Determination of Neomycin B and Impurities Using HPAE-IPAD

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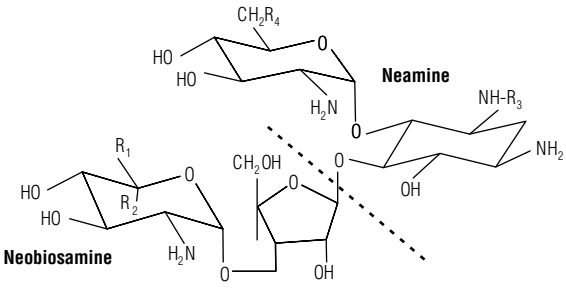
INTRODUCTION

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



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

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

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

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

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

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

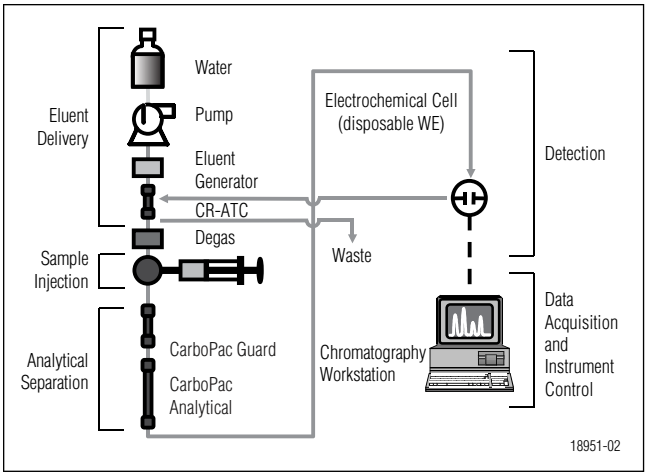

Figure 2. HPAE-PAD system for Neomycin determinations.

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

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

EQUIPMENT

Dionex BioLC system consisting of:

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

Chromeleon® Chromatography Management Software

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

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

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

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

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

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Standards

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

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

Samples

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

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

CONDITIONS

Method

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

AAA-Direct Waveform for the ED50*

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

Reference electrode in pH mode.

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

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

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

Stock Standards and Drug Substance

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

Neosporin Extraction

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

RESULTS AND DISCUSSION

Separation

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

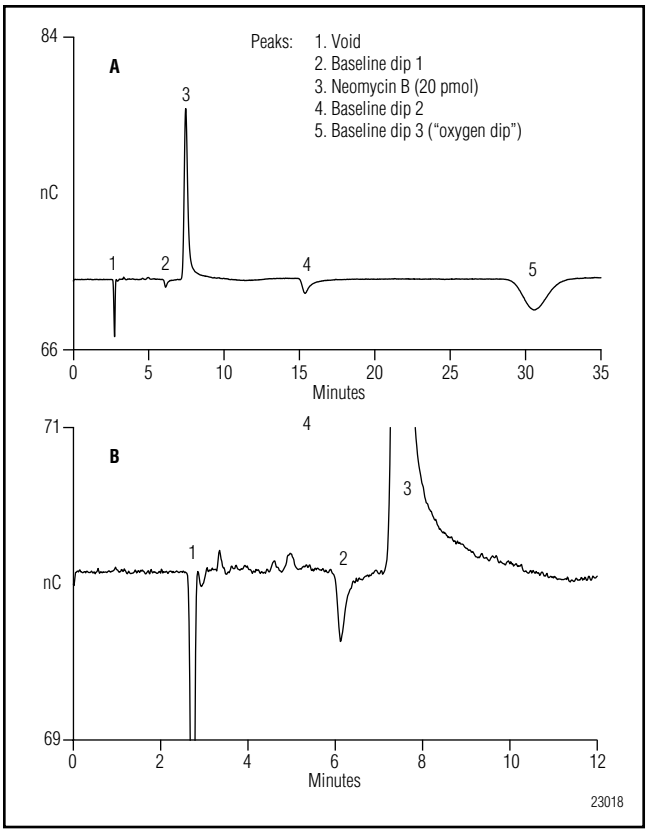

Figure 3. Determination of Neomycin B (1.0 µM, 20-µL injection) using eluent generation (2.40 mM KOH).

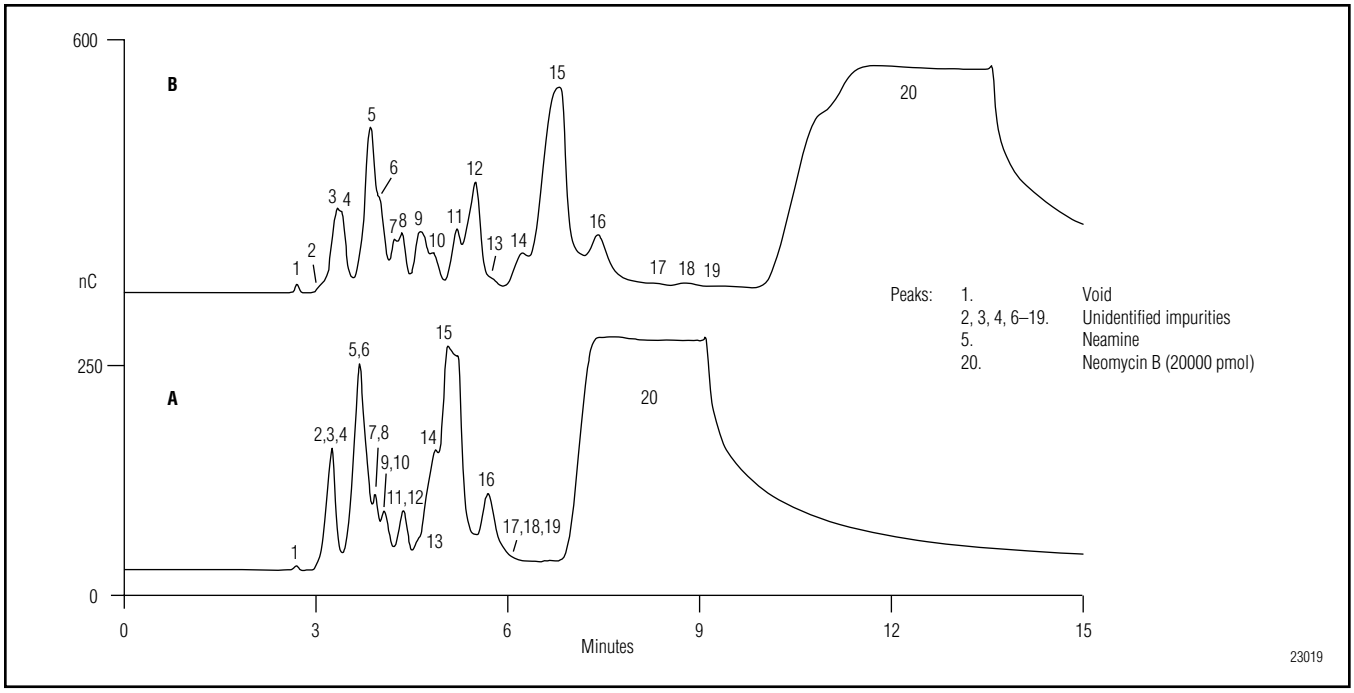

Figure 4. Separation of Neomycin B and impurities is highly dependent on eluent concentration. Comparison of the resolution of Neomycin B (1 mM, 20000 pmol) and impurities in commercial grade Neomycin sulfate separated using 2.40 mM KOH (chromatogram A) and 2.16 mM KOH (chromatogram B). Neomycin B (peak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

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

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

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

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

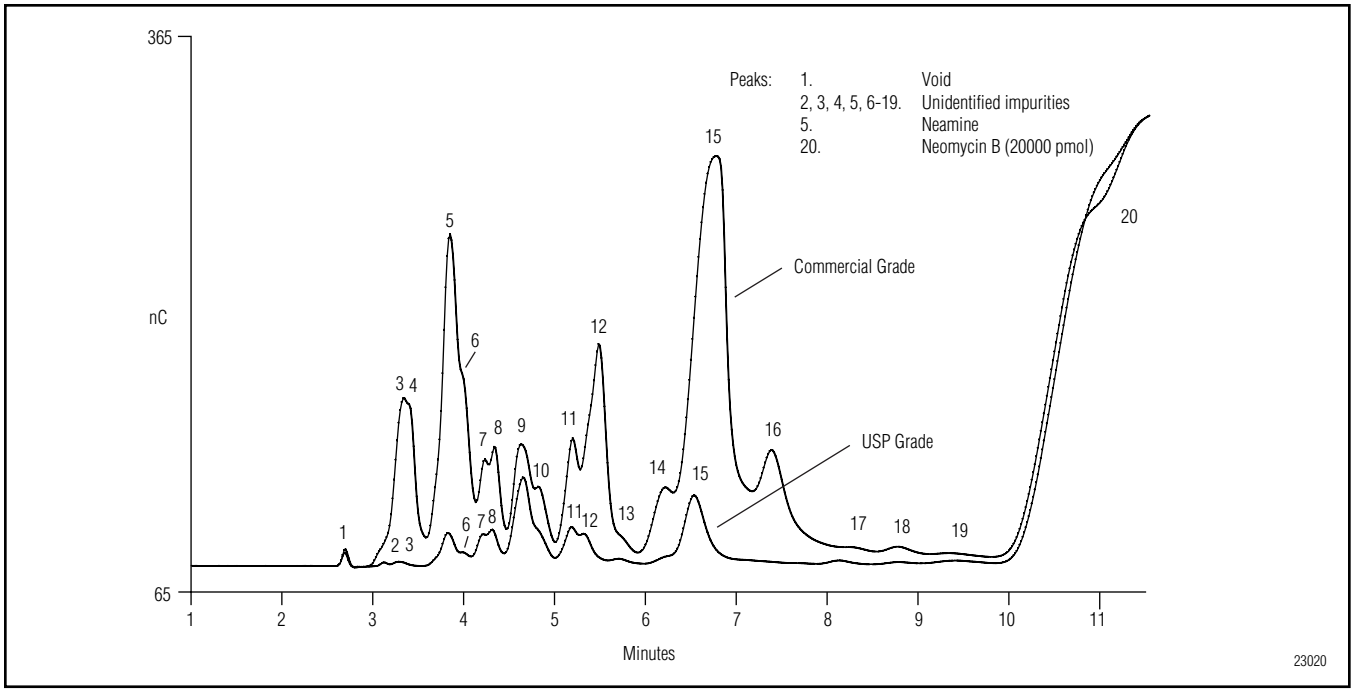

Figure 5. Comparison of the impurities found in USP and commercial grade Neomycin B (1 mM, 2 x 10^4 pmol) separated using 2.16 mM KOH. Neomycin B (peak 20) is injected at a concentration above its upper limit of detection.

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

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

Detection

Linearity

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

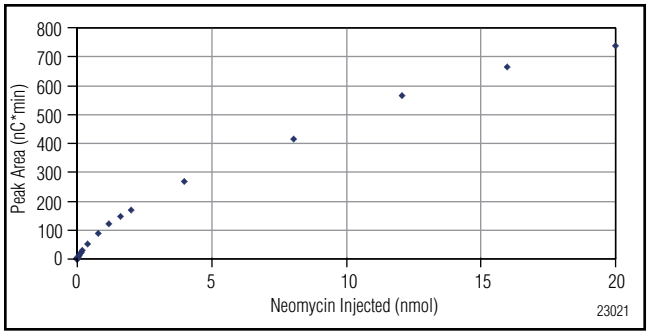

Figure 6. The relationship of peak area (mean) to nmol of Neomycin B injected for estimation of linear range.

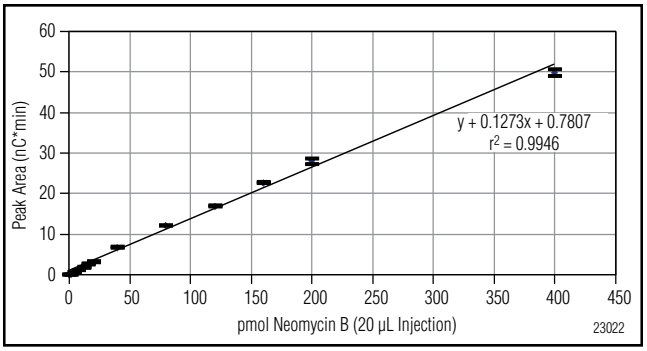

Figure 7. The linear relationship of Neomycin B peak area (mean ± SD) within its estimated linear range.

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

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

Lower Limits of Detection and Quantification

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

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

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

* 20-µL injections

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

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

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

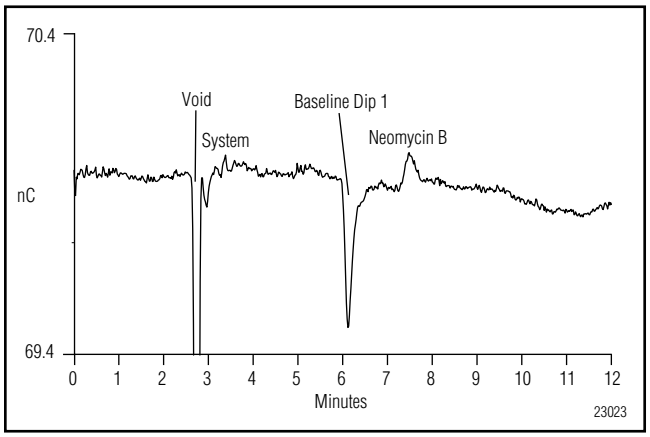

Figure 8. 0.20 pmol Neomycin B (0.010 µM, 20 µL) at its lower limit of detection.

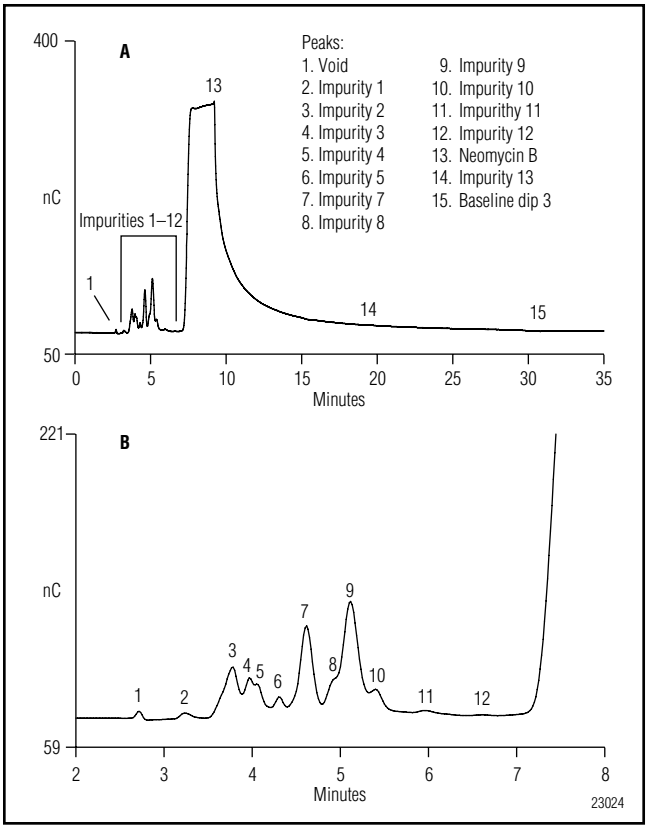

Figure 9. Determination of impurities when Neomycin B is analyzed outside the upper end of its linear range (0.50 mg/mL, 20-µL injection).

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

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

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

Precision

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

Retention Time

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

Peak Area

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

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

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

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

Robustness

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

Eluent Concentration

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

Column Temperature

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

Flow Rate

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

Sample Matrix

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

Column Reproducibility

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

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

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

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

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

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

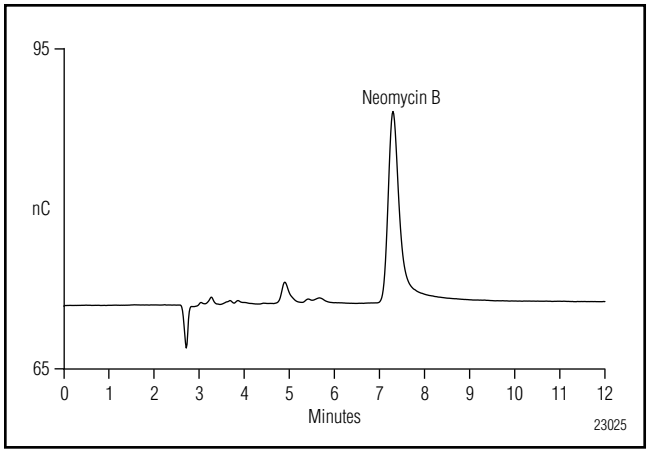

Figure 10. Determination of Neomycin B (2.12 µM, 25 µL injection) extracted from 25.6-mg Neosporin topical ointment with 1 mL water, and diluted 85-fold.

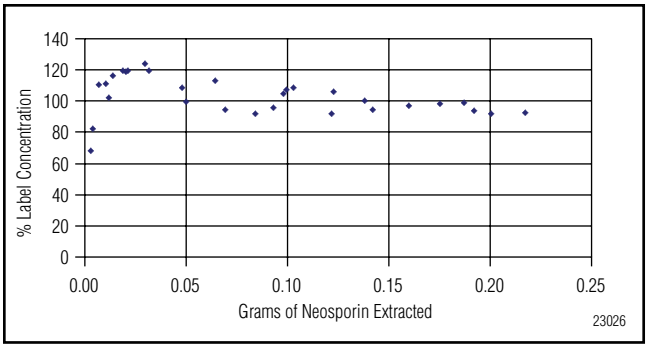

Figure 11. The relationship of Neomycin B yield and the mass of Neosporin ointment extracted.

Analysis of Formulations

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

Instrument Operational Considerations

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

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

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

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

CONCLUSION

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

REFERENCES

1. *Physicians' Desk Reference.* Medical Economics Company, Inc. PDR 44th Edition, Edward R. Barnhart, publisher, Oradell, NJ, **1990**.
2. Swart, E. A.; Romano, A. H.; Waksman, S. A. *Proc. Soc. Exp. Biol. Med.* **1950**, *73*, 376.
3. Lemieux, R. U.; Kullnig, R. K.; Moir, R. Y. *J. Am. Chem. Soc.* **1958**, *80*, 2237.
4. Rinehart, K. L. *The Neomycins and Related Antibiotics. Chemistry of Microbial Products.* E. R. Squibb Lectures on Chemistry of Microbial Products. Institute of Microbiology. John Wiley & Sons, Inc.; NY, **1964**.
5. United States Pharmacopeia, The National Formulary. *Neomycin Sulfate*. USP 29, NF 24, **2006**, p. 1491.
6. European Pharmacopia (EP). Fifth Edition, Neomycin Sulphate. Section 0197. The Council of Europe; Strasbourg, France, **2005**; www.pheur.org.
7. Szunyog, J.; Adams, E.; Roets, E.; Hoogmartens, J. Analysis of Tobramycin by Liquid Chromatography with Pulsed Electrochemical Detection. *J. Pharm. Biomed. Anal.* **2000**, *23*, 891–896.
8. Polta, J. A.; Johnson, D. C.; Merkel, K. E. Liquid-Chromatographic Separation of Aminoglycosides with Pulsed Amperometric Detection. *J. Chromatogr.* **1985**, *324*, 407–414.
9. Adams, E.; Schepers, R.; Roets, E.; Hoogmartens, J. Determination of Neomycin Sulfate by Liquid Chromatography with Pulsed Electrochemical Detection. *J. Chromatogr., A.* **1996**, *741*, 233–240.
10. *Neomycin in Topical Lotions.* Application Note 66 (LPN 034289-01, June 1991), Dionex Corporation, Sunnyvale, CA.
11. *Determination of Tobramycin and Impurities Using HPAE-PAD*. Application Note 61 (LPN 1626, July 2004), Dionex Corporation, Sunnyvale, CA.
12. Hanko, V. P.; Rohrer, J. S. Determination of Tobramycin and Impurities Using High-Performance Anion-Exchange Chromatography with Integrated Pulsed Amperometric Detection. *J. Pharm. Biomed. Anal.* **2006**, *40*, 1006–1012.
13. Cheng, J.; Jandik, P.; Avdalovic, N. Development and Characterization of Microfabricated Disposable Gold Working Electrodes for High-Performance Ion Chromatography and Integrated Pulsed Amperometric Detection. *Anal. Chem.* **2003**, *75*, 572–579.

14. Cheng, J., Jandik, P., and Avdalovic, N. Use of Disposable Gold Working Electrodes for Cation Chromatography-Integrated Pulsed Amperometric Detection of Sulfur-Containing Amino Acids. *J. Chromatogr., A*. **2003**, *997*, 73–78.
15. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline. Text on Validation of Analytical Procedures. Q2A. Recommended for Adoption at Step 4 of the ICH Process on 24 October **1994** by the ICH Steering Committee; www.ICH.org.
16. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Methodology. Q2B. Recommended for Adoption at Step 4 of the ICH Process on 6 November **1996** by the ICH Steering Committee; www.ICH.org.
17. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). Guidance for Industry. Analytical Procedures and Method Validation. Chemistry, Manufacturing, and Controls Documentation. Draft Guidance. August, **2000**; http://www.fda.gov/cder/guidance/2396dft.htm.
18. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Reviewer Guidance. Validation of Chromatographic Methods. November, **1994**; http://www.fda.gov/cder/guidance/cmc3.pdf.
19. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Guideline for Submitting Samples and Analytical Data for Methods Validation. February, **1987**; http://www.fda.gov/cder/guidance/ameth.htm.
20. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Guidance for Industry. Bioanalytical Method Validation. May, **2001**; http://www.fda.gov/cder/guidance/4252fnl.pdf.
21. United States Pharmacopeia, The National Formulary. “<1225> Validation of Compendial Methods.” USP 29, NF 24; **2006**, 3050–3052.
22. United States Pharmacopeia, The National Formulary. “<621> Chromatography.” USP 29, NF 24; **2006**, 2639–2651.
23. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline. Impurities in New Drug Substances Q3A(R). Attachment 1. Thresholds. Recommended for Adoption at Step 4 of the ICH Process on 7 February 2002 by the ICH Steering Committee; www.ICH.org.
24. Product Manual. *AAA-Direct* Amino Acid Analysis System. Document No. 031481; Dionex Corporation, Sunnyvale, CA.
25. MedlinePlus; United States National Library of Medicine; National Institutes of Health; Bethesda, MD; http://www.nlm.nih.gov/medlineplus/druginfo/uspdi/202396.html#Brands.
26. United States Pharmacopeia, The National Formulary. Neomycin and Polymyxin B Sulfates and Bacitracin Zinc Ointment.” USP 29, NF 24; **2006**, 1503.

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