribose, sucrose, raffinose, and maltose to make a 100 mg/L standard mix solution. Add methanol to this mix at a 10 g/L concentration. Dilute serially with water to final desired concentrations. This study used the following concentrations: 90, 80, 70, 60, 50, 40, 30, 20, 10, 8, 6, 4, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.08, 0.06, and 0.04 mg/L. Methanol concentrations ranged from 9 to 0.004 g/L after dilution. Prepare two standard solutions at concentrations of 1 g/L. One solution contained 2,3-butanediol, erythritol, arabitol, galactitol, arabinose, galactose, and ribose. The other solution contained glycerol, rhamnose, sorbitol, mannitol, glucose, lactose, sucrose, raffinose, and maltose. Dilute these solutions to 0.8, 0.6, 0.4, and 0.2 g/L.

SAMPLE PREPARATION

Yeast Fermentation Broth Culture—Standard Media

In a sterile 500-mL Erlenmeyer flask, dissolve 10 g of Bacto YPD broth (DIFCO Laboratories, Cat. No. 0428-17-5) in 200 mL of purified sterile water. Bacto YPD broth contains 2 g of Bacto Yeast Extract, 4 g of Bacto Peptone, and 4 g of dextrose (glucose) per 10 g. Dissolve 1.0 g of yeast (*Saccharomyces cerevisiae*; Bakers Yeast type II; Sigma Chemical Co., Cat. No. 45C-2) in the YPD broth. Cap the flask with a vented rubber stopper. Incubate the culture in a 37 °C shaking water bath (500–600 rpm) for 24 hours. Remove aliquots at designated time points and place on ice.

For this study, samples were taken after the addition of yeast at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 24 hour intervals. The incubation began when yeast was added to the media. Aliquots were centrifuged at 14,000 × g for 10 minutes and diluted 100-fold in purified water. Diluted supernatant (10 µL) was analyzed directly.

Yeast Fermentation Broth Culture—Modified Multiple Carbohydrate Media

In a sterile 500-mL Erlenmeyer flask, dissolve 2 g of Bacto Yeast Extract (DIFCO Laboratories, Cat. No. 0127-15-1), 4 g of Bacto Peptone (DIFCO Laboratories, Cat. No. 0118-15-2), and 4 g of carbohydrates (0.4 g each of glucose, sucrose, maltose, lactose, galactose, sorbitol, ribose, arabinose, rhamnose, and raffinose) in 200 mL of purified sterile water. Dissolve 1.0 g of yeast (Saccharomyces cerevisiae; Bakers Yeast type II; and Sigma Chemical Co., Cat. No. 45C-2) in the YPD broth. Incubate and sample the culture as described for the standard media.

E. coli Fermentation Broth Culture—Standard Media

Dissolve LB broth to a concentration of 25 g/L with water, heat to a boil, and autoclave for 15 minutes at 121 psi. LB broth contains 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per 25 g. Incubate and sample the culture as described for the yeast standard media.

RESULTS AND DISCUSSION

Selectivity

CarboPac MA1

Figure 1 shows the separation of alcohols (2,3-butanediol, ethanol, methanol), glycols (glycerol), alditols (erythritol, arabitol, sorbitol, galactitol, mannitol), and carbohydrates (rhamnose, arabinose, glucose, galactose, lactose, sucrose, raffinose, maltose) commonly found in fermentation broths using a CarboPac MA1 column set with 480 mM sodium hydroxide eluent flowing at 0.4 mL/min. The alcohols, sugar alcohols (alditols), glycols, and carbohydrates are well resolved. Maltose elutes last at about 60 minutes.

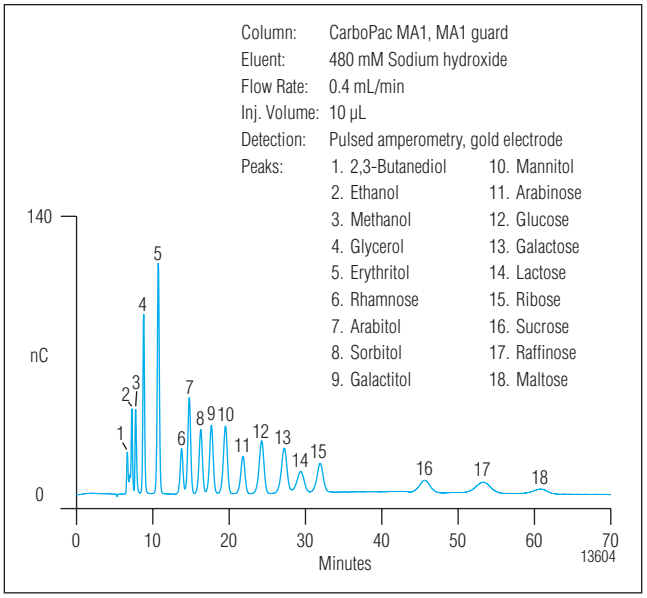

Figure 1. Common carbohydrates, alditols, alcohols, and glycols found in fermentation broths separated on the CarboPac MA1 column with pulsed amperometry.

The retention times of common fermentation broth carbohydrates under these conditions are listed in Table 1. Generally, alcohols elute first, followed by glycols, alditols, monosaccharides, disaccharides, and trisaccharides. Ethanol, methanol, and 2,3-butanediol are resolved, but the ethanol response is about 570 times less than the glucose response, and methanol is about 3600 times less responsive than glucose by mass. This can be advantageous when relatively large amounts of ethanol or methanol are produced, such as in the manufacture of alcoholic beverages and in the generation of alternative energy sources. Some large carbohydrates such as maltotriose could not be eluted under these conditions and are best analyzed using the CarboPac PA1 column.

Table 1. Retention Times for Carbohydrates and Alcohols on the CarboPac MA1

Analyte	Retention Time (min)
2,3-Butanediol	6.6
Ethanol	7.3
Methanol	7.7
Glycerol	8.7
Erythritol	10.7
Rhamnose	13.6
Fucose	13.8
Arabitol	14.7
Galactosamine	14.7
Glucosamine	15.3
Sorbitol	16.1
Trehalose	17.0
Galactitol	17.6
Ribitol	17.8
Mannitol	19.4
2-Deoxy-D-Glucose	20.2
Mannose	21.6
Arabinose	21.6
Glucose	24.0
Xylose	24.6
Galactose	27.0
Maltitol	27.7
Lactose	28.9
Fructose	29.0
Ribose	31.5
Cellobiose	43.2
Sucrose	44.9
Raffinose	51.8
Maltose	59.4
Maltotriose	>60.0

CarboPac PA1

Figure 2 shows the analysis of common fermentation broth alcohols, glycols, alditols, and carbohydrates using the CarboPac PA1 column. The elution order of the PA1 is similar to the MA1. Alcohols elute first, followed by glycols, alditols, and mono-, di-, and trisaccharides. At elevated eluent strengths (e.g., 100–250 mM sodium hydroxide), many larger carbohydrates separate in under 20 minutes. For example, maltotriose, which elutes after 60 minutes on the MA1, elutes at 25.8 minutes with a 250 mM sodium hydroxide eluent on the PA1. Under these conditions, the early eluting peaks (e.g., 2,3-butanediol, methanol, and ethanol) coelute.

Table 2 summarizes the retention times for common fermentation analytes using the PA1 column at 16, 50, 100, and 250 mM sodium hydroxide eluent conditions with 1 mL/min flow rates. These results demonstrate that adjustment of the eluent strength modifies column selectivity, sometimes changing analyte elution order. For example, at 16 mM sodium hydroxide, sucrose eluted slightly before ribose; yet at 50, 100, or 250 mM, ribose eluted significantly ahead of sucrose. Therefore, some separations can be improved by adjusting eluent strength. Adjusting eluent strengh can increase or decrease the peak area response of some analytes. Generally, response increases with increased eluent strength, but can decrease for some analytes. A decrease in response at elevated eluent strengths is probably due to hydroxide ions competing with analytes for sites on the electrode surface.

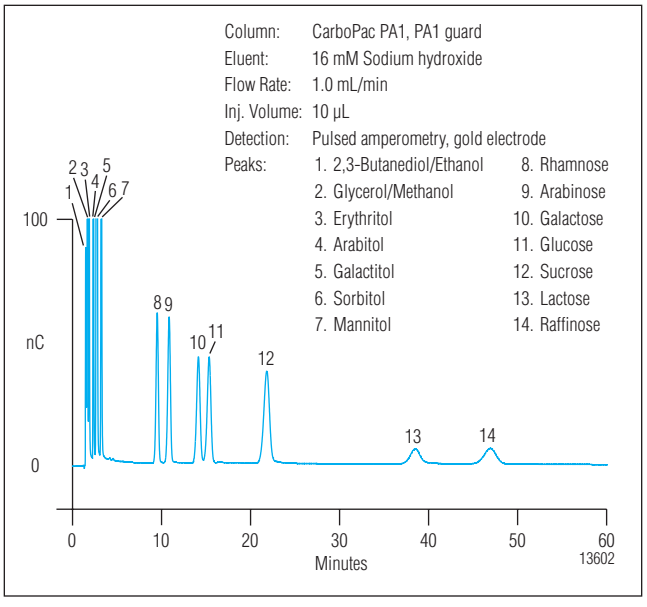

Figure 2. Common carbohydrates, alditols, alcohols, and glycols found in fermentation broths separated on the CarboPac PA1 column.

Table 2 CarboPac PA1 with PA1 Guard Retention Times (Minutes)

Analyte	Sodium Hydroxide Concentration at 1.0 mL/min			
	16 mM	50 mM	100 mM	250 mM
2,3-Butanediol	1.5	1.5	1.5	1.5
Ethanol	1.6	1.6	1.6	1.6
Glycerol	1.7	1.7	1.7	1.7
Methanol	1.7	1.7	1.7	1.7
Erythritol	1.9	1.8	1.8	1.8
Arabitol	2.4	2.3	2.2	2.1
Galactitol	2.7	2.5	2.5	2.3
Ribitol	2.8	2.6	2.5	2.3
Sorbitol	2.8	2.6	2.5	2.2
Mannitol	3.3	3.0	2.8	2.4
Trehalose	3.7	3.3	3.1	2.6
Fucose	5.5	4.0	3.2	2.3
Maltitol	8.9	7.1	5.9	3.9
2-Deoxy-D-Glucose	9.3	6.2	4.6	3.0
Rhamnose	9.6	5.4	3.8	2.5
Galactosamine	10.7	6.2	4.4	2.7
Arabinose	10.9	6.6	4.6	2.9
Glucosamine	12.7	6.9	4.7	2.8
Galactose	14.3	8.4	5.8	3.3
Glucose	15.5	8.7	5.8	3.3
Mannose	16.9	8.6	5.5	3.1
Xylose	17.3	9.2	6.0	3.3
Fructose	20.4	10.3	6.5	3.6
Sucrose	21.7	15.4	10.9	6.0
Ribose	22.0	11.1	7.0	3.8
Lactose	38.5	18.9	10.7	5.0
Raffinose	47.1	31.0	21.1	9.3
Cellobiose	>60	31.8	17.7	6.9
Maltose	>60	55.7	27.0	9.5
Maltotriose	>60	>60	43.5	25.8

The CarboPac PA1 is similar to the CarboPac PA10. The PA10 is solvent-compatible and has better resolution between amino and neutral sugars. In some cases, the PA10 has a slightly different selectivity. For example, sucrose and fructose coelute on the PA10 at an eluent strength of 16–18 mM sodium hydroxide, but are well resolved on the PA1 column. At low eluent strengths, sucrose and ribose coelute on the PA1 column, but are resolved on the PA10 column, especially at lower sodium hydroxide concentrations (10 mM).

Figures 1 and 2 and Tables 1 and 2 show that the CarboPac MA1 and PA1 columns have different selectivities and therefore different strengths for determining the alcohols and carbohydrates in fermentation broths. Column choice will be dictated by the analytes, their concentrations, and the desired analysis time.

METHOD DETECTION LIMITS

The method detection limits (MDL) for a 10-µL injection of common fermentation broth constituents using the MA1 column are shown in Table 3. The MDL is defined as the minimum concentration required to produce a peak height signal-to-noise ratio of 3. The MDL can be further decreased by increasing the injection volume above the 10-µL injection volume used in this Application Note, and by using smoothing algorithms available in PeakNet software.[11–13] Detection limits generally increase with longer retention times because of peak broadening. Methanol and ethanol have higher detection limits because their response factors are lower.

LINEARITY

Glycerol, 2,3-butanediol, erythritol, rhamnose, arabitol, sorbitol, galactitol, mannitol, arabinose, glucose, galactose, lactose, ribose, sucrose, raffinose, and maltose standards ranging from 0.1 to 1000 mg/L (1 to 10,000 ng) were injected (n=2 to 3 per concentration) onto a CarboPac MA1 column. Figure 3 shows that the method was linear for 2,3-butanediol, rhamnose, arabitol, sorbitol, mannitol, arabinose, glucose, galactose, lactose, ribose, sucrose, raffinose, and maltose over this range (r^2=0.998–0.999). Glycerol was linear over the range of

Table 3. Estimated Detection Limits* Using the CarboPac MA1 with Pulsed Amperometry

Analyte	ng	µg/L**
2,3-Butanediol	1	100
Ethanol	300	30000
Methanol	7000	700000
Glycerol	0.4	40
Erythritol	0.2	20
Rhamnose	1	100
Arabitol	0.5	50
Sorbitol	0.8	80
Galactitol	0.7	70
Mannitol	0.7	70
Arabinose	1	100
Glucose	0.9	90
Galactose	1	100
Lactose	2	200
Ribose	1	100
Sucrose	4	400
Raffinose	5	500
Maltose	9	900

*Lower Limit of Detection is Based on 3 X Baseline Noise
**10 µL Injections

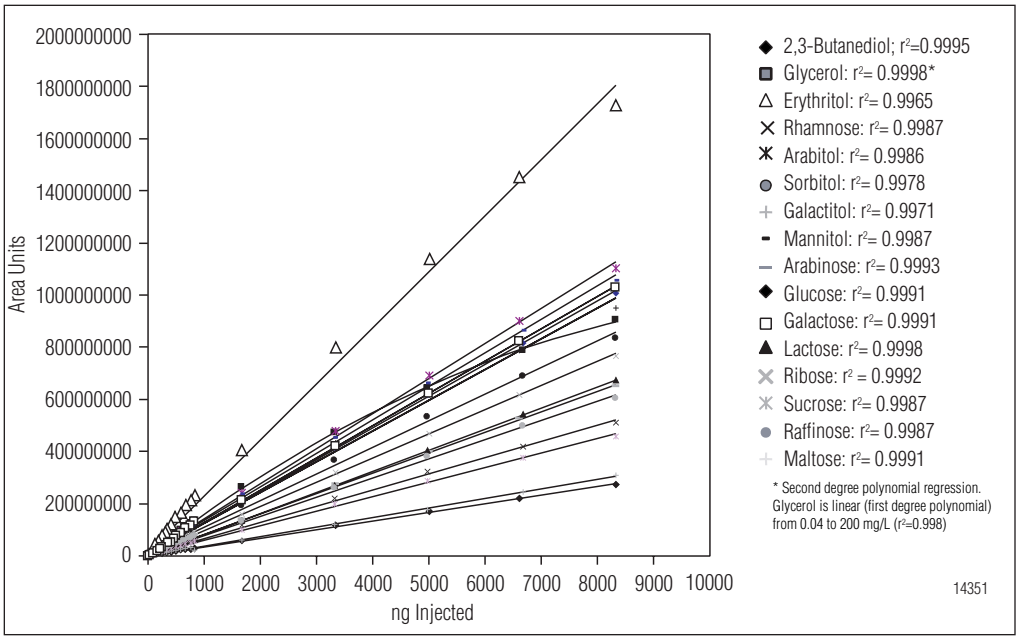

Figure 3. Method linearity using the CarboPac MA1 with pulsed amperometric detection.

0.04–200 mg/L (0.04–2000 ng, r^2=0.998); erythritol over the range of 0.04–100 mg/L (0.4–1000 ng, r^2=0.999); and galactitol over the range of 0.07–100 mg/L (0.7–1000 ng, r^2=0.998). For the range of 0.1–1000 mg/L (1–10,000 ng), glycerol, erythritol, and galactitol deviated from linearity (r^2=0.987, 0.997, 0.997, respectively). Using a second order polynomial regression, the r^2 for glycerol, erythritol, and galactitol was 1.000. For all analytes, linearity was demonstrated over at least three orders of magnitude, and for most analytes, over four orders of magnitude. Broad linear ranges help reduce the need to dilute the sample and repeat the analysis when components vary greatly in concentration.

PRECISION AND STABILITY

Peak area and retention time RSDs were determined for replicate injections of common carbohydrates, alditols, alcohols, and glycols spiked into yeast fermentation broth. Common fermentation broth carbohydrate and alcohol standards were added (10 mg/L) to heat-treated yeast fermentation broth culture supernatant and then analyzed over 48 hours (10 µL per injection, 42 injections) on the MA1 column. Results for precision are presented in Table 4.

Figures 4 and 5 show the stability of peak area and retention time for fermentation broths analyzed over 48 hours. At this concentration, peak area RSDs ranged from 2 to 7%, and retention time RSDs ranged from 0.2 to 0.4%. Precision is affected by concentration (i.e., RSD values increase as the concentrations approach the MDL).

Table 4. Peak Area and Retention Time Precision over 48 Hours, RSD (%)

Analyte	Peak Area Units	Retention Time
2,3-Butanediol	2.4	0.2
Ethanol	2.7	0.2
Glycerol	2.0	0.2
Erythritol	3.4	0.2
Rhamnose	1.8	0.2
Arabitol	3.0	0.2
Sorbitol	2.7	0.2
Galactitol	2.7	0.3
Mannitol	2.7	0.3
Arabinose	3.1	0.3
Glucose	3.3	0.2
Galactose	3.5	0.3
Lactose	3.6	0.3
Ribose	3.1	0.3
Raffinose	4.8	0.4
Maltose	6.8	0.4

DETERMINATION OF CARBOHYDRATES, ALDITOLS, ALCOHOLS, AND GLYCOLS IN FERMENTATION BROTH CULTURES

Yeast (*Saccharomyces cerevisiae*) Culture

Yeast was grown in Bacto YPD broth at 37 ˚C for up to 24 hours. Figure 6 shows the separation of fermentation broth ingredients in a yeast culture at the beginning (Figure 6A) and after 24 hours (Figure 6B) of incubation. At the beginning of the culture, the glucose (dextrose) component was prominent. Ethanol was found at a relatively high concentration, along with trace levels of glycerol, erythritol, rhamnose, trehalose, arabinose, and cellobiose. During the first 3 hours, glucose levels decreased, and after 3 hours glucose was not detected (data not shown).

Glycerol increased over the same time period and remained constant after 3 hours. Ethanol concentration remained constant up to 7 hours. Between 7 and 24 hours, ethanol concentration decreased, presumably due to evaporative losses. Erythritol and rhamnose concentrations did not change, cellobiose concentration decreased by 50%, and trehalose and arabinose were depleted between 7 and 24 hours.

When the cell culture broth was modified to contain ten different carbohydrates and alditols, at the same total carbohydrate concentration as the standard Bacto YPD broth, it was apparent that yeast prefer to use certain carbohydrates over others, and that some carbohydrates or alditols could not be used as a carbon source during the 24-hour incubation period. Figure 7 shows the concentration of broth components over 24 hours. Glucose and raffinose were metabolized within the first hour. After one hour, the yeast began to consume maltose and galactose. Rhamnose, sorbitol, arabinose, lactose, and ribose were either unchanged or decreased slightly over 24 hours. Glycerol increased for the first hour, and then leveled off. Sucrose could not be measured, even at 0.15 hours (9 min) of incubation, which was the earliest time point possible due to the time required for full yeast dissolution. Sucrose was probably digested by the extracellular enzyme invertase, which is present in large amounts in the dried yeast. Invertase will cleave sucrose into its monosaccharides, glucose and fructose. Glucose was measured at levels higher than expected at the first time point, which supports this hypothesis.

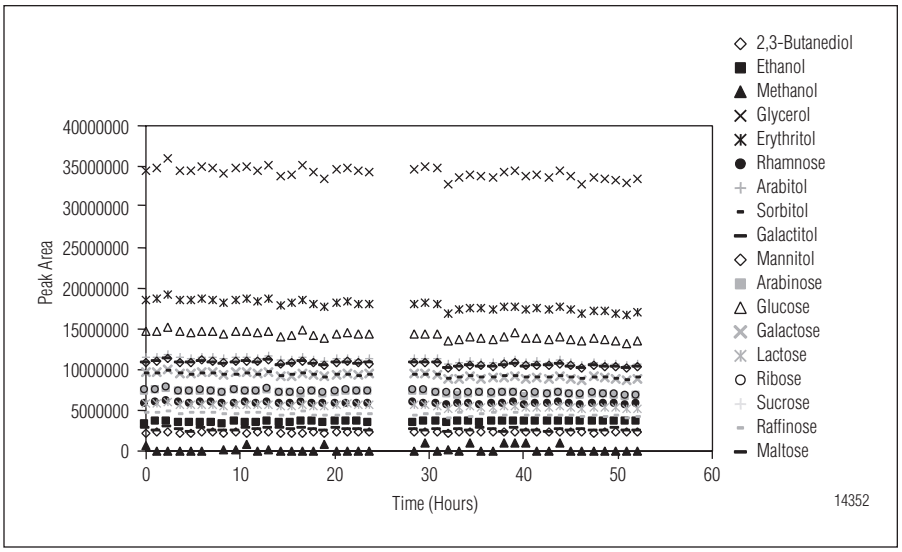

Figure 4. Stability of peak area over 48 hours for fermentation broth analysis. Injections 25 through 28 were standards in water.

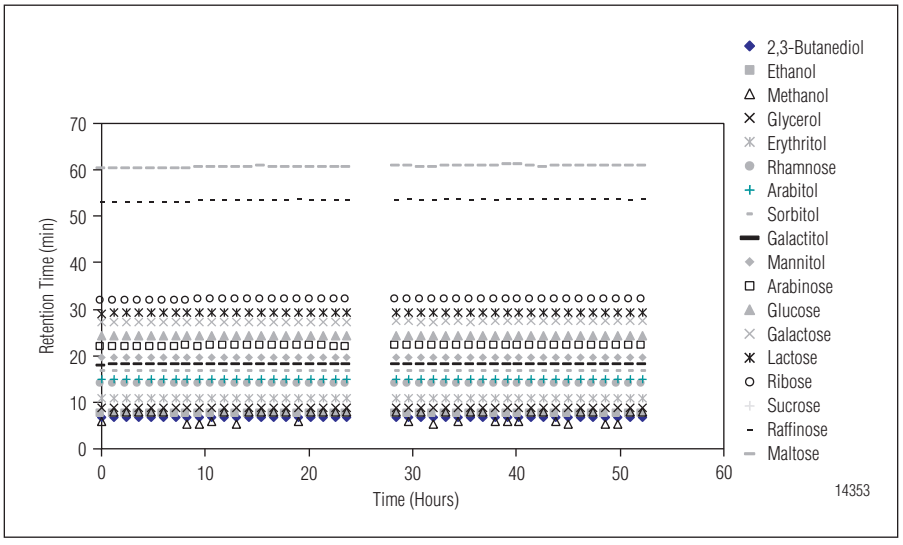

Figure 5. Stability of retention time over 48 hours for fermentation broth analysis. Injections 25 through 28 were standards in water.

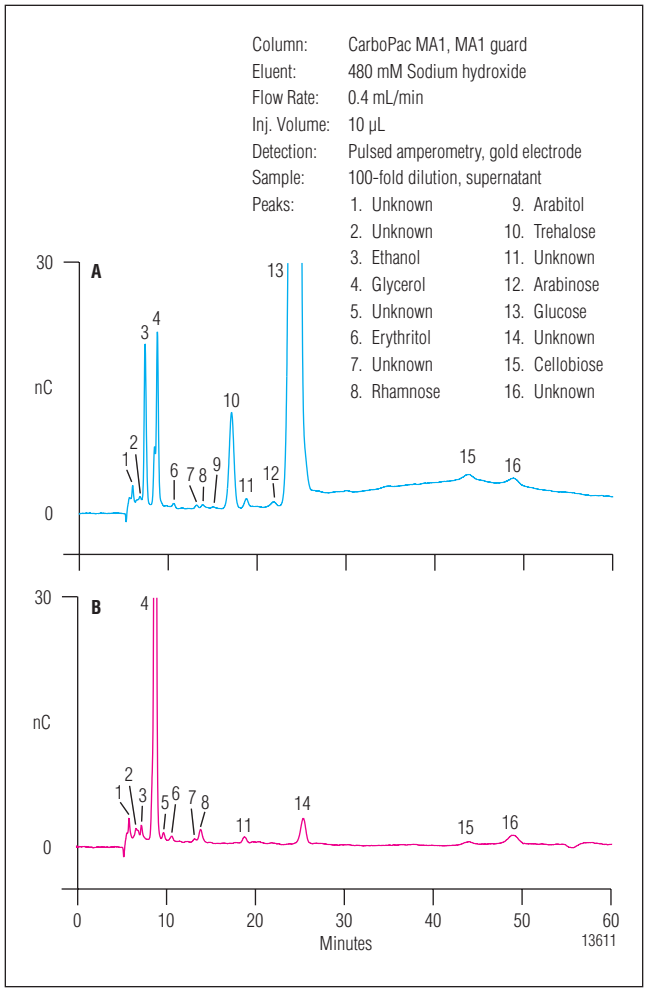

Figure 6. Saccharomyces cerevisiae *fermentation broth culture using the CarboPac MA1 column, at (A) 0 hours of incubation and (B) 24 hours of incubation.*

E. coli Culture

E. coli was grown on LB broth for 24 hours at 37 °C. Figure 8 shows that only trace levels of carbohydrates were found in this media. Erythritol, arabitol, arabinose, lactose, and maltose were identified by retention time in the starting media. No glucose was measured. After 24 hours, trace levels of 2,3-butanediol, erythritol, mannitol, glucose, and galactose were measured. Many unidentified peaks were consumed during this incubation period.

CONCLUSION

These results show that both yeast and bacterial fermentation broths can be analyzed for carbohydrate composition using high-performance anion-exchange chromatography and pulsed amperometry. Two columns (CarboPac MA1 and CarboPac PA1) are available for the analysis of fermentation broth carbohydrates, alcohols, alditols, and glycols. The MA1 provides excellent separation of early eluting compounds such as alcohols, glycols, alditols, and monosaccharides. The run times are long for more complex carbohydrates such as trisaccharides. Separations using the PA1 are faster and can effectively separate di- and trisaccharides. Complex mixtures of carbohydrates and alditols can be monitored simultaneously, providing the analyst with information that is needed to optimize the fermentation.

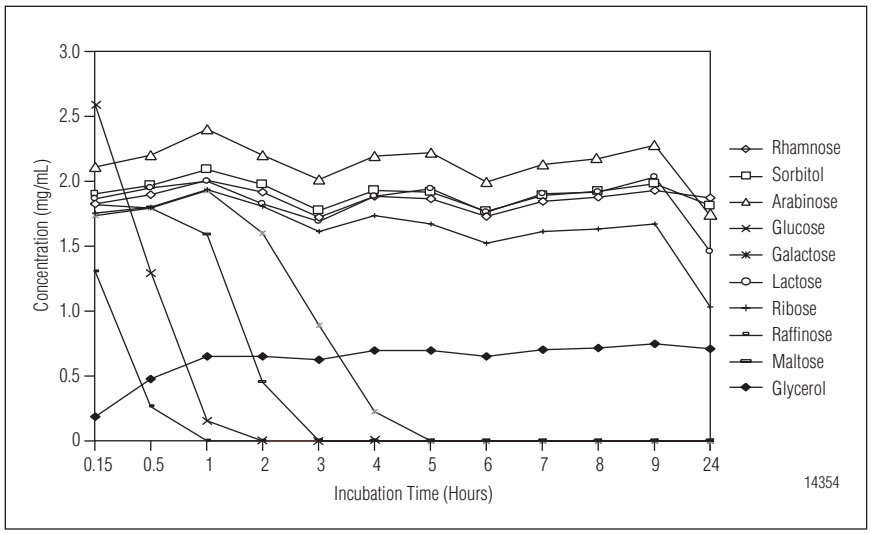

Figure 7. Saccharomyces cerevisiae *culture grown on fermentation broth consisting of multiple carbohydrates and alditols, analyzed on a CarboPac MA1 with integrated amperometry.*

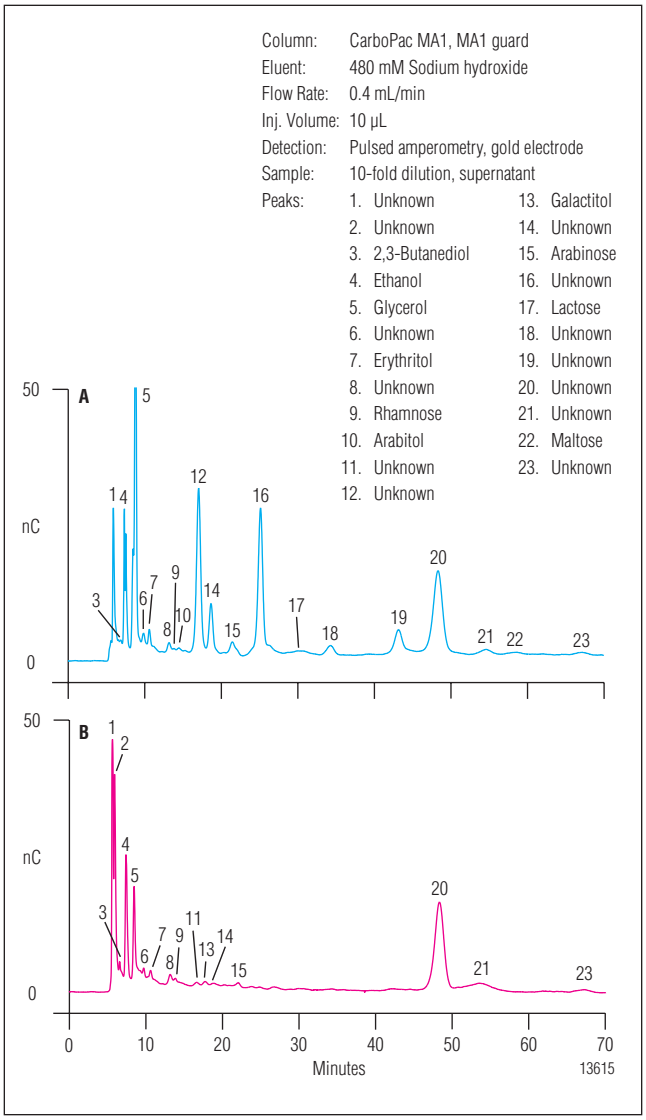

Figure 8. E. coli *fermentation broth culture using the CarboPac MA1 column, at (A) 0 hours of incubation and (B) 24 hours of incubation.*

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LIST OF SUPPLIERS

Aldrich Chemical Company, Inc., 1001 West Saint Paul Avenue, P.O. Box 355, Milwaukee, Wisconsin, 53233, USA. Tel.: 1-800-558-9160.

J.T. Baker Incorporated, 222 Red School Lane, Phillipsburg, New Jersey, 08865, USA. Tel.: 1-800-582-2537.

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EM Science, P.O. Box 70, 480 Democrat Road, Gibbstown, New Jersey, 08027, USA. Tel.: 1-800-222-0342.

Fisher Scientific, 711 Forbes Avenue, Pittsburgh, Pennsylvania, 15219-4785, USA. Tel.: 1-800-766-7000.

Pfanstiehl Laboratories, Inc., 1219 Glen Rock Avenue, Waukegan, Illinois, 60085-0439, USA. Tel.: 1-800-383-0126.

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178, USA. Tel.: 1-800-325-3010.

Determination of Ten Active Ingredients in Sunscreen-Containing Products in a Single Injection

INTRODUCTION

To prevent skin damage from the sun's radiation, many skin care products, such as lipstick, makeup, and lotions contain one or more compounds to block UV radiation. The lotions containing these compounds are commonly referred to as sunscreens and other products that contain one or more of these compounds are said to contain sunscreen. The active ingredients in sunscreens are usually aromatic compounds conjugated with carbonyl groups (e.g. homosalate) and rather than literally blocking the UV radiation, they absorb it and release it as lower-energy UV radiation. The FDA allows over 15 different compounds to be used as the active ingredient in sunscreens and sunscreen-containing products. Additional compounds have been approved for use in the European Union and other parts of the world.

In this Application Note (AN) we developed a separation of the following 10 compounds used in sunscreen: 2-phenyl-benzimidazole-5-sulfonic acid, benzophenone-3, diethylamino-hydroxybenzoylhexyl benzoate, 4-methylbenzylidine-camphor, octocrylene, methylanthranilate, octyl-methoxycinnamate, butyl-methoxydibenzoylmethane, octyl-salicylate, and homosalate. A manufacturer of sunscreen products chose these 10 compounds and requested baseline resolution of all 10 in a single injection.

Using a 3-µm Acclaim® 120 C18 column with an ethanol-containing mobile phase we were able to baseline resolve all 10 compounds in less than 12 min. This method successfully determined subsets of these 10 compounds in a lipstick, a cosmetic powder, and a lotion provided by the manufacturer. The Acclaim 120 C18 column paired with a Dionex UltiMate® 3000 system is an ideal platform for developing methods to determine sunscreen ingredients in a variety of products.

EQUIPMENT

Dionex UltiMate 3000RS chromatography system consisting of:

- SRD-3600 Solvent Rack with integrated vacuum degasser
- HPG-3400RS Binary gradient pump with 200 µL static mixer kit (P/N 6040.5150)
- WPS-3000RS split loop sampler with 100 µL sample loop
- TCC-3000RS Thermostatted column compartment
- DAD-3000RS Diode array detector

Chromeleon® Chromatography Data System, Version 6.80 SP5

REAGENTS AND STANDARDS

Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistivity or better

Absolute ethanol ($C2H_5OH$), AR grade (LAB-SCAN)

Methanol (CH_3OH), HPLC grade (LAB-SCAN)

Glacial acetic acid (CH_3COOH), AR grade (LAB-SCAN)

2-Phenyl-Benzimidazole-5-sulfonic acid (PHS)

Benzophenone-3 (B-3)

Diethylamino-hydroxybenzoylhexyl benzoate (DHHB)

4-Methylbenzylidine-camphor (4-MBC)

Octocrylene (OCR)

Methylanthranilate (MA)

Octyl-methoxycinnamate (OMC)

Butyl-methoxydibenzoylmethane (BMDM)

Octyl-salicylate (OS)

Homosalate (HMS)

CHROMATOGRAPHIC CONDITIONS

Column:	Acclaim 120 C18 3 µm, 4.6 × 100 mm (P/N 059132)
Eluent:	A: 0.8% Acetic acid B: Ethanol
Eluent Gradient:	25% B from -5 to 1 min, 25 to 80% from 1 to 1.5 min, and 80% B from 1.5 to 11.5 min
Flow rate:	0.7 mL/min
Column Temp.:	25 ºC
Inj. Volume:	5 µL
Detection:	UV, 310 and 354 nm, Wavelength scanning 250–600 nm
Backpressure:	2600–2900 psi

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent A

0.8% Acetic acid

Add approximately 100 mL deionized water to a 1000 mL volumetric flask, pipet 8 mL of glacial acetic acid to the same volumetric flask, bring to volume with deionized water, and mix.

Standards

Stock Standard Solutions

To prepare a 1000 mg/L stock standard for each of ten compounds, weigh 0.1 g of the compound, add to a 100 mL breaker, add 70 mL of methanol, and place in an ultrasonic bath for 10 min to ensure dissolution. Move this solution to a 100 mL volumetric flask and bring it to volume with methanol.

Working Standard Solutions

To prepare the five mixed standard solutions with analyte concentrations of 10, 25, 35, 50, and 75 mg/L, pipet 100, 250, 350, 500, and 750 µL of the individual stock standards into 100 mL volumetric flasks and bring to volume with methanol. Filter each standard with a 0.2 µm nylon filter prior to analysis.

Sample Preparation

Three products containing sunscreen compounds were provided by a customer. These products were a lotion, a lip balm, and a cosmetic powder. The customer also provided versions of these products without sunscreen compounds and, in this note, we refer to these as placebo products. Accurately weigh 0.1 g of sample and place in a 100 mL breaker. Add 70 mL of methanol, and place in an ultrasonic bath for 10 min to ensure dissolution. Move the sample solution to a 100 mL volumetric flask and bring to volume with methanol. Filter this sample solution with a 0.2 µm nylon filter prior to analysis.

RESULTS AND DISCUSSION

Separation

The 10 compounds in this study are all ideal candidates for reversed-phase chromatography with UV detection. A spectral scan of the ten compounds revealed that eight of them would be ideally detected at 310 nm and the other two at 354 nm. We chose the Acclaim 120 C18 column because it contains small-pore, high-purity, low-metal content silica with high C18 surface coverage (i.e. high carbon load), ideal for developing high resolution separations of compounds typically determined by reversed-phase chromatography. Using a methanol/acetic acid mobile phase we were unable to achieve a separation with all resolution factors 2.0 or greater.Switching to an ethanol/acetic acid mobile phase yielded the required separation (Figure 1). The

Table 1. Resolution and Peak Purity of the Ten Sunscreen Ingredient Standards in an Injection of a Mixed Standard (35 mg/L) with Detection at 310 nm (Wavelength Scanning 250–600 nm for Peak Purity)

Compound	Resolution* (USP)	Match	RSD Match	PPI (nm)	RSD PPI
2-Phenyl-benzimidazole -5-sulfonic acid (PHS)	20.53	1000	0.02	279.6	0.01
Benzophenone-3 (B-3)	7.17	1000	0.11	284.4	0.04
Diethylamino-hydroxybenzoylhexyl-benzoate (DHHB)	3.64	1000	0.39	335.6	0.11
4-Methylbenzylidine-camphor (4-MBC)	3.07	1000	0.21	281.0	0.07
Octocrylene (OCR)	2.57	999	0.74	285.0	0.25
Methylanthranilate (MA)	2.93	976	5.56	313.5	0.88
Octyl-methoxycinnamate (OMC)	2.52	999	0.38	284.7	0.12
Butyl-methoxydibenzoylmethane (BMDM)	4.38	1000	0.20	332.9	0.06
Octyl-salicylate (OS)	3.32	986	3.37	283.8	0.59
Homosalate (HMS)	n.a.	995	1.22	285.5	0.20

* All values in this table were calculated by Chromeleon.

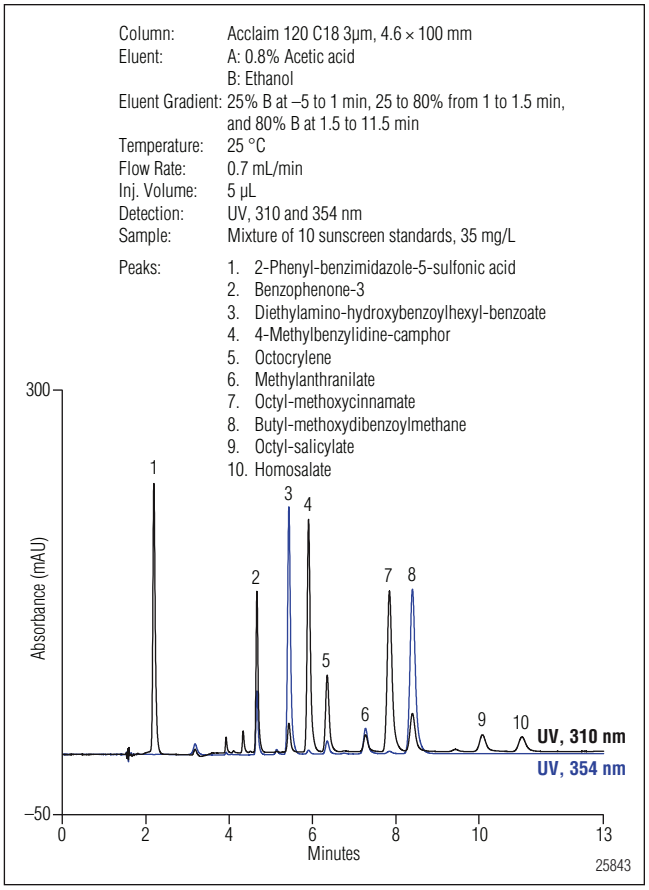


Figure 1. Chromatogram of a mix of 10 sunscreen ingredient standards with detection at 310 nm (Black) and 354 nm (Blue).

resolution of all 10 components is greater than 2.5. Spectral matching of each peak compared to the spectral library (loaded by making single injections of a each of the standards) showed high purity of all ten peaks (Table 1). The low RSD of the peak purity index (PPI) of all ten peaks also indicates peak purity.

Table 2. Calibration Data from Chromeleon for 10 Sunscreen Ingredient Standards at 310 nm, Unless Otherwise Noted

Compound	Cal.Type	Points	R-Squared	Slope
PHS	Lin	5	0.9994	0.4408
B-3	Lin	5	0.9994	0.2347
DHHB	Lin	5	0.9983	0.0578
DHHB – 354 nm	Lin	5	0.9993	0.4991
4-MBC	Lin	5	0.9992	0.5007
OCR	Lin	5	0.9992	0.1876
MA	Lin	5	0.9994	0.0517
OMC	Lin	5	0.9992	0.5248
BMDM	Lin	5	0.9989	0.1352
BMDM- 354 nm	Lin	5	0.9993	0.6049
OS	Lin	5	0.9986	0.0678
HMS	Lin	5	0.9989	0.0681

Method Calibration

Before sample analysis, the 10 sunscreen compounds was separated at 5 concentrations: 10, 25, 35, 50, and 75 mg/L, and the data used to prepare a calibration curve that was forced through the origin. Table 2 displays the calibration data and shows a good linear fit for all ten compounds between 0 and 75 mg/L.

Sample Analysis

The manufacturer provided three products containing sunscreen compounds, a lipstick, a cosmetic powder, and a lotion. They also supplied the same products without added sunscreen compounds, referred to here as placebo products. We analyzed each of the placebo products after sample preparation to determine if there were any peaks from the sample that would interfere with sample analysis. None of the three products contained interfering compounds. Figure 2 shows the chromatogram of the cosmetic powder placebo. Chromatography of the lipstick and lotion placebos was indistinguishable from Figure 2.

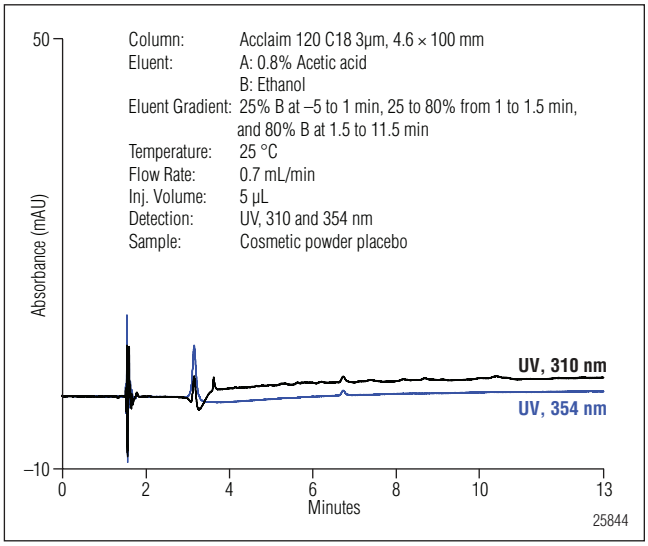


Figure 2. Chromatography of a cosmetic powder placebo with detection at 310 nm (Black) and 354 nm (Blue). The other 2 placebos yielded the same result.

To evaluate recovery, we spiked each placebo product with the 35 mg/L mixed standard. Table 3 shows that there was excellent recovery of all 10 compounds from each of the 3 samples, suggesting that this method is accurate for the determination of these compounds in the three products. Figure 3 shows chromatography of the lotion placebo product spiked with the 35 mg/L mixed standard. Chromatography of the spiked lipstick and cosmetic powder placebos was nearly identical to Figure 3.

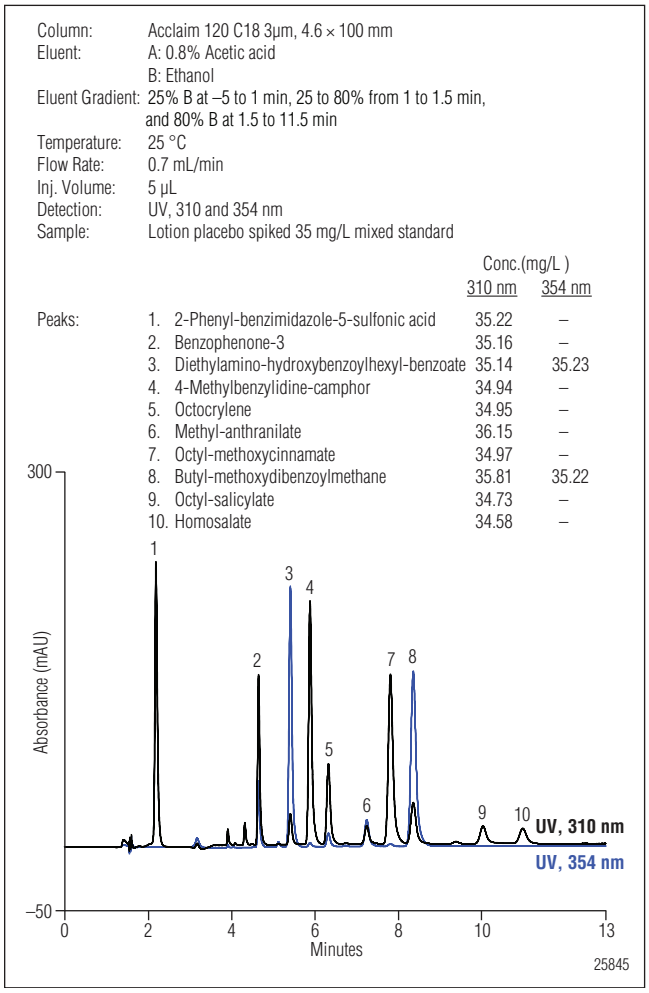


Figure 3. Overlay of three injections of a lotion placebo spiked with a 35 mg/L mixed standard with detection at 310 nm (Black) and 354 nm (Blue)

Finally we determined the amounts of the 10 sunscreen ingredients in 3 injections of each of the 3 products. The cosmetic powder sample was found to contain MA, OMC, and OS (Figure 4), the lotion sample contained PHS, B-3, 4-MBC, OMC, OS, and HMS (Figure 5), and the lipstick sample contained PHS, B-3 DHHB, 4-MBC, OMC, BMDM, OS, and HMS (Figure 6). Table 4 summarizes the amount of each sunscreen compound found in each sample.

Table 3. Recovery Results for the Spiked (35 mg/L) Cosmetic Powder, Lotion, and Lipstick Placebo Samples													
		Concentration (mg/L) Determined at 310 nm and 354 nm When Noted											
		PHS	B-3	DHHB	DHHB (354 nm)	4-MBC	OCR	MA	OMC	BMDM	BMDM (354 nm)	OS	HMS
Cosmetic Powder Placebo	Average*	35.07	36.05	35.80	36.00	35.76	35.82	37.25	35.89	34.52	33.94	37.45	35.46
	RSD	0.27	0.23	0.74	0.17	0.23	0.37	1.06	0.24	0.25	0.34	0.39	0.34
	%Recovery	100.2	103.0	102.3	102.9	102.2	102.3	106.4	102.5	98.60	97.00	107.0	101.3
Lotion Placebo	Average*	35.22	35.16	35.14	35.23	34.94	34.95	36.15	34.97	35.81	35.22	34.73	34.58
	RSD	0.11	0.02	0.12	0.12	0.01	0.03	0.60	0.03	0.09	0.02	0.22	0.14
	%Recovery	100.6	100.5	100.4	100.7	99.83	99.86	103.3	99.90	102.3	100.63	99.23	98.80
Lipstick Placebo	Average*	37.09	35.55	35.71	35.54	35.22	35.30	35.63	35.27	35.95	35.45	35.89	34.91
	RSD	0.07	0.08	0.12	0.13	0.12	0.12	0.74	0.14	0.26	0.30	0.04	0.39
	%Recovery	106.0	101.6	102.0	101.5	100.6	100.9	101.8	100.8	102.7	101.3	102.5	99.74

*Three injections were made of each sample.

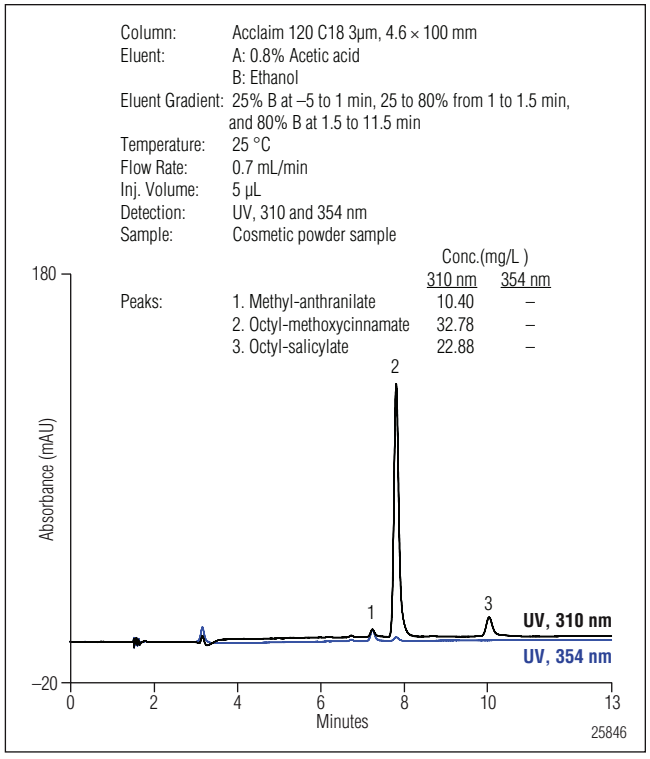


Figure 4. Overlay of three injections of the cosmetic powder sample with detection at 310 nm (Black) and 354 nm (Blue).

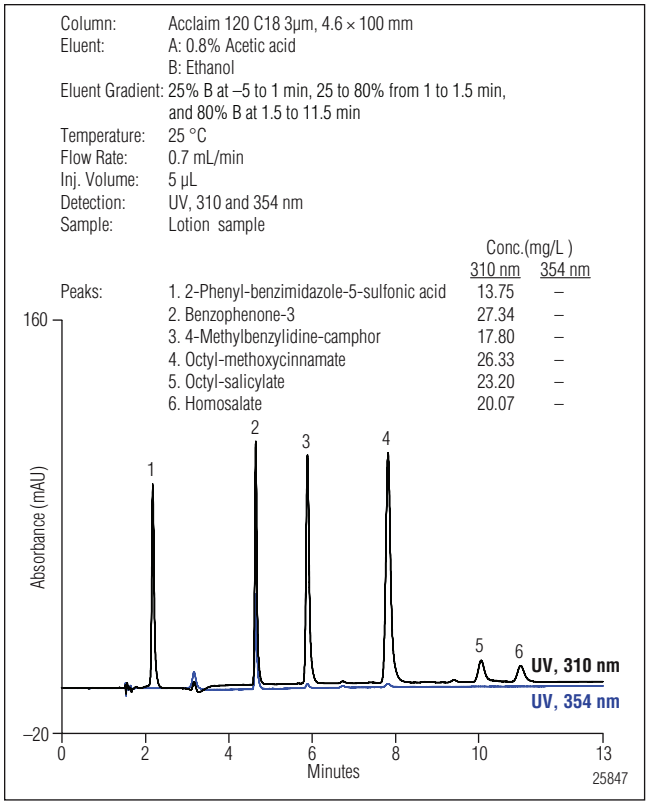


Figure 5. Overlay of three injections of the lotion sample with detection at 310 nm (Black) and 354 nm (Blue).

Table 4. Determination of Sunscreen Ingredients in Cosmetic Powder, Lotion, and Lipstick Samples													
		Concentration (mg/L) Determined at 310 nm and 354 nm When Noted											
		PHS	B-3	DHHB	DHHB (354 nm)	4-MBC	OCR	MA	OMC	BMDM	BMDM (354 nm)	OS	HMS
Cosmetic Powder Sample	Average*	N.A	N.A	N.A	N.A	N.A	N.A	10.40	32.78	N.A	N.A	22.88	N.A
	RSD	N.A	N.A	N.A	N.A	N.A	N.A	3.30	0.12	N.A	N.A	0.21	N.A
	%W/W	N.A	N.A	N.A	N.A	N.A	N.A	1.04	3.28	N.A	N.A	2.29	N.A
Lotion Sample	Average*	13.75	27.34	N.A	N.A	17.80	N.A	N.A	26.33	N.A	N.A	23.20	20.07
	RSD	0.74	0.20	N.A	N.A	0.08	N.A	N.A	0.08	N.A	N.A	0.17	0.19
	%W/W	1.38	2.73	N.A	N.A	1.78	N.A	N.A	2.63	N.A	N.A	2.32	2.01
Lipstick Sample	Average*	26.87	27.37	N.A	0.32	26.99	N.A	N.A	24.11	27.39	26.99	34.00	30.39
	RSD	0.33	0.32	N.A	2.15	0.15	N.A	N.A	0.31	0.50	0.28	0.68	0.56
	%W/W	2.69	2.74	N.A	0.03	2.70	N.A	N.A	2.41	2.74	2.70	3.40	3.04

*Three injections were made of each sample.

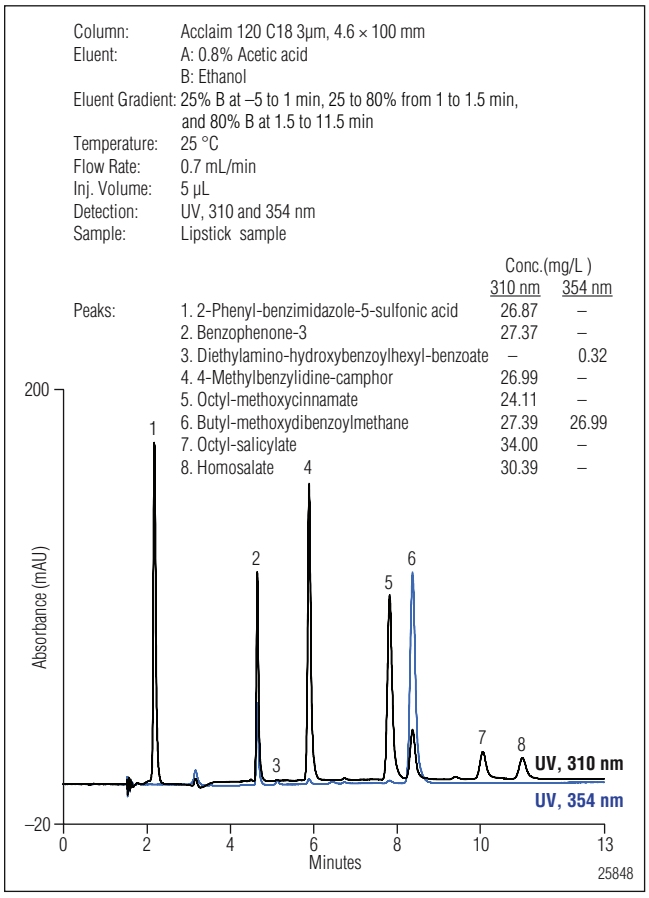


Figure 6. Overlay of three injections of the lipstick sample with detection at 310 nm (Black) and 354 nm (Blue).

CONCLUSION

This application note shows that 10 sunscreen compounds are baseline resolved in less than 12 min using an Acclaim 120 C18 column on an UltiMate 3000 system. This method accurately determines these compounds in a cosmetic power, a lotion and a lipstick.

Isocratic Separation of Basic, Neutral, and Acidic Molecules on the Acclaim Mixed-Mode Column

INTRODUCTION

The separation of different types of molecules in mixtures containing bases, neutrals and acids can prove to be challenging. The novel column chemistry of the Acclaim Mixed-Mode WAX-1 results in a multi-mode separation mechanism that includes hydrophobic, anion-exchange and cation-exclusion interactions. This column can separate all these molecules with baseline resolution.

METHOD

In this application, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate a mixture of 11 basic, neutral and acidic molecules. The column was maintained at 30 °C. The separation is performed under isocratic conditions using an acetonitrile/buffer (1:1) mobile phase at a flow rate of 1.0 mL/min. The buffer was composed of 6.8 g KH_2PO_4 and 0.5 g NaP_2O_7*10 H_2O in 1000 mL water, adjusted to pH 6.0 with NaOH. A 5 µL injection was made and the method yielded a total run time under 20 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of all 11 components in the mixture. This separation is clearly suitable for quantitatively assaying a wide variety of molecules in a single analysis.

Note: Pyrophosphate in the buffer suppresses interference from metal ions.

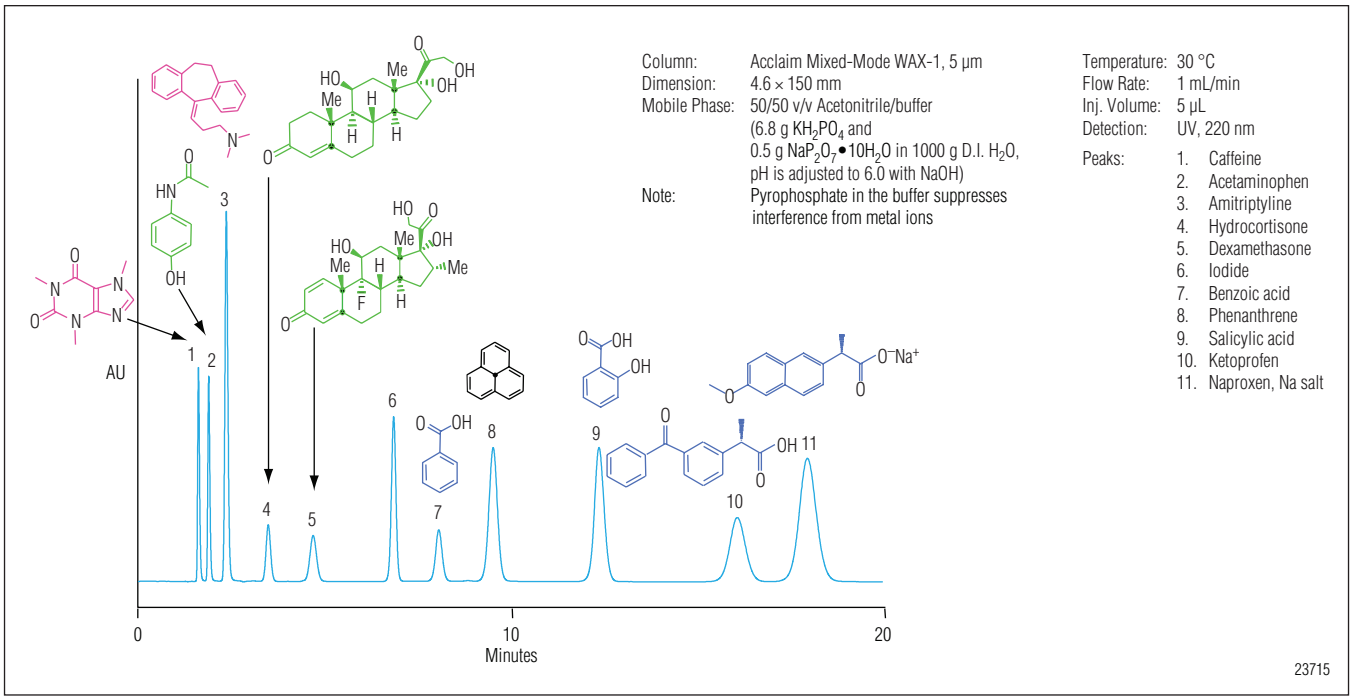


Figure 1. Isocratic separation of basic, neutral, and acidic molecules on the Acclaim Mixed-Mode WAX-1 column.

Gradient Separation of Basic, Neutral, and Acidic Pharmaceuticals on the Acclaim Mixed-Mode WAX-1 Column

INTRODUCTION

The separation of different types of molecules in mixtures containing bases, neutrals and acids can prove to be challenging. The novel column chemistry of the Acclaim Mixed-Mode WAX-1 column results in a multi-mode separation mechanism that includes hydrophobic, anion-exchange and cation-exclusion interactions. This column can separate all these molecules with baseline resolution.

METHOD

In this application, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate a mixture of 16 basic, neutral and acidic molecules. The column was maintained at 30 °C. The separation is performed under ternary gradient conditions. Mobile phase A is acetonitrile, mobile phase B is water and mobile phase C is 150 mM phosphate buffer adjusted to pH 6.0 with NaOH. The 1.0 mL/min. flow rate yielded a total run time under 40 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown in Figure 1, this ternary gradient method provides baseline separation of all 16 components in the mixture. This separation is clearly suitable for quantitatively assaying a wide variety of molecules in a single analysis.

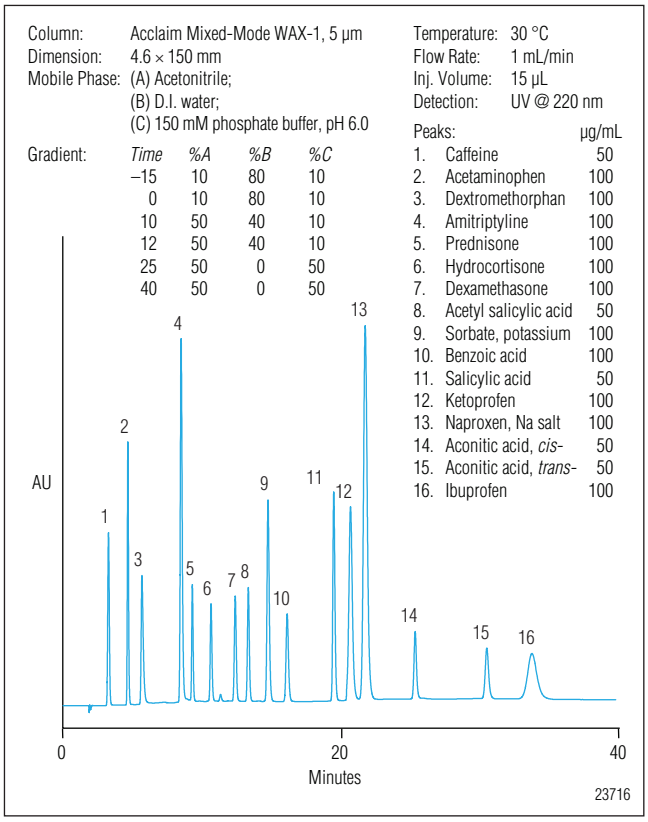


Figure 1. Gradient separation of basic, neutral, and acidic pharmaceuticals on the Acclaim Mixed-Mode WAX-1 column.

Analysis of Triton X-100 Using LC-ESI-MS

INTRODUCTION

Triton X-100 (octylphenol ethoxylate) is one of the most commonly used nonionic detergents, and its residues are ubiquitous. The Acclaim Surfactant column's superior resolution of ethoxylated compounds combines with the sensitivity and specificity of ESI-MS to give a uniquely powerful tool for characterizing this surfactant.

METHOD

In this application, a 5 µm Acclaim Surfactant column with the dimensions of 4.6 × 150 mm is used to separate a solution of Triton-X. The column was maintained at 25 °C. The separation is performed under isocratic conditions using an acetonitrile/ammonium acetate buffer (45:55) at a flow rate of 1.0 mL/min. The buffer was composed of 50 mM NH_2OAc, adjusted to pH 5.4. A 25 µL injection was made and the method yielded a total run time under 20 min. Detection is accomplished using ESI-MS monitoring a number of single ion masses.

RESULTS

As shown in Figure 1, the unique selectivity of the Acclaim Surfactant column is well suited to resolve the wide range of polymers found in nonionic detergents, such as Triton-X. This separation is clearly suitable for confirming the presence or absence of this common chemical.

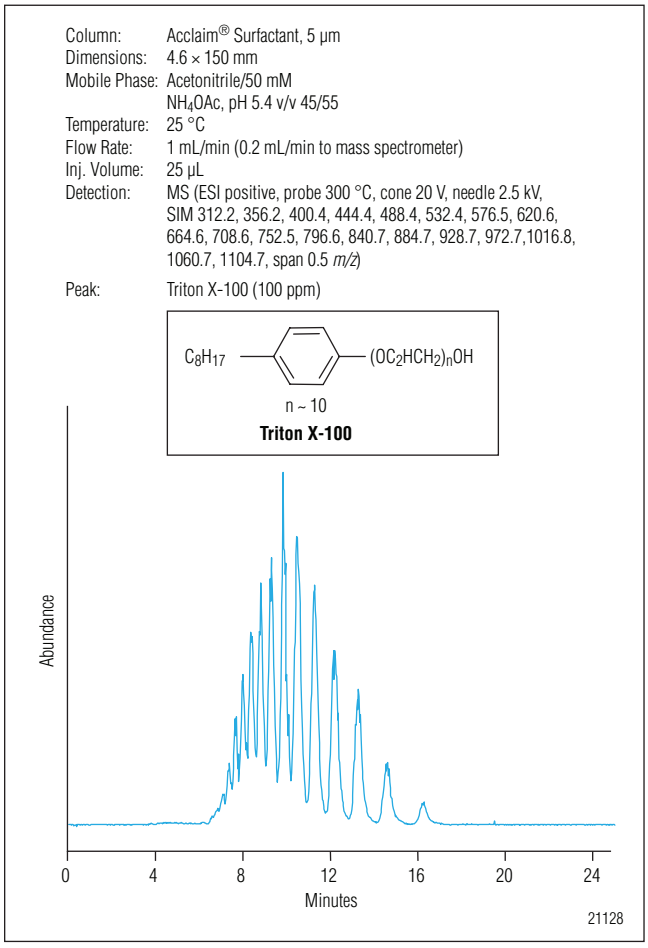
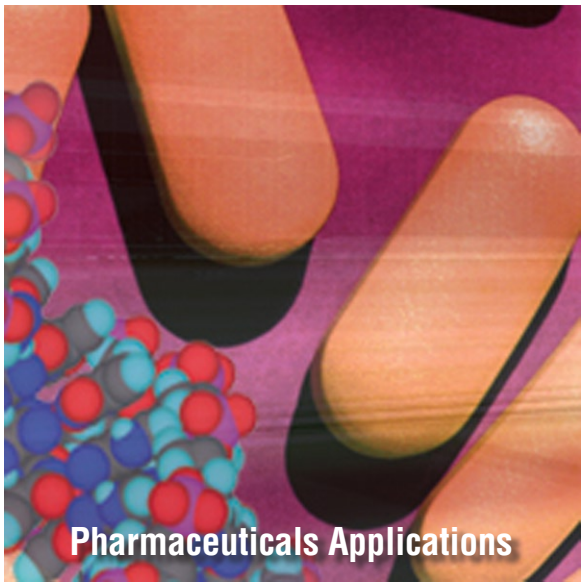


Figure 1. Analysis of Triton X-100 using LC-ESI-MS.

DIONEX

Part VI: Column Selection Guide



DIONEX

Column Selection Guide

Silica Columns

			Reversed-Phase LC					Specialty LC								
			Acclaim 120 C18	Acclaim 120 C8	Acclaim 300 C18	Acclaim Polar Advantage (PA)	Acclaim Polar Advantage II (PA2)	Acclaim Mixed-Mode WAX	Acclaim Mixed-Mode WCX	Acclaim Mixed-Mode HILIC	Acclaim Organic Acid	Acclaim Surfactant	Acclaim Explosives E1	Acclaim Explosives E2	Example Applications
General Applications	Neutral	High hydrophobicity	√	√	√	√	√	√	√	√					fat-soluble vitamins, dyes
		Intermediate hydrophobicity	√	√	√	√	√	√	√	√					steroids, phthalates, phenolics
		Low hydrophobicity				√	√	√	√	√					acetaminophen, urea, polyethylene glycols
	Anions	High hydrophobicity	√	√	√	√	√	√	√	√					NSAIDs, phospholipids
		Intermediate hydrophobicity	√	√	√	√	√	√		√					aspirin, PFOA, preservatives
		Low hydrophobicity				√	√	√		√					organic acids, food dyes
	Cations	High hydrophobicity	√	√	√	√	√	√	√	√					antidepressants
		Intermediate hydrophobicity	√	√	√	√	√		√	√					beta blockers, benzidines, alkaloids
		Low hydrophobicity							√	√					antacids, pseudoephedrine, amino sugars
	Amphoteric/ Zwitterions	Small molecules				√	√		√	√					amino acids, aspartame
		Peptides			√		√								
	Mixtures	Neutrals and acids	√			√	√	√							artificial sweeteners
		Neutrals and bases	√			√	√		√						cough syrup
		Acids and bases						√	√						drug active ingredient with counterion
		Neutrals, acids, and bases						√	√						combination pain relievers
Specific Applications	Surfactants	Anionic					√					√			SDS, LAS, laureth sulfates
		Cationic										√			benzylalkonium, quaternary amines
		Non-ionic								√		√			Triton, Brij
		Amphoteric										√			cocoamidopropyl betaine
		Hydrotropes										√			xylenesulfonates
		Surfactant blends										√			consumer products
	Organic Acids	Hydrophobic						√			√				aromatic acids, fatty acids
		Hydrophilic						√			√				small organic acids
	Pesticides/ Herbicides	Phenoxy	√	√	√	√	√								
		Triazine	√	√	√	√	√								
		Glyphosate						√							
		Carbamates (EPA 531)	√	√		√									
	Other	Explosives (EPA 8330)											√	√	
		Phenols				√									
		Carbonyl compounds (CARB 1004)												√	
		Vitamin analysis				√	√								

See also the Acclaim Library of related applications on www.dionex.com under Literature, Application Notes (right side of the page).

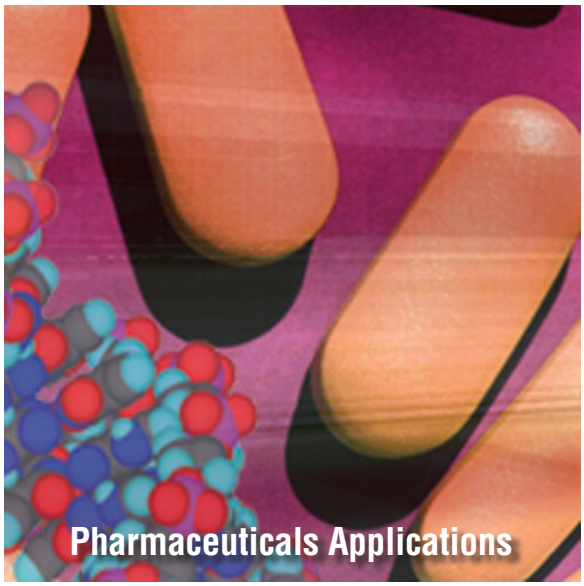
Polymer Columns

		IonPac AS23	IonPac AS22	IonPac AS14/A	IonPac AS12A	IonPac AS9/HC/SC	IonPac AS4A/SC	IonPac AS24	IonPac AS21	IonPac AS20	IonPac AS19	IonPac AS18	IonPac AS17-C	IonPac AS16	IonPac AS15	IonPac AS11(-HC)	IonPac AS10	IonPac AS7	IonPac AS5	IonPac FastAnion IIIA	OmniPac PAX100	OmniPac PAX500	IonPac CS18
ANIONS	Inorganic Anions	√	√	√	√	√	√	√		√	√	√	√		√	√	√						
	Oxyhalides	√			√	√		√			√												
	Bromate	√				√		√			√												
	Perchlorate								√	√				√		√							
	Organic Acids												√		√	√	√						
	Phosphoric/Citric Acids																			√			
	Poly/High-Valence Anions									√				√		√		√	√				
	Hydrophobic Anions									√				√		√							
	Hydrophobic/Halogenated Anions									√						√					√		
	Anionic Neutral Molecules							√	√	√	√											√	
CATIONS	Inorganic Cations																						√
	Sodium/Ammonium																						
	Amines/Polyvalent Amines																						√
	Aliphatic/Aromatic Amines																						
	Alkanol/Ethhanolamines																						√
	Biogenic Amines																						√
	Transition/Lanthanide Metals																						
	Hydrophobic Cations																						√
	Cationic Neutral Molecules																						
BIO-MOLECULES	Amino Acids																						
	Phosphorylated Amino Acids																						
	Amino Sugars																						
	Oligoscccharides																						
	Mono-/Di-Saccharides																						
	Glycoproteins																						
	Alditols/Aldoses mono/di Saccharides																						
	ds Nucleic Acids																						
	Single-Stranded Oligonucleotides																						
	Peptides																						
	Proteins																						
	Metal-binding Proteins																						
	Monoclonal antibodies																						
ORGANIC MOLECULES	Aliphatic Organic Acids																						
	Alcohols																						
	Borate																						
	Large Molecules, Anions																						
	Small Molecules																						
	Small Molecules/LC-MS																						
	Polar/Non-Polar Small Molecules																						
	Hydrophobic/Aliphatic Organic Acids																						
	Surfactant Formulations																						
	Explosives/EPA 8330																						
MODE	Anion Exchange / Carbonate	√	√	√	√	√	√																
	Anion Exchange / Hydroxide							√	√	√	√	√	√	√	√	√	√	√	√				
	Cation Exchange																						√
	Multi-Mode																				√	√	
	Affinity																						
	Ion Exclusion																						
	Reversed Phase																						
	Anion Exchange/Other																						

		IonPac CS17	IonPac CS16	IonPac CS15	IonPac CS14	IonPac CS12A	IonPac CS11	IonPac CS10	IonPac CS5A	OmniPac PCX100	OmniPac PCX500	AminoPac PA10	AminoPac PA1	CarboPac PA200	CarboPac PA100	CarboPac PA20	CarboPac PA10	CarboPac PA1	CarboPac MA1	DNAPac 200	DNAPac 100	ProPac WAX/SAX	ProPac WCX/SCX	ProPac IMAC	ProPac HIC	ProPac PA1	ProSwift	IonPac ICE-AS6	IonPac ICE-AS1	IonPac ICE-Borate	IonPac NS1
ANIONS	Inorganic Anions																														
	Oxyhalides																														
	Bromate																														
	Perchlorate																														
	Organic Acids																														
	Phosphoric/Citric Acids																														
	Poly/High-Valence Anions																														
	Hydrophobic Anions																														
	Hydrophobic/Halogenated Anions																														
	Anionic Neutral Molecules																														
CATIONS	Inorganic Cations	√	√	√	√	√	√	√																							
	Sodium/Ammonium		√	√		√																									
	Amines/Polyvalent Amines	√																													
	Aliphatic/Aromatic Amines	√			√	√																									
	Alkanol/Ethhanolamines	√		√																											
	Biogenic Amines	√																													
	Transition/Lanthanide Metals								√																						
	Hydrophobic Cations	√			√					√																					
	Cationic Neutral Molecules										√																				
BIO-MOLECULES	Amino Acids											√	√																		
	Phosphorylated Amino Acids											√																			
	Amino Sugars											√		√	√	√	√														
	Oligoscccharides													√	√			√	√												
	Mono-/Di-Saccharides															√	√	√	√												
	Glycoproteins																				√		√	√			√				
	Alditols/Aldoses mono/di Saccharides															√	√	√	√												
	ds Nucleic Acids											√	√							√	√										
	Single-Stranded Oligonucleotides											√	√														√				
	Peptides																							√	√	√	√				
	Proteins																					√	√	√	√	√	√				
	Metal-binding Proteins																							√							
	Monoclonal antibodies													√	√			√		√	√	√	√	√	√	√	√				
ORGANIC MOLECULES	Aliphatic Organic Acids																											√	√		√
	Alcohols																											√	√		
	Borate																													√	
	Large Molecules, Anions																														√
	Small Molecules																														
	Small Molecules/LC-MS																														
	Polar/Non-Polar Small Molecules																														
	Hydrophobic/Aliphatic Organic Acids																														
	Surfactant Formulations																														√
	Explosives/EPA 8330																														
MODE	Anion Exchange / Carbonate																														
	Anion Exchange / Hydroxide													√	√	√	√	√	√												
	Cation Exchange	√	√	√	√	√	√	√	√														√			√	√				
	Multi-Mode									√	√																				
	Affinity																							√							
	Ion Exclusion																											√	√	√	
	Reversed Phase																								√	√	√				√
	Anion Exchange/Other											√	√							√	√	√				√	√				

DIONEX

Part VII: Transferring HPLC Methods to UHPLC



Easy Method Transfer from HPLC to RSLC with the Dionex Method Speed-Up Calculator

INTRODUCTION

The goal of every chromatographic optimization is a method that sufficiently resolves all peaks of interest in as short a time as possible. The evolution of packing materials and instrument performance has extended chromatographic separations to new limits: ultrahigh-performance liquid chromatography (UHPLC).

The new Dionex UltiMate® 3000 Rapid Separation LC (RSLC) system is ideal for ultrafast, high-resolution LC. The RSLC system was designed for ultrafast separations with flow rates up to 5 mL/min at pressures up to 800 bar (11,600 psi) for the entire flow-rate range. This industry-leading flow-pressure footprint ensures the highest flexibility possible; from conventional to ultrahigh-resolution to ultrahigh-speed methods. The RSLC system, with autosampler cycle times of only 15 seconds, oven temperatures up to 110 °C, and data collection rates up to 100 Hz (even when acquiring UV-Vis spectra), sets the standard for UHPLC performance. Acclaim® RSLC columns with a 2.2 µm particle size complete the RSLC dimension.

A successful transfer from an HPLC method to an RSLC method requires recalculation of the chromatographic parameters. Underlying chromatographic principles have to be considered to find the appropriate parameters for a method transfer. With the Method Speed-up Calculator, Dionex offers an electronic tool that streamlines the process of optimum method transfer. This technical note describes the theory behind the Method Speed-Up Calculator and the application of this interactive, multi-language tool, illustrated with an exemplary method transfer from a conventional LC separation to an RSLC separation. You may obtain a copy of this calculator from your Dionex representative.

METHOD SPEED-UP STRATEGY

The purpose of method speed-up is to achieve sufficient resolution in the shortest possible time. The strategy is to maintain the resolving power of the application by using shorter columns packed with smaller particles. The theory for this approach is based on chromatographic mechanisms, found in almost every chromatography text book. The following fundamental chromatographic equations are applied by the Method Speed-Up Calculator for the method transfer from conventional to ultrafast methods.

The separation efficiency of a method is stated by the peak capacity *P*, which describes the number of peaks that can be resolved in a given time period. The peak capacity is defined by the run time divided by the average peak width. Hence, a small peak width is essential for a fast method with high separation efficiency. The peak width is proportional to the inverse square root of the number of theoretical plates N generated by the column. Taking into account the length of the column, its efficiency can also be expressed by the height equivalent to a theoretical plate *H*. The relationship between plate height H and plate number *N* of a column with the length L is given by Formula 1.

Formula 1: $$N = \frac{L}{H}$$

Low height equivalents will therefore generate a high number of theoretical plates, and hence small peak width for high peak capacity is gained. Which factors define *H*? For an answer, the processes inside the column have to be considered, which are expressed by the Van Deemter equation (Formula 2).

Formula 2: $$H = A + \frac{B}{u} + C \cdot u$$

The Eddy diffusion *A* describes the mobile phase movement along different random paths through the stationary phase, resulting in broadening of the analyte band. The longitudinal diffusion of the analyte against the flow rate is expressed by the term *B*. Term *C* describes the resistance of the analyte to mass transfer into the pores of the stationary phase. This results in higher band broadening with increasing velocity of the mobile phase. The well-known Van Deemter plots of plate height *H* against the linear velocity of the mobile phase are useful in determining the optimum mobile phase flow rate for highest column efficiency with lowest plate heights. A simplification of the Van Deemter equation, according to Halász[1] (Formula 3), describes the relationship between column efficiency (expressed in plate height *H*), particle size d_p (in µm) and velocity of mobile phase *u* (in mm/s):

Formula 3: $$H = 2 \cdot d_p + \frac{6}{u} + \frac{d_p^2 \cdot u}{20}$$

The plots of plate height H against velocity u depending on the particle sizes dp of the stationary phase (see Figure 1, top) demonstrate visually the key function of small particle sizes in the method speed-up strategy: The smaller the particles, the smaller the plate height and therefore the better the separation efficiency. An efficiency equivalent to larger particle columns can be achieved by using shorter columns and therefore shorter run times.

Another benefit with use of smaller particles is shown for the 2 µm particles in Figure 1: Due to improved mass transfer with small particle packings, further acceleration of mobile phases beyond the optimal flow rate with minimal change in the plate height is possible.

Optimum flow rates and minimum achievable plate heights can be calculated by setting the first derivative of the Halász equation to zero. The optimal linear velocity (in mm/s) is then calculated by Formula 4.

Formula 4: $$u_{opt} = \sqrt{\frac{B}{C}} = \frac{10.95}{d_p}$$

The minimum achievable plate height as a function of particle size is calculated by insertion of Formula 4 in Formula 3, resulting in Formula 5.

Formula 5: $$H_{min} \approx 3 \cdot d_p$$

Chromatographers typically prefer resolution over theoretical plates as a measure of the separation quality. The achievable resolution *R* of a method is directly proportional to the square root of the theoretical plate number as can be seen in Formula 6. *k* is the retention factor of the analyte and *k* the selectivity.

Formula 6: $$R = \frac{1}{4} \cdot \sqrt{N} \cdot \frac{k_2}{1 + k_2} \cdot \frac{\alpha - 1}{\alpha}$$

If the column length is kept constant and the particle size is decreased, the resolution of the analytes improves. Figure 1, bottom, demonstrates this effect using 5 µm and 2 µm particles.

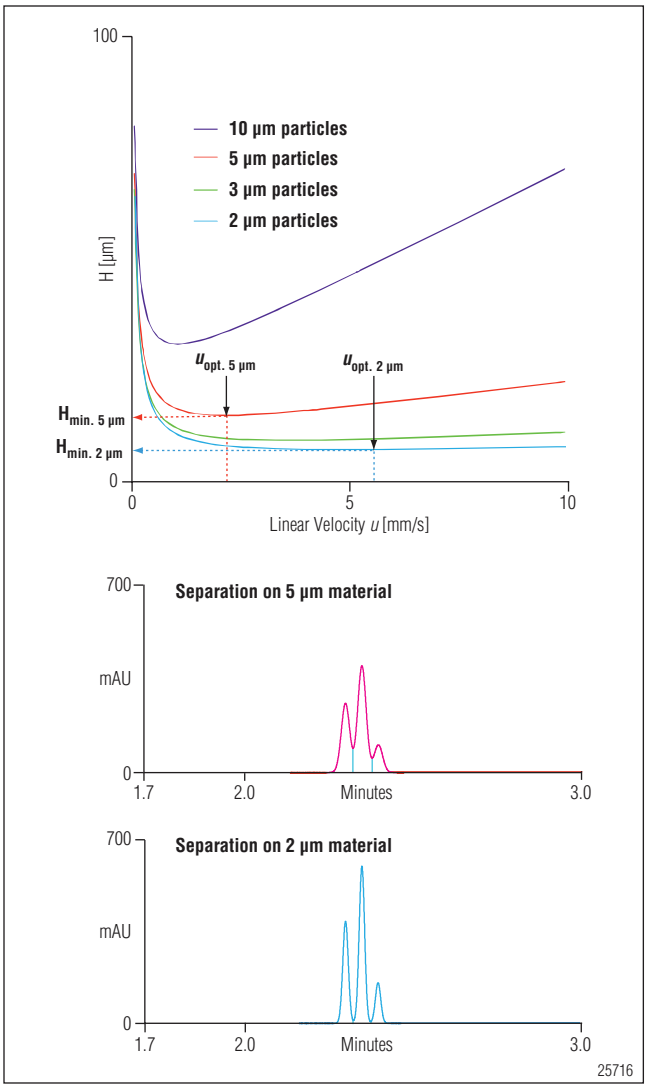


Figure 1. Smaller particles provide more theoretical plates and more resolution, demonstrated by the improved separation of three peaks (bottom) and smaller minimum plate heights H *in the Van Deemter plot (top). At linear velocities higher than uopt,* H *increases more slowly when using smaller particles, allowing higher flow rates and therefore faster separations while keeping separation efficiency almost constant. The speed-up potential of small particles is revealed by the Van Deemter plots (top) of plate height* H *against linear velocity u of mobile phase: Reducing the particle size allows higher flow rates and shorter columns because of the decreased minimum plate height and increased optimum velocity. Consequently, smaller peak width and improved resolution are the result (bottom).*

When transferring a gradient method, the scaling of the gradient profile to the new column format and flow rate has to be considered to maintain the separation performance. The theoretical background was introduced by L. Snyder[2] and is known as the gradient volume principle. The gradient volume is defined as the mobile phase volume that flows through the column at a defined gradient time t_G. Analytes are considered to elute at constant eluent composition. Keeping the ratio between the gradient volume and the column volume constant therefore results in a correct gradient transfer to a different column format.

Taking into account the changed flow rates F and column volume (with diameter d_c and length L), the gradient time intervals t_G of the new methods are calculated with Formula 7.

Formula 7: $$t_{G,new} = t_{G,old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{L_{new}}{L_{old}} \cdot \left(\frac{d_{c,new}}{d_{c,old}} \right)^2$$

An easy transfer of method parameters can be achieved by using the Dionex Method Speed-Up Calculator (Figure 2), which incorporates all the overwhelming theory and makes manual calculations unnecessary. This technical note describes the easy method transfer of an example separation applying the calculator. Just some prerequisites described in the following section have to be taken into account.

PREREQUISITES

The Method Speed-Up Calculator is a universal tool and not specific for Dionex products. Nevertheless, some prerequisites have to be considered for a successful method transfer, which is demonstrated in this technical note by the separation of seven soft drink additives.

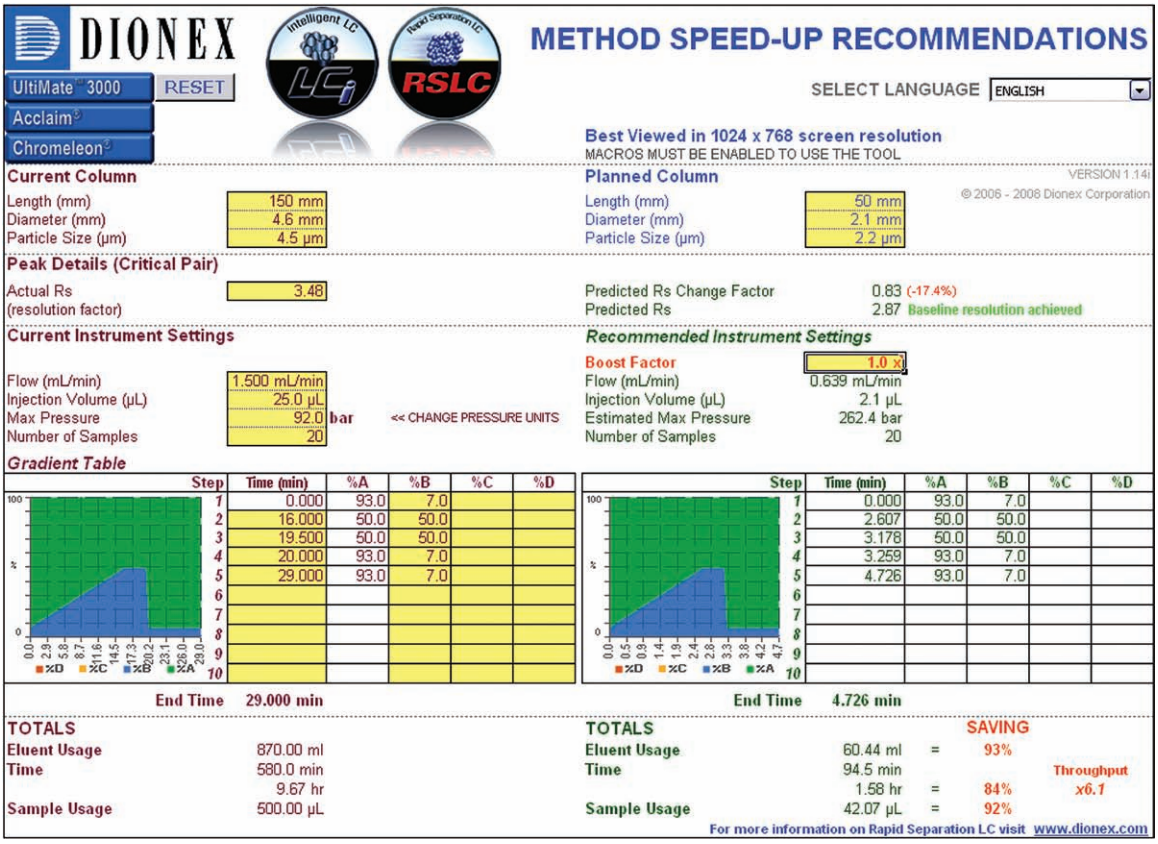


Figure 2. The Dionex Method Speed-Up Calculator transfers a conventional (current) HPLC method to a new (planned) RSLC method.

Column Dimension

First, the transfer of a conventional method to an RSLC method requires the selection of an adequate column filled with smaller particles. The RSLC method is predicted best if the selectivity of the stationary phase is maintained. Therefore, a column from the same manufacturer and with nominally identical surface modification is favoured for an exact method transfer. If this is not possible, a column with the same nominal stationary phase is the best choice. The separation is made faster by using shorter columns, but the column should still offer sufficient column efficiency to allow at least a baseline separation of analytes. Table 1 gives an overview of the theoretical plates expected by different column length and particle diameter size combinations using Dionex Acclaim column particle sizes. Note that column manufacturers typically fill columns designated 5 µm with particle sizes 4–5 µm. Dionex Acclaim 5 µm columns are actually filled with 4.5 µm particles. This is reflected in the table.

Table 1. Theoretical Plates Depending on Column Length and Particle Diameter (Calculated Using Formula 5)

	Theoretical Plates N		
Particle size	4.5 µm	3 µm	2.2 µm
Column length: 250 mm	18518	27778	37879
150 mm	11111	16667	22727
100 mm	7407	11111	15152
75 mm	5555	8333	11364
50 mm	3703	5556	7576

If the resolution of the original separation is higher than required, columns can be shortened. Keeping the column length constant while using smaller particles improves the resolution. Reducing the column diameter does not shorten the analysis time but decreases mobile phase consumption and sample volume. Taking into account an elevated temperature, smaller column inner diameters reduce the risk of thermal mismatch.

System Requirements

Smaller particles generate higher backpressure. The linear velocity of the mobile phase has to be increased while decreasing the particle size to work within the Van Deemter optimum. The UltiMate 3000 RSLC system perfectly supports this approach with its high maximum operation pressure of 800 bar (11,600 psi). This maximum pressure is constant over the entire flow rate range of up to 5 mL/min, providing additional potential to speed up applications even further by increasing the flow rate.

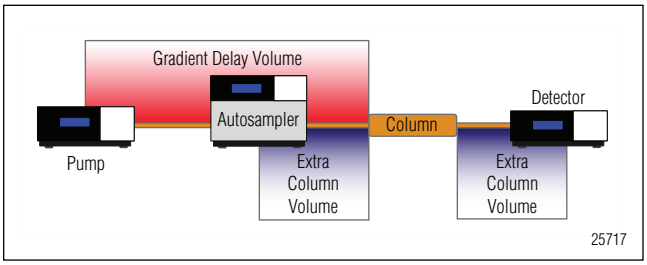


Figure 3. Gradient delay volume and extra column volume of an HPLC system. Both play an important role in method speed-up.

For fast gradient methods, the gradient delay volume (GDV) plays a crucial role. The GDV is defined as the volume between the first point of mixing and the head of the column. The GDV becomes increasingly important with fast, steep gradients and low flow rate applications as it affects the time taken for the gradient to reach the head of the column. The larger the GDV, the longer the initial isocratic hold at the beginning of the separation. Typically, this leads to later peak elution times than calculated. Early eluting peaks are affected most. In addition, the GDV increases the time needed for the equilibration time at the end of a sample and therefore increases the total cycle time. A general rule is to keep the gradient steepness and the ratio of GDV to column volume constant when transferring a standard method into a fast LC method. This will maintain the selectivity of the original method.[3]

The GDV can be adjusted to the column volume by installing appropriate mixer kits to the RSLC pump (see Table 2), which contributes most to the GDV. Typically, 100 µL or 200 µL mixers are good starting points when operating a small volume column in an RSLC system.

Another option is to switch the sample loop of the split-loop autosampler out of the flow path. The GDV is then reduced by the sample loop volume in the so-called bypass mode. The GDV of a standard sample loop of the RSLC autosampler is 150 µL, the micro injection loop has a 50 µL GDV.

Table 2. Mixer Kits Available for UltiMate 3000 RSLC System to Adapt GDV of Pump

Mixer Kit	GDV pump
Mixer kit 6040.5000	35 µL
Static mixer kit 6040.5100	100 µL
Static mixer kit 6040.5150	200 µL

Besides the gradient delay volume, the extra column volume is an important parameter for fast LC methods. The extra column volume is the volume in the system through which the sample passes and hence contributes to the band broadening of the analyte peak (Figure 3). The extra column volume of an optimized LC system should be below $^{1}/_{10}$th of the peak volume. Therefore the length and inner diameter of the tubing connections from injector to column and column to detector should be as small as possible. Special care has to be taken while installing the fittings to avoid dead volumes. In addition, the volume of the flow cell has to be adapted to the peak volumes eluting from the RSLC column. If possible, the flow cell detection volume should not exceed $^{1}/_{10}$th of the peak volume.

Detector Settings

When transferring a conventional method to an RSLC method, the detector settings have a significant impact on the detector performance. The data collection rate and time constant have to be adapted to the narrower peak shapes. In general, each peak should be defined by at least 30 data points. The data collection rate and time constant settings are typically interrelated to optimize the amount of data points per peak and reduce short-term noise while still maintaining peak height, symmetry, and resolution.

The Chromeleon® Chromatography Management Software has a wizard to automatically calculate the best settings, based on the input of the minimum peak width at half height of the chromatogram. This width is best determined by running the application once at maximum data rate and shortest time constant. The obtained peak width may then be entered into the wizard for optimization of the detection settings. Refer to the detector operation manual for further details.

METHOD SPEED-UP USING THE CALCULATOR

Separation Example

Separation was performed on an UltiMate 3000 RSLC system consisting of a HPG-3200RS Binary Rapid Separation Pump, a WPS-3000RS Rapid Separation Well Plate Sampler with analytical sample loop (100 µL), a TCC-3000RS Rapid Separation Thermostatted Column Compartment with precolumn heater (2 µL), and a VWD-3400RS Variable Wavelength Detector with semi-micro flow cell (2.5 µL). Chromeleon Chromatography Management Software (version 6.80, SR5) was used for both controlling the instrument and reporting the data. The modules were connected with stainless steel micro capillaries, 0.01" ID, $^{1}/_{16}$" OD when applying the conventional LC method, 0.007" and 0.005" ID, $^{1}/_{16}$" OD when applying the RSLC methods. A standard mixture of seven common soft drink additives was separated by gradient elution at 45 °C on two different columns:

- Conventional HPLC Column: Acclaim 120, C18, 5 µm, 4.6 × 150 mm column, (P/N 059148)
- Rapid Separation Column: Acclaim RSLC 120, C18, 2.2 µm, 2.1 × 50 mm column (P/N 068981).

The UV absorbance wavelength at 210 nm was recorded at 5 Hz using the 4.6 × 150 mm column and at 25 Hz and 50 Hz using the 2.1 × 50 mm column. Further method details such as flow rate, injection volume, and gradient table of conventional and RSLC methods are described in the following section. The parameters for the method transfer were calculated with the Dionex Method Speed-Up Calculator (version 1.14i).

The conventional separation of seven soft drink additives is shown in Figure 4A. With the Method Speed-Up Calculator, the method was transferred successfully to RSLC methods (Figure 4B and C) at two different flow rates. The easy method transfer with this universal tool is described below.

Column Selection for Appropriate Resolution

The column for method speed-up must provide sufficient efficiency to resolve the most critical pairs. In this example, separating peaks 5 and 6 is most challenging. A first selection of the planned column dimensions can be made by considering the theoretical plates according to Table 1. The 4.6 × 150 mm, 5 µm column is actually filled with 4.5 µm particles. Therefore, it provides 11,111 theoretical plates. On this column, the

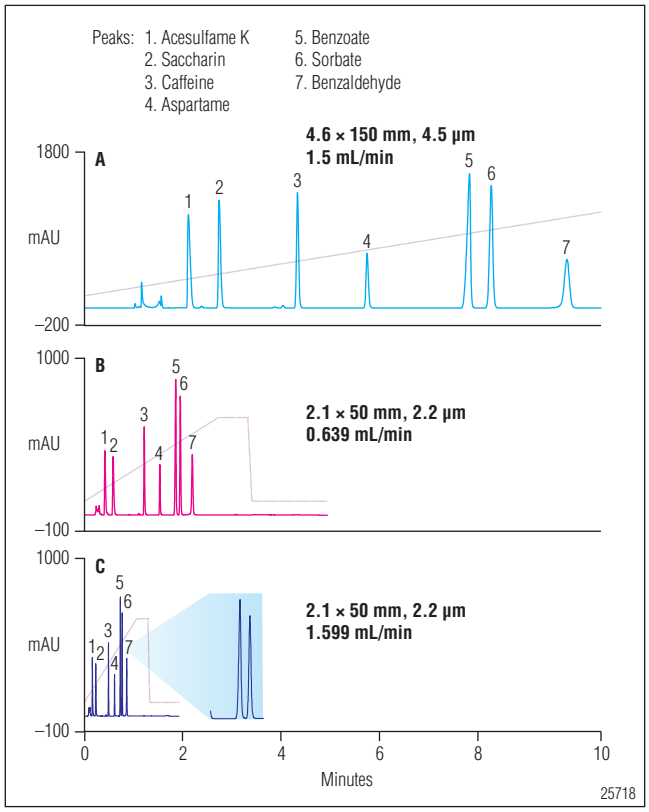


Figure 4. Method transfer with the Method Speed-Up Calculator from A) a conventional LC separation on an Acclaim 5 µm particle column, to B) and C) RSLC separations on an Acclaim 2.2 µm particle column.

resolution is $R_{(5,6)}$=3.48. This resolution is sufficiently high to select a fast LC column with fewer theoretical plates for the speed up. Therefore, a 2.1 × 50 mm, 2.2 µm column with 7579 plates was selected.

The first values to be entered into the yellow field of the Method Speed-Up Calculator are the current column dimension, planned column dimension, and the resolution of the critical pair. To obtain the most accurate method transfer, use the particle sizes listed in the manufacturer's column specifications sheet instead of the nominal size, which may be different. Dionex Acclaim columns with a nominal particle size of 5 µm are actually filled with 4.5 µm particles, and this value should be used to achieve a precise method transfer calculation. This has a positive impact on the performance and pressure predictions for the planned column. Based on the assumption of unchanged stationary phase chemistry, the calculator then predicts the resolution provided by the new method (Figure 5).

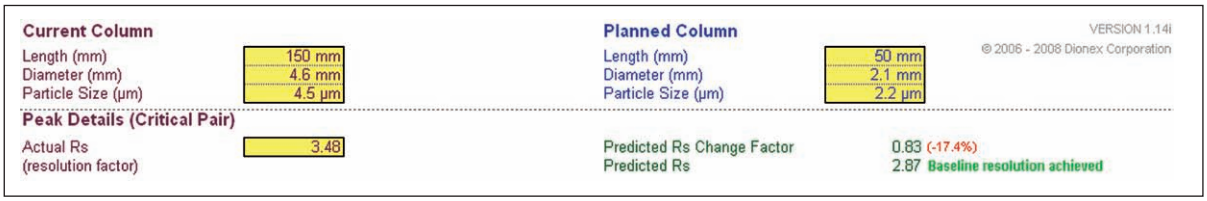


Figure 5. Column selection considering the resolution of the critical pair.

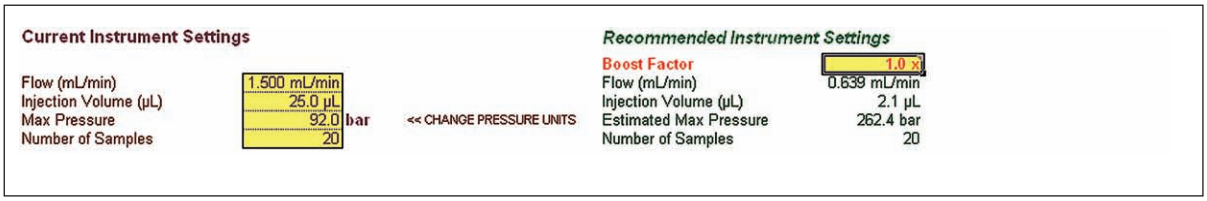


Figure 6. The flow rate, injection volume and backpressure of the current method are scaled to the new column dimension.

In the example in Figure 5, the predicted resolution between benzoate and sorbate is 2.87. With a resolution of $R \geq 1.5$, the message "Baseline resolution achieved" pops up. This indicates that a successful method transfer with enough resolution is possible with the planned column. If R is smaller than 1.5, the red warning "Baseline is not resolved" appears. Note that the resolution calculation is performed only if the boost factor BF is 1, otherwise it is disabled. The function of the boost factor is described in the Adjust Flow Rate section.

Instrument Settings

The next section of the Method Speed-Up Calculator considers basic instrument settings. These are flow rate, injection volume, and system backpressure of the current method (Figure 6). In addition to these values, the detector settings have to be considered as described in the earlier section "Detector Settings". Furthermore, the throughput gain with the new method can be calculated if the number of samples to be run is entered.

Adjust Flow Rate

As explained by Van Deemter theory, smaller particle phases need higher linear velocities to provide optimal separation efficiency. Consequently, the Dionex Method Speed-Up Calculator automatically optimizes the linear velocity by the ratio of particle sizes of the current and planned method. In addition, the new flow rate is scaled to the change of column cross section if the column inner diameter changed. This keeps the linear velocity of the mobile phase constant. A boost factor (*BF*) can be entered to multiply the flow rate for a further decrease in separation time. If the calculated resolution with $BF=1$ predicts sufficient separation, the method can be accelerated by increasing the boost factor and therefore increasing the flow rate. Figure 1 shows that applying linear velocities beyond the optimum is no problem with smaller particle phases, as they do not significantly loose plates in this region. Note that the resolution calculation of the Method Speed-Up Calculator is disabled for $BF \neq 1$.

For the separation at hand, the flow rate is scaled from 1.5 mL/min to 0.639 mL/min when changing from an Acclaim 4.6 × 150 mm, 4.5 µm column to a 2.1 × 50 mm, 2.2 µm column (see Figure 6), adapting the linear velocity to the column dimensions and the particle size. The predicted resolution between peak 5 and 6 for the planned column is $R=2.87$. The actual resolution achieved is $R=2.91$, almost as calculated (chromatogram B in Figure 4).

A Boost Factor of 2.5 was entered for further acceleration of the method (Figure 7). The method was then performed with a flow rate of 1.599 mL/min, and resolution of the critical pair was still sufficient at $R=2.56$ (see zoom in chromatogram C in Figure 4).

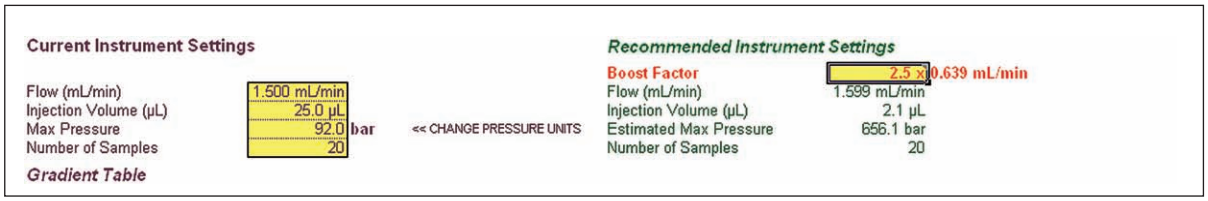


Figure 7. The new flow rate is further accelerated by applying the Boost Factor of 2.5.

Scale Injection Volume

The injection volume has to be adapted to the new column dimension to achieve similar peak heights by equivalent mass loading. Therefore the injection plug has to be scaled to the change of column cross section. In addition, shorter columns with smaller particles cause a reduced zone dilution. Consequently, sharper peaks compared to longer columns are expected. The new injection volume $V_{inj,new}$ is then calculated by Formula 8, taking a changed cross section and reduced band broadening by changed particle diameter into account.

Formula 8: $$V_{inj,new} = V_{inj,old} \cdot \left(\frac{d_{c,new}}{d_{c,old}}\right)^2 \cdot \sqrt{\frac{L_{new} \cdot d_{p,new}}{L_{old} \cdot d_{p,old}}}$$

Generally, it is recommended that a smaller flow cell be used with the RSLC method to minimize the extra column volume. Also, the difference in path length of different flow cell sizes has to be taken into account while scaling the injection volume. In the example of the soft drink analysis, the injection volume is scaled from 25 µL to 2.1 µL when replacing the Acclaim 4.6 × 150 mm, 4.5 µm column with a 2.1 × 50 mm, 2.2 µm column (see Figure 6).

Predicted Backpressure

Speeding-up the current method by decreasing particle size and column diameter and increasing flow rate means elevating the maximum generated backpressure. The pressure drop across a column can be approximated by the Kozeny-Carman formula.[4] The pressure drop of the new method is predicted by the calculator considering changes in column cross section, flow rate, and particle size and is multiplied by the boost factor. The viscosity of mobile phase is considered constant during method transfer. The calculated pressure is only an approximation and does not take into account nominal and actual particle size distribution depending on column manufacturer. If the predicted maximum pressure is above 800 bar (11,600 psi) the warning "Exceeds pressure limit RSLC" is shown, indicating the upper pressure limit of the UltiMate 3000 RSLC system. However, in the case the method is transferred to a third party system, its pressure specification has to be considered.

In the example of the soft drink analysis, the actual pressure increases from 92 bar to 182 bar with *BF*=1 on the 2.1 × 50 mm column, and to 460 bar for the RSLC method with *BF*=2.5. The pressures predicted by the Method Speed-Up Calculator are 262 bar and 656 bar, respectively. The pressure calculation takes into account the change of the size of the column packing material. In a speed up situation, the pressure is also influenced by other factors such as particle size distribution, system fluidics pressure, change of flow cell, etc. When multiplication factors such as the boost factor are used, the difference between calculated and real pressure is pronounced. The pressure calculation is meant to give an orientation, what flow rates might be feasible on the planned column. However, it should be confirmed by applying the flow on the column.

Adapt Gradient Table

The gradient profile has to be adapted to the changed column dimensions and flow rate following the gradient-volume principle. The gradient steps of the current method are entered into the yellow fields of the gradient table. The calculator then scales the gradient step intervals appropriately and creates the gradient table of the new method.

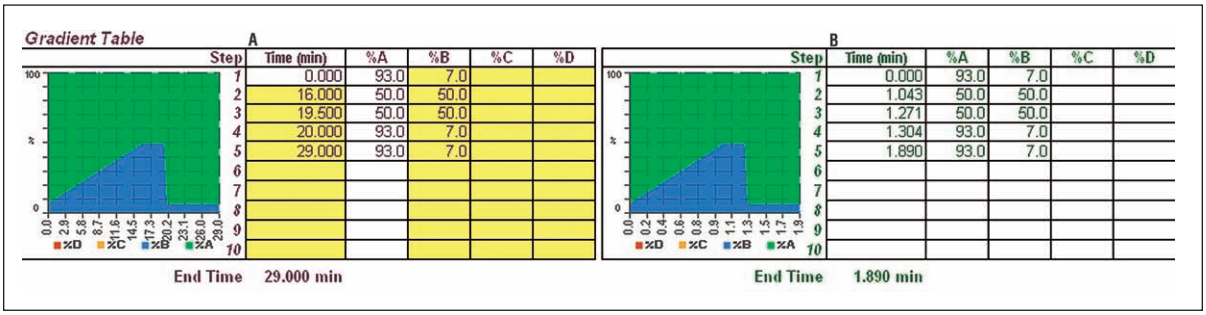


Figure 8. The gradient table of the current method (A) is adapted to the boosted method (B) according to the gradient-volume principle.

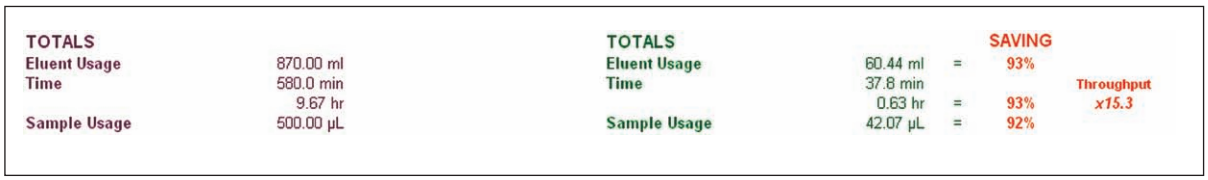


Figure 9. The absolute values for analysis time, eluent usage, and sample usage of the current (purple) and planned (green) method are calculated by the Method Speed-Up Calculator. The savings of eluent, sample, and time due to the method transfer are highlighted.

The adapted gradient table for the soft drink analysis while using a boost factor *BF*=1 is shown in Figure 8. According to the gradient-volume principle, the total run time is reduced from 29.0 min to 4.95 min by taking into account the changed column volume from a 4.6 × 150 mm, 5 µm (4.5 µm particles entered) to a 2.1 × 50 mm, 2.2 µm column and the flow rate reduction from 1.5 mL/min to 0.639 mL/min. The separation time was further reduced to 1.89 min by using boost factor *BF*=2.5. Gradient time steps were adapted accordingly. The comparison of the peak elution order displayed in Figure 4 shows that the separation performance of the gradient was maintained during method transfer.

Consumption and Savings

Why speed-up methods? To separate analyte peaks faster and at the same time reduce the mobile phase and sample volume consumption. Those three advantages of a method speed-up are indicated in the Method Speed-Up Calculator sheet right below the gradient table. The absolute values for the time, eluent, and sample usage are calculated taking the numbers of samples entered into the current instrument settings section of the calculation sheet into account (see Figure 6).

Regarding the soft drink analysis example, geometrical scaling of the method from the conventional column to the RSLC method means saving 93% of eluent and 92% of sample. The sample throughput increases 6.1-fold using *BF*=1. The higher flow rate at *BF*=2.5 results in a 15.3-fold increased throughput compared to the conventional LC method (Figure 9).

CONCLUSION

Fast method development or increased sample throughput are major challenges of most analytical laboratories. A systematic method speed-up is accomplished by reducing the particle size, shortening the column length, and increasing the linear velocity of the mobile phase. The Dionex Method Speed-Up Calculator automatically applies these rules and scales the conventional LC parameters to the conditions of the RSLC method. The interactive electronic tool is universally applicable. New instrument settings are predicted and gradient tables are adapted for optimum performance for the new method. The benefit of the method transfer is summarized by the integrated calculation of savings in time, eluent and sample. In addition, users can benefit from getting results earlier and thereby reducing the time to market. The Dionex Method Speed-Up Calculator is part of Dionex's total RSLC solution, which further consists of the industry leading UltiMate 3000 RSLC system, powerful Chromeleon Chromatography Management Software, and high-efficiency Acclaim RSLC columns.

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