# Pharmaceutical Applications Notebook

Antibiotics

Thermo SCIENTIFIC

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# Introduction to Pharmaceuticals

The pharmaceutical industry is the largest consumer of high-performance liquid chromatography (HPLC) instrumentation. In drug discovery, HPLC and ion chromatography (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 quality assurance/quality control (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.

Thermo Fisher Scientific 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.

***Thermo Scientific and Dionex Integrated Systems***

Dionex Products are now a part of the Thermo Scientific brand, creating exciting new possibilities for scientific analysis. Now, leading capabilities in liquid chromatography (LC), IC, and sample preparation are together in one portfolio with those in mass spectrometry (MS). Combining Dionex's leadership in chromatography with Thermo Scientific's leadership position in mass spec, a new range of powerful and simplified workflow solutions now becomes possible.

For more information on how the new line-up of Thermo Scientific products can expand your capabilities and provide the tools for new possibilities, choose one of our integrated solutions:

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- Liquid Chromatography and Mass Spectrometry
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# UltiMate 3000 UHPLC+ Systems

***Best-in-class HPLC systems for all your chromatography needs***

Thermo Scientific Dionex UltiMate 3000 UHPLC+ Systems provide excellent chromatographic performance while maintaining easy, reliable operation. The basic and standard analytical systems offer ultra HPLC (UHPLC) compatibility across all modules, ensuring maximum performance for all users and all laboratories. Covering flow rates from 20 nL/min to 10 mL/min with an industry-leading range of pumping, sampling, and detection modules, UltiMate™ 3000 UHPLC+ Systems provide solutions from nano to semipreparative, from conventional LC to UHPLC.

- Superior chromatographic performance
- UHPLC design philosophy throughout nano, standard analytical, and rapid separation liquid chromotography (RSLC)
- 620 bar (9,000 psi) and 100 Hz data rate set a new benchmark for basic and standard analytical systems
- RSLC systems go up to 1000 bar and data rates up to 200 Hz
- ×2 Dual System for increased productivity solutions in routine analysis
- Fully UHPLC compatible advanced chromatographic techniques
- Thermo Scientific Dionex Viper and nanoViper–the first truly universal, fingertight fitting system even at UHPLC pressures

Thermo Fisher Scientific is the only HPLC company uniquely focused on making UHPLC technology available to all users, all laboratories, and for all analytes.

*Rapid Separation LC Systems:* The extended flow-pressure footprint of the RSLC system provides the performance for ultrafast high-resolution and conventional LC applications.

*RSLCnano Systems:* The Rapid Separation nano LC System (RSLCnano) provides the power for high-resolution and fast chromatography in nano, capillary, and micro LC.

*Standard LC Systems:* Choose from a wide variety of standard LC systems for demanding LC applications at nano, capillary, micro, analytical, and semipreparative flow rates.

*Basic LC Systems*: UltiMate 3000 Basic LC Systems are UHPLC compatible and provide reliable, high-performance solutions to fit your bench space and your budget.

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

For ion analysis, nothing compares to a Thermo Fisher Scientific ion chromatography 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. And with your IC purchase, you get more than just an instrument—you get a complete solution based on modern technology and world-class support.

- Thermo Scientific Dionex ICS-5000: The world's first capillary IC system
- Dionex ICS-2100: Award-winning integrated Reagent-Free™ IC system
- Dionex ICS-1600: Standard integrated IC system
- Dionex ICS-1100: Basic integrated IC system
- Dionex ICS-900: Starter line IC system

Ranging from the Dionex ICS-900 to the ICS-5000, these IC systems cover the entire range of IC needs and budgets and come with superior support and service worldwide.

*Dionex ICS-5000:* Developed with flexibility, modularity, and ease-of-use in mind, the Dionex ICS-5000 combines the highest sensitivity with convenience

*Dionex ICS-2100:* An integrated Reagent-Free IC (RFIC™) system for electrolytically generated isocratic and gradient separations with conductivity detection, now with electrolytic sample preparation.

*Dionex ICS-1600:* The Dionex ICS-1600 combines high sensitivity with convenience. Now ready for eluent regeneration, with available dual-valve configuration for automated sample preparation.

*Dionex ICS-1100:* With dual-piston pumping and electrolytic suppression. Now ready for eluent regeneration, with available dual-valve configuration for automated sample preparation.

*Dionex ICS-900:* Can routinely analyze multiple anions and cations in 10–15 min—fully automated with Displacement Chemical Regeneration (DCR).

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# MS Instruments

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

Thermo Fisher Scientific provides advanced integrated IC/MS and LC/MS solutions with superior ease-of-use and modest price and space requirements. UltiMate 3000 System Wellness technology and automatic MS calibration allow continuous operation with minimal maintenance. The Dionex ICS-5000 instrument and the family of RFIC systems automatically remove mobile phase ions for effort-free transition to MS detection.

- Thermo Scientific MSQ Plus mass spectrometer, the smallest and most sensitive single quadrupole on the market for LC and IC
- Self-cleaning ion source for low-maintenance operation
- Thermo Scientific Dionex 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™ mass spectrometer, PC datasystem, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) probe inlets, and vaccum system

You no longer need two software packages to operate your LC/MS system. Chromeleon™ LC/MS software provides single-software method setup and instrument control; powerful UV, conductivity, and MS data analysis; and fully integrated reporting.

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# Chromeleon 7 Chromatography Data System Software

***The fastest way to get from samples to results.***

Discover Chromeleon software version 7, the chromatography software that streamlines your path from samples to results. Get rich, intelligent functionality and outstanding usability at the same time with Chromeleon software version 7—the Simply Intelligent™ chromatography software.

- Enjoy a modern, intuitive user interface designed around the principle of operational simplicity
- Streamline laboratory processes and eliminate errors with eWorkflows, which enable anyone to perform a complete analysis perfectly with just a few clicks
- Access your instruments, data, and eWorkflows instantly in the Chromeleon Console
- Locate and collate results quickly and easily using powerful built-in database query features
- Interpret multiple chromatograms at a glance using MiniPlots
- Find everything you need to view, analyze, and report data in the Chromatography Studio
- Accelerate analyses and learn more from your data through dynamic, interactive displays
- Deliver customized reports using the built-in Excel-compatible speadsheet

Chromeleon software version 7 is a forward-looking solution to your long-term chromatography data needs. It is developed using the most modern software tools and technologies, and innovative features will continue to be added for many years to come.

The Cobra™ integration wizard uses an advanced mathematical algorithm to define peaks. This ensures that noise and shifting baselines are no longer a challenge in difficult chromatograms. When peaks are not fully resolved, the SmartPeaks™ integration assistant visually displays integration options. Once a treatment is selected, the appropriate parameters are automatically included in the processing method.

Chromeleon software version 7 ensures data integrity and reliability with a suite of compliance tools. Compliance tools provide sophisticated user management, protected database structures, and a detailed interactive audit trail and versioning system.

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# Process Analytical Systems and Software

***Improve your process by improving your process monitoring with a Thermo Scientific Dionex on-line IC or HPLC system***

Our process analytical systems provide timely results by moving liquid chromatography-based measurements on-line. Information from the Thermo Scientific Dionex Integral process analyzer can help reduce process variability, improve efficiency, and reduce downtime. These systems provide comprehensive, precise, accurate information faster than is possible with laboratory-based results. From the lab to the factory floor, your plant's performance will benefit from the information provided by on-line LC.

- Characterize your samples completely with multicomponent analysis
- Reduce sample collection time and resources with automated multipoint sampling
- Improve your process control with more timely results
- See more analytes with unique detection capabilities
- 25 years of experience providing on-line IC and HPLC capabilities to a wide range of industries
- The Thermo Scientific Integral Migration Path approach lets you choose the systems that best meets your needs

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

*Integral:* The Integral Migration Path approach: System solutions wherever you need them: lab, pilot plant, or manufacturing

*Chromeleon Process Analytical (PA) Software:* Chromeleon PA software provides unique capabilities to support on-line IC or HPLC analysis

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# 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 the Thermo Scientific Dionex Accelerated Solvent Extractor (ASE) System or AutoTrace 280 Solid-Phase Extraction (SPE) instrument.

The Dionex ASE™ system is dramatically faster than Soxhlet, sonication, and other extraction methods, and uses significantly less solvent and labor. Accelerated solvent extraction methods are accepted and established in the environmental, pharmaceutical, foods, polymers, and consumer product industries.

Dionex ASE systems are also 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

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

# Analysis of Antibiotics

Pharmaceutical Applications Notebook

# 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]

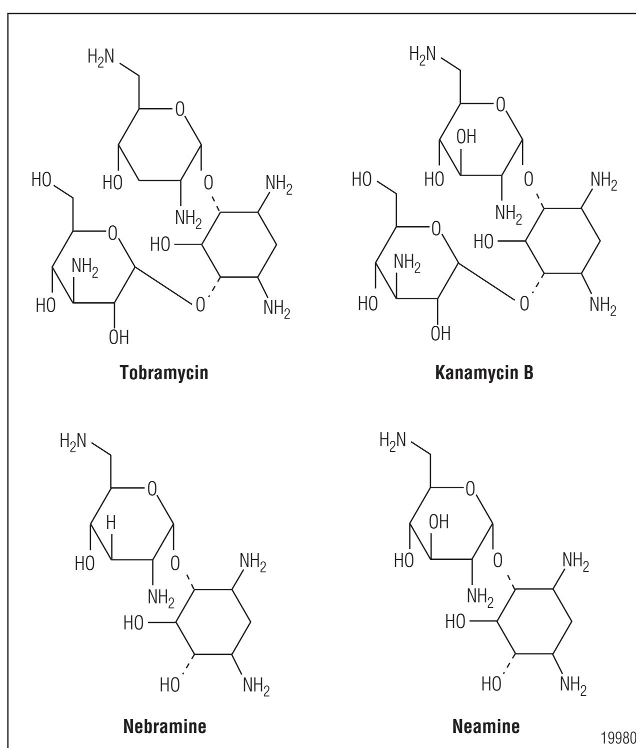


*Figure 1. Chemical structures of tobramycin and known impurities (kanamycin, nebramine, and neamine).*

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.

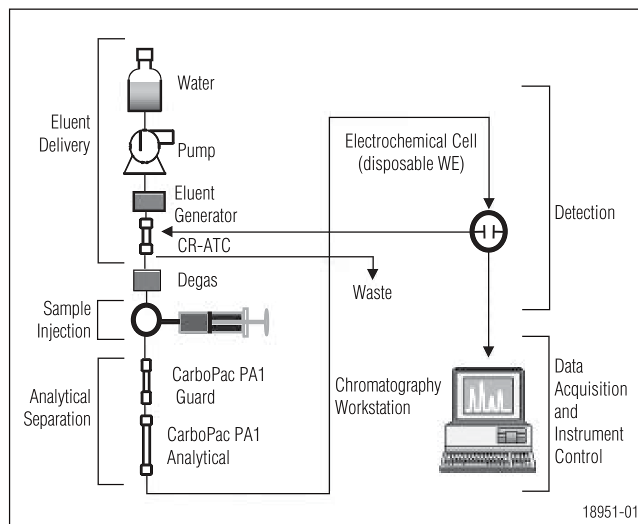


*Figure 2. HPAE-PAD system for tobramycin determinations.*

## 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.*

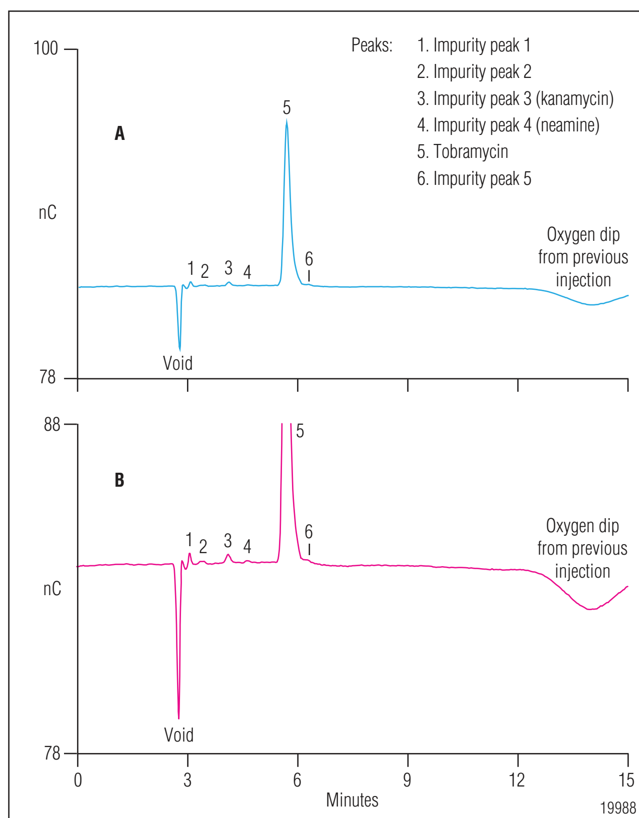


*Figure 3. Determination of tobramycin (1.07 µM, 20-µL injection) using eluent generation (2 mM KOH) with 0.5 mL/min flow rate, 30 °C column temperature, and* AAA-Direct *waveform. Full view (A) and expanded view of baseline (B).*

## 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 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.

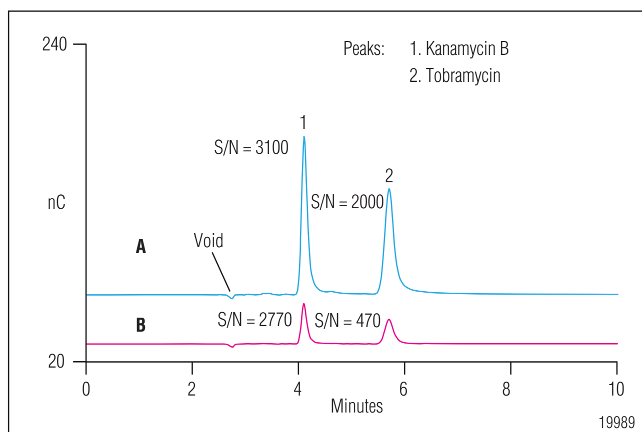


*Figure 4. Comparison of 10 µM tobramycin and kanamycin B peaks (20-µL injection) using the* AAA-Direct *waveform (A) and the carbohydrate waveform (B).*

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.

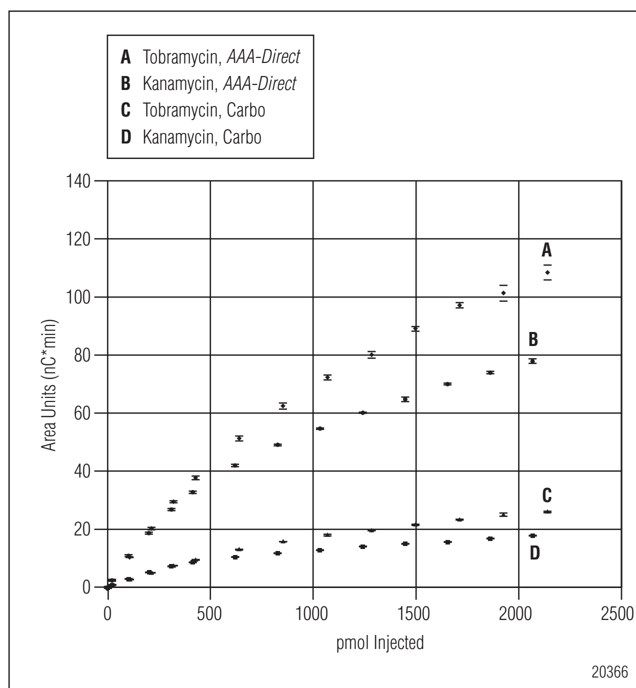


*Figure 5. The relationship of peak area (mean ± SD, n = 4 injections each concentration) to mass of tobramycin and kanamycin B injected using the carbohydrate and* AAA-Direct *waveforms for estimation of linear range.*

### *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

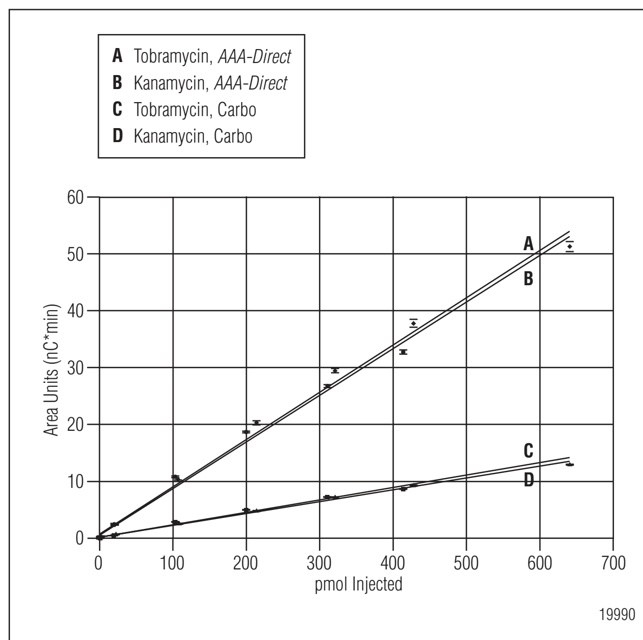


*Figure 6. The linear relationship of tobramycin and kanamycin B peak area (mean ± SD, n = 4 injections each concentration) within their estimated linear range using the carbohydrate and* AAA-Direct *waveforms.*

***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.

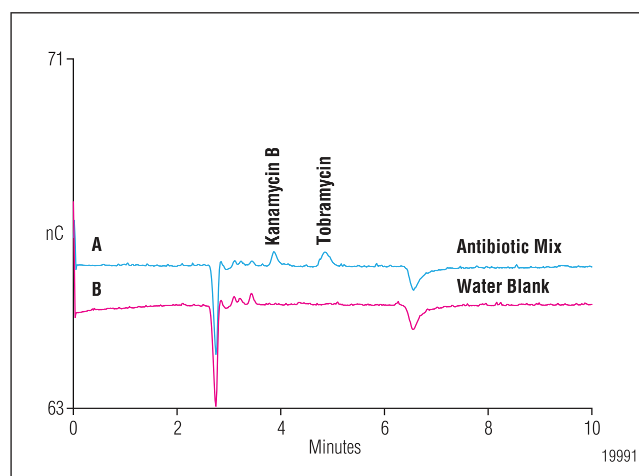


*Figure 7. Determination of 0.22 pmol tobramycin (0.011 µM) and 0.20 pmol kanamycin B (0.010 µM) near their lower limits of detection (20-µL injection).*

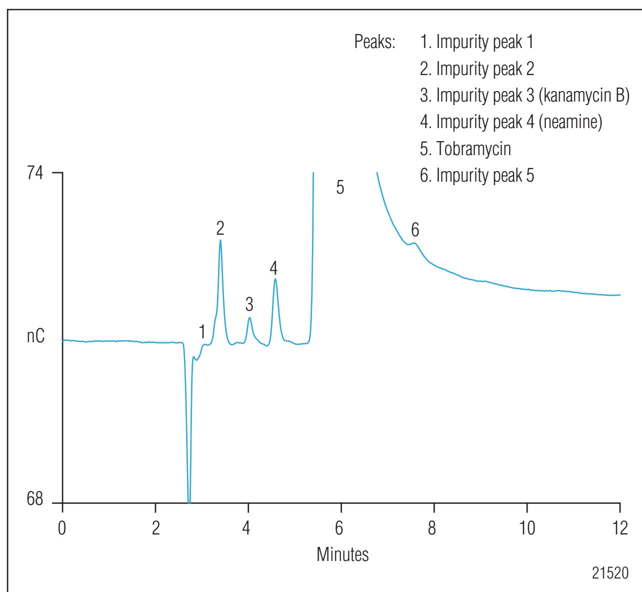


*Figure 8. Determination of impurities when tobramycin is analyzed at the upper limit of linearity (0.038 mg/mL, 20-µL injection) using the* AAA-Direct *waveform.*

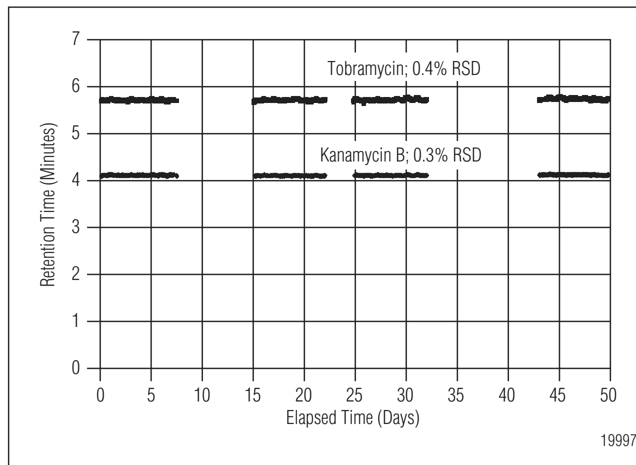


*Figure 9. Reproducibility of tobramycin and kanamycin B retention time over 50 days using the eluent generator. Intervals without data represent periods when the system was either shut down, idle, or used for other tobramycin experiments.*

## 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).

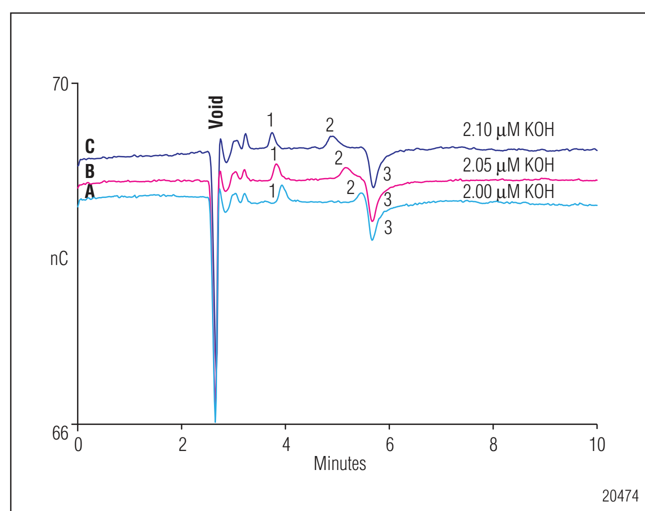


*Figure 10. Effect of minor adjustments in KOH concentration on the separation of the tobramycin (peak 2) from the first baseline dip (peak 3). Mixture of tobramycin (0.011 µM) and kanamycin B (peak 1; 0.010 µM) near their lower limits of detection using (A) 2.00 mM KOH, (B) 2.05 mM KOH, and (C) 2.10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

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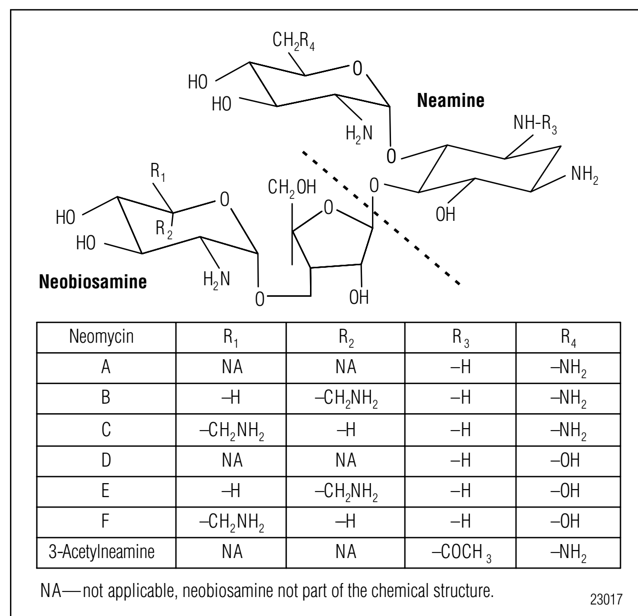
World Health Organization (WHO) Collaborating Centre for Chemical Reference Substances; Apoteket AB; Produktion & Laboratories; Centrallaboratoriet, ACL; PrismavAgen 2; SE-141 75 Kungens Kurva, Sweden, Fax: + 46 8 740 60 40, who.apl@apoteket.se, www.who.int/medicines/strategy/quality_safety/trs917annl.pdf.

# 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.

*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.

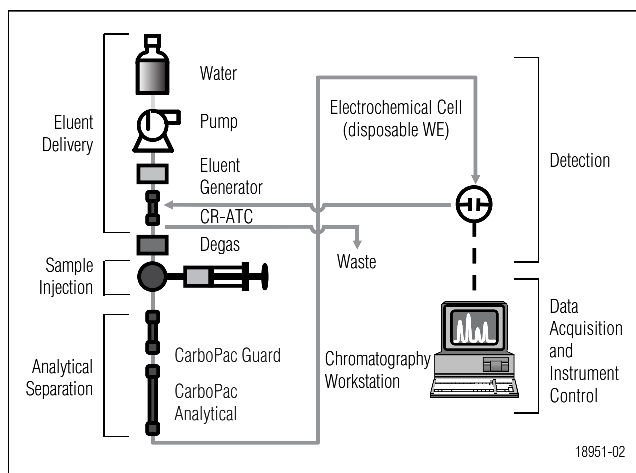



*Figure 2. HPAE-PAD system for Neomycin determinations.*

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

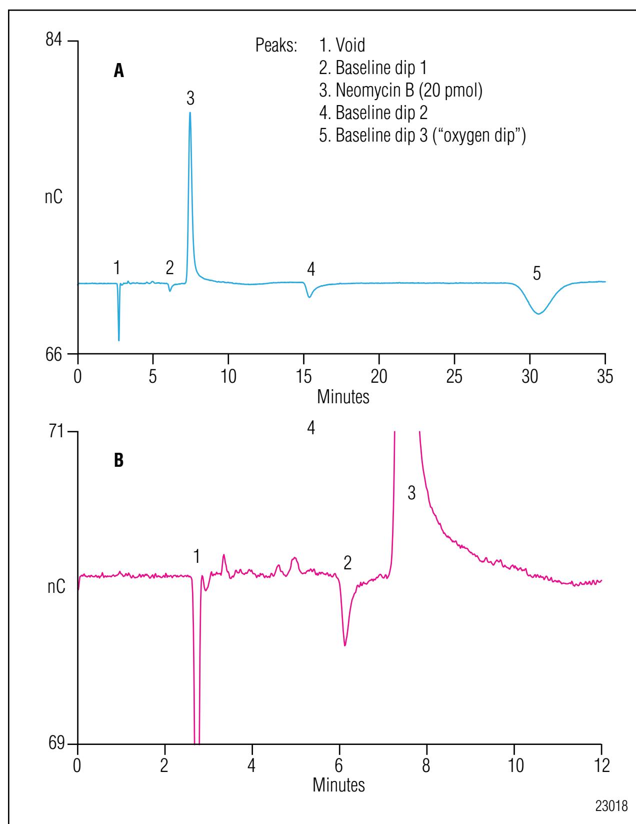



*Figure 3. Determination of Neomycin B (1.0 µM, 20-µL injection) using eluent generation (2.40 mM KOH).*

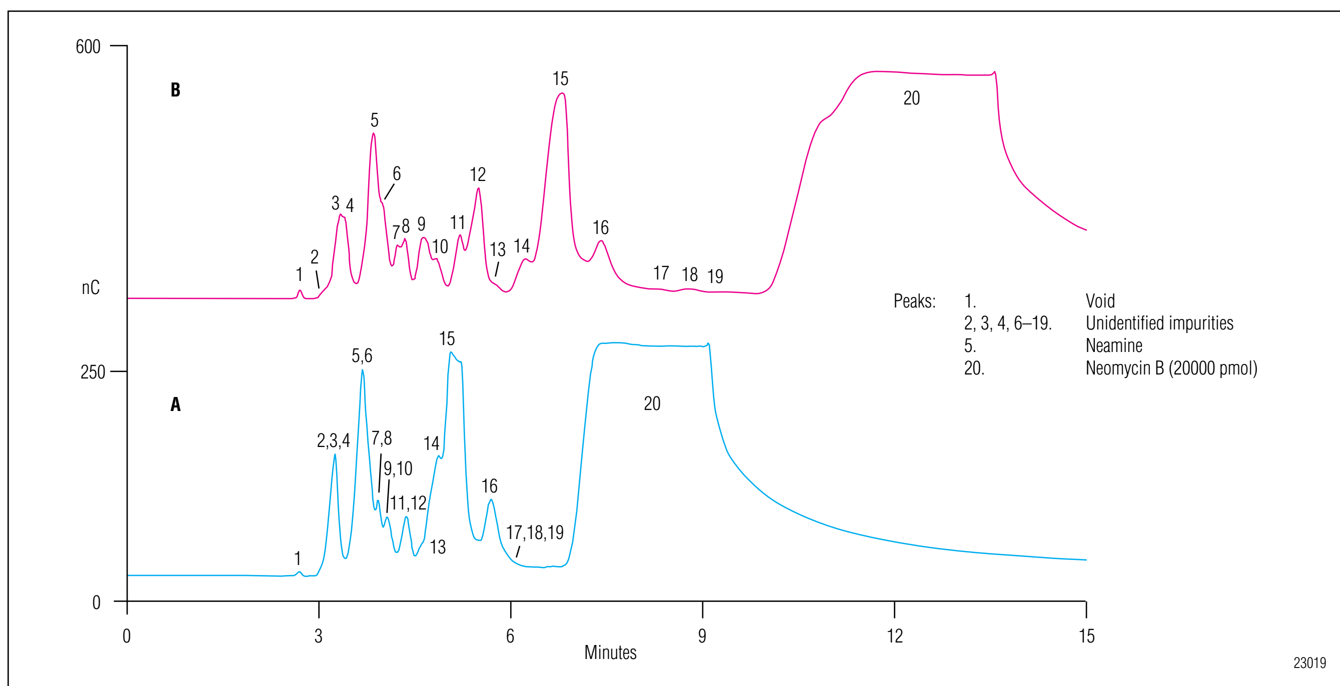



*Figure 4. Separation of Neomycin B and impurities is highly dependent on eluent concentration. Comparison of the resolution of Neomycin B (1 mM, 20000 pmol) and impurities in commercial grade Neomycin sulfate separated using 2.40 mM KOH (chromatogram A) and 2.16 mM KOH (chromatogram B). Neomycin B (peak 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 |

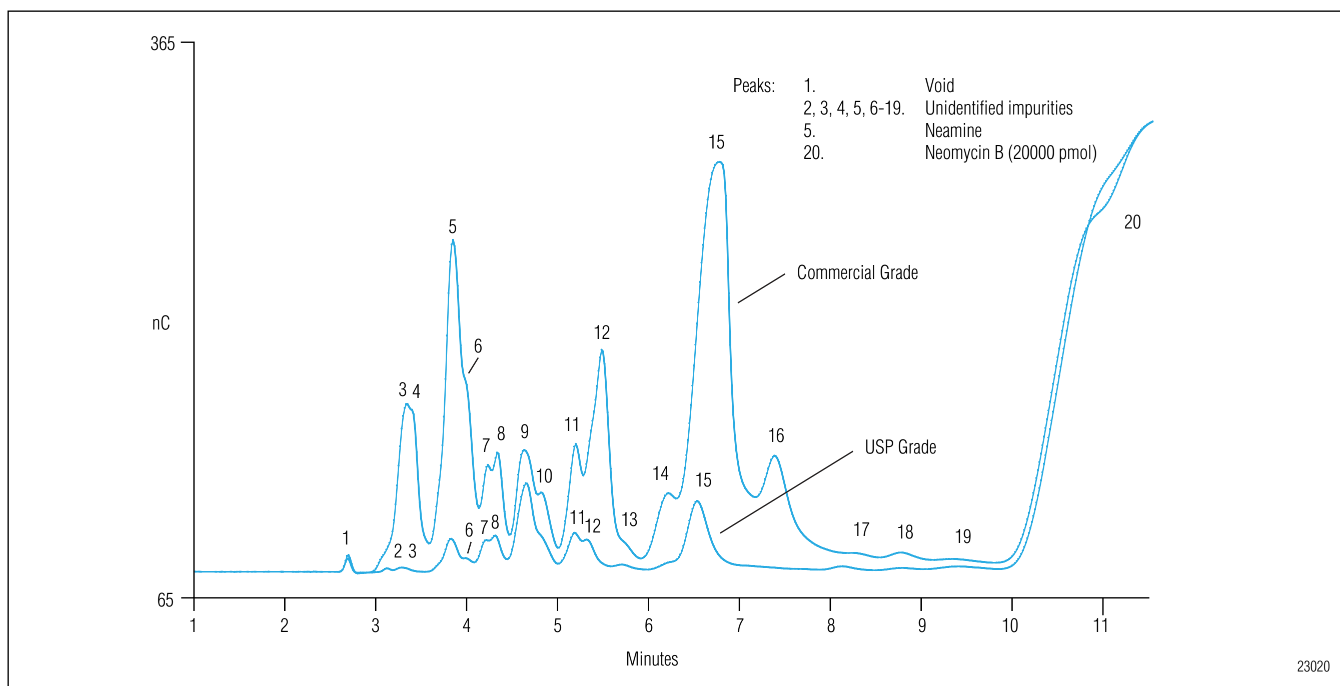



*Figure 5. Comparison of the impurities found in USP and commercial grade Neomycin B (1 mM, 2 x $10^4$ pmol) separated using 2.16 mM KOH. Neomycin B (peak 20) is injected at a concentration above its upper limit of detection.*

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

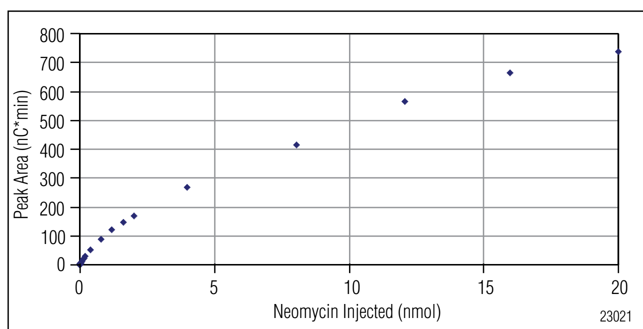


*Figure 6. The relationship of peak area (mean) to nmol of Neomycin B injected for estimation of linear range.*

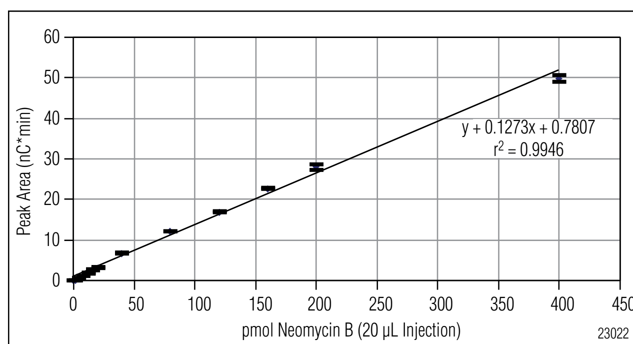


*Figure 7. The linear relationship of Neomycin B peak area (mean ± SD) within its estimated linear range.*

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.

| 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

### *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

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

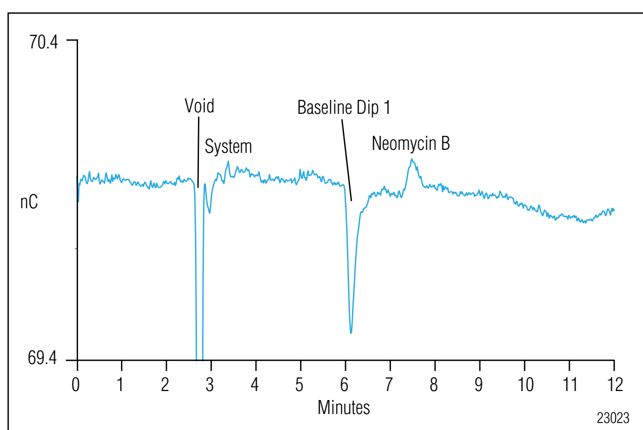



*Figure 8. 0.20 pmol Neomycin B (0.010 µM, 20 µL) at its lower limit of detection.*

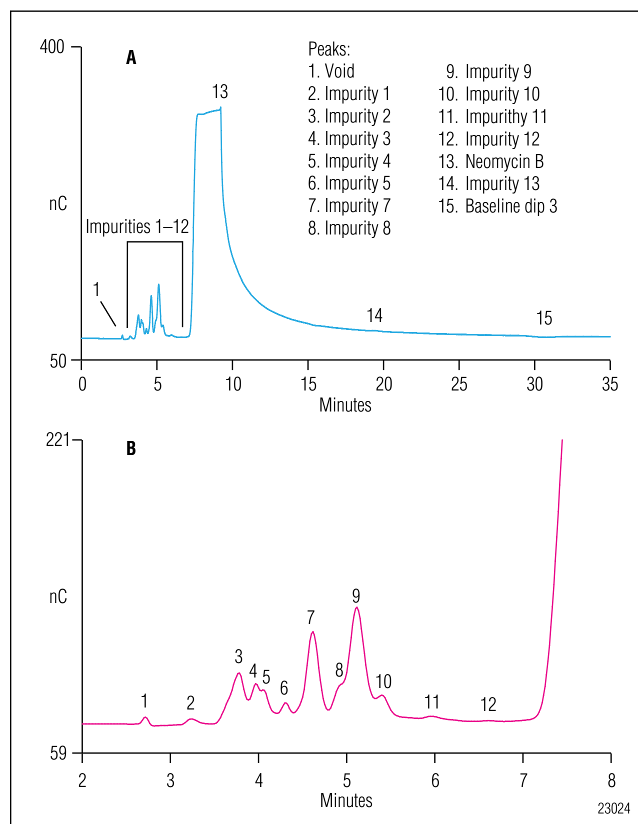



*Figure 9. Determination of impurities when Neomycin B is analyzed outside the upper end of its linear range (0.50 mg/mL, 20-µL injection).*

≤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.

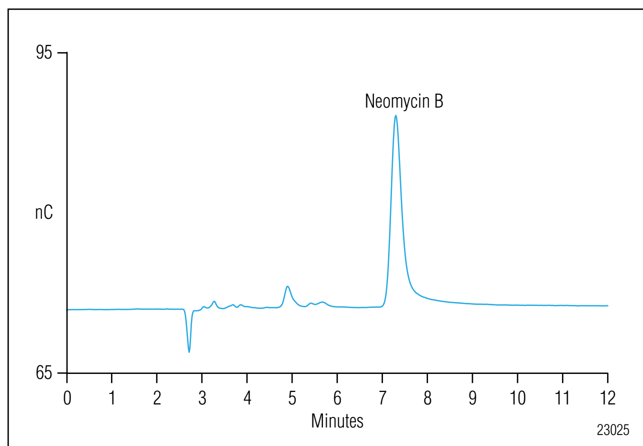



*Figure 10. Determination of Neomycin B (2.12 µM, 25 µL injection) extracted from 25.6-mg Neosporin topical ointment with 1 mL water, and diluted 85-fold.*

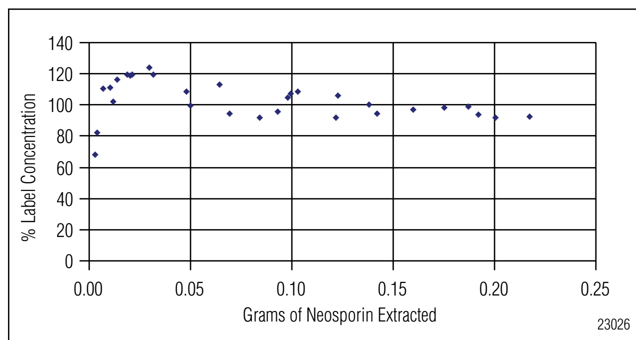



*Figure 11. The relationship of Neomycin B yield and the mass of Neosporin ointment extracted.*

## 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 °C. 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 startup, 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<br>B: 500 mM sodium acetate, pH 3.75<br>C: 90% acetonitrile<br>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)<br>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.

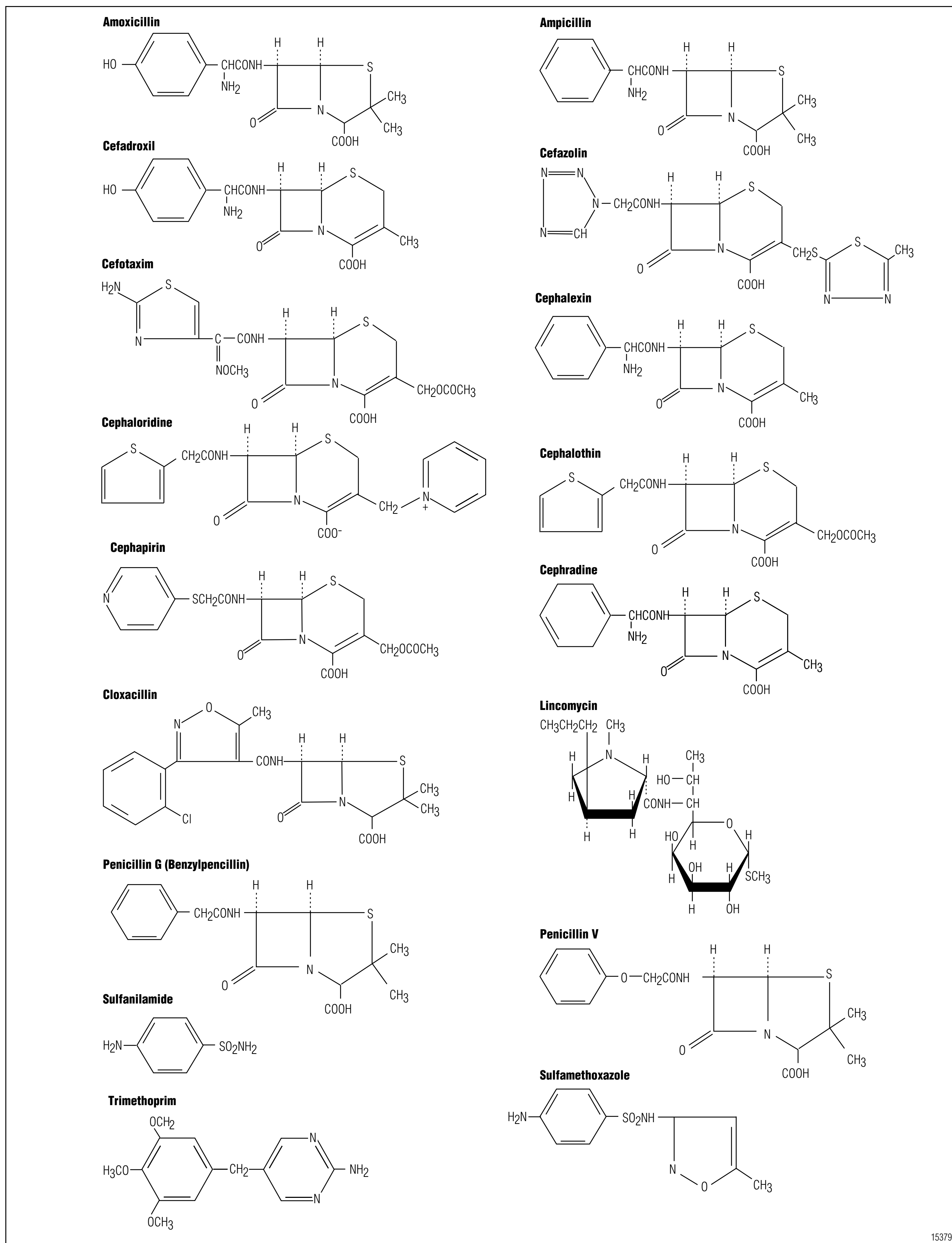


*Figure 1. Chemical structures of antibiotics*

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

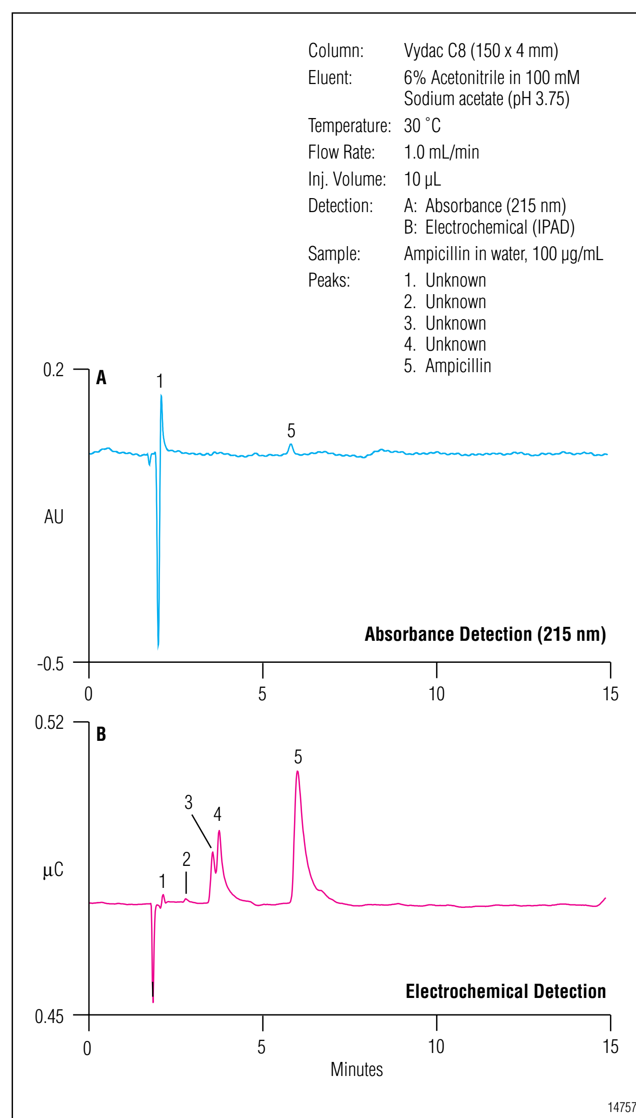


*Figure 2. Ampicillin detected by (A) absorbance at 215 nm and (B) IPAD.*

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.

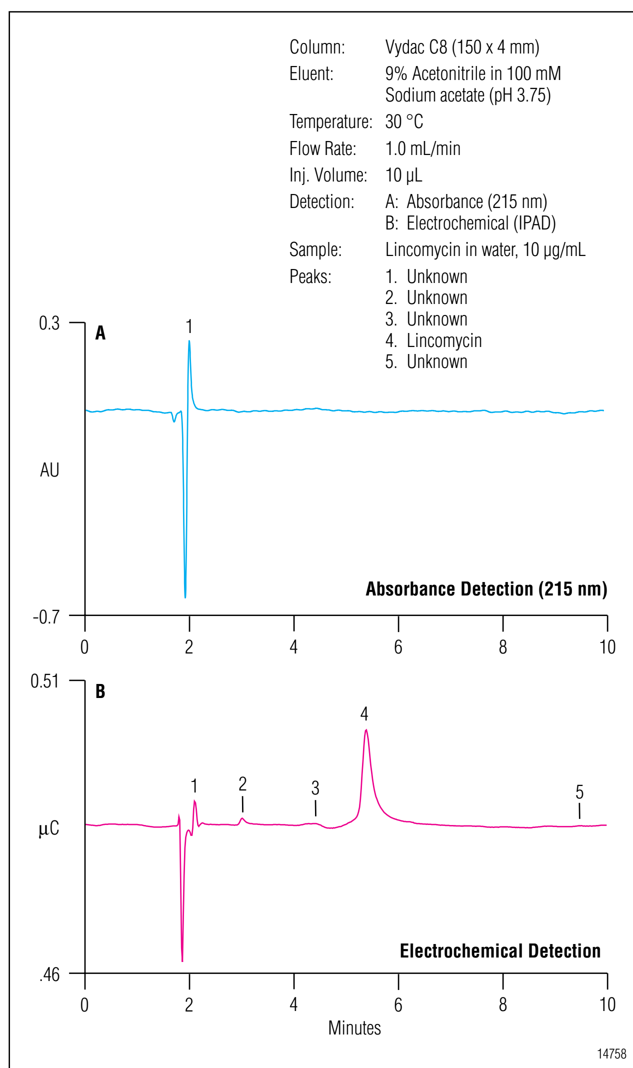


*Figure 3. Lincomycin detected by (A) absorbance at 215 nm and (B) IPAD.*

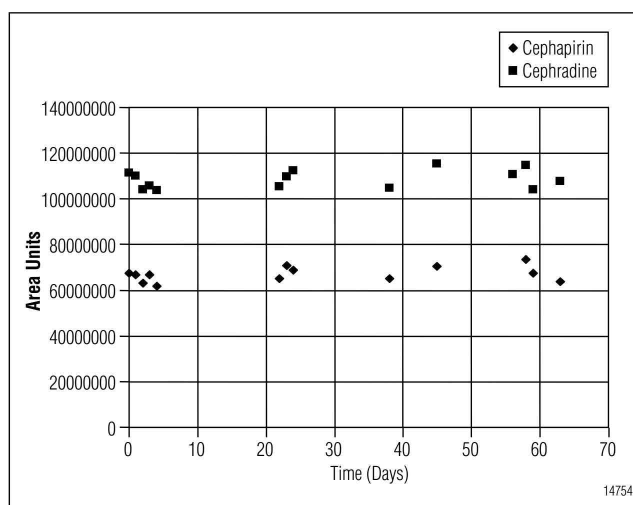


*Figure 4. Stability of electrochemical response over 64 days.*

| 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.

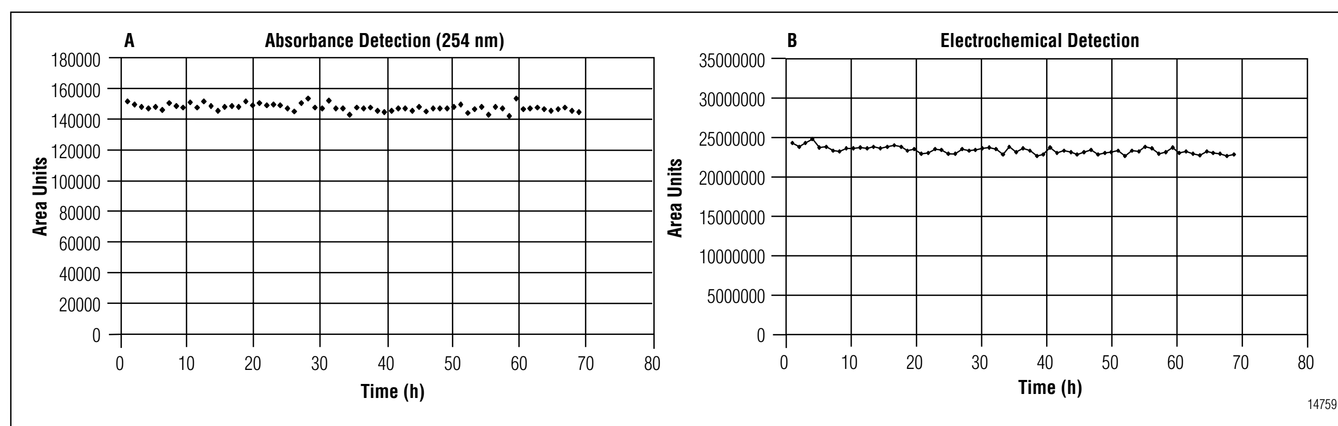


*Figure 5. Monitoring cephradine (10 µg/mL) stability in water at ambient temperature by (A) absorbance detection and (B) IPAD.*

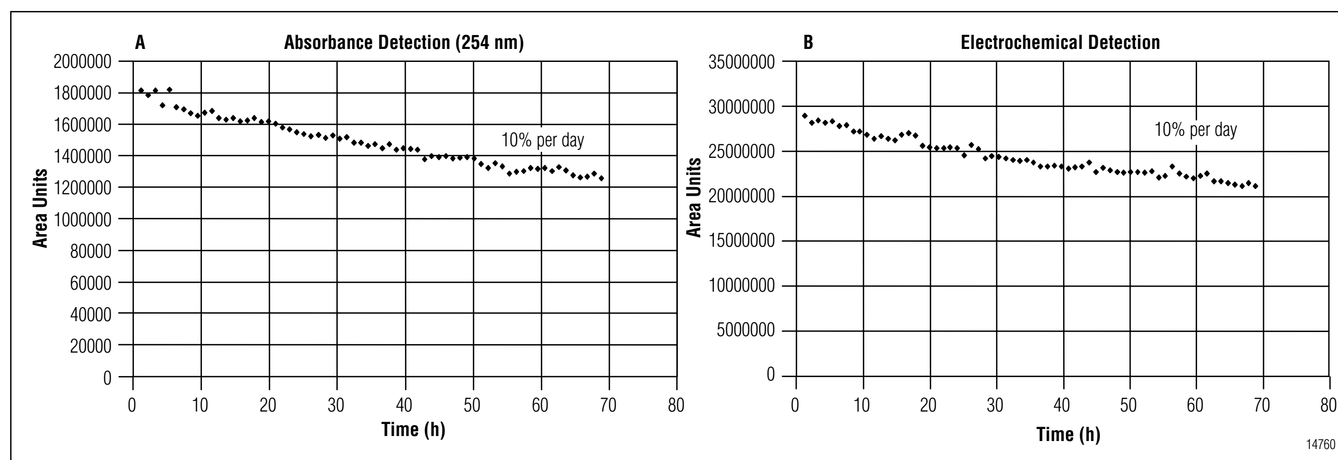


*Figure 6. Monitoring cephapirin (10 µg/mL) stability in water at ambient temperature by (A) absorbance detection and (B) IPAD.*

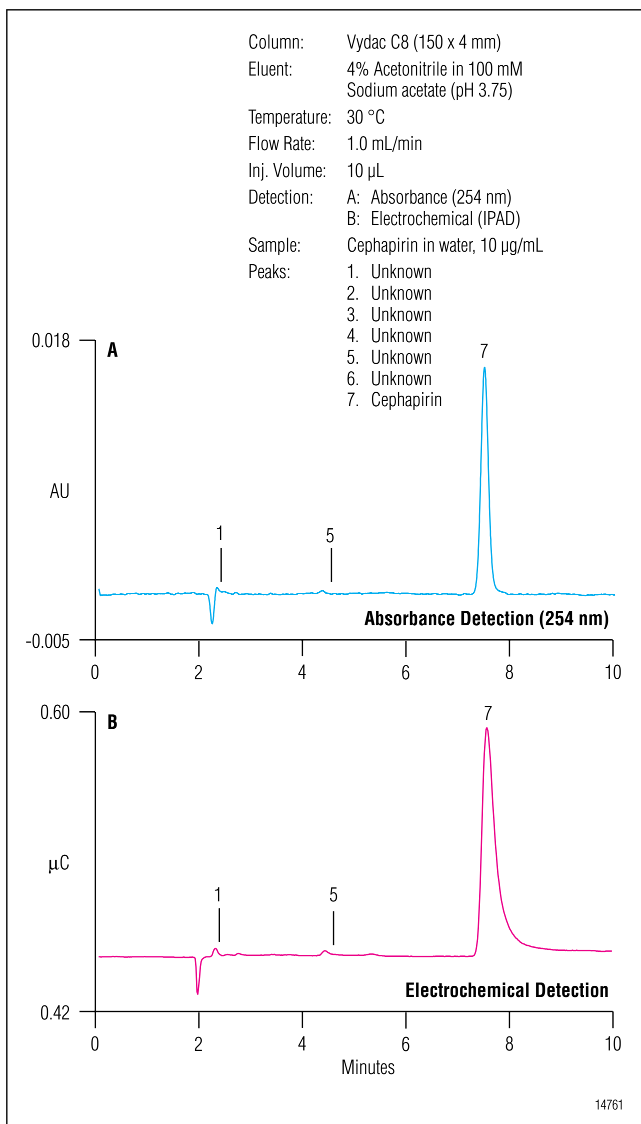


*Figure 7. Chromatograms of cephapirin by (A) absorbance detection and (B) IPAD.*

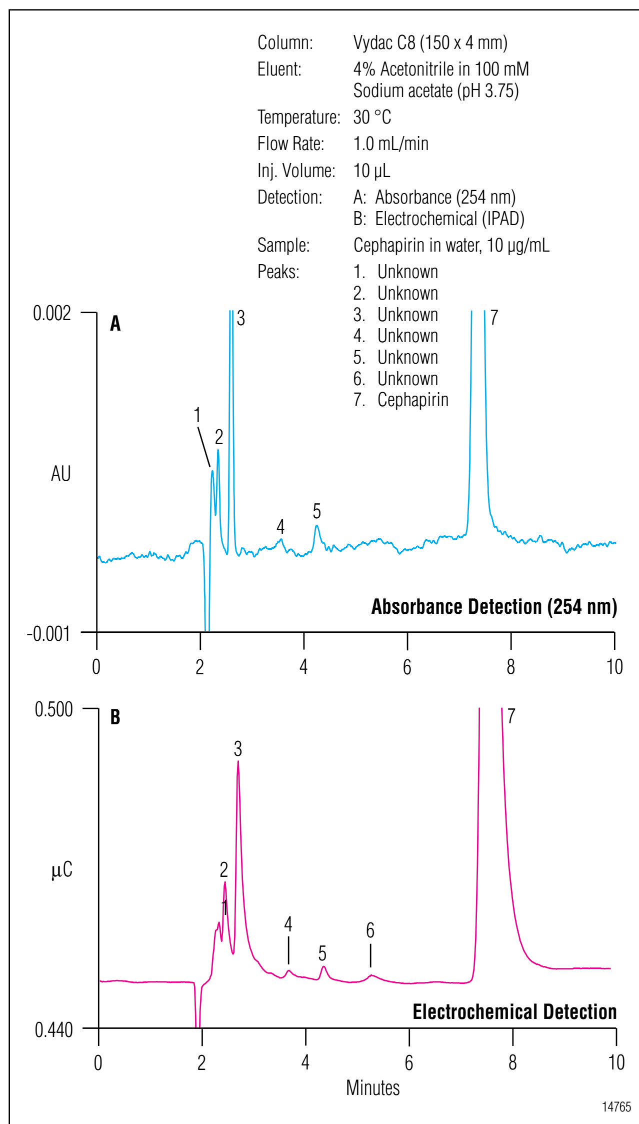


*Figure 8. Chromatograms of cephapirin after 69-h incubation in water at ambient temperature by (A) absorbance detection and (B) IPAD.*

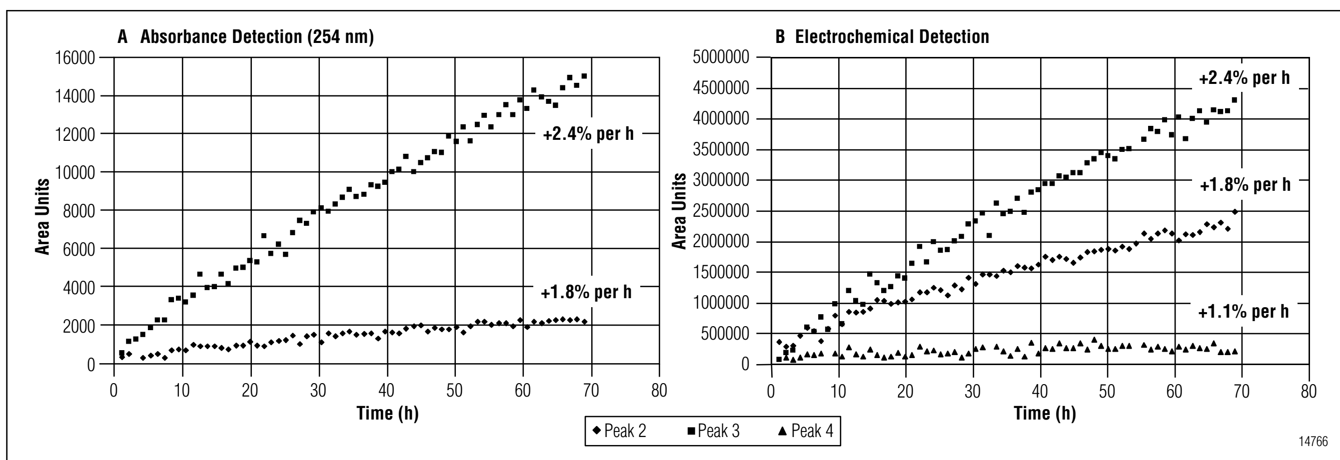


*Figure 9. Monitoring cephapirin (10 µg/mL) decomposition products by (A) absorbance detection and (B) IPAD.*

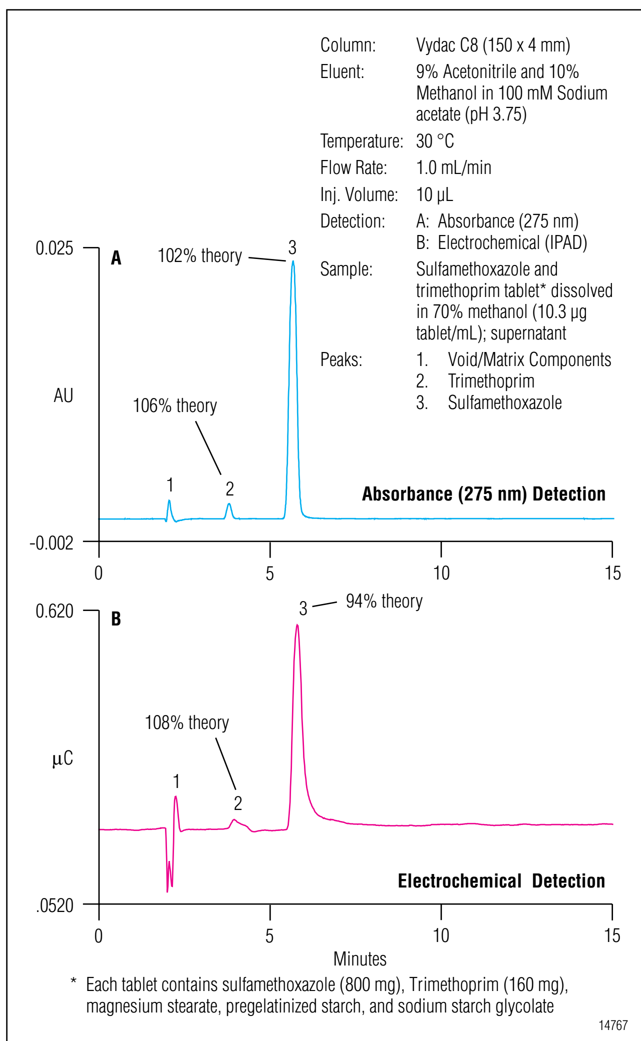


*Figure 10. Recovery of sulfamethoxazole and trimethoprim from a tablet formulation by (A) absorbance detection and (B) IPAD.*

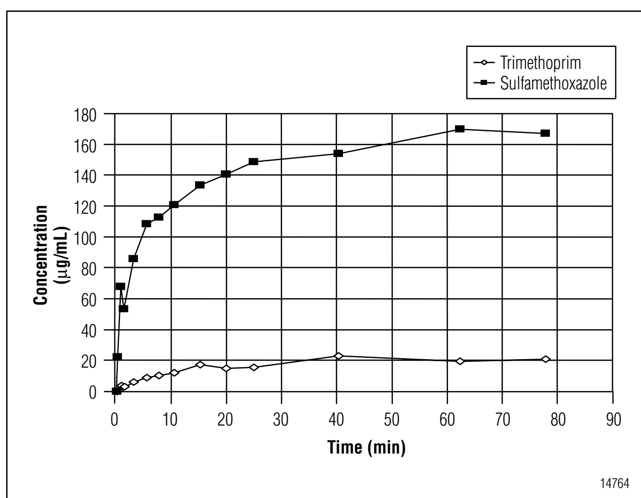


*Figure 11. Determination of the dissolution of sulfamethoxazole and trimethoprim from a tablet formulation by IPAD.*

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 | | | | | | | | | | | | | | | | | |

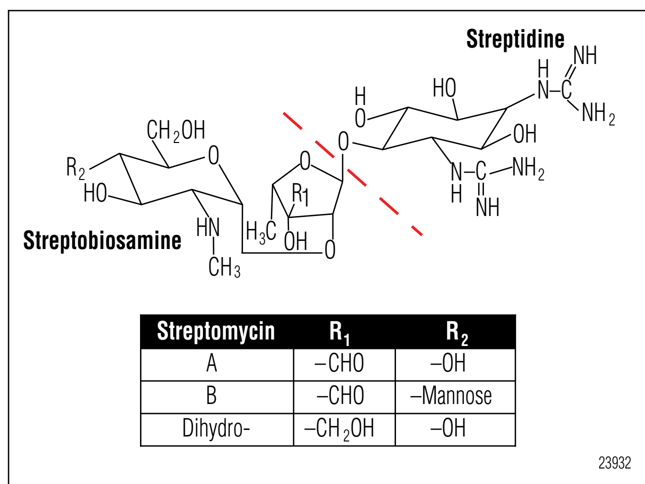
*Appendix 1. Selectivity of sulfur-containing antibiotics on a Vydac C8 Column.*

# 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 |

*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:***

$$\frac{100.92\text{ mg dried streptomycin SO}_4}{1.0023\text{ g H}_2\text{O}} \times 1.000\text{ g/mL} = 100.69\text{ mg/mL}$$

***(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):

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

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:

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

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.

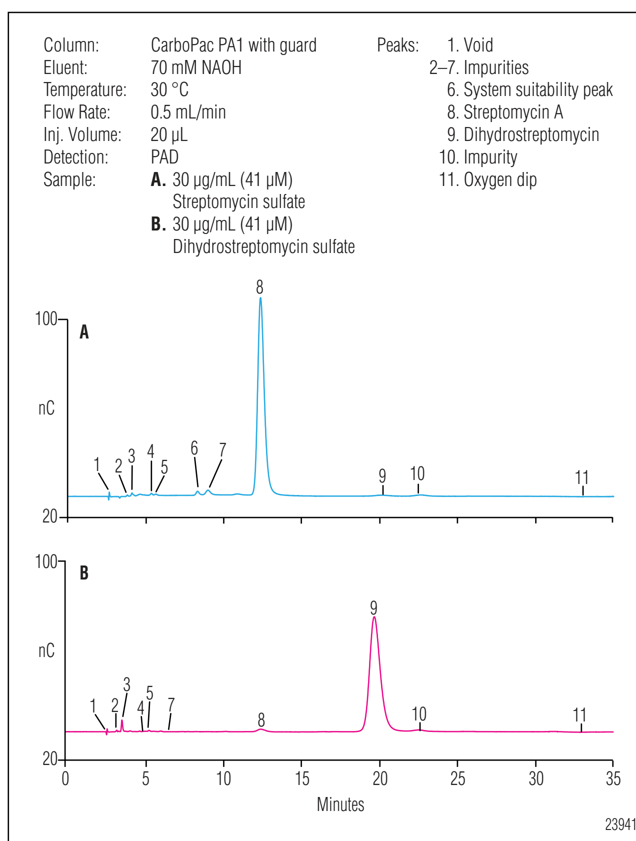



*Figure 2. Determination of streptomycin A and dihydrostreptomycin.*

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.

**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).

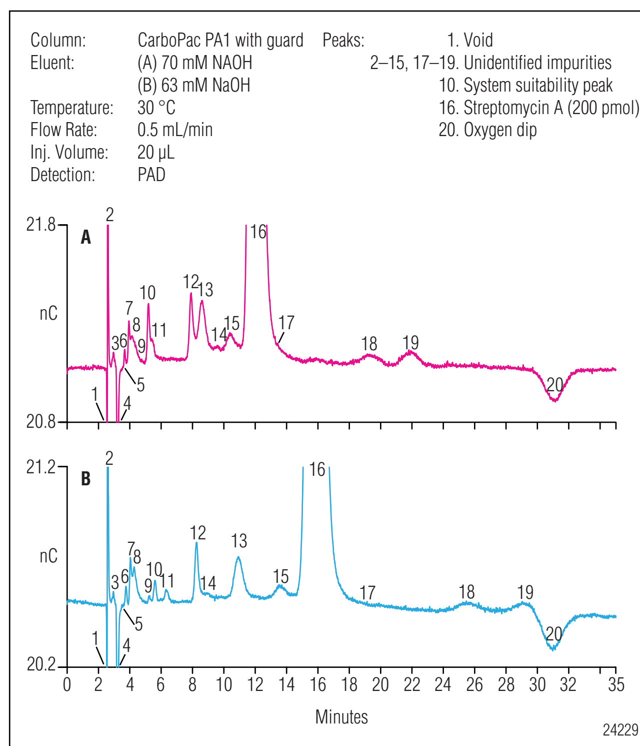



*Figure 3. Comparison of USP streptomycin at 70- and 63-mM NaOH eluent concentrations.*

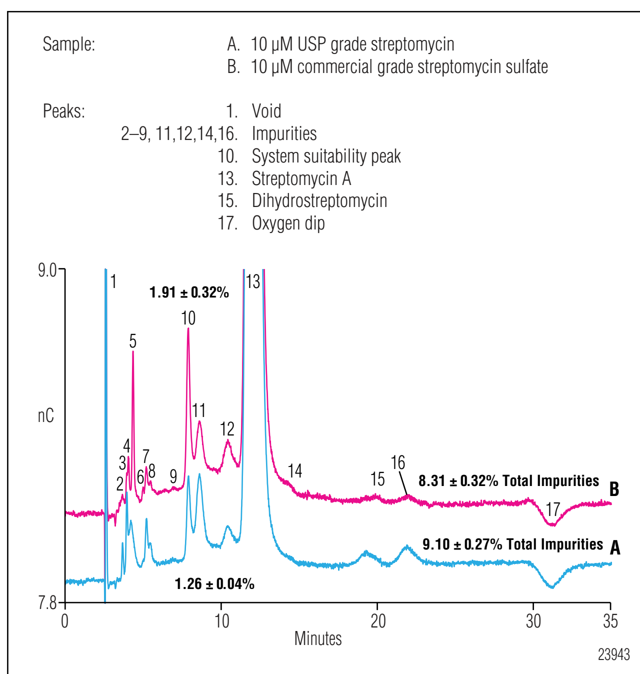



*Figure 4. Comparison of USP and a second commercial source of streptomycin sulfate at a 70-mM NaOH eluent concentration.*

## 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.

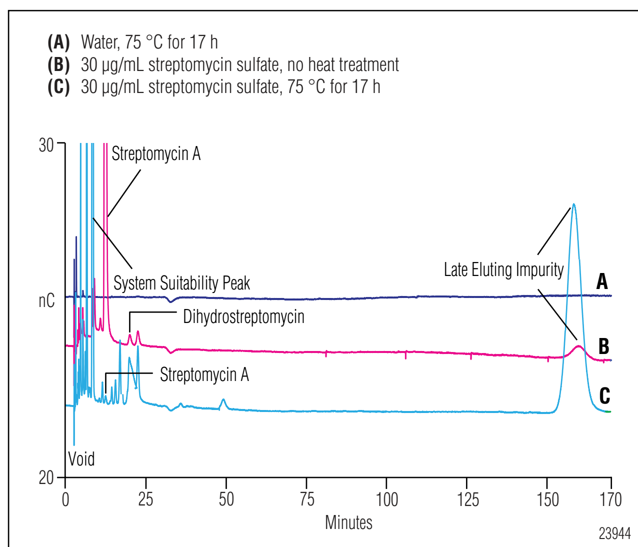



*Figure 5. Late eluting thermal degradation peak.*

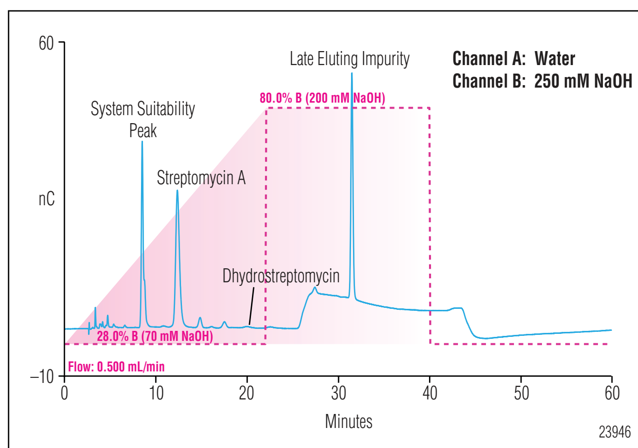



*Figure 6. Use of a different elution program to more quickly elute the thermal degradation peak.*

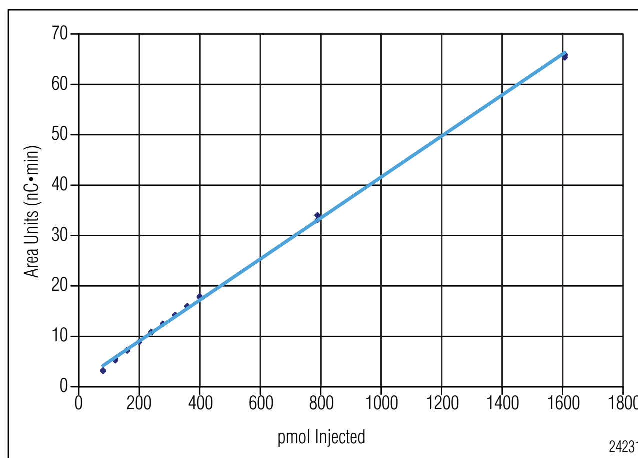



*Figure 7. Linearity of streptomycin within the range of 80–1600 pmol (4–80 µM, 20 µL injection).*

### *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<br>2 | 2.9% | 10.49% | 0.39%<br>–0.32% | 9.56%<br>8.79% |
| Chemist 2 | 1<br>2 | 2.9% | 10.29% | 0.66%<br>0.34% | 9.86%<br>9.50% |
| Chemist 3 | 1<br>2 | 2.9% | 10.36% | 0.79%<br>–0.95% | 10.00%<br>8.10% |
| Chemist 4 | 1<br>2 | 2.9% | 10.36% | –1.27%<br>0.41% | 7.75%<br>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

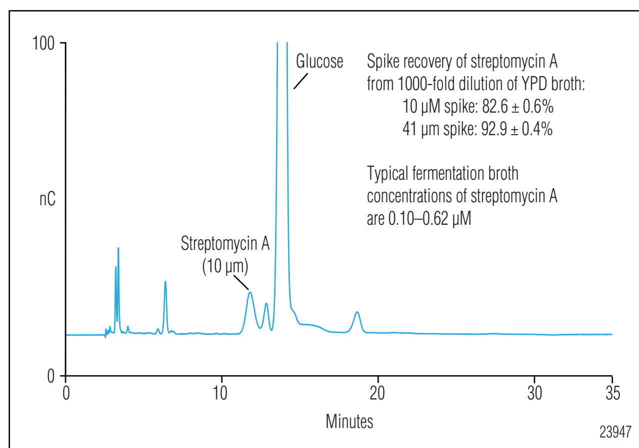



*Figure 8. Determination of streptomycin A in YPD fermentation broth.*

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]

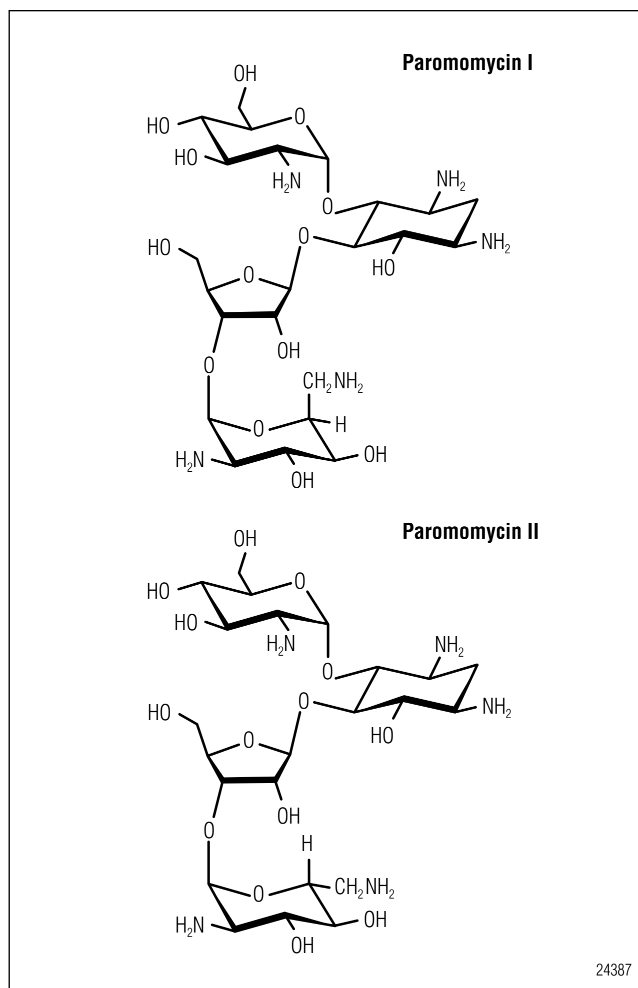


*Figure 1. Paromomycin I and II.*

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)<br>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)<br>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.

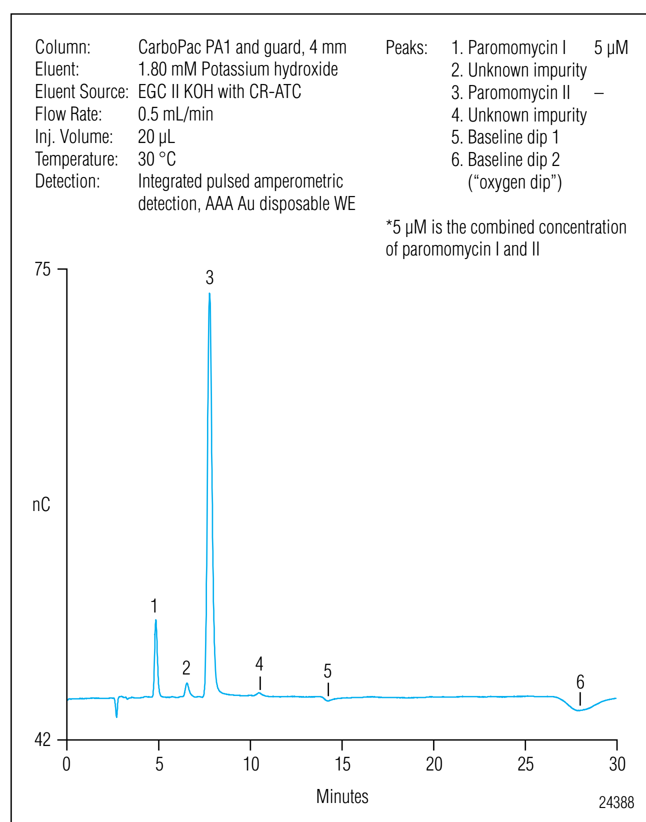


*Figure 2. Determination of paromomycin (5 µM) using the CarboPac PA1 column with eluent generation.*

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.

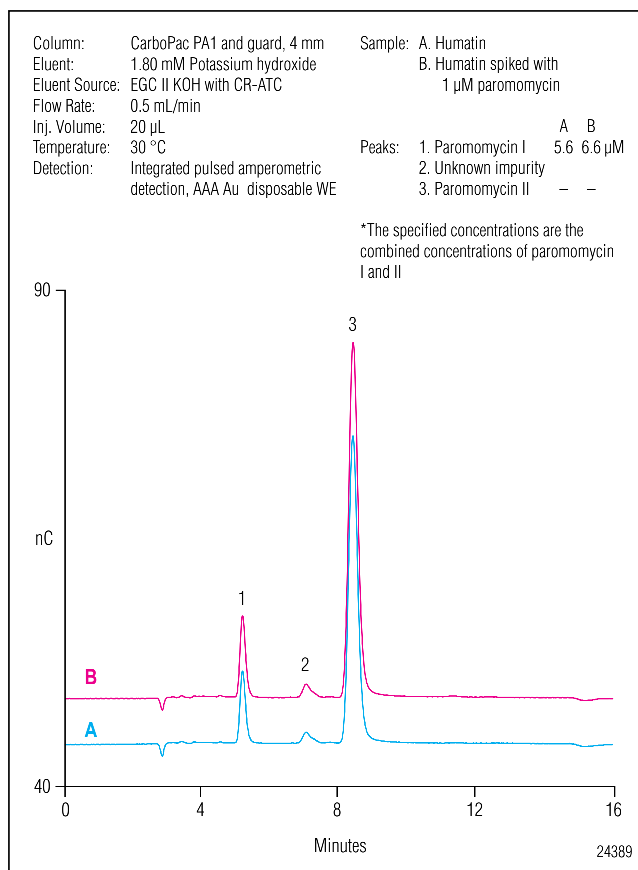


*Figure 3. Comparison of A) unspiked and B) spiked Humatin sample containing paromomycin.*

## 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.

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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.

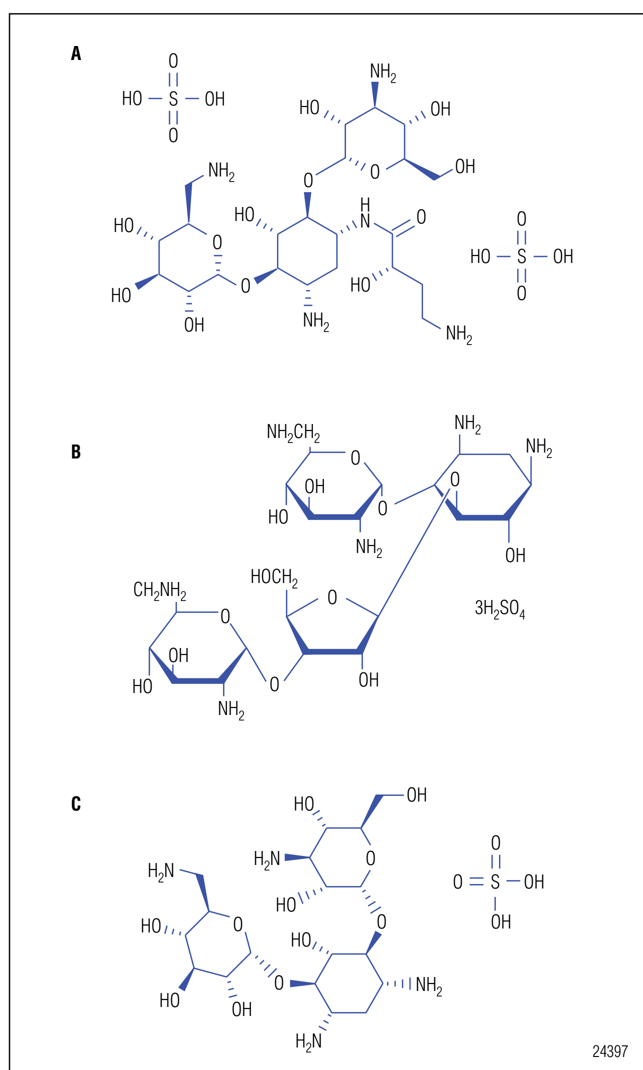

*Figure 1. Chemical structures of some aminoglycoside sulfate compounds: A) amikacin disulfate, B) neomycin B trisulfate, C) kanamycin A sulfate.*

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 $Na_4P_2O_7 \cdot 10 H_2O$, 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

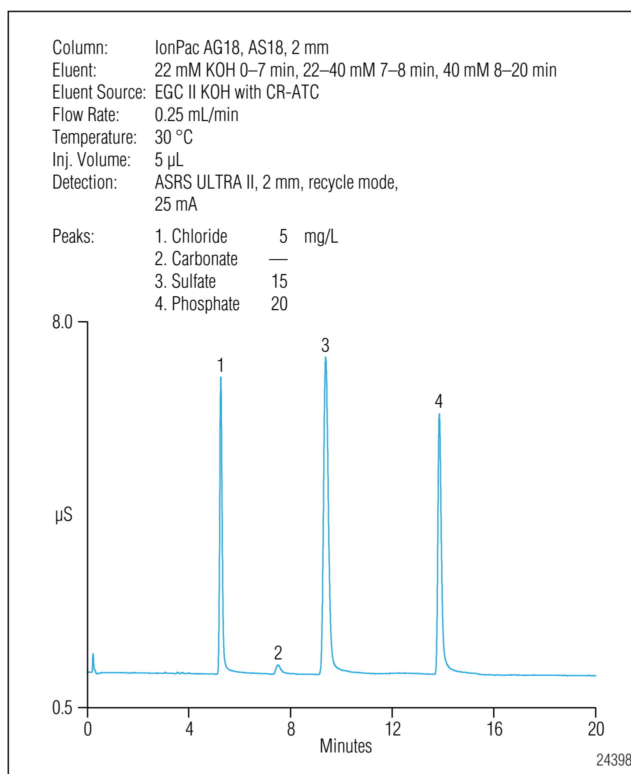


*Figure 2. Separation of target anions on the IonPac AS18 column.*

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.

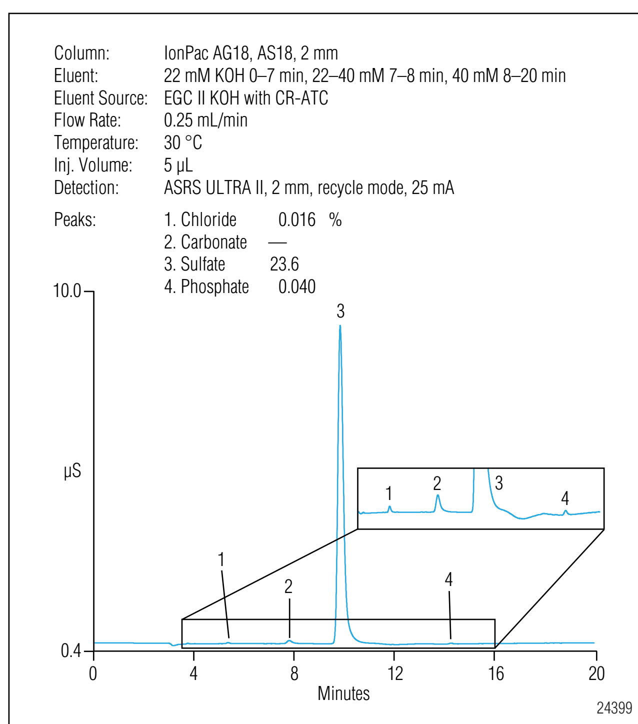


*Figure 3. Separation of sulfate counter ion and anionic impurities in USP-grade paromomycin sulfate.*

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,

| Sample | Theoretical Sulfate (%) | Experimental Sulfate (%) | Acetate (%) | Chloride (%) | Phosphate (%) | Pyrophosphate (%) |
|---|---|---|---|---|---|---|
| Humatin | 23.7 | 24.7 | 0.080 | 0.025 | 0.23 | 0.035 |

Table 5. Percentages of Sulfate Counter Ion and Anionic Impurities Detected in Humatin Using Method 2

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.

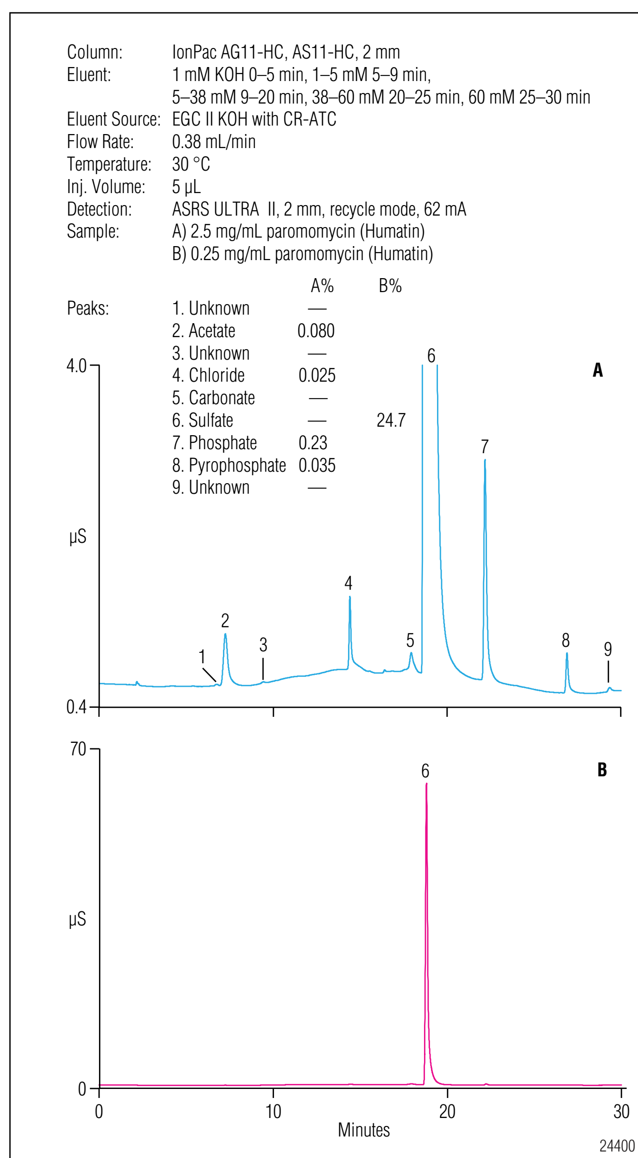


*Figure 4. Separation of sulfate counter ion and anionic impurities in Humatin using the IonPac AS11-HC.*

## 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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19. *Determination of Neomycin B and Impurities using HPAE-IPAD.* Application Note 66 (LPN 1828, July 2006), Dionex Corporation, Sunnyvale, CA.
20. *Assay of Paromomycin Using HPAE-IPAD.* Application Note 186 (LPN 1942, August 2007), Dionex Corporation, Sunnyvale, CA.

# 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]

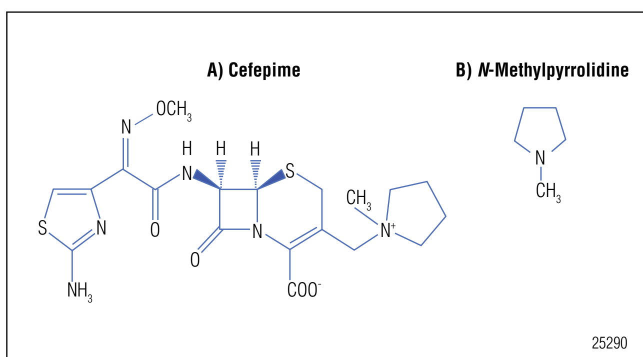


*Figure 1. Chemical structures of A) cefepime and B)* N-*methyl-pyrrolidine.*

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)<br>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)<br>(50 °C was used for the system suitability test)<br>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.

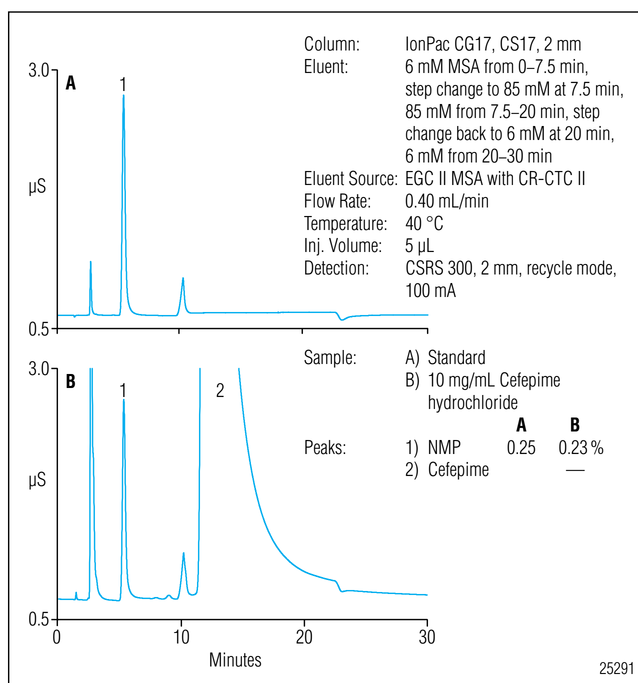


*Figure 2. Comparison of the separation of NMP in A) a standard solution and B) cefepime hydrochloride.*

### 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.)

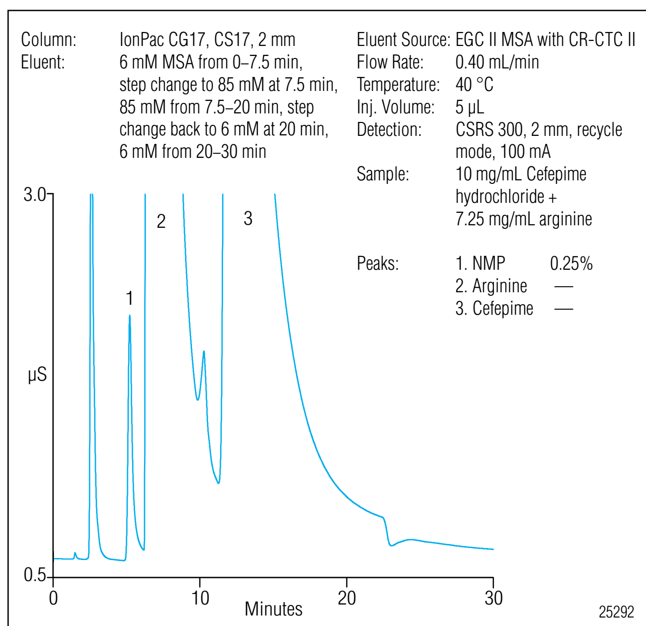


*Figure 3. Determination of NMP in a simulated Cefepime for Injection sample.*

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.

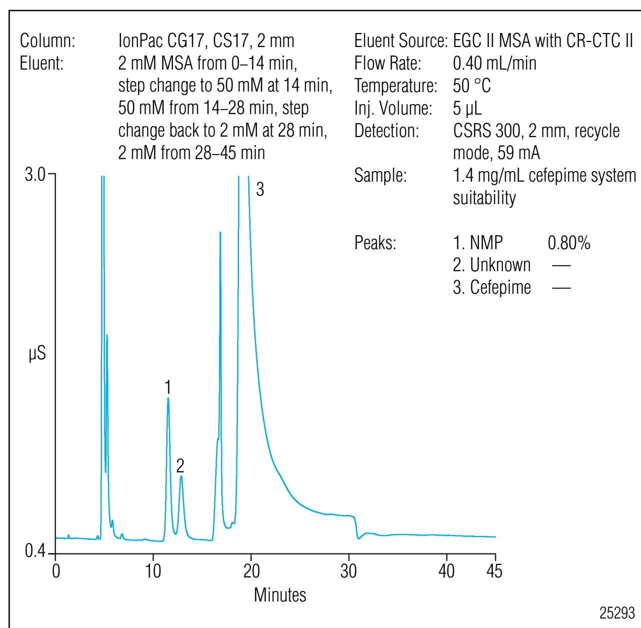


*Figure 4. Determination of NMP in USP Cefepime Hydrochloride System Suitability RS sample.*

| 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 |

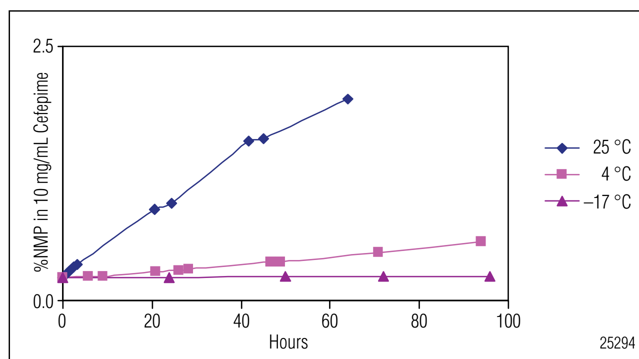


*Figure 5. Stability of NMP in USP Cefepime Hydrochloride RS sample.*

### 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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### *SUPPLIERS*

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

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

# 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

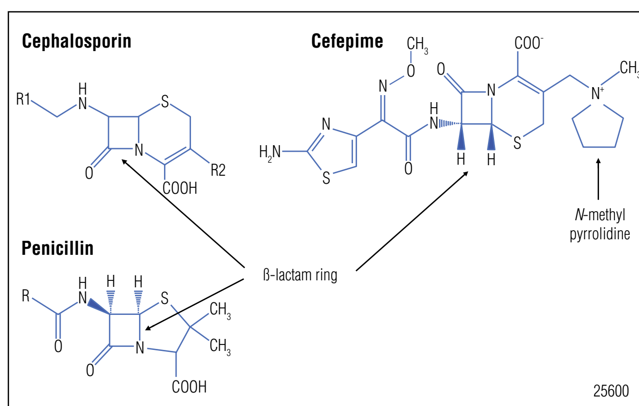


*Figure 1. Penicillin, cephalosporin, and cefepime.*

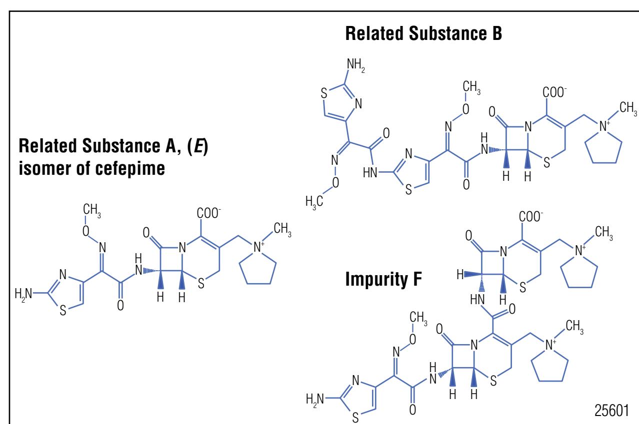


*Figure 2. Cefepime-related substances.*

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**<br>A: 90/10 5 mM potassium phosphate/acetonitrile<br>B: 50/50 5 mM potassium phosphate/acetonitrile<br>-or-<br>**Increased Resolution Method**<br>A: 94/6 5 mM potassium phosphate/acetonitrile<br>B: 50/50 5 mM potassium phosphate/acetonitrile |
| Gradient: | **USP Method**<br>100% A for 10 min, 0–50% B in 20 min, 50% B for 5 min, 9 min of equilibration prior to injection<br>-or-<br>**Shortened Runtime Method**<br>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.

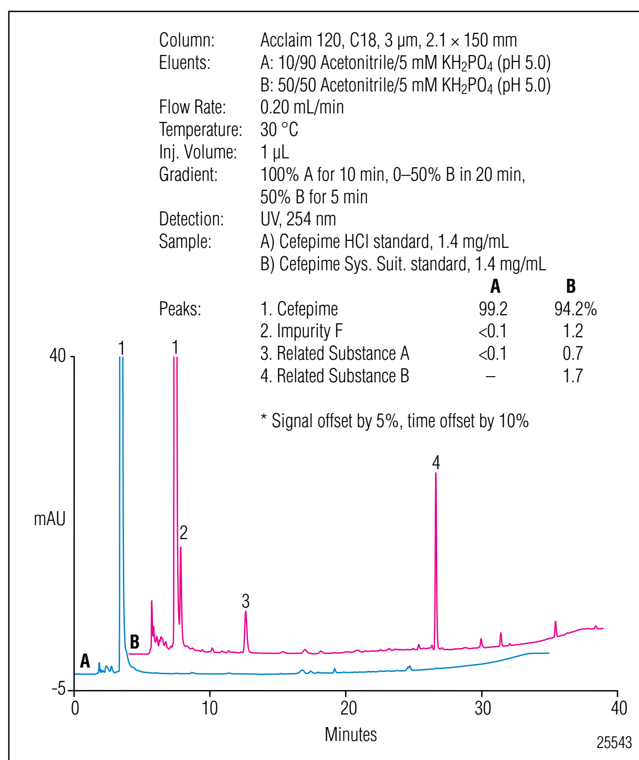


*Figure 3. Separation of cefepime and Cefepime System Suitibility Standard.*

### 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.

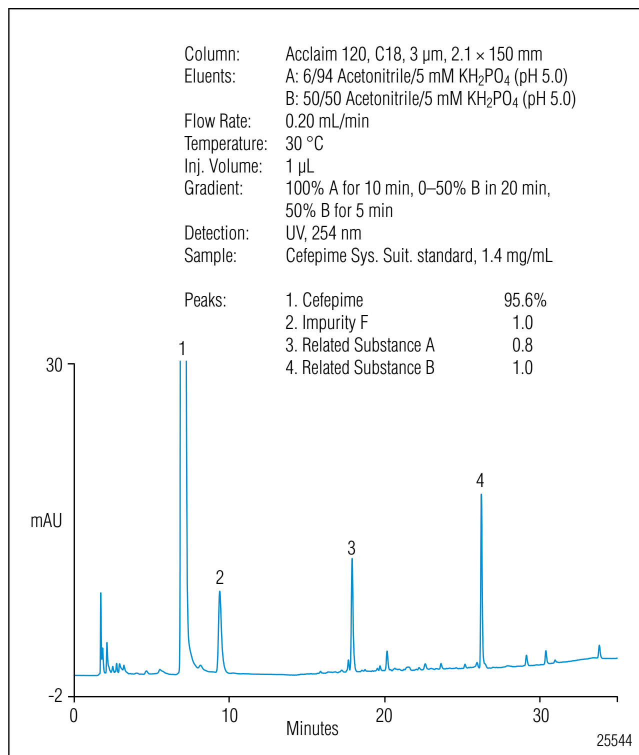


*Figure 4. Resolution improvement with 6% acetonitrile in mobile phase A.*

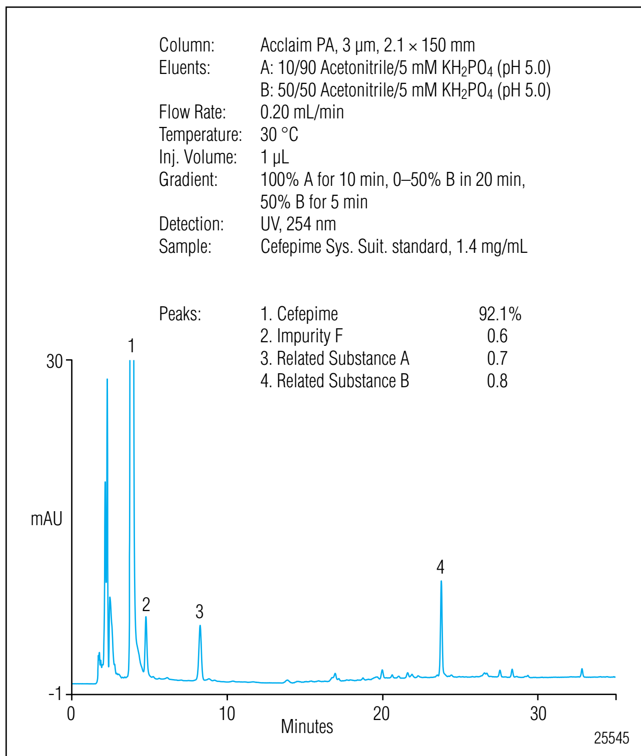


*Figure 5. Improved resolution using the Acclaim PA column.*

## 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 |

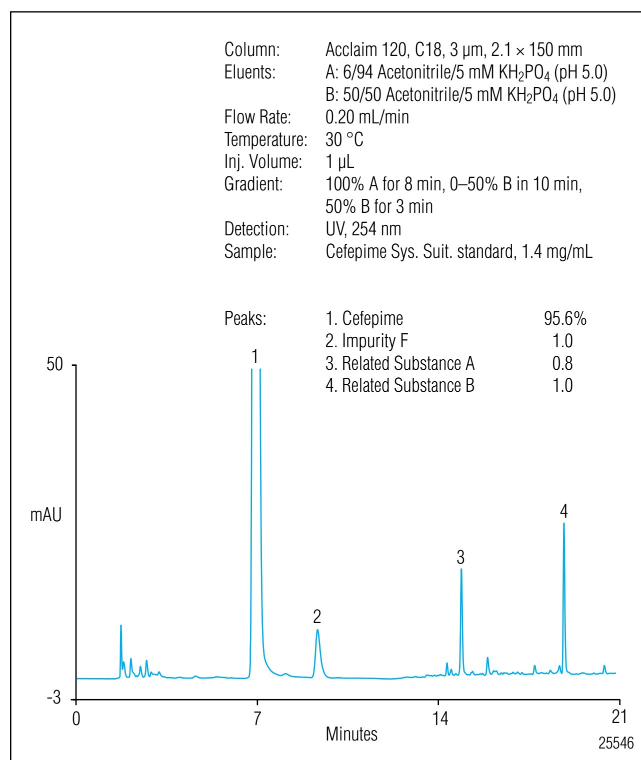


*Figure 6. Separation with a rapid gradient using the Acclaim 120 C18 column*

| 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.

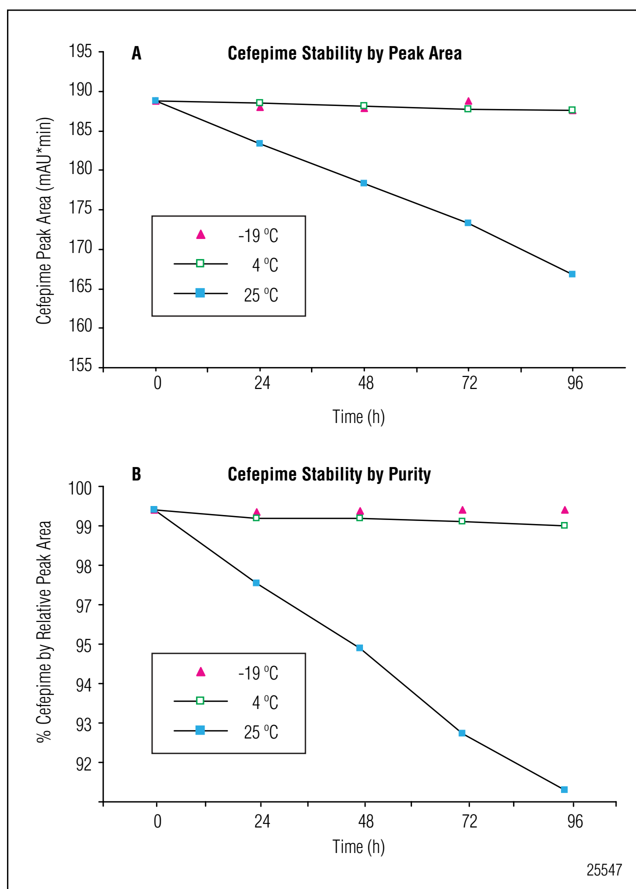


*Figure 7. Stability of cefepime over 96 h. A) Absolute cefepime peak area. B) Relative cefepime peak area.*

## 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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## *SUPPLIERS*

VWR, 1310 Goshen Parkway,
West Chester, PA 19380 USA.
Tel: 800-932-5000. www.vwr.com

Fisher Scientific, One Liberty Lane,
Hampton, NH 03842 USA.
Tel: 800-766-7000. www.fishersci.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

# Sulfonamide Antibiotics on Acclaim 120 C18 RSLC

## 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, pre-dating 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 8 sulfonamide antibiotics in a single run of only 3 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.

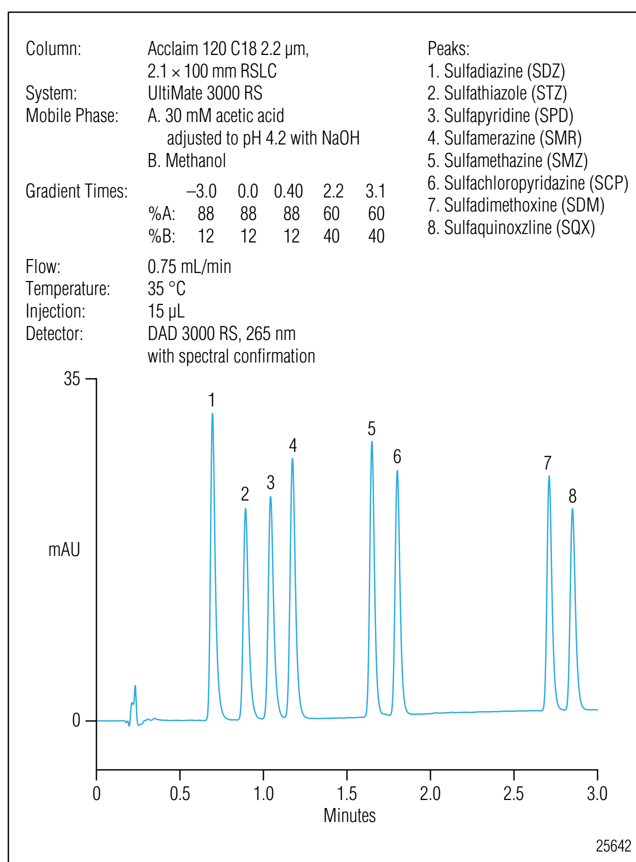


*Figure 1. Sulfonamide antibiotics on Acclaim 120 C18 RSLC.*

# Rapid HPLC Separation of Multiclass Antibiotics in Food and Water

## INTRODUCTION

The use of antibiotics on livestock, aquaculture, and bee husbandry helps maintain health and provides other benefits such as improved disease resistance, increased production, and in some cases, reduction in foodborne pathogens. However, antibiotic residues in foods can cause undesirable side effects such as idiosyncratic aplastic anemia, production of antibiotic-resistant bacteria, and the reduction of indigenous microbiota found in the human digestive tract. In addition, the wastes of these animals can lead to antibiotics being present in our water. Organizations responsible for food safety perform assays for the presence of allowed and banned antibiotics as part of their surveillance activities. This application brief demonstrates a reproducible Rapid Separation Liquid Chromatography (RSLC) method for the separation of polyketide (tetracycline and oxytetracycline), macrolide (tylosin), phenicol (chloramphenicol), nitrofuran (nitrofurantoin), and sulfonamide (sulfathiazole) classes of antibiotics with an MS-compatible mobile phase (Figure 1, Table 1).

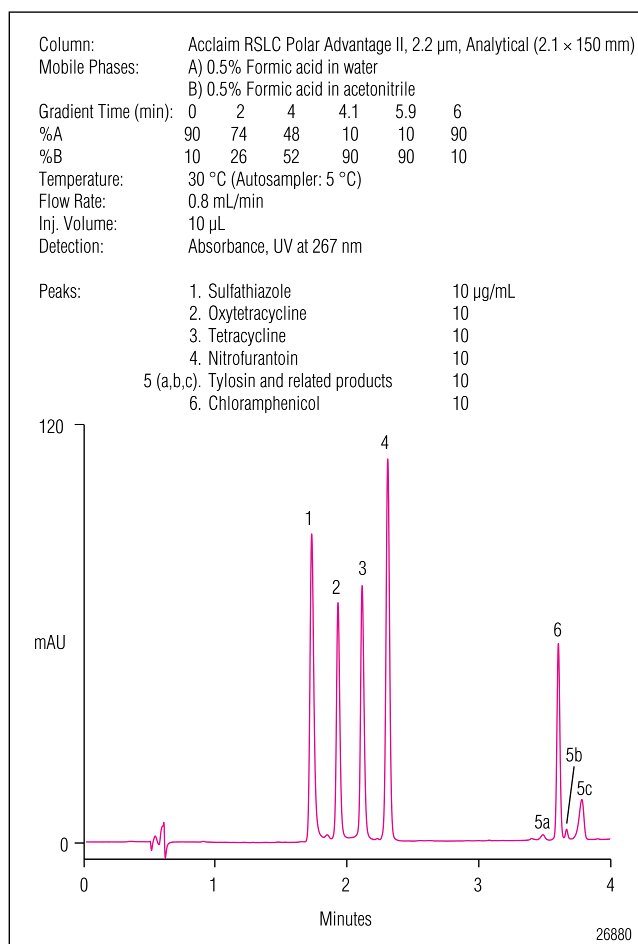


*Figure 1. Separation of multiple classes of antibiotics using the Dionex RSLC system and UV-diode array detection at 267 nm.*

## *CONDITIONS*

System: UltiMate® 3000 RSLC consisting of HPG-3200 Binary pump, SRD-3200 solvent rack with degasser channels, WPS-3000TRS thermostatted in-line split-loop autosampler, TCC-3000RS themostatted column compartment, and DAD-3000RS diode array detector with semi-micro (2.5 µL, 7 mm) SS flow cell

Column: Acclaim® RSLC Polar Advantage II (2.1 x 150 mm packed with 2.2 µm particles, 120 Å average pore size), P/N 071401

Mobile Phases: A = 0.5% formic acid in water (pH 2.4)
B = 0.5% formic acid in acetonitrile

Gradient:

| Time (min) | %A | %B |
|---|---|---|
| 0.00 | 90.0 | 10.0 |
| 2.00 | 74.0 | 26.0 |
| 4.00 | 48.0 | 52.0 |
| 4.10 | 10.0 | 90.0 |
| 5.90 | 10.0 | 90.0 |
| 6.00 | 90.0 | 10.0 |

Maximum pressure – 685 bar (9900 psi)

Flow Rate: 0.80 mL/min

Inj. Volume: 10.0 µL

Temperatures: 30 ºC (column)
5 ºC (autosampler)

Detection: 267, 278, 287, 355, 367 nm (based on UV spectra of individual antibiotics)
Bandwidth – 11 nm each
Data collection rate – 25 Hz
Response time – 0.20 s

**Table 1. Retention Time Precision for the Separation of Multiclass Antibiotics [a, b]**

| Peak | Antibiotic | RT (min) | RSD |
|---|---|---|---|
| 1 | Sulfathiazole | 1.725 | 0.13 |
| 2 | Oxytetracycline | 1.925 | 0.11 |
| 3 | Tetracycline | 2.109 | 0.12 |
| 4 | Nitrofurantoin | 2.305 | 0.12 |
| 5a | Tylosin & Related Products | 3.492 | 0.12 |
| 5b | | 3.673 | 0.11 |
| 5c | | 3.789 | 0.11 |
| 6 | Chloramphenicol | 3.610 | 0.12 |

[a] Acclaim RSLC Polar Advantage II column
[b] n = 10 replicates

# Determination of *N*-Methylpyrrolidine in Cefepime with Nonsuppressed Conductivity Detection

## INTRODUCTION

Cefepime (Figure 1A) is a semi-synthetic, fourth-generation cephalosporin with low toxicity and a broad antimicrobial activity range against Gram-negative and Gram-positive bacteria.[1, 2] However, this antibiotic is thermally unstable and will rapidly degrade at temperatures ≥ 25 °C and slowly degrade at lower temperatures (e.g., 4 °C).[3] One of the degradation products is *N*-methylpyrrolidine (NMP, Figure 1B).[3] The primary concerns with NMP formation are loss of cefepime potency and potential toxicity to patients. Although NMP is metabolized to the N-oxide and cleared rapidly,[4] the potential for side effects is still a health concern. Therefore, the determination of NMP in cefepime is critical to assess the purity of the pharmaceutical product due to potential toxicity of NMP to patients.

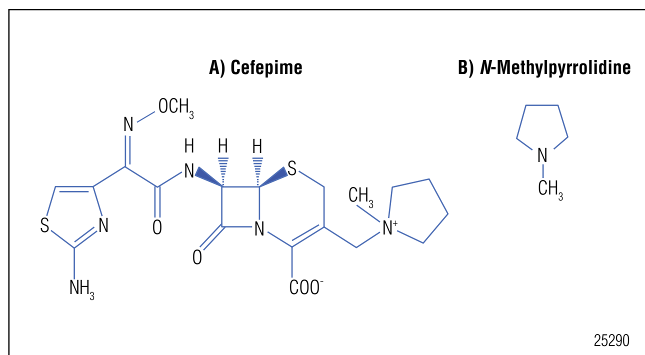


*Figure 1. Chemical structures of A) cefepime and B)* N-*methylpyrrolidine.*

Cefepime and its related compounds have been characterized by HPLC with UV detection,[5] whereas the NMP degradation product in cefepime has been determined by cation-exchange chromatography with suppressed conductivity detection using a Reagent-Free Ion Chromatography (RFIC™) system.[6] The U.S. Pharmacopeia (USP) has proposed improving compendial methods for determining the limit of NMP in Cefepime Hydrochloride[7] and Cefepime for Injection[8] by eliminating column rinse and re-equilibration steps, thereby increasing sample throughput from 3–4 h to 60 min per sample.[9, 10]

This work describes the determination of NMP by cation-exchange chromatography with nonsuppressed conductivity detection.[11] The results meet the USP criteria for Organic Impurities, Procedure 1 (Limit of *N*-Methylpyrrolidine) in the proposed methods for Cefepime Hydrochloride[9] and Cefepime for Injection.[10]

## EQUIPMENT

Dionex ICS-2100 system* including:

- Single isocratic pump
- Vacuum degasser
- High pressure, 6-port injector
- Column heater enclosure
- Conductivity cell detector
- EO Eluent Organizer, including pressure regulator, and 2 L plastic bottle

AS Autosampler with sample tray temperature control and 2 mL vial tray

Chromeleon® 6.8 or greater Chromatography Data System (CDS) software

Helium or nitrogen, 4.5-grade (99.995%) or better, <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

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

*This application can also be run using an ICS-1100, -1600, -3000, or -5000 system.

## CONSUMABLES

IonPac® SCG1 column, 4 × 50 mm (Dionex P/N 061523)

IonPac SCS1 column, 4 × 250 mm (Dionex P/N 061521)

IonPac Mixer (Dionex P/N 063443)

## REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher, filtered and degassed

Nitric acid, Ultrex II ultrapure reagent (VWR P/N JT6901-5)

Acetonitrile, UV (VWR P/N BJ015-4)

Cefepime Hydrochloride Reference Standard (USP P/N 1097636), Lot H0G278 was used in this study.

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

*N*-Methylpyrrolidine, 97% (Sigma-Aldrich P/N M79204)

## CONDITIONS

Columns: IonPac SCG1 4 × 50 mm (Dionex P/N 061523)
IonPac SCS1 4 × 250 mm (Dionex P/N 079809)

Eluent: 10 mM nitric acid/5% acetonitrile

Flow Rate: 1.00 mL/min

Inj. Volume: 10 µL (full loop)

Column Temp.: 30 °C

Detector Temp.: 40 °C

Detection: Nonsuppressed conductivity

Background: ~3300 µS

Noise: Typically <20 nS/min

Backpressure: ~2300 psi

## ELUENT AND STANDARDS PREPARATION

### Eluent Solution

Fill a 2 L glass volumetric flask to the mark with filtered and degassed deionized (DI) water. Remove 101.2 mL of DI water, add 1.2 mL of concentrated nitric acid (70%, 15.8 N), and mix thoroughly. Add acetonitrile with mixing to the mark to produce 2 L of eluent.

### Standard and Sample Solvent Solution

The NMP stock, calibration standards, and cefepime surrogate sample solutions use 2 mM nitric acid as the diluent. To prepare 1 L of 2 mM nitric acid, add 500 g of filtered and degassed DI water to a tared glass bottle, add 0.127 mL of concentrated nitric acid to the flask, mix well, and add enough filtered and degassed DI water to make 1.0 kg of solution.

### Standard NMP Solution

To prepare a 1000 µg/mL NMP solution, add 19 mL of 2 mM nitric acid solvent to a tared glass scintillation vial, accurately add 0.0200 g NMP solution to the vial beneath a well-ventilated fume hood, and add enough 2 mM nitric acid solvent to prepare 20 g of solution. Store the stock solution at 4 ºC when not in use.
Make all subsequent dilutions of NMP stock solution gravimetrically with 2 mM nitric acid for generating the calibration curve and system precision. Store at 4 ºC.

## *SAMPLE PREPARATION*

### Cefepime Hydrochloride Samples

Prepare 5 mg/mL cefepime matrix solution based on the cefepime assay result listed on the USP Reference Standard label (0.865 mg cefepime/mg solid in Lot H0G278). For this study, accurately weigh 0.0289 g Cefepime Hydrochloride into a tared, 20 mL glass scintillation vial and dissolve in 2 mM nitric acid to produce 5.0 g of a 5 mg/mL cefepime solution. Mix to dissolve the solid, then immediately withdraw 1.0 mL of the matrix solution and place the aliquot in a 1.5 mL glass autosampler vial. This vial then should be placed into the AS sample tray cooled to 4–6 ºC and analyzed immediately to minimize sample degradation. To determine the method's accuracy and precision, reweigh the scintillation vial, add an appropriate weight of 1000 µg/mL NMP solution to produce a working sample of 15 µg/mL NMP in 5 mg/mL cefepime, and immediately analyze the solution.

### Cefepime for Injection Simulated Samples

Precision of the simulated Cefepime for Injection samples was evaluated. In Cefepime for Injection, 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. Prepare 5.0 g of 5 mg/mL cefepime solution in 2 mM nitric acid as described above. Based on the expected 0.827 mg arginine/mg arginine hydrochloride, add 0.0219 mg arginine hydrochloride to the cefepime solution. Mix, immediately withdraw 1.0 mL of this matrix solution, place the aliquot in a 1.5 mL glass autosampler vial, and place the vial into the AS sample tray cooled to 4–6 ºC. Start the sequence soon after placing the vials in the autosampler.

## *RESULTS AND DISCUSSION*

### Chromatography

The IonPac SCS1 column is a low-capacity, silica-based, weak cation-exchange column functionalized with carboxylic acid groups.[12] Typical recommended eluent conditions for this column are 3 mM MSA at 1 mL/min to elute six common cations in about 35 min (guard and analytical columns). In the proposed USP method, the

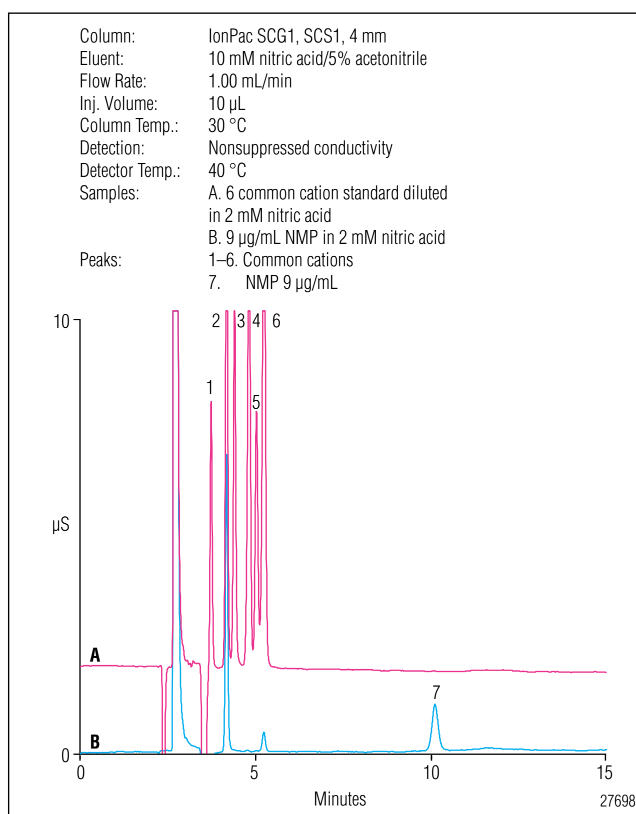


*Figure 2. Chromatography of A) six common cation standard and B) 15 ug/mL NMP standard dissolved in 2 mM nitric acid.*

described eluent composition consists of 10 mM nitric acid with 5% acetonitrile. The organic solvent reduces the hydrophobic interactions of cefepime with the stationary phase to improve sample throughput, whereas higher acid concentrations decrease the retention of alkali and alkaline earth metals on the column. However, an increase in the hydronium ions in the eluent produces a proportional increase in the background conductivity and, therefore, baseline noise, which reduces the sensitivity of the method. Despite the reduction in sensitivity, the method retains the ability to quantify at or below the USP specification for NMP in Cefepime Hydrochloride. Figure 2A demonstrates the separation of common cations on the IonPac SCS1 column using the eluent conditions described in the USP method. As shown, the retention time of these cations is significantly reduced relative to the standard conditions with 3 mM MSA. Figure 2B shows the separation of NMP eluting at 10 min as a sharp peak with an asymmetry of 1.1 and a plate count > 15000.

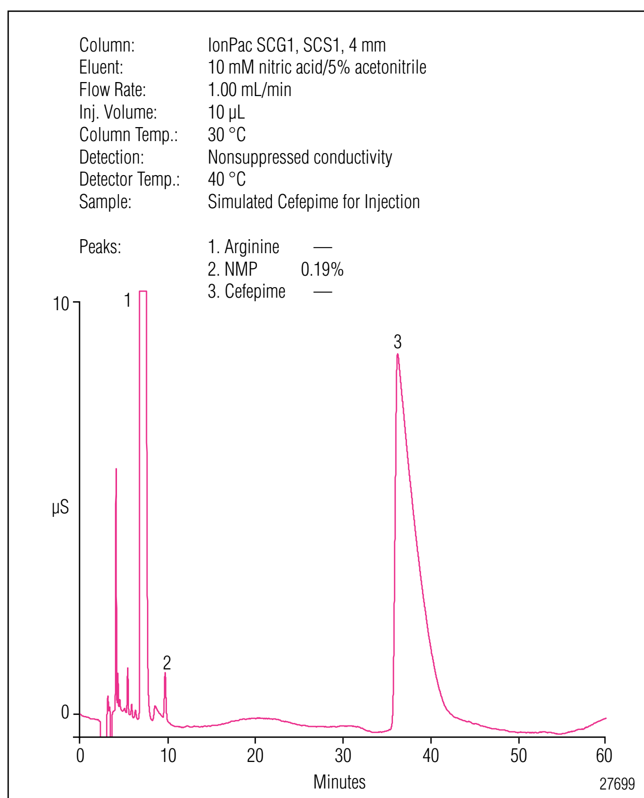


*Figure 3. Determination of NMP in simulated Cefepime for Injection sample.*

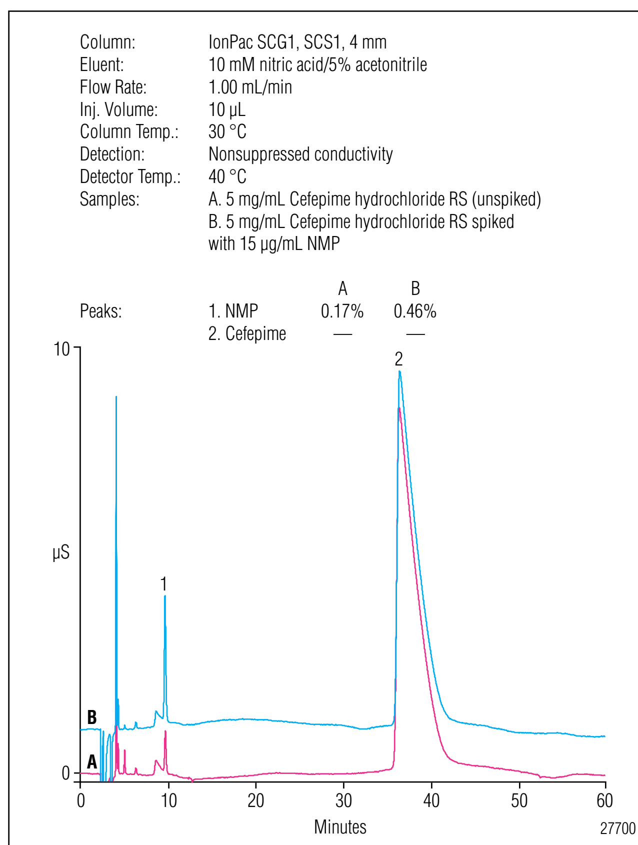


*Figure 4. Comparison of A) unspiked and B) 15 µg/mL (0.3%) NMP-spiked solutions of 5 mg/mL cefepime in 2 mM nitric acid.*

Figure 3 shows the separation of NMP in a simulated Cefepime for Injection sample, which also contains relatively high concentrations of arginine. As shown, arginine and NMP are well resolved ($R_s = 8.2$) on the IonPac SCS1 column using the conditions specified in the USP monograph. This chromatogram also demonstrates that cefepime elutes completely within 6× the NMP retention time limit as specified in the USP proposed monograph, thus avoiding cefepime carryover.[9, 10] However, the total analysis time is twice as long as the IC method described in Dionex AN 199.[6]

### Limit of Detection, Limit of Quantification, and Linearity

The USP General Chapter on validation of compendial methods <1225> specifies a signal-to-noise (S/N) ratio of three for the limit of detection (LOD) and 10 for the limit of quantification (LOQ).[13] Baseline noise was determined to be 18 nS by averaging the peak-to-peak noise of seven system (no injection) blanks over two 1 min windows centered on the NMP retention time. Peak heights from triplicate injections of standards in 2 mM nitric acid were plotted versus NMP concentration. The LOD and LOQ estimates for NMP were 0.5 and 1.6 µg/mL, respectively, corresponding to 0.01 and 0.032% in 5 mg/mL cefepime. The LOQ estimate was 10-fold lower than the 0.3% acceptance criterion cutoff level for Cefepime Hydrochloride and more than 30-fold lower than the 1.0% acceptance criterion cutoff level for Cefepime for Injection.

To determine method linearity, calibration standards were prepared at nine concentration levels in the range of 5–100 µg/mL NMP in 2 mM nitric acid, corresponding to 0.1–2.0% in 5 mg/mL cefepime. A plot of peak area versus concentration produced a correlation coefficient ($r^2$) value of 0.9996 using a linear least squares regression fit. The relative standard deviation of the measured peak areas based on the calibration curve was < 1.8%.

| Table 1. Retention Time and Peak Area Precisions for *N*-Methylpyrrolidine in Cefepime Samples | | | | | | |
|---|---|---|---|---|---|---|
| Sample[a] | N | NMP Conc. | Average Retention Time (min) | Retention Time RSD | Average Peak Area (µS*min) | Peak Area RSD |
| 15 µg/mL NMP Standard | 6 | 15.0 µg/mL | 9.742 | 0.02 | 0.3686 | 0.4 |
| USP Cefepime Hydrochloride RS[b] | 6 | 0.18% | 9.715 | 0.06 | 0.2033 | 1.1 |
| Simulated Cefepime for Injection[c] | 6 | 0.19% | 9.704 | 0.13 | 0.2248 | 1.6 |

[a] - 2 mM nitric acid solvent
[b] - 5 mg/mL cefepime
[c] - 5 mg/mL cefepime + 3.6 mg/mL arginine

### Accuracy and Precision

Method accuracy was evaluated by spiking 14.9 µg/mL of NMP into a 5 mg/mL cefepime sample solution (Figure 4). The unspiked cefepime sample contained 8.41 µg/mL (0.17%) NMP, which was below the acceptance criterion cutoff value. After correcting for the amount of NMP in the cefepime sample, the average recovery for three replicates was 98.7 ± 1.0%.

Table 1 summarizes the results of three sets of repeatability experiments. System precision refers to repeatability for a standard without cefepime or cefepime/arginine mixture present. Excellent system precisions for NMP retention time (0.02%) and peak area (0.36%) were measured for an NMP concentration of 15 µg/mL. Precision of NMP retention time and peak area was also determined for Cefepime HCl and Cefepime for Injection samples. Retention time and peak area repeatabilities were 0.06% and 1.1%, respectively, for NMP in 5 mg/mL cefepime and 0.13% and 1.6% for NMP in 5 mg/mL Cefepime for Injection containing 3.6 mg/mL arginine. Cefepime sample instability was observed when stored in the autosampler tray at 4 ºC over the 6.1 h time interval needed to run the six replicates of each of the two cefepime-containing samples. NMP peak areas increased 2.7% for the Cefepime Hydrochloride sample and 3.9% for the Cefepime for Injection surrogate sample. Using the IC-based method described in AN 199 will result in faster sample throughput, producing less cefepime decomposition during analysis of a given sample.

## *CONCLUSION*

This work presents the determination of *N*-methylpyrrolidine, a cefepime decomposition product, using a silica-based, weak cation-exchange column coupled with nonsuppressed conductivity detection. Results for LOD, LOQ, linear calibration range, spike recovery, retention time precision, and peak area precision determinations show that this instrumental configuration fulfills acceptance criteria for determining NMP in Cefepime Hydrochloride and Cefepime for Injection samples. Cefepime decomposition during sample analysis can be decreased and eluent preparation can be simplified by using the method described in AN 199.

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*Validation of Compendial Methods;* United States Pharmacopeia, The National Formulary: General Chapter <1225>, USP 33, NF 28, 2010.

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

VWR International, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A. Tel: 1-800-932-5000. www.vwr.com

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

# Determination of 2-Ethylhexanoic Acid Impurity in Clavulanate

## INTRODUCTION

Clavulanic acid is a potent beta-lactamase inhibitor that when used in combination with penicillin and cephalosporin antibiotics, increases their effectiveness by counteracting bacterial resistance.[1, 2] Clavulanic acid is produced from *Streptomyces clavuligerus* and then isolated by acidifying and extracting the cold, clarified culture medium with ethyl acetate.[3] Further preparation of clavulanic acid is needed to increase purity, minimize aqueous hydrolysis, and convert the acid to a stable form used in the pharmaceutical product.[4] Potassium clavulanate is more stable than the free acid or other salts such as the sodium, calcium, or magnesium analogs. One possible secondary purification approach uses a non-aqueous precipitation of potassium clavulanate by adding the potassium salt of 2-ethylhexanoic acid in isopropanol to the primary isolation extract.[5] Although 2-ethylhexanoic acid is soluble in the organic solvents, a small fraction may co-precipitate with the potassium clavulanate active pharmaceutical ingredient (API).

The United States Pharmacopeia (USP) monograph for potassium clavulanate describes a procedure to determine 2-ethylhexanoic acid in a 75 mg/mL solution of the API in a strong acid that is extracted into organic solvent and analyzed by gas chromatography with flame ionization detection.[6] The work shown here describes a simpler approach for determining 2-ethylhexanoic acid in clavulanate using a Reagent-Free™ Ion Chromatography (RFIC™) system. Clavulanate USP reference standard, prepared at 0.5 mg/mL in deionized water, was spiked with 2-ethylhexanoic acid and injected directly on an IonPac® AS11 column without additional sample pretreatment. The target analyte was separated from clavulanate-related peaks using electrolytically generated potassium hydroxide eluent and measured using suppressed conductivity detection.

## EQUIPMENT

Dionex ICS-2100 system* including:

- Single isocratic pump
- Vacuum degasser
- Eluent generator
- High pressure, 6-port injector
- Column heater enclosure
- Conductivity cell and detector
- EO Eluent Organizer, including pressure regulator, and 2 L plastic bottle
- AS Autosampler and 2 mL vial tray
- Chromeleon® Chromatography Data System (CDS) Software Version 6.8 or 7
- Helium or nitrogen; 4.5-grade (99.995%) or better, < 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 Polypropylene injection vials with caps (Vial Kit, Dionex P/N 061696)

*This application can be run using any Dionex RFIC system

## CONSUMABLES

EluGen II KOH Cartridge (Dionex P/N 058900)

CR-ATC (Dionex P/N 060477)

ASRS® 300 suppressor, 2 mm (Dionex P/N 064555)

IonPac AG11 column, 2 × 50 mm (Dionex P/N 044079)

IonPac AS11 column, 2 × 250 mm (Dionex P/N 044077)

## REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher, filtered and degassed

Clavulanate Lithium Reference Standard (USP P/N 1134426)

2-Ethylhexanoic acid, 99% (VWR P/N 101226-112)

## CONDITIONS

Columns: IonPac AG11, 2 × 50 mm (Dionex P/N 044079)
IonPac AS11, 2 × 250 mm (Dionex P/N 044077)

Eluent: 3 mM KOH from -10 to 0 min (column equilibration), 3 mM KOH from 0 to 10 min (separation), 3 to 60 mM KOH from 10 to 10.1 min, 60 mM KOH from 10.1 to 20.1 min (column cleanup)

Flow Rate: 0.25 mL/min

Inj. Volume: 5 µL

Column Temp.: 30 °C

Detection: Suppressed conductivity, ASRS 300 suppressor, 2 mm (Dionex P/N 064555), recycle mode, 2 mA suppressor current during equilibration and separation, switch to 38 mA during column cleanup

Background: <1 µS at sample injection

Noise: <2 nS/min

Backpressure: 2500 psi

Run Time: 20.1 min

| Time (min) | [KOH] (mM) | Suppressor Current (mA) | Comment |
|---|---|---|---|
| -10 | 3 | 2 | Equilibrate column |
| 0 | 3 | 2 | Inject sample |
| 10 | 3 | 2 | Finish separation |
| 10.1 | 60 | 38 | Start column cleanup |
| 20.1 | 60 | 38 | Complete column cleanup |

## STANDARDS AND SURROGATE SAMPLE SOLUTIONS

### 500 µg/mL Potassium Clavulanate Matrix Solution

Dissolve 0.0432 g of lithium clavulanate reference standard in 100.0 mL of degassed deionized water to make 432 µg/mL of solution, which is equivalent to 500 µg/mL of the potassium salt form (the factor for converting equimolar amounts of lithium salt to potassium salt is 1.157). Store in a high-density polyethylene or polypropylene bottle at 4 ºC.

### 1000 µg/mL 2-Ethylhexanoic Acid Stock Solution

Dissolve 0.1000 g of 2-ethylhexanoic acid in ~ 75 mL of degassed deionized water by stirring vigorously overnight at 30 to 35 ºC. Let the solution cool to room temperature, remove the stirring bar, and wash the stirring bar with water into the solution, making sure that the total solution volume does not exceed 100 mL. Add degassed deionized water for a total solution volume of 100.0 mL. Store in a high-density polyethylene or polypropylene bottle at 4 ºC.

Make all subsequent dilutions of 2-ethylhexanoic acid stock standard gravimetrically with degassed deionized water when generating standards for calibration and limit of detection/limit of quantification (LOQ/LOQ) studies, or with 500 µg/mL potassium clavulanate matrix solution when generating surrogate samples for accuracy and precision studies.

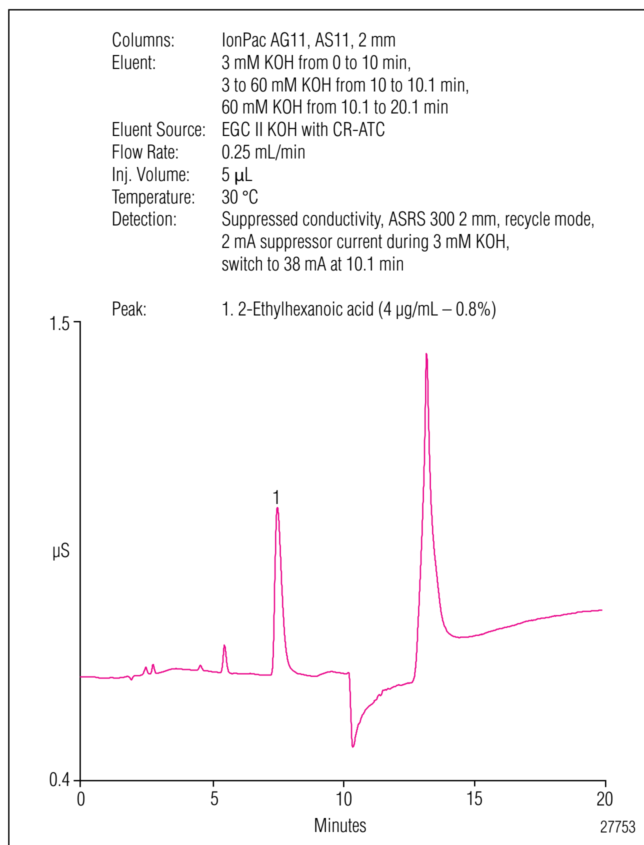


*Figure 1. Chromatogram of 4 µg/mL 2-ethylhexanoic acid in DI water.*

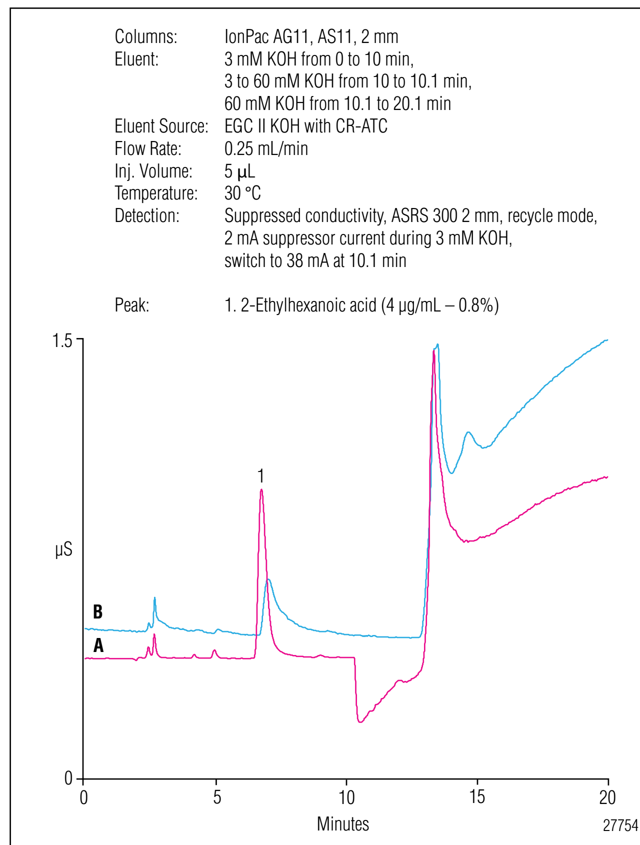


*Figure 2. The effect of high versus low suppressor current during the separation of 2-ethylhexanoic acid. Trace A: 2 mA with step-up to 38 mA at 10 min; Trace B: 38 mA constant current.*

## RESULTS AND DISCUSSION

### Chromatography

In AU 157, the IonPac AS17 column was used to separate potential anionic contaminants, such as 2-ethylhexanoic acid, in electronic component extracts.[7] Although the IonPac AS17 column previously was a good choice for 2-ethylhexanoic acid determinations in clean, well-characterized matrices, it was not chosen for this application because of its low capacity.[8] The IonPac AS11 column was selected for its ability to separate a wide range of inorganic and organic anions in complex matrices.[9] In addition, the IonPac AS11 column has 30% higher capacity than the IonPac AS17 column and lower surface hydrophobicity, a property that is expected to produce better peak shape for 2-ethylhexanoic acid.

Figure 1 shows a chromatogram of a 4 µg/mL 2-ethylhexanoic acid standard prepared in deionized water. This concentration corresponds to the 0.8% acceptance criterion cutoff value specified in the USP monograph when the potassium clavulanate concentration is 500 µg/mL.[6] The chromatographic features in Figure 1 observed after 10 min were also present in the system (no injection) and DI water blanks. The baseline dip was due to increasing the suppressor current at 10.1 min (Trace A in Figure 2). When a higher suppressor current was maintained throughout the chromatographic analysis, significant peak tailing for 2-ethylhexanoic acid was observed (Trace B in Figure 2). Therefore, the authors strongly recommend using the optimal current setting for the corresponding KOH concentrations.

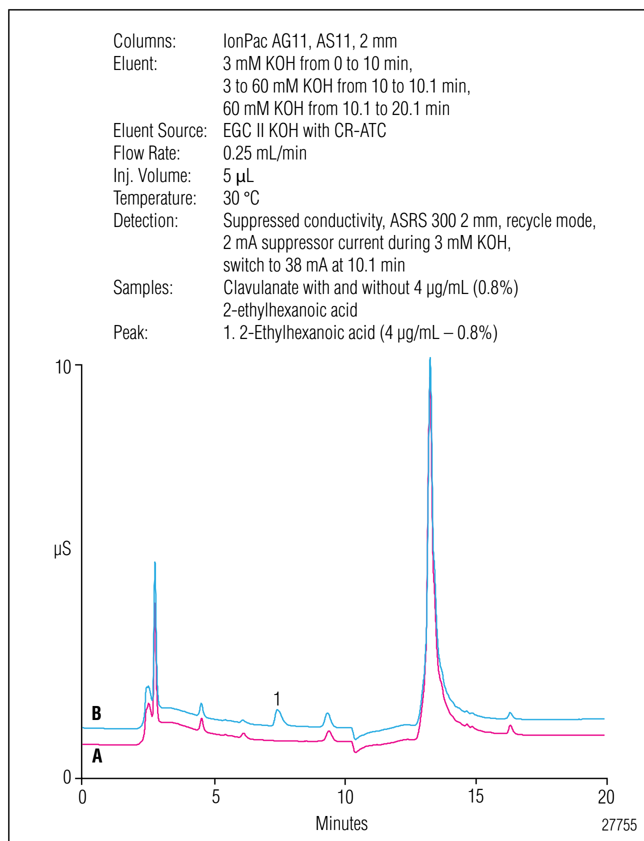


*Figure 3. Chromatographic comparison of clavulanate A) without and B) with 4 µg/mL (0.8%) 2-ethylhexanoic acid.*

Potassium clavulanate and its decomposition products do not interfere with the chromatography of 2-ethylhexanoic acid (Figure 3). The 10 min cleanup with 60 mM KOH was determined to be sufficient to elute the matrix components from the column and, therefore, prevent carryover in subsequent injections.

### Limit of Detection, Limit of Quantification, and Linearity

The USP general chapter for validation <1225> suggests a signal-to-noise (S/N) ratio of three for LOD and 10 for LOQ.[10] Baseline noise was determined to be 1.4 nS by measuring the peak-to-peak noise of seven system (no injection) blanks over a 2 min window centered on the retention time of 2-ethylhexanoic acid. Peak heights from triplicate injections of standards in DI water were plotted versus 2-ethylhexanoic acid concentration. The LOD and LOQ estimates for 2-ethylhexanoic acid were 0.036 and 0.12 µg/mL, respectively, corresponding to 0.0072 and 0.024% in 500 µg/mL potassium clavulanate. The LOQ estimate is more than 30-fold lower than the 0.8% acceptance criterion cutoff level.

**Table 1. Retention Time and Peak Area Precisions for 0.8% 2-Ethylhexanoic Acid in Clavulanate API[a]**

| Day | N | Average Retention Time (min) | Retention Time RSD | Average Peak Area (µS*min) | Peak Area RSD |
|---|---|---|---|---|---|
| 1 | 15 | 7.047 | 0.11 | 0.1154 | 2.0 |
| 2 | 15 | 7.010 | 0.18 | 0.1140 | 1.2 |
| 3 | 15 | 6.968 | 0.13 | 0.1141 | 0.9 |
| 4 | 15 | 6.927 | 0.06 | 0.1141 | 1.1 |
| 5 | 15 | 6.898 | 0.16 | 0.1126 | 0.7 |

[a] 2-Ethylhexanoic acid concentration at the acceptance criterion cutoff level.

To determine method linearity, triplicate injections were made of calibration standards prepared at seven concentration levels in the range of 1 to 7 µg/mL of 2-ethylhexanoic acid, corresponding to 0.2 to 1.4% in 500 µg/mL potassium clavulanate. A plot of peak area versus concentration produced a correlation coefficient ($r^2$) value of 0.9991 using a linear least squares regression fit. The relative standard deviation of the measured peak areas from the areas predicted by the calibration equation was < 1.5%.

### Accuracy and Precision

Method accuracy was evaluated by spiking 2-ethylhexanoic acid at three different concentrations in 500 µg/mL potassium clavulanate matrix solutions. Spiked 2-ethylhexanoic acid concentrations were 2.0 µg/mL (0.40%), 4.0 µg/mL (0.80%), and 6.0 µg/mL (1.2%). The average recoveries for seven replicates were 94.1 ± 1.7%, 99.0 ± 2.2%, and 100.0 ± 1.0%, respectively. No measurable peak area was detected at the 2-ethylhexanoic acid retention time with potassium clavulanate matrix blank injections.

Method reproducibility was evaluated with replicate injections of 4.0 µg/mL (0.80%) 2-ethylhexanoic acid in potassium clavulanate (the acceptance criterion cutoff value). Retention times and peak area precisions for 2-ethylhexanoic acid were determined from 15 replicate injections per day for 5 days. Table 1 shows daily retention time and peak area values and their respective RSDs. Peak area RSDs ranged from 0.7 to 2.0%, whereas the retention times for 2-ethylhexanoic acid trended lower on a daily basis by 0.4%, implying loss of column capacity. Running a daily check sample and adjusting the 2-ethylhexanoic acid retention time

window in Chromeleon software will assure proper peak assignment and integration for this potential impurity. If the 2-ethylhexanoic acid retention time decrease exceeds internal quality control requirements, replace the guard column. If no requirements are specified, replace the guard column after no more than 150 injections. For long-term storage (> 1 week), flush the column set with 100 mM sodium borate solution as described in the IonPac AS11 column product manual.[9]

## PRECAUTIONS

Contact of clavulanate powder or 2-ethylhexanoic acid with eyes, skin, and respiratory tract causes irritation. The 2-ethylhexanoic acid also may be teratogenic. Wear protective gloves, chemical safety goggles, and a laboratory coat. To dispose of these materials, contact a licensed waste disposal service. Read the material safety data sheet for these compounds before use.

Extensive use of the column may cause performance degradation, such as loss of capacity, loss of resolution, shortened retention times, higher noise and background, spurious peaks, and peak tailing. Prescribed column cleanings did not restore 2-ethylhexanoic acid retention time. For more information on column troubleshooting, see the IonPac AS11 column product manual.[9]

## CONCLUSION

This work presents an IC method for the detection and quantification of 2-ethylhexanoic acid, a potential impurity in potassium clavulanate API incorporated during sample purification. Results for LOD/LOQ, linear calibration range, spike recovery, retention time precision, and peak area precision determinations show that IC is an accurate and reproducible technique to determine 2-ethylhexanoic acid in clavulanate below the 0.8% acceptance criteria. The method uses 150-fold lower concentration of the API and eliminates the need for solvent extraction per the current USP monograph, thus consuming less of the API and simplifying sample preparation.

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10. United States Pharmacopeia. The National Formulary. General Chapter <1225>, V*alidation of Compendial Methods*. USP 33, NF 28, 2010.

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DIONEX

# Application Note 267

# Analysis of the Aminoglycoside Antibiotics Kanamycin and Amikacin Matches USP Requirements

## INTRODUCTION

Kanamycin and amikacin are broad-spectrum aminoglycoside antibiotics that are closely related (Figure 1). Kanamycin, used to treat a wide variety of serious gram-negative-bacterial infections, is purified from fermentation of *Streptomyces kanamyceticus* and is usually formulated as a sulfate in both oral and intravenous forms. Kanamycin, like many other aminoglycosides, can have oto- and nephrotoxic side effects, so the patient needs to be closely monitored after kanamycin administration.[1] The main component of purified kanamycin is kanamycin A, and the minor structurally related constituents are kanamycin B, C, and D (Figure 1).

Amikacin is commonly administered parenterally for the treatment of gram-negative infections resistant to kanamycin, gentamicin, or tobramycin. Compared to other aminoglycosides, the amikacin molecule has fewer sites susceptible to enzymatic reaction. Amikacin is synthesized by acylation of the amino group of kanamycin A with L-(-)-γ-amino-α-hydroxybutyric acid (L-HABA). As a result, kanamycin A and L-HABA are expected impurities in commercial amikacin samples.

The purity of these antibiotics must be determined and must meet specified criteria before clinical use. The detection of aminoglycosides is not straightforward because they do not have a significant UV-absorbing chromophore. Paper chromatography, ion-exclusion chromatography, gas-liquid chromatography after silylation, and reversed-phase LC with derivatization have been reported for the analysis of kanamycin purity.[2]

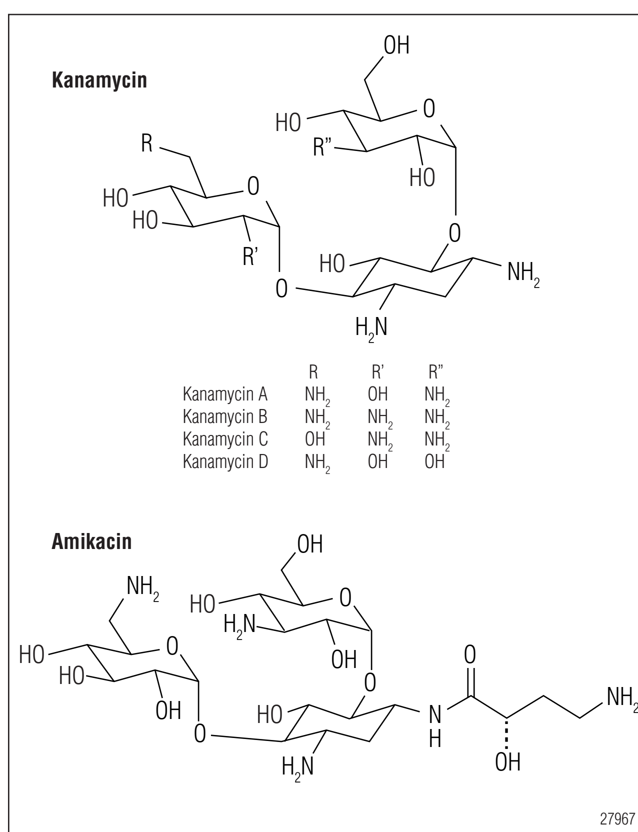


Figure 1. Chemical structures of kanamycin A, B, C, D, and amikacin.

Detection methods using pre- or postcolumn chemical derivatization have been used for amikacin.[3] However, these techniques are time consuming and require considerable sample preparation.

Aminoglycosides can be oxidized and detected by amperometry, a robust detection technique with a broad linear range and low detection limits.[4] High-performance anion-exchange chromatography in combination with pulsed amperometric detection (HPAE-PAD) provides a sensitive and reliable analytical method for aminoglycoside antibiotics.[5-7] The United States Pharmacopeia (USP) monographs for kanamycin and amikacin drug substances both use HPAE-PAD for assay.[8, 9] The same assay methods are also used for the assay of kanamycin and amikacin drug products.[10]

The work shown here evaluates the HPAE-PAD assay method described in the USP monographs for kanamycin and amikacin[8,9] and describes a revised HPAE-PAD method using a CarboPac® MA1 column and disposable Au-on-polytetrafluoroethylene (PTFE) working electrodes for the analysis of kanamycin and amikacin. Key parameters evaluated are precision, linearity, and resolution. The revised method meets or exceeds the USP requirements for peak resolution, tailing (also referred to as peak asymmetry), and precision. The use of disposable electrodes provides the benefits of shorter equilibration time and greater electrode-to-electrode reproducibility. Compared to other disposable Au electrodes, the Au-on-PTFE electrodes have longer lifetimes and can operate at higher hydroxide concentrations. The described method provides good sensitivity, high sample throughput, and retention time reproducibility for kanamycin and amikacin.

## EQUIPMENT

Dionex ICS-3000 or -5000 system including:

Gradient or Isocratic Pump

DC Detector/Chromatography Module

20 µL Injection loop

Electrochemical Detector (P/N 061718)

Disposable Au-on-PTFE Working Electrode (P/N 066480, package of 6)

Ag/AgCl Reference Electrode (P/N 061879)

2 mil PTFE gaskets (P/N 060141)

AS Autosampler

Chromeleon® Chromatography Data System (CDS) software

Eluent Organizer, including 2 L plastic bottles and pressure regulator

Polypropylene injection vials with caps (0.3 mL vial kit, Dionex P/N 055428)

Nalgene® 125 mL HDPE narrow mouth bottles (VWR P/N 16057-062)

Nalgene 250 mL HDPE narrow mouth bottles (VWR P/N 16057-109)

Nalgene 250 mL 0.2 µm nylon filter units (VWR P/N 28199-371)

Nalgene 1000 mL 0.2 µm nylon filter units (VWR P/N 28198-514)

## REAGENTS AND STANDARDS

### Reagents

Deionized water, Type I reagent grade, 18 MΩ-cm resistivity or better, filtered through a 0.2 µm filter immediately before use

### Standards

Kanamycin sulfate (Sigma-Aldrich Chemical Co. Cat # K4000)

Kanamycin sulfate reference standard (USP Cat # 1355006)

Amikacin disulfate salt (Sigma-Aldrich Chemical Co. Cat # A1774)

Amikacin reference standard (USP Cat # 1019508)

Sodium hydroxide 50% (w/w) (Fisher Scientific Cat # SS254-500)

| Carbohydrate Waveform | | |
|---|---|---|
| **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 | |

## CONDITIONS

### Method

Columns: CarboPac MA1 Analytical, 4 × 250 mm (P/N 044066)
CarboPac MA1 Guard, 4 × 50 mm (P/N 044067)

Flow Rate: 0.5 mL/min

Inj. Volume: 20 µL (full loop)

Temperature: 30 °C

Back Pressure: 1500 psi

Eluent: 115 mM NaOH

Detection: PAD (Au)

Background: 30–70 nC

### Reference Electrode

Mode: Ag/AgCl mode

Noise: 30 pC

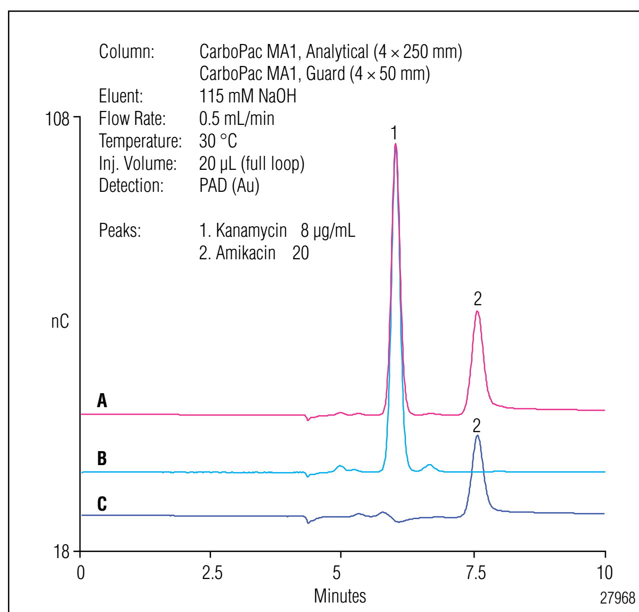


*Figure 2. Typical chromatograms of (A) resolution solution (kanamycin 0.008 mg/mL and amikacin 0.02 mg/mL), (B) commercial kanamycin A sulfate sample, and (C) commercial amikacin sample.*

## PREPARATION OF SOLUTIONS AND REAGENTS

### Eluent Solutions

*115 mM Sodium Hydroxide*

It is essential to use high-quality water of high resistivity (18 MΩ-cm) containing as little dissolved carbon dioxide as possible. Biological contamination must be absent. Obtain source water 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. It is extremely important to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the column, 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. Sodium hydroxide (50% w/w) is much lower in carbonate and is the recommended source for sodium hydroxide.

Dilute 5.9 mL of a 50% (w/w) sodium hydroxide into 994.1 mL of thoroughly degassed water to yield a 115 mM sodium hydroxide solution. Maintain the eluents under 34 to 55 kPa (5 to 8 psi) of nitrogen at all times to reduce diffusion of atmospheric carbon dioxide and minimize microbial contamination.[11]

### Stock and Sample Preparation

Place solid kanamycin and amikacin reference standards in plastic vials and dissolve in deionized water to 0.16 mg/mL and 0.2 mg/mL stock standards, respectively. Further dilute the stock solutions to 0.008 mg/mL (kanamycin sulfate) and 0.02 mg/mL (amikacin) with DI water. Maintain the solutions frozen at -40 °C until needed. Prepare the research grade kanamycin and amikacin samples similarly.

## RESULTS AND DISCUSSION

### Separation

Figure 2 shows the separation of kanamycin and amikacin on the CarboPac MA1 column. The relative retention times are 1 for kanamycin and 1.3 for amikacin. Peak resolution between kanamycin and amikacin is >4, exceeding the USP requirement of 3. The asymmetry for both kanamycin and amikacin is 1.1 (USP requires <2). The total analysis time is 10 min, providing high sample throughput. A small baseline dip is seen at ~6 min and it co-elutes with kanamycin. However, at the concentrations tested, the contribution of the dip is insignificant (<3%).

### Ruggedness

The variance due to different columns was tested by comparing results from columns from two different lots. Columns from different lots gave similar results. In addition, other waveforms (USP and *AAA-Direct*) were evaluated. The waveform reported for assay methods for kanamycin and amikacin in their USP monographs gave similar results. However, the USP waveform is not recommended for the disposable Au electrodes because the electrode will fail in less than 24 h. The *AAA-Direct* waveform also provides similar results (data not shown).

### Linear Range

Linearity was investigated in the range of 2 to 16 µg/mL for kanamycin and 4 to 40 µg/mL for amikacin. The highest concentration investigated for both the aminoglycosides was twice the concentration used in the USP assay method. The correlation coefficient was 0.9993 for kanamycin and 0.9991 for amikacin (Table 1).

### Precision

The RSD for retention time was 0.16 for kanamycin and 0.07 for amikacin for nine replicate injections (USP requirements <0.3%). The between-day precision was 0.01 for kanamycin and 0.07 for amikacin. The intra-day peak area precisions were 0.99 for kanamycin and 1.2 for amikacin (Table 1). The between-day peak area precisions were 1.3 and 2.3 for kanamycin and amikacin, respectively. The high precision suggests that this method can be used to analyze relatively pure aminoglycoside antibiotics like kanamycin and amikacin without column regeneration.

### Product Degradation

Degradation of kanamycin and amikacin may occur during manufacturing, formulation, shipping, and storage. Degradation is monitored to evaluate the potency and quality of the active pharmaceutical ingredient. Pharmaceutical product stability is studied by exposing the product to acidic or basic conditions. Elevated temperatures are used to accelerate these studies.

**Table 1. Calibrations for Precisions and Precisions for Kanamycin Sulfate and Amikacin (n = 9 injections)**

| Analyte | Range (µg/L) | Corr. Coeff. ($r^2$) | RT (min) | RT Precision (RSD) | Peak Area (nC*min) | Peak Area Precision (RSD) |
|---|---|---|---|---|---|---|
| Kanamycin Sulfate | 2–16 | 0.9993 | 6.00 | 0.16 | 11.11 | 0.99 |
| Amikacin | 4–40 | 0.9991 | 7.6 | 0.07 | 5.35 | 1.2 |

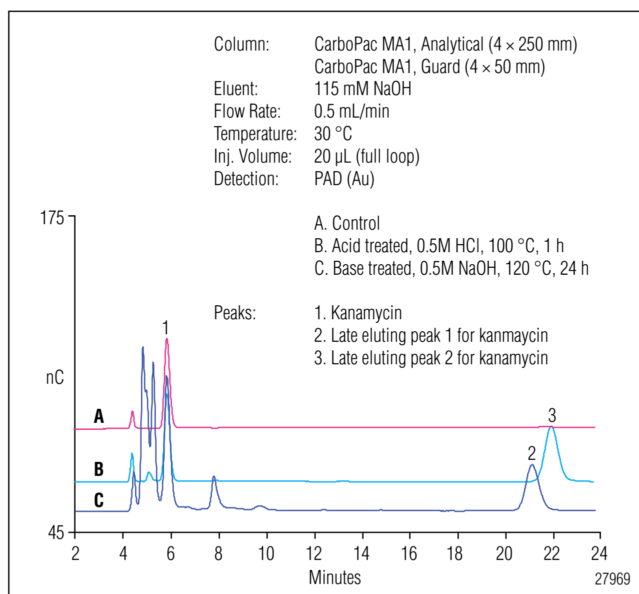


*Figure 3. An accelerated stability study of kanamycin using (B) forced acid and (C) base degradation of kanamycin.*

Kanamycin and amikacin were treated with high acid (0.5 M HCl) at 100 °C for 1 h, or base (0.5 M NaOH) at 120 °C for 24 h and 2 h, respectively. The samples were adjusted to neutral pH prior to analysis. Figure 3 shows the degradation products for kanamycin, and their relative amounts under acidic and basic conditions. Most of the degradation products elute within 10 min. Interestingly, there are late-eluting peaks at ~21 (Figure 3, peak 2) and 22 min (Figure 3, peak 3) in samples degraded under basic and acidic conditions, respectively. Similar late-eluting thermal decomposition product has been reported for the aminoglycoside drug streptomycin.[12] The identity of the late-eluting peak is not known, but the long retention time may interfere with subsequent injections if a shorter run time is used.

Figure 4 shows the degradation product for amikacin under acidic and basic conditions. First, there is a peak eluting at 6 min (same retention time as kanamycin, peak 1 in Figure 4). This suggests that under basic conditions amikacin loses its acetylated group, resulting in a kanamycin-like molecule. Second, no intact amikacin is detected in samples exposed to basic conditions. Thirdly, similar to kanamycin, amikacin also exhibits late-eluting peaks at ~20.5 (Figure 4, peak 3) and 22 min (Figure 4, peak 4) under basic and acidic degradation conditions, respectively. Peak 4 (Figure 4) for amikacin can be similar to peak 3 (Figure 3) in kanamycin, suggesting that the late-eluting species formed in both under acid-degradation conditions are similar. Additionally, the resolution of the closely eluting peaks can be improved if a lower column temperature (20 °C) is used (data not shown). These results show the capability of the HPAE-PAD method to be used in stability assays for these aminoglycoside antibiotics.

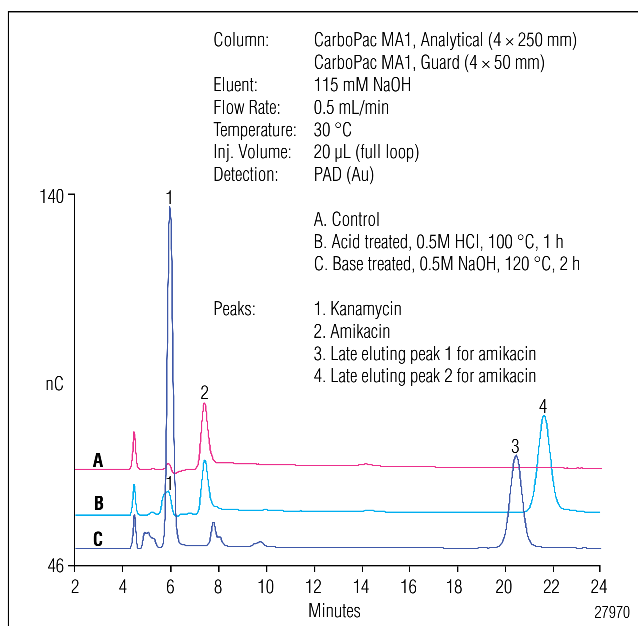


*Figure 4. An accelerated stability study of amikacin using (B) forced acid and (C) base degradation of amikacin.*

### Accuracy

To evaluate accuracy, recoveries were determined in acid-degraded samples spiked with kanamycin and amikacin. The acid-degraded samples were adjusted to neutral pH prior to being spiked. Recovery was 80% for kanamycin and 86% for amikacin, suggesting that the method is accurate.

## CONCLUSION

This work describes an HPAE-PAD method for the analysis of two closely related aminoglycoside antibiotics, kanamycin A and amikacin. The method uses the CarboPac MA1 column with hydroxide eluent. The disposable gold working electrode provides consistently high detector response, assuring greater instrument-to-instrument and lab-to-lab reproducibility. The method is shown to be accurate and reliable and meets the USP requirements for peak resolution, tailing (asymmetry), and reproducibility.

## *SUPPLIERS*

Fisher Scientific, 2000 Park Lane Drive, Pittsburgh, PA 15275, U.S.A. Tel: 800.766.7000

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A. Tel: 800-932-5000.

Sigma-Aldrich Chemical Co., P.O. Box 2060, Milwaukee, WI 53201, U.S.A. Tel: 800-558-9160.

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Dionex Corporation, *Determination of Streptomycin and Impurities Using HPAE-PAD*. Application Note 181, LPN 1887, 2007, Sunnyvale, CA.

DIONEX

Application Note 288

# New HPLC and UHPLC Assay Methods for Tetracycline Hydrochloride and 4-Epianhydrotetracycline Hydrochloride Impurity in Tetracycline Drug Products

## INTRODUCTION

Tetracycline (TC) is a common antibiotic used to fight bacterial infections. It is used to treat urinary tract infections, acne, gonorrhea, and other conditions. Tetracycline yeilds a toxic degradation product, 4-epianhydrotetracycline (EATC). The United States Pharmacopeia and National Formulary (USP-NF) contains General Chapter 226 that prescribes an assay for EATC impurity in TC.[1] The monograph for epitetracycline and three other monographs for drug products containing tetracycline hydrochloride (TC-HCl) refer to General Chapter 226.[2–5] This is an antiquated method—certainly a target for modernization—that uses a self-packed column eluted with chloroform. The EATC impurity elutes as a yellow band and is detected by its visible absorbance.

The TC-HCl drug substance monograph[6] and some TC-HCl-containing drug products do not refer to General Chapter 226 to determine the amount of EATC but instead use a high-performance liquid chromatography (HPLC) method. This method, which is used both to determine the amount of EATC and to assay TC, uses a 4.6 × 250 mm, 5–10 µm L7 (C8) packing column, a mobile phase containing ammonium oxalate/dimethylformamide/dibasic ammonium phosphate pH 7.6–7.7, and a 280 nm detection wavelength. This assay method is also a target for modernization because it uses the undesirable mobile phase component dimethylformamide, the dimethylformamide/ammonium oxalate/dibasic ammonium phosphate pH 7.6–7.7 mobile phase is tedious to prepare, and it uses a large resin bead size HPLC column.

This study reports HPLC and ultra HPLC (UHPLC) methods for assay of EATC in TC-containing drug products. These methods use Thermo Scientific Acclaim™ Polar Advantage II (PA2) columns. The HPLC method uses a 4.6 × 150 mm column packed with 3 µm resin and an acetonitrile/ammonium dihydrogen orthophosphate pH 2.2 mobile phase. The Acclaim PA2 column is made with high-purity spherical silica and—unlike a standard C8 column—can be used in a pH range of 1.5–10.

After developing the HPLC method, it was transferred to a UHPLC system using a 2.1 × 100 mm column packed with 2.2 µm packing material. This reduced the run time from 8 to 2 min. Both methods were evaluated using a TC-HCl drug product (TC-HCl capsules). The results from both methods exceed the specifications of the HPLC method in the USP TC-HCl monograph.

## EQUIPMENT

Thermo Scientific Dionex UltiMate™ 3000 system including:

- Integrated Vacuum Degasser SRD-3600 Solvent Rack
- HPG-3400RS Pump
- WPS-3000TRS Split-Loop Sampler
- TCC-3000RS Column Compartment
- DAD-3000RS Diode Array Detector

| | |
|---|---|
| Sample Loop Size: | 100 µL (conventional HPLC)<br>25 µL (UHPLC) |
| Mixer: | 400 µL (conventional HPLC)<br>200 µL (UHPLC) |
| Flow Cell: | 13 µL SST (conventional, HPLC)<br>2.5 µL SST (UHPLC) |

Thermo Scientific Dionex Chromeleon™ Chromatography Data System (CDS) Software Version 6.80, SR10

## CONDITIONS

### Conventional HPLC

| | |
|---|---|
| Column: | Acclaim PA2, 3 µm, 4.6 × 150 mm |
| Mobile Phase: | A: 20 mM $NH_4H_2PO_4$, pH 2.2<br>B: 50% $CH_3CN$ in<br>20 mM $NH_4H_2PO_4$, pH 2.2 |
| Gradient: | 30% B from –5 to 0 min, ramp to 100% B in 6 min, hold 100% B for 1 min, and return to 30% B in 0.5 min |
| Flow Rate: | 1.0 mL/min |
| Inj. Volume: | 5 µL |
| Column Temp.: | 40 °C |
| Sample Tray Temp.: | 10 °C |
| Detection: | UV 280 nm,<br>data collection rate 5 Hz,<br>response time 2.0 s |

### UHPLC

| | |
|---|---|
| Column: | Acclaim RSLC PA2, 2.2 µm, 2.1 × 100 mm |
| Mobile Phase: | A: 20 mM $NH_4H_2PO_4$, pH 2.2<br>B: 50% $CH_3CN$ in<br>20 mM $NH_4H_2PO_4$, pH 2.2 |
| Gradient: | 30% B from –1 to 0 min, ramp to 100% B in 1.4 min, hold 100% B for 0.2 min, and return to 30% B in 0.1 min |
| Flow Rate: | 0.6 mL/min |
| Inj. Volume: | 1 µL |
| Column Temp.: | 40 °C |
| Sample Tray Temp.: | 10 °C |
| Detection: | UV 280 nm,<br>data collection rate 25 Hz,<br>response time 0.5 s |

## REAGENTS AND STANDARDS

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

Acetonitrile ($CH_3CN$), HPLC grade (LAB-SCAN)

Ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$), AR grade (Ajax)

Orthophosphoric acid, 85%, AR grade (ASP Finechem)

Tetracycline hydrochloride (TC-HCl), ≥ 95% (Sigma Aldrich)

4-Epianhydrotetracycline hydrochloride (EATC), 96.1% (Fluka)

## PREPARATION OF SOLUTIONS AND REAGENTS

### Mobile Phase A: 20 mM $NH_4H_2PO_4$, pH 2.2

Dissolve 2.3 g $NH_4H_2PO_4$ in 700 mL DI water. Transfer this solution to a 1 L volumetric flask and add DI water to bring to volume. Adjust to pH 2.2 with orthophosphoric acid (add ~3 mL of 85% orthophosphoric acid for 1 L preparation). Filter with a 0.2 µm filter (Whatman,® Cellulose Acetate, Cat. No. 7001 0004).

#### Mobile Phase B: 50% $CH_3CN$ in 20 mM $NH_4H_2PO_4$

Mix 500 mL $CH_3CN$ with 500 mL of 20 mM $NH_4H_2PO_4$, pH 2.2. Filter with a 0.2 µm filter.

#### TC-HCl Stock Standard Solution, 10,000 mg/L

Weigh 526 mg TC-HCl in a 50 mL volumetric flask. Add ~25 mL of mobile phase A and place in an ultrasonic bath until completely dissolved. Bring to volume with mobile phase A and mix well.

#### EATC Stock Standard Solution, 1000 mg/L

Weigh 10 mg EATC in a 10 mL volumetric flask. Add ~5 mL of mobile phase A. Place in an ultrasonic bath until completely dissolved. Bring to volume with mobile phase A and mix well.

#### Standard Solution for System Suitability Testing (Mixture of 100 mg/L TC and 25 mg/L EATC): Resolution Solution

Pipet 250 µL of TC-HCl stock standard solution and 625 µL of EATC stock standard solution into a 25 mL volumetric flask. Bring to volume with mobile phase A and mix well.

#### TC-HCl Working Standard Solutions

Transfer the appropriate volume of TC-HCl stock standard solution into a 10 mL volumetric flask and bring to volume with mobile phase A. A list of TC-HCl working standard solutions prepared in this study is presented in Table 1.

#### EATC Working Standard Solutions

Transfer the appropriate volume of EATC stock standard solution into a 25 mL volumetric flask and bring to volume with mobile phase A. The list of EATC working standard solutions prepared in this study is presented in Table 1.

#### Sample Preparation

Dissolve a capsule in 1 L of mobile phase A. Filter with a 0.2 µL syringe filter prior to injection.

Note: Prepare all standard and sample solutions just before analysis.

### *RESULTS AND DISCUSSION*

#### Separation

The Acclaim PA2 column contains a polar-embedded stationary phase that has similar selectivity to a standard C8 or C18 stationary phase but can be used under both lower and higher pH conditions as well as with highly aqueous conditions. Given these properties, the Acclaim PA2 column was chosen for developing a method for determining EATC in TC-HCl-containing drug products. Compared to the L7 column used in the TC-HCl monograph, a column smaller in terms of both length and resin bead size (3 µm, 4.6 × 150 mm) was chosen. With this column and a gradient separation using an ammonium dihydrogen phosphate pH 2.2/acetonitrile mobile phase, TC and EATC were well resolved (Figure 1). This separation exhibited a change in selectivity compared to the HPLC method in the TC-HCl monograph.

**Table 1. Preparation of Working Standard Solutions**

| Compound | Stock Standard Solution Concentration (mg/L) | Stock Standard Solution Volume (µL) | | | | | Working Standard Solution Concentration (mg/L) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L1 | L2 | L3 | L4 | L5 | L1 | L2 | L3 | L4 | L5 |
| TC-HCl* | 10,000 | 200 | 300 | 400 | 500 | 600 | 200 | 300 | 400 | 500 | 600 |
| EATC** | 1000 | 12.5 | 25 | 125 | 250 | 375 | 0.5 | 1 | 5 | 10 | 15 |

*Volume used to prepare 10 mL of working standard solution

**Volume used to prepare 25 mL of working standard solution

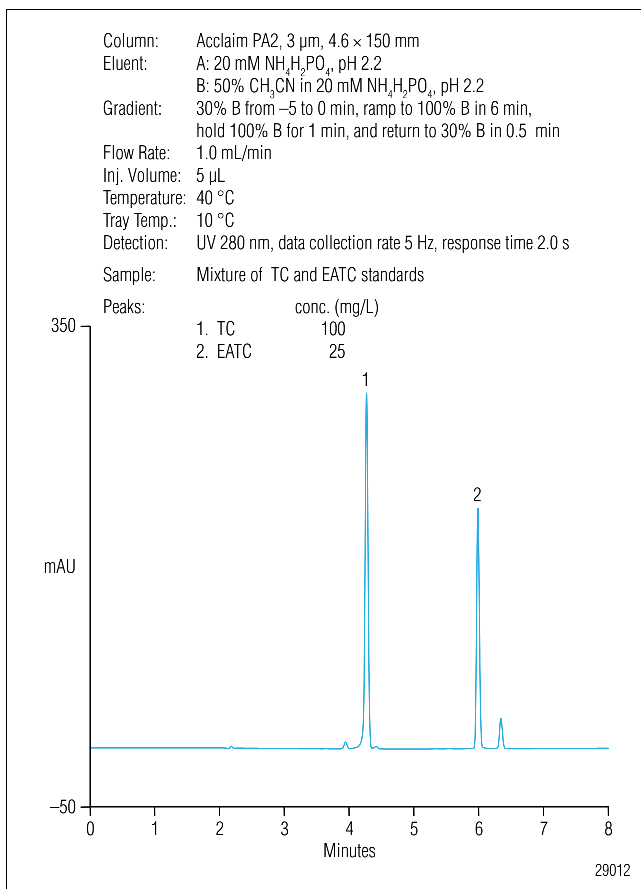


*Figure 1. Chromatogram of the SST solution obtained using the HPLC method.*

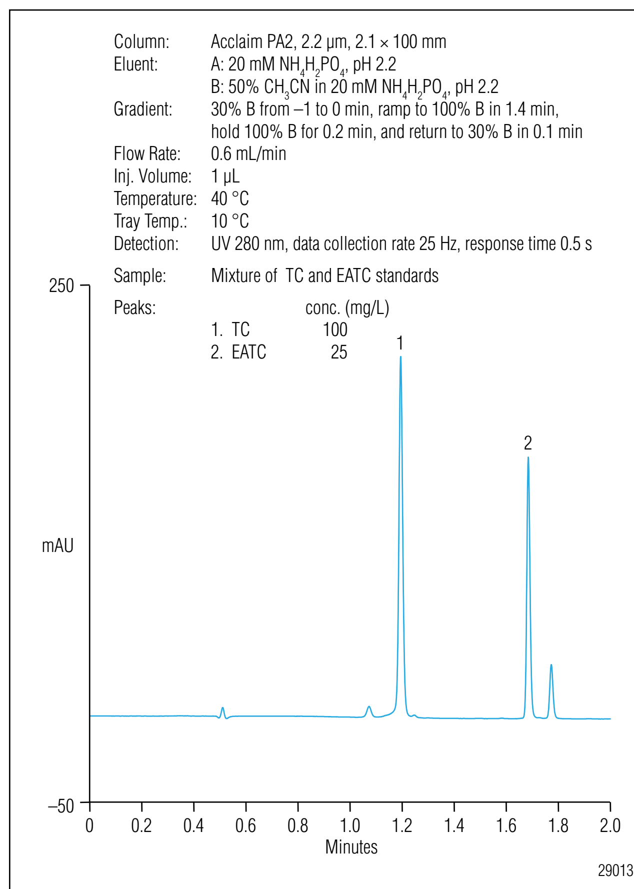


*Figure 2. Chromatogram of the SST solution obtained using the UHPLC method.*

In the monograph, the relative retention times are 0.9 for EATC and 1 for TC. The separation in Figure 1 shows EATC eluting well after TC with a relative retention time of 1.4 min. The peak eluting before TC originates with the TC standard and the peak eluting after EATC originates with the EATC standard. The large retention time gap between TC and EATC makes the HPLC method ideal for transferring to a UHPLC system and accelerating it using a smaller Acclaim PA2 column format with smaller resin size packing material. The flow rate, column length, injection volume, and flow cell size are also reduced (Figure 2). With the UHPLC method, the run time is reduced from 8 to 2 min. The retention time gap and the higher pressure limits of the UHPLC system allow the flow rate to be reduced less than the proportional amount (i. e., a faster flow rate is possible). This results in a 75% reduction in run time while providing a 40% reduction in mobile phase consumption. The HPLC method uses significantly less mobile phase than the HPLC method in the TC-HCl monograph, which uses a 2 mL/min flow rate and a 25 cm column.

### System Suitability Testing

System suitability testing (SST) must be performed before analysis of a drug substance or drug product. Prepare a mixed standard of TC and EATC at concentrations specified in the USP TC-HCl monograph and inject five times. The monograph specifies that resolution between TC and EATC is not less than 1.2 in the resolution solution and the relative standard deviation (RSD) of peak response is not more than (NMT) 2 when analyzing a 0.5 mg/mL solution of TC-HCl. The resolutions of the HPLC and UHPLC methods are 20.8 and 19.9, respectively, exceeding the USP specification. The RSDs of TC peak area response in the resolution solution, where the concentration is only 0.1 mg/mL instead of the less challenging 0.5 mg/mL, are 0.60% and 0.45% from HPLC and UHPLC methods, respectively. These results also exceed the monograph specification.

**Table 2. Reproducibility of Five SST Solution Injections Obtained from the HPLC and UHPLC Methods**

| Inj.No. | Area (mAU*min) | | | |
|---|---|---|---|---|
| | HPLC | | UHPLC | |
| | TC | EATC | TC | EATC |
| 1 | 16.05 | 10.03 | 3.644 | 2.185 |
| 2 | 16.08 | 10.03 | 3.645 | 2.177 |
| 3 | 16.07 | 10.03 | 3.656 | 2.194 |
| 4 | 16.18 | 10.01 | 3.616 | 2.171 |
| 5 | 16.28 | 10.04 | 3.656 | 2.187 |
| Average | 16.13 | 10.03 | 3.643 | 2.183 |
| RSD | 0.60 | 0.11 | 0.45 | 0.41 |
| Resolution Between TC and EATC (USP) | 20.8 | | 19.9 | |
| Relative Retention Time | 1 | 1.4 | 1 | 1.4 |

**Table 3. Calibration Concentrations and Calibration Results**

| Compound | Calibration Concentration (mg/L) | | | | | Calibration Results | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L1 | L2 | L3 | L4 | L5 | HPLC | | | | UHPLC | | | |
| | | | | | | Points | $r^2$ | Offset | Slope | Points | $r^2$ | Offset | Slope |
| TC | 200 | 300 | 400 | 500 | 600 | 15 | 0.99968 | 3.4928 | 0.1505 | 15 | 0.99965 | 0.8230 | 0.0337 |
| EATC | 0.5 | 1 | 5 | 10 | 15 | 15 | 0.99996 | 0.0091 | 0.3908 | 15 | 0.99996 | 0.0005 | 0.0866 |

Additional results of the SST of the HPLC and UHPLC methods are shown in Table 2. The chromatography of the resolution solution by the HPLC and UHPLC methods is shown in Figures 1 and 2, respectively.

### Method Detection Limits

To determine the sensitivity of both methods for determining EATC, the method detection limit (MDL) of EATC was studied. The calculated MDLs of the HPLC and UHPLC methods for EATC were 0.002 and 0.014 mg/L, respectively. These were calculated from seven injections of 0.01 and 0.05 mg/L standards for the HPLC and UHPLC methods, respectively, using the equation:

$$\text{MDL} = t(\text{n-1}, 0.99)\ (\text{S})$$

where $t$(n-1, 0.99) = the Student's $t$ value appropriate for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom, S = standard deviation of the replicate analyses.

The following estimates of 3× signal-to-noise (S/N) were calculated from the concentration used for the MDL studies:

- For the HPLC method (0.01 mg/L), S/N was ~5, MDL based on S/N was 0.006 mg/L
- For the UHPLC method (0.05 mg/L), S/N was ~10, MDL based on S/N was 0.015 mg/L

### Method Calibration

Both methods were calibrated before performing sample analysis. Five-point calibration curves were constructed for both TC and EATC separately, due to a small amount of EATC present in the TC-HCl standard. Standard concentrations and calibration results for both methods are shown in Table 3.

| Method | Capsule | TC | | | EATC | | |
|---|---|---|---|---|---|---|---|
| | | Average (mg/L) | RSD | % Assay (90–125%) | Average (mg/L) | RSD | % Concentration (NMT 3%) |
| HPLC | 1 | 495 | 0.05 | 99.0 | 0.57 | 0.19 | 0.057 |
| | 2 | 480 | 0.04 | 96.0 | 0.71 | 0.20 | 0.071 |
| | 3 | 480 | 0.09 | 96.0 | 0.77 | 0.27 | 0.077 |
| UHPLC | 1 | 497 | 0.27 | 99.4 | 0.98 | 0.88 | 0.098 |
| | 2 | 510 | 0.59 | 102 | 0.82 | 0.81 | 0.082 |
| | 3 | 482 | 0.42 | 96.4 | 0.75 | 0.43 | 0.075 |

Table 4. Sample Results Obtained Using Each Method (Five Injections/Capsule)

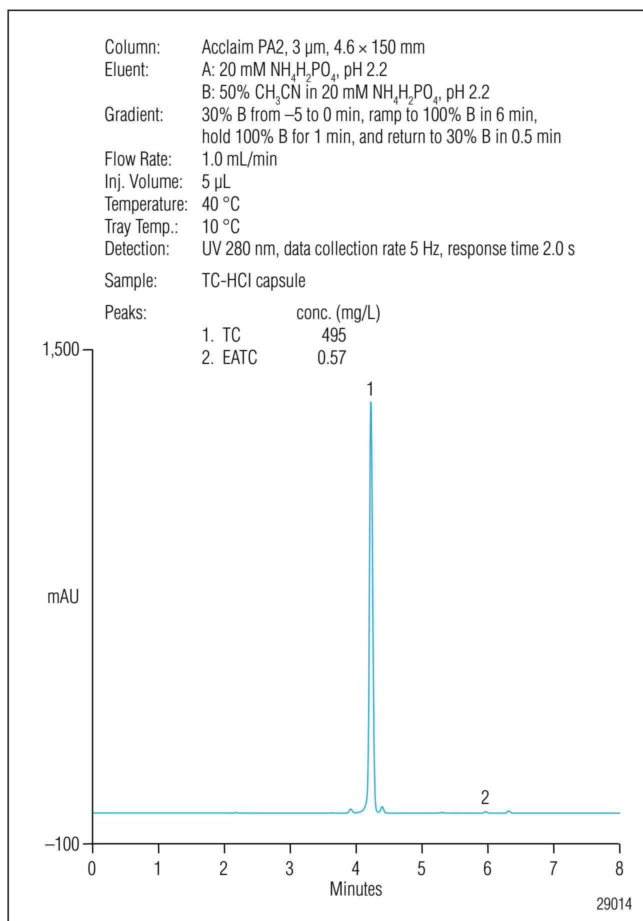


*Figure 3. Chromatogram of the TC-HCl capsule sample obtained using the HPLC method.*

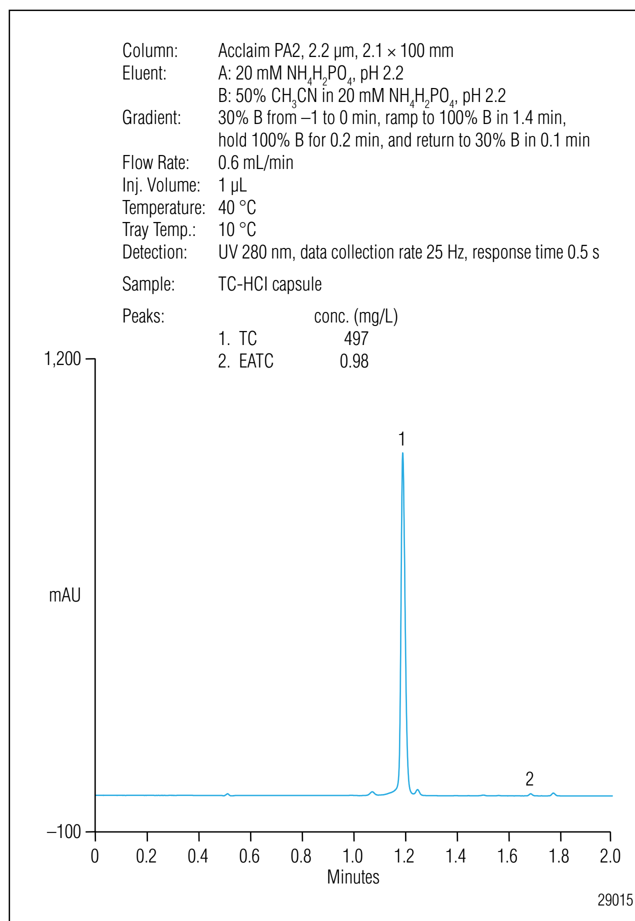


*Figure 4. Chromatogram of the TC-HCl capsule sample obtained using the UHPLC method.*

## Sample Analysis

A TC drug product (TC-HCl capsules) was purchased from a pharmacy. The sample label indicated that a capsule contained 500 mg of TC. To assay this product, three sample preparations were performed and each sample preparation was injected five times.

The assay results (Table 4) obtained from both methods show that the capsules have the appropriate amount of TC to meet the USP specified range (90–125% of the labeled amount).[7] The capsules also met the monograph requirement of NMT 3% EATC (Table 4). Figures 3 and 4 show chromatography of the sample obtained using the HPLC and UHPLC methods, respectively.

## CONCLUSION

This study shows HPLC and UHPLC methods that can be used to assay TC and EATC in a TC-HCl capsule. The results from both methods meet or exceed the criteria in the appropriate USP monographs. Both methods are faster and show higher resolution than the current methods. They also generate less waste and use less hazardous organic solvents. These methods may be applicable to other TC-HCl-containing drug products.

## REFERENCES

1. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), General Chapter 226, p 132.
2. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Epitetracycline Hydrochloride, p 2707.
3. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride and Novobiocin Sodium Tablets, p 4391.
4. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride, Novobiocin Sodium, and Prednisolone Tablets, p 4391.
5. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride and Nystatin Capsules, p 4392.
6. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride, p 4385.
7. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride Capsules, p 4386.

# Determination of Tobramycin in Crude and In-Process Production Samples During Manufacturing Using HPAE-IPAD

## INTRODUCTION

Tobramycin is purified from the products of an actinomycete *Streptomyces tenebrarius* fermentation. These fermentation broths typically consist of cell culture media, a very complex sample matrix that includes salts. The product of microbial fermentation is carbamoyl-tobramycin, which is converted to tobramycin during manufacture using ammonium hydroxide hydrolysis. Tobramycin is further purified by extraction in alcohol, crystallization, and then separation by large scale (e.g., 1000 L columns) ion-exchange chromatography.

Application Note 61 (AN 61) describes a method for determination of tobramycin and its impurities in commercially available finished products (standards) using Reagent-Free™ high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAE-IPAD). Detection using IPAD is sensitive and direct, while eluent generation provides the benefits of high reproducibility and ease-of-use.1 The tobramycin samples assayed in AN 61 are highly purified, and do not have appreciable amounts of salt or other sample components that can interfere with the assay. High salt content in tobramycin samples can shorten retention times and broaden peaks. This update describes how tobramycin and its typical impurities can be determined in manufacturing process matrices using the same HPAE-IPAD method described in AN 61. The same method is used to assay tobramycin that is intentionally degraded, demonstrating its suitability as a stability indicating method.

## *EQUIPMENT*

Dionex ICS-3000 ion chromatography system consisting of:

- Gradient or Isocratic Pump, with vacuum degas option and GM-4 gradient mixer (P/N 049135)
- Electrochemical Detector with AAA-Direct™ Certified (Au) Disposable Electrodes (P/N 060082, package of 6; 060140, package of 24) and combination pH/Ag/AgCl Reference Electrode (P/N 044198 for ED40 and ED50, or 061879 for ICS-3000 systems)
- EG Eluent Generator with KOH eluent generator cartridge (EluGen® II Hydroxide; P/N 053921)
- Vacuum Degas Conversion Kit (P/N 055431 for older systems or 063353 for current systems)
- CR-ATC, Continuously Regenerated Anion Trap Column (P/N 060477)
- Autosampler with 20 µL injection loop
- Eluent Organizer, including four 2 L plastic bottles and pressure regulator
- Chromeleon® Chromatography Data System
- 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)

## *REAGENTS AND STANDARDS*

### Reagents

Deionized water, 18 MW-cm resistance or higher

### Standards

Tobramycin (Sigma-Aldrich Chemical Co.; Cat. # T-4014)

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

Kanamycin A (Sigma-Aldrich Chemical Co.; Cat# K-1637)

Kanamycin B (also known as bekanamycin sulfate; Sigma-Aldrich Chemical Co.; Cat# B-5264)

## *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

Inj. Volume: 20 µL (full loop)

Temperature: 30 °C

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

Eluent Generation Method: 2.00 mM KOH; isocratic, 15 min run time or longer as needed

Detection: Integrated pulsed amperometry

Background: 33–96 nC

Reference Electrode Mode: pH

Waveform: *AAA-Direct* waveform

| 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 | |

## *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 or high-purity nitrogen at all times to reduce diffusion of atmospheric carbon dioxide and opportunistic microorganisms.

### Standards

Solid tobramycin, kanamycin B, kanamycin A, and neamine standards were placed in plastic vials and dissolved in deionized water to a 10 mg/mL concentration and diluted for use as described in AN 61.[1]

Note: Tobramycin, and to a lesser extent kanamycin B, kanamycin A, and neamine, 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.

### Samples

All samples were generously provided by Dr. Harry H. Liu, Ph.D. of Crick Pharma, Inc. (Cambridge, MA, 02140, USA) and Chengmin Zheng, Shaorong Zhang, Xiaojie Liu, and Xinping Tang of Livzon New North River Pharmaceutical Co., Ltd. (Qingyuan, Guangdong, China). For each of the following samples received, 100 mg of solid was reconstituted in 10 mL of water, and then serially diluted with water to 5 µg/mL for HPAE-IPAD analysis. Moisture and salt contents were unknown for each sample received, and therefore no corrections were applied for potency. No additional drying was performed prior to the reconstitution of each of the four samples below.

1. Fermentation broth—20 mL of *Streptomyces tenebrarius* fermentation broth in growth media, vacuum dried.
2. Hydrolyzed fermentation broth—15 mL *Streptomyces tenebrarius* fermentation broth hydrolyzed with 6 M ammonium hydroxide and vacuum dried.
3. Crude tobramycin—Tobramycin isolated from hydrolyzed fermentation broth, partially purified by ion-exchange chromatography and crystallization in alcohol, vacuum dried.
4. Finished tobramycin—Tobramycin purified with additional alcoholic crystallization steps, vacuum dried. Two separate batches were provided.

## RESULTS AND DISCUSSION

The manufacture of tobramycin starts with the fermentation of a strain of the actinomycete *Streptomyces tenebrarius* in a proprietary growth media optimized for production of the nebramycin complex. Besides the microorganisms, this fermentation broth is a complex matrix typically consisting of one or more carbon and nitrogen sources, essential metabolic precursors, co-factors, and various salts required for cell growth, metabolic intermediates, waste products, and the desired nebramycin complex consisting of carbamoyl-tobramycin, among other related substances.

Free tobramycin is not produced by *Streptomyces tenebrarius,* and therefore alkaline hydrolysis of the fermentation broth is required to chemically convert the carbamoyl-tobramycin to tobramycin. Hydrolysis also converts other related substances present in the broth, such as carbamoyl-kanamycin B, another nebramycin complex ingredient produced by the microorganism. The 3 N ammonium hydroxide used for alkaline hydrolysis is removed during evaporation to dryness under vacuum.

This dry residue is a very impure form of tobramycin. Initial isolation of tobramycin from impurities is accomplished using both ion-exchange chromatography and crystallization in alcohol. The tobramycin material produced from this process is known as crude tobramycin. Further purification is accomplished using additional ion-exchange and crystallization steps, and leads to a highly purified finished tobramycin that complies with USP and other standard-setting organizations' purity criteria.

Finished products chemically degrade by different pathways. Under acidic or basic conditions, tobramycin degrades into smaller subunits that retain electrochemical activity, but still lack a good chromophore. These degradation products have been previously described.[2-4]

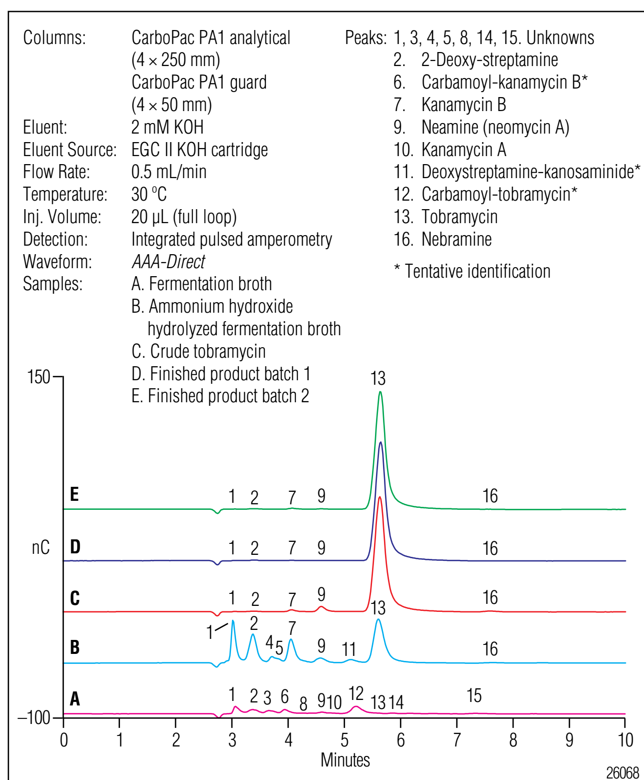


*Figure 1. Determination of tobramycin and impurities in manufacturing process intermediates and final products.*

### Fermentation Broth

The culture medium towards the end of the fermentation process, just prior to harvest, should consist of carbamoyl-kanamycin B and carbamoyl-tobramycin, with only trace levels of kanamycin B and tobramycin.4 The HPAE-IPAD analysis of this sample (Figure 1, Trace A) confirms the presence of only trace levels of kanamycin B (peak 7) and tobramycin (peak 13). The tentative identification of the carbamoyl-kanamycin B and carbamoyl-tobramycin peaks were previously described as peaks 6 and 12, respectively.[2] We found a total of 10 peaks in this chromatogram, more than the three factors described by Stark et al.[4] Among these we found neamine (neomycin A, peak 9, a degradation component of kanamycin B), and kanamycin A (peak 10). This chromatogram showed no interferences, shifts in retention times, or peak broadening that would occur if these samples were not suited for HPAE-IPAD analysis. This method successfully analyzes fermentation broths used for the manufacture of tobramycin, and the same or similar method should be suitable for assaying the fermentation broths of other aminoglycoside antibiotics.

### Base-Hydrolyzed Broth Concentrate

Hydrolysis of the concentrated broth used in the manufacture of tobramycin with 3 N ammonium hydroxide converts carbamoyl-kanamycin B and carbamoyl-tobramycin to kanamycin B and tobramycin, respectively. Chromatography of this sample shows the appearance of kanamycin B and tobramycin peaks (Figure 1, Trace B, peaks 7 and 13, respectively), and seven other peaks (peaks 1, 2, 4, 5, 9, 11, and 16). Because salt was not removed in this manufacturing step, this sample represents a potential challenge for this method to determine tobramycin. No retention time shifts or peak broadening was observed, demonstrating the suitability of HPAE-IPAD for this analysis.

### Crude Tobramycin

Crude tobramycin, the result of ion-exchange chromatography and crystallization techniques for the isolation of tobramycin, is expected to contain fewer and lower amounts of the impurities found in the hydrolyzed fermentation broth. Figure 1, Trace C shows the separation of tobramycin from these impurities in this crude sample, and clearly shows a purer product.

### Finished Tobramycin (Final Product)

The finished product, the result of further ion-exchange and crystallization steps, is expected to be more purified than the crude material. The chromatograms for two finished product batches (Figure 1, Traces D and E) both showed significantly lower amounts of the detected impurity peaks compared to the crude material (Figure 1, Trace C). The calculated percentage for the sum of the six impurities, expressed as tobramycin peak area equivalents, ranged from 0.6 to 0.9% for the two finished batches, while the sum of the six impurity peaks in the crude material was 4.9%. These results again demonstrate the capability of this method to determine tobramyin in process intermediates and final products, and to evaluate the quality of the product throughout the process. The method's sensitivity allows these samples to be diluted so that other sample ingredients (e.g., salt) do not interfere. The chromatograms of these finished batches are similar to the final product, as evaluated in AN 61.[1] This indicates that the industrial processes involved in manufacturing tobramycin are probably similar, and that the results presented in this update, for this process, would be applicable to other manufacturers.

### Product Degradation

Degradation of tobramycin may occur during storage, or if it is subjected to a new process as part of a new pharmaceutical formulation. Degradation should be monitored to assess the potency and quality of the drug product. Two common tests used for monitoring the stability of pharmaceutical products involve exposure to acidic or basic conditions. Elevated temperatures are used to accelerate these studies.

Finished tobramycin (batch #1) was treated with high concentrations (0.5 M) of hydrochloric acid and sodium hydroxide at 100 °C for 1 h and 120 °C for 24 h, respectively.[2] Figure 2 shows the degradation products formed from this study, and the loss of tobramycin peak area (Figure 2, Trace A), and the gain in peak areas for its known degradation products (Figure 2, Traces B and C).[2,3,5] These results show the capability of HPAE-IPAD for use in a stability indicating assay for tobramycin, and for evaluating the drug for its associated degradation products.

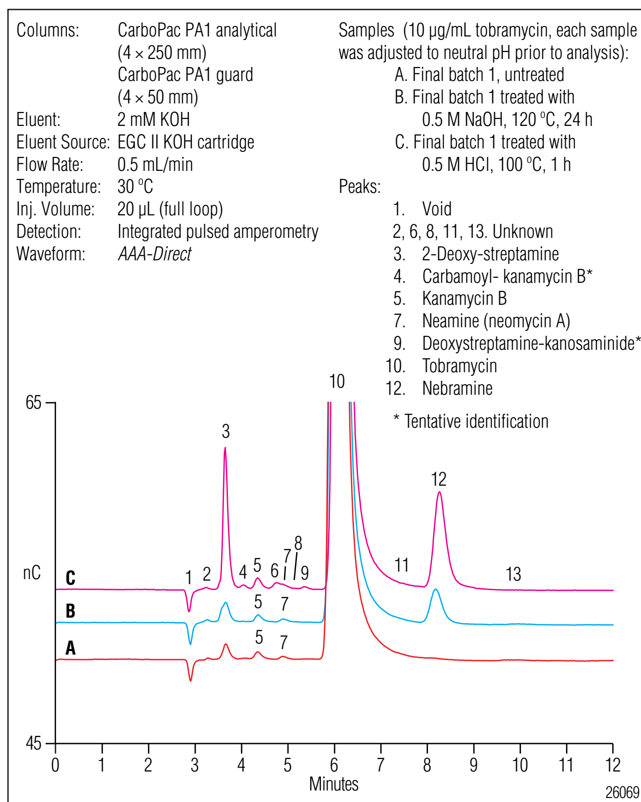


*Figure 2. An accelerated stability study using forced acid and base decomposition of finished tobramycin (10 µg/mL).*

## CONCLUSION

Based on comparison of chromatograms for tobramycin materials collected during different stages in the manufacturing process, this chromatographic method can accurately assess the quality of tobramycin during in-process production. This was demonstrated by the progressive decreases in the amount of impurities found for the process samples moving forward through the manufacturing process and analyzed by HPAE-IPAD. The two finished commercial batches of tobramycin exhibited the highest level of purity, and the trace levels of the impurities showed nearly identical profiles, although some differences in the relative proportions of the impurity peaks were observed. These profiles also closely resembled that obtained from a different commercial source (presented in AN 61), suggesting the manufacturing process for tobramycin is similar throughout the industry. Forced degradation of tobramycin under both high and low pH conditions demonstrated that this method can be used as a stability indicating assay.

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6. Hanko, V.P.; Rohrer, J.S. Determination of Tobramycin and Impurities Using High-Performance Anion-Exchange Chromatography with Integrated Pulsed Amperometric Detection. *J. Pharm. Biomed. Anal.* **2006,** *40,* 1006–1012.


## LIST OF SUPPLIERS

J.T. Baker Incorporated, 222 Red School Lane, Phillipsburg, New Jersey, 08865, U.S.A., 1-800-582-2537.

Fisher Scientific, 711 Forbes Avenue, Pittsburgh, Pennsylvania, 15219-4785, U.S.A., 1-800-766-7000.

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

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

# Column Selection Guide

Pharmaceutical Applications Notebook

# Thermo Scientific Acclaim Column Selection Guide

Please refer to www.thermoscientific.com/dionex for more information

| | | | Reversed-Phase (RP) | | | | | | Mixed-Mode | | | HILIC | | Application-Specific | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Acclaim 120 C18 | Acclaim 120 C8 | Acclaim 300 C18 | Acclaim Polar Advantage (PA) | Acclaim Polar Advantage II (PA2) | Acclaim Phenyl-1 | Acclaim Trinity P1 | Acclaim Mixed-Mode WAX-1 | Acclaim Mixed-Mode WCX-1 | Acclaim Mixed-Mode HILIC-1 | Acclaim HILIC-10 | Acclaim Organic Acid | Acclaim Surfactant | Acclaim Explosives E1 | Acclaim Explosives E2 | Acclaim Carbamate | ***Example Applications*** |
| ***General Applications*** | ***Neutral Molecules*** | High hydrophobicity | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | | | | | *Fat-soluble vitamins, PAHs, glycerides* |
| | | Intermediate hydrophobicity | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | | | | | | *Steroids, phthalates, phenolics* |
| | | Low hydrophobicity | ● | | | ● | ● | | | | | ● | ● | | | | | | *Acetaminophen, urea, polyethylene glycols* |
| | ***Anionic Molecules*** | High hydrophobicity | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | | | | | | *NSAIDs, phospholipids* |
| | | Intermediate hydrophobicity | ● | ● | ● | ● | ● | ● | ● | ● | | ● | | | | | | | *Asprin, alkyl acids, aromatic acids* |
| | | Low hydrophobicity | | | | ● | | | ● | ● | | ● | ● | | | | | | *Small organic acids, e.g. acetic acids* |
| | ***Cationic Molecules*** | High hydrophobicity | ● | ● | ● | ● | ● | ● | | ● | ● | ● | | | | | | | *Antidepressants* |
| | | Intermediate hydrophobicity | ● | ● | ● | ● | ● | ● | ● | | ● | ● | | | | | | | *Beta blockers, benzidines, alkaloids* |
| | | Low hydrophobicity | ● | | | ● | | | ● | | ● | ● | ● | | | | | | *Antacids, pseudoephedrine, amino sugars* |
| | ***Amphoteric/ Zwitterionic Molecules*** | High hydrophobicity | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | | | | | | *Phospholipids* |
| | | Intermediate hydrophobicity | ● | ● | ● | ● | ● | ● | | | ● | | | | | | | | *Amphoteric surfactants, peptides* |
| | | Low hydrophobicity | | | | ● | ● | | ● | ● | ● | ● | ● | | | | | | *Amino acids, aspartame, small peptides* |
| | ***Mixtures of Neutral, Anionic, Cationic Molecules*** | 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 | | | | | | | | | | | | | ● | | | | *Quats, benzylalkonium in medicines* |
| | | Nonionic | ● | ● | ● | ● | ● | | | | | ● | | | ● | | | | *Triton X-100 in washing tank* |
| | | Amphoteric | ● | ● | ● | ● | ● | | | | | | | | ● | | | | *Cocoamidopropyl betaine* |
| | | Hydrotropes | | | | | | | | | | | | | ● | | | | *Xylenesulfonates in handsoap* |
| | | Surfactant blends | | | | | | | | | | | | | ● | | | | *Noionic and anionic surfactants* |
| | ***Organic Acids*** | Hydrophobic | | | | | | | ● | ● | | | | ● | | | | | *Aromatic acids, fatty acids* |
| | | Hydrophilic | | | | | | | ● | ● | | | | ● | | | | | *Organic acids in soft drinks, pharmaceuticals* |
| | ***Environmental Contaminants*** | Explosives | | | | | | | | | | | | | | ● | ● | | *U.S. EPA Method 8330, 8330B* |
| | | Carbonyl compounds | | | | | | | | | | | | | | | ● | | *U.S. EPA 1667, 555, OT-11; CA CARB 1004* |
| | | Phenols | ● | | | ● | | | | | | | | | | | | | *Compounds regulated by U.S. EPA 604* |
| | | Chlorinated/Phenoxy acids | | | | ● | | | | | | | | | | | | | *U.S. EPA Method 555* |
| | | Triazines | ● | | | ● | | | | | | | | | | | | | *Compounds regulated by U.S. EPA 619* |
| | | Nitrosamines | | | | ● | | | | | | | | | | | | | *Compounds regulated by U.S. EPA 8270* |
| | | Benzidines | ● | | | ● | | | | | | | | | | | | | *U.S. EPA Method 605* |
| | | Perfluorinated acids | | | | ● | | | | | | | | | | | | | *Dionex TN73* |
| | | Microcystins | ● | | | | | | | | | | | | | | | | *ISO 20179* |
| | | Isocyanates | | | | | ● | | | | | ● | | | | | | | *U.S. OSHA Methods 42, 47* |
| | | Carbamate insecticides | | | | | | | | | | | | | | | | ● | *U.S. EPA Method 531.2* |
| | ***Vitamins*** | Water-soluble vitamins | | | | ● | ● | | ● | | | | | | | | | | *Vitamins in dietary supplements* |
| | | Fat-soluble vitamins | ● | ● | ● | ● | ● | ● | | ● | | | | | | | | | *Vitamin pills* |
| | ***Pharmaceutical Counterions*** | Anions | | | | | | | ● | ● | | | | | | | | | *Inorgaic anions and organic acids in drugs* |
| | | Cations | | | | | | | ● | | ● | | | | | | | | *Inorgaic cations and organic bases in drugs* |
| | | Mixture of Anions and Cations | | | | | | | ● | | | | | | | | | | *Screening of pharmaceutical counterions* |
| | | API and counterions | | | | | | | ● | | | | | | | | | | *Naproxen $Na^+$ salt, metformin $Cl^-$ salt, etc.* |

# Transferring HPLC Methods to UHPLC

Pharmaceutical Applications Notebook

DIONEX

# Technical Note 75

# 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.

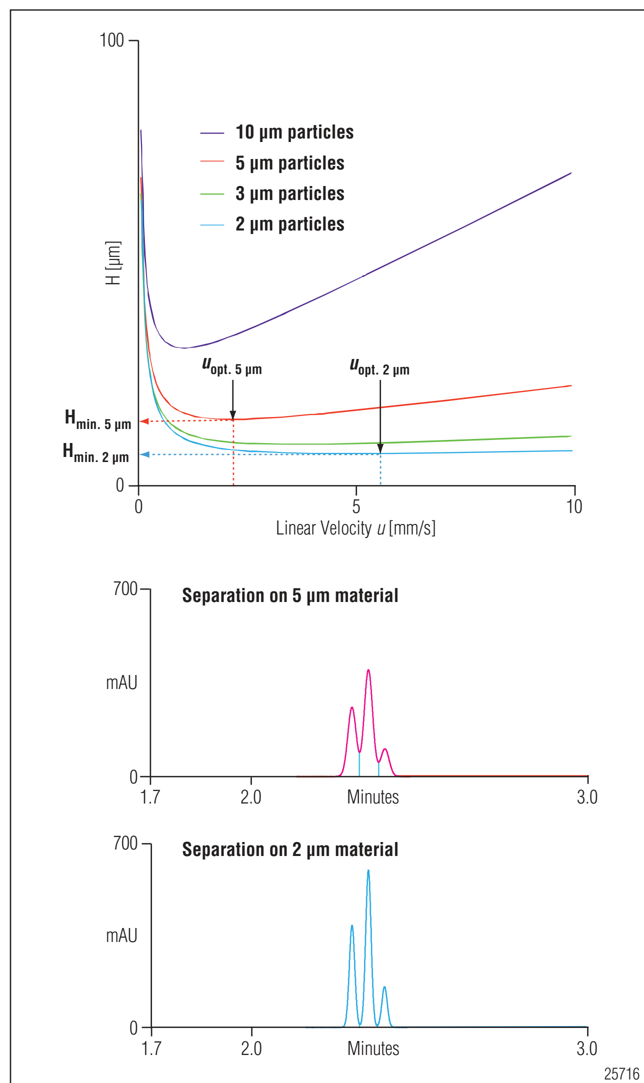



*Figure 1. Smaller particles provide more theoretical plates and more resolution, demonstrated by the improved separation of three peaks (bottom) and smaller minimum plate heights* H *in the Van Deemter plot (top). At linear velocities higher than uopt,* H *increases more slowly when using smaller particles, 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.

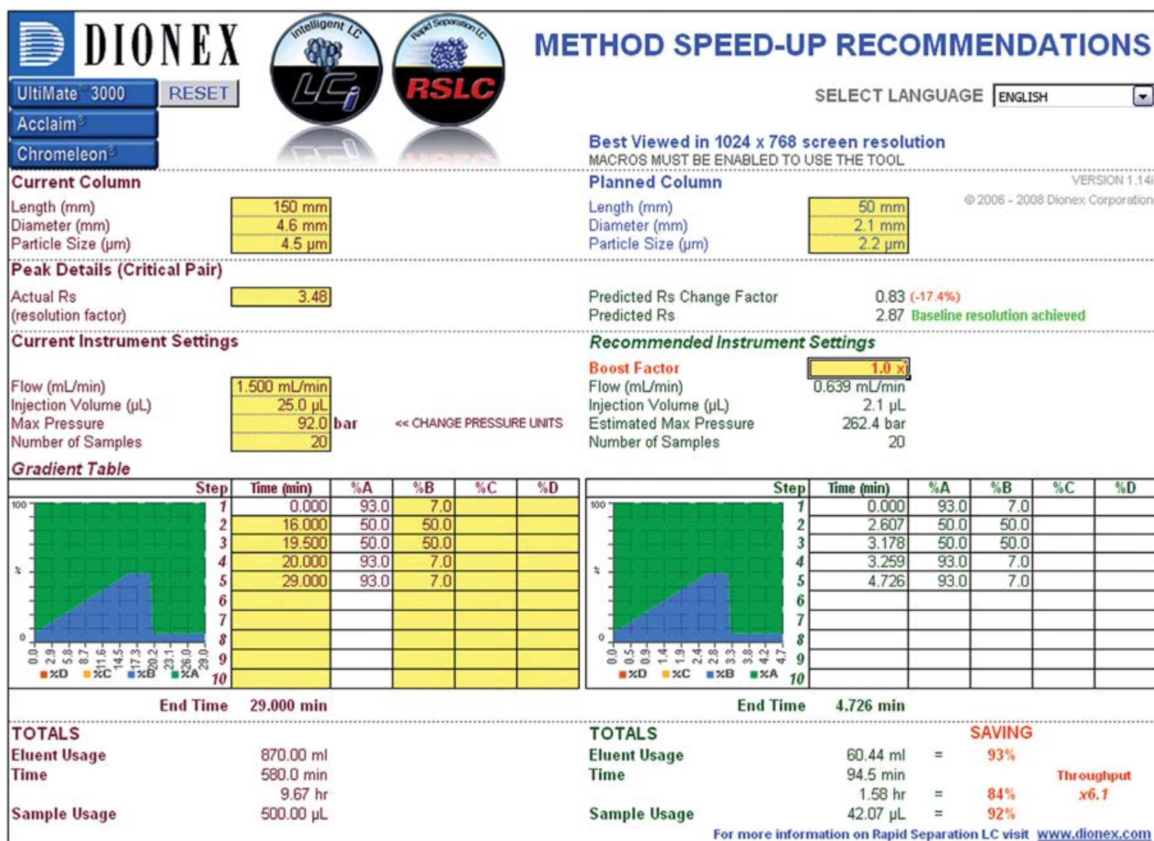



*Figure 2. The Dionex Method Speed-Up Calculator transfers a conventional (current) HPLC method to a new (planned) RSLC method.*

### 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.

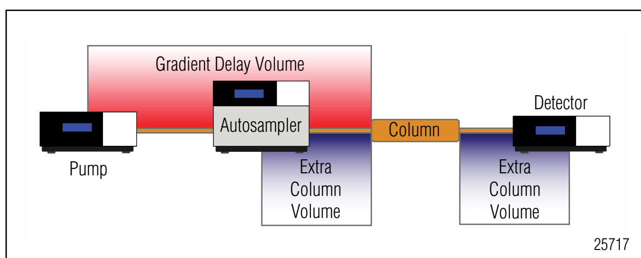



*Figure 3. Gradient delay volume and extra column volume of an HPLC system. Both play an important role in method speed-up.*

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

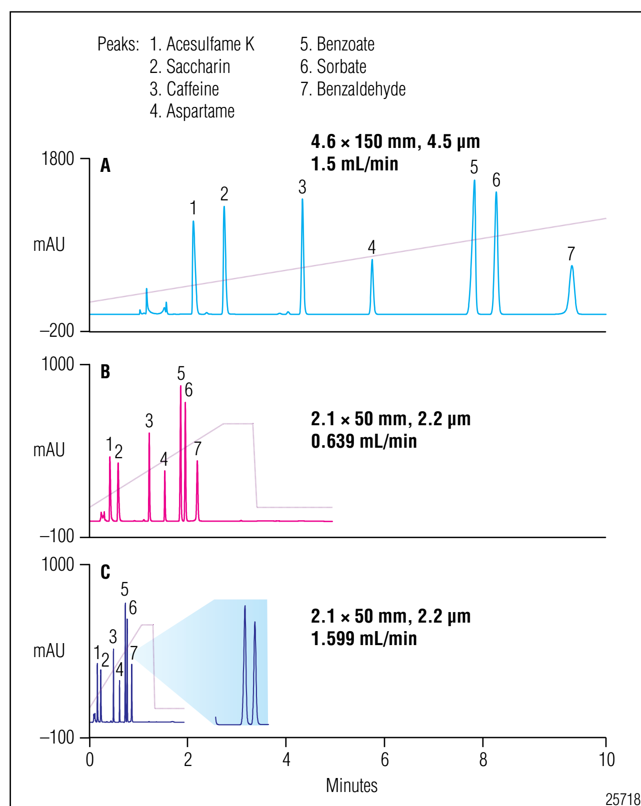



*Figure 4. Method transfer with the Method Speed-Up Calculator from A) a conventional LC separation on an Acclaim 5 µm particle column, to B) and C) RSLC separations on an Acclaim 2.2 µm particle column.*

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).

| Current Column | | Planned Column | | VERSION 1.14i © 2006 - 2008 Dionex Corporation |
|---|---|---|---|---|
| Length (mm) | 150 mm | Length (mm) | 50 mm | |
| Diameter (mm) | 4.6 mm | Diameter (mm) | 2.1 mm | |
| Particle Size (µm) | 4.5 µm | Particle Size (µm) | 2.2 µm | |
| **Peak Details (Critical Pair)** | | | | |
| Actual Rs (resolution factor) | 3.48 | Predicted Rs Change Factor | 0.83 (-17.4%) | |
| | | Predicted Rs | 2.87 Baseline resolution achieved | |

*Figure 5. Column selection considering the resolution of the critical pair.*

| Current Instrument Settings | | | Recommended Instrument Settings | |
|---|---|---|---|---|
| | | | Boost Factor | 1.0 x |
| Flow (mL/min) | 1.500 mL/min | | Flow (mL/min) | 0.639 mL/min |
| Injection Volume (µL) | 25.0 µL | | Injection Volume (µL) | 2.1 µL |
| Max Pressure | 92.0 bar | << CHANGE PRESSURE UNITS | Estimated Max Pressure | 262.4 bar |
| Number of Samples | 20 | | Number of Samples | 20 |

*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).

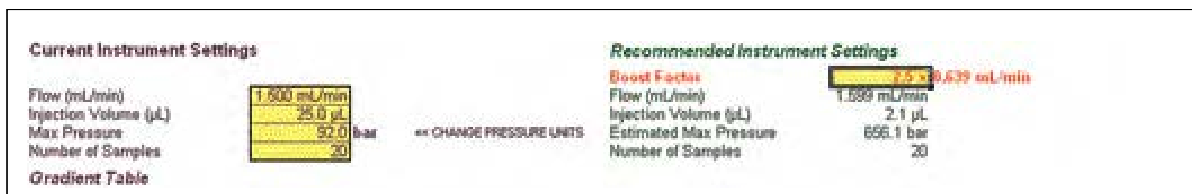



*Figure 7. The new flow rate is further accelerated by applying the Boost Factor of 2.5.*

### 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).

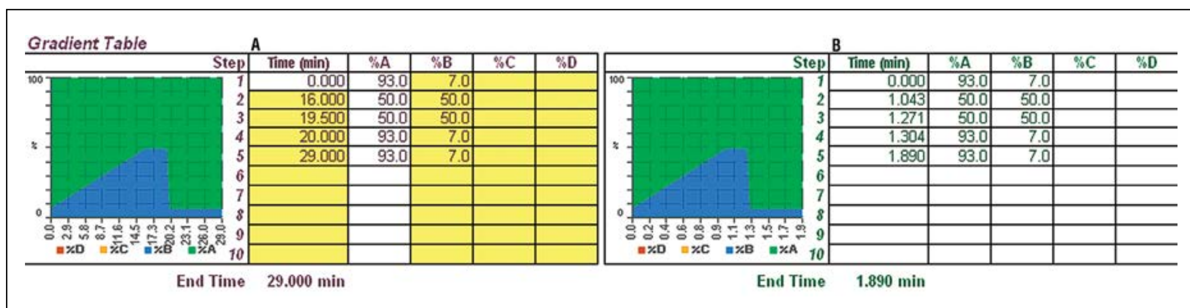
### Predicted Backpressure

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.



Gradient Table

A

| Step | Time (min) | %A | %B | %C | %D |
|---|---|---|---|---|---|
| 1 | 0.000 | 93.0 | 7.0 | | |
| 2 | 16.000 | 50.0 | 50.0 | | |
| 3 | 19.500 | 50.0 | 50.0 | | |
| 4 | 20.000 | 93.0 | 7.0 | | |
| 5 | 29.000 | 93.0 | 7.0 | | |
| 6 | | | | | |
| 7 | | | | | |
| 8 | | | | | |
| 9 | | | | | |
| 10 | | | | | |

End Time 29.000 min

B

| Step | Time (min) | %A | %B | %C | %D |
|---|---|---|---|---|---|
| 1 | 0.000 | 93.0 | 7.0 | | |
| 2 | 1.043 | 50.0 | 50.0 | | |
| 3 | 1.271 | 50.0 | 50.0 | | |
| 4 | 1.304 | 93.0 | 7.0 | | |
| 5 | 1.890 | 93.0 | 7.0 | | |
| 6 | | | | | |
| 7 | | | | | |
| 8 | | | | | |
| 9 | | | | | |
| 10 | | | | | |

End Time 1.890 min

*Figure 8. The gradient table of the current method (A) is adapted to the boosted method (B) according to the gradient-volume principle.*

| TOTALS | | TOTALS | | | SAVING | |
|---|---|---|---|---|---|---|
| Eluent Usage | 870.00 ml | Eluent Usage | 60.44 ml | = | 93% | |
| Time | 580.0 min | Time | 37.8 min | | | Throughput |
| | 9.67 hr | | 0.63 hr | = | 93% | x15.3 |
| Sample Usage | 500.00 µL | Sample Usage | 42.07 µL | = | 92% | |

*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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