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Determination of Streptomycin and Impurities Using HPAE-PAD

INTRODUCTION

Streptomycin is a water-soluble aminoglycoside antibiotic purified from the fermentation of the actinomycete *Streptomyces griseus* and used for intravenous administration¹ to treat infections. Streptomycin must be determined and all impurities must meet specified limits before a manufactured lot is used clinically. The current United States Pharmacopeia (USP 30, NF 25) compendial method for streptomycin sulfate measures streptomycin A as the primary antibiotic.^{2,3} One of the thermal degradation products serves as a measure for system suitability. Streptomycin, also known as Streptomycin A, or O-2-Deoxy-2-(methylamino)- α -L-glucopyranosyl-(1 \rightarrow 2)-O-5-deoxy-3-C-formyl- α -L-lyxofuranosyl-(1 \rightarrow 2)-*N,N'*-bis(aminoiminomethyl)-D-streptamine, is the main antibiotic component of *S. griseus* fermentation broth, but also contains a less abundant form of streptomycin: mannosidostreptomycin, also known as streptomycin B. Unless otherwise noted, streptomycin in this note refers to streptomycin A. The precursors of streptomycin biosynthesis also occur during fermentation: streptidine and streptobiosamine (formed from streptose and *N*-methyl-L-glucosamine).⁴⁻⁸ These and other compounds may result

from chemical degradation during manufacture or storage.⁹⁻¹² Acid hydrolysis of streptomycin yields streptidine and streptobiosamine. Alkaline hydrolysis of streptomycin yields maltol. Thermal degradation of streptomycin, above 70 °C, produces streptidine and streptobiosamine, neither of which is commercially available. Figure 1 shows the chemical structure of streptomycin A and its major impurities. The system suitability peak used in the USP method is unidentified, but may be the less charged of the two thermal degradation products, streptobiosamine.

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

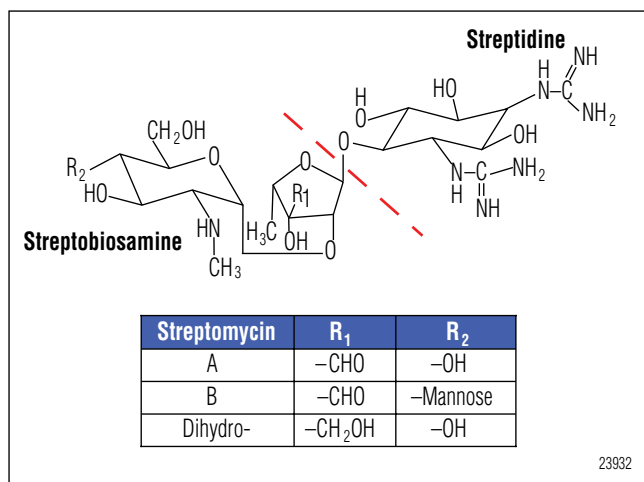


Figure 1. Chemical structures of streptomycin A and some known impurities.

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

In this application note, we use an ICS-3000 system with PAD to run the USP Compendial Method for the assay of streptomycin sulfate. We show key performance parameters, including precision in determining streptomycin purity, limits of detection, linearity, and ruggedness, in a manner consistent with requirements of normal method validation.¹⁸⁻²⁴ We use disposable gold (Au) working electrodes to improve electrode-to-electrode (and system-to-system) reproducibility of streptomycin A electrochemical response. Disposable Au working electrodes are manufactured in a manner that improves electrode-to-electrode reproducibility.^{17,25,26} We demonstrate HPAE-PAD tech-

nology for streptomycin A purity analysis and its feasibility for determinations in a fermentation broth. Finally, we evaluate streptomycin A purity per the requirements of the International Conference on Harmonization (for new drug substances).²⁷

EQUIPMENT

Dionex ICS-3000 Ion Chromatography system with:

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

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

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

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

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

Chromeleon® Chromatography Workstation

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

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

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

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

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

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 M Ω -cm resistance or higher

Standards

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

Samples

Streptomycin A (Streptomycin Sulfate; Sigma-Aldrich)
Bacto[®] YPD Broth (Pfizer Consumer Healthcare, BD Laboratories, Cat# 0428-17-5)

CONDITIONS

Method:

Columns: CarboPac PA1 Analytical, 4 x 250 mm (P/N 035391)

CarboPac PA1 Guard, 4 x 50 mm (P/N 043096)

Eluent Channel A: Water

Eluent Channel B: 250 mM NaOH

Flow Rate: 0.5 mL/min

Inj. Volume: 20 μ L (full loop)

Temperature: 30 $^{\circ}$ C column
25 $^{\circ}$ C detector compartment

Detection: Pulsed amperometry, carbohydrate certified disposable Au working electrodes (P/N 060139)

Isocratic Program:

Separating Eluent: 70 mM NaOH

Program: 72% A + 28% B

Run time: 35 min

Background: 7.9–32 nC

Typical System

Operating

Backpressure: 800–950 psi

Gradient Program:

Separating Eluent: 70 mM NaOH and 200 mM NaOH

Program: 72% A + 28% B for 22 min, then step to 20% A + 80% B for 18 min, then step to 60% A + 40% B for 20 min, for reequilibration to starting conditions

Run time: 60 min

Background: 7.9–36 nC

Typical System

Operating

Backpressure: 800–970 psi

Carbohydrate Waveform for the ED*

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

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

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

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

Water (Eluent Channel A)

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

250 mM Sodium Hydroxide (Eluent Channel B)

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

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

Stock Standards

Place solid streptomycin sulfate and dihydrostreptomycin sulfate in plastic microcentrifuge vials with screw caps (Sarstedt) and weigh them. The label for the USP material indicates that the material should be dried prior to use, using vacuum pressure not exceeding 5 mm (5 Torr) of mercury at 60 °C for 3 h. Simultaneously centrifuge, heat, and dry the preweighed solid samples using a SpeedVac Evaporator at 0.35–0.60 Torr of vacuum for 20–24 h, set to 50 °C. Within 1 min of completion of the drying, tightly seal and reweigh the vials to calculate the percent moisture content of the solid material. Dissolve the anhydrous solid in a weighed amount of deionized water (~1.0 mL) to obtain an accurate concentration of 100 mg dried solid/mL (assume density of H₂O = 1.000 g/mL). Adjust the 100 mg/mL streptomycin sulfate concentration to the streptomycin A base concentration using the reported percent sulfate stated on the manufacturer's Certificate of Analysis. Calculate the molar concentration using the molecular weight for the streptomycin base. An example of these calculations follows:

Certificate of Analysis information for streptomycin sulfate:

Sulfate:	18.7%
Potency:	758 IU/mg dried solid (based on the Third International Standard)
Loss On Drying:	2.9%
Molecular Weight (streptomycin sulfate):	728.69
Molecular Formula:	C ₂₁ H ₃₉ N ₇ O ₁₂ – 1.5H ₂ SO ₄
Wet weight of solid (weight before SpeedVac drying):	115.34 mg
Dry weight of solid (weight after SpeedVac drying):	100.92 mg

(1) Calculation of percent moisture content:

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

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

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

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

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

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

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

b) Calculation of mg/mL streptomycin base concentration:

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

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

Most formula weights reported by commercial vendors of streptomycin sulfate are erroneously referred to as molecular weights. Streptomycin A is ionic and may contain a counter-ion, typically sulfate, that varies depending on manufacturing. Therefore, it is important to differentiate between mass concentrations (e.g., μg/mL) that include the anion and those that do not. In the example here, 100 mg/mL of streptomycin

sulfate is equal to 77 mg/mL streptomycin base.

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

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

Also, the theoretical concentration of sulfate is then $1.5 \times 133 \text{ mM} = 200 \text{ mM}$ sulfate. Another point of occasional confusion is the formula for streptomycin sesquisulfate, which actually is the same as streptomycin sulfate. This formula is occasionally expressed as $(\text{C}_{21}\text{H}_{39}\text{N}_7\text{O}_{12})_2 \cdot 3\text{H}_2\text{SO}_4$ with a formula weight of 1457.4, but the presence of two moles of streptomycin base may be overlooked, resulting in incorrect molarity calculations.³⁰

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

System Suitability Sample Preparation

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

Streptomycin and Dihydrostreptomycin Degradation Study

Evaluate streptomycin and dihydrostreptomycin for time-dependent changes in impurity content by exposure to elevated temperature. Incubate aliquots of $41 \mu\text{M}$ streptomycin and dihydrostreptomycin in water at 75°C for 0, 60 min, and 24 h. Evaluate the treated samples for changes in purity.

YPD Broth Media

Dissolve 1.0 g Bacto Yeast Extract-Peptone-Dextrose (YPD) Broth in 20.0 mL aseptically filtered ($0.2 \mu\text{m}$, nylon) water. Centrifuge an aliquot at $16,000 \times g$ for 10 min and dilute 1000-fold in purified water. For spike recovery determinations, add concentrated streptomycin to the supernatant during dilution to final concentrations of 10 and $41 \mu\text{M}$. Directly analyze the diluted supernatant.

INSTRUMENT OPERATION

Wash columns with 200–250 mM NaOH for 1 h to restore streptomycin A retention time after installing a column and for weekly column maintenance when analyses are made without column regeneration after each injection. The application of 200 mM NaOH changes system equilibrium, and reequilibration at 70 mM NaOH for >2 h is recommended to achieve high precision. For most work, however, commence injections after 15 min. Retention time stability is observed 3 h from the start of column reequilibration, at which time retention is increased by 2.4%. Complete stability of retention time is observed after 10 h, at which time retention is increased by 3.0% from start of equilibration. When the pump has been turned off for longer than 1 day, regenerate the column with 200–250 mM NaOH for 1–2 h, and reequilibrate with 70 mM NaOH for 2 h before analyzing samples.

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

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

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

RESULTS AND DISCUSSION

Separation

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

Separation of streptomycin A and its impurities is highly dependent on eluent concentration. Table 1 shows the effect of NaOH eluent concentration on the retention time of streptomycin A. The greatest effect on retention was observed between 50 and 77 mM, where very minor changes in hydroxide concentration produced large changes in retention times. Figure 3 compares the resolution of impurity peaks for injections of 10 μM USP grade streptomycin A using 63 mM (chromatogram A) with

70 mM NaOH (chromatogram B). The 10% reduction in eluent concentration from the USP Monograph Method increases the retention time of streptomycin A, reducing throughput and increasing peak tailing; however, the separation of impurities is improved.

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

The impurity peak at 8 min (Figure 3, chromatogram A, peak 12) was identified as the USP system suitability

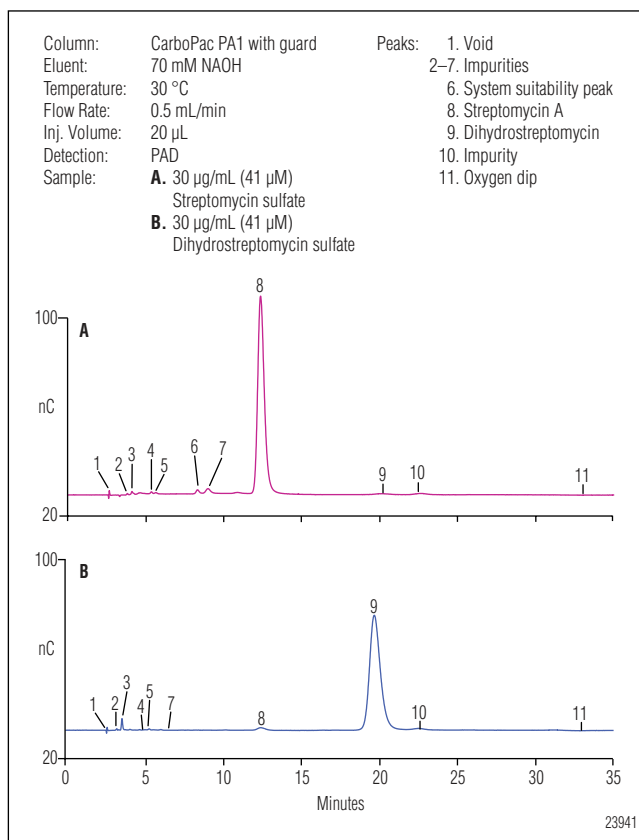


Figure 2. Determination of streptomycin A and dihydrostreptomycin.

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

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

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

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

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

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

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

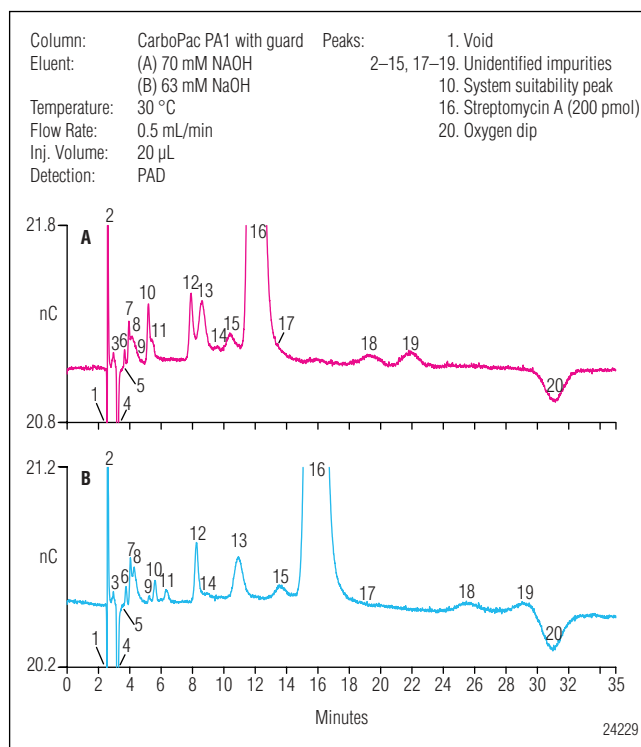


Figure 3. Comparison of USP streptomycin at 70- and 63-mM NaOH eluent concentrations.

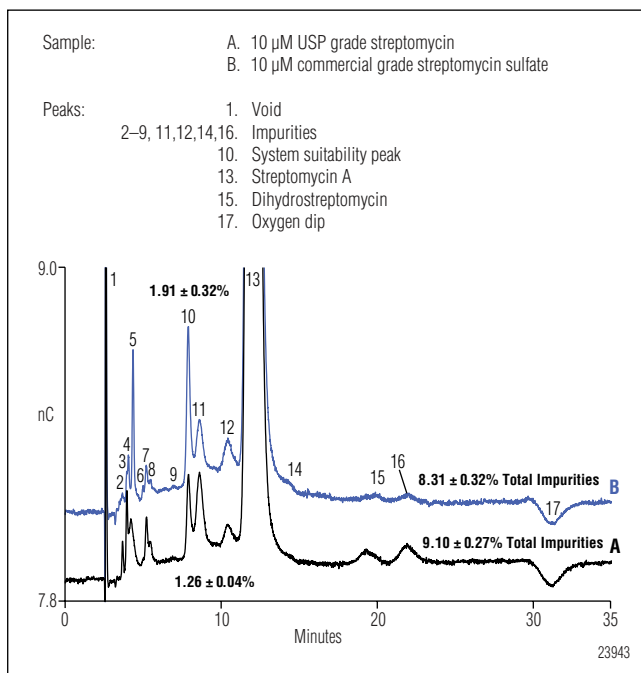


Figure 4. Comparison of USP and a second commercial source of streptomycin sulfate at a 70-mM NaOH eluent concentration.

Detection

Linear Range

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

Linearity

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

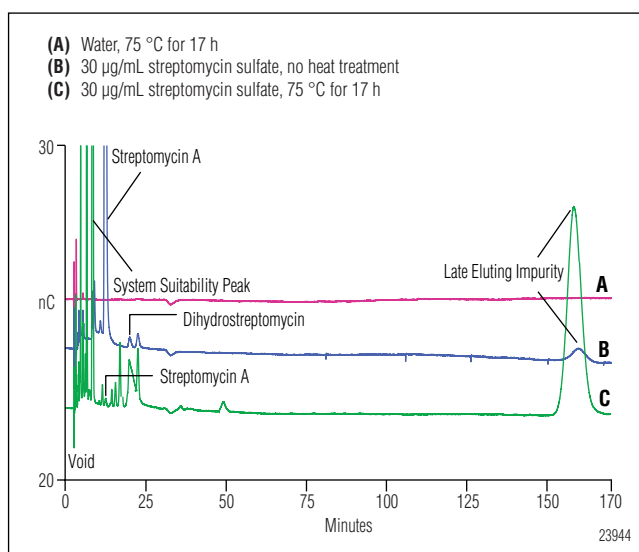


Figure 5. Late eluting thermal degradation peak.

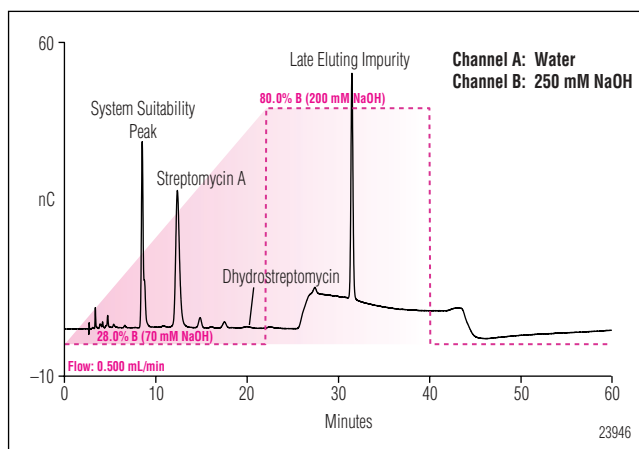


Figure 6. Use of a different elution program to more quickly elute the thermal degradation peak.

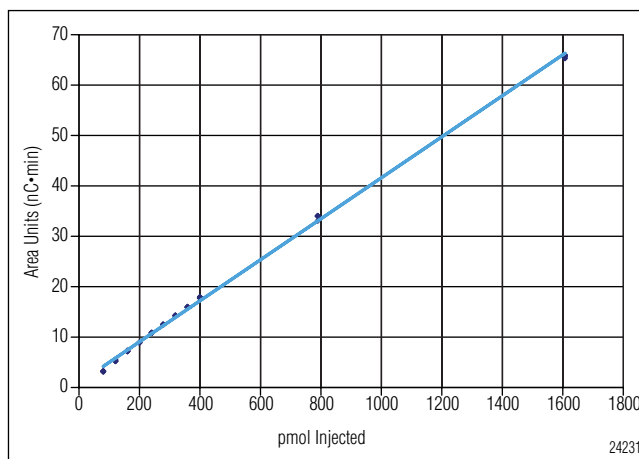


Figure 7. Linearity of streptomycin within the range of 80–1600 pmol (4–80 μM , 20 μL injection).

Lower Limits of Detection and Quantification

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

Precision

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

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

* 20- μL injections

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

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

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

* 20- μL injections of $10 \mu\text{M}$ streptomycin A

Intraday results for eluent prepared by separate chemists on separate days

ACCURACY

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

Sample Preparation Error

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

The second source of error is the moisture content. The manufacturers and distributors of streptomycin provide data for the percent moisture content of each lot. Depending on the storage container, age, humidity of the different storage locations, and the initial drying method used by the manufacturer, we find moisture content changes from the time it was first assayed. This change is of particular concern for streptomycin sulfate and other aminoglycoside antibiotics because they are hygroscopic. Any increase in moisture content of the solid streptomycin sulfate from the amount stated for the material in its Certificate of Analysis reduces the accuracy of the concentration by that same percentage. Table 4 shows the results for moisture content of the same streptomycin sulfate material, preweighed and redried by four different chemists using a previously unopened bottle. The moisture content for these four preparations ranged from 10.3% to 10.5%—7.4% to 7.6% greater than the moisture content of 2.9% provided by the Certificate of Analysis (C of A). In addition, the USP streptomycin sulfate had a measured moisture content of 9.1%; a difference in 4.1% from the 5% stated on its C of A. When the commercial material is analyzed using this HPAE-PAD method both with and without predrying, the error in accuracy of the dried material averaged 0.01%, while the undried material had an average error of 9.1% (see Table 4). Because

Table 4. Effect of Moisture Content on Accuracy

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

the USP glass vials appear sufficiently sealed, either the moisture content changed prior to their sealing, or the drying method used for its manufacture was not as effective as the SpeedVac method (using 0.5 torr of vacuum, 20–24 h, 50 °C).

The third source of error, salt content, was previously discussed in the section titled *Preparation of Solutions and Reagents, Stock Standards*. The percent of salt present in the streptomycin is an important factor used in the calculation of the streptomycin base concentration. The mass percentage of sulfate is theoretically 20.19% of streptomycin sulfate, assuming exactly 1.5 moles of sulfate per mole of streptomycin base. The presence of different types of salts can alter this percentage. For this reason, an accurate measure of the anionic salts presence in the anhydrous streptomycin sulfate material can assure an accurate potency factor of the material is used during sample preparation. Application Note 190³³ shows how ion chromatography with suppressed conductivity can be used to obtain a profile of the different major salts present in aminoglycoside antibiotics and help make accurate determinations of their potency. Using the ICS-3000 system with dual pump and dual detector, both the aminoglycoside base (using HPAE-PAD) and the salt composition (using IC) of the material are determined simultaneously.

Calibration Error

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

Spike Recovery

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

Purity

The USP Monographs describe eight categories of impurities in official chemical material: foreign substances, residual solvents, toxic impurities, concomitant components, signal impurities, ordinary impurities, related substances, and process contaminants.³³ This method is useful for many toxic impurities, concomitant components, ordinary impurities, related substances, and some process contaminants. For determination of process contaminants such as chloride, sulfate, bromate, and other inorganic and some organic anions, Application Note 190³⁰ may be useful. Streptomycin A purity was determined by comparing two different commercial sources of streptomycin sulfate, and evaluating its thermal and

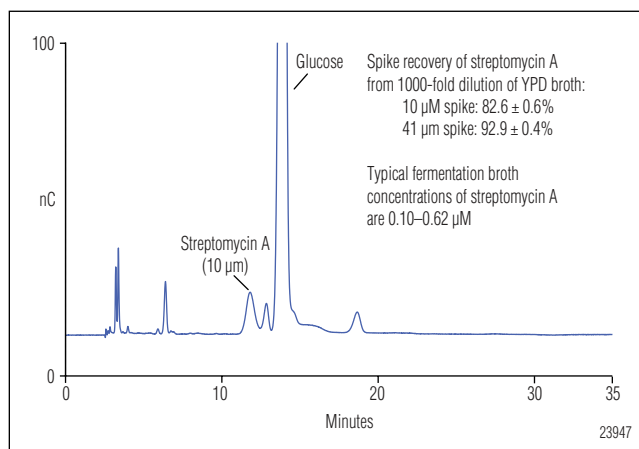


Figure 8. Determination of streptomycin A in YPD fermentation broth.

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

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

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

Ruggedness

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

Eluent Concentration

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

Column Temperature

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

Detector Compartment Temperature

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

Flow Rate

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

Column Reproducibility

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

CONCLUSION

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

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