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

INTRODUCTION

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

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

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

Aminoglycosides are a large and diverse class of

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

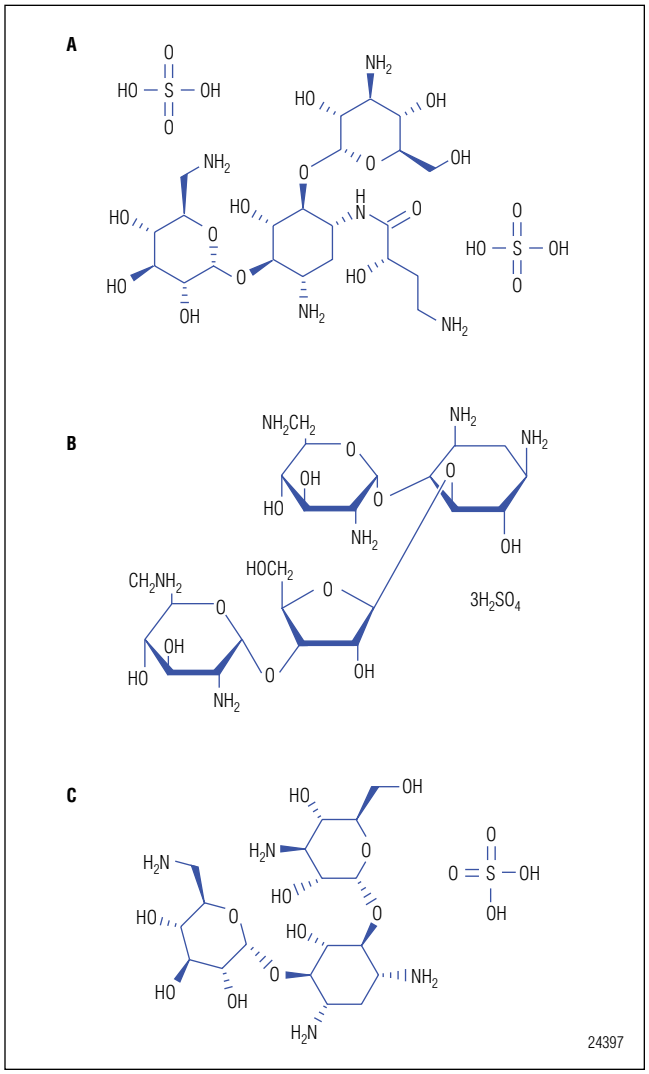

Figure 1. Chemical structures of some aminoglycoside sulfate compounds: A) amikacin disulfate, B) neomycin B trisulfate, C) kanamycin A sulfate.

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

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

EQUIPMENT

Dionex ICS-3000 RFIC system consisting of:

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

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

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

Chromeleon® 6.8 Chromatography Workstation

REAGENTS AND STANDARDS

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

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

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

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

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

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

Samples

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

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

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

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

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

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

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

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

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

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

CONDITIONS

Method 1

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

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

METHOD 2

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

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

PREPARATION OF SOLUTIONS AND REAGENTS

Stock Standard Solutions

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

Primary Dilution Standards

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

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

Calibration Standards

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

SAMPLE PREPARATION

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

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

RESULTS AND DISCUSSION

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

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

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

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

Method 1

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

Table 1. Calibration Data and Detection Limits Using Method 1

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

[a]LODs estimated from 3 x S/N

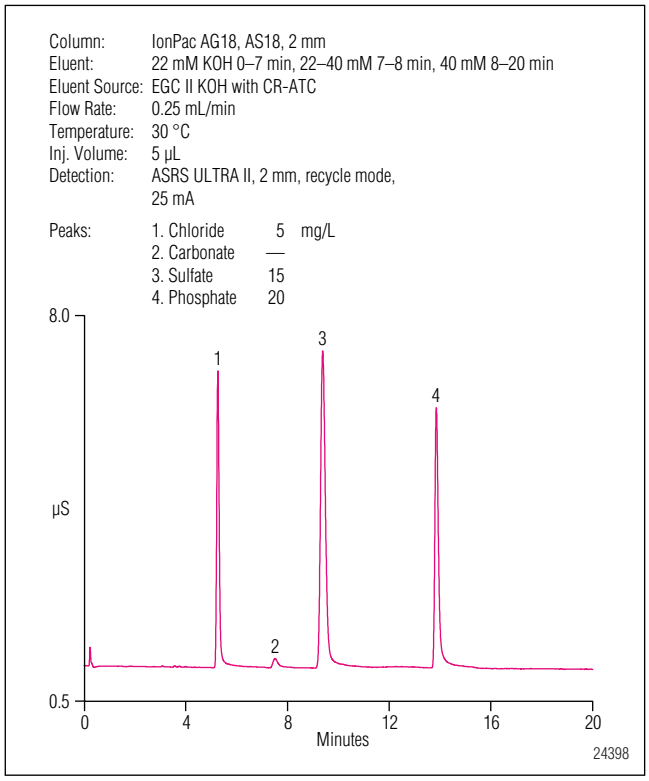

Figure 2. Separation of target anions on the IonPac AS18 column.

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

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

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

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

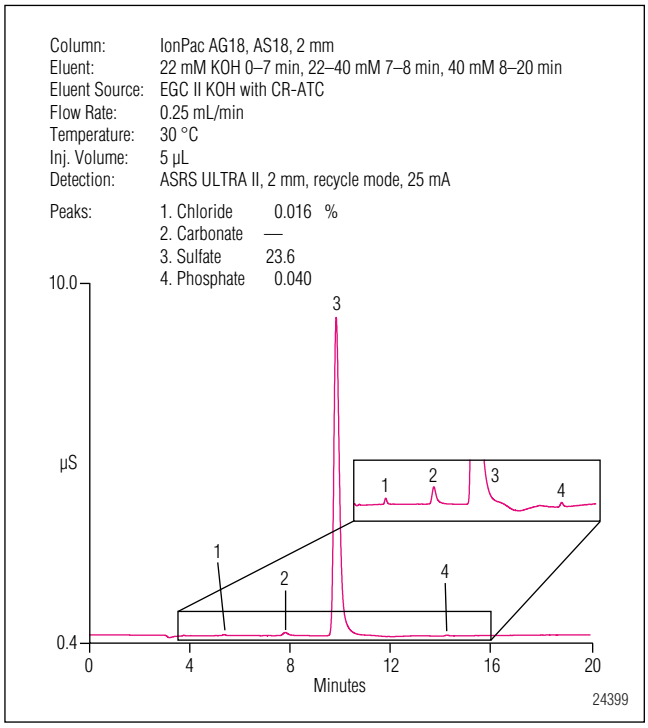

Figure 3. Separation of sulfate counter ion and anionic impurities in USP-grade paromomycin sulfate.

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

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

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

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

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

Table 4: Calibration Data and Detection Limits Using Method 2

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

[a]LODs estimated from 3 x S/N

Method 2

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

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

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

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

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

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

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

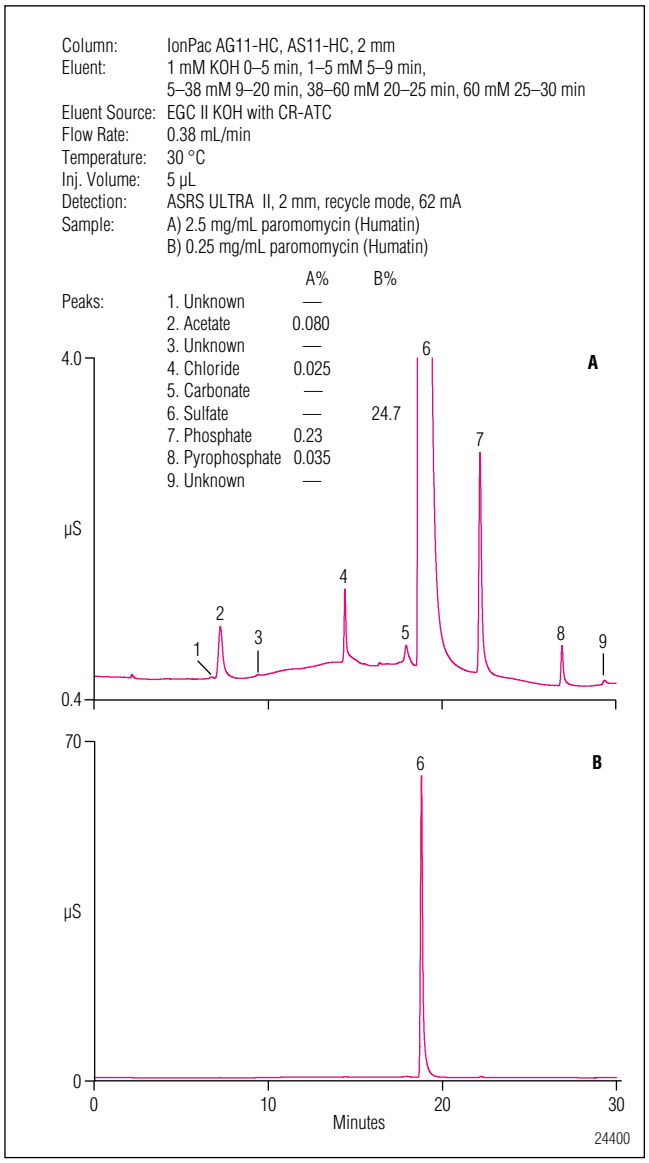

Figure 4. Separation of sulfate counter ion and anionic impurities in Humatin using the IonPac AS11-HC.

CONCLUSION

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

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

LIST OF SUPPLIERS

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

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

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

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