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Determination of neomycin sulfate and impurities using high-performance anion-exchange chromatography with integrated pulsed amperometric detection

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Abstract

Neomycin B is one of a class of aminoglycoside antibiotics that lack a good chromophore, and is therefore difficult to determine using reversed-phase HPLC with absorbance detection. This is especially true for determining the quantity of each impurity. We show that neomycin sulfate and its major impurities, including neamine (neomycin A), can be separated on a strong anion-exchange column using a weak potassium hydroxide eluent (2.40 mM) at a column temperature of 30 °C, and directly detected by integrated pulsed amperometric detection (IPAD). The resolution (United States Pharmacopeia (USP) definition) between neomycin B and the closest major impurity ranged from 6.56 and 7.45 over 10 days of consecutive analysis (7.24 ± 0.10, $n = 836$ injections). Due to the difficulty of producing weak hydroxide eluents of the required purity (i.e. carbonate-free), this method depends on automatic eluent generation to ensure method ruggedness. This method exhibited good long-term (10 days, 822 injections) retention time stability with a R.S.D. of 0.6%. Peak area R.S.D. (10 μM) was 1.3%. Method robustness was evaluated by intentionally varying the flow rate, eluent concentration, column temperature, and column. The spike recoveries of neomycin B from extractions of three different topical ointments and cream formulations ranged from 95 to 100%. The measured concentration of neomycin B in these formulations ranged from 119 to 154% of the label concentration. The R.S.D. for the measured concentration of one of the formulations tested over three separate days, $n = 11$ extracts, was 3.2%. Based on the results of these evaluations, we believe this method can be used for neomycin sulfate identity, assay, and purity.

Keywords: HPAE-PAD; HPAE-IPAD; Neomycin A; Neomycin B; Neamine; Aminoglycoside; Antibiotic; Amperometric detection; Anion-exchange; Chromatography

1. Introduction

Neomycin sulfate, also known as bekanamycin sulfate, is a water-soluble complex of aminoglycoside antibiotics used in a variety of pharmaceutical applications including ophthalmic, topical, oral, and intravenous administrations (e.g. Neosporin®, NeoDecadron®, PediOtic®) [1].[1] Neomycin is purified from the fermentation of the actinomycete *Streptomyces fradiae* [2–4]. Neomycin B (also known as framycetin) is the principle 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), and neomycin F (paromomycin II). The acetylation of neomycins A, B, and C also occurs during fermentation, lowering the antibiotic potency (LP = low potency), and these compounds have been designated as neomycin LP-A (also known as mono-*N*-acetyl-neamine or 3-acetylneamine), neomycin LP-B (mono-*N*-acetyl-neomycin B, or LP-1 in early publications), and 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 [5,6]. Other impurities may result from chemical degradation during manufacture or storage [7]. For example, acid hydrolysis of neomycin B yields

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[1] Neosporin is a registered trademark of Pfizer Consumer Healthcare (Morris Plains, NJ 07950). NeoDecadron is a registered trademark of Merck & Company, Inc. (West Point, NJ 19486). PediOtic is a registered trademark of Monarch Pharmaceuticals, Inc. (Bristol, TN 37620). AAA-Direct is a trademark, and CarboPac, Chromeleon, and EluGen are registered trademarks of Dionex Corporation (Sunnyvale, CA 94088).

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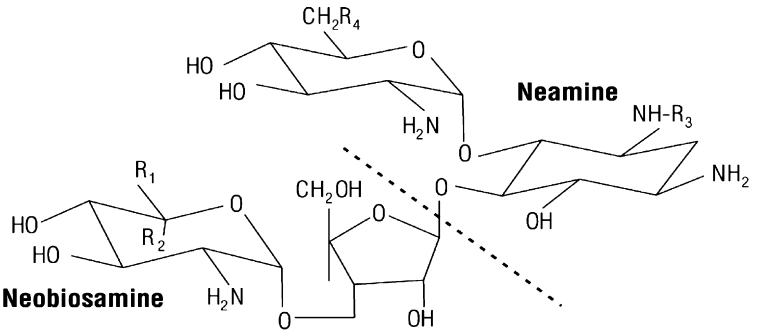

Neomycin	R_1	R_2	R_3	R_4
A	NA	NA	–H	$–NH_2$
B	–H	$–CH_2NH_2$	–H	$–NH_2$
C	$–CH_2NH_2$	–H	–H	$–NH_2$
D	NA	NA	–H	–OH
E	–H	$–CH_2NH_2$	–H	–OH
F	$–CH_2NH_2$	–H	–H	–OH
3-Acetylneamine	NA	NA	$–COCH_3$	$–NH_2$

NA—not applicable

Fig. 1. Chemical structures of neomycin B and known impurities neomycin A (neamine), C, D, E, F, and acetylneamine. Neomycin A, D, and 3-acetylneamine do not contain the neobiosamine structure.

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 (fifth edition) monograph for the *Europoean Pharmacopoeia* (EP) compendial method for neomycin sulphate and framycetin sulphate measures neomycin B as the primary antibiotic, with neomycin A, C, D, E, F, A-LP, B-LP as impurities [8]. Henceforth in this document, the term "impurities" will mean all substances present in neomycin sulfate that are not neomycin B sulfate. The chemical structures of neomycin B and its major impurities are shown in Fig. 1. The molecular weights of neomycin B and C, and neomycin E and F, are identical and therefore limit the usefulness of mass spectroscopy for peak identification. These aminoglycosides, like most carbohydrates, lack a good chromophore, and therefore require high concentrations to be detected by UV absorbance. Many manufacturing process-intermediates and ingredients of final pharmaceutical formulations are chromophoric and can interfere with the direct detection of neomycin B and its impurities by absorbance. Refractive index detection has similar limitations. For these reasons, most methods for identity, assay, quality, and purity of neomycin B require some type of chemical derivatization to increase detection sensitivity. The current official USP monograph describes two derivatization methods for neomycin sulfate identification [9]. A microbial technique is used for quantification. The compendial method has no tests for quality or purity. Many of these techniques are labor-intensive, involving materials hazardous to both laboratory personnel and the environment. The absence of a test for quality and purity is consistent with the *Code of Federal Regulations* (*CFR*), title 21, chapter 1, subchapter D, part 444.42 and 444.42a for neomycin sulfate and sterile neomycin sulfate, respectively; however, conflicts with the U.S. food and drug administration requirements for good manufacturing practices (GMP) where identity, strength, quality, and purity must be determined for drug substances described in the *Code of Federal Regulations* (*CFR*), title 21, chapter 1, subchapter C, part 211.160b, and *Certification of Antibiotic Drugs* (431.10d). The lack of a suitable method to evaluate quality and purity is also non-compliant with the international conference on harmonisation (ICH) harmonised tripartite guideline [10]. The USP does provide chromatographic purity tests for other similar aminoglycoside antibiotics (e.g. tobramycin [11], kanamycin sulfate [12]). When official methods are not available to meet GMP requirements, alternative methods must be adopted.

Derivatization techniques have the associated limitations of varying derivatization efficiencies and reagent instabilities that compromise method ruggedness. These techniques also require handling and disposal of hazardous materials. Neomycin B and its major impurities may be oxidized under alkaline conditions and directly detected by pulsed amperometric detection (PAD), which has a broad linear range and very low detection limits for aminoglycoside antibiotics [13–16]. The HPLC method specified by the *European Pharmacopia* (EP) procedures for neomycin sulphate [8] and framycetin sulphate [17] identity, assay, and purity uses a non-alkaline mobile phase and requires a post-column addition of NaOH for PAD. This requires an additional pump and dilutes eluting peaks, reducing method sensitivity compared to a method with a sufficiently alkaline eluent.

High-performance anion-exchange chromatography (HPAE) with PAD is an established technique for carbohydrate analysis. HPAE-PAD has been used for neomycin determinations [18], and for other aminoglycoside antibiotic determinations [12,14,19–23]. HPAE with integrated pulsed amperometric detection (IPAD, similar to PAD but includes current integra-

tion through varying voltage potentials), an established technique for amino acid analysis, has also been used for other aminoglycoside antibiotic determinations for improved sensitivity [16,24]. All published methods require weak sodium hydroxide eluents (1–10 mM) that are difficult to prepare reproducibly without varying amounts of carbonate contamination that adversely affect retention time precision. This problem has limited the adoption of HPAE-PAD for neomycin determinations. To address this limitation, we added two commercially available devices to an HPAE-IPAD system, an eluent generator (EG), and a continuously regenerated anion trap column (CR-ATC). The EG automatically prepares precise concentrations of KOH eluent from water and a potassium electrolyte solution using electrolysis. The electrolysis occurs without exposure to atmospheric gases, and therefore with a significant reduction of carbonate contamination. The minor amounts of carbonate, as well as borate and other contaminating anions from the supply water, are removed by the CR-ATC installed after the EG. Consequently, the normal variability in hydroxide concentration associated with manual eluent preparation and the variability of carbonate contamination are essentially eliminated, leading to highly reproducible retention times and peak area. We previously demonstrated that a similar system could be used for the analysis of tobramyin [16]. Here we used this system to generate a 2.40 mM KOH eluent for a fast rugged assay of neomycin B and its major known impurities. To achieve the best sensitivity, we used an amino acid (IPAD) waveform instead of the waveform typically used for carbohydrate analysis. For the best reproducibility we used disposable gold working electrodes. Disposable gold working electrodes are manufactured in a manner that improves electrode to electrode reproducibility [25–27]. In this paper, we evaluated the performance of our method in terms of ruggedness and the ability to measure low levels of the major known impurities of neomycin B. We evaluated neomycin B purity and demonstrated the capability of this method for determination of neomycin B in three different topical over-the-counter pharmaceutical formulations containing, among other ingredients, polymyxin B sulfate and bacitracin zinc [28]. Overall, the described set-up demonstrated good sensitivity, good sample throughput (15 min per run), high retention time reproducibility, and good spike recovery of neomycin B from the pharmaceutical products.

2. Experimental

2.1. Standards

Solid commercial grade neomycin sulfate (cat. #N1876, Sigma–Aldrich Chemical Co., St. Louis, MO, USA) and USP grade neomycin sulfate (reference standard #45800, USP, Rockville, MD, USA) were placed in plastic vials and dissolved in deionized water to 10-mg/mL. Neamine (also known as neomycin A hydrochloride; cat. #9930354, International Chemical Reference Substances, World Health Organization Collaborating Centre for Chemical Substances, Prismavägen, Stockholm) standard was received pre-weighed in sealed ampules, and the required amount of deionized water was added to make a 0.5 mg/mL (1.5 mM) standard. The masses of moisture, sulfate, and impurities, as stated on the manufacturer's certificate of analysis, were subtracted from the measured mass to improve solution concentration accuracy. These solutions were diluted with water (gravimetrically) to yield the desired stock mixture concentrations. The solutions were maintained frozen at −40 °C until needed. Masses of 0.02, 0.04, 0.08, 0.12, 0.16, 0.20, 2, 4, 8, 12, 16, 20, 40, 80, 120, 160, 200, 400, 800, 1200, 1600, 2000, 4000, 8000, 12 000, 16 000, and 20 000 pmol neomycin B USP reference material were injected four times each for linear range studies. Only the results for amounts ≤200 pmol neomycin B were used for linearity studies. Polypropylene injection vials and other labware were used to reduce possible adsorptive losses, and ensure accurate results.

2.2. Samples

Original Neosporin and Maximum Strength Neosporin + Pain Relief (Pfizer Consumer Healthcare, Morris Plains, NJ), and Walgreens Triple Antibiotic PLUS (Walgreen Co., Deerfield, IL) in amounts ranging from 17 to 32 mg were each placed in a 1.5-mL polypropylene microcentrifuge vial with a detachable screw cap, and combined with 1.00 mL water. The mass of the ointment and water were documented gravimetrically. The sealed vial was placed in an 80 °C heating block for 2.5 min, vortexed (at highest setting), and then heated an additional 2.5 min. After 5 min, the melted ointment was again vortexed continuously for 5 min, and then placed in the refrigerator for >1 h. The chilled extract was centrifuged at 16 000 × g in a microcentrifuge for 10 min, the supernatant separated from an upper fat layer using a Pasteur pipette pre-rinsed with water, and transferred to another vial. This extract (31–59-fold dilution of starting sample) was diluted 85.4-fold with water gravimetrically to accurately calculate the dilution. A 20-μL aliquot of the diluted extract was analyzed by HPAE-IPAD to determine the neomycin B concentration. The pharmaceutical products tested contained 3.5 mg neomycin B per gram based on product labeling and therefore 17–32 mg extracted in 1-mL were expected to yield 96.8–182 μM neomycin B. For spike recovery experiments, the 1.00 mL water used for extraction was replaced with 1.00 mL of a 600-μM neomycin B standard (USP Reference Material). After 84.5-fold dilution of these extracts, the expected neomycin B concentrations ranged from 1.13 to 2.15 μM for the non-spiked samples, and a total of 8.23–9.25 μM for the spiked samples.

2.3. Chromatography

The chromatography system consisted of Dionex GP50 gradient pump with degas option and GM-4 gradient mixer, EG50 eluent generator with EGC II KOH eluent generator cartridge (EluGen® II Hydroxide), EG40/50 vacuum degas conversion kit, CR-ATC, ED50A electrochemical detector, AS50 autosampler, AS50 thermal compartment, and Chromeleon® chromatography workstation (Dionex Corporation, Sunnyvale, CA, USA). Neomycin and its impurities were separated with a CarboPac™ PA1 (4 mm × 250 mm, Dionex Corporation) anion-exchange column (USP designation L46) with its guard (4 mm × 50 mm).

Table 1
Effect of eluent concentration on retention times (min)

Peak identity	KOH eluent concentration (mM)							
	100	75	50	25	10	5	2	1
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

The electrochemical waveform was +0.13 V from 0.00 to 0.04 s, +0.33 V from 0.05 to 0.21 s, +0.55 V from 0.22 s to 0.46 s, +0.33 V from 0.47 s to 0.56 s, −1.67 V from 0.57 s to 0.58 s, +0.93 V at 0.59 s, and +0.13 V at 0.60 s, using the pH reference mode with current integrated between 0.21 and 0.56 s for detection. We used *AAA-Direct*™-certified disposable gold working electrodes (replaced every 7 days) [25]. Neomycin B and its impurities were separated using 2.40 mM KOH, produced by the eluent generator, at a flow rate of 0.50 mL/min and a column temperature of 30 °C. This method used a 20-μL injection and had a run time of 15 min. The column set was washed once a week with 100 mM KOH for 60 min and re-equilibrated overnight to 2.40 mM to restore retention times to their initial values.

3. Results and discussion

3.1. Specificity

3.1.1. Separation

The retention times of neamine and neomycin B remain relatively unchanged through the eluent concentration range of 5–100 mM KOH, eluting near the void. Below 5 mM, retention times were affected significantly by eluent concentration (Table 1). The location of a void dip at 2.7–2.9 min, baseline dip at 15–16 min, and dip due to dissolved oxygen at 30–32 min, remained unchanged with varying hydroxide eluent concentrations. We selected 2.40 mM KOH for routine analysis as a compromise between rapid elution of neomycin B, resolution of impurities, and the resolution of neomycin B from either the baseline dip or the oxygen dips in the baseline. Fig. 2 shows the separation of 200 pmol neomycin B (peak 13), 40 pmol neamine (peak 4), and 10 unidentified impurities (peaks 2, 3, 5–12). Peak 9 was presumed to be neomycin C because it was the major impurity peak listed in commercial grade neomycin sulfate. No commercial source of neomycin C exists to confirm this. When the concentration of a commercial grade neomycin B was increased to 1 mM, at least 13 impurity peaks were observed, and decreasing eluent strength to 2.16 mM KOH enabled 18 impurity peaks to be observed. The resolution (USP definition) between neomycin B and peak 9, presumed to be neomycin C, ranged from 6.56 and 7.45 (7.24 ± 0.10, $n = 836$ injections) over 10 days of consecutive injections without any column regeneration using 2.40 mM KOH. The resolution between neomycin B and peak 10 ranged from 6.20 and 7.61 (7.09 ± 0.26). The resolution between neomycin B and peaks 11 and 12 (Fig. 3) were 2.38 and 1.65, respectively. No resolution specification is stated in the USP monographs for neomycin sulfate; however, the EP liquid chromatographic method [8,33] states a resolution specification of ≥2.0 between neomycin B and neomycin C and

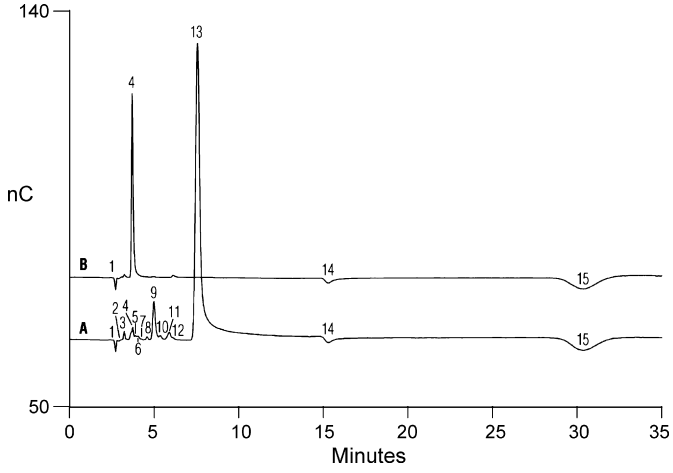

Fig. 2. Determination of 200 pmol USP grade neomycin B (chromatogram A) and 40 pmol neamine (chromatogram B) using a CarboPac PA1 column with a 2.40 mM KOH eluent (produced by an eluent generator) at 0.5 mL/min, 20 μL injections, a 30 °C column temperature, and IPAD. Peak 1: void; peaks 2, 3, 5–12: unknown impurities; peak 4: neamine; peak 13: neomycin B; peak 14: baseline dip; peak 15: oxygen dip.

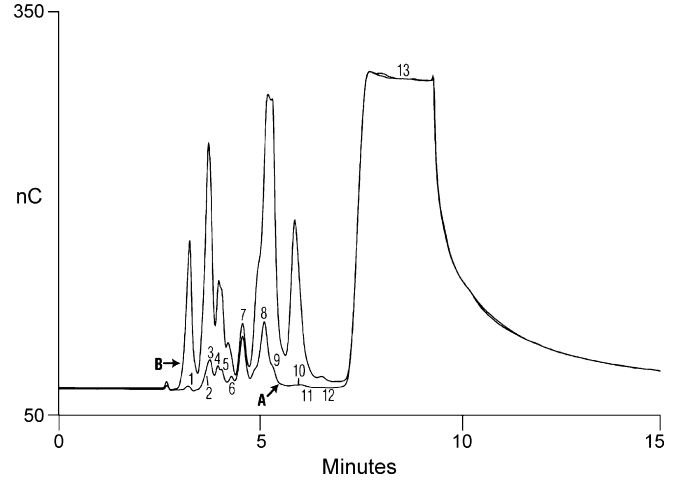

Fig. 3. Determination of impurities (peaks 1–12) when neomycin B (peak 13; 1.0 mM) is injected outside its calculated linear range. Comparison of USP grade (chromatogram A), and commercial grade (chromatogram B) materials. Peak 3 is neamine, and peak 8 is presumed to be neomycin C. Impurity peak 8 in the commercial grade material is also outside its linear range.

allows mobile phase concentration adjustment to achieve this required resolution. The method described here meets the EP specification.

3.1.2. Detection

The EP method specifies a gold working electrode using a three-potential pulsed amperometric waveform with detection at +0.05 V, oxidative cleaning at +0.75 V, and gold oxide reduction at −0.15 V, but does not specify the durations of these steps. These voltages are identical to a pulsed amperometric detection (PAD) waveform that in earlier years was used for carbohydrate determinations (Waveform B; Dionex Technical Note 21) [29]. The strong positive (oxidative) cleaning potential of this waveform led to the loss of gold from the surface, resulting in a loss in peak area response over long-term use [30]. Since the development of the EP method, the recommended waveform for carbohydrate applications has been revised [29,30] to produce a more stable long-term response. This revised waveform (Waveform A; Dionex Technical Note 21) [29] is a four-potential program that uses negative (reductive) cleaning rather than oxidative cleaning, preserving the gold working electrode surface. Unlike the older three-potential waveform, this waveform is fully compatible with disposable gold working electrodes, which only have a thin layer of gold. While we found the four-potential waveform was suitable for aminoglycoside determinations [24], here we used an integrated pulsed amperometric detection (IPAD) waveform originally developed for amino acid determinations. An IPAD waveform detects an analyte using more than the one potential used in a PAD waveform. We chose this waveform because the signal-to-noise ratio was determined to be two to four times greater than the four-potential waveform for tobramycin [24], a similar aminoglycoside. This was also true for neomycin and enabled us to achieve maximum sensitivity for neomycin and its impurities.

3.2. Linearity

3.2.1. Range

For estimation of the upper and lower limits of the linear range, the technique of Cassidy and Janoski [31] and Snyder et al. [32] was used. The linear range was calculated using a plot that relates amount of neomycin B injected to its peak area or height response factors (ratio of peak area/mass or height/mass injected). The region of the plot showing the highest and constant response factor values was averaged, and then a 10% deviation from this averaged value was calculated. The upper and lower limits of the linear range were the amounts of neomycin B where the observed response factors are within 10% of the average response factor. We chose a 10% deviation as our upper and lower limits. The neomycin B peak area linearity ranged from 4 to 200 pmol. The peak height linearity ranged from 6 to 100 pmol.

3.2.2. Linearity

The linear relationship (first degree polynomial regression) of peak area to injected masses of 4, 8, 12, 16, 20, 40, 80, 120, 160, and 200 pmol (0.2–10 μM) neomycin B produced an r^2 value of 0.9987, y-intercept was 0.3519 nC min, and slope was 0.1394 nC min/pmol. The known 12–200 pmol amounts were compared against the calculated amounts of neomycin B using the slope and y-intercept, and errors ranged from −13 to +13%. For 4 and 8 pmol, the calculated errors were −63 and −24%, respectively. When the amounts of neomycin B were calculated using the average response factor instead of the slope and y-intercept derived from linear regression, the errors ranged from −9 to +9% for the 4–200 pmol known amounts. Better results (lower error) were attained using a separate low-level average response factor for amounts below 4 pmol.

3.3. Lower limits of detection and quantification

The concentration (or mass injected) of neomycin B at the lower limit of detection (LOD) was calculated from three times the average peak-to-peak noise (pC units, a height value), divided by the average peak height response factor for neomycin B within its linear region. At this concentration, signal-to-noise ratio equals 3. The lower limit of quantification (LOQ) was calculated as 10 times the average peak-to-peak noise. Baseline noise ranged from 13 to 81 pC (mean 35 pC). The LOD and LOQ were 0.21 and 0.72 pmol, respectively, for a 20-μL injection of neomycin B.

3.4. Determination of neomycin B purity

When neomycin is analyzed at the upper limit of its calculated linear range (200 pmol), a 0.2 pmol LOD is equivalent to 0.1 mol% impurity of neomycin B. This percent neomycin B impurity 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 can be detected by injecting neomycin B at concentrations outside its linear range. Injecting 20 μL of 1.0 mM neomycin B (20 nmol, Fig. 3) decreases the lower detectable percentage of a neomycin B impurity 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 the percent impurity calculation. No current USP specification exists for the purity of neomycin sulfate, while the EP monograph requires ≤2% neamine (neomycin A) and neomycin C between 3 and 15%, any other impurity ≤5%, and total of other impurities ≤15% [8]. For framycetin sulphate, the EP monograph requires ≤1% neamine (neomycin A), neomycin C ≤3%, and total of other impurities ≤3% [33]. This method easily achieves the EP neomycin B impurity levels by injecting neomycin B at the upper limit of its linear range.

Table 2 presents the measured impurities found in the two sources of neomycin B analyzed in this paper, as pmol neomycin B equivalents with their corresponding percents. Using the injection shown in Fig. 3, the largest single impurity peak at 5.22 min (peak 8), presumed to be neomycin C in the USP Reference Material, was a 0.67% impurity of neomycin B. The sum of all impurities (peaks 1–12) was 1.51%. Neamine (peak 3), was 0.20%. Peak 12, a trace impurity, was only detected with 1 mM injections of neomycin B. For the less pure commercial neomycin B material, the largest single measured impurity peak

Table 2
Comparison of impurity content of two sources of neomycin sulfate

Impurity peak no.:	3[a]	8[a]	1, 2, 4, 5, 6, 7, 9, 10[a], 11, 12	
Retention time (min)	3.75	5.22		
Peak identity:	Neamine (%)	Neomycin C[b] (%)	All other unidentified impurities (%)	Total impurities (%)
USP grade neomycin B (1.00 mM) (% impurity)				
Mean	0.200	0.671	0.635	1.51
S.D.	0.009	0.050	0.059	0.05
Commercial grade neomycin B (1.00 mM) (% impurity)				
Mean	1.16	5.10	5.11	11.37
S.D.	0.02	0.03	0.02	0.03

[a] Impurity peaks 3, 8 and 10 exceeded the 10 μM upper limit of linearity (>200 pmol) for the commercial material at the 1.00-mM neomycin B injected concentration, and required a five-fold dilution for their determinations. All values presented for these peaks in the commercial material were obtained using a 200-μM neomycin B injected concentration, and corrected for the five-fold dilution.
[b] Suspected neomycin C peak.

at 5.22 min (peak 8; presumed to be neomycin C) was a 5.1% impurity of neomycin B, neamine was 1.16%, and the sum of all impurities (peaks 1–12) was 11.37%. Table 3 shows the relationship of mass of neomycin B injected to the measured level of total impurities, for replicate trials performed on separate days. The calculated total % impurity remained consistent through the concentration range of 0.051–1.00 mM neomycin B injected for both the USP and commercial grade materials, and from day-to-day.

A reduction of the eluent concentration to 2.16 mM KOH increased resolution of impurities but decreased the symmetry of the neomycin B peak. The number of impurity peaks increased from 12 to 20. This increased resolution did not alter the measurement of total impurity compared to the 2.40 mM KOH eluent. Using 2.16 mM KOH and 1.00 mM neomycin B, we determined that the total impurity was $1.66 \pm 0.03\%$ for the USP reference material, and $10.99 \pm 0.02\%$ for the commercial material.

Table 3
Total % impurity

Trial	Injected concentration (mM)	Mean	R.S.D. (%)
USP grade neomycin			
1	1.000	1.4	1.5
2	1.000	1.2	2.1
3	1.000	1.5	3.2
1	0.503	1.2	5.8
1	0.202	1.8	2.6
2	0.203	1.6	1.4
1	0.101	1.5	6.0
1	0.051	1.4	6.6
Commercial grade neomycin			
1	1.000	11.4	0.72
2	1.000	11.4	0.25
1	0.499	11.4	1.0
1	0.207	11.9	0.41
2	0.203	11.6	0.49
1	0.100	11.8	0.68
1	0.051	12.0	0.96

3.5. Accuracy

Method accuracy was determined by recovery experiments. 20 μM neomycin B was augmented with known concentrations of neamine ranging from 0.05 to 0.4 μM, and 1.0 mM neomycin B was augmented with 2 μM neamine. The results of these experiments are summarized in Table 4. The recovery of neamine from neomycin B ranged from 76 to 88%. We previously published that an increase in kanamycin B recovery from tobramycin [16] was attained by acidification in 2 mM HCl. Only a slight increase in neamine recovery at low concentrations was found using an identical treatment (Table 4). Acidification increased recovery 10–15%, but caused a 32–46% loss in neamine peak area response, and therefore was not used in this study, nor recommended. The accuracy of this method for neomycin B in pharmaceutical products was also evaluated, and is described in Section 3.8.

3.6. Precision and reproducibility

3.6.1. Peak retention time

The average retention time for neomycin B (10 μM) over 10 days (822 injections) was 7.45 ± 0.05 min. The retention time R.S.D. for each 24 h period ranged from 0.23 to 0.43% (except 1.3% on the first day), with no upward or downward trend. This

Table 4
Recovery of trace neamine from Neomycin B

Neomycin B concentration (μM)	Neamine concentration (μM)	Recovery (%)		
		Mean	S.D.	*N*
Without acidification				
1000	2.00	86.1	1.4	5
20.0	0.05	87.6	7.5	4
20.0	0.20	75.5	2.7	4
20.0	0.40	85.5	1.5	4
With 2 mM HCl acidification				
1000	2.00	87.5	1.3	4
20.0	0.05	121.1	72.1	4
20.0	0.20	103.9	12.4	4
20.0	0.40	98.5	10.4	4

Table 5
Summary of results for robustness

Treatment	Retention time (min)		Peak area (nC min)	
	Mean ± S.D.	% Change	Mean ± S.D.	% Change
Flow rate (mL/min)				
0.55 (+10%)	6.76 ± 0.01	−9.4	28.0 ± 0.4	−4.4
0.50 (0%)	7.47 ± 0.02	0.0	29.3 ± 1.0	0.0
0.45 (−10%)	8.23 ± 0.02	10.2	30.7 ± 0.2	4.9
Eluent concentration (mM)				
2.64 (+10%)	5.89 ± 0.01	−21.4	30.7 ± 1.0	1.8
2.40 (0%)	7.50 ± 0.02	0.0	30.2 ± 0.4	0.0
2.16 (−10%)	10.90 ± 0.03	45.4	26.1 ± 0.2	−13.6
Column temperature (°C)				
33 (+10%)	7.66 ± 0.02	2.7	31.3 ± 1.0	5.4
30 (0%)	7.45 ± 0.02	0.0	29.6 ± 0.5	0.0
27 (−10%)	7.28 ± 0.02	−2.4	28.1 ± 0.8	−5.4
Sample salt (NaCl) concentration (mM)				
0	7.47 ± 0.03	0.0	30.0 ± 0.7	0.0
1	7.45 ± 0.02	−0.3	31.6 ± 1.0	5.3
10	7.50 ± 0.03	0.5	33.3 ± 0.5	10.9
20	7.64 ± 0.02	2.3	31.0 ± 0.3	3.2
40	7.88 ± 0.04	5.5	29.9 ± 0.3	−0.6
60	8.22 ± 0.03	10.0	29.9 ± 0.4	−0.6
80	8.57 ± 0.04	14.7	27.9 ± 0.4	−7.2
100	8.94 ± 0.04	19.7	26.6 ± 0.3	−11.3

high retention time precision was attributed to the automated production of KOH. When analyzing a relatively pure preparation of neomycin, this method can be used without column regeneration for at least 10 days.

3.6.2. Peak area and height

The mean peak area for neomycin B (10 μM) injected for 10 days (822 injections) was 29.92 ± 0.40 nC min. Peak height was 81.86 ± 1.45 nC. Daily (24 h) peak area R.S.D. ranged from 0.79 to 1.7%; daily peak height R.S.D. ranged from 0.65 to 2.8%. The high retention time and response reproducibility indicate that this method is suitably rugged for this application.

3.7. Robustness

3.7.1. Eluent concentration

A 10% increase in KOH (2.64 mM) produced a retention time decrease to 5.89 ± 0.01 min (−21% from 2.40 mM); while a 10% decrease in KOH (2.16 mM) produced a retention time increase to 10.90 ± 0.03 min (+45%). These results are summarized in Table 5. With either change, neomycin B and the nearest prior major peak (presumed to be neomycin C) were still resolved ($R = 5.43 \pm 0.06$ and 7.98 ± 0.10 for a 10% eluent concentration increase and decrease, respectively). A 10% increase in KOH produced a peak area increase of 1.8%; while a 10% decrease in KOH produced an area decrease of 14%. Amperometric peak area for aminoglycosides (and carbohydrates) is dependent on eluent pH. Background response and baseline noise were unchanged. The large % change in retention time, resolution, peak area and peak height for a relatively small change in KOH eluent concentration demonstrated the importance of producing a consistent eluent concentration, which was achieved with automated eluent generation. Poor retention time reproducibility was observed when attempting to use manually prepared 2.40 mM NaOH.

3.7.2. Column temperature

At the recommended operating temperature of 30 °C, the retention time for neomycin B was 7.45 min. At 33 °C (10% increase), the retention time was 7.66 min (2.7% increase), while at 27 °C (10% decrease), the retention time was 7.28 min (2.4% decrease). These results are summarized in Table 5. These changes did not impact separation. A 10% increase in temperature increased peak area 5.4%; a 10% decrease in temperature decreased area 5.4%. A 10% increase in temperature increased background 8% and a 10% decrease in temperature decreased background 5%. Noise was unaffected by 10% temperature changes. The temperature of the electrochemical cell was not intentionally altered.

3.7.3. Flow rate

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 (10% increase) and 0.45 mL/min (10% decrease), the retention times were 6.76 min (9.4% decrease) and 8.23 min (10.2% increase), respectively. The EP required resolution factor was still maintained with either a 10% increase or decrease. At 10% higher flow rate, peak area decreased 4.4% and at 10% lower flow rate, peak area increased 4.9%. These results are summarized in Table 5. Noise was unaffected by a 10% change in flow rate.

Table 6
Retention time (min) reproducibility of separate analytical columns

CarboPac PA1 column serial number[a]	Void		Neamine		Neomycin B		Baseline dip		Oxygen dip	
	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
10964 (manufactured January 2003)	2.74 ± 0.01	0.38	3.77 ± 0.00	0.12	7.69 ± 0.02	0.24	16.33 ± 0.01	0.05	32.68 ± 0.02	0.05
10154 (manufactured December 2001)	2.66 ± 0.01	0.47	3.66 ± 0.01	0.25	7.40 ± 0.03	0.39	15.49 ± 0.01	0.06	30.94 ± 0.02	0.06
11553 (manufactured June 2003)	2.73 ± 0.01	0.19	3.72 ± 0.01	0.29	7.52 ± 0.04	0.48	15.35 ± 0.01	0.05	30.56 ± 0.02	0.06
9270 (manufactured October 2000)	2.72 ± 0.01	0.34	3.50 ± 0.01	0.26	6.74 ± 0.01	0.18	15.68 ± 0.01	0.03	31.23 ± 0.03	0.09
Mean	2.71		3.66		7.34		15.71		31.35	
S.D.	0.03		0.12		0.41		0.44		0.93	
R.S.D. (%)	1.2		3.3		5.6		2.8		3.0	
Range	2.74		3.77		7.69		16.33		32.68	
	2.66		3.50		6.74		15.35		30.56	

[a] Column 11553 was used in this paper. Columns S/N 10964 and 10154 were used previously for other applications prior to this work. Column S/N 9270 was previously unused. $N = 8$ injections/column.

3.7.4. Salt matrix effects

With water as sample solvent (recommended), the retention time for neomycin B was 7.47 min. The retention time remained unchanged from 1 to 10 mN NaCl. At 20 mN NaCl, the retention time increased 2.3%, at 40 mN increased 5.5%, and at 60 mN increased 10%. The peak area increased from 5 to 11% through the range of 1 to 10 mN NaCl. Above 10 mN NaCl, the peak area trended in a decreasing manner, where at 40–60 mN the peak area returned to that of water. At 80 and 100 mN NaCl, the peak area decreased 7 and 11%, respectively. These results are summarized in Table 5. Neomycin B peak asymmetry (AIA definition) [34] was 2.01 in water, and the symmetry improved with increasing [NaCl]. At 10 mN NaCl, peak asymmetry was 1.20, and at 20 mN was 0.96. At higher salt concentrations peak fronting occurred, and asymmetry values dropped below 1.00. Peak efficiency (EP definition) [35] was 4800 in water, and the theoretical plates decreased with increasing [NaCl]. The theoretical plates decreased 10% at 7 mN NaCl, and 20% at 60 mN. The results in Table 5 suggest that sample salt must be considered for samples known to contain concentrations above approximately 10 mN. Accuracy is improved by preparing calibration standards in identical salt matrices. Because IPAD is a highly sensitive detection technique, all current commercially available pharmaceutical products containing neomycin B must be diluted approximately 1000-fold to attain the desired target concentration of 5–10 μM, which also dilutes their salt concentrations below 10 mN.

3.7.5. Column reproducibility

Table 6 compares the retention times for four separate columns. These columns were produced over a period of 4 years. Little variation was observed. The neomycin B retention time R.S.D. for four columns was 5.6%, and 3.3% for neamine. The retention times of the void peak ranged from 2.66 to 2.74 min, the baseline dip ranged from 15.4 to 16.3 min and the oxygen dip ranged from 30.6 to 32.7 min. The times for these three events are a constant for each column and do not vary with eluent concentration. High analyte resolution was achieved on each column.

3.8. Applications

For this study we evaluated three neomycin-formulated pharmaceuticals containing other active and inactive ingredients. Original Neosporin is a topical antibiotic ointment consisting of the active ingredients neomycin (3.5 mg/g of ointment), bacitacin (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. Maximum Strength Neosporin + Pain Relief is a topical cream consisting of the active ingredients neomycin (3.5 mg/g of cream), polymyxin B (10000 units/g), pramoxine HCl (10 mg/g) and the inactive ingredients emulsified wax, methylparaben, mineral oil, propylene glycol, and white petrolatum. Walgreens Triple Antibiotic PLUS is a topical antibiotic ointment consisting of the active ingredients neomycin (3.5 mg/g of ointment), bacitacin zinc

Table 7
Percent recovery of neomycin B extracted from ointments, cream, and from water

Treatment	Extract #	Ointment/cream (mg)	Mean ± S.D. (n = 4 injections of each extract)	R.S.D. (%)	Mean ± S.D. (within each treatment)	R.S.D. (%)
Neomycin B in water	1	0	95.9 ± 0.73	0.76	101.8 ± 5.8	5.7
	2	0	97.9 ± 1.3	1.3		
	3	0	108 ± 1.3	1.2		
	4	0	105 ± 1.3	1.2		
Original Neosporin®	1	24.6	96.0 ± 1.2	1.2	99.6 ± 2.5	2.5
	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		
Neosporin + Pain Relief	1	15.5	97.7 ± 0.67	0.7	95.2 ± 3.9	4.1
	2	28.9	90.7 ± 1.8	1.3		
	3	24.0	97.3 ± 0.70	0.7		
Walgreens Triple Antibiotic PLUS	1	21.9	96.4 ± 0.9	0.7	96.3 ± 1.6	1.6
	2	27.9	94.7 ± 1.8	0.6		
	3	18.1	97.8 ± 1.4	1.4		

(500 units/g), polymyxin B sulfate (10000 units/g), and pramoxine HCl (10 mg/g) and the inactive ingredient white petrolatum. The largely water insoluble inactive ingredients and the presence of other antibiotics makes these challenging mixtures to analyze by LC and thus these materials are ideal model pharmaceutical formulations to test method suitability. Fig. 4 shows chromatograms of neomycin B recovered from each of the three non-spiked pharmaceutical extracts. Neomycin B recovery was dependent on the mass of ointment extracted, and when the mass extracted was limited to the range of 17–32 mg, optimal spike recovery of 99.6 ± 2.5% was obtained for five separate extracts of Original Neosporin (Table 7). The recovery of neomycin B in

Table 8
Determination of neomycin B concentrations in ointments and creams

Trial day	Extract #	mg Neomycin B/g ointment or cream			
		Mean ± S.D. (n = 4 injections of each extract)	R.S.D. (%)	Mean ± S.D. (within each day)	R.S.D. (%)
Original Neosporin® (13.7–31.6 mg used for extraction)					
1	1	4.08 ± 0.09	2.2	4.12 ± 0.17	4.2
	2	3.97 ± 0.04	1.0		
	3	4.31 ± 0.09	2.2		
	4	3.94 ± 0.09	2.4		
	5	4.28 ± 0.06	1.4		
2	1	4.17 ± 0.05	1.1	4.17 ± 0.02	0.36
	2	4.16 ± 0.12	2.9		
	3	4.19 ± 0.05	1.2		
3	1	4.17 ± 0.20	4.8	4.20 ± 0.14	3.4
	2	4.07 ± 0.02	0.4		
	3	4.35 ± 0.14	3.2		
Between days		4.15 ± 0.13	3.2		
Neosporin® + Pain Relief (20.8–29.8 mg)					
1	1	5.24 ± 0.15	2.8	5.02 ± 0.21	4.2
	2	4.74 ± 0.04	0.76		
	3	5.13 ± 0.13	2.5		
	4	4.86 ± 0.11	2.2		
	5	5.11 ± 0.07	1.4		
Walgreens Triple Antibiotic PLUS (19.3–29.0 mg)					
1	1	5.34 ± 0.12	2.3	5.40 ± 0.18	3.3
	2	5.37 ± 0.06	1.1		
	3	5.59 ± 0.07	1.2		
	4	5.56 ± 0.06	1.2		
	5	5.16 ± 0.05	1.0		

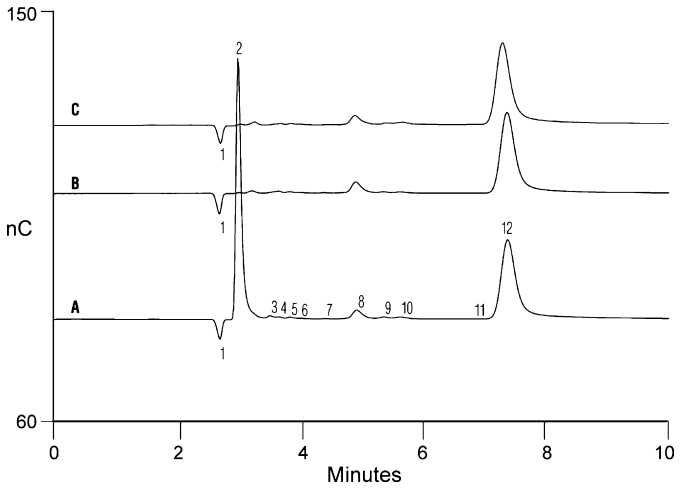

Fig. 4. Determination of neomycin B in Neosporin® + Pain Relief (chromatogram A), Walgreens Triple Antibiotic PLUS (chromatogram B), and Original Neosporin® (chromatogram C). Peak 1, void; peak 2, propylene glycol; peaks 3–11, neomycin B impurities and pharmaceutical ingredients; peak 12, neomycin B.

water, extracted using the same procedure used for the ointments and cream was 101.8 ± 2.5%. The neomycin B concentration in Original Neosporin was 4.15 ± 0.13 mg/g of ointment (3.2% R.S.D.) (Table 8) over three trials conducted over three separate days, $n = 11$ extracts. The label of this product states an expected 3.5-mg/g concentration, and our measured level is 18.6% greater than expected. USP specifications allow ointments of this type to be not less than 90.0 and not more than 130.0% of the label value [28], 3.15–4.55 mg/g. Our measurements show this product to be within these specifications. The neomycin B concentration in maximum strength Neosporin + Pain Relief was 5.02 ± 0.21 mg/g of ointment (4.2% R.S.D.) (Table 8) over a single trial, $n = 5$ extracts; spike recovery was 95.2 ± 3.9% (4.1% R.S.D.), $n = 3$ extracts (Table 7). The neomycin B concentration in Walgreens Triple Antibiotic PLUS was 5.40 ± 0.18 mg/g of ointment (3.3% R.S.D.) (Table 8) over a single trial, $n = 5$ extracts and spike recovery was 96.3 ± 1.6% (1.6% R.S.D.), $n = 3$ extracts (Table 7). Our measurements show maximum strength Neosporin + Pain Relief exceeded the USP specifications [36] of 90.0–130.0% of the label value (3.15–4.55 mg/g). Walgreens Triple Antibiotic PLUS also exceeded the USP specifications [28] of 90.0–130.0% of the labelled value.

4. Summary

HPAE-IPAD with eluent generation was used to determine neomycin B and its impurities. This method was fast (<15 min per analysis) and demonstrated high retention time and peak area precision. Electrolytic eluent generation ensures reproducibility and ruggedness with respect to retention time, peak resolution, and peak area. By intentionally varying method parameters, and testing neomycin-containing pharmaceutical products, the method was shown to be rugged for the intended application of neomycin identity, purity, and assay.

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