Pharmaceutical Applications Notebook

Controlled Drugs

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
- Sample Preparation and Mass Spectrometry

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.

IC and RFIC Systems

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).

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.

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.

Process Analytical Systems and Software

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

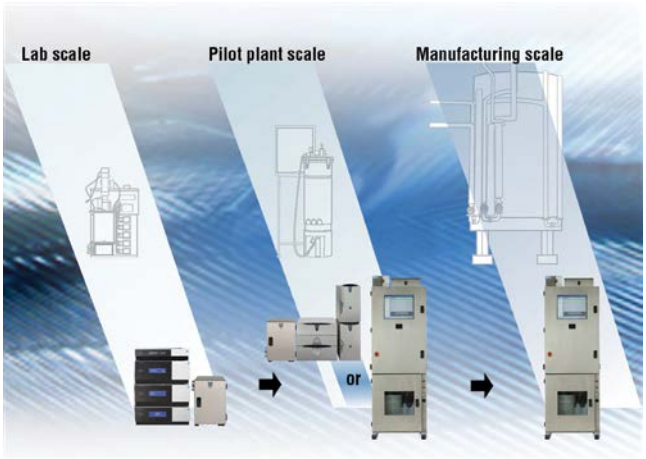
Our process analytical systems provide timely results by moving liquid chromatography-based measurements on-line. Information from the Thermo Scientific Dionex Integral process analyzer can help reduce process variability, improve efficiency, and reduce downtime. These systems provide comprehensive, 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



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 Controlled Drugs

Pharmaceutical Applications Notebook

Determination of Bethanechol by Ion Chromatography

INTRODUCTION

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

EQUIPMENT

Dionex DX-600 ion chromatography system consisting of:

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

REAGENTS AND STANDARDS

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

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

Bethanechol chloride (U.S. Pharmacopeia)

CONDITIONS

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

PREPARATION OF SOLUTIONS AND REAGENTS

Reagent Water

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

Eluent Solution

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

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

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

Stock Standard Solutions

1000 mg/L Bethanechol Standard Solution

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

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

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

Working Standard Solutions

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

SYSTEM PREPARATION AND SETUP

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

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

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

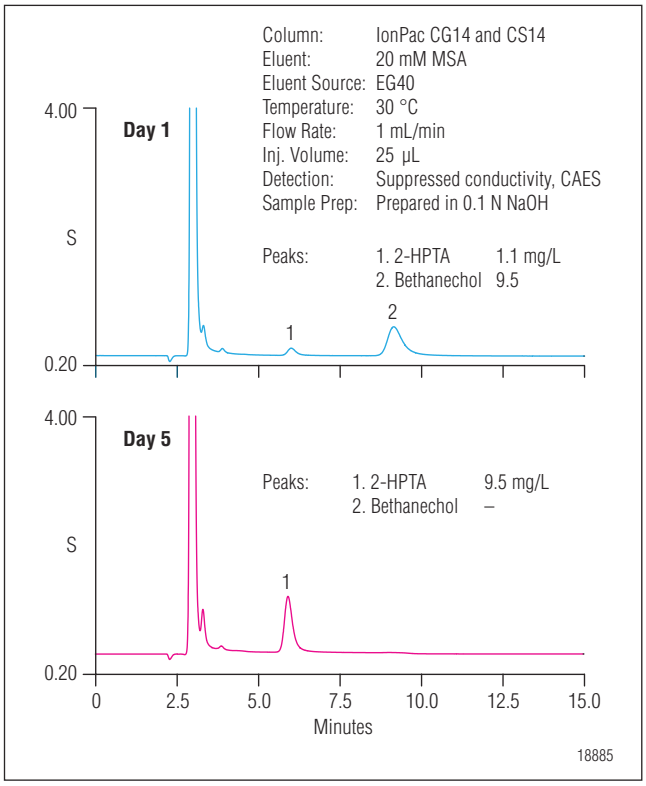


Figure 1. Conversion of Bethanechol to 2-HPTA in the presence of NaOH.

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

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

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

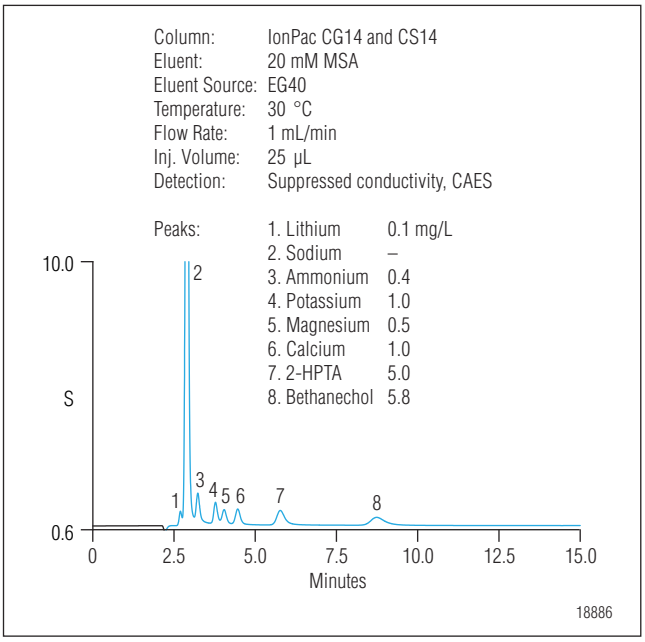


Figure 2. Separation of Bethanechol and 2-HPTA from common inorganic cations using the IonPac CS14.

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

RESULTS AND DISCUSSION

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

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

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

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

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

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

SUMMARY

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

APPENDIX

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

REFERENCE

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

SUPPLIER

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

Determination of Nevirapine Using HPLC with UV Detection

INTRODUCTION

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

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

EQUIPMENT

Dionex UltiMate® 3000 Intelligent LC system:

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

Chromeleon® 6.70 chromatography management system

REAGENTS AND STANDARDS

Acetonitrile, Fisher HPLC Grade or equivalent

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

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

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

Thymine, 99% from Sigma

PREPARATION OF MOBILE PHASE AND STANDARDS

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

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

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

PREPARATION OF SAMPLES

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

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

CHROMATOGRAPHIC CONDITIONS

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

Gradient Table

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

RESULTS AND DISCUSSION

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

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

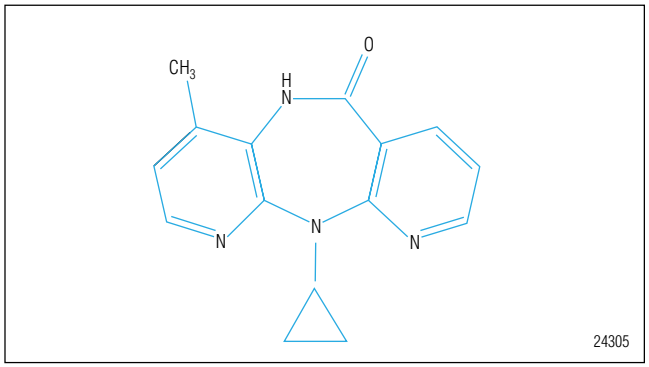


Figure 1. Chemical structure of nevirapine.

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

Resolution

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

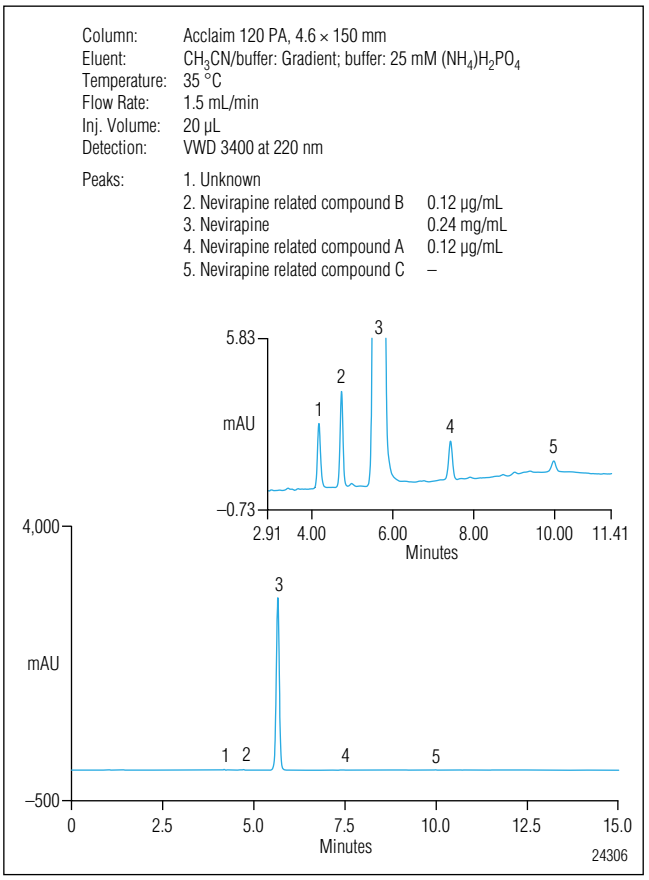


Figure 2. Chromatogram of nevirapine and its related compounds.

Reproducibility

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

Linearity

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

$$y = 1095.6x$$

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

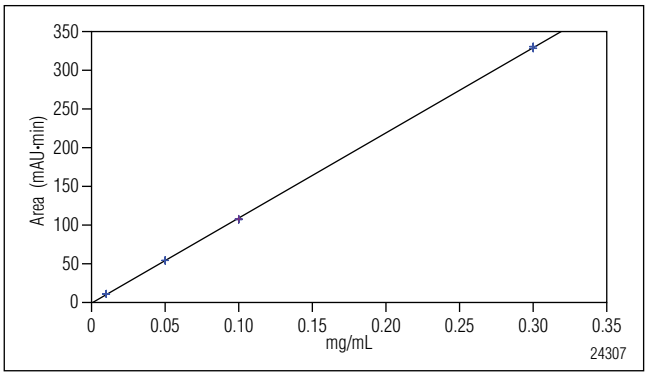


Figure 3. Linearity of nevirapine (n = 6).

Table 1. Data of Nevirapine Standards for Calibration

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

Sample Analysis

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

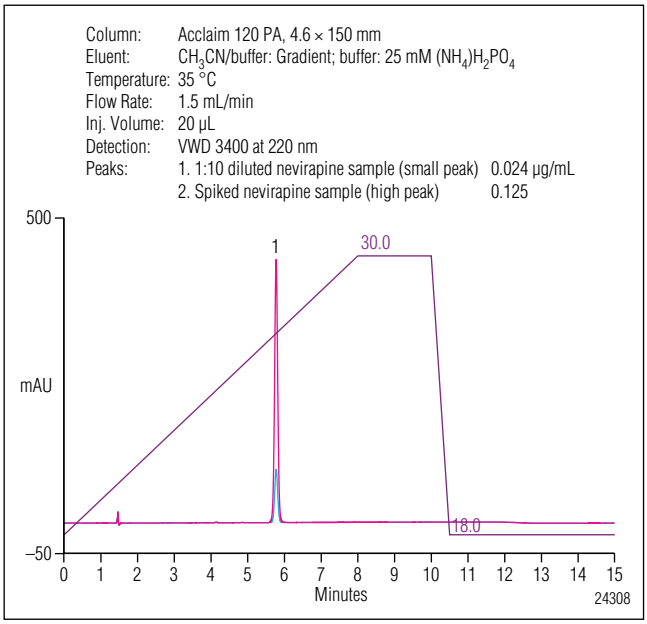


Figure 4. Overlay of chromatograms of 1:10 diluted nevirapine sample with and without a nevirapine spike.

Application to the Analysis of Other NNRTIs

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

Faster Analysis of Nevirapine

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

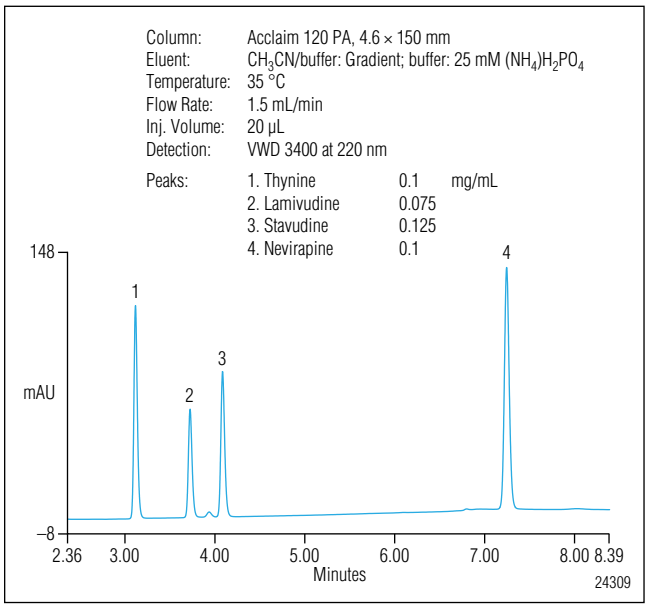


Figure 5. Separation of thymine, lamivudine, stavudine, and nevirapine.

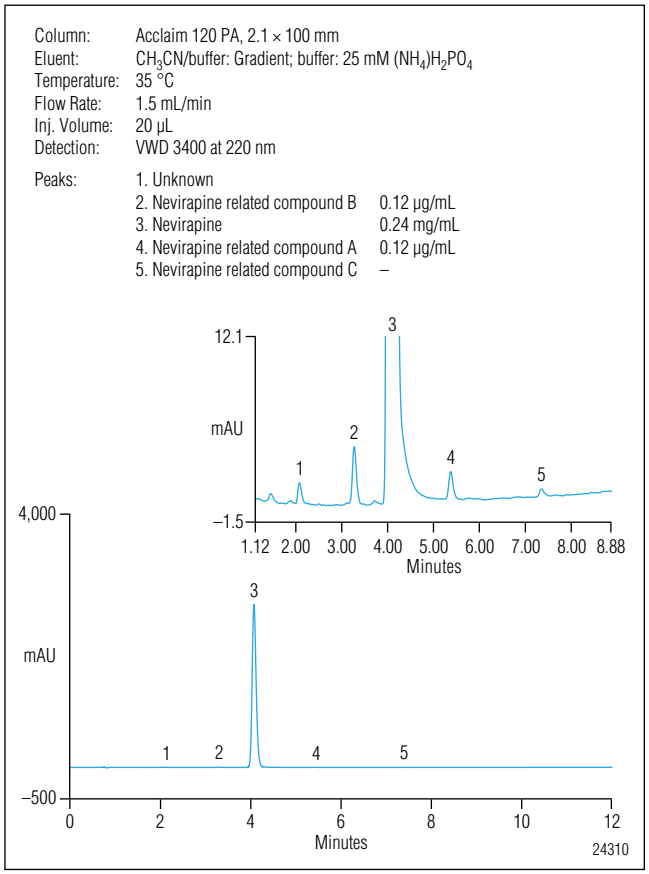


Figure 6. Fast separation of nevirapine and its related compounds, A and B, on a Summit® HPG HPLC system (modified for narrow bore analysis: using microflow kit and 2.5-µL flow cell) using an Acclaim PA column (2.1 × 100 mm). The inset is an enlarged view of the main chromatogram.

Performance of the UltiMate 3000 Intelligent LC System

Simultaneous Determination of Nevirapine and Related Compounds with Different Concentrations

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

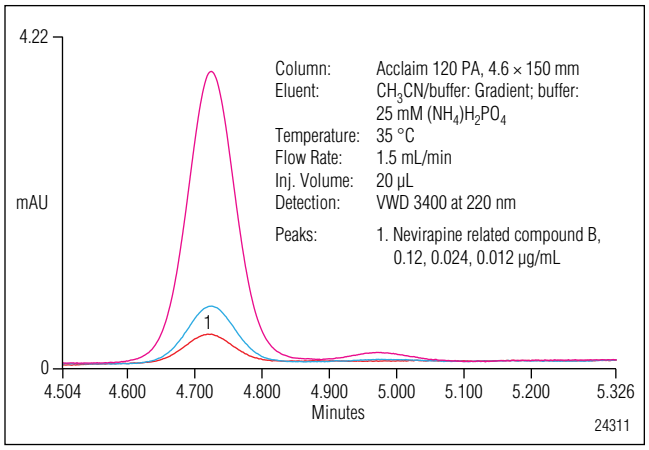


Figure 7. Overlay chromatograms demonstrating trace-level detection of nevirapine-related compound B.

Table 2. Related Data of Impurities Performance Test*

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

* Mean value of six determinations

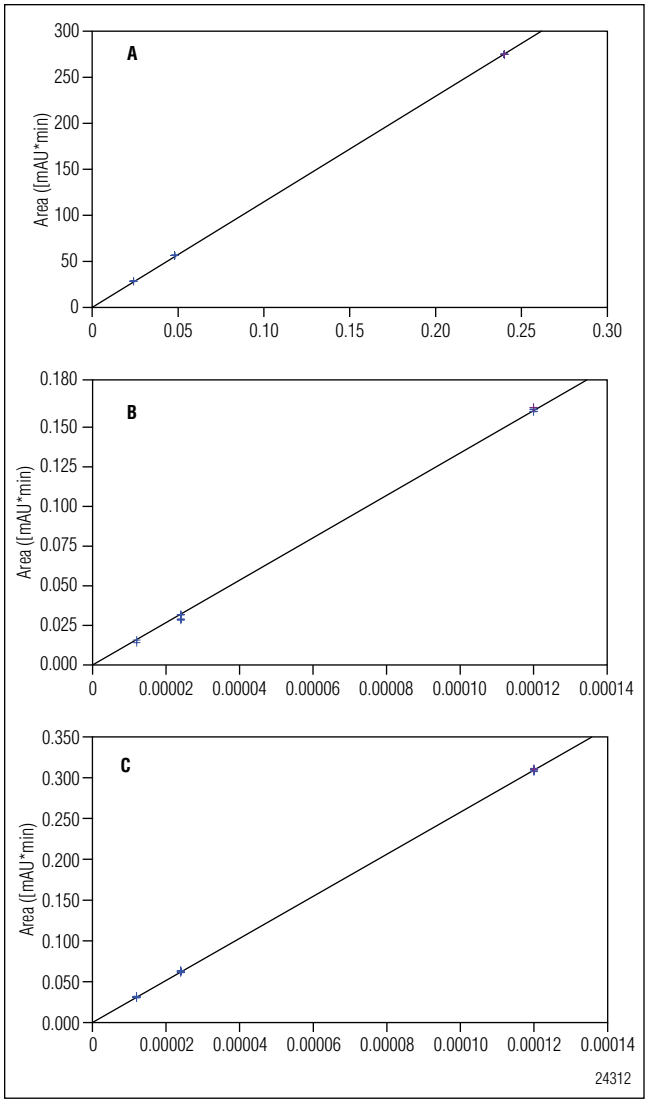


Figure 8. Linearity of (A) nevirapine, (B) related compound B, and (C) related compound A (n = 6).

Carry-Over Performance of the WPS-3000

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

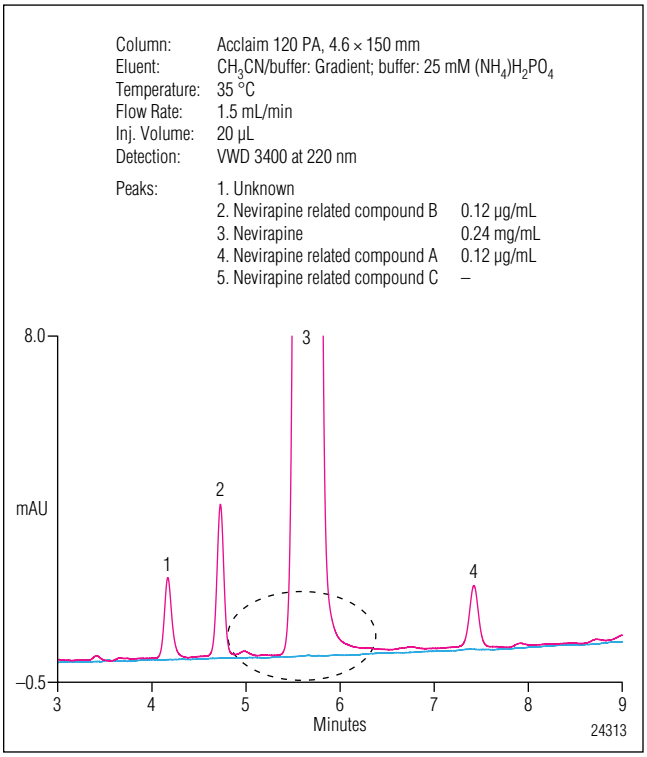


Figure 9. Carry-over test on theWPS-3000 autosampler. Original nevirapine sample solution and blank solution (25 mM $NH_4H_2PO_4$ buffer) were injected in series.

CONCLUSION

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

PRECAUTIONS

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

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2. Gulick, R.M.; Mellors, J.M.; Havlir, D.; et al. *N. Engl. J. Med.* **1997;** 337, 734–739.
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6. Dionex Corporation, *Acclaim Catalog* **2006;** 18.

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

INTRODUCTION

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

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

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

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

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

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

EQUIPMENT

ICS-2000 (Dionex P/N 061098)

AS Autosampler

Chromeleon® 6.8 SP2 Chromatography Workstation

CONSUMABLES AND REAGENTS

CR-CTC II (Dionex P/N 066202)

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

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

Carbachol Chloride (USP reference standard P/N 1092009)

Bethanechol Chloride (USP reference standard P/N 1071009)

Six Cation Standard II (Dionex P/N 046070)

Dimethylamine (Fluka P/N 38960)

Alcon OPTI-FREE® RepleniSH™ Multipurpose Disinfecting Lens Solution

Bausch & Lomb Gentle Sensitive Eyes® Plus Saline Solution

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

CONDITIONS

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

ELUENT SOLUTION

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

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

STOCK STANDARD SOLUTIONS

1000 mg/L Carbachol Solution

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

1000 mg/L Choline Solution

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

1000 mg/L Bethanechol Solution

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

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

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

1000 mg/L Dimethylamine Solution

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

WORKING STANDARD SOLUTIONS

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

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

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

INTERFERENCE STUDIES

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

SAMPLES

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

SYSTEM PREPARATION AND SETUP

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

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

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

Chromatography and Interference Studies

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

Determination of Linearity for Carbachol and Choline

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

Table 1. Linear Range for Carbachol and Choline

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

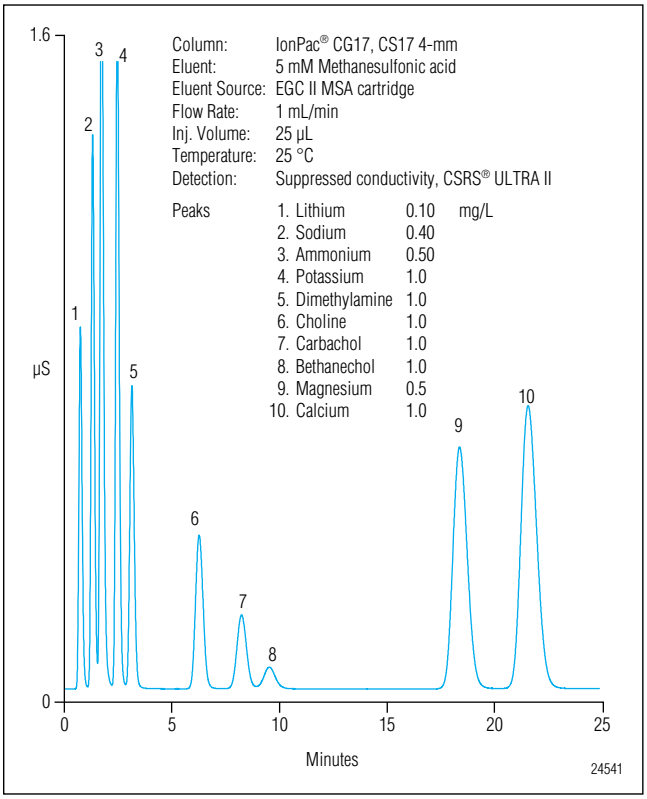

Figure 1. Separation of 1 mg/L carbachol, choline, and bethanechol with a mixed cation standard.

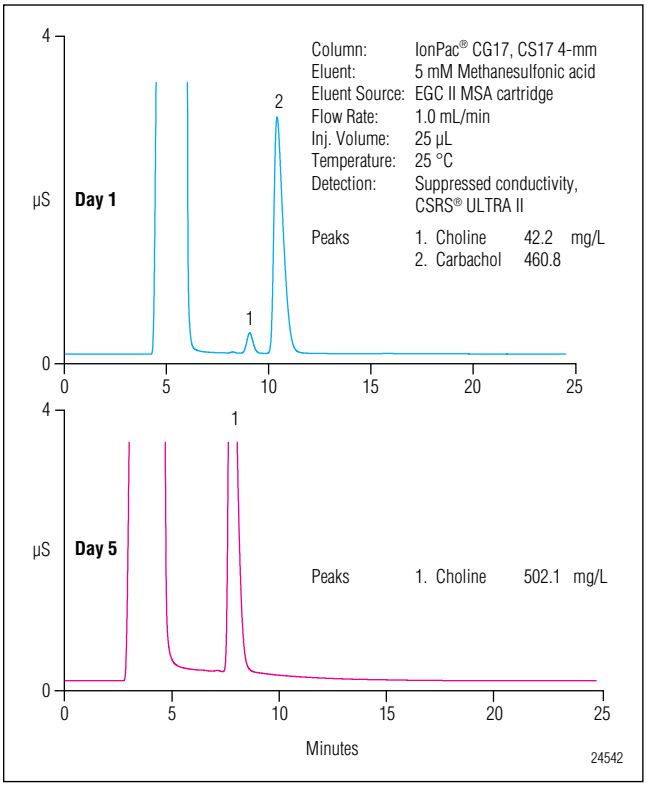

Figure 2. Conversion of carbachol to choline in the presence of 0.1 N NaOH.

Minimum Detection Limit (MDL) for Carbachol and Choline

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

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

Table 2. Determination of Carbachol and Choline MDLs

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

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

SAMPLE ANALYSIS

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

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

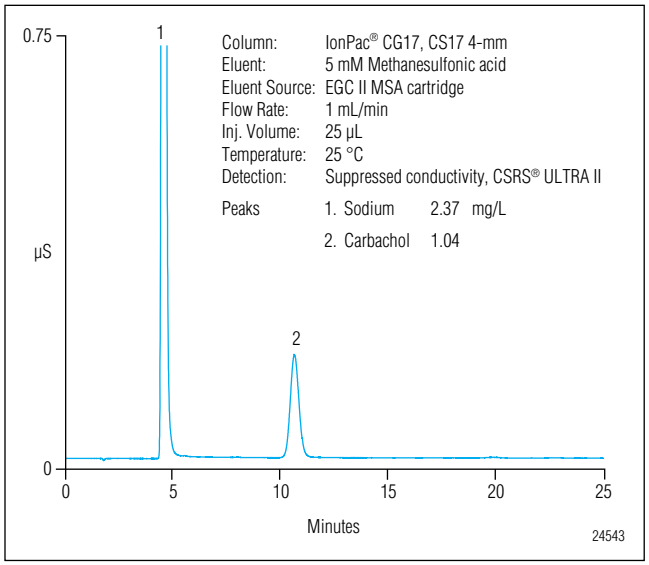

Figure 3. Alcon Lens Solution (diluted 1:1000) spiked with 1 mg/L of carbachol.

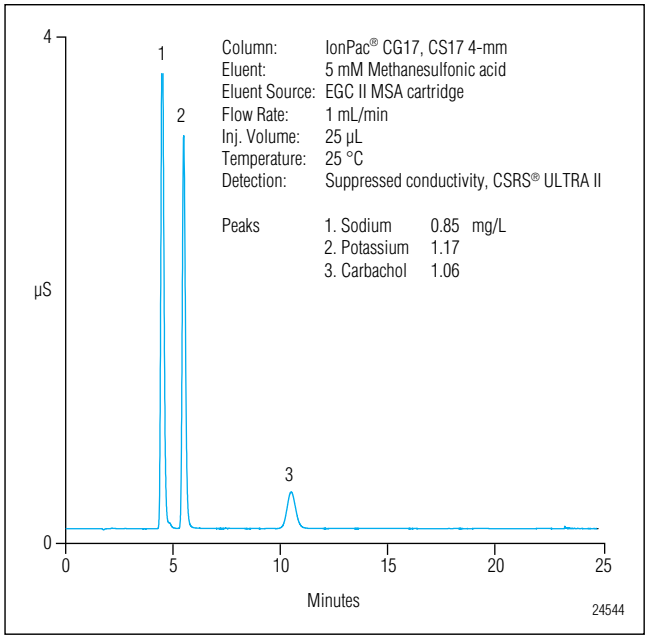

Figure 4. Saline solution (diluted 1:1000) spiked with 1 mg/L of carbachol.

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

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

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

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

Table 4. Recovery of Carbachol from Ophthalmic Samples

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

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

Determination of Bethanechol

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

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

Table 5. Reproducibility

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

Table 6. Linear Range for Bethanechol and 2-HPTA

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

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

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

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

PRECAUTIONS

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

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

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

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

SUMMARY

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

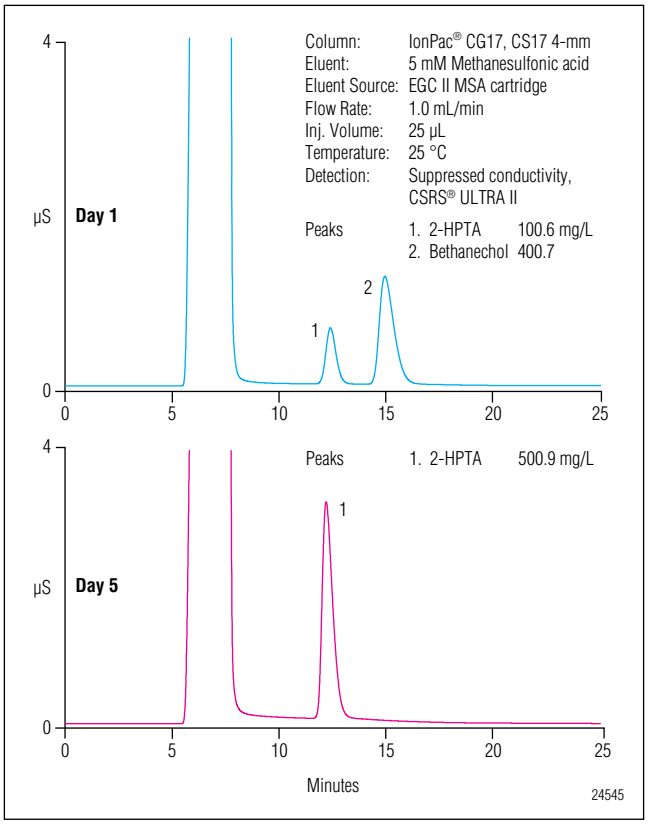

Figure 5. Conversion of bethanechol to 2-HPTA in the presence of 0.1 N NaOH.

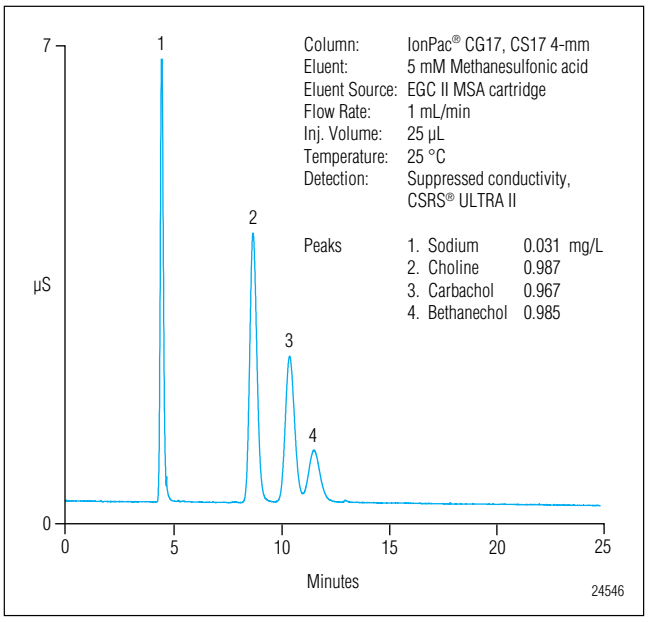

Figure 6. Separation of choline from carbachol and bethanechol in lens solution.

SUPPLIER

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

REFERENCES

1. Determination of Carbachol in Aqueous Solutions J. Pharm Sci. V 58, 1969, 602-604.
2. U.S Pharmacopeia 29 NF 24, 2006.
3. Application Note 70, Choline and Acetylcholine, Dionex Corporation. LPN 034516.
4. Application Note 124, Determination of Choline in Dry Milk and Infant Formula, Dionex Corporation. LPN 1054.
5. Application Note 148, Determination of Bethanechol by Ion Chromatography Dionex Corporation. LPN 1510.

Determination of Verapamil Hydrochloride Purity Using the Acclaim PA Column

INTRODUCTION

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

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

EQUIPMENT

UltiMate® 3000 HPLC

HPG 3400 pump with SRD 3400 degasser

WPS 3000 TSL autosampler

TCC 3000 thermostatted column compartment

VWD-3400 UV-Vis detector

Chromeleon® 6.80 SP1 Chromatography Workstation

REAGENTS AND STANDARDS

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

Acetonitrile (CH_3CN), Fisher, HPLC grade

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

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

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

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

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

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

PREPARATION OF REAGENTS AND STANDARDS

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

System suitability solution:

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

Standard solution and test solution:

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

CHROMATOGRAPHIC CONDITIONS

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

Temperature: 35 °C

Inj. Volume: 5 µL

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

B: CH_3CN

Detection: Absorbance at 278 nm

Gradient Table:

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

RESULTS AND DISCUSSION

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

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

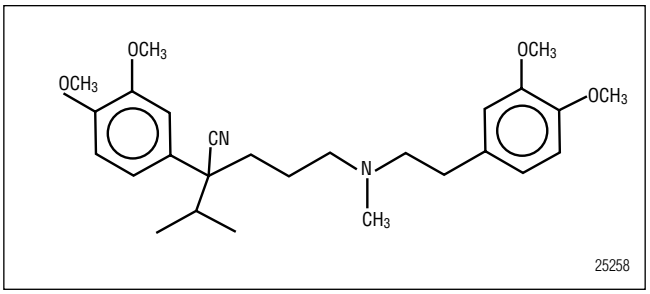

Figure 1. Structure of verapamil.

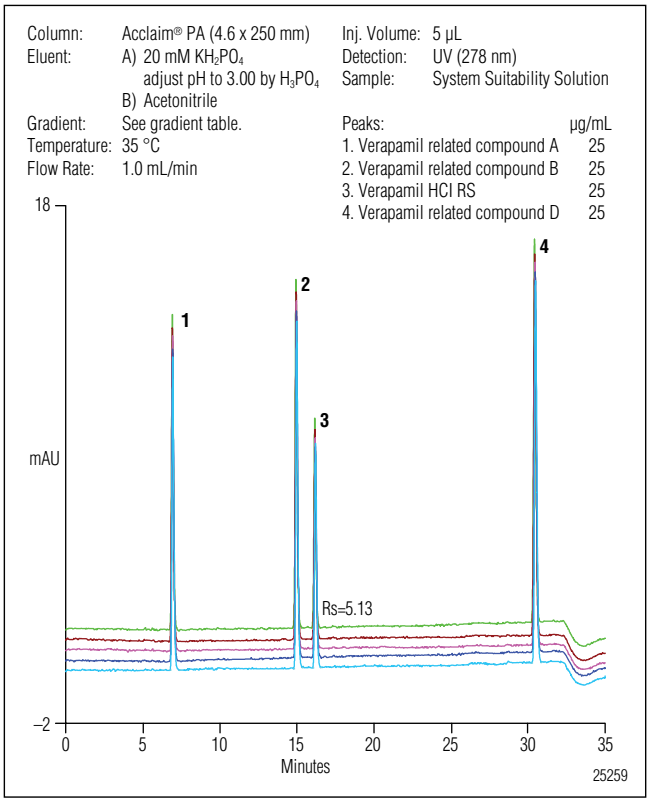

Figure 2. Overlay of five chromatograms of the system suitability solution.

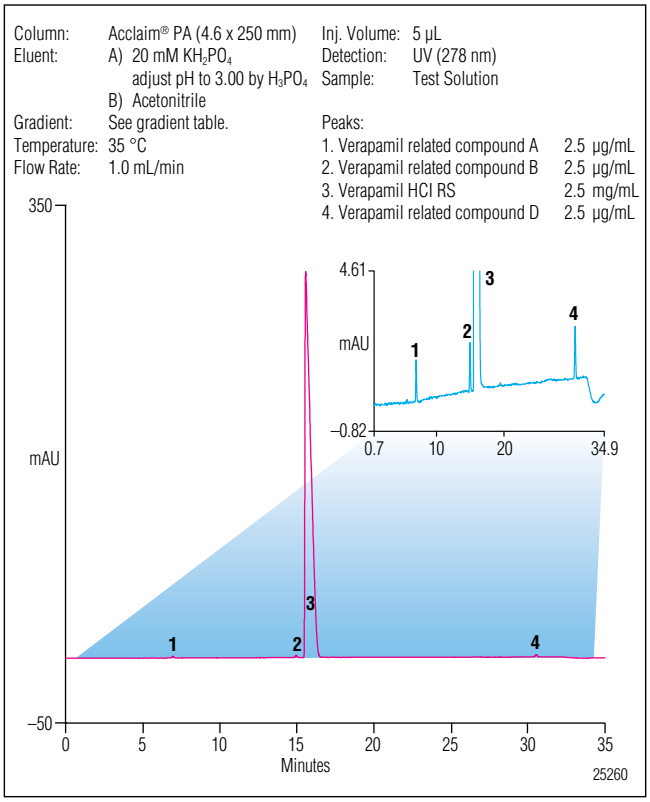

Figure 3. Chromatogram of the test solution.

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

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

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

Effect of Mobile Phase pH

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

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

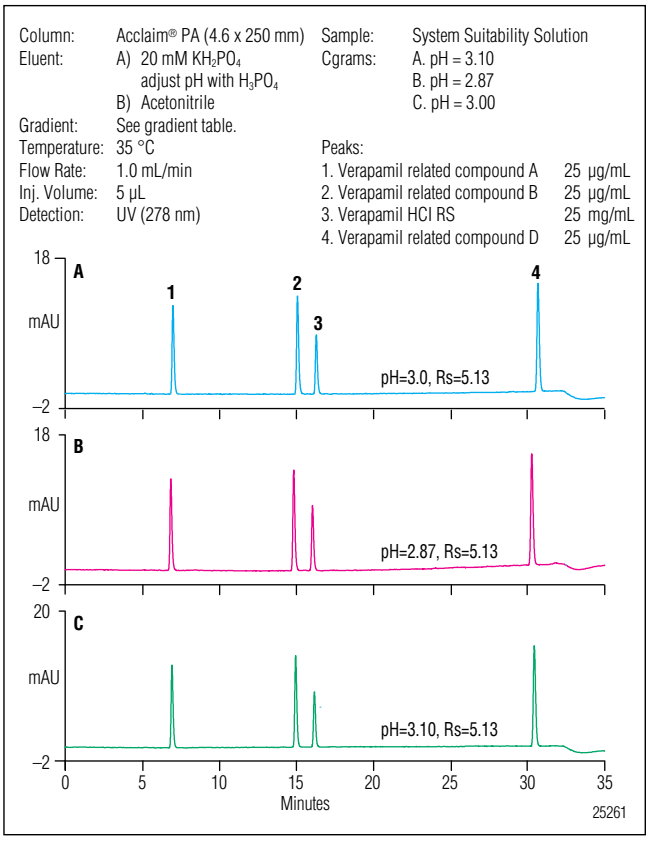

Figure 4. Overlay of chromatograms of the system suitability solution analyzed with different pH mobile phases. Peaks: 1) verapamil-related compound A; 2) verapamil-related compound B; 3) verapamil HCl RS, 4) verapamil-related compound D.

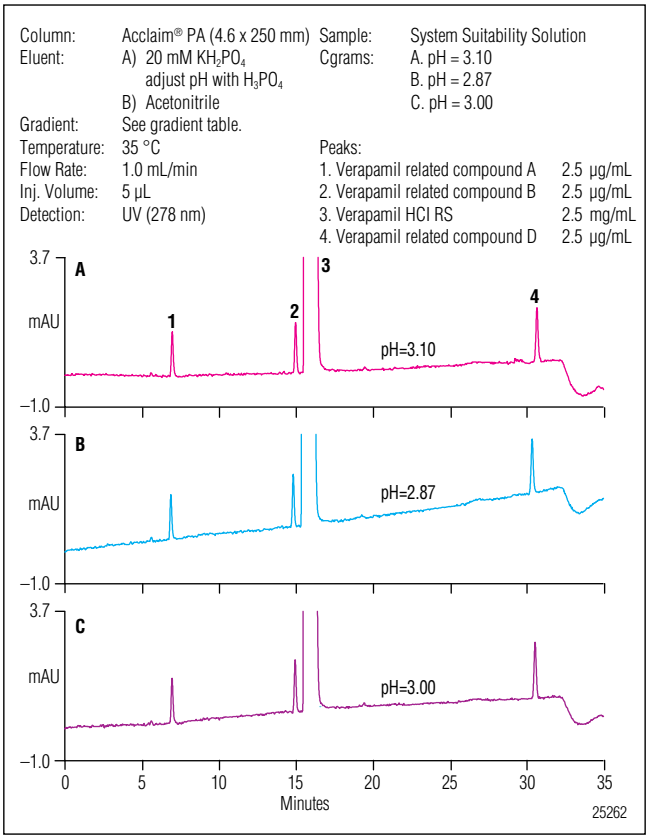

Figure 5. Analysis of the test solution with different pH mobile phases. Peaks: 1) verapamil-related compound A (2.5 µg/mL); 2) verapamil-related compound B (2.5 µg/mL), 3) verapamil HCl RS (2.5 mg/mL); 4) verapamil-related compound D (2.5 µg/mL).

REFERENCES

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

Determination of Glucosamine in Dietary Supplements Using HPAE-PAD

INTRODUCTION

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

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

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

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

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

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

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

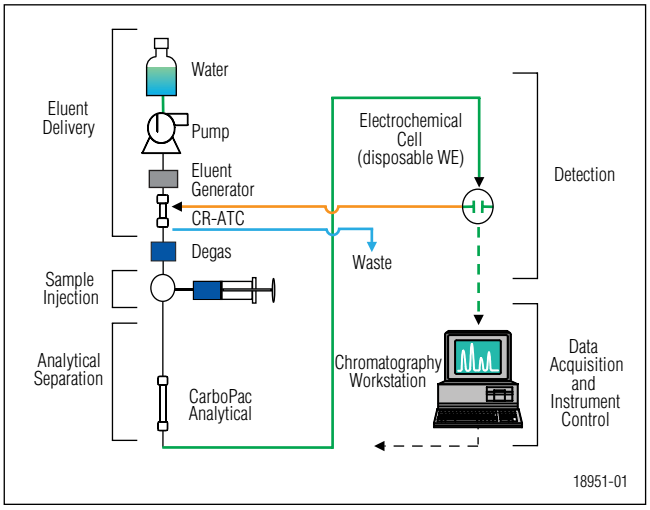

Figure 1. HPAE-PAD system for glucosamine determinations.

EQUIPMENT

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

DP Dual Gradient or SP Single Gradient Pump, with the EG/DP/SP Vacuum Degas Conversion Kit (P/N 063353) and GM-4 Gradient Mixer (P/N 049135)

Eluent Generator with EGC II KOH eluent generator cartridge (EluGen® II Hydroxide; P/N 058900) and Continuously Regenerated Anion Trap Column (CR-ATC; P/N 060477)

DC Detector/Chromatography module equipped with single or dual temperature zones, injection valve(s) and 10 µL injection loop, ED Electrochemical Detector (P/N 061718), ED cell and spacer block (P/N 061756) with combination pH/Ag/AgCl Reference Electrode (P/N 061879) and Carbohydrate Disposable Au Working Electrodes (P/N 060139, package of 6; 060216, package of 24)

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

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

Chromeleon® Chromatography Management Software

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

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

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

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

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

REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher

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

Sucrose (Thermo Fisher Scientific; P/N S5500)

Glucose (Sigma-Aldrich; P/N G5250)

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

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

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

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

Mannitol (Sigma-Aldrich; P/N M9546)

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

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

SAMPLES

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

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

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

**One can contains 237 mL of liquid

CONDITIONS

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

Carbohydrate 4-Potential Waveform for the ED

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

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

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

Instrument Operational Considerations

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

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

PREPARATION OF REAGENTS AND STANDARDS

Eluents

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

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

Stock Standards

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

SAMPLE PREPARATION

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

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

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

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

Separation

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

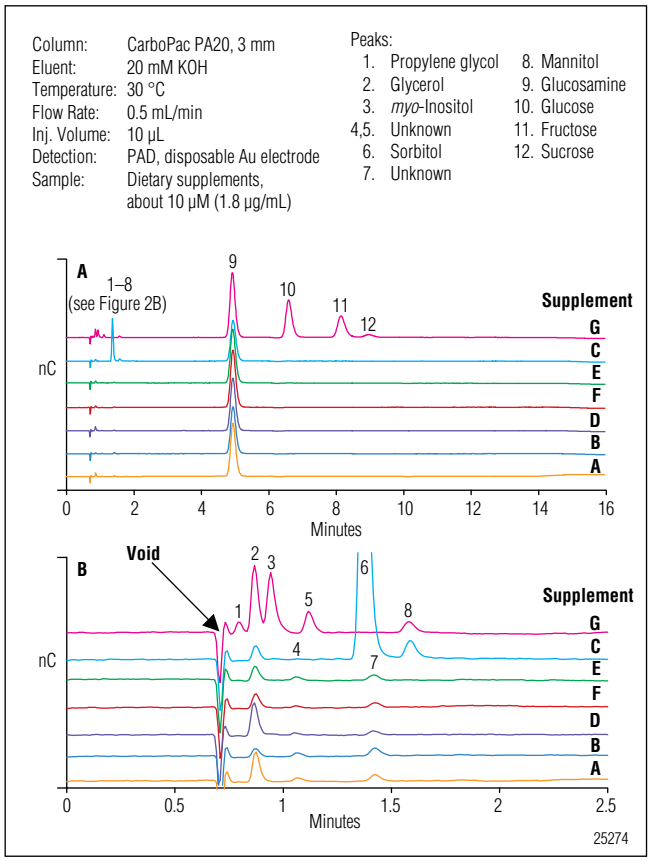

Figure 2. HPAE-PAD analysis of GlcN-containing dietary supplements. Seven dietary supplement samples diluted to approximately 10 µM (1.8 µg/mL) GlcN, 10-µL injection. A) Full chromatogram. B) Expanded early RT region of the chromatogram.

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

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

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

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

.

Detection

Linearity

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

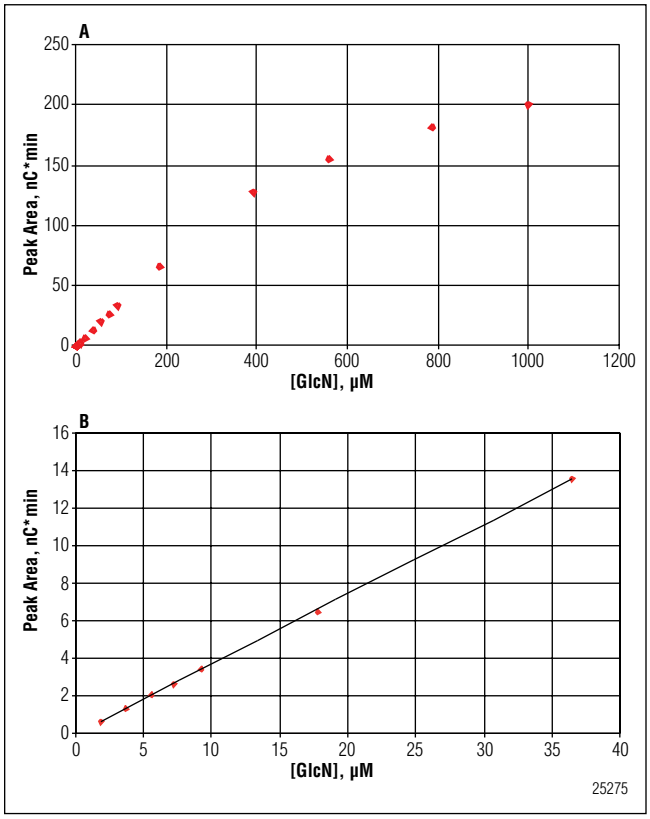

Figure 3. The relationship of peak area (mean) to glucosamine concentration injected for estimation of linear range (n = 8). A) Wide range curve. B) Narrower range used for GlcN quantification.

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

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

Precision

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

Retention Time

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

Peak Area

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

Accuracy

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

Table 3. Determination of Glucosamine in Dietary Supplement Samples

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

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

Table 4. Determination of Other Substances Detected in Dietary Supplements

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

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

Application

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

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

CONCLUSION

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

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SUPPLIERS

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

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

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

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

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

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

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

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

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

INTRODUCTION

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

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

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

EQUIPMENT

UltiMate® 3000 HPLC system:

HPG 3400A pump with SRD 3400 Solvent Rack with degasser

TCC-3000 thermostatted column compartment

WPS-3000TSL autosampler

VWD-3400 UV-Vis detector

Chromeleon® 6.80 SP2b Chromatography Workstation

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

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

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

REAGENTS AND STANDARDS

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

Acetonitrile (CH_3CN), HPLC grade, Fisher

Allantoin, purum, ≥ 98%, Fluka

Urea, analytical grade, SCRC, China

n-Heptane, analytical grade, SCRC, China

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

Ammonium acetate, analytical grade, SCRC, China

Diatomaceous earth, Hyflo Super Cel®, Sigma-Aldrich

CHROMATOGRAPHIC CONDITIONS

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

PREPARATION OF STANDARDS

Stock Standard Solutions

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

Mixed Working Standard Solutions

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

Table 1. Concentration of the Mixed Working Standard Solutions

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

SAMPLE PREPARATION

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

RESULTS AND DISCUSSION

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

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

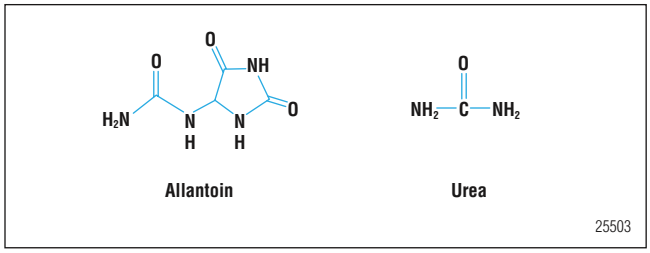

Figure 1. Structures of allantoin and urea.

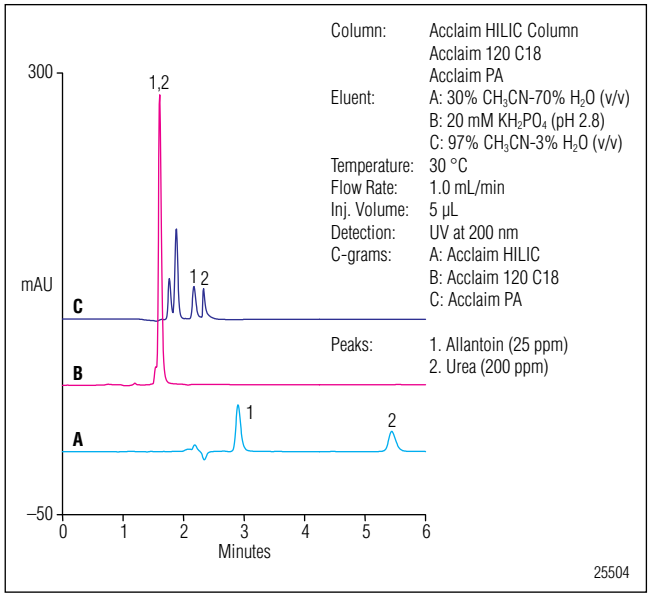

Figure 2. Chromatograms of allantoin and urea on A) Acclaim Mixed-Mode HILIC, B) 120 C18, and C) PA columns.

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

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

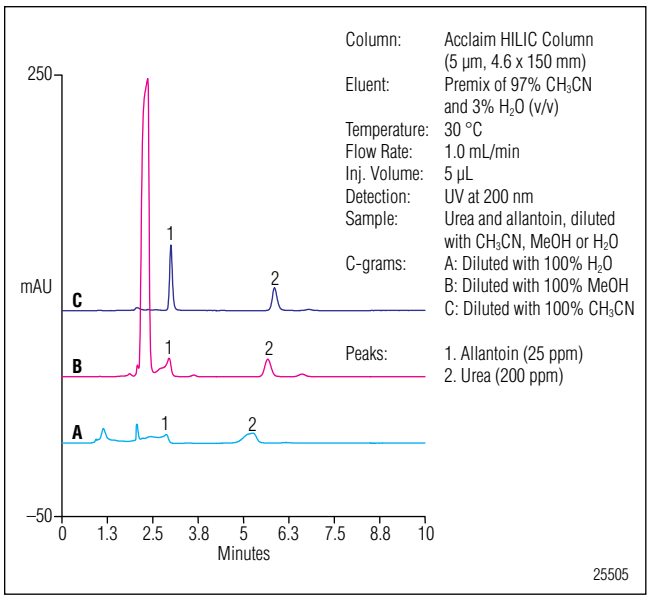

Figure 3. Chromatograms of allantoin and urea diluted with A) water, B) methanol, and C) acetonitrile.

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

Influence of Mobile Phase Buffer on HILIC Chromatography

Phosphate buffers are not recommended due to precipitation in the highly organic mobile phases commonly used in HILIC. The buffers usually used with HILIC columns are ammonium formate, ammonium acetate, formic acid, and phosphoric acid. We evaluated the influences of the pH value and concentration of an acetic acid-ammonium acetate buffer on HILIC chromatography. The pH value of the acetic acid-ammonium acetate buffer was adjusted by changing the proportion of acetic acid and ammonium acetate. As shown in Figure 6, although the retention time of allantoin

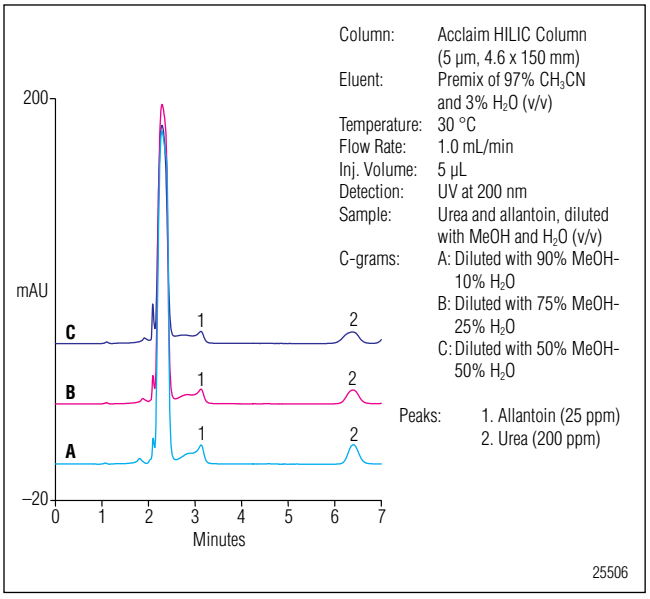

Figure 4. Chromatograms of allantoin and urea diluted with methanol/water with differing proportions.

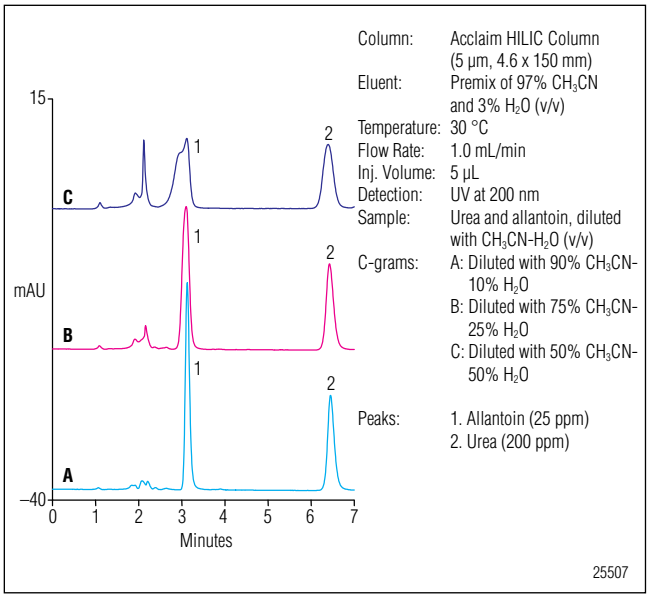

Figure 5. Chromatograms of allantoin and urea diluted with acetonitrile/water with differening proportions.

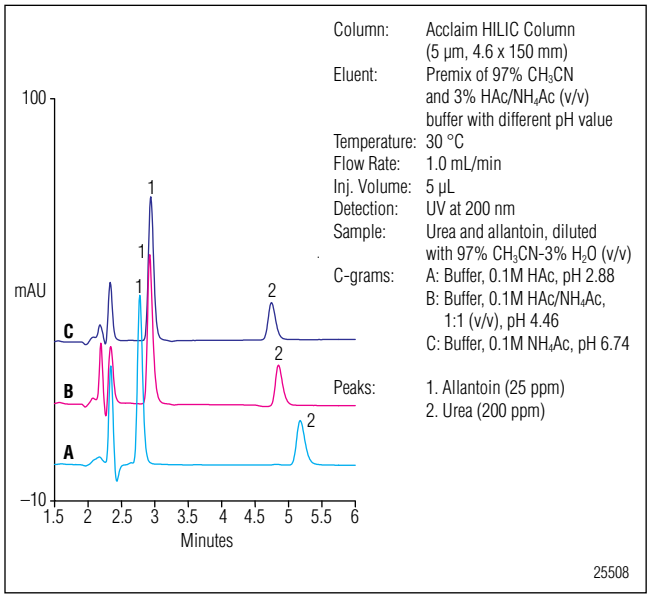

Figure 6. Chromatograms of allantoin and urea when the buffers in mobile phase are at different pH values.

increases and that of urea decreases with increasing buffer pH, their peak shapes are maintained. Figure 7 shows chromatograms of allantoin and urea when the ammonium acetate concentration in the mobile phase changes from 100 to 0 mM (100% H_2O). Peak shapes are also maintained with changes in the buffer concentration, even in the absence of buffer. However, for some ionic compounds, e.g., benzoate, a buffer in the mobile phase is needed for good peak shape and retention (Figure 8).

Reproducibility, Linearity and Detection Limits

Prior to sample analysis, we estimated the reproducibility by making seven replicate injections of a mixed standard solution with concentrations of 25 mg/L for allantoin and 200 mg/L for urea. The RSDs for retention time were both 0.000, and the RSDs for peak area were 0.178 for allantoin and 0.379 for urea.

Calibration linearity for allantoin and urea was investigated by making replicate injections of a mixed standard prepared at six different concentrations. The external standard method was used to calculate the calibration curve and to quantify these compounds in samples. Table 2 shows the data from the calibration as calculated in Chromeleon. The single-sided Student's test method was used for estimating method detection limits (MDL). These data are also reported in Table 2.

Table 2. Calibration Data as Reported by Chromeleon and MDLs for the Two Analytes

Analyte	Equation	r	RSD	MDL (mg/L)
Allantoin	A = 0.1362 c + 0.0163	0.9998	1.6408	0.66
Urea	A = 0.0111 c + 0.0039	0.9990	2.9740	11.6

Note: The single-sided Student's test method (at the 99% confidence limit) was used for determining MDL, where the standard deviation (SD) of the peak area of seven injections is multiplied by 3.14 to yield the MDL.

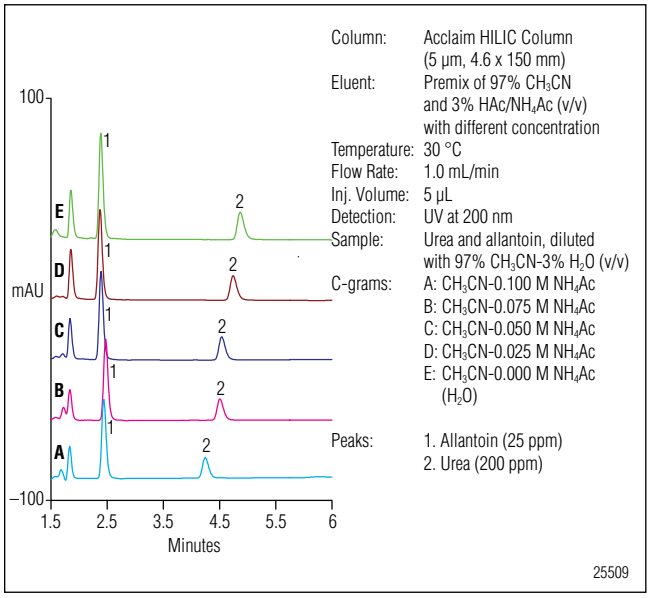

Figure 7. Chromatograms of allantoin and urea diluted when changing buffer (NH_4Ac) concentration in mobile phase

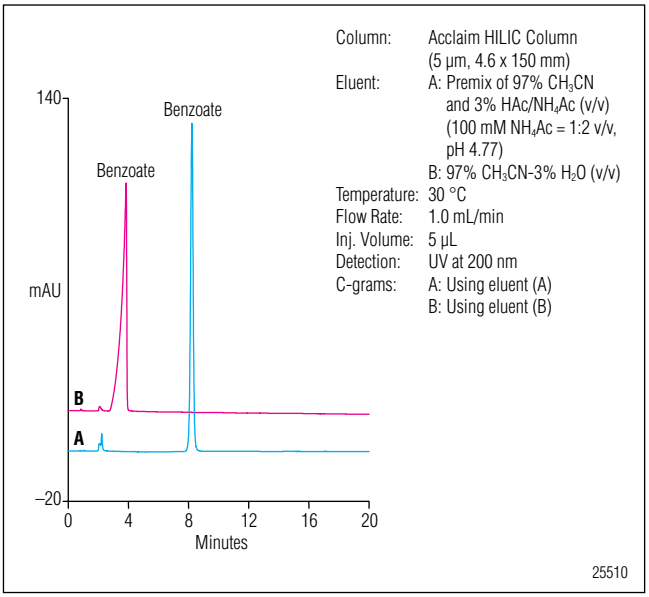

Figure 8. Chromatograms of benzoate on the Acclaim Mixed-Mode HILIC column using different mobile phases.

Sample Analysis

Allantoin and urea are added to cosmetic products for skin protection and regeneration, especially for the treatment of dry skin. We analyzed two different cream samples. Figure 9 shows chromatograms of a blank, sample 2, and the same sample spiked with standards. The amounts of allantoin and urea in each sample and the spike recovery from sample 2 are summarized in Table 3. Urea was found in both of the samples, and allantoin was found in sample 2.

CONCLUSION

For HILIC, sharp symmetric peaks for polar compounds are obtained using 90% acetonitrile, 10% water as the sample diluent. Our recommended buffer for HILIC mobile phases is ammonium acetate. For the determination of urea and allantoin, the pH value of the buffer does not have a significant influence on peak shape. However, keeping a certain buffer concentration in the mobile phase can yield better peak shape for some ionic polar compounds (e.g., for benzoate). Our experiments demonstrate that the Acclaim Mixed-Mode HILIC column is suitable for separation of highly polar compounds such as allantoin and urea and their determination in cosmetic products.

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2. Mant, C.T., Litowski, J.R., Hodges, R.S. *J. Chromatogr. A* **1998,** *816,* 65.
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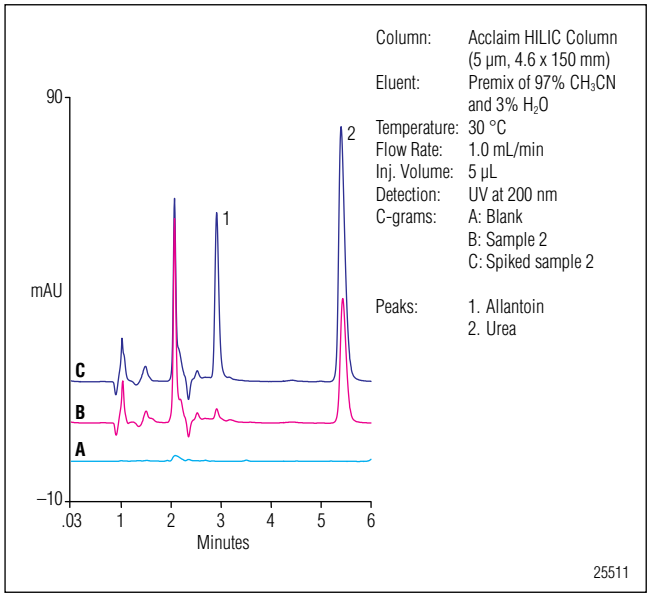

Figure 9. Overlay of chromatograms of the blank, sample 2 and spiked sample 2.

Table 3. Analysis Results for Cosmetic Products

Analyte	Sample 1	Sample 2			
	Detected (g/Kg)	Detected (g/Kg)	Added (g/Kg)	Found (g/Kg)	Recovery (g/Kg)
Allantoin	ND	0.37	5.00	4.92	98.4
Urea	53.5	76.6	90.0	87.6	97.3

Note: 1. One sample and spiked sample were prepared, respectively, and 3 injections were made for each.
2. Detected = Measured Value of sample x Diluted fold
3. Found = Measured value of spiked sample — Measure value of sample
4. ND = "not detected"

Analysis of Sumatriptan in Cerebro-spinal Fluid Using Capillary LC/MS/MS

INTRODUCTION

Sumatriptan helps to relieve headache pain and associated symptoms of migraine (nausea, vomiting, sensitivity to light and sound). It also helps to constrict dilated blood vessels that may contribute to development of migraines. Most analysis techniques for the separation, detection and quantification of sumatriptan are based on conventional high-performance liquid-chromatography techniques. Here the gain in sensitivity is demonstrated by using capillary LC/MS/MS.

RESULTS AND DISCUSSION

The sample preparation steps for sumatriptan in cerebro spinal fluid (CSF) prior to LC/MS analysis is straight forward and demonstrated in the Figure 1. The UltiMate®/FAMOS™ Capillary LC System was equipped with a 300 µm I.D. x 15 cm column packed with C18, 3 µm stationary phase at flow rate of 5 µL/min. Mobile phase A: 0.1% aqueous formic acid, B: acetonitrile. Gradient: 5 to 60% B in 8 min. MS/MS analysis was conducted with a triple quadrupole MS equipped with an electrospray interface.

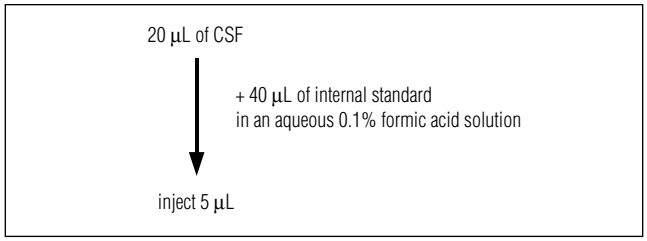

Figure 1. Schematics of sample handling prior to capillary LC/MS/MS.

Figure 2 shows multiple reaction monitoring (MRM) chromatograms of sumatriptan analysis by means of Capillary and conventional HPLC. The upper trace corresponds to a chromatogram taken when 1.25 pg of sumatriptan was injected on column and the lower trace shows the limits of detection achieved with conventional HPLC, which is approximately equal to 300 pg. The overall gain in sensitivity is approximately a 700 fold. For spiked CSF samples, the peak area ratios of analyte to internal standard were linear over the calibration range 0.5–15 ng/µL. A typical calibration curve is shown in the Figure 3. The limit of quantitation was found to be equal to 300 pg/µL.

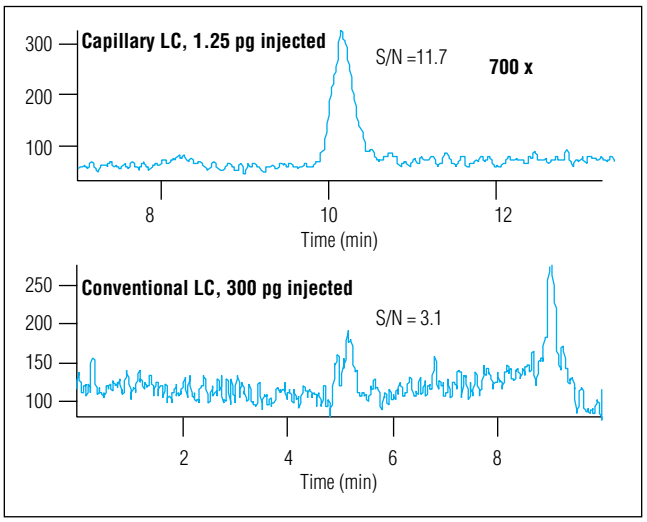

Figure 2. Capillary (top) and conventional (bottom) LC/MRM chromatograms of sumatriptan. Gradient conditions are given in the text.

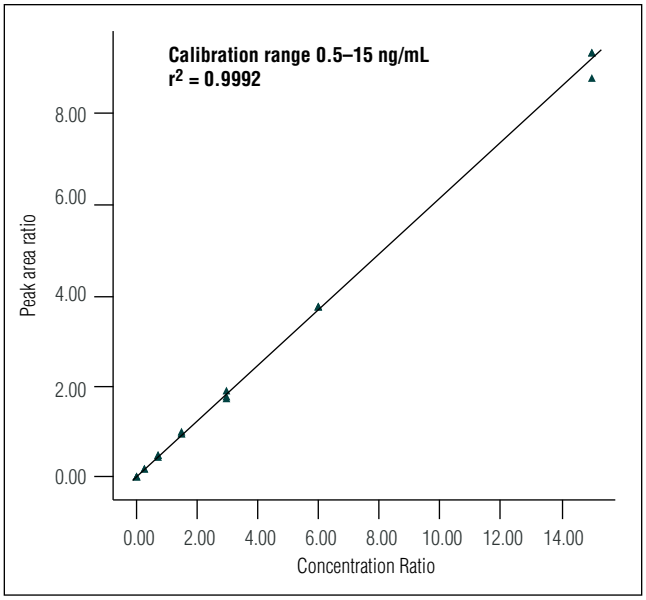

Figure 3. Calibration curve for the analysis of sumatriptan in CSF.[1]

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1. Courtesy Prof. G. Moneti, CIMS, University of Florence, Italy

Budesonide and Related Substances on Acclaim 300 C18

INTRODUCTION

Budesonide is a highly effective steroidal antihistamine decongestant, and is available as a mixture of two epimers. The safety of budenoside-based pharmaceuticals depends on careful monitoring of impurities associated with the epimers. The related substances are decomposition products and synthetic byproducts. The Acclaim 300 C18 column is a direct replacement for the column in the referenced method.

METHOD

In this application, 6 µL of a degraded Budesonide sample (500 µg/mL) is injected onto a 3 µm Acclaim 300 C18 column with the dimensions of 4.6 × 150 mm. The isocratic separation of the budesonide epimers from the decomposition products and synthetic byproducts is accomplished using a mobile phase of 66% aqueous and 34% organic. The aqueous portion is 0.1% phosphoric acid and the organic portion is a mixture of acetonitrile: ethanol (15:1). The total run time is roughly 16 min. Detection is accomplished using UV absorbance at 240 nm.

RESULTS

As shown in Figure 1, the method provides baseline resolution of the two major components as well as the 8 impurities. This separation is clearly suitable for assaying Budesonide.

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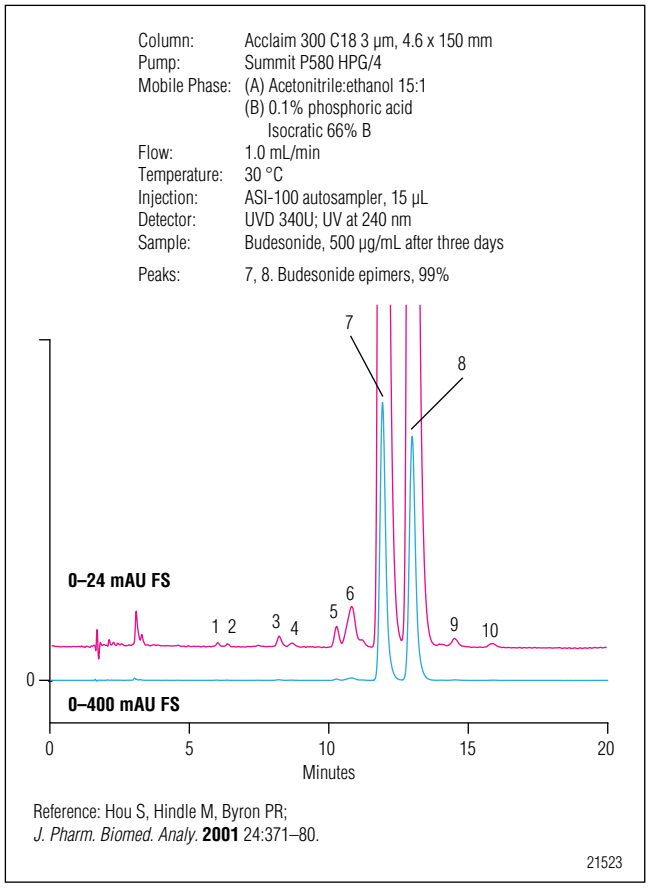


Figure 1. Budesonide and related substances.

Glucocorticosteroids in Serum on Acclaim 120 C18

INTRODUCTION

Accurate analysis of glucocorti-costeroids, both natural and synthetic, is necessary for monitoring the health of patients with a number of medical conditions. The referenced method is widely used in clinical laboratories. The Acclaim 120 C18 column is a direct substitute for the referenced column.

SAMPLE PREPARATION

The sample is prepared by extracting bovine serum (alkalized with sodium hydroxide) with ethyl acetate and injecting 60 µL.

METHOD

A 5 µm Acclaim 120 C18 column with the dimensions of 4.6 × 250 mm is used to separate 6 glucocorticosteroids from the internal standard, Fludrodcortisone. The separation is performed under isocratic conditions using methanol:THF:water (3:25:72) at a flow rate of 1.0 mL/min.The total run time for the analysis is under 25 min. Detection is accomplished using UV absorbance at 240 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of the 6 glucocorticosteroids with a wide window for the internal standard. This separation is clearly suitable for quantitatively assaying a number of glucocorticosteroids.

REFERENCE

1. McWhinney B C, Ward G, Hickman P E; *Clin. Chem,* **1996,** *42:* 979-81.

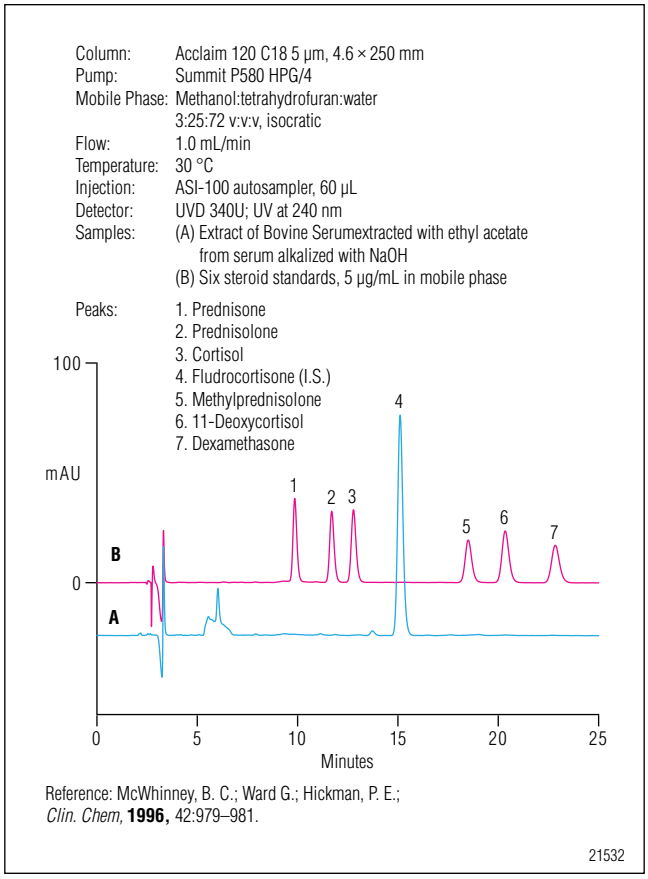


Figure 1. Glucocorticosteroids in serum on Acclaim 120 C18.

Propafenone and Related Substances Using Acclaim PA

INTRODUCTION

Propafenone is a sodium channel blocker used to treat irregular heart rhythm. This analysis of the formulated drug resolves the known impurities and degradation products.

SAMPLE PREPARATION

In this application brief, sample preparation is accomplished by initially dissolving the sample in methanol and then further diluting 1 mL of the sample in 40% mobile phase A and 60% mobile phase B.

METHOD

20 µL of sample is injected onto a 5 µm Acclaim PA column with the dimensions of 4.6 × 150 mm. A gradient solvent system at a flow rate of 1.0 mL/min is used to separate the early eluting propafenone from 5 related substances in 30 min. Mobile phase A consists of 10 mM ammonium acetate at pH 2.4. Mobile phase B is methanol. The gradient starts out with a 5 min ramp from 20% to 50% B followed with a 5 min ramp to 60% B. Mobile phase B is increased to 70% at 15 min and 95% at 30 min before returning to 20% for column re-equilibration. Detection is accomplished using UV absorbance at 249 nm.

RESULTS

As shown in Figure 1, the method provides more than adequate resolution of propafenone and the 5 related substances. This separation is clearly suitable for assaying propafenone.

ACKNOWLEDGEMENTS

Data courtesy of V. Bhate of Analytical Solutions

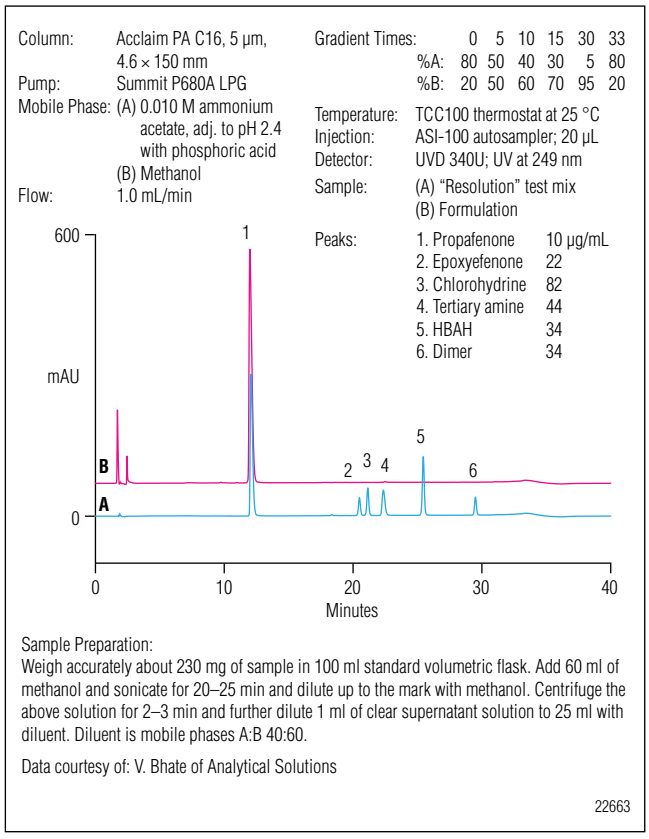


Figure 1. Propafenone and related substances using Acclaim PA.

Loperamide in Anti-diarrhea Tablets on Acclaim 120 C8

INTRODUCTION

Loperamide is the active ingredient in many formulations used to treat diarrhea. The U.S. Pharmacopeia assay specifies an L7 column be used for this analysis. Here the Acclaim 120 C8 is used for determination of this compound.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving ground up time-release tablets in a solution of 5% H_3PO_4, methanol and water. After sonication, followed by filtration, 20 µL of the sample is injected.

METHOD

In this application, a 3 µm Acclaim 120 C8 column with the dimensions of 4.6 × 150 mm is used to separate loperamide hydrochloride. The column was maintained at 30 °C. The separation is performed under isocratic conditions at a flow rate of 1.2 mL/min. The mobile phase consisted of 45% acetonitrile and 55% buffer. The buffer was composed of 3.0 g of Et_3N HCl plus 1.0 mL of H_3PO_4 in 550 mL water. The method yielded a total run time of just over 5 min with the detection of the compound using UV absorbance at 214 nm.

RESULTS

As shown in Figure 1, this method provides excellent retention of the loperamide peak. Clearly this separation is suitable for the quantitative assaying of loperamide in anti-diarrheal tablets.

REFERENCE

1 USP-31 NF-26, page 2545 (2008).

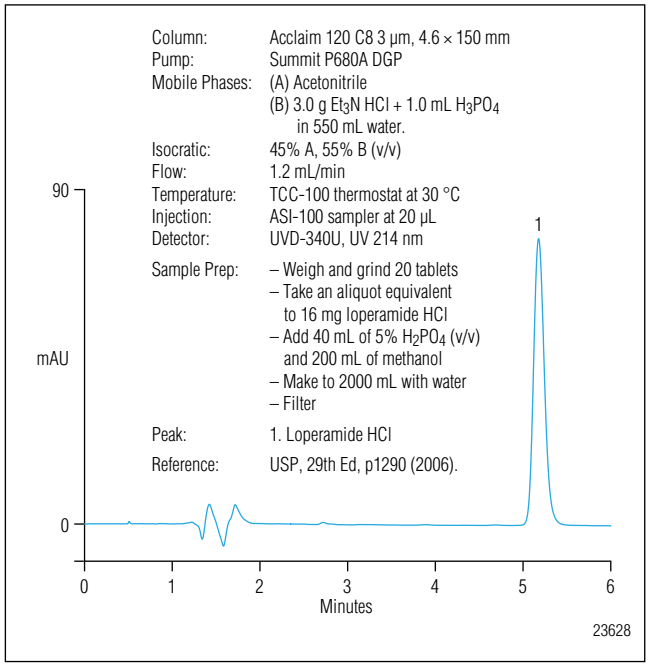


Figure 1. Loperamide in anti-diarrhea tablets on Acclaim 120 C8.

Simultaneous Analysis of a Basic Drug and Its Counterion (Trimipramine)

INTRODUCTION

Retaining a hydrophobic base and its hydrophilic counterion can prove very difficult using conventional reversed-phase columns. The Acclaim Mixed-Mode WAX-1 retains the hydrophobic base by reversed-phase interaction, and the hydrophilic counterion by anion-exchange interaction. In this way, it can eliminate a separate test for the two components.

SAMPLE PREPARATION

In this application, the sample is prepared by dissolving trimipramine maleate in mobile phase at a concentration of approximately 0.5 mg/mL. 2.5 µL of sample is injected on column.

METHOD

For this analysis, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate trimipramine from its counterion, maleate. The separation is performed under isocratic conditions using an acetonitrile/buffer (30:70) mobile phase at a flow rate of 1.0 mL/min.The buffer was 50 mM phosphate at pH 6.0. The total run time for the analysis is under 7 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown Figure 1, this HPLC method produces a chromatogram with a the trimipramine peak well-resolved from its maleate counterion peak. This separation is clearly suitable for quantitatively assaying both trimipramine and its counterion in a single analysis.

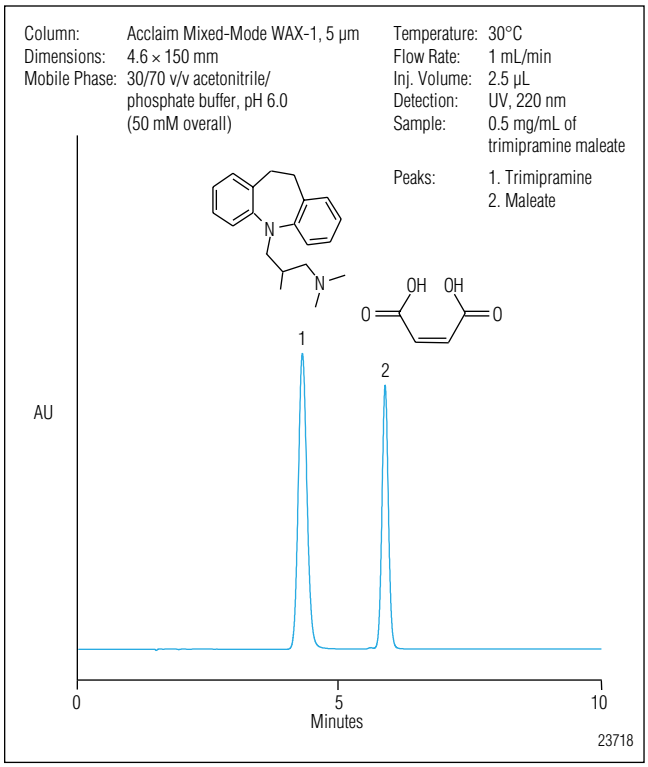
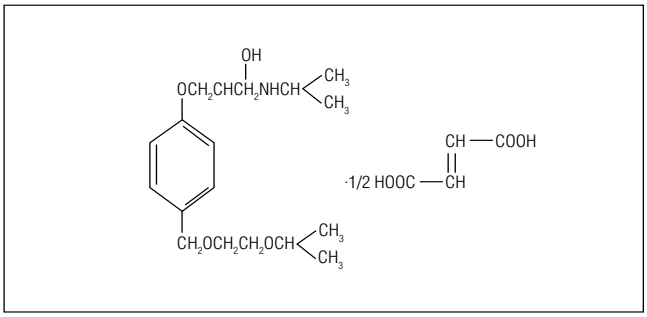


Figure 1. Simultaneous analysis of a basic drug and its counterion (trimipramine).

Simultaneous Separation of Bisoprolol and Its Counterion Fumarate

Bisoprolol fumarate is a synthetic ß1-selective (cardioselective) adrenoceptor-blocking agent. Many drug substances are prepared as salts to promote solubility, improve stability, and/or improve bioavailability. For bisoprolol fumarate, bisoprolol is the active ingredient and fumarate is the counterion. Chemically, it is a 1-[4-[[2-(1-Methylethoxy) ethoxy] methyl] phenoxy]-3-[(1-methylethyl) amino]-2-propanol hemifumarate salt (structure shown below).



HPLC is usually only used to determine the active ingredient, bisoprolol, of bisoprolol fumarate.[1, 2] The simultaneous determination of its counterion, fumarate, has not been reported due to its weak retention on a conventional reversed-phase (RP) C18 column. The Acclaim® Mixed-Mode WAX-1 column, which combines anion-exchange and RP properties, has been used for determination of iodide in drinking water,[3] corrosion inhibitors in engine coolants,[4] additives in carbonated beverages,[5] and simultaneous determination of a peptide and its acetate counterion.[6] This column is a good choice for the simultaneous separation of bisoprolol and fumarate.

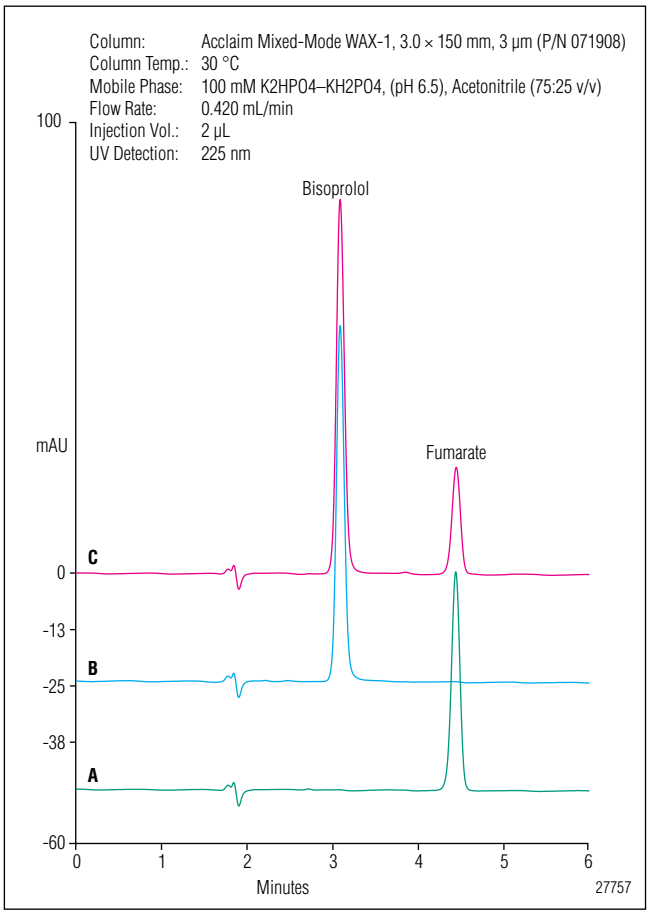


Figure 1. Overlay of chromatograms of A) fumarate standard (50 ppm), B) bisoprolol standard (100 ppm), and C) bisoprolol fumarate tablet.

The work shown here describes an efficient method for simultaneously separating bisoprolol and fumarate in a tablet sample. The method uses an Acclaim Mixed-Mode WAX-1 column (3.0 × 150 mm, 3 µm) with a phosphate buffer (100 mM of K_2HPO_4–KH_2PO_4, pH 6.5) and acetonitrile (75:25, v/v) mobile phase at 0.42 mL/min flow rate, and a detection wavelength of 225 nm. Figure 1 shows the overlay of chromatograms of a bisoprolol fumarate tablet sample and the standards. In a single injection, the analyst can determine both the active pharmaceutical ingredient and its counterion.

EQUIPMENT

Dionex UltiMate® 3000 Rapid Separation Liquid Chromatography (RSLC) system including:

HPG-3400RS Pump

WPS-3000RS Autosampler

TCC-3000RS Thermostatted Column Compartment

DAD-3000RS UV-vis Diode Array Detector

Chromeleon® Chromatography Data System (CDS) software Version 6.80 SR9

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2. Patel. L.J.; Suhagia, B.N.; Shah, P.B.; Shah, R.R. Simultaneous Estimation of Bisoprolol Fumarate and Hydrochlorothiazide in Tablet Dosage Form by RP-HPLC Method. *India J. Pharm. Sci.* **2006**, *68*, 635.
3. Dionex Corporation, *Determination of Iodide in a Nutritional Supplement Using an Acclaim Mixed-Mode WAX-1 Column.* Application Brief 111, LPN 2538, 2010, Sunnyvale, CA.
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5. Dionex Corporation, *Determination of Additives in Carbonated Beverages*. Application Note 193, LPN 1961, 2007, Sunnyvale, CA.
6. Dionex Corporation, *Simultaneous Determination of Pharmaceutical Peptides and Acetate by HPLC with UV Detection Using the Acclaim Mixed-Mode WAX-1 Column.* Application Note 234, LPN 2291, 2009, Sunnyvale, CA.

Rapid Separation of Paclitaxel and Related Compounds in Paclitaxel Injection

INTRODUCTION

Paclitaxel was approved as Taxol® by the National Cancer Institute (NCI) in 1991 for the treatment of ovarian cancer. An analysis of paclitaxel and related compounds—cephalomannine (related compound A), 10-deacetyl-7-epipaclitaxel (related compound B), and 7-epipaclitaxel (related compound C) (structures shown in Figure 1)—by reversed-phase (RP) HPLC was published by both the United States Pharmacopeia (USP) and the Chinese Pharmacopoeia (CP). These methods each required over 70 min.[1,2]

There are several RP-HPLC assays for paclitaxel in the literature;[3-6] however, all of them are also relatively long (15 to 35 min). Therefore, researchers interested in the rapid separation of paclitaxel and related compounds developed a UHPLC method,[7] but the separation did not include paclitaxel-related compound B, which is required by the USP. Use of an UltiMate® 3000 RSLC system and an Acclaim® RSLC C18 column packed with smaller particles is an easy way to increase speed and peak capacity. Therefore, the authors used this system to create an UHPLC method for the analysis of paclitaxel and related compounds including related compound B.

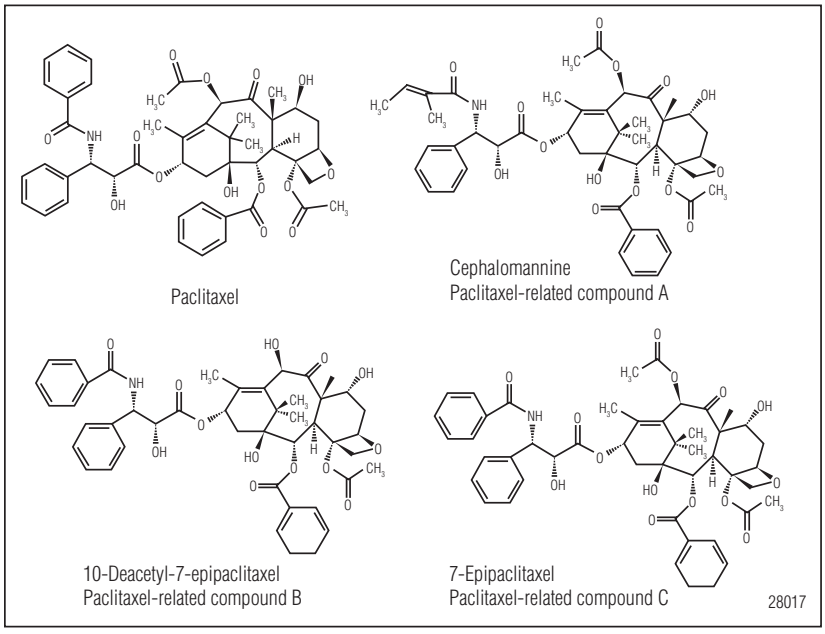

Figure 1. Structures of paclitaxel and related compounds.

The work shown here describes a rapid and efficient UHPLC method to separate paclitaxel and related compounds in a Paclitaxel Injection sample. The method uses an Acclaim RSLC 120 C18 column (2.1 × 100 mm, 2.2 µm) with a water/acetonitrile/methanol gradient mobile phase (Table 1) at a flow rate of 0.42 mL/min, and a detection wavelength of 227 nm. Figure 2 shows an overlay of a chromatogram of a Paclitaxel Injection sample on that of a mixture of standards.

This improved UHPLC method achieves a baseline separation within 6 min. The resolution values (R_s>2.2) were greater than those required in the USP and CP methods, (not less than [NLT] 1.2 for paclitaxel and paclitaxel-related compound B, and NLT 1.0 for paclitaxel-related compound A and paclitaxel-related compound B).

One difference worth noting is the order of elution for paclitaxel and related compound B. Initial experiments using a larger Acclaim 120 C18 column (4.6 × 250 mm, 5 µm) with a water/acetonitrile binary gradient, as described in the USP and CP methods, showed related compound B eluting before paclitaxel. However, with the smaller Acclaim 120 C18 RSLC column (2.1 × 100 mm, 2.2 µm) and water/acetonitrile/methanol ternary gradient, related compound B elutes after paclitaxel.

The peak area relative standard deviation (RSD) for five replicate injections is ≤0.45%, demonstrating good performance for the rapid separation of paclitaxel and related compounds using an UltiMate 3000 RSLC system and an Acclaim C18 column packed with smaller particles.

With this improved UHPLC method, it was found that the methanol/acetonitrile/water ternary gradient dramatically reduced the analysis time by an order of magnitude while still meeting USP and CP requirements for peak resolution and peak area reproducibility.

Table 1. Gradient for the Separation of Paclitaxel and Related Compounds

Time (min)	Flow Rate (mL/min)	H_2O (%)	Acetonitrile/Methanol (40:60, v/v) (%)
-2.0	0.42	40	60
0.0		40	60
4.4		28	72
4.5		20	80
6.0		20	80

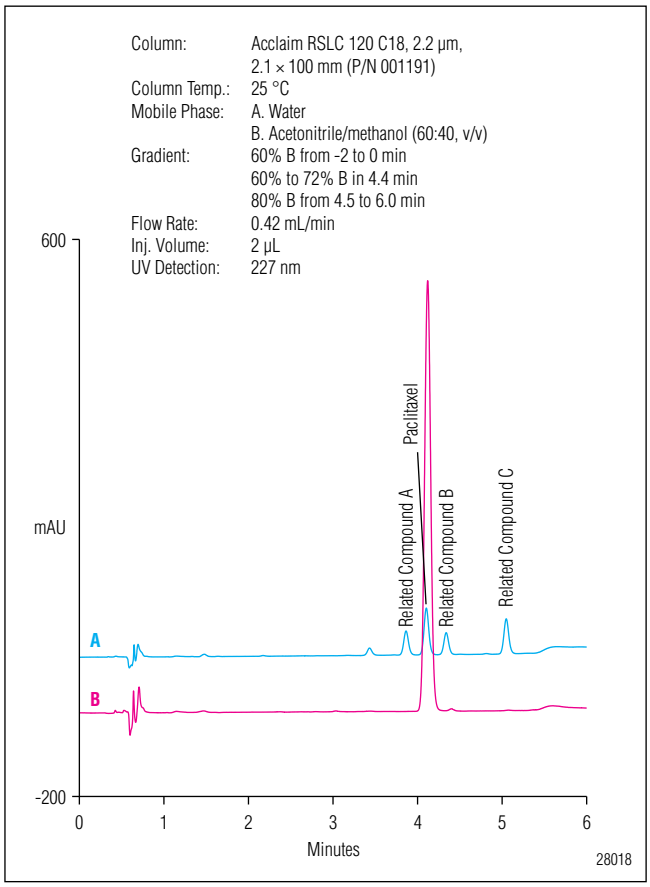

Figure 2. Overlay of chromatograms of (A) mixture of paclitaxel and related compounds standards (5 µg/mL for each) and (B) Paclitaxel Injection sample.

EQUIPMENT

Dionex UltiMate 3000 RSLC system including:

HPG-3400RS Pump

WPS-3000RS Autosampler

TCC-3000RS Thermostatted Column Compartment

DAD-3000RS UV-vis Detector

Chromeleon® Chromatography Data System (CDS) software version 6.80 SR9

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Separation of Etoposide Using an Acclaim Phenyl-1 Column

INTRODUCTION

Etoposide, a semisynthetic derivative of podophyllotoxin, is used in treating patients with a variety of malignant tumors.[1] Its analysis by reversed-phase high-performance liquid chromatography (HPLC) has been published by the United States Pharmacopeia (USP) and Chinese Pharmacopoeia (CP).[2,3]

The USP related compounds method uses a 50 min gradient on a column containing packing L11. The USP column packing L11 is defined as phenyl groups chemically bonded to porous silica particles 1.5 to 10 µm in diameter. The particle size requirement in the USP etoposide monograph for the related compounds method is less than 5 µm in diameter. The resolution (R_s) between etoposide and propylparaben required in the related compounds test should be not less than 1.1. The CP method is similar to the USP method but adds a requirement that the retention time (t_R) of etoposide be approximately 25 min.

The work shown here describes a separation of etoposide using an Acclaim® Phenyl-1 column. The Phenyl-1 column is based on covalent modification of high-purity, spherical, porous silica particles (3 µm), with a specially designed silane ligand-bearing proprietary alkyl aromatic functionality.[4]

Figure 1A shows chromatograms of etoposide and propylparaben following the USP method. The R_s between etoposide and propylparaben is 8.7, much better than required in the USP method; and the t_R of etoposide is close to 25 min, which meets the requirement in the CP method.

Figure 1B shows a faster and simpler method for the separation of etoposide and propylparaben using the Phenyl-1 column with isocratic elution. The separation of etoposide is completed within 7 min with excellent resolution (R_s = 5.2) between etoposide and propylparaben.

EQUIPMENT

Dionex UltiMate® 3000 RSLC system including:

- HPG 3400RS pump
- WPS 3000RS autosampler
- TCC-3000RS thermostatted column compartment
- DAD-3000RS UV-vis detector

Chromeleon® Chromatography Data System (CDS) software version 6.80 SR9

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Column: Acclaim Phenyl-1 (4.6 × 150 mm, 3 µm)
Column Temp.: 30 °C
Mobile Phase Panel A: Solution 1) CH_3CN/20 mM NaAc (pH 4.0), 20:80 (v/v)
Solution 2) CH_3CN/20 mM NaAc (pH 4.0), 40:60 (v/v)

Gradient Time	*Solution 1 (%)*	*Solution 2 (%)*
0	100	0
0-15	100	0
15-30	100→40	0→60
30-40	40	60
40-42	40→0	60→100
42-45	0	400
45-47	0→100	100→0
47-50	100	0

Mobile Phase Panel B: Solution 2) CH_3CN/20 mM NaAc (pH 4.0), 40:60 (v/v)
Flow Rate: 1.5 mL/min
Injection Volume: 10 µL
Detection: UV, 254 nm

Chromatograms: a) Blank (solution 1)
b) Etoposide (100 µg/mL)
c) Mix of etoposide (90 µg/mL) and propylparaben (8 µg/mL))

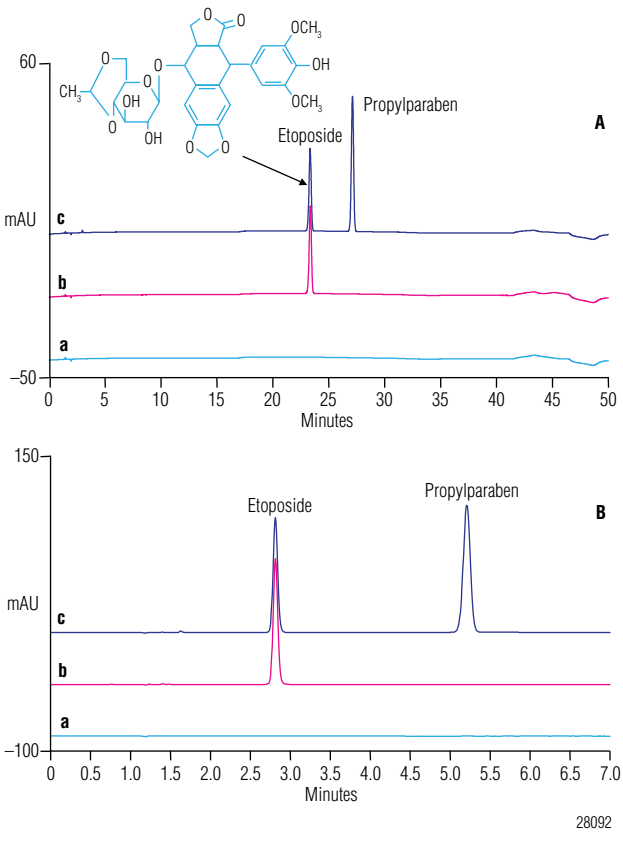

Figure 1. Chromatograms of etoposide following (A) the USP method and (B) isocratic method, using the Acclaim Phenyl-1 column.

UHPLC Separation of Nine Corticosteroids in Under Four Minutes

INTRODUCTION

Corticosteroids are a class of steroid hormones that are involved in the regulation of a variety of biological processes, and consequently are used to treat a wide range of maladies. For example, they are used to treat conditions caused by an overactive immune system (e.g., allergies), control inflammation, treat skin rashes, and reduce the growth of cancerous tumors. Corticosteroids are produced by the cortex of the adrenal gland, but there are synthetic compounds that act like a corticosteroid in the body, and are therefore also considered corticosteroids (e.g., dexamethasone).

This work shows separation of a set of nine corticosteroids: prednisone, prednisolone, cortisone, hydrocortisone, methylprednisone, betamethasone, dexamethasone, triamcinolone acetate, and cortisone acetate using an Acclaim® 120 C18 column. Good separation of these nine compounds is achieved using isocratic conditions with a water/THF/methanol gradient mobile phase. Different column formats can be used to execute this separation, depending on the analyst's needs for separation speed and mobile phase conservation.

This experiment first investigated a ternary gradient separation using the Acclaim 120 C18, 5 µm, 4.6 × 250 mm column with commonly used reversed-phase mobile phase components: water, methanol, and acetonitrile. Seven of the nine corticosteroids were separated, while cortisone and prednisolone coeluted (Figure 1).

Although these conditions may be acceptable if neither cortisone nor prednisolone are expected in the sample, an alternative method is needed to separate all nine compounds. To achieve separation of these steroids, acetonitrile was replaced by THF in the mobile phase.

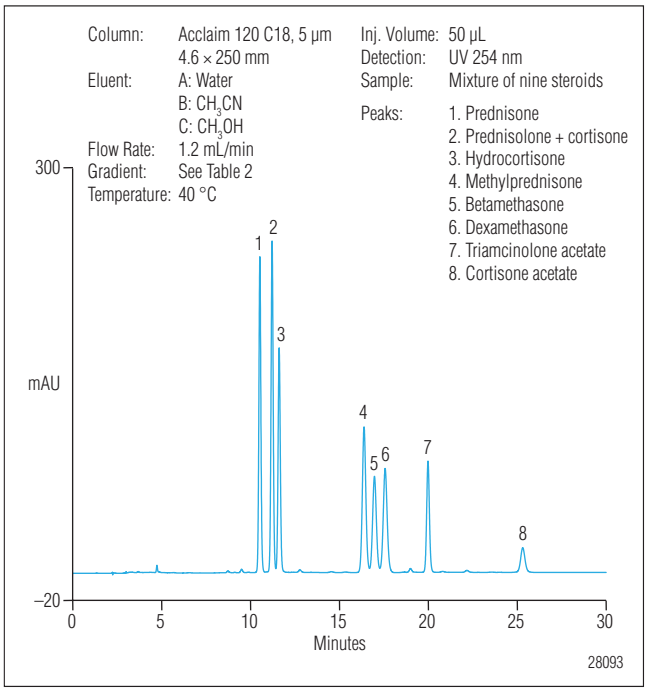

Figure 1. Separation of a mixture of nine corticosteroids using Condition 1.

Using isocratic conditions and increasing the column temperature to 50 °C, the nine steroids were well resolved (Figure 2). The same separation was achieved in less time and with reduced mobile phase consumption by switching to a 3 µm, 4.6 × 150 mm column (Figure 3). Figure 3 also shows that to achieve the separation, a 2 µL precolumn heater was necessary so that there was no thermal mismatch between the mobile phase and column.

To achieve a faster separation and further reduce mobile phase consumption, the separation was adapted to a 2.2 µm Acclaim Rapid Separation Liquid Chromatography (RSLC) 120 C18, 2.1 × 100 mm column (Figure 4). Because the heat exchange rate of the RSLC column is sufficiently fast due to the small internal diameter of 2.1 mm, a 2 µL precolumn heater was not needed. This separation required only four min and the column pressure was 8500 psi (586 bar). Chromatographic parameters from the four column formats are compared in Table 1 and show there is no sacrifice in performance in changing column format to reduce analysis time and mobile phase consumption.

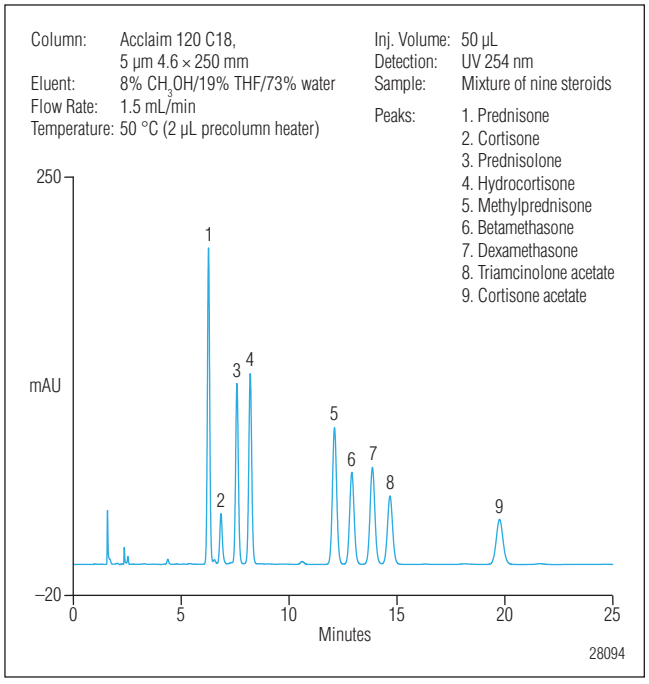

Figure 2. Separation of a mixture of nine corticosteroids using Condition 2.

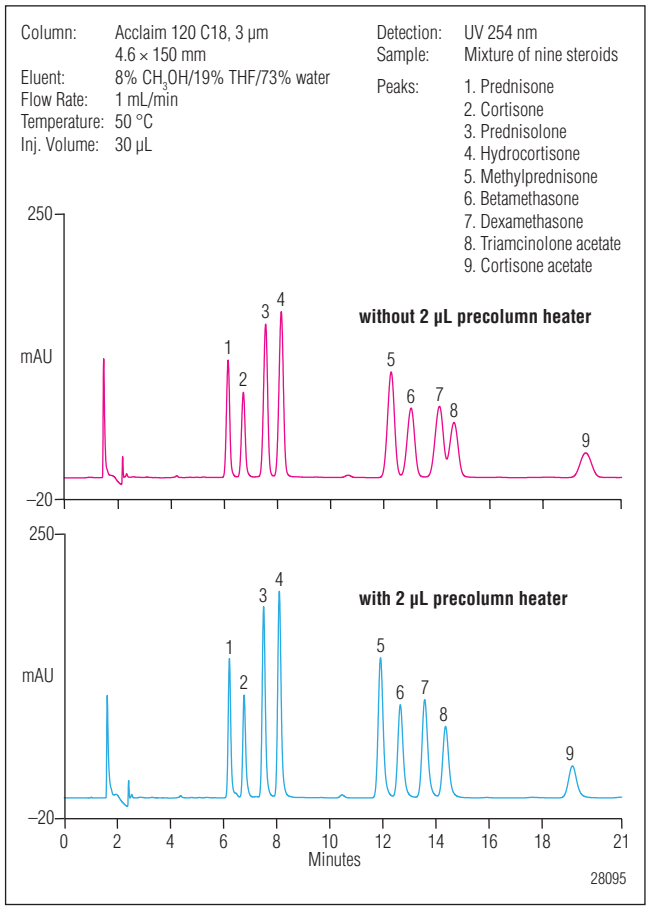

Figure 3. Separations of a mixture of nine corticosteroids using Condition 3 with and without a 2 µL precolumn heater.

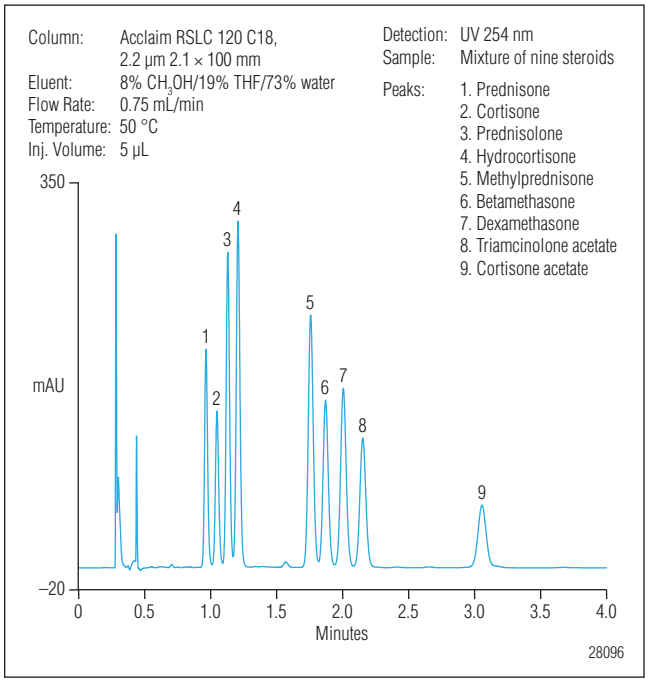

Figure 4. Separation of a mixture of nine corticosteroids using Condition 4.

Table 1. Peak Analysis Results of a Mixture of Nine Corticosteroids Obtained from Chromatography Conditions 1, 2, 3, and 4								
	Condition 1		Condition 2		Condition 3*		Condition 4	
Steroid	Resolution (USP)	Asymmetry (USP)	Resolution (USP)	Asymmetry (USP)	Resolution (USP)	Asymmetry (USP)	Resolution (USP)	Asymmetry (USP)
Prednisone	3.20	1.06	2.81	1.03	2.96	1.23	1.93	1.09
Cortisone	1.77	1.03	3.29	1.16	3.59	1.31	1.53	1.05
Prednisolone	1.77**	1.03**	2.54	1.05	2.64	1.22	1.57	1.08
Hydrocortisone	16.17	1.09	8.46	1.03	8.94	1.23	8.98	1.12
Methylprednisone	1.57	1.02	2.14	1.04	2.12	1.21	1.76	1.06
Betamethasone	1.55	1.05	2.38	1.05	2.45	1.20	1.71	1.05
Dexamethasone	7.02	1.09	1.92	1.05	1.96	1.18	2.05	1.06
Triamcinolone acetate	14.53	1.06	9.98	1.05	9.73	1.19	9.47	1.06
Cortisone acetate	n.a.	1.06	n.a.	1.05	n.a.	1.18	n.a.	1.08

*Condition 3 data relates to the separation with the 2 µL precolumn heater. **With Condition 1, cortisone and prednisolone coelute.

EQUIPMENT

Dionex UltiMate® 3000 RSLC system including:

- SRD-3600 Integrated Vacuum Degasser
- DGP-3600RS Dual-Gradient Pump with 400 µL static mixer
- WPS-3000RS Split-Loop Sampler with 100 µL sample loop
- TCC-3000RS Thermostatted Column Compartment
- DAD-3000RS Diode Array Detector
- Semi-Analytical flow cell (5 µL, 7 mm) (P/N 6082.0200)
- Semi-Micro flow cell (2.5 µL, 7 mm) (P/N 6082.0300)

CHROMATOGRAPHIC CONDITIONS

Condition 1

Column: Acclaim 120 C18, 5 µm 4.6 × 250 mm

Mobile Phase: A; Water
B; CH_3CN
C; CH_3OH

Flow Rate: 1.2 mL/min

Gradient: See Table 2

Column Temp.: 40 °C

Inj. Volume: 50 µL

Detection: UV 254 nm
Semi-analytical SST flow cell (5 µL, 7 mm)
Data collection rate, 5 Hz
Response time, 0.5 s

Table 2. Gradient Table			
Time (min)	% A	% B	% C
–5	80	10	10
0	80	10	10
0.1	50	10	40
2	50	10	40
5	67	30	3
15	67	30	3
16	62	35	3
30	62	35	3

Condition 2

Column: Acclaim 120 C18, 5 µm 4.6 × 250 mm

Mobile Phase: 8% CH_3OH/19% THF/73% water

Flow Rate: 1.5 mL/min

Column Temp.: 50 °C (2 µL precolumn heater is required)

Inj. Volume: 50 µL

Detection: UV 254 nm
Semi-analytical (5 µL, 7 mm) SST flow cell
Data collection rate, 5 Hz
Response time, 0.5 s

Condition 3

Column:	Acclaim 120 C18, 3 µm 4.6 × 150 mm
Mobile Phase:	8% CH_3OH/19% THF/73% water
Flow Rate:	1.0 mL/min
Column Temp.:	50 °C (2 µL precolumn heater is required)
Inj. Volume:	30 µL
Detection:	UV 254 nm Semi-analytical SST flow cell (5 µL, 7 mm) Data collection rate, 5 Hz Response time, 0.5 s

Condition 4

Column:	Acclaim RSLC 120 C18, 2.2 µm 2.1 × 100 mm
Mobile Phase:	8% CH_3OH/19% THF/ 73% water
Flow Rate:	0.75 mL/min
Column Temp.:	50 °C
Inj. Volume:	5 µL
Detection:	UV 254 nm, Semi-Micro SST flow cell (2.5 µL, 7 mm) Data collection rate, 25 Hz Response time, 0.2 s

Determination of Methacholine Chloride and Potential Impurities Using a Reagent-Free Ion Chromatography System

INTRODUCTION

Methacholine chloride [2-(acetyloxy)-N,N,N-trimethyl-1-propanaminium chloride] is a synthetic analogue of the neurotransmitter acetylcholine. Its principal use is as a parasympathomimetic broncho-constrictor agent to assess bronchial asthma of subjects in respiratory function labs and epidemiological field studies.[1] Methacholine chloride powder is dissolved in 0.9% sodium chloride (with or without the presence of 0.4% phenol used to prevent biological growth) at 16 or 25 g/L.[2, 3] After serial dilutions of this initial solution, each methacholine chloride solution is nebulized and inhaled by the subjects, followed by an evaluation of pulmonary function.

The accuracy of this inhalation challenge test depends on the methacholine concentration. However, methacholine has been shown to decompose over time.[4] Methacholine hydrolysis will yield □-methylcholine and acetic acid. Acetylcholine, a possible synthetic impurity, may also be present in the powder. These choline-containing impurities may affect the biological response in an inhalation challenge study.

Methacholine chloride purity and stability have been determined using a colorimetric assay[5] and ion-pairing HPLC.[4,5] Neither of these methods has been used to determine potential impurities in methacholine chloride solutions. Choline and its analogs have been determined in various matrices using ion chromatography (IC). Carbachol chloride and bethanechol chloride, plus their alkaline decomposition products (choline and 2-hydroxypropyltrimethylammonium chloride, respectively), have been determined in ophthalmic solutions after sample dilution using a Reagent-Free™ Ion Chromatography (RFIC™) system.[6] These cholinergic agents were well resolved from other commonly occurring inorganic cations. The method demonstrated high precision, high recovery, and excellent day-to-day reproducibility. Other application notes showed the feasibility of determining bethanachol and 2-hydroxypropyltrimethylammonium in prescription and non-prescription medications,[7] choline in milk and infant formula,[8] and choline and acetylcholine in a vitamin and mineral formulation.[9] The work shown here demonstrates that an RFIC system can determine methacholine, acetylcholine, and β-methylcholine with good precision and accuracy. Anion IC is used to determine acetate. The first method can be used to assay methacholine, and the combination of both methods can be used to determine methacholine's related substances.

EQUIPMENT

Dionex ICS-2100 system* comprising:

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

AS Autosampler and 2 mL vial tray

Chromeleon® Chromatography Data System (CDS) 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])

* The application in this note can be run using any Dionex RFIC system.

CONSUMABLES

Cation Analysis

EluGen II MSA Cartridge (Dionex P/N 058902)

CR-CTC II (Dionex P/N 066262)

CSRS® 300, 4 mm (Dionex P/N 064556)

IonPac® CG17, 4 × 50 mm (Dionex P/N 060560)

IonPac CS17, 4 × 250 mm (Dionex P/N 060557)

Anion analysis

ASRS® 300, 4 mm (Dionex P/N 064554)

IonPac AG22, 4 × 50 mm (Dionex P/N 064139)

IonPac AS22, 4 × 250 mm (Dionex P/N 064141)

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Sodium chloride, ultrapure (VWR P/N JT4058-1)

Phenol, SigmaUltra (Sigma P/N P5566)

Sodium hydroxide, 50% w/w (Thermo Fisher Scientific P/N SS254)

Hydrochloric acid, Ultrex® II (VWR P/N JT6900-5)

Sodium carbonate concentrate, 0.5 M (Dionex P/N 037162)

Sodium bicarbonate concentrate, 0.5 M (Dionex P/N 037163) or

IonPac AS22 eluent concentrate, 100× (Dionex P/N 063965)

Standards

Sodium acetate, anhydrous (Sigma P/N 71183)

Methacholine chloride (USP P/N 1396364)

Acetylcholine chloride (USP P/N 1008501)

CONDITIONS

Cations

Columns:	IonPac CG17, 4 × 50 mm (Dionex P/N 060560) IonPac CS17, 4 × 250 mm (Dionex P/N 060557)
Eluent:	5 mM methanesulfonic acid
Flow Rate:	1.0 mL/min
Inj. Volume:	25 µL (full loop)
Column Temp.:	30 °C
Detector Temp.:	35 °C
Detection:	Suppressed conductivity, CSRS 300, 4 mm (Dionex P/N 064556), recycle mode, 15 mA suppressor current
Background:	< 1 µS
Noise:	< 0.5 nS/min
Backpressure:	2500 psi
Run Time:	26 min

Anions

Columns:	IonPac AG22 4 × 50 mm (Dionex P/N 064139) IonPac AS22 4 × 250 mm (Dionex P/N 064141)
Eluent:	4.5 mM Sodium carbonate/ 1.4 mM sodium bicarbonate
Flow Rate:	1.2 mL/min
Inj. Volume:	25 µL (full loop)
Column Temp.:	30 °C
Detector Temp.:	35 °C
Detection:	Suppressed conductivity, ASRS 300 4 mm (Dionex P/N 064554), recycle mode, 31 mA suppressor current
Background:	20 to 23 µS
Noise:	< 5 nS/min
Backpressure:	1600 psi
Run Time:	15 min

ELUENT SOLUTIONS

Cation Determinations

5 mM methanesulfonic acid (MSA) was generated on-line electrolytically using an Eluent Generator with an MSA EluGen cartridge. Fill the eluent reservoir with degassed deionized water and maintain an inert nitrogen or helium atmosphere of 3 to 5 psi in the eluent reservoir. Alternately, manually prepared MSA may be used. Prepare a 1.0 N stock solution by adding 96.10 g of MSA to a 1 L volumetric flask containing approximately 500 mL of deionized water. Bring to volume with deionized water and mix thoroughly. Prepare 5 mM MSA by diluting 5 mL of the 1 N MSA stock solution to 1 L with degassed deionized water.

Anion Determinations

The 4.5 mM carbonate/1.4 mM bicarbonate eluent solution was manually prepared by adding 18.90 g of 0.5 M sodium carbonate and 5.88 g of 0.5 M sodium bicarbonate solutions to a 2 L volumetric flask and diluting to volume with degassed deionized water. Alternately, 21.0 g of IonPac AS22 Eluent Concentrate can be added to a 2 L volumetric flask and diluted to volume with degassed deionized water. It is also possible to generate eluent on-line electrolytically using an Eluent Generator module, Carbonate EluGen cartridge (Dionex P/N 058904), Electrolytic pH Modifier (Dionex P/N 063175) to generate bicarbonate, and a 4 mm Carbonate Mixer Kit (Dionex P/N 079943).

STANDARDS AND SURROGATE SAMPLE SOLUTIONS

1000 mg/L Methacholine Solution

Dissolve 0.500 g of methacholine chloride reference standard in degassed deionized water to make 409.3 g of solution. Store in a high-density polyethylene or polypropylene bottle at 4 ºC.

1000 mg/L Acetylcholine Solution

Dissolve 0.200 g of acetylcholine chloride reference standard in degassed deionized water to make 160.9 g of solution. Store in a high-density polyethylene or polypropylene bottle at 4 ºC.

500 mg/L β-Methylcholine Solution

Dilute 0.26 mL of 50% sodium hydroxide solution with 15.8 mL water. Add 33.9 mL of the 1000 mg/L methacholine stock standard to the diluted sodium hydroxide solution. Allow the resulting mixture to react overnight to hydrolyze methacholine to form β-methylcholine and acetate. Acidify the mixture with the minimum volume of 1 N hydrochloric acid needed to lower the pH below 4 (~ 0.1 mL of HCl/mL of alkaline β-methylcholine solution). Store in a high-density polyethylene or polypropylene bottle at 4 ºC.

1000 mg/L Acetate Solution

Add 0.1390 g sodium acetate to a 1 L volumetric flask and dilute to volume with degassed deionized water. Store at 4 ºC.

Matrix Stock Solutions for Methacholine Assay

To prepare 0.9% sodium chloride matrix, add 0.900 g of sodium chloride to a 100 mL volumetric flask and dilute to volume with deionized water. To prepare 0.9% sodium chloride–0.4% phenol matrix, add 0.900 g of sodium chloride and 0.400 g of phenol to a 100 mL volumetric flask, then dilute to volume with deionized water. Store at 4 ºC.

Matrix Stock Solutions for Methacholine Impurities Determination

Prepare 1 L of 120 mg/L sodium chloride. Immediately before preparing working stock and surrogate sample solutions, mix three parts of the sodium chloride solution with one part of the 1000 mg/L methacholine solution to yield a 250 mg/L methacholine in 90 mg/L sodium chloride solution. Prepare 1 L of 120 mg/L sodium chloride–53 mg/L phenol. Immediately before preparing working stock and surrogate sample solutions, mix 3 parts of the sodium chloride–phenol solution with 1 part of the 1000 mg/L methacholine solution to yield a 250 mg/L methacholine in 90 mg/L sodium chloride–40 mg/L phenol solution. Store matrix solutions at 4 ºC.

Make all subsequent dilutions of stock standards gravimetrically when generating standards for calibration and MDL studies and when generating surrogate samples for accuracy and precision studies.

RESULTS AND DISCUSSION

Chromatography

To test system suitability for the determination of methacholine and its possible choline-containing impurities in the presence of commonly occurring cations, a chromatographic trace for β-methylcholine, acetylcholine, and methacholine was compared to the chromatogram for a mixture of lithium, sodium, ammonium, potassium, magnesium, and calcium ions. As shown in Figure 1, all of the components are well separated. The large sodium peak is a result of the base-catalyzed hydrolysis of methacholine to form β-methylcholine and acetate. The reaction in 0.1 N NaOH occurs rapidly and was shown to be complete in less than 16 h (Figure 2). After acidification of the reaction products, this mixture was combined with methacholine and acetylcholine standards to create the 3-component sample in Figure 1.

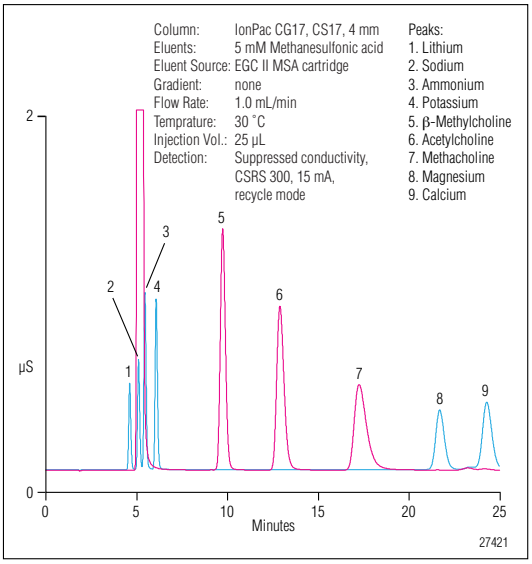

Figure 1. Overlay of β-methylcholine, acetylcholine, and methacholine with a mixed-cation standard.

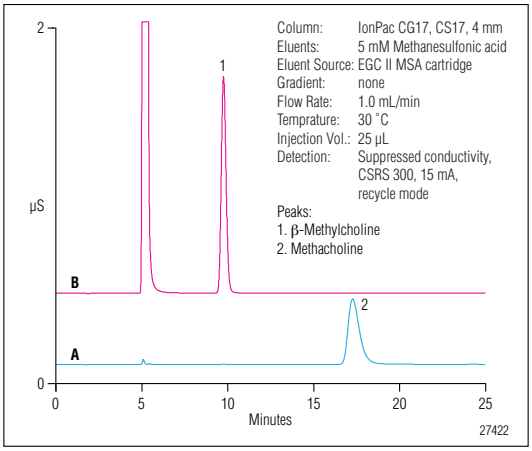

Figure 2. Conversion of methacholine to β-methylcholine in the presence of 0.1 N NaOH. Trace A, time = 0 h; and trace B, time = 16 h.

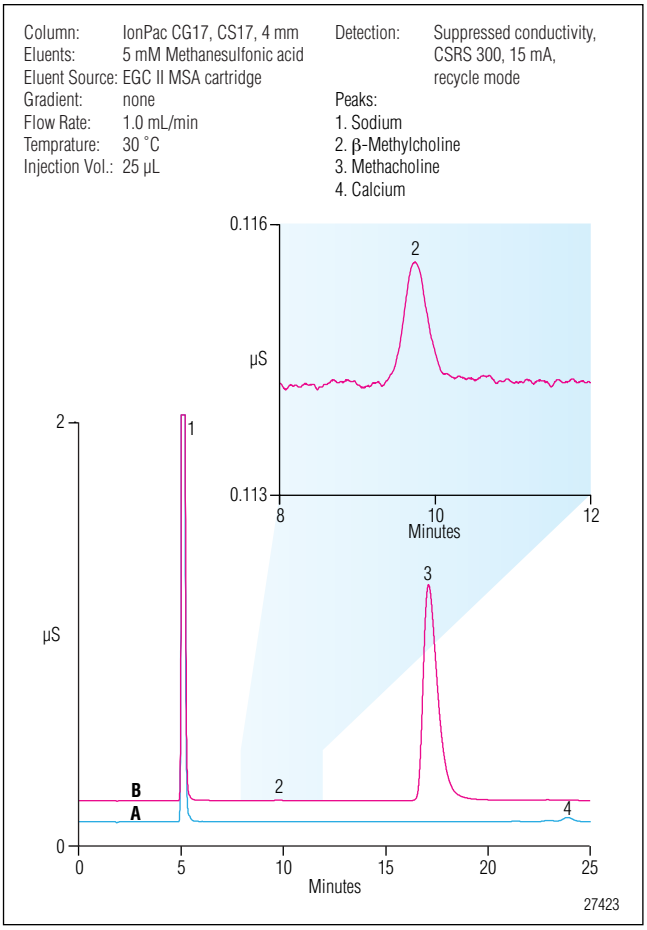

Figure 3. Cations in 1000:1 dilution in 0.9% NaCl + 0.4% phenol matrix. Trace A, matrix blank; trace B, 25 mg/L methacholine in diluted matrix (β-methylcholine conc. ~ 0.1% of methacholine). The inset shows the 8 to 12 min region.

Methacholine Linearity

The method for methacholine assay includes diluting the initial solution (16 or 25 g/L methacholine in 0.9% sodium chloride with or without 0.4% phenol) 1000-fold with filtered deionized water. Figure 3 shows a chromatogram from a sample in sodium chloride/phenol matrix after 1000-fold dilution.

A trace amount of β-methylcholine, formed from methacholine hydrolysis, can be detected (Figure 3 inset). Calibration data were generated for methacholine over a 5 to 50 mg/L range. Excellent linearity was observed in this calibration range with a correlation coefficient of 0.99995 (Table 1).

Determination of Linearity and MDL for Possible Methacholine Impurities

Calibration data were collected for β-methylcholine, acetylcholine, and acetate in deionized water. Table 1 shows the calibration results for these three possible impurities. Calibration ranges covered three orders of magnitude. Excellent correlation coefficients (> 0.9998) were observed.

Table 1. Calibration Results for Methacholine and Possible Impurities[a]

Analyte	Range (mg/L)	Correlation Coefficient (r^2)	Offset (µS min)	Slope	RSD
Methacholine	5–50	0.99995	-0.0315	0.0360	0.42
Acetylcholine	0.1–100	0.99991	-0.0159	0.0403	1.6
β-Methylcholine	0.1–100	0.99997	-0.0171	0.0503	0.89
Acetate	0.1–50	0.99983	0.0011	0.0148	2.0

[a] Triplicate injections of solutions prepared in deionized water.

MDL estimates were based on the system blank (no injection). Signal noise was calculated over a four-minute interval centered on the analyte's retention time, converted to analyte concentration using a peak height vs. concentration calibration curve, and multiplied by three to calculate the MDL estimate. Table 2 shows that MDL estimates for β-methylcholine and acetylcholine are at single-digit µg/L levels while the estimate for acetate MDL is 75 µg/L.

Table 2. Estimated Method Detection Limits for Possible Methacholine Impurities[a,b]

Analyte	MDL (µg/L)
Acetylcholine	8
β-Methylcholine	5
Acetate	75

[a] Noise determined from average of 8 replicates of system (no injection) blanks.

[b] Calculated as 3x noise over 4-min interval centered at analyte RT.

Recovery of Methacholine and Possible Methacholine Impurities

Recovery and precision studies were performed in simulated matrices. The 1000-fold dilution protocol for methacholine assay is described above. Impurity determination involves a 100-fold dilution with deionized water of 25 g/L methacholine in 0.9% sodium chloride with or without 0.4% phenol. Final matrix composition for possible methacholine impurity determination is 250 mg/L methacholine in 90 mg/L sodium chloride with or without 40 mg/L phenol.

Table 3. Recoveries of Methacholine and Possible Impurities in Simulated Matrices[a]					
Analyte	**Matrix[b]**	**Spiking Level (mg/L)**	**Molar % of Methacholine[c]**	**Recovery (%)**	**Precision (RSD)**
Methacholine	9 mg/L NaCl	10	—	97.2	0.4
		25	—	98.5	0.3
		45	—	99.2	0.2
	9 mg/L NaCl 4 mg/L phenol	10	—	97.2	0.4
		25	—	98.1	0.1
		45	—	99.9	0.2
Acetylcholine	250 mg/L methacholine 90 mg/L NaCl	2.5	1.1	95.0	0.4
		25	11	97.1	0.2
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	1.1	85.4	0.2
		25	11	97.2	0.2
β-Methylcholine*	250 mg/L methacholine 90 mg/L NaCl	2.5	1.4	88.7	0.9
		25	14	98.3	0.1
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	1.4	84.7	0.3
		25	14	96.7	0.2
Acetate*	250 mg/L methacholine 90 mg/L NaCl	2.5	2.7	88.7	0.9
		25	27	98.1	0.2
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	2.7	92.4	0.8
		25	27	99.8	0.1

[a] Seven replicates of blanks and spiked matrices.

[b] Diluted 1000-fold for methacholine assay and 100-fold for impurities analysis.

[c] Converting mass ratio to molar ratio based on impurity ion's molecular weight.

* Measured concentrations of β-Methylcholine and acetate in blank at ~ 0.4 mg/L.

Table 3 summarizes recovery results for methacholine and possible impurities. Methacholine recoveries range from 97 to100% for the three spiking levels. The three possible impurity analytes were spiked at 2.5 and 25 mg/L in the 100-fold diluted sample matrix. The lower spiking amount corresponds to 1 to 3% of the methacholine present in the matrix (Table 3). Recoveries at 2.5 mg/L spiking range from 85 to 95%. Amounts of β-methylcholine and acetate in the matrix blanks (0.4 mg/L) may be overestimated and contribute to lower than expected recoveries at spiking amounts of 2.5 mg/L. A peak for β-methylcholine is observed in both the matrix blank and acetylcholine-spiked matrix in Figure 4. Recoveries of possible impurities spiked at 25 mg/L ranged from 97 to 100%. Impurity determinations can be performed at amounts approaching 1% of the methacholine in solution.

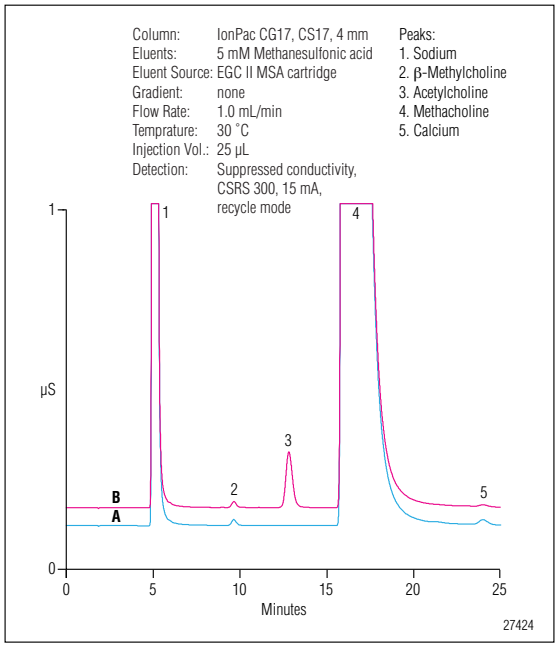

Figure 4. Cations in 100:1 dilution of 25 g/L methacholine + 0.9% NaCl + 0.4% phenol matrix. Trace A, matrix blank; trace B, 2.5 mg/L acetylcholine in diluted matrix.

Table 4. Retention Time and Peak Area Reproducibilities for Methacholine and Possible Impurities in Simulated Matrices[a]

Analyte (conc.)	Matrix[b]	Average RT, min (RSD)	Average Peak Area, µS min (RSD)
Methacholine (25 mg/L)	9 mg/L NaCl	17.019 (0.040)	0.8190 (0.31)
	9 mg/L NaCl 4 mg/L phenol	17.013 (0.052)	0.8359 (0.40)
Acetylcholine (10 mg/L)	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	12.772 (0.060)	0.3647 (0.45)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	12.780 (0.047)	0.3634 (0.42)
β-Methylcholine (10 mg/L)	250 mg/L methacholine 90 mg/L NaCl	9.642 (0.091)	0.4938 (0.75)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	9.638 (0.10)	0.4960 (0.70)
Acetate (2.5 mg/L)	250 mg/L methacholine 90 mg/L NaCl	3.249 (0.34)	0.0438 (1.1)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	3.248 (0.28)	0.0441 (0.85)

[a] Eight replicates per day in each matrix collected over a five day period.

[b] Samples diluted 1000-fold for methacholine assay and 100-fold for impurities analysis.

Retention Time and Peak Area Precision

Table 4 summarizes the results of a five-day study of retention time and peak area reproducibilities for methacholine and possible impurities. Eight replicates for each target analyte in each of the two sample matrices were injected daily. No trending in daily averages was observed. Highly reproducible retention time and peak areas were observed for the choline-based analytes. Acetate determination yielded somewhat lower retention time and peak area reproducibility. Lower acetate retention-time reproducibility may be due to use of manually prepared carbonate/bicarbonate eluent, compared to the electrolytically produced eluent for the cation determinations.

PRECAUTIONS

Methacholine and acetylcholine chlorides are neurotransmitters and can affect several of the body's systems. Contact of the powder with eyes, skin, and respiratory tract causes severe irritation. Wear protective gloves, chemical safety goggles, and a laboratory coat when working with these materials. Methacholine should never be administered orally or by injection because serious toxic reactions can occur. In addition, β-Methylcholine can be irritating to eyes, respiratory system, and skin. To dispose of these materials, contact a licensed waste disposal service. Read the material safety data sheet for these compounds before using them.

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. For more information on column troubleshooting, see the *IonPac CS17 Column Product Manual* (Dionex Document No. 031877).

CONCLUSION

This work presents IC-based methods for the detection and quantification of methacholine and possible impurities in solutions used to assess bronchial asthma of subjects evaluated in respiratory function labs and epidemiological field studies. Methacholine assay involves diluting the original solution (dissolved in 0.9% sodium chloride with or without 0.4% phenol) 1000-fold with deionized water, then analyzing the sample on a cation RFIC system. Acetylcholine and β-methylcholine are determined after a 100-fold dilution of the original solution using the same system. Acetate, another possible impurity, is determined by anion IC. These methods demonstrate high precision and accuracy for both methacholine and possible impurities present in amounts approaching 1%.

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8. Dionex Corporation. *Determination of Choline in Dry Milk and Infant Formula*. Application Note 124, LPN 1054-01, 2002, Sunnyvale, CA.
9. Dionex Corporation. *Choline and Acetylcholine.* Application Note 70, LPN 034516, 1991, Sunnyvale, CA.

SUPPLIERS

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

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

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

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

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

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

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

HPLC Assay Method for Drug Products Containing Anti-Tuberculosis Active Pharmaceutical Ingredients

INTRODUCTION

Isoniazid, pyrazinamide, rifampicin, and ethambutol are anti-tuberculosis compounds. The standard treatment for tuberculosis (TB) is to treat the patient with a combination of these four compounds for two months, followed by isoniazid and rifampicin alone for an additional four months. Depending on the state of infection, ethambutol may be omitted from the treatment.[1] These compounds are used in combination because they have different modes of action. For more than 50 years, TB has been treated with combination drug therapy and there are a number of available combination drug products with different drug contents and composition.

The United States Pharmacopeia (USP) 32 NF27 contains a monograph for isoniazid, pyrazinamide, rifampicin, and ethambutol hydrochloride tablets.[2] The monograph has two assay methods for this drug product. One method is for an assay of isoniazid, pyrazinamide, and rifampicin and the other for ethambutol. Both are HPLC methods.

The work shown here reports a single HPLC assay method that accurately determines these four compounds. The method is evaluated using two drug products. One drug product is a tablet containing all four compounds and the other drug product contains three of the compounds (no ethambutol). The 10 min HPLC method accurately determines all compounds of interest in both drug products. This method saves time, reduces mobile phase consumption, and reduces waste. Further savings and waste reduction are possible with an ultra HPLC (UHPLC) method that requires less than 2 min per injection.

EQUIPMENT

Dionex UltiMate® 3000 system including:

Equipment	Conventional HPLC	UHPLC
Integrated vacuum degasser solvent rack	SRD-3600	SRD-3600
Pump	DGP-3600A	HPG-3400RS
Split-loop sampler	WPS-3000TSL	WPS-3000TRS
Column compartment	TCC-3200	TCC-3000RS
Diode array detector	PDA-3000	DAD-3000RS
Sample loop size	100 µL	100 µL*
Mixer	Standard	200 µL Static mixer kit
Flow cell	13 µL SST	2.5 µL SST
Chromeleon® Chromatography Data System (CDS) software version	6.80 SR7	6.80 SR7

*While the data collected in this AN used a 100 µL loop, a smaller loop (e.g. 20 µL) would be more appropriate.

REAGENTS AND STANDARDS

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

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

Sodium dihydrogen orthophosphate, AR grade (Ajax)

Triethylamine (TEA), AR grade (Fisher)

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

Isoniazid, (101.30%), (provided by customer)

Pyrazinamide, (99.91%), (provided by customer)

Ethambutol, (98.10%), (provided by customer)

Rifampicin, (96.92%), (provided by customer)

CONDITIONS

Conventional HPLC

Column:	Acclaim® Polar Advantage II (PA2), 3 µm 4.6 × 150 mm (P/N 063191) Acclaim PA2 Guard, 5 µm 4.3 × 10 mm (P/N 063195) Acclaim Guard Kit (P/N 059526)
Mobile Phase:	A: 8% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8 B: 50% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8
Flow Rate:	1.0 mL/min
Gradient:	100% A from -5 to 3 min, ramp to 100% B in 0.5 min, and hold 100% B for 7 min
Column Temp.:	35 °C
Inj. Volume:	5 µL
Detection:	Channel_1 UV-vis_1 at 200 nm and 337 nm at 5 min Channel_2 UV-vis_2 at 238 nm Wavelength scanning 190 to 800 nm Data collection rate 5 Hz, rise time 0.5 s

UHPLC

Column:	Acclaim PA2, 2.2 µm 2.1 × 100 mm (P/N 068990) EXP™ Pre-Column Ultra High Pressure Filter Cartridges, 0.2µm (P/N 15-04-03097, Optimize Technologies) EXP Filter Holder with EXP™ Titanium Hybrid Ferrule (P/N 15-04-03837, Optimize Technologies)
Mobile Phase:	A: 4% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8 B: 50% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8
Flow Rate:	1.0 mL/min
Gradient:	100% A from -2.5 to 0.5 min, ramp to 100% B in 0.1 min, and hold 100% B for 1.2 min
Column Temp.:	35 °C
Inj. Volume:	1.5 µL
Detection:	Channel_1 UV-vis_1 at 200 nm and 337 nm at 1 min Channel_2 UV-vis_2 at 238 nm Wavelength scanning 190 to 800 nm Data collection rate 25 Hz, response time 0.2 s

PREPARATION OF SOLUTIONS AND REAGENTS

20 mM NaH_2PO_4 pH 6.8 plus 1.5 mL TEA

Dissolve 3.12 g NaH_2PO_4 in 700 mL water, add 1.5 mL TEA, and mix well. Transfer this solution into a 1 L volumetric flask and add water to bring to volume. Adjust to pH 6.8 with orthophosphoric acid.

Mobile Phases

Mobile Phase A (Conventional HPLC)

Mix 80 mL CH_3CN with 920 mL of 20 mM NaH_2PO_4 TEA pH 6.8. Filter with a 0.2 µM filter.

Mobile Phase A (UHPLC)

Mix 40 mL CH_3CN with 960 mL of of 20 mM NaH_2PO_4 TEA pH 6.8. Filter with a 0.2 µM filter.

Mobile Phase B

Mix 500 mL CH_3CN with 500 mL of 20 mM NaH_2PO_4 TEA pH 6.8. Filter with a 0.2 µM filter.

Stock Standard Solutions

Accurately weigh 20 mg, 110 mg, 74 mg, and 110 mg isoniazid, pyazinamide, ethambutol, and rifampicin, respectively, into a 50 mL beaker. Add 5 mL CH_3CN and 20 mL mobile phase A. Stir and place in an ultrasonic bath until dissolution is complete. Transfer this solution to a 50 mL volumetric flask, rinse the beaker with mobile phase A a few times, and transfer into the same volumetric flask. Add mobile phase A to bring to volume. Table 1 shows the concentration of the stock standard solution.

Working Standard Solutions

Pipet 1, 1.5, and 2 mL stock standard solution into separate 10 mL volumetric flasks. Add mobile phase A to bring to volume. Table 1 shows the concentrations of the working standard solutions.

Note: Prepare stock and working standard solutions just before analysis.

SAMPLE PREPARATION

The authors analyzed two different anti-tuberculosis drug samples (referred to as Samples A and B) and these drugs had different compositions. Sample A contained four active pharmaceutical ingredients (API): isoniazid, pyrazinamide, ethambutol, and rifampicin. Sample B had only three of the APIs; it lacked ethambutol. Table 2 reports the content of each drug and API concentration after sample preparation.

Sample A

1. Grind a tablet of Sample A and transfer into a 50 mL beaker. Add 5 mL CH_3CN and 20 mL mobile phase A. Stir and place in an ultrasonic bath until dissolution is complete. Transfer this solution into a 100 mL volumetric flask, rinse the beaker with mobile phase A a few times, and transfer into the same volumetric flask. Add mobile phase A to bring to volume.
2. Pipet 0.75 mL of this sample solution into a 10 mL volumetric flask and add mobile phase A to bring to volume. Filter with a 0.2 µm filter.

Sample B

1. Prepare a tablet in the same manner as step 1 for Sample A.
2. Pipet 1 mL of this sample solution into a 10 mL volumetric flask and add mobile phase B to bring to volume. Filter with a 0.2 µm filter.

Note: Prepare samples on the day of analysis.

Table 1. Concentrations of Stock and Working Standard Solutions

Compound	Concentration of Stock Standard Solution (mg/L)	Stock Standard Solution Volume (mL)*			Working Standard Solution Concentration (mg/L)		
		L1	L2	L3	L1	L2	L3
Isoniazid	405	1	1.5	2	40.5	60.8	81.0
Pyrazinamide	2198	1	1.5	2	220	330	440
Ethambutol	1452	1	1.5	2	145	218	290
Rifampicin	775	1	1.5	2	78	116	155

*Volume used to prepare 10 mL of working standard solution

Table 2. Tablet Content and Sample Concentration after Sample Preparation

Compound	Sample A		Sample B	
	Tablet Content (mg/tablet)	Calculated Concentration after Sample Preparation (mg/L)	Tablet Content (mg/tablet)	Calculated Concentration after Sample Preparation (mg/L)
Isoniazid	75	56.3	80	80
Pyrazinamide	400	300	250	250
Ethambutol	275	206	—	—
Rifampicin	150	113	120	120

Table 3. Tablet Weights		
Tablet No.	Tablet Weight (g)	
	Sample A	Sample B
1	1.23	0.71
2	1.19	0.72
3	1.20	0.70
Average	1.21	0.71
Total Weight of APIs (g)	0.91	0.45
Placebo Weight (g)	0.30	0.26

Table 4. Spiked Sample Concentrations				
Compound	Spiked Standard Amount (mg)		Calculated Spiked Concentration after Sample Preparation (mg/L)	
	In 0.3 g of Sample A Placebo	In 0.26 g of Sample B Placebo	Spiked Sample A Placebo	Spiked Sample B Placebo
Isoniazid	75	80	57	81
Pyrazinamide	400	250	300	250
Ethambutol	275	—	202	—
Rifampicin	150	120	109	116

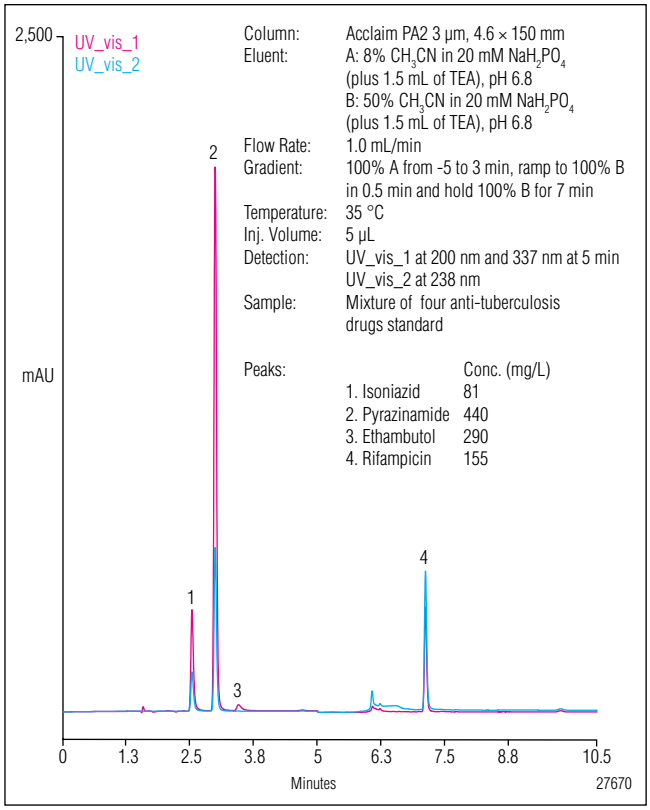

Figure 1. Chromatograms of a standard containing four anti-tuberculosis drugs.

Spiked Placebo Sample

To calculate the placebo weight for each sample, average the weights of the three tablets and subtract the average total API weight of those tablets to obtain the average placebo weight (Table 3). Use the same placebo weight for each sample in Table 3 for the spiked placebo sample preparation. Spike standards (dry) into the placebo to achieve API content similar to the tablet content. Table 4 shows the amount of standards added to each sample placebo and the calculated concentration after sample preparation.

RESULTS AND DISCUSSION

Separation and Detection

The goal of this work was to create one method to determine all four APIs in the combination drug product. The authors started by reviewing the USP monograph for rifampin, isoniazid, pyrazinamide, and ethambutol hydrochloride tablets. The USP monograph has two assay methods. The first assay is for isoniazid, pyrazinamide, and rifampicin. It is an HPLC assay that calls for a 4.6 × 250 mm, 5 µm L1 column, a sodium phosphate buffer pH 6.8/CH_3CN eluent, and a 238 nm detection wavelength. The second assay, which is also an HPLC assay, is for ethambutol. This assay calls for a 4.6 × 150 mm, 5 µm L10 column, triethylamine pH 7/CH_3CN eluent, and a 200 nm detection wavelength. To create a single method for all four APIs, the authors used an Acclaim PA2 (3 µm, 4.6 × 150 mm) column with a sodium phosphate plus triethylamine pH 6.8/CH_3CN eluent. Figure 1 shows a separation of all four compounds in 10 min. The compounds were detected with two UV detection channels. Channel 1 (UV-vis_1) detects compounds by absorbance at 200 nm for the first 5 min and at 337 nm for the final five min. Channel 2 (UV-vis_2) detects at 238 nm. Ethambutol is not detected at 238 nm.

Table 5. Calibration Results at UV-vis_1 as Reported by Chromeleon Software

Compound	Cal. Type	Points	r^2	Offset	Slope
Isoniazid	LOff	3	0.99999	-0.3938	0.2920
Pyrazinamide	LOff	3	0.99979	3.1730	0.2684
Ethambutol	LOff	3	0.99938	-0.1861	0.0088
Rifampicin	LOff	3	0.99999	-0.4919	0.1338

Table 6. Calibration Results at UV-vis_2 as Reported by Chromeleon Software

Compound	Cal. Type	Points	r^2	Offset	Slope
Isoniazid	LOff	3	0.99994	-0.1826	0.1153
Pyrazinamide	LOff	3	0.99998	-0.4231	0.0812
Ethambutol	—	—	—	—	—
Rifampicin	LOff	3	0.99998	-0.7014	0.1801

Method Calibration

After optimizing sample preparation to determine that all compounds can be detected in each of the two samples, three-point calibration curves were prepared using the two UV channels with the diode array detector. The calibration data in Table 5 show linear peak area response for each detected compound in channel 1 and Table 6 shows linear peak area response for each detected compound in channel 2.

Sample Analysis

Customers provided Samples A and B as well as products without the APIs, referred to as Sample A Placebo and Sample B Placebo. Three tablets for each sample were prepared and three injections of each prepared tablet were made to evaluate the reproducibility of sample preparation, injection, and tablet content. Chromatograms for Samples A and B are shown in Figures 2 and 3, respectively. Sample A contained the expected four APIs, whereas Sample B contained the expected three APIs. Neither tablet contained compounds that interfere with determination of the four APIs. To determine if the four peaks were pure, the photodiode array detector was used for the standard separation. The authors injected single component standards, collected the spectral data, and entered it into the spectral library.

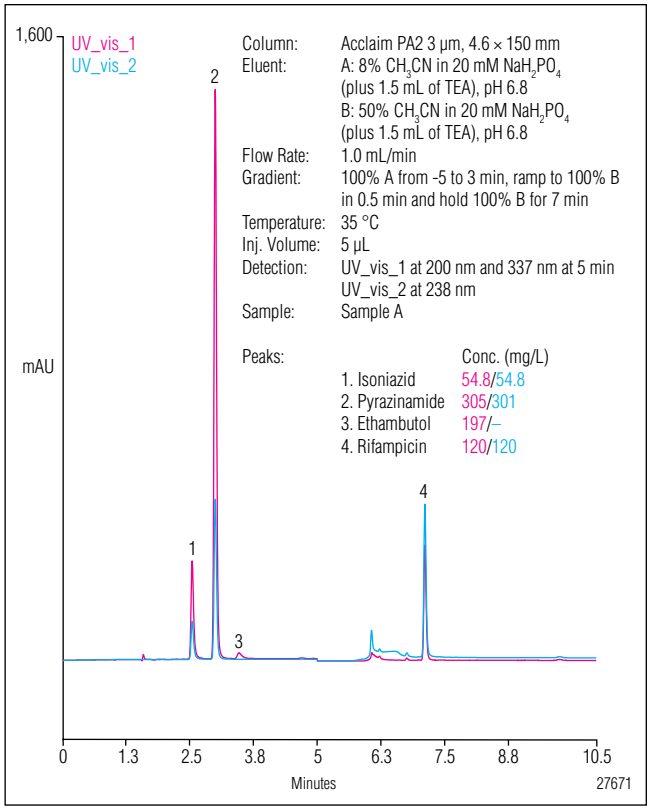

Figure 2. Example chromatograms of Sample A.

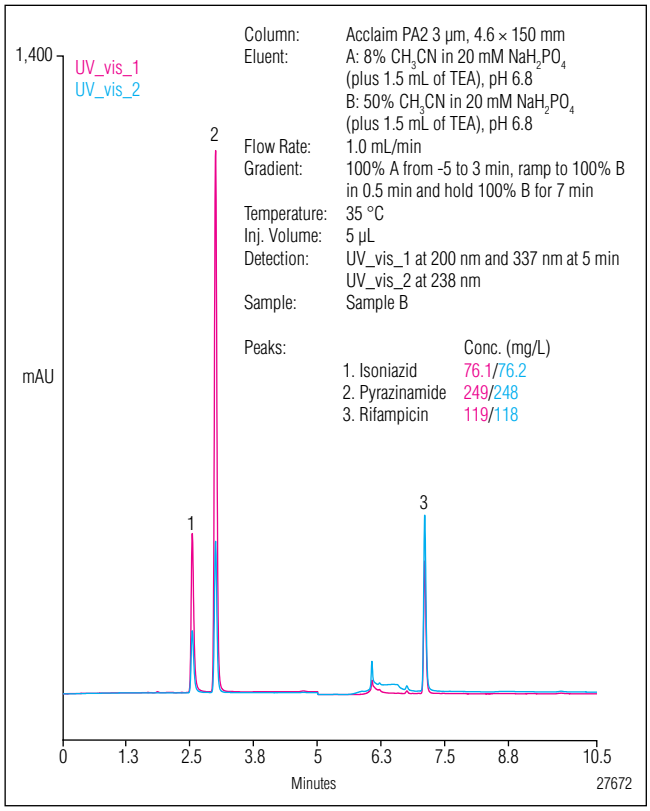

Figure 3. Example chromatograms of Sample B.

Table 7. Peak Purity, Resolution, and Peak Analysis Results for the Standard							
Compound	**Resolution* (USP)**	**Peak Purity Match**	**RSD Peak Purity Match**	**Peak Purity Index**	**RSD Peak Purity Index**	**Asymmetry**	**Plates (USP)**
Isoniazid	4.98	1000	0.47	215.9	0.22	1.42	12410
Pyrazinamide	3.58	1000	0.51	226.2	0.23	1.14	16446
Ethambutol	29.92	995	1.78	194.3	0.80	1.94	7244
Rifampicin	n.a.	998	3.45	284.3	1.21	1.20	—

Table 8. Peak Purity, Resolution, and Peak Analysis Results for the Samples									
Sample	**Compound**	**Peak Purity Match**	**RSD Peak Purity Match**	**Peak Purity Index**	**RSD Peak Purity Index**	**Resolution* (USP)**	**Asymmetry**	**Plates (USP)**	**Match with Library**
Sample A	Isoniazid	1000	0.49	215.9	0.23	4.96	1.38	12185	1000
	Pyrazinamide	1000	0.58	226.2	0.26	3.76	1.16	17480	1000
	Ethambutol	993	2.57	195.0	1.20	30.46	1.81	7705	999
	Rifampicin	997	4.32	284.7	1.50	n.a.	1.16	—	997
Sample B	Isoniazid	1000	0.37	215.9	0.17	4.94	1.37	11999	1000
	Pyrazinamide	1000	0.50	226.3	0.22	47.02	1.14	17053	1000
	Ethambutol	—	—	—	—	—	—	—	—
	Rifampicin	998	2.25	286.0	0.77	n.a.	1.20	—	996

*Calculation is based on USP and is compared to the next main peak.

The data in Table 7 suggest that each peak in the standard was pure. Table 8 shows that all peaks in Samples A and B were pure (judging by the values calculated from the spectral data) and all peaks had very good spectral match with the data entered into library, also suggesting that the peaks in the samples were pure.

Table 9. Average Found Concentration from Three Injections for Three Tablets at UV-vis_1											
Sample	Compound	Calculated Concentration (mg/L)	Tablet 1			Tablet 2			Tablet 3		
			Average	RSD	% Content	Average	RSD	% Content	Average	RSD	% Content
A	Isonaizid	56.3	54.8	0.47	97.34	53.3	0.13	97.26	51.3	0.42	91.12
	Pyrazinamide	300	305	0.69	101.7	303	0.09	101.0	288	0.25	96.00
	Ethambutol	206	197	0.77	95.63	198	0.65	96.12	201	0.65	97.57
	Rifampicin	113	120	0.50	106.2	115	0.28	101.8	120	0.21	106.2
B	Isonaizid	80	76.1	0.08	95.13	76.2	0.16	95.25	73.2	0.42	91.50
	Pyrazinamide	250	249	0.23	99.60	251	0.09	100.4	246	0.37	98.4
	Ethambutol	—	—	—	—	—	—	—	—	—	—
	Rifampicin	120	119	0.15	99.17	125	0.11	104.2	115	0.14	95.83

Table 10. Average Found Concentration from Three Injections for Three Tablets at UV-vis_2											
Sample	Compound	Calculated Concentration (mg/L)	Tablet 1			Tablet 2			Tablet 3		
			Average	RSD	% Content	Average	RSD	% Content	Average	RSD	% Content
A	Isonaizid	56.3	54.8	0.51	97.34	53.2	0.13	94.49	51.4	0.24	91.30
	Pyrazinamide	300	301	0.53	100.3	298	0.10	99.33	284	0.27	94.67
	Ethambutol	206	—	—	—	—	—	—	—	—	—
	Rifampicin	113	120	0.52	106.2	115	0.28	101.8	120	0.18	106.2
B	Isonaizid	80	76.2	0.08	95.25	76.3	0.13	95.38	73.2	0.09	91.5
	Pyrazinamide	250	248	0.15	99.20	249	0.07	99.60	244	0.18	97.6
	Ethambutol	—	—	—	—	—	—	—	—	—	—
	Rifampicin	120	118	0.16	98.33	125	0.12	104.2	115	0.18	95.83

The averaged concentration of APIs in each sample tablet and the RSDs (< 1% for each tablet using both wavelength channels, as shown in Tables 9 and 10) showed good reproducibility of the method and injection. The amounts of each API were compared to the labeled value; for each API for each tablet of Sample A, the amount was between 90 and 110%. The USP monograph for this product specifies that there should be not less than (NLT) 90% and not more than (NMT) 110% of the API in the drug product. The assay demonstrated that each tablet met the USP criteria. The three-API product, Sample B, also passed the NLT 90% and NMT 110% criteria.

To evaluate method accuracy in another manner, the recoveries of APIs added to the sample placebos were determined. Sample placebos without added APIs were also prepared and analyzed with the HPLC method and no interfering compounds were observed (Figure 4). The same amounts of APIs were added to the placebo as shown on the sample label for the drug products. The spiked placebo samples were prepared and three injections were made for each sample. The averaged found concentration in each spiked placebo sample was compared to calculated concentration to determine recovery.

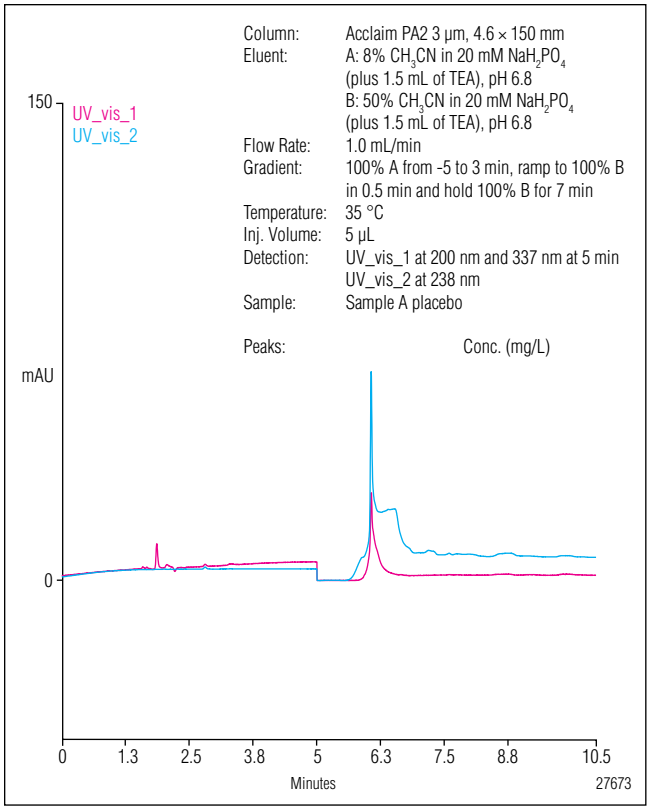

Figure 4. Example chromatograms of Sample A Placebo (chromatograms for Sample B Placebo were equivalent to Sample A Placebo).

Table 11. Recovery at UV-vis_1					
Sample	**Compound**	**Calculated Spiked Concentration (mg/L)**	**Average Found Concentration (mg/L)**	**RSD**	**Recovery**
Spiked Sample A Placebo	Isonaizid	57	55.8	0.06	97.89
	Pyrazinamide	300	299	0.18	99.67
	Ethambutol	202	197	0.17	97.52
	Rifampicin	109	107	0.05	98.17
Spiked Sample B Placebo	Isonaizid	81	78.3	0.11	96.67
	Pyrazinamide	250	250	0.06	100.0
	Ethambutol	—	—	—	—
	Rifampicin	116	110	0.12	94.83

Table 12. Recovery at UV-vis_2					
Sample	**Compound**	**Calculated Spiked Concentration (mg/L)**	**Average Found Concentration (mg/L)**	**RSD**	**Recovery**
Spiked Sample A Placebo	Isonaizid	57	55.8	0.05	97.89
	Pyrazinamide	300	295	0.08	98.33
	Ethambutol	202	—	—	—
	Rifampicin	109	107	0.10	98.17
Spiked Sample B Placebo	Isonaizid	81	78.4	0.06	96.79
	Pyrazinamide	250	248	0.09	99.20
	Ethambutol	—	—	—	—
	Rifampicin	116	109	0.13	93.97

The recoveries in Spiked Placebo Sample A were 97.52 to 99.67% at UV-vis_1 and 97.89 to 98.33% at UV-vis_2, and recoveries in Spiked Placebo Sample B were 94.83 to 100% at UV-vis_1 and 93.97 to 99.20% at UV-vis_2 (Tables 11 and 12). This experiment also indicated that the single HPLC method was accurate for all four APIs.

Faster Analysis

This method can be made faster by using a smaller column format and a smaller particle size. In this work (using the Acclaim RSLC PA2, 2.2 µm, 2.1 × 100 mm column), faster separation was complete in less than 2 min with a system backpressure of ~ 570 bar. Figure 5 shows a chromatogram of faster separation of the four-API standard. Figures 6 and 7 show that faster separation also successfully analyzed the samples.

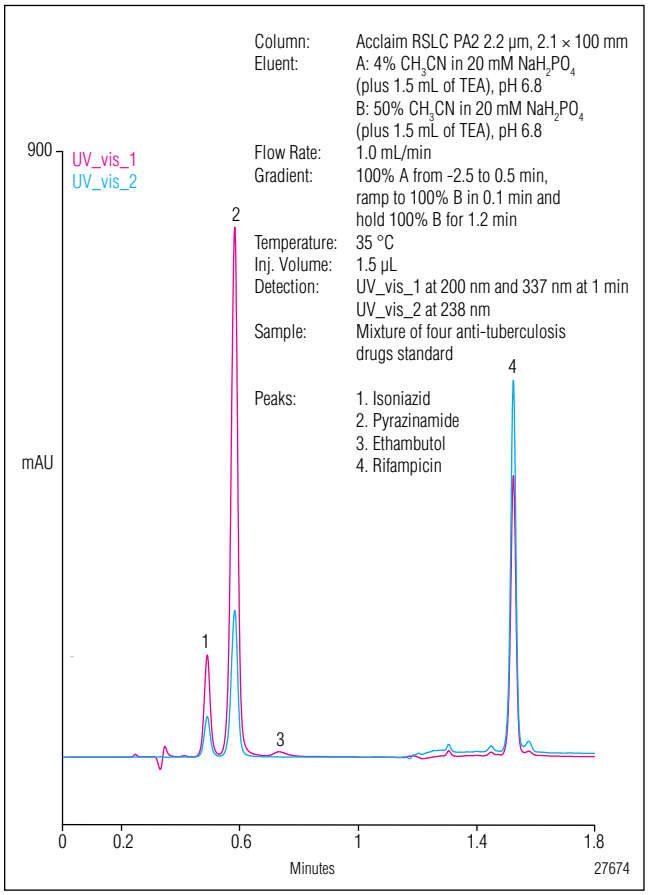

Figure 5. Faster separation of a standard containing four anti-tuberculosis drugs.

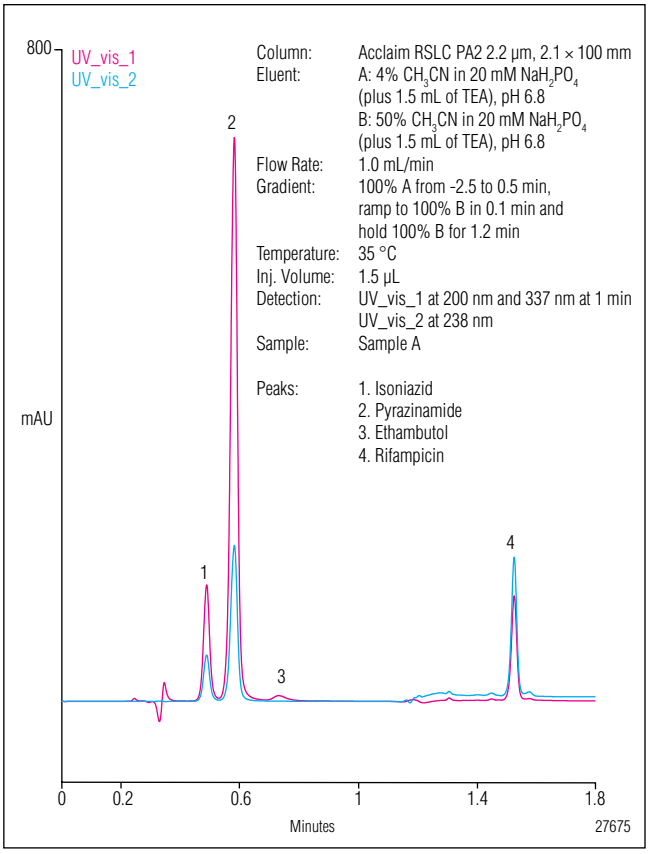

Figure 6. Faster separation of Sample A.

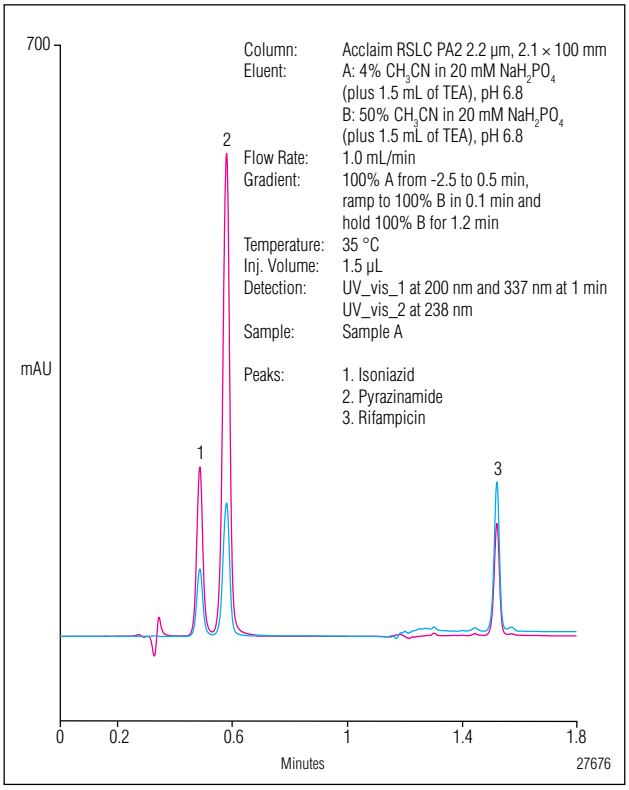

Figure 7. Faster separation of Sample B.

Table 13. Peak Purity, Resolution, and Peak Analysis Results for the Standard (UHPLC)							
Compound	**Resolution* (USP)**	**Peak Purity Match**	**RSD Peak Purity Match**	**Peak Purity Index**	**RSD Peak Purity Index**	**Asymmetry**	**Plates (USP)**
Isoniazid	2.37	1000	0.12	216.8	0.06	1.00	2775
Pyrazinamide	2.61	1000	0.24	228.5	0.11	0.94	3226
Ethambutol	14.82	989	4.25	196.3	1.97	1.42	1573
Rifampicin	n.a.	1000	0.32	284.9	0.11	0.93	—

Table 14. Peak Purity, Resolution, and Peak Analysis Results for the Samples (UHPLC)									
Sample	**Compound**	**Peak Purity Match**	**RSD Peak Purity Match**	**Peak Purity Index**	**RSD Peak Purity Index**	**Resolution* (USP)**	**Asymmetry**	**Plates (USP)**	**Match with Library**
Sample A	Isoniazid	1000	0.13	216.8	0.06	2.36	1.01	2726	1000
	Pyrazinamide	1000	0.24	228.5	0.11	2.65	0.93	3156	1000
	Ethambutol	989	2.56	195.7	1.02	15.17	1.38	1701	999
	Rifampicin	1000	0.27	284.8	0.09	n.a.	0.93	—	1000
Sample B	Isoniazid	1000	0.13	216.8	0.06	2.33	1.02	2610	1000
	Pyrazinamide	1000	0.25	228.6	0.11	24.79	0.94	3084	1000
	Ethambutol	—	—	—	—	—	—	—	—
	Rifampicin	1000	0.39	285.0	0.14	n.a.	0.93	—	1000

*Calculation is based on USP and is compared to the next main peak.

The peak purity and spectral match data for the standard and samples are displayed in Tables 13 and 14 (spectra from the standard compounds were added to the library for sample analysis) and support the visual observation from Figures 6 and 7 that a successful analysis was achieved with the fast method.

CONCLUSION

This work shows that a single HPLC method can be used to assay the four APIs in a combination drug tablet used to treat TB. This 10 min method saves time as well as mobile phase, compared to the two HPLC assay methods described in the USP monograph for this product. The analysis of two drug products yielded acceptable percentage contents, as judged by the limits in the USP monograph for the four-API drug product. Method accuracy was also tested by spiking standards in the sample placebos and measuring the recovery; there were good recoveries for both samples. The analysis time can be accelerated with the faster method. This separation was complete in < 2 min, providing high throughput sample analysis.

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http://en.wikipedia.org/wiki/Tuberculosis_treatment, accessed September 30, 2010.

United States Pharmacopeia, 32 NF 27, 2009.

Sensitive and Fast Determination of Endothall in Water Samples by IC-MS/MS

INTRODUCTION

Endothall is a widely used herbicide for both terrestrial and aquatic weeds. Major uses of endothall include defoliation of cotton, the control of aquatic weeds and algae, and as a dessicating agent for lucerne and potatoes. Human exposure to endothall in excess of the maximum contamination level (MCL) may cause gastrointestinal problems. Endothall is regulated by the United States Environmental Protection Agency (EPA) with an MCL at 0.1 mg/L or 100 ppb for drinking water;[1] and the California EPA developed the Public Health Goal (PHG) of 0.58 mg/L or 580 ppb for this compound.[2]

Current analytical methods for quantitation of endothall in water samples are described in EPA method 548.1[3] as gas chromatography with mass spectrometry or flame ionization detection (GC-MS or GC-FID). These methods involve ion-exchange solid phase extraction, sample enrichment, and dimethyl ester derivatization, followed by a 20 min GC separation and MS or FID detection.

This study describes the direct analysis of trace-level endothall in water samples by ion chromatography mass spectrometry (IC-MS). Water samples were directly injected without labor-intensive sample preparation and chromatographic separation was achieved in 10 min, thus significantly improving method throughput. An MS/MS instrument was operated in selected reaction monitoring (SRM) mode requiring minimal sample cleanup and ensuring highly sensitive (low ppb) and selective quantitation. Isotope labeled glutaric acid (glutaric acid–d_6) was used as an internal standard to ensure quantitation accuracy. This method has been successfully used for quantification of endothall in various water matrices including fresh creek water, lake water, and high salt content lake water. Method performance parameters such as linearity, calibration range, precision, accuracy, detection limits, and recovery were evaluated and will be presented in this application note.

EQUIPMENT

Dionex ICS-2000 or ICS-2100 Reagent-Free™ Ion Chromatography (RFIC™) system

TSQ Quantum Access™ triple quadrupole mass spectrometer

XCalibur® 2.1 with integrated Dionex Chromatography Mass Spectrometry Link (DCMS^{Link} ™) 2.8 for instrument control, data acquisition and processing, and report generation

Two Dionex AXP-MS auxiliary pumps

CHROMATOGRAPHIC CONDITIONS

Column: IonPac® AS16 and AG16 hydroxide selective anion-exchange columns (2 mm)

Column Temp.: 30 °C

Flow Rate: 400 µL/min

Eluent Source: EGC II KOH with Continuously-Regenerated Anion Trap Column (CR-ATC), 2 mm

Eluent: Hydroxide gradient

Time/min	*Conc./mM*
–4.0	15
0.0	15
5.0	15
6.0	80
9.0	80
9.5	15
10.0	5

Solvent: 200 µL/min acetonitrile delivered by an AXP-MS pump

Detection: 1st detector: Suppressed Conductivity with Anion Self-Regenerating Suppressor® (ASRS®), 2 mm (external water at 0.5 mL/min delivered by an AXP-MS pump)
2nd detector: TSQ Quantum Access Mass Spectrometer

MASS SPECTROMETRIC CONDITIONS

Interface: Negative Electrospray Ionization (ESI)

Spray Voltage: 3500 V

Sheath Gas: 50 Arbitrary units

Auxiliary Gas: 30 Arbitrary units

Capillary Temp.: 350 °C

Collision Gas: Argon at 1.5 mTorr

SRM Acquisition: 4.2 to 6 min

Operating Mode: Selected Reaction Monitoring (SRM)

Analyte	*Parent Ion (m/z)*	*Product Ion (m/z)*	*Collision Energy (V)*
Endothall-1	185	141	17
Endothall-2	185	123	19
Glutaric Acid-d_6	137	74	21

REAGENTS AND STANDARDS

Endothall standard solution, 1 mg/mL in methanol (AccuStandard P/N P-183S-10XT)

Isotope labeled internal standard (IStd) glutaric acid-d_6 (C/D/N Isotopes P/N D-5227)

Seven anion standard solution with various concentrations from 20 ppm (fluoride) to 150 ppm (sulfate and phosphate) (Dionex P/N 056933)

Acetonitrile (HPLC grade, Burdick & Jackson)

Deionized (DI) water with 18.2 MΩ-cm resistance (Millipore Coporation)

The chemical structures of endothall and isotope labeled internal standard glutaric acid-d_6 are shown in Figure 1.

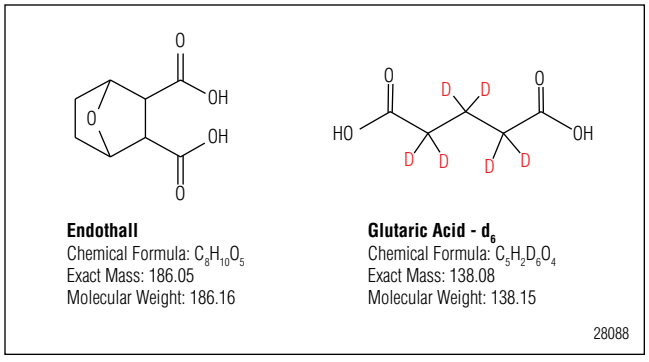

Figure 1. Chemical structures of studied compounds.

STANDARD PREPARATION

Dilute endothall standard in DI water to 10 ppm and 1 ppm as the two working standards to prepare calibration standards. Dilute the internal standard stock solution (1000 ppm) to 10 ppm for calibration standard preparation and for spiking unknown samples.

Prepare calibration standards by serial dilution from the working standards at 10 concentrations: 1 ppb, 2 ppb, 5 ppb, 10 ppb, 20 ppb, 50 ppb, 100 ppb, 200 ppb, 500 ppb, and 1000 ppb with the IStd spiked at 100 ppb for each concentration with the presence of diluted (100× dilution) seven common anions (fluoride 0.2 ppm, chloride 0.3 ppm, nitrite 1 ppm, bromide 1 ppm, nitrate 1 ppm, phosphate 1.5 ppm, and sulfate 1.5 ppm).

SAMPLE PREPARATION

Ground water samples were collected from a local creek and Lake Tahoe west shore. A lake water sample with a high salt content was kindly provided by a customer. These samples were spiked with IStd at 100 ppb and injected directly for ion chromatography tandem mass spectrometry (IC-MS/MS) quantification. (For samples with observed particulates, filtration is required prior to injection.) The salty water sample was diluted with DI water at a 1 to 10 ratio (v/v) due to the observed high concentration of ionic species.

RESULTS AND DISCUSSION

Chromatography

As shown in Figure 1, endothall is a dicarboxylic acid. IC is the preferred separation technique for ionic or ionizable compounds; therefore, it was selected in this study for the separation of endothall from interferences. For IC-MS analysis, the major interferences are inorganic matrix ions such as chloride and sulfate, and other ionizable organics at high concentration. The goals of chromatographic separation were to achieve sufficient chromatographic resolution for endothall from common anions, and to have a short run time to improve method throughput.

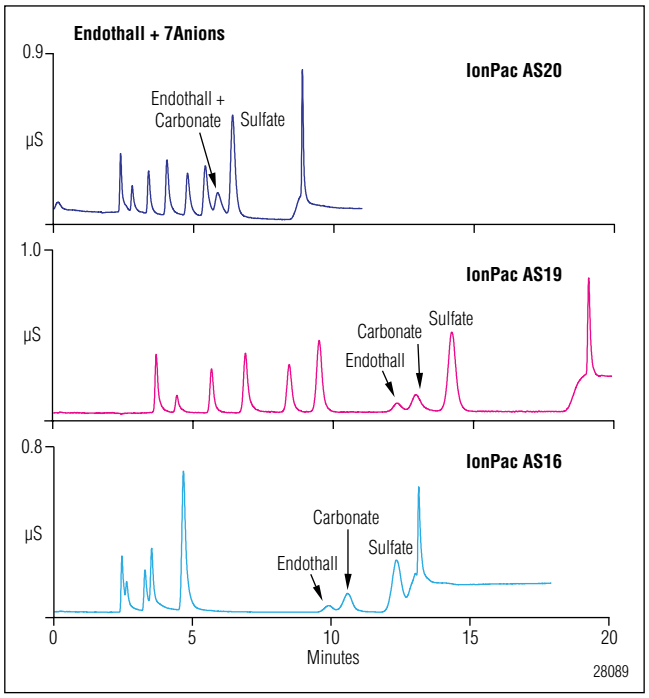

Figure 2. Column selection for endothall separation.

In this study, several high-capacity anion-exchange columns were evaluated for their selectivity; namely, the IonPac AS20, AS19, and AS16 columns. The IonPac AS16 column exhibited the best performance by: 1) providing a wide elution window for endothall between chloride and sulfate, and 2) requiring substantially less chromatographic run time than the IonPac AS19 column. Note that the IonPac AS19 column would be a better choice for simultaneous quantification of endothall and seven anion profiling, as it provided complete chromatographic resolution for all analytes (Figure 2).

CHROMATOGRAPHIC CONDITIONS

System:	ICS-2000 or ICS-2100 RFIC system
Column:	IonPac AS16 and AG16 (2 mm)
Column Temp.:	30 °C
Flow Rate:	400 µL/min
Eluent Source:	EGC II KOH with CR-ATC (2 mm)
Eluent:	Hydroxide gradient

Time/min	*Conc./mM*
–4.0	15
0.0	15
5.0	15
6.0	80
9.0	80
9.5	15
10.0	5

Solvent:	200 µL/min acetonitrile delivered by an AXP-MS pump
Detection:	1st detector: Suppressed Conductivity using ASRS 300 (2 mm) (external water at 0.5 mL/min delivered by an AXP-MS pump) 2nd detector: TSQ Quantum Access Mass Spectrometer

MASS SPECTROMETRIC CONDITIONS

System:	TSQ Quantum Access triple quadrupole mass spectrometer
Interface:	Negative Electrospray Ionization (ESI)
Spray Voltage:	3500 V
Sheath Gas:	50 Arbitrary units
Auxiliary Gas:	30 Arbitrary units
Capillary Temp.:	350 °C
Collision Gas:	Argon at 1.5 mTorr
SRM Acquisition:	4.2 to 6 min
Operating Mode:	Selected Reaction Monitoring (SRM)

Analyte	*Parent Ion (m/z)*	*Product Ion (m/z)*	*Collision Energy (V)*
Endothall-1	185	141	17
Endothall-2	185	123	19
Glutaric Acid-d_6	137	74	21

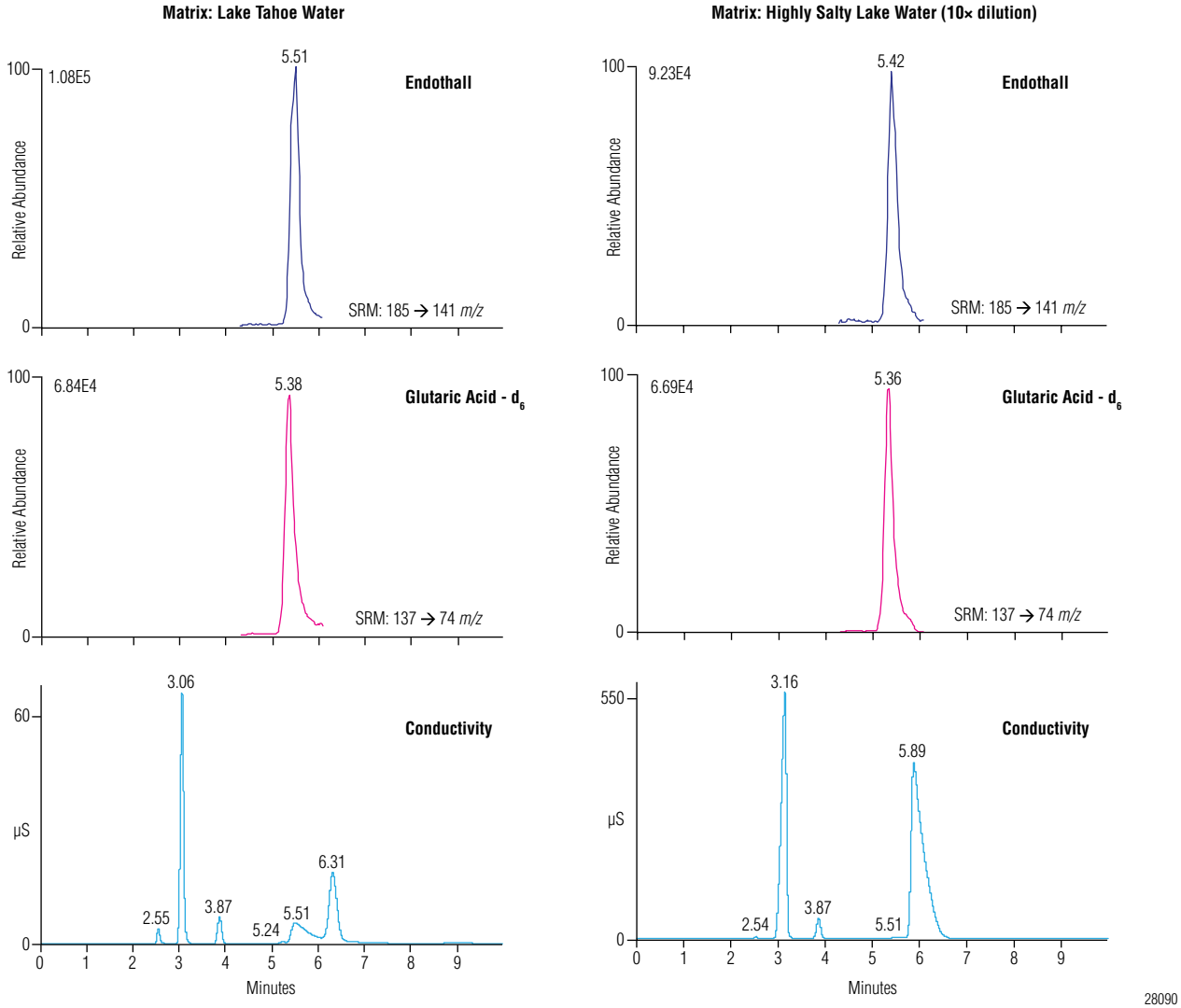

Figure 3. Conductivity and SRM chromatograms of endothall spiked in different samples. Left, 5 ppb endothall spiked in Lake Tahoe water; right, 50 ppb endothall in salty lake water with 10-fold dilution.

Mass Spectrometry

A TSQ Quantum Access triple quadrupole mass spectrometer was selected in this study for sensitivity and selectivity, which allowed minimum sample preparation such as cleanup and enrichment. A matrix diversion valve was used to divert high concentrations of inorganic anions to waste to prevent MS entrance fouling and to maintain long-term system stability. It is important to adjust the diversion window for specific matrices to ensure the analyte signal is collected by the MS and inorganic salts are diverted to waste.

As seen in Figure 3, two different matrices, Lake Tahoe water (LTW) and highly salty lake water (HSW), were spiked with endothall at 5 ppb and analyzed by this method. The HSW is a highly salty water sample (~1000 ppm sulfate, estimated by conductivity peak area), and the retention time of endothall in this 10× diluted matrix was slightly shifted (0.09 min earlier than in the LTW matrix). In addition, the retention time for sulfate shifted significantly earlier by 0.42 min; thus, the diversion window had to be adjusted accordingly to avoid MS entrance contamination. In rare situations where dilution of the original sample is not applicable, such as when endothall is present in very low concentration in a high salt matrix, the gradient program will have to be adjusted, i.e. decrease the gradient slope from 5 to 9 min, in order to increase the resolution of endothall from sulfate (as seen in Figure 2).

The quantitation of endothall was carried out in SRM mode, the precursor ion was observed as the deprotonated molecular ion $[M\text{-}H]^-$ at 185 *m/z*, and the predominant product ion was observed as $[M\text{-}H\text{-}CO_2]^-$ at 141 *m/z* and used as the quantitative SRM transition; another characteristic product ion was also observed as $[M\text{-}H\text{-}CO_2\text{-}H_2O]^-$ at 123 *m/z* with less intensity and was used as a confirmative SRM transition. Isotope labeled glutaric acid-d_6 was used as the internal standard due to its similarity in chemical structure and chromatographic retention to endothall.

Because the IC eluent is 100% aqueous post-suppression, acetonitrile was introduced as the desolvation solvent to assist desolvation/ionization and was delivered by an auxiliary pump at 0.2 mL/min.

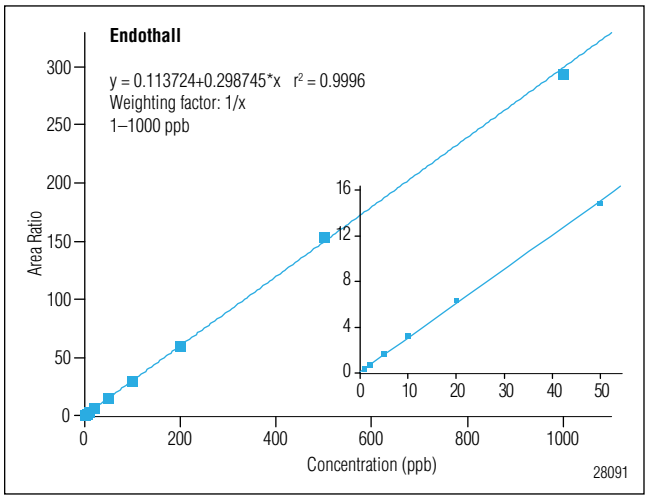

Figure 4. Calibration curve of endothall using glutaric acid-d_6 as an internal standard.

Method Performance

Typical conductivity and SRM chromatograms are shown in Figure 3. Sulfate, chloride, and nitrate were observed as the main anionic species in tested ground water, and were chromatographically separated from the target analyte, endothall, and the internal standard, glutarate-d_6. The MS/MS instrument operated in SRM mode provided sensitive and selective detection: each target analyte was observed as a single peak in each SRM channel with excellent intensity.

This method was used to determine endothall in various water samples mentioned in the Sample Preparation section, and no quantifiable level of target analyte was observed in any of the tested samples. Thus, these three matrices were used to evaluate the recovery from matrices.

Method performance was evaluated against quality parameters such as linearity, reproducibility, precision, accuracy, detection limits, and recovery from matrix. Calibration was performed by analyzing calibration standards at 10 concentrations from 1 to 1000 ppb using internal calibration with IStd at 100 ppb in each standard. Excellent linearity was achieved through three orders of magnitude with the coefficient of determination (r^2) at 0.9996. The calibration curve is shown in Figure 4, and the insert shows the calibration curve at lower concentrations.

Table 1. Recovery of Endothall from Three Matrices						
Sample	**5 ppb**			**500 ppb**		
	Mean	**Standard Deviation**	**% Recovery**	**Mean**	**Standard Deviation**	**% Recovery**
Creek Water	5.00	0.15	100	551	31.6	110
LTW	5.20	0.08	104	540	7.32	108
HSW	4.76	0.06	95.1	535	50.3	107

Precision and accuracy were evaluated by seven replicate assays of a standard at 5 ppb, with the calculated mean at 4.89 ppb (97.8% accuracy) with standard deviation at 0.18 (3.63% RSD). The method detection limit (MDL) was calculated following the equation MDL= s × *t*, where s is the standard deviation and *t* is the Student's *t* at 99% confidence interval. The calculated MDL is 0.56 ppb in DI water (n =7).

Recovery was evaluated by spiking three samples (Creek Water, LTW, and a HSW [10-fold dilution])at two levels: 5 ppb and 500 ppb with IStd spiked at 100 ppb. The results are summarized in Table 1.

CONCLUSION

A fast and sensitive IC-MS/MS method was presented for the quantitative determination of trace level endothall in environmental water samples. The detection limit was estimated at 0.56 ppb, and linear response was observed from 1 ppb to 1000 ppb. The significantly improved sensitivity enables direct analysis of water samples without labor intensive sample enrichment and derivatization. The total chromatographic run time was halved from the 20 min GC run in EPA Method 548.1.

REFERENCES

1. United States Environmental Protection Agency, *Basic Information about Endothall in Drinking Water*. http://water.epa.gov/drink/contaminants/basicinformation/endothall.cfm (accessed September, 2010).
2. Pesticide and Environmental Toxicology Section, Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, *Public Health Goal for ENDOTHALL in Drinking Water,* December 1997.
3. United States Environmental Protection Agency, *Method 548.1: Determination of Endothall in Drinking Water by Ion-Exchange Extraction, Acidic Methanol Methylation and Gas Chromatography/ Mass Spectrometry.* Available from http://www.epa.gov/waterscience/methods/method/files/548_1.pdf (accessed January 31, 2011).

Mass Spectrometry Compatible Separation of Itraconazole and Related Substances by UHPLC

INTRODUCTION

An improved analysis method is now available for itraconazole (structure shown in Figure 1A), one member of a family of triazole antifungal agents. Azoles share the same antifungal mechanism that prevents formation of the ergosterol necessary for the fungi's cell wall. As a broad spectrum antifungal, itraconazole is approved by the United States Food and Drug Administration (FDA) for systemic mycoses, onychomycoses, blastomycosis, histoplasmosis, and aspergellosis in patients who cannot tolerate amphotericin B.

The United States Pharmacopeia (USP) analysis method for itraconazole and its related substances uses high-performance liquid chromatography (HPLC) with single wavelength UV or diode array detection (DAD).[1] Tetrabutylammonium hydrogen sulfate (TBAHS) is added to the mobile phase to improve itraconazole retention and peak shape, possibly by decreasing the effect of the stationary phase's residual silanols. The analysis method for itraconazole from the China Pharmacopoeia (CP) is similar to the USP method.[2] Generally, any impurity of a synthetic drug that is greater than 0.1% must have its structure elucidated. This is often accomplished with the aid of mass spectrometry (MS) and many times with liquid chromatography-mass spectrometry (LC-MS). A MS-compatible mobile phase is needed for LC-MS, and the USP and CP mobile phases for the itraconazole analysis method are not MS compatible due to the TBAHS.

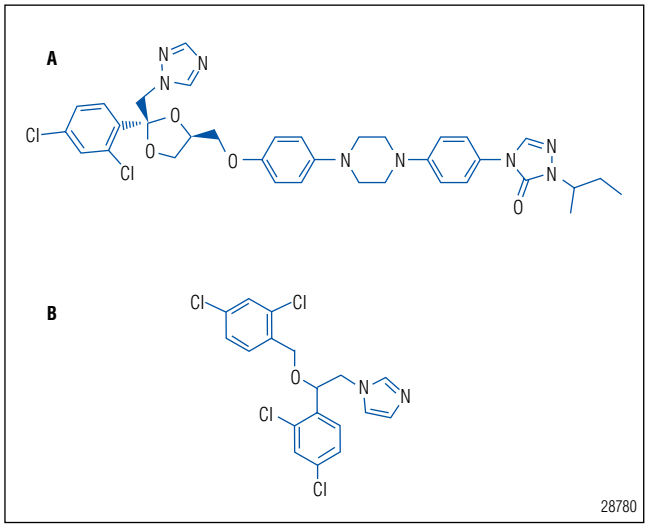

Figure 1. Structures of itraconazole A) and miconazole B).

Though a minor concern, quaternary ammonium salt ion-pairing agents can damage silica-based packing materials. In addition to the MS-incompatible mobile phase, the CP method for itraconazole analysis requires 50 min. Therefore, there are good reasons to try to improve the itraconazole analysis method.

A literature search reveals HPLC methods to determine the concentration of itraconazole and hydroxy-itraconazole in plasma using an MS-incompatible mobile phase.[3-5] Dionex published an on-line solid-phase extraction (SPE)-LC method for analysis of multiple drugs, including itraconazole, using ammonium acetate as the mobile phase buffer.[6]

All of these methods were designed to determine itraconazole in plasma samples and were not concerned with itraconazole impurities. In pharmaceutical companies, separation and quantitation of trace amounts of impurities are usually the focus of method development. The HPLC methods reported in the literature also did not determine miconazole (structure shown in Figure 1B), which is an important reference substance for resolution control in the pharmacopeia methods.

This work demonstrates an efficient program for analyzing itraconazole and its related substances without an ion-pairing agent. The study also investigates the chromatographic behavior of itraconazole and miconazole under different diluent constitutions and/or volumes. The effect of buffer constitution and concentration on peak shape is also discussed.

The separation was performed on the Thermo Scientific Acclaim® 120 C18 Rapid Separation LC (RSLC) column and Thermo Scientific Dionex UltiMate® 3000 HPLC system with DAD detection using a mobile phase comprised of an acetate buffer, water, and acetonitrile. The analysis was completed within 15 min. The method was also compatible with MS and was executed as an LC-MS method to identify the related substances.

EQUIPMENT

Dionex UltiMate 3000 quaternary Rapid Separation LC (RSLC) system including:

- LPG 3400RS Pump with a 350 µL mixer
- WPS 3000TRS Autosampler with a 100 µL sample loop
- TCC-3000 Thermostatted Column Compartment (preheater not used)
- DAD-3000RS Diode Array Detector with a 13 µL flow cell

Thermo Scientific MSQ Plus™ single quadrupole mass spectrometer (MS) with an ESI source

Thermo Scientific Dionex Chromeleon® 6.80 SR9 Chromatography Workstation

Mettler Toledo AL-204 analytical balance, Mettler-Toledo Instruments (Shanghai) Co., Shanghai, China

REAGENTS AND STANDARDS

Water, from Milli-Q® Gradient A 10

Acetonitrile (CH_3CN), HPLC grade, Fisher

Methanol (CH_3OH), HPLC grade, Fisher

Ammonium acetate (NH_4Ac), analytical grade, SCRC, China

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

Formic acid, analytical grade, SCRC, China

Tetrahydrofuran (THF), HPLC grade, SCRC, China

Tetrabutylammonium hydrogen sulfate (TBAHS), HPLC grade, J&K Scientific, China

Itraconazole (99.3%), NICPBP, China

Miconazole nitrate salt, Sigma

CHROMATOGRAPHIC CONDITIONS

Analytical Column:	Acclaim RSLC 120, C18, 2.2 µm, 2.1 × 100 mm, P/N 068982
Column Temp.:	30 °C
Mobile Phase:	HAc/NH_4Ac buffer to CH_3CN gradients (details shown in figures)
Flow Rate:	0.45 mL/min
Inj. Volume:	2 or 10 µL
UV Detection:	Absorbance at 225 nm

MSQ-PLUS MS CONDITIONS

Ionization Mode:	ESI
Operating Mode:	Positive Scan
Probe Temp.:	400 °C
Needle Voltage:	2000 V
Mass Range:	400–1000 amu
Scan Time:	0.3 sec
Cone Voltage:	80 V
Nebulizer Gas:	Nitrogen at 75 psi

PREPARATION OF STANDARDS

Stock Standard Solutions

Dissolve accurately weighed 10 mg of itraconazole in 10 mL diluent (a mixture of CH_3OH and THF, 4:1, v/v). The concentration of itraconazole is 1 mg/mL. Prepare a 1 mg/mL solution of miconazole in the same manner.

PREPARATION OF SAMPLES

Itraconazole Capsule Sample

Itraconazole capsules, made by Xian-Janssen Pharmaceutical LTD, China, were purchased from a local pharmacy.

Dissolve the accurately weighed contents of an itraconazole capsule equivalent to 50 mg itraconazole in 250 mL CH_3OH/THF mixture (4:1, v/v). (The capsule used in this work contained 100 mg of itraconazole, so half the weight of the capsule contents was used.) Sonicate to aid dissolution and filter the solution through a 0.45 µm filter (Millex®-HV) and store at room temperature.

Itraconazole Capsule Hydrolysis Sample

Dissolve the accurately weighed contents of an itraconazole capsule equivalent to 20 mg itraconazole (20% of the capsule contents for the sample used in this work) in 1 mL formic acid. Incubate the solution in a 60 °C water bath for 3 h. Dilute the solution with 9 mL CH_3OH/THF mixture (4:1, v/v). Store the sample at room temperature for at least 24 h before analysis.

RESULTS AND DISCUSSION

Development of an Improved Itraconazole Analysis Method

Both the itraconazole method from the CP (4.6 mm diameter column) and an improved method (2.1 mm diameter column) were used to separate itraconazole and its related substances (Figures 2A and 2B). The faster method separated at least as many itraconazole related substances as the CP method. Initially, the same sample volume and diluent used in the USP and CP methods were also used in the faster method, and the itraconazole peak exhibited fronting. The THF in the sample diluent seemed to impact peak fronting because the fronting increased when more THF was present in the diluent.

Miconazole—the resolution reference substance for itraconazole in the USP—fronted in the same manner as itraconazole when TBAHS was used in the mobile phase. In contrast, itraconazole was influenced more heavily than miconazole by the diluent constitution when NH_4Ac/HAc buffer replaced TBAHS in the solvent system. As shown in Figure 3, a 10 µL itraconazole injection in a 1:1 methanol:THF diluent led to obvious itraconazole peak fronting, whereas no fronting was observed for miconazole. When the composition of the dilute was changed to 4:1 methanol:THF, fronting was eliminated. Reducing the injection volume to 2 µL eliminated peak fronting even when 1:1 methanol:THF was used.

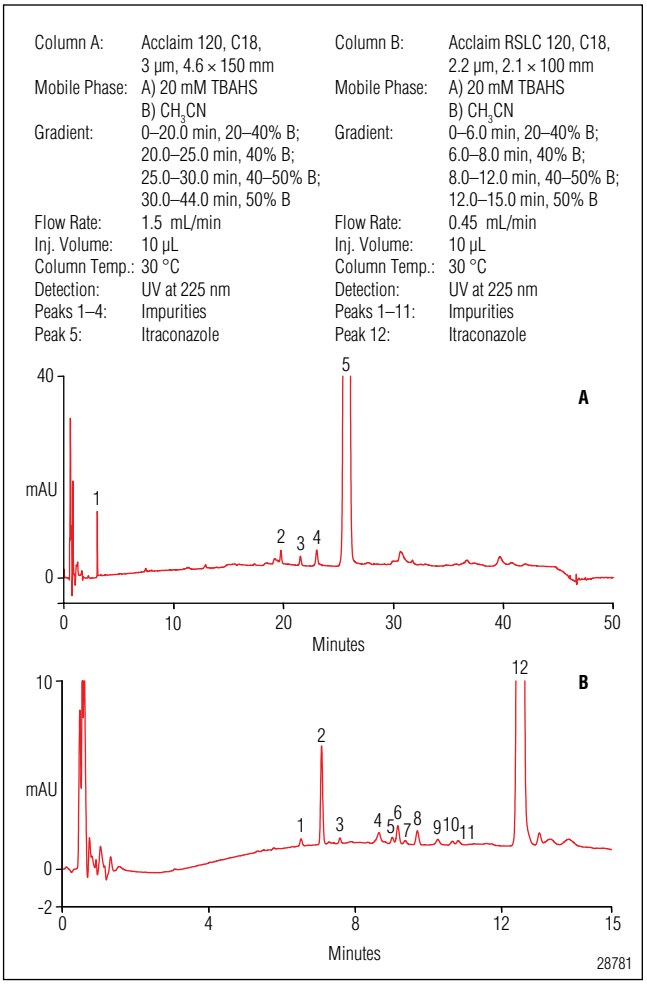

Figure 2. Chromatograms of an itraconazole standard using A) method from the CP and B) modified method with a shorter run time.

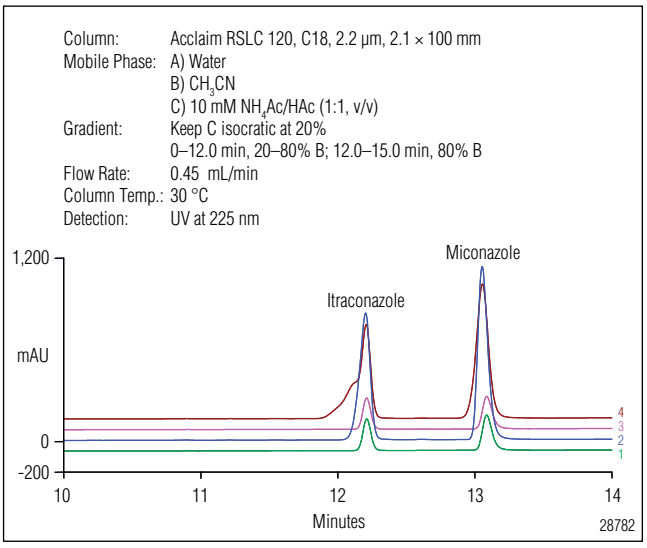

Figure 3. Overlay of chromatograms of itraconazole and miconazole using diluents as 1) CH_3OH and THF (4:1, v/v) with 2 µL injection; 2) CH_3OH and THF (4:1, v/v) with 10 µL injection; 3) CH_3OH and THF (1:1, v/v) with 2 µL injection; and 4) CH_3OH and THF (1:1, v/v) with 10 µL injection.

Effect of pH on Retention Times and Peak Shapes of Itraconazole and Miconazole

The pH of the mobile phase was adjusted by changing the ratio of HAc to NH_4Ac. HPLC analysis was performed using the following ratios of HAc to NH_4Ac: 19:1, 15:5, 10:10, 5:15, and 1:19. The retention time of miconazole decreased significantly when the solvent system was more acidic (Figure 4). The asymmetry of miconazole peak increased as retention time decreased. In contrast, neither itraconazole retention time nor peak shape were affected significantly by mobile phase pH.

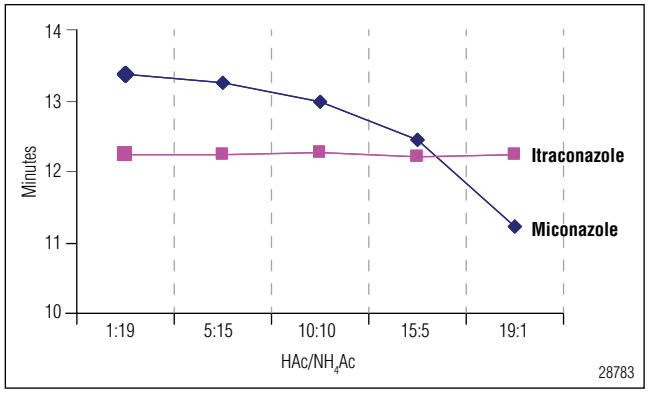

Figure 4. Comparison of retention times of itraconazole and miconazole at different pH values (buffer concentration: 20 mM). The corresponding pH values of the HAc/NH_4Ac buffer are 5.92, 5.15, 4.54, 4.25, and 3.26.

Effect of Buffer Concentration on Peak Shape of Itraconazole and Miconazole

One objective of this work was to develop a method that removed TBAHS from the mobile phase. Initially this was attempted using only water and acetonitrile in bottles A and B, respectively. Without TBAHS, the miconazole peak disappeared whereas itraconazole remained intact. A gradually increasing concentration of buffer (NH_4Ac/HAc) was added to the mobile phase to improve miconazole peak shape. Miconazole, with its basic moiety as imidazole, is a medium-strong base. In contrast to itraconazole (which is a very weak base, considering its 1,2,4-triazole structure), miconazole may have stronger interaction with silanol residues. This explains why a higher concentration of TBAHS is needed to keep miconazole peak shape, compared to itraconazole. TBAHS can decrease the effect of silanol residues by either TBA–silanol interaction or the acidic effect of the HSO_{-4} to inhibit silanol proton dissociation. As shown in Figures 5A and 5B, the buffer concentration significantly affected the miconazole peak shape. A low buffer concentration of 0.1 mM was enough to keep the itraconazole peak intact whereas the miconazole peak shape was distorted.

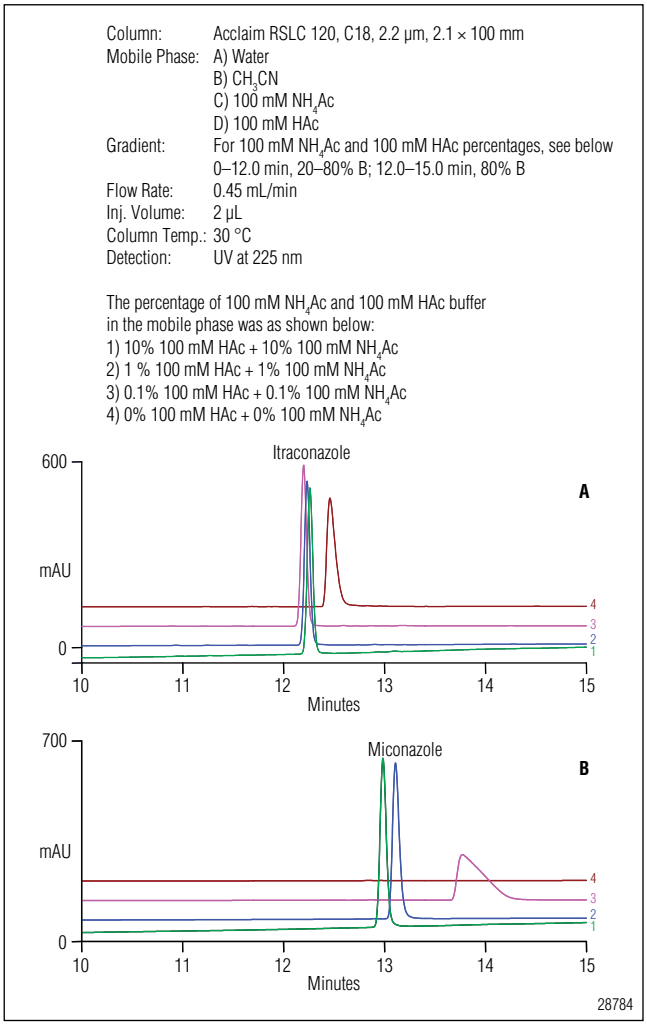

Figure 5. Overlay of chromatograms of A) itraconazole and B) miconazole using a mobile phase with different buffer concentrations.

Separation of Itraconazole and Its Related Substances

After establishing the improved method that eliminated the ion-pairing agent, an itraconazole standard, a miconazole standard, an itraconazole acid hydrolysis sample, and a commercial itraconazole capsule were analyzed (Figure 6A). The negative sloping baseline is attributed to the different absorption coefficient of ammonium acetate buffer (bottle A) and acetonitrile (bottle B) at 225 nm. The USP uses miconazole as a resolution control whereas the CP does not have a similar resolution control. As an alternative for resolution control, itraconazole was intentionally acid hydrolyzed to generate impurities that can be used as controls to judge resolution.[2] For comparison, the same samples were also analyzed by the method with the ion-pairing agent (Figure 6B).

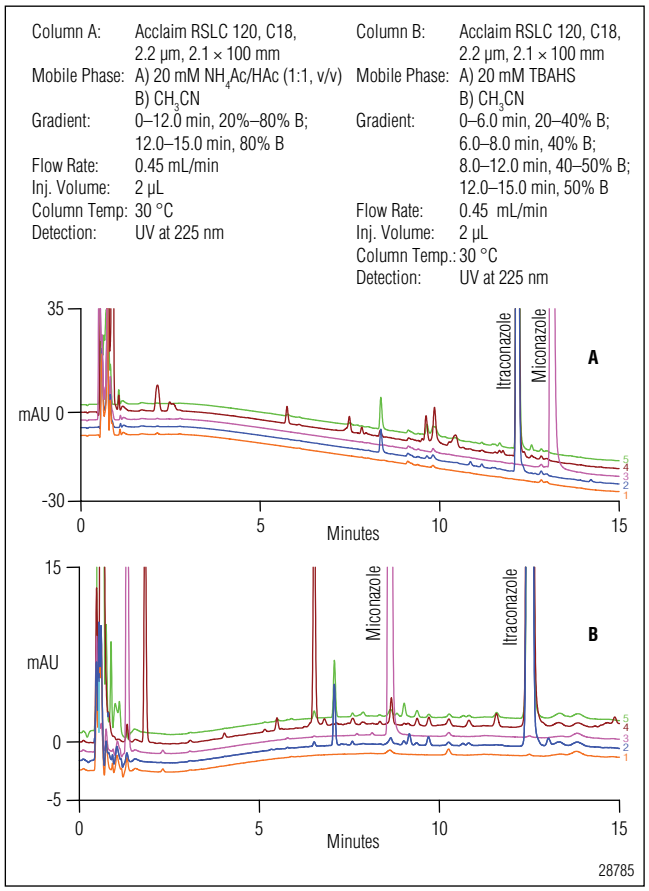

Figure 6. Overlay of chromatograms of 1) solvent A, 2) itraconazole standard, 3) miconazole standard, 4) itraconazole acid hydrolysis, and 5) itraconazole capsule sample with A) NH_4Ac/HAc and B) TBAHS in the mobile phase.

Sample Analysis by LC-MS

The USP describes seven impurities in the itraconazole standard. They are the 4-methoxy derivative, the 4-triazolyl isomer, the propyl analog, isopropyl analog, an itraconazole epimer, the n-butyl isomer, and the didioxolanyl analog with *m/z* of 408, 705, 691, 691, 705, 705, and 960 respectively.[1] As shown in Figure 7, two peaks with *m/z* of 705.1 were observed in itraconazole by analysis in the total ion chromatogram (TIC) mode. In the more sensitive selected ion monitoring (SIM) mode, four peaks with *m/z* of 705.1 were observed, which correlated to itraconazole, the 4-triazolyl isomer, the epimer, and the n-butyl isomer. Also in TIC mode, two peaks with *m/z* of 691.1 were found which matched perfectly with the propyl and isopropyl analogs of itraconazole.[1]

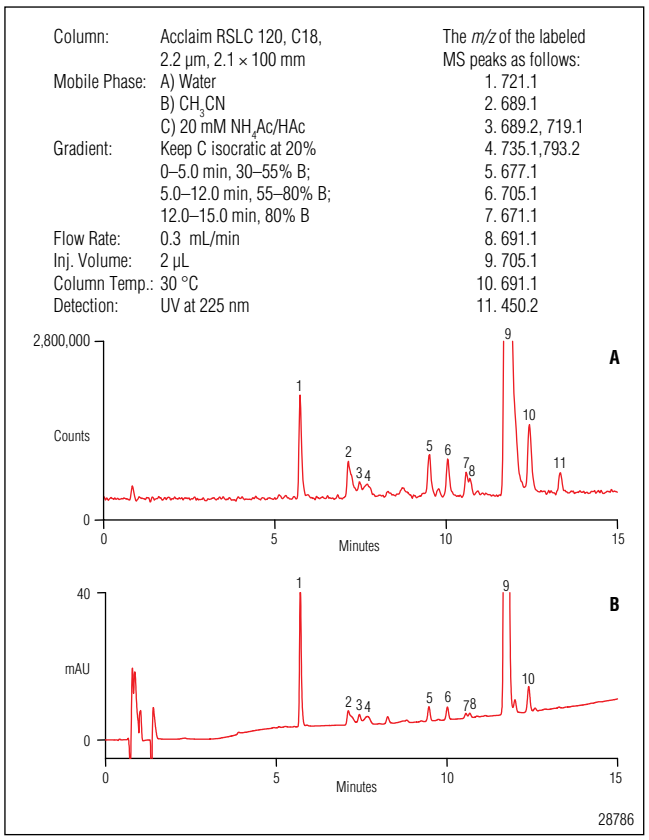

Figure 7. A) MS (TIC) chromatogram and B) UV chromatogram of the itraconazole standard.

All USP-indicated *m/z* values were observed using the LC-MS method in SIM mode. Due to the abundance of ^{37}Cl as 32.5% of ^{35}Cl, a specific isotopic MS distribution pattern was expected for itraconazole, which contains two chlorines. Theoretically, the height of the 707.1 peak would be 65% of the 705.1 peak, and the 709.1 peak height would be 10.5% of the 705.1 peak. This prediction was fully confirmed by the data shown in Figure 8.

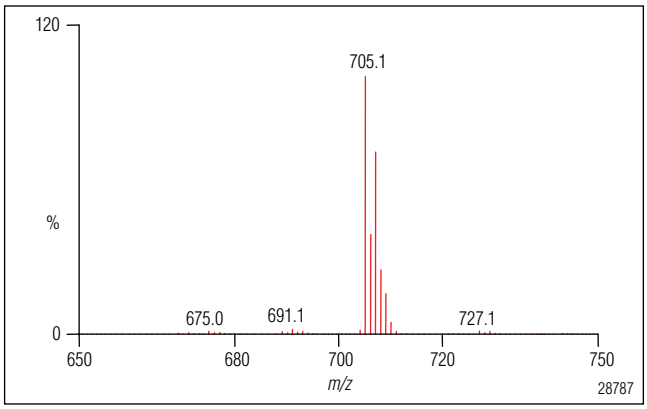

Figure 8. Isotopic MS peak distribution of itraconazole.

CONCLUSION

This application replaces a lengthy HPLC method that includes an ion-pairing agent with an efficient MS-compatible method that saves valuable time and resources in the analysis of itraconazole and its related substances. The Dionex Acclaim RSLC C18 column exhibits similar separation ability for itraconazole-related substances using NH_4Ac/HAc buffer in mobile phase as when using TBAHS. Sample diluent constitution, volume, solvent pH, and solvent ionic strength affect the chromatography of either itraconazole, miconazole, or both; therefore, these parameters were optimized to yield the best chromatography for both compounds. Use of LC-MS detected all the impurities indicated by USP including isomers having the same molecular weight.

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Determination of Ethyl Sulfate Impurity in Indinavir Sulfate Drug Using Ion Chromatography

INTRODUCTION

Indinavir sulfate is a specific and potent inhibitor of HIV-1 protease and is widely used in the treatment of AIDS.[1] Indinavir sulfate is prepared by dissolving indinavir base in anhydrous ethanol that is then treated with sulfuric acid. At ambient temperature, a mixture of ethanol and sulfuric acid reacts to yield monoethylsulfate (ethyl sulfate). Therefore, treatment of indinavir base in anhydrous ethanol with sulfuric acid is performed at a controlled temperature of less than 0 °C to avoid the formation of ethyl sulfate.[1] However, it is very important for manufacturers of drug products to monitor the level of anticipated process-related and degradation impurities before commercial release to prove the consistency of the manufacturing process. It is also important to monitor drug stability during storage.

Ion chromatography (IC) has been successfully used to measure ionic drug degradation products and process-related impurities. *N*-methylpyrrolidine, a degradation product of cefipime, has been measured in cefipime and simulated Cefipime for Injection.[2,3] In addition, IC also has been used determine the process impurity ethylhexanoate in clavulanate.[4]

The work here shows an IC method to measure ethyl sulfate in indinavir sulfate. This method uses a Thermo Scientific Dionex IonPac® AS12A column with a carbonate/bicarbonate eluent to separate ethyl sulfate from other anions, including sulfate. The eluent was produced using an eluent generator to improve ease of use and retention time reproducibility. Reproducible chromatography is demonstrated without the labor or possible error of eluent preparation, and without the need to prepare sulfuric acid solutions for suppressed conductivity detection.

EQUIPMENT

Thermo Scientific Dionex ICS-3000 or Dionex ICS-5000 system including:*

- DP Dual Pump
- DC Detector/Chromatography module with dual-temperature zone equipped with 6-port injection valves
- EG Eluent Generator module
- EPM Electrolytic pH Modifier

Thermo Scientific Dionex Chromeleon® Chromatography Data System software, Version 6.80, SR9

*A Dionex ICS-2100 or other Dionex IC system featuring eluent generation also can be used. Alternately, if the eluents are manually prepared, any Dionex IC system can be used.

REAGENTS AND STANDARDS

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

Dionex Seven Anion Standard II (P/N 57590)

Sulfuric acid, 98% (H_2SO_4, Merck)

Ethanol (Absolute), AR grade (C_2H_5OH, RCI Labscan)

PREPARATION OF SOLUTIONS AND REAGENTS

Carbonate (2.7 mM)/Bicarbonate (0.3 mM) Eluent

Generate carbonate/bicarbonate eluent on-line by pumping high-quality DI water (18 MΩ-cm resistivity or better) through the Thermo Scientific Dionex EluGen EGC II K_2CO_3 Cartridge and EPM. Use Chromeleon software to control the concentration of carbonate/bicarbonate. Add backpressure tubing to achieve 2000–2300 psi system backpressure, which allows the EG degasser to work properly. Refer to the Dionex ICS-3000 operator's manual for how to add backpressure. Alternately, prepare the eluent manually by pipetting 5.4 mL of 0.5 M Na_2CO_3 (P/N 037162) and 0.6 mL of 0.5 M $NaHCO_3$ (P/N 037163) into a 1 L volumetric flask and dilute to volume with DI water.

Eluent generation was used to produce the data in this study.

Ethyl Sulfate Stock Solution

Transfer 0.5 mL ethanol into a 100 mL volumetric flask. Slowly add 0.5 mL 98% sulfuric acid to the same volumetric flask. Gently swirl the flask to mix and bring to volume with DI water. Note: this preparation must be performed in a fume hood while wearing goggles and protective clothing.

Mixture of Seven Anion Secondary Standard

Add 0.5 mL Dionex Seven Anion Standard Stock Solution to a 10 mL volumetric flask and bring to volume with DI water.

Ethyl Sulfate Standard Solution

Add 0.1 mL ethyl sulfate stock solution to a 25 mL volumetric flask and bring to volume with DI water.

Sample Preparation

Dissolve a capsule of the indinavir sulfate drug in a 10 mL volumetric flask with DI water. Filter the sample solution with a 0.45 µm syringe filter.

CONDITIONS

Column:	Dionex IonPac AS12A (4 × 200 mm) (P/N 046034)
Guard:	Dionex IonPac AG12A (4 × 50 mm) (P/N 079801)
Eluent Source:	Dionex EluGen EGC II K_2CO_3 cartridge (P/N 058904) with EPM Electrolytic pH Modifier and EGC Carbonate Mixer, 4 mm (P/N 079943)
Eluent:	2.7 mM Carbonate/0.3 mM bicarbonate
Flow Rate:	1.5 mL/min
Inj. Volume:	20 µL
Column Temp.:	30 ºC
Pressure:	~2300 psi
Detection:	Suppressed conductivity, Thermo Scientific Dionex ASRS® 300, 4 mm (P/N 064554), recycle mode, current 25 mA
Background:	~11 µS

RESULTS AND DISCUSSION

Ethyl Sulfate Production

Ethyl sulfate is not commercially available, but it can be easily produced in the laboratory by mixing sulfuric acid and ethanol.[5] Ethyl sulfate was prepared as described in the Ethyl Sulfate Stock Solution section, and ultimately used to prepare the Ethyl Sulfate Standard Solution used for the chromatography in this study. IC was used to judge the success of ethyl sulfate production. The Ethyl Sulfate Standard Solution was injected into the IC system and the chromatography was compared to ethanol and sulfuric acid (diluted in the same manner as was the Ethyl Sulfate Stock Solution to prepare the Ethyl Sulfate Standard Solution [Figure 1]). The Ethyl Sulfate Standard Solution exhibited two peaks: one at approximately 5 min and another at the retention time of sulfate. No peaks were found in the ethanol solution and only a peak corresponding to sulfate was found in the sulfuric acid solution. Therefore, the peak at approximately 5 min was assumed to be ethyl sulfate.

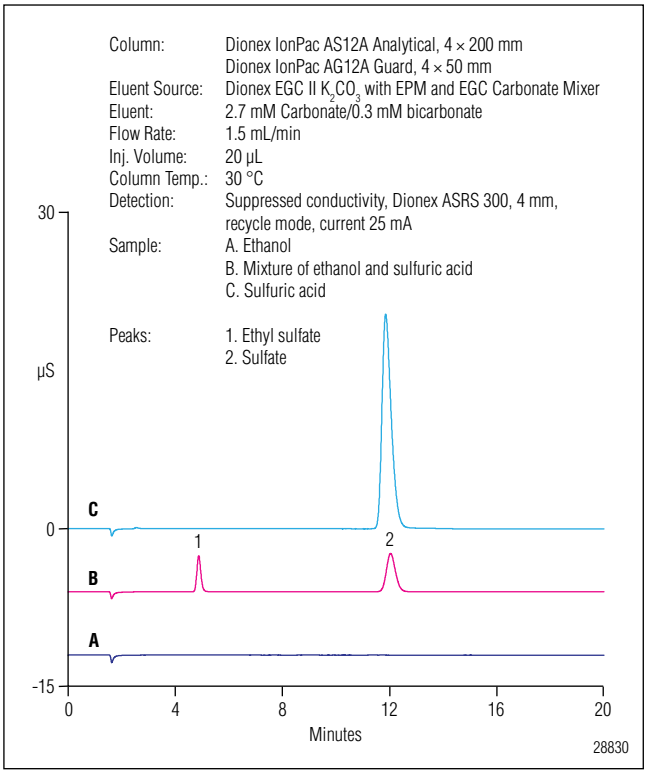

Figure 1. Overlay of chromatograms of ethanol, sulfuric acid, and a mixture of ethanol and sulfuric acid.

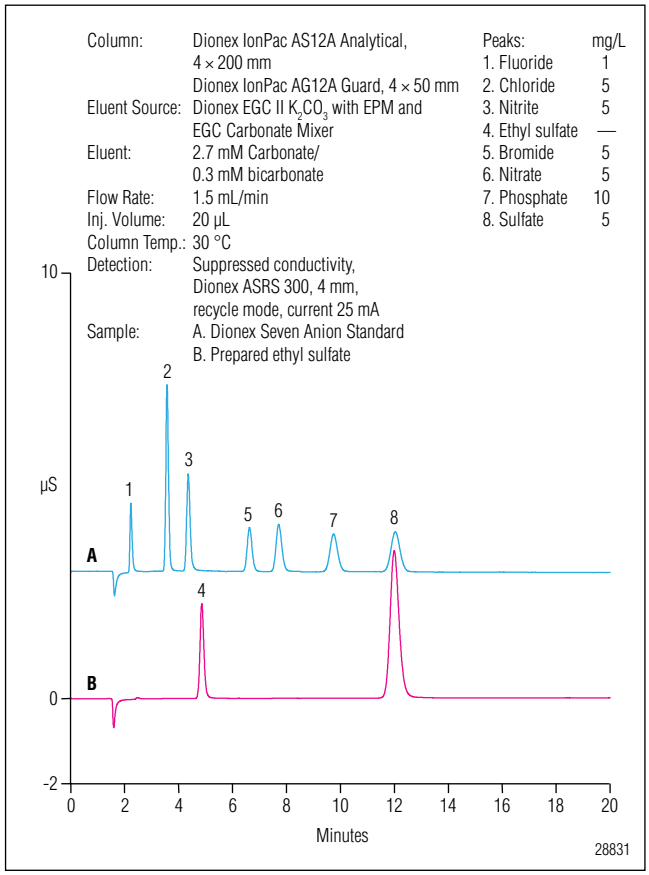

Figure 2. Chromatograms of a Dionex Seven Anion Standard and the prepared ethyl sulfate.

Separation

The Dionex IonPac AS22, AS23, and AS12A columns were evaluated to determine the best separation of ethyl sulfate from the other inorganic anions. The Dionex IonPac AS12A column provided the best separation. Figure 2 shows chromatograms of a mixture of seven common inorganic anions and the prepared Ethyl Sulfate Standard Solution. Note that ethyl sulfate was well resolved from the other anions. The Ethyl Sulfate Standard Solution was injected five times to evaluate stability. The peak area was stable and there was no noticeable increase or decrease in retention time (Table 1).

Table 1. Area of Ethyl Sulfate Standard Solution

Injection No.	Area (µS*min)
1	0.3793
2	0.3736
3	0.3736
4	0.3836
5	0.3761
Average	**0.3772**
% RSD	**1.14**

Sample Analysis

A customer kindly provided the indinavir sulfate final drug product in the form of capsules that had been stored on a shelf for a long period of time prior to the analysis reported here. Five capsules of sample were prepared and each sample was injected five times into the IC system. Figure 3 shows the chromatography of one of the capsule samples. The putative ethyl sulfate peak was well resolved from the other peaks in the sample. Spiking the sample solution with the Ethyl Sulfate Standard Solution confirmed that the peak in the sample had the same retention time as the peak in the standard (Figure 4). The position of the peak tentatively identified as ethyl sulfate relative to sulfate and other peaks in the capsule sample was also consistent with a peak identified as ethyl sulfate in a publication that uses a longer IC method.[1] Peak area data from the 25 sample injections are shown in Table 2. These data show that ethyl sulfate was found in all five capsules and the peak area response was reproducible. There was also a consistent amount of ethyl sulfate in the five capsules.

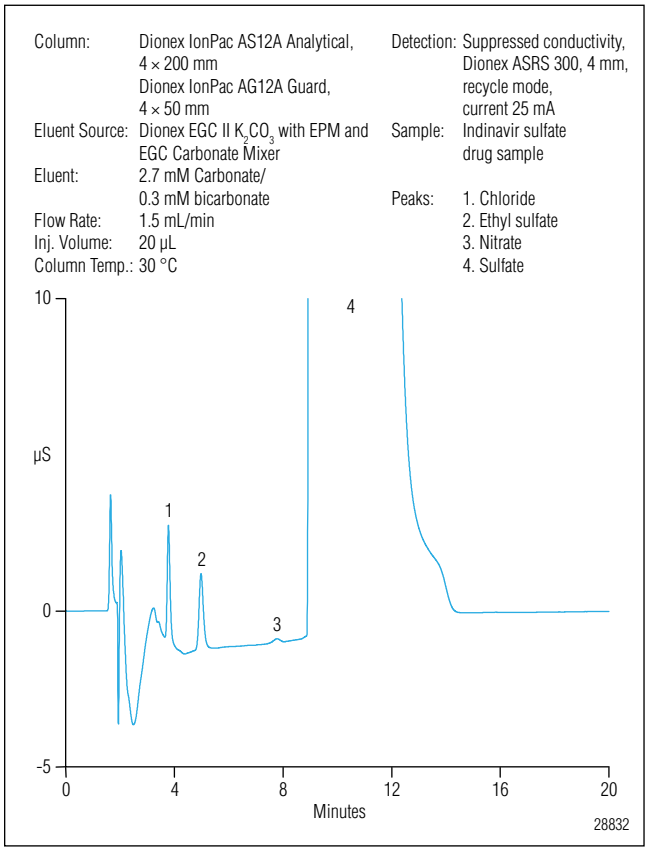

Figure 3. Chromatogram of the indinavir sulfate drug sample.

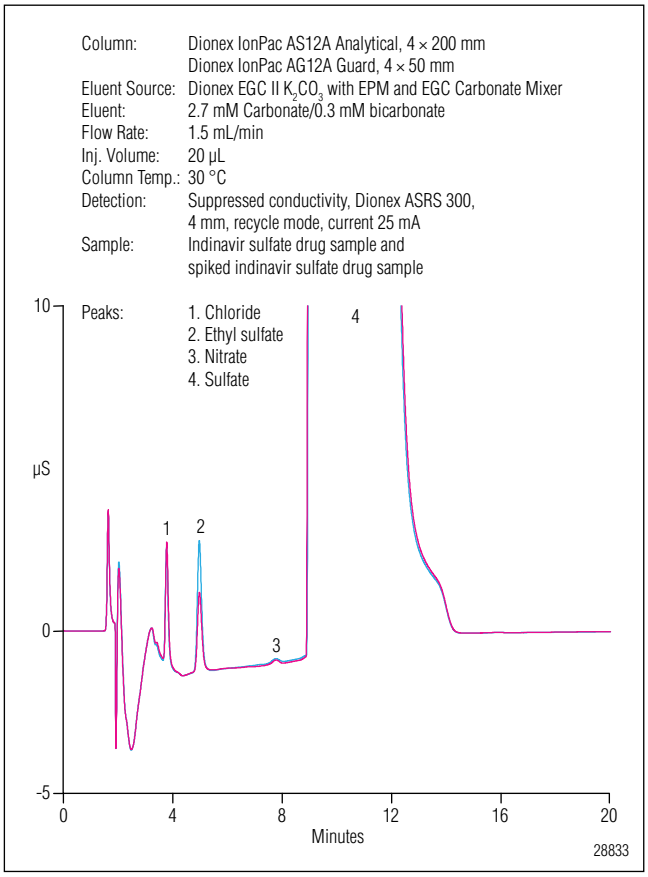

Figure 4. Overlay chromatograms of the indinavir sulfate and spiked indinavir sulfate sample.

Table 2. Area of Ethyl Sulfate in Samples

Injection No.	Area (µS*min)				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	0.5063	0.5189	0.4852	0.5037	0.4988
2	0.5015	0.5104	0.4812	0.4941	0.4994
3	0.4993	0.5115	0.4816	0.4930	0.4953
4	0.5098	0.5083	0.4827	0.4870	0.4919
5	0.5054	0.5087	0.4807	0.4887	0.4895
Average	**0.5045**	**0.5116**	**0.4823**	**0.4933**	**0.4950**
RSD	**0.82%**	**0.84%**	**0.38%**	**1.32%**	**0.87 %**

CONCLUSION

This work shows IC separation of ethyl sulfate in an indinavir sulfate drug sample. This method only requires the addition of DI water to the IC system to deliver reproducible chromatography while avoiding the time, labor, and potential error of manual eluent preparation. This method uses an electrolytic suppressor in recycle mode, and therefore does not require the analyst to prepare sulfuric acid solutions for eluent suppression prior to conductivity detection.

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Evaluation of the USP Risedronate Sodium Assay

INTRODUCTION

Bisphosphonates are a class of molecules that consist of two phosphonate groups linked to a carbon atom. These molecules are analogues of pyrophosphates that were found to be biologically active in 1969[1] and currently these analogues are widely used in the treatment of bone-resorption diseases, such as osteoporosis.[2] Three generations of bisphosphonate products have been developed for the treatment of bone-resorption diseases; each generation more potent than its predecessor. Risedronate is a third-generation bisphosphonate, which is 5000 times more potent than a first-generation bisphosphonate.[3]

Risedronate (Figure 1) is a pyridinyl bisphosphonate and is one of the most popular first-line drugs available for the prevention and treatment of osteoporosis, a debilitating disease estimated by the National Osteoporosis Foundation to affect 61 million Americans age 50 and older by 2020.[4] Risedronate is available as a tablet for oral administration that contains the equivalent of 5, 30, 35, or 75 mg of anhydrous risedronate sodium in the hemi-pentahydrate form with small amounts of the monohydrate. In October 2010, a second-generation risedronate in a delayed-release formulation was approved by the U.S. Food and Drug Administration (FDA).

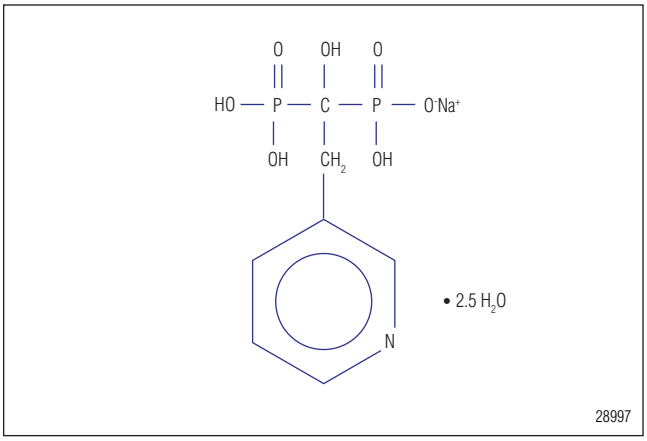

Figure 1. Risedronate sodium hemi-pentahydrate chemical structure.

Risedronate poses analytical challenges for reversed-phase (RP) high-performance liquid chromatography (HPLC) due to the presence of two polar phosphonate groups. This makes retention on commonly used RP columns (e.g., the Thermo Scientific Acclaim™ 120 C18 column) difficult. In addition, the metal chelation property of risedronate can cause poor peak shape and analyte recovery in systems that are not metal-free. Chelating agents, such as edetate disodium (EDTA), are added to the mobile phase to prevent metal contaminants from chelating with risedronate.

In addition, ion-pairing reagents typically are added to the mobile phase to enable retention and separation on a RP column. However, ion-pairing RP methods typically are not as robust as ion-exchange methods.[5] The multiple interactions between the ion-pairing reagent and the different phases, longer equilibration times, and the need for dedicating columns for specific ion-pair applications, increases the complexity of ion-pairing methods compared to ion-exchange methods.

Assay of the active pharmaceutical ingredient (API) and determining the presence of impurities and other related compounds is critical to ensure the formulation is safe and effective. The U.S. Pharmacopeia (USP) monograph describes an ion chromatography (IC) method to assay risedronate in the drug substance and product (Actonel, 35 mg tablets). This study evaluates and validates the USP monograph method for risedronate analysis and identifies the critical points of the method for successful chromatography.

The method specifies the USP L48 column with an EDTA eluent at pH 9.5 ± 0.1 followed by UV detection at 263 nm.[6] The Thermo Scientific Dionex IonPac™ AS7 column meets the description of USP L48[7] and is used in this method. EDTA in the mobile phase prevents risedronate from chelating with metals and improves peak shape. The Dionex IonPac AS7 column is a high-capacity, high-efficiency, hydrophobic, anion-exchange column specifically developed for the analysis of a wide range of polyvalent anions, including polyphosphonates, hexavalent chromium, cyanide, and iodide. This study demonstrates the linearity, detection limits, precision, and accuracy of the assayed amount of risedronate in the drug substance and drug product, thus showing the method to be simple, rugged, and accurate.

EQUIPMENT

Thermo Scientific Dionex ICS-3000 system* including:

SP Single Pump or DP Dual Pump module

DC Detector/Chromatography module

(single- or dual-temperature zone configuration)

AS Autosampler with 2 mL vial tray (PN 062481)

Dionex ICS Variable Wavelength Detector (VWD), single or multiple wavelength with deuterium lamp (P/N 066347) and 11 µL, 10 mm path length PEEK™ cell (P/N 066346)

Thermo Scientific Dionex Chromeleon™ software, 6.8 or higher

Polypropylene injection vials with caps, 1.5 mL (Vial Kit, P/N 061696)

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

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

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

*A Dionex ICS-5000 system can also be used for this application.

REAGENTS AND STANDARDS

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

pH buffer, 7.00 (VWR P/N BDH5046-500ML)

pH buffer, 10.00 (VWR P/N BDH5072-500ML)

Risedronate sodium, hemi-pentahydrate, USP RS

$C_7H_{10}NNaO_7P_2 \bullet 2.5\ H_2O$, F.W. 350.13 (US Pharmacopeia Cat # 1604610)

USP Risedronate Related Compound A RS,

F.W. 283.12 (US Pharmacopeia Cat # 1604621)

USP Risedronate Related Compound C RS,

F.W. 267.11 (US Pharmacopeia Cat # 1604643)

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

Edetate disodium [EDTA, disodium salt, dihydrate USP grade (VWR Cat # 1395-04)]

Samples

Drug substance: Risedronate sodium, hemi-pentahydrate

Drug product: Tablets containing 35 mg risedronate sodium per tablet.

Samples were donated by a pharmaceutical company.

CONDITIONS

Column:	Dionex IonPac AS7 analytical, 4 × 250 mm (P/N 035393) Dionex IonPac AG7 guard, 4 × 50 mm (P/N 035394)
Mobile Phase:	4.8 mM EDTA, pH = 9.5 ± 0.1
Flow Rate:	0.8 mL/min
Inj. Volume:	20 µL
Temperature:	25 °C
Detection:	UV absorbance, 263 nm
System Backpressure:	~1430 psi
Noise:	~ 0.016 mAU
Run Time:	20 min

PREPARATION OF SOLUTIONS AND REAGENTS

Sodium Hydroxide, 5 M

To prepare 5 M sodium hydroxide (NaOH) solution, pipette 26.2 ml of 50% w/w NaOH into a 100 mL polypropylene volumetric flask containing approximately 50 mL of DI water, then dilute to the mark with DI water. Stir gently to mix well.

Mobile Phase

Weigh and transfer 1.80 g of EDTA to a 1 L polypropylene bottle and dilute to a final weight of 1000 g with degassed DI water. Mix the solution until all the EDTA is dissolved. Adjust the pH of the resulting 4.8 mM EDTA disodium solution to 9.5 ± 0.1 by adding 1.16 mL of 5 M NaOH solution. Set aside 500 mL of the mobile phase to use as diluent.

PREPARATION OF STANDARDS AND SAMPLE SOLUTIONS

Risedronate sodium can be either a monohydrate containing one molecule of water or a hemi-pentahydrate containing 2.5 molecules of water. The hemi-pentahydrate form has 12.9% water, which equals 0.871 g of anhydrous risedronate sodium per 1.0 g of risedronate sodium hemi-pentahydrate. This must be considered when preparing the standards and samples on an anhydrous basis. All standard and sample solutions are stable for at least 30 days when stored at 4 °C.

DRUG SUBSTANCE ASSAY

USP Related Compound A Stock Solution

Weigh 10.0 mg of compound A into a 20 mL polypropylene bottle and tare the balance. Add 10.0 g of diluent to make 1.0 mg/mL USP risedronate-related compound A stock solution. Close the bottle cap and agitate using a vortex mixer for approximately 1 min to dissolve the solid material.

USP Risedronate Standard

Weigh 11.5 mg of USP risedronate sodium hemi-pentahydrate into a 20 mL polypropylene bottle and tare the balance. Add 1.0 g of 1 mg/mL USP related compound A stock solution and bring the final weight to 10.0 g with diluent. Close the bottle cap and agitate using vortex mixer for 1 min to obtain a homogenous solution of 1.0 mg/mL anhydrous risedronate sodium with 0.1 mg/mL of USP related compound A.

Assay Preparation

Weigh 11.5 mg of risedronate sodium hemi-pentahydrate sample into a 20 ml polypropylene bottle and tare the balance. Add 10 g of diluent to make a sample solution of approximately 1.0 mg/mL anhydrous risedronate sodium. Agitate using a vortex mixer for approximately 1 min to dissolve the solid material.

DRUG PRODUCT ASSAY

USP Risedronate Sodium Stock Standard

Weigh 11.5 mg of USP risedronate sodium hemi-pentahydrate into a 20 mL polypropylene bottle, tare the balance, and add 10.0 g of diluent. Close the bottle cap and agitate using vortex mixer for 1 min to obtain a homogenous solution of 1.0 mg/mL anhydrous risedronate sodium.

USP-Related Compound C Stock Solution

Weigh 10.0 mg of USP risedronate-related compound C into a 20 mL polypropylene bottle and tare the balance. Add 10.0 g of diluent to obtain 1.0 mg/mL USP risedronate-related compound C stock solution. Close the bottle cap and agitate using vortex mixer for approximately 1 min to ensure the solid is completely dissolved.

System Suitability Standard

Transfer 1.5 mL of risedronate sodium stock solution and 75 µL of USP risedronate-related compound C stock solution into a 20 mL polypropylene bottle, tare the balance, and add 10.0 g of diluent. Close the bottle cap and agitate using vortex mixer to obtain a homogenous solution of 0.15 mg/mL anhydrous risedronate sodium with 7.5 µg/mL of related compound C.

USP Risedronate Sodium Working Standard

Transfer 1.0 mL of risedronate sodium stock solution into a 20 mL polypropylene bottle, tare the balance, and bring the final weight to 10.0 g with diluent. Close the bottle cap and agitate using vortex mixer for a few seconds to obtain a homogenous solution of 0.1 mg/mL anhydrous risedronate sodium.

Assay Preparation

Risedronate sodium tablets are available in 5, 30, 35, or 75 mg strength dosage forms. For this study, 35 mg strength tablets were used. To assay risedronate in tablets, dissolve 5 tablets in 350 g diluent by shaking continuously for 10 min, then sonicate for 5 min to obtain a 0.5 mg/mL solution. Decant approximately 10 mL of the supernatant into a centrifuge tube, discarding the insoluble material, and centrifuge at 5000 rpm for 15 min. Dilute 2 mL of the supernatant from the centrifuge tube to 10 mL with diluent to obtain a final solution of 0.1 mg/mL of risedronate sodium.

Precautions

Use polypropylene bottles instead of glassware for mobile phase preparation. Calcium and other metal ions can leach from glassware, making it difficult to accurately adjust the pH of the EDTA solution. In addition, maintaining the mobile phase pH within the specified range and adding a consistent volume of NaOH to adjust pH is critical to avoid retention time shifts and decreased resolution between risedronate and its related compounds. If the resolution does not meet the USP specification, verify that the mobile phase pH is within 9.5 ± 0.1. Due to the strong chelation character of risedronate with metals, risedronate determination requires a metal-free system for efficient peak shapes.

RESULTS AND DISCUSSION

Separation and Detection

Separation of risedronate from its related substances is achieved on a Dionex IonPac AS7 (4 × 250 mm) column with 4.8 mM EDTA mobile phase adjusted to pH 9.5. The Dionex IonPac AS7 column is specially designed for determining polyvalent anions, such as bisphosphonates. At pH 9.5 ± 0.1, risedronate is readily ionized into a polyvalent anion and EDTA is predominantly trivalent. Separation is likely achieved by the trivalent EDTA anion eluting the polyvalent risedronate anion. The EDTA in the mobile phase also helps prevent metal contaminants from chelating with risedronate. Risedronate and its related compounds elute within 15 min and the detection is by UV absorbance at 263 nm.

Figure 2, trace A, shows the separation of risedronate from related compound C, and trace B shows the separation of risedronate and its related compound A using the conditions described in the monograph. The retention times of related compound C, related compound A, and risedronate were approximately 9.4, 10.2, and 11.9 min, respectively. Figure 3 shows a risedronate peak in the drug product (Actonel®, 35 mg tablet) preparation.

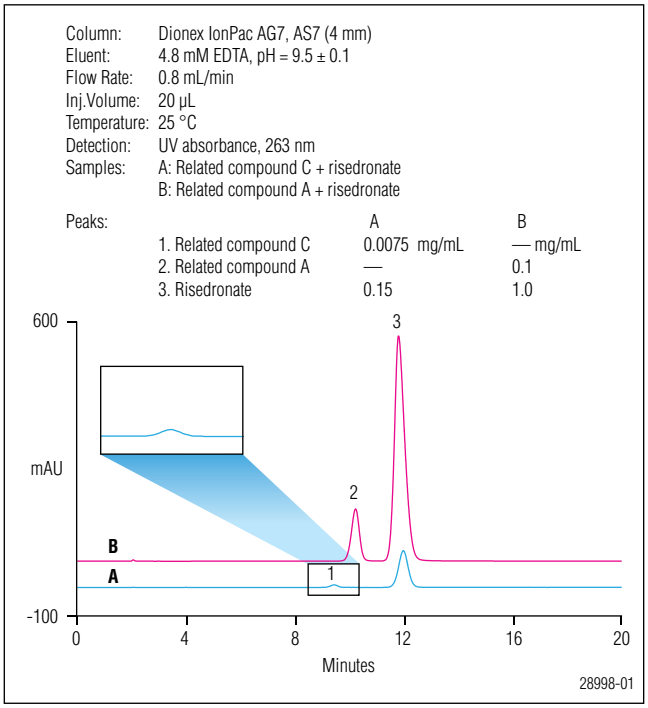

Figure 2. Overlay of chromatograms of risedronate standard with related compound C in trace A and related compound A in trace B.

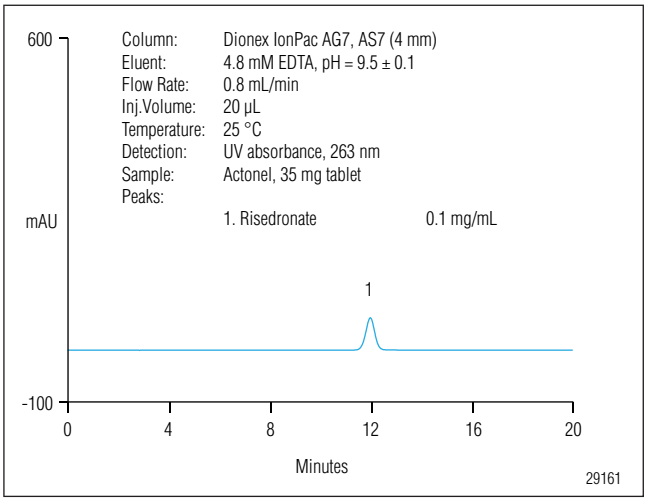

Figure 3. Chromatogram of Actonel, 35 mg tablet preparation showing risedronate.

The USP specifies values for the assay amount, resolution between risedronate and its related compounds, tailing factor, and peak area RSD in both drug product and drug substance monographs. All these parameters were evaluated and proven to meet or exceed the USP specifications. To obtain the specified resolution of ≥2.3 between related compound A and risedronate in the analysis of the drug substance, it is critical to prepare the mobile phase as outlined here. Typically, the amount of 5 M NaOH solution required to adjust the mobile phase pH to 9.5 ± 0.1 is less than 1.2 mL. If more NaOH is required to obtain the correct pH, there is a possibility of not meeting the USP resolution specification. In this case, the mobile phase should be prepared fresh with attention to the amount of NaOH added. Peak asymmetry or tailing factor for risedronate was 1.3, which meets the USP specification of ≤1.5. A tailing factor >1.5 can indicate metal contamination in the solutions and/or the system.

For three replicate injections of risedronate standard, peak area precision of 0.32% was obtained, which is within the USP specification limit of ≤1.0%. The USP specifications and the experimental values obtained are presented in Table 1.

Table 1. Specifications

Drug Substance				
	Tailing Factor	**Resolution[a]**	**% RSD[c]**	**% Risedronate**
USP Spec.	≤ 1.5	≥ 2.3	≤ 1.0	98.0%–102.0%
Experimental	1.3	2.5	0.32	99.6%
Drug Product				
		Resolution[b]	**% RSD[c]**	**% Risedronate**
USP Spec.		≥ 2.5	≤ 1.0	90.0%–110.0%
Experimental		4.4	0.19	98.3%

[a]Related compound A and risedronate

[b]Related compound C and risedronate

[c]n = 3

Linearity, Limit of Detection (LOD), and Limit of Quantification (LOQ)

To determine linearity, the system was calibrated using duplicate injections of five concentrations covering the range of 0.5 to 1.5 mg/mL anhydrous risedronate sodium for the drug substance and 0.05 to 0.15 mg/mL anhydrous risedronate sodium for the drug product. Peak area response was plotted versus the risedronate concentration, and linear regression analysis was performed. The baseline noise for LOD was determined by measuring the peak-to-peak noise in a representative 1 min segment of the baseline where no peaks elute. The method demonstrated a very low LOD and LOQ of 0.08 µg/mL and 0.3 µg/mL, respectively, for risedronate. Table 2 shows the linearity, LOD, and LOQ for risedronate determined in the drug substance and drug product using UV detection.

Table 2. Linearity, LOD, LOQ for Risedronate

	Analyte	**Range (mg/mL)**	**Coefficient of Determination (r^2)**	**LOD (µg/mL)**	**LOQ (µg/mL)**
Substance	Risedronate	0.5–1.5	1.00	0.08	0.3
Product	Risedronate	0.05–0.15	0.9999	0.08	0.3

Accuracy and Precision

Method accuracy was tested by preparing risedronate sodium drug substance and tablets (drug product) in triplicates at 75%, 100%, and 125% of the monograph concentration, which is 1.0 mg/mL anhydrous risedronate for the drug substance and 0.1 mg/mL for the drug product. The % assay for the samples was calculated using the following formula found in both the USP monographs:

$$\% \text{ risedronate} = 100\ (C_s/C_u)\ (r_u/r_s)$$

C_s is concentration, in mg/mL, of anhydrous R_S in the standard preparation.

C_u is concentration, in mg/mL, of anhydrous R_S in assay preparation.

r_u is assay preparation peak area response.

r_s is standard preparation peak area response.

The average percent assay of risedronate in the drug substance prepared at 0.75, 1.00, and 1.25 mg/mL anhydrous risedronate is 99.5%, 99.6%, and 99.1%, respectively. Average percent assay of risedronate in the drug product (tablets) prepared at 0.075, 0.100, and 0.125 mg/mL anhydrous risedronate is 97.1%, 98.3 %, and 97.7%, respectively. The USP monograph specifies % assay for risedronate in the drug substance to be 100.0 ± 2.0% and in the drug product to be 100.0 ± 10.0%. All the values obtained in this validation successfully meet the specifications.

Method precision was calculated from the peak area values at three different concentrations. For both substance and product analysis, the method shows a peak area precision of <1.0% at the test concentration (100%) and <2.0% for concentrations at 75% and 125% of the test method concentration. The accuracy and precision values are reported in Tables 3 and 4.

Table 3. Accuracy and Precision for the Determination of Risedronate in the Drug Substance

Drug Substance Preparation	Concentration (mg/mL)	Average Peak Area Response (mAU*min)	% RSD	% Risedronate	Average % Assay
75%–1	0.75	172.1	1.0	98.6	99.5 ± 0.8
75%–2	0.75	175.6		99.9	
75%–3	0.75	174.8		99.9	
100%–1	1.00	233.7	0.3	99.9	99.6 ± 0.5
100%–2	1.00	233.3		99.9	
100%–3	1.01	234.7		99.0	
125%–1	1.25	289.5	1.0	99.6	99.1 ± 0.9
125%–2	1.25	284.5		98.0	
125%–3	1.25	289.6		99.6	
1.01 mg/mL anhydrous USP risedronate standard response 235.9 mAU*min				**Average**	**99.4**
				Std. Dev.	**0.3**

Table 4. Accuracy and Precision for the Determination of Risedronate in the Drug Product (Tablet)					
Drug Product Preparation	**Concentration (mg/mL)**	**Average Peak Area Response (mAU*min)**	**% RSD**	**% Risedronate**	**Average % Assay**
75%–1	0.075	17.5	2.0	98.6	97.1 ± 1.5
75%–2	0.075	17.3		97.2	
75%–3	0.075	16.8		95.6	
100%–1	0.100	23.1	0.6	98.0	98.3 ± 0.6
100%–2	0.100	23.1		98.0	
100%–3	0.100	23.3		99.0	
125%–1	0.125	29.0	1.8	98.8	97.7 ± 1.8
125%–2	0.125	29.0		98.8	
125%–3	0.125	28.1		95.6	
0.101 mg/mL anhydrous USP risedronate standard response 23.8 mAU*min				**Average**	**97.7**
				Std. Dev.	**0.6**

Method Robustness

According to the International Conference on Harmonization (ICH): "The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage." [8,9] Although not required in the USP monograph, this method was evaluated for its ability to pass USP specifications with slight changes in the parameters that can be expected during routine analysis. Column temperature, mobile phase pH, and the condition of the column were variables selected for this study. Column temperature was varied by ± 2 °C from the control temperature of 25 °C and the pH was varied within the USP specified range of 9.5 ± 0.1 units. In addition, two Dionex IonPac AS7 columns from the same manufactured lot were compared for this analysis. The results from this study are presented in Table 5. All the USP specifications were still met despite the variations in the test method parameters. Resolution between related compound A and risedronate is affected most significantly by these variations.

Table 5. Robustness for the Analysis of the Drug Substance								
Parameter	**Resolution[a] (spec ≥2.3)**	**Difference (%)**	**Resolution[b] (spec ≥2.5)**	**Difference (%)**	**Tailing Factor[c] (spec ≤1.5)**	**Difference (%)**	**Risedronate Peak Area (mAU*min)**	**Difference (%)**
Column Temperature								
23 °C	3.0	+20	4.5	+2	1.18	-8	233.1	-1
25 °C	2.5	—	4.4	—	1.28	—	236.5	—
27 °C	2.9	+16	4.6	+5	1.25	-2	232.7	-2
Mobile Phase pH								
9.38	3.4	+36	4.7	+7	1.15	-10	231.5	-2
9.52	2.5	—	4.4	—	1.28	—	236.5	—
9.63	2.6	+4	4.4	0	1.28	0	231.0	-2
Column Condition								
Column 1 After ~ 550 Injections	2.5	—	4.4	—	1.28	—	236.5	—
Column 2	3.3	+32	5.1	+16	1.45	+13	236.7	+0.1

[a]Resolution between related compound A and risedronate

[b]Resolution between related compound C and risedronate

[c]Tailing factor of risedronate peak

CONCLUSION

The USP monograph for risedronate sodium was used to assay the API in the drug substance and product, which is available from 5–75 mg strength tablets. This method is based on an anion-exchange separation with a 4 × 250 mm Dionex IonPac AS7 (USP L48) column using EDTA eluent to resolve risedronate from related compounds. As shown in Table 1, the results exceeded the USP monograph specifications. The method separated risedronate in <15 min and it was well resolved from related compounds A and C. Although not specifically required in the USP monograph, the method ruggedness was also evaluated by varying the column temperature, eluent pH, and using different Dionex IonPac AS7 columns from the same lot. For each variable in the range tested, the results met or exceeded the USP specifications to demonstrate method ruggedness. The results from this study demonstrated a simple, rugged, and accurate method to assay risedronate sodium in the drug substance and product. Therefore, the method is suitable for quality control labs to ensure that the drug meets the approved specifications.

SUPPLIERS

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

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

U.S. Pharmacopeia, 12601 Twinbrook Parkway, Rockville, Maryland 20852-1790, U.S.A. Tel: 800-227-8772. http://www.usp.org

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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					Example Applications
			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	
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

Easy Method Transfer from HPLC to RSLC with the Dionex Method Speed-Up Calculator

INTRODUCTION

The goal of every chromatographic optimization is a method that sufficiently resolves all peaks of interest in as short a time as possible. The evolution of packing materials and instrument performance has extended chromatographic separations to new limits: ultrahigh-performance liquid chromatography (UHPLC).

The new Dionex UltiMate® 3000 Rapid Separation LC (RSLC) system is ideal for ultrafast, high-resolution LC. The RSLC system was designed for ultrafast separations with flow rates up to 5 mL/min at pressures up to 800 bar (11,600 psi) for the entire flow-rate range. This industry-leading flow-pressure footprint ensures the highest flexibility possible; from conventional to ultrahigh-resolution to ultrahigh-speed methods. The RSLC system, with autosampler cycle times of only 15 seconds, oven temperatures up to 110 °C, and data collection rates up to 100 Hz (even when acquiring UV-Vis spectra), sets the standard for UHPLC performance. Acclaim® RSLC columns with a 2.2 µm particle size complete the RSLC dimension.

A successful transfer from an HPLC method to an RSLC method requires recalculation of the chromatographic parameters. Underlying chromatographic principles have to be considered to find the appropriate parameters for a method transfer. With the Method Speed-up Calculator, Dionex offers an electronic tool that streamlines the process of optimum method transfer. This technical note describes the theory behind the Method Speed-Up Calculator and the application of this interactive, multi-language tool, illustrated with an exemplary method transfer from a conventional LC separation to an RSLC separation. You may obtain a copy of this calculator from your Dionex representative.

METHOD SPEED-UP STRATEGY

The purpose of method speed-up is to achieve sufficient resolution in the shortest possible time. The strategy is to maintain the resolving power of the application by using shorter columns packed with smaller particles. The theory for this approach is based on chromatographic mechanisms, found in almost every chromatography text book. The following fundamental chromatographic equations are applied by the Method Speed-Up Calculator for the method transfer from conventional to ultrafast methods.

The separation efficiency of a method is stated by the peak capacity *P*, which describes the number of peaks that can be resolved in a given time period. The peak capacity is defined by the run time divided by the average peak width. Hence, a small peak width is essential for a fast method with high separation efficiency. The peak width is proportional to the inverse square root of the number of theoretical plates N generated by the column. Taking into account the length of the column, its efficiency can also be expressed by the height equivalent to a theoretical plate *H*. The relationship between plate height H and plate number *N* of a column with the length L is given by Formula 1.

Formula 1: $$N = \frac{L}{H}$$

Low height equivalents will therefore generate a high number of theoretical plates, and hence small peak width for high peak capacity is gained. Which factors define *H*? For an answer, the processes inside the column have to be considered, which are expressed by the Van Deemter equation (Formula 2).

Formula 2: $$H = A + \frac{B}{u} + C \cdot u$$

The Eddy diffusion *A* describes the mobile phase movement along different random paths through the stationary phase, resulting in broadening of the analyte band. The longitudinal diffusion of the analyte against the flow rate is expressed by the term *B*. Term *C* describes the resistance of the analyte to mass transfer into the pores of the stationary phase. This results in higher band broadening with increasing velocity of the mobile phase. The well-known Van Deemter plots of plate height *H* against the linear velocity of the mobile phase are useful in determining the optimum mobile phase flow rate for highest column efficiency with lowest plate heights. A simplification of the Van Deemter equation, according to Halász[1] (Formula 3), describes the relationship between column efficiency (expressed in plate height *H*), particle size d_p (in µm) and velocity of mobile phase *u* (in mm/s):

Formula 3: $$H = 2 \cdot d_p + \frac{6}{u} + \frac{d_p^2 \cdot u}{20}$$

The plots of plate height H against velocity u depending on the particle sizes dp of the stationary phase (see Figure 1, top) demonstrate visually the key function of small particle sizes in the method speed-up strategy: The smaller the particles, the smaller the plate height and therefore the better the separation efficiency. An efficiency equivalent to larger particle columns can be achieved by using shorter columns and therefore shorter run times.

Another benefit with use of smaller particles is shown for the 2 µm particles in Figure 1: Due to improved mass transfer with small particle packings, further acceleration of mobile phases beyond the optimal flow rate with minimal change in the plate height is possible.

Optimum flow rates and minimum achievable plate heights can be calculated by setting the first derivative of the Halász equation to zero. The optimal linear velocity (in mm/s) is then calculated by Formula 4.

Formula 4: $$u_{opt} = \sqrt{\frac{B}{C}} = \frac{10.95}{d_p}$$

The minimum achievable plate height as a function of particle size is calculated by insertion of Formula 4 in Formula 3, resulting in Formula 5.

Formula 5: $$H_{min} \approx 3 \cdot d_p$$

Chromatographers typically prefer resolution over theoretical plates as a measure of the separation quality. The achievable resolution *R* of a method is directly proportional to the square root of the theoretical plate number as can be seen in Formula 6. *k* is the retention factor of the analyte and *k* the selectivity.

Formula 6: $$R = \frac{1}{4} \cdot \sqrt{N} \cdot \frac{k_2}{1 + k_2} \cdot \frac{\alpha - 1}{\alpha}$$

If the column length is kept constant and the particle size is decreased, the resolution of the analytes improves. Figure 1, bottom, demonstrates this effect using 5 µm and 2 µm particles.

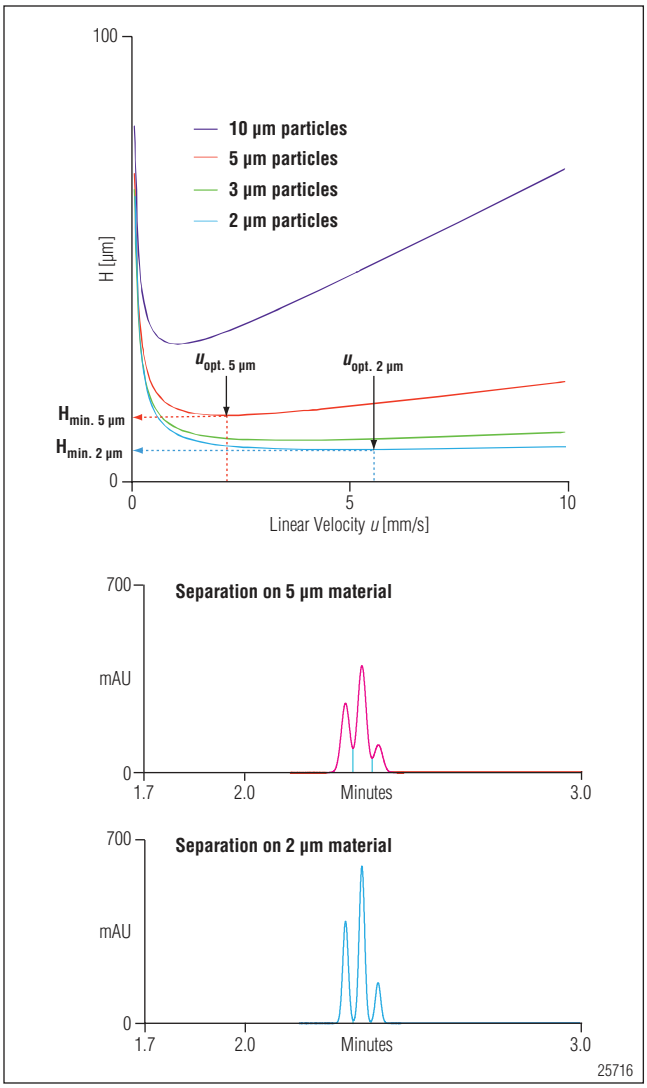

Figure 1. Smaller particles provide more theoretical plates and more resolution, demonstrated by the improved separation of three peaks (bottom) and smaller minimum plate heights H *in the Van Deemter plot (top). At linear velocities higher than uopt,* H *increases more slowly when using smaller particles, allowing higher flow rates and therefore faster separations while keeping separation efficiency almost constant. The speed-up potential of small particles is revealed by the Van Deemter plots (top) of plate height* H *against linear velocity u of mobile phase: Reducing the particle size allows higher flow rates and shorter columns because of the decreased minimum plate height and increased optimum velocity. Consequently, smaller peak width and improved resolution are the result (bottom).*

When transferring a gradient method, the scaling of the gradient profile to the new column format and flow rate has to be considered to maintain the separation performance. The theoretical background was introduced by L. Snyder[2] and is known as the gradient volume principle. The gradient volume is defined as the mobile phase volume that flows through the column at a defined gradient time t_G. Analytes are considered to elute at constant eluent composition. Keeping the ratio between the gradient volume and the column volume constant therefore results in a correct gradient transfer to a different column format.

Taking into account the changed flow rates F and column volume (with diameter d_c and length L), the gradient time intervals t_G of the new methods are calculated with Formula 7.

Formula 7: $$t_{G,new} = t_{G,old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{L_{new}}{L_{old}} \cdot \left(\frac{d_{c,new}}{d_{c,old}} \right)^2$$

An easy transfer of method parameters can be achieved by using the Dionex Method Speed-Up Calculator (Figure 2), which incorporates all the overwhelming theory and makes manual calculations unnecessary. This technical note describes the easy method transfer of an example separation applying the calculator. Just some prerequisites described in the following section have to be taken into account.

PREREQUISITES

The Method Speed-Up Calculator is a universal tool and not specific for Dionex products. Nevertheless, some prerequisites have to be considered for a successful method transfer, which is demonstrated in this technical note by the separation of seven soft drink additives.

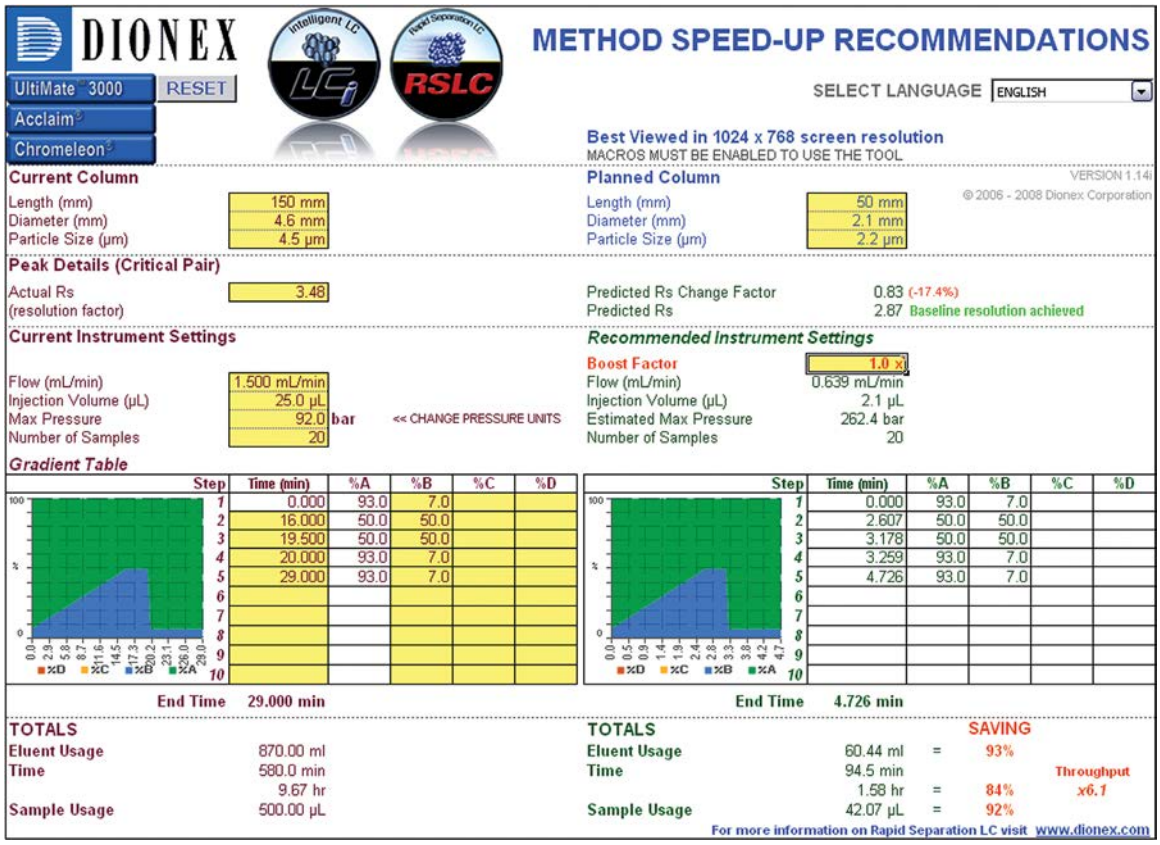

Figure 2. The Dionex Method Speed-Up Calculator transfers a conventional (current) HPLC method to a new (planned) RSLC method.

Column Dimension

First, the transfer of a conventional method to an RSLC method requires the selection of an adequate column filled with smaller particles. The RSLC method is predicted best if the selectivity of the stationary phase is maintained. Therefore, a column from the same manufacturer and with nominally identical surface modification is favoured for an exact method transfer. If this is not possible, a column with the same nominal stationary phase is the best choice. The separation is made faster by using shorter columns, but the column should still offer sufficient column efficiency to allow at least a baseline separation of analytes. Table 1 gives an overview of the theoretical plates expected by different column length and particle diameter size combinations using Dionex Acclaim column particle sizes. Note that column manufacturers typically fill columns designated 5 µm with particle sizes 4–5 µm. Dionex Acclaim 5 µm columns are actually filled with 4.5 µm particles. This is reflected in the table.

Table 1. Theoretical Plates Depending on Column Length and Particle Diameter (Calculated Using Formula 5)

	Theoretical Plates N		
Particle size	4.5 µm	3 µm	2.2 µm
Column length: 250 mm	18518	27778	37879
150 mm	11111	16667	22727
100 mm	7407	11111	15152
75 mm	5555	8333	11364
50 mm	3703	5556	7576

If the resolution of the original separation is higher than required, columns can be shortened. Keeping the column length constant while using smaller particles improves the resolution. Reducing the column diameter does not shorten the analysis time but decreases mobile phase consumption and sample volume. Taking into account an elevated temperature, smaller column inner diameters reduce the risk of thermal mismatch.

System Requirements

Smaller particles generate higher backpressure. The linear velocity of the mobile phase has to be increased while decreasing the particle size to work within the Van Deemter optimum. The UltiMate 3000 RSLC system perfectly supports this approach with its high maximum operation pressure of 800 bar (11,600 psi). This maximum pressure is constant over the entire flow rate range of up to 5 mL/min, providing additional potential to speed up applications even further by increasing the flow rate.

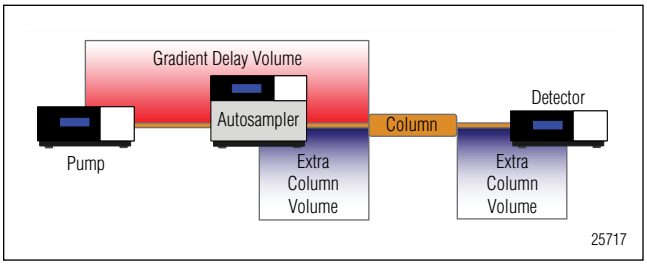

Figure 3. Gradient delay volume and extra column volume of an HPLC system. Both play an important role in method speed-up.

For fast gradient methods, the gradient delay volume (GDV) plays a crucial role. The GDV is defined as the volume between the first point of mixing and the head of the column. The GDV becomes increasingly important with fast, steep gradients and low flow rate applications as it affects the time taken for the gradient to reach the head of the column. The larger the GDV, the longer the initial isocratic hold at the beginning of the separation. Typically, this leads to later peak elution times than calculated. Early eluting peaks are affected most. In addition, the GDV increases the time needed for the equilibration time at the end of a sample and therefore increases the total cycle time. A general rule is to keep the gradient steepness and the ratio of GDV to column volume constant when transferring a standard method into a fast LC method. This will maintain the selectivity of the original method.[3]

The GDV can be adjusted to the column volume by installing appropriate mixer kits to the RSLC pump (see Table 2), which contributes most to the GDV. Typically, 100 µL or 200 µL mixers are good starting points when operating a small volume column in an RSLC system.

Another option is to switch the sample loop of the split-loop autosampler out of the flow path. The GDV is then reduced by the sample loop volume in the so-called bypass mode. The GDV of a standard sample loop of the RSLC autosampler is 150 µL, the micro injection loop has a 50 µL GDV.

Table 2. Mixer Kits Available for UltiMate 3000 RSLC System to Adapt GDV of Pump

Mixer Kit	GDV pump
Mixer kit 6040.5000	35 µL
Static mixer kit 6040.5100	100 µL
Static mixer kit 6040.5150	200 µL

Besides the gradient delay volume, the extra column volume is an important parameter for fast LC methods. The extra column volume is the volume in the system through which the sample passes and hence contributes to the band broadening of the analyte peak (Figure 3). The extra column volume of an optimized LC system should be below $^{1}/_{10}$th of the peak volume. Therefore the length and inner diameter of the tubing connections from injector to column and column to detector should be as small as possible. Special care has to be taken while installing the fittings to avoid dead volumes. In addition, the volume of the flow cell has to be adapted to the peak volumes eluting from the RSLC column. If possible, the flow cell detection volume should not exceed $^{1}/_{10}$th of the peak volume.

Detector Settings

When transferring a conventional method to an RSLC method, the detector settings have a significant impact on the detector performance. The data collection rate and time constant have to be adapted to the narrower peak shapes. In general, each peak should be defined by at least 30 data points. The data collection rate and time constant settings are typically interrelated to optimize the amount of data points per peak and reduce short-term noise while still maintaining peak height, symmetry, and resolution.

The Chromeleon® Chromatography Management Software has a wizard to automatically calculate the best settings, based on the input of the minimum peak width at half height of the chromatogram. This width is best determined by running the application once at maximum data rate and shortest time constant. The obtained peak width may then be entered into the wizard for optimization of the detection settings. Refer to the detector operation manual for further details.

METHOD SPEED-UP USING THE CALCULATOR

Separation Example

Separation was performed on an UltiMate 3000 RSLC system consisting of a HPG-3200RS Binary Rapid Separation Pump, a WPS-3000RS Rapid Separation Well Plate Sampler with analytical sample loop (100 µL), a TCC-3000RS Rapid Separation Thermostatted Column Compartment with precolumn heater (2 µL), and a VWD-3400RS Variable Wavelength Detector with semi-micro flow cell (2.5 µL). Chromeleon Chromatography Management Software (version 6.80, SR5) was used for both controlling the instrument and reporting the data. The modules were connected with stainless steel micro capillaries, 0.01" ID, $^{1}/_{16}$" OD when applying the conventional LC method, 0.007" and 0.005" ID, $^{1}/_{16}$" OD when applying the RSLC methods. A standard mixture of seven common soft drink additives was separated by gradient elution at 45 °C on two different columns:

- Conventional HPLC Column: Acclaim 120, C18, 5 µm, 4.6 × 150 mm column, (P/N 059148)
- Rapid Separation Column: Acclaim RSLC 120, C18, 2.2 µm, 2.1 × 50 mm column (P/N 068981).

The UV absorbance wavelength at 210 nm was recorded at 5 Hz using the 4.6 × 150 mm column and at 25 Hz and 50 Hz using the 2.1 × 50 mm column. Further method details such as flow rate, injection volume, and gradient table of conventional and RSLC methods are described in the following section. The parameters for the method transfer were calculated with the Dionex Method Speed-Up Calculator (version 1.14i).

The conventional separation of seven soft drink additives is shown in Figure 4A. With the Method Speed-Up Calculator, the method was transferred successfully to RSLC methods (Figure 4B and C) at two different flow rates. The easy method transfer with this universal tool is described below.

Column Selection for Appropriate Resolution

The column for method speed-up must provide sufficient efficiency to resolve the most critical pairs. In this example, separating peaks 5 and 6 is most challenging. A first selection of the planned column dimensions can be made by considering the theoretical plates according to Table 1. The 4.6 × 150 mm, 5 µm column is actually filled with 4.5 µm particles. Therefore, it provides 11,111 theoretical plates. On this column, the

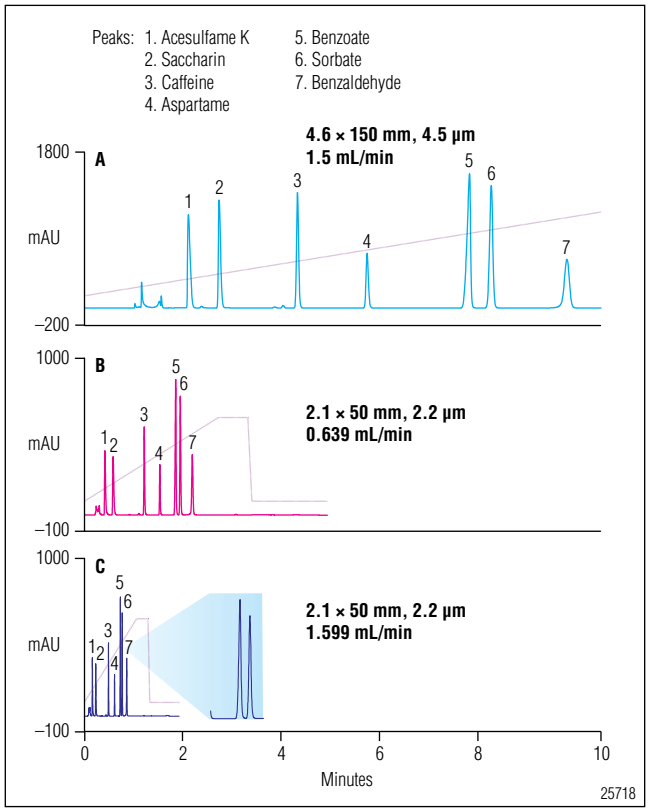

Figure 4. Method transfer with the Method Speed-Up Calculator from A) a conventional LC separation on an Acclaim 5 µm particle column, to B) and C) RSLC separations on an Acclaim 2.2 µm particle column.

resolution is $R_{(5,6)}$=3.48. This resolution is sufficiently high to select a fast LC column with fewer theoretical plates for the speed up. Therefore, a 2.1 × 50 mm, 2.2 µm column with 7579 plates was selected.

The first values to be entered into the yellow field of the Method Speed-Up Calculator are the current column dimension, planned column dimension, and the resolution of the critical pair. To obtain the most accurate method transfer, use the particle sizes listed in the manufacturer's column specifications sheet instead of the nominal size, which may be different. Dionex Acclaim columns with a nominal particle size of 5 µm are actually filled with 4.5 µm particles, and this value should be used to achieve a precise method transfer calculation. This has a positive impact on the performance and pressure predictions for the planned column. Based on the assumption of unchanged stationary phase chemistry, the calculator then predicts the resolution provided by the new method (Figure 5).

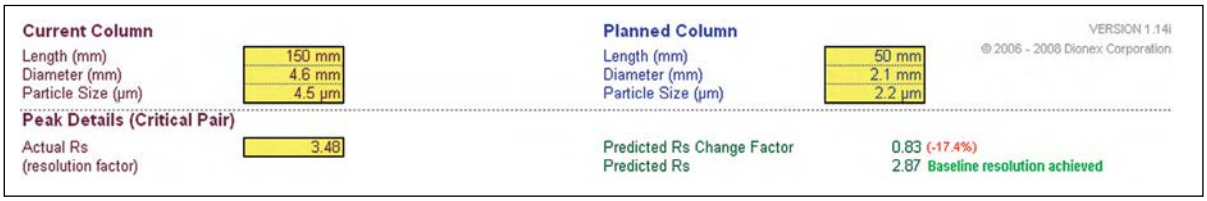

Figure 5. Column selection considering the resolution of the critical pair.

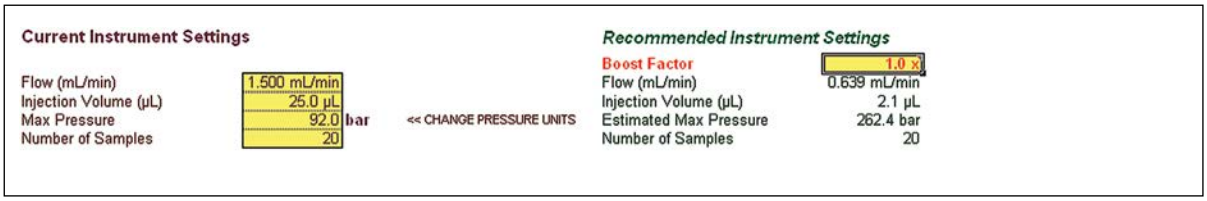

Figure 6. The flow rate, injection volume and backpressure of the current method are scaled to the new column dimension.

In the example in Figure 5, the predicted resolution between benzoate and sorbate is 2.87. With a resolution of $R \geq 1.5$, the message "Baseline resolution achieved" pops up. This indicates that a successful method transfer with enough resolution is possible with the planned column. If R is smaller than 1.5, the red warning "Baseline is not resolved" appears. Note that the resolution calculation is performed only if the boost factor BF is 1, otherwise it is disabled. The function of the boost factor is described in the Adjust Flow Rate section.

Instrument Settings

The next section of the Method Speed-Up Calculator considers basic instrument settings. These are flow rate, injection volume, and system backpressure of the current method (Figure 6). In addition to these values, the detector settings have to be considered as described in the earlier section "Detector Settings". Furthermore, the throughput gain with the new method can be calculated if the number of samples to be run is entered.

Adjust Flow Rate

As explained by Van Deemter theory, smaller particle phases need higher linear velocities to provide optimal separation efficiency. Consequently, the Dionex Method Speed-Up Calculator automatically optimizes the linear velocity by the ratio of particle sizes of the current and planned method. In addition, the new flow rate is scaled to the change of column cross section if the column inner diameter changed. This keeps the linear velocity of the mobile phase constant. A boost factor (*BF*) can be entered to multiply the flow rate for a further decrease in separation time. If the calculated resolution with $BF=1$ predicts sufficient separation, the method can be accelerated by increasing the boost factor and therefore increasing the flow rate. Figure 1 shows that applying linear velocities beyond the optimum is no problem with smaller particle phases, as they do not significantly loose plates in this region. Note that the resolution calculation of the Method Speed-Up Calculator is disabled for $BF \neq 1$.

For the separation at hand, the flow rate is scaled from 1.5 mL/min to 0.639 mL/min when changing from an Acclaim 4.6 × 150 mm, 4.5 µm column to a 2.1 × 50 mm, 2.2 µm column (see Figure 6), adapting the linear velocity to the column dimensions and the particle size. The predicted resolution between peak 5 and 6 for the planned column is $R=2.87$. The actual resolution achieved is $R=2.91$, almost as calculated (chromatogram B in Figure 4).

A Boost Factor of 2.5 was entered for further acceleration of the method (Figure 7). The method was then performed with a flow rate of 1.599 mL/min, and resolution of the critical pair was still sufficient at $R=2.56$ (see zoom in chromatogram C in Figure 4).

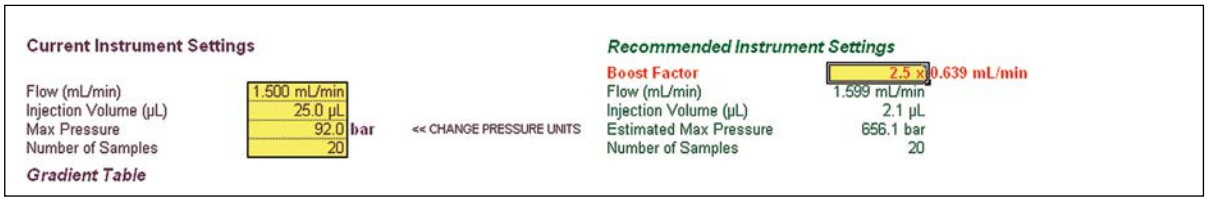

Figure 7. The new flow rate is further accelerated by applying the Boost Factor of 2.5.

Scale Injection Volume

The injection volume has to be adapted to the new column dimension to achieve similar peak heights by equivalent mass loading. Therefore the injection plug has to be scaled to the change of column cross section. In addition, shorter columns with smaller particles cause a reduced zone dilution. Consequently, sharper peaks compared to longer columns are expected. The new injection volume $V_{inj,new}$ is then calculated by Formula 8, taking a changed cross section and reduced band broadening by changed particle diameter into account.

Formula 8: $$V_{inj,new} = V_{inj,old} \cdot \left(\frac{d_{c,new}}{d_{c,old}}\right)^2 \cdot \sqrt{\frac{L_{new} \cdot d_{p,new}}{L_{old} \cdot d_{p,old}}}$$

Generally, it is recommended that a smaller flow cell be used with the RSLC method to minimize the extra column volume. Also, the difference in path length of different flow cell sizes has to be taken into account while scaling the injection volume. In the example of the soft drink analysis, the injection volume is scaled from 25 µL to 2.1 µL when replacing the Acclaim 4.6 × 150 mm, 4.5 µm column with a 2.1 × 50 mm, 2.2 µm column (see Figure 6).

Predicted Backpressure

Speeding-up the current method by decreasing particle size and column diameter and increasing flow rate means elevating the maximum generated backpressure. The pressure drop across a column can be approximated by the Kozeny-Carman formula.[4] The pressure drop of the new method is predicted by the calculator considering changes in column cross section, flow rate, and particle size and is multiplied by the boost factor. The viscosity of mobile phase is considered constant during method transfer. The calculated pressure is only an approximation and does not take into account nominal and actual particle size distribution depending on column manufacturer. If the predicted maximum pressure is above 800 bar (11,600 psi) the warning "Exceeds pressure limit RSLC" is shown, indicating the upper pressure limit of the UltiMate 3000 RSLC system. However, in the case the method is transferred to a third party system, its pressure specification has to be considered.

In the example of the soft drink analysis, the actual pressure increases from 92 bar to 182 bar with *BF*=1 on the 2.1 × 50 mm column, and to 460 bar for the RSLC method with *BF*=2.5. The pressures predicted by the Method Speed-Up Calculator are 262 bar and 656 bar, respectively. The pressure calculation takes into account the change of the size of the column packing material. In a speed up situation, the pressure is also influenced by other factors such as particle size distribution, system fluidics pressure, change of flow cell, etc. When multiplication factors such as the boost factor are used, the difference between calculated and real pressure is pronounced. The pressure calculation is meant to give an orientation, what flow rates might be feasible on the planned column. However, it should be confirmed by applying the flow on the column.

Adapt Gradient Table

The gradient profile has to be adapted to the changed column dimensions and flow rate following the gradient-volume principle. The gradient steps of the current method are entered into the yellow fields of the gradient table. The calculator then scales the gradient step intervals appropriately and creates the gradient table of the new method.

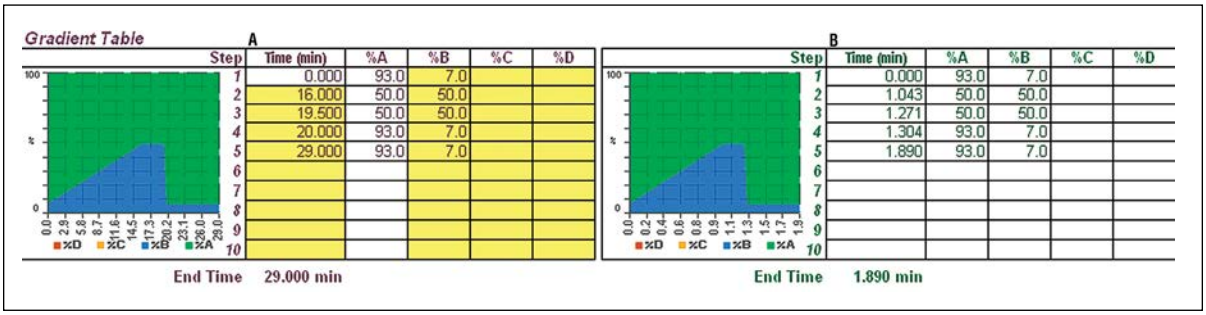

Figure 8. The gradient table of the current method (A) is adapted to the boosted method (B) according to the gradient-volume principle.

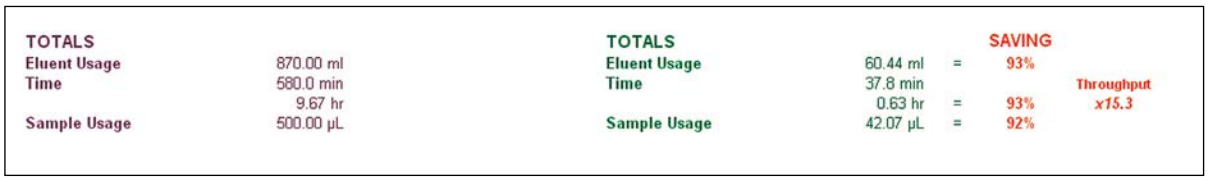

Figure 9. The absolute values for analysis time, eluent usage, and sample usage of the current (purple) and planned (green) method are calculated by the Method Speed-Up Calculator. The savings of eluent, sample, and time due to the method transfer are highlighted.

The adapted gradient table for the soft drink analysis while using a boost factor *BF*=1 is shown in Figure 8. According to the gradient-volume principle, the total run time is reduced from 29.0 min to 4.95 min by taking into account the changed column volume from a 4.6 × 150 mm, 5 µm (4.5 µm particles entered) to a 2.1 × 50 mm, 2.2 µm column and the flow rate reduction from 1.5 mL/min to 0.639 mL/min. The separation time was further reduced to 1.89 min by using boost factor *BF*=2.5. Gradient time steps were adapted accordingly. The comparison of the peak elution order displayed in Figure 4 shows that the separation performance of the gradient was maintained during method transfer.

Consumption and Savings

Why speed-up methods? To separate analyte peaks faster and at the same time reduce the mobile phase and sample volume consumption. Those three advantages of a method speed-up are indicated in the Method Speed-Up Calculator sheet right below the gradient table. The absolute values for the time, eluent, and sample usage are calculated taking the numbers of samples entered into the current instrument settings section of the calculation sheet into account (see Figure 6).

Regarding the soft drink analysis example, geometrical scaling of the method from the conventional column to the RSLC method means saving 93% of eluent and 92% of sample. The sample throughput increases 6.1-fold using *BF*=1. The higher flow rate at *BF*=2.5 results in a 15.3-fold increased throughput compared to the conventional LC method (Figure 9).

CONCLUSION

Fast method development or increased sample throughput are major challenges of most analytical laboratories. A systematic method speed-up is accomplished by reducing the particle size, shortening the column length, and increasing the linear velocity of the mobile phase. The Dionex Method Speed-Up Calculator automatically applies these rules and scales the conventional LC parameters to the conditions of the RSLC method. The interactive electronic tool is universally applicable. New instrument settings are predicted and gradient tables are adapted for optimum performance for the new method. The benefit of the method transfer is summarized by the integrated calculation of savings in time, eluent and sample. In addition, users can benefit from getting results earlier and thereby reducing the time to market. The Dionex Method Speed-Up Calculator is part of Dionex's total RSLC solution, which further consists of the industry leading UltiMate 3000 RSLC system, powerful Chromeleon Chromatography Management Software, and high-efficiency Acclaim RSLC columns.

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