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Suitability of a liquid chromatography assay of neomycin sulfate to replace the microbiological assay for neomycin in USP Monographs☆

Valoran P. Hanko*, Jeffrey S. Rohrer

Dionex Corporation, 1214 Oakmead Parkway, Sunnyvale, CA 94088-3603, USA

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ABSTRACT

The current USP National Formulary contains 65 Monographs for drug formulations containing neomycin. All 65 Monographs prescribe a bioassay for neomycin assay. This bioassay, based on cell culture, is labor intensive, has poor precision, and cannot be adapted for purity or identification. High-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAE-IPAD), a liquid chromatography technique, has been shown to be suitable for neomycin purity analysis and neomycin assay of an over-the-counter first aid cream (Hanko and Rohrer [17]). Here we propose that an HPAE-IPAD assay can replace the bioassay in the 65 neomycin-containing Monographs. We applied the HPAE-IPAD assay to four neomycin-containing drug products representing the four classes of formulations found in the 65 Monographs, liquid, solid, suspension, and cream. Each drug was analyzed with two chromatography systems, and on 3 separate days. For all products, HPAE-IPAD measurements were precise and accurate with respect to the label concentrations. There was also high accuracy for spike recovery of neomycin from the four drug products throughout 70–150% of the labeled concentration. These results suggest that an HPAE-IPAD assay would be an accurate assay for neomycin, and would be faster and more precise than the current bioassay.

1. Introduction

Neomycin is a water-soluble complex of aminoglycosides produced from the fermentation of the actinomycete *Streptomyces fradiae* [1–4]. Neomycin B (also known as framycetin) is the principal component of the complex, and has the highest antibiotic activity. Neomycin B is purified from the fermentation complex, and the free base is coupled with sulfate counter-ions, which is then used in a variety of antibiotic pharmaceutical products, labeled as containing neomycin sulfate. These product applications include ophthalmic, topical, oral, and intravenous administrations. The current USP Monographs specify that an assay for neomycin sulfate and all neomycin sulfate-containing pharmaceutical products be performed using an antibiotics microbial assay with *Klebsiella pneumoniae* or *Staphylococcus epidermidis* as the test organism [5,6]. Microbial assays are labor intensive and drug potency is normally measured in units of activity, relative to a designated federal master standard [7]. Inter- and intra-assay variables impact reliability. Each test requires 16–24 h to prepare the inoculums, and either 16–18 h to incubate cylinder plates or 4–5 h to incubate test tubes for the turbidimetric method. No purity information can be obtained using the microbial assay, but antibiotic impurities can produce errors in the measured activity, thereby compromising method accuracy with respect to the measurement of just neomycin B activity.

In 2002, the Council of Europe revised the European Pharmapoeia (EP) official monograph for neomycin sulfate and framycetin sulfate from a bioassay to a liquid chromatographic (LC) method for

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* Corresponding author. Tel.: +1 650 269 5367; fax: +1 650 857 9415.
E-mail address: v.hanko@att.net (V.P. Hanko).

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identity, assay, and purity [8,9]. The LC methods use pulsed amperometric detection (PAD) with a gold working electrode. Neomycin B and its major impurities belong to a class of compounds, including carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds, that can be oxidized and therefore directly detected by amperometry. PAD has a broad linear range and very low detection limits for aminoglycoside antibiotics [10–19]. The LC method specified by the EP requires a non-alkaline mobile phase; therefore the pH must be elevated through post-column addition of NaOH to achieve detection. The post-column addition requires an additional pump and dilutes eluting peaks through a reaction coil, causing a reduction in method sensitivity compared to a method with a sufficiently alkaline eluent. In addition to the complication of a post-column setup, there are concerns about the reproducibility of the method based on the choice of an older PAD waveform that is known to have reduced response with use, and possible issues resulting from an inadequate description of the electrochemical conditions [19]. In Ref. [19], the authors had to alter the eluent conditions to reproduce the reported chromatography, and they adapted the method to another column to improve the chromatography. Our experience with the EP method suggests that there are problems with varying quality of eluent components, some difficulty in eluent preparation, and possible issues with column lifetime.

High-performance anion-exchange chromatography (HPAE) is a technique capable of separating aminoglycoside antibiotics and their impurities [13–18]. The USP Compendial Method for streptomycin currently uses HPAE-PAD to assay this aminoglycoside antibiotic [20]. The CarboPac® PA1 anion-exchange column (USP packing L46) separates neomycin B and its impurities using an alkaline mobile phase, necessary for amperometric detection. In previous publications [15,17], we evaluated accuracy, precision, lower limits of detection, linearity, and ruggedness, in a manner consistent with the requirements of USP method validation [21]. We demonstrated the capability of HPAE-IPAD for the determination of neomycin B in three different topical over-the-counter pharmaceutical formulations also containing pramoxine HCl, Polymyxin B sulfate, and Bacitracin Zinc among the active ingredients; and emulsified wax, methylparaben, mineral oil, propylene glycol, cocoa butter, cottonseed oil, olive oil, sodium pyruvate, vitamin E, and white petrolatum among the inactive ingredients [22]. Overall, the method demonstrated good sensitivity, good sample throughput (15 min per sample), and high retention time reproducibility. Spike recovery of neomycin B from these two ointments and one cream ranged from 95 to 100%, and the measured concentrations closely agreed with their respective label concentrations. The same method can be used to evaluate the purity of neomycin sulfate. Although this previous publication demonstrated good performance for the assay of neomycin sulfate contained within what we considered to be a challenging formulation, the USP National Formulary has 65 Monographs of neomycin sulfate-containing formulations, and we did not demonstrate that HPAE-IPAD was applicable to the other classes of formulations among the 65.

We reviewed all USP formulations and identified four major classes based on the methods required to prepare samples to obtain accurate measurements. We chose a commercial product to evaluate from each class. The four classes and chosen products were (1) solid (e.g., tablets and powder): Neo-Rx Neomycin Sulfate; (2) liquids (e.g., sterile injectables or irrigating solutions): Neosporin G.U. Irrigant; (3) suspensions: Cortisporin® Ophthalmic Suspension; and (4) ointments and creams: Original Neosporin Neomycin and Polymyxin B and Bacitracin Zinc First Aid Ointment. We tested these four representative pharmaceuticals for accuracy and precision using two different chromatographic systems, with assays on each conducted on 3 separate days, and found the method to be suitable for this application. Based on these results, we believe this method is suitable for assay of all 65 neomycin sulfate-containing formulations described in the USP National Formulary.

2. Experimental

2.1. Reference standard

Neomycin sulfate (782 mg standard free base per gram dry solid material, Reference Standard #45800, Lot No. L3E135; USP, Rockville, MD, USA), MW 614.65 (free base), CAS [1405–10–3].

2.2. Drug substance

Neomycin sulfate (701 mg standard free base per gram dry solid material, serving as drug substance; Cat# N5285-25G, 25 g, Lot No. 061K08921; Sigma–Aldrich Chemical Co, St. Louis, MO, USA). Formula: $C_{23}H_{46}N_6O_{13}\cdot 3H_2SO_4\cdot xH_2O$, FW 908.9, MW = 614.65 (free base), CAS [1405-10-3]. *Certificate of analysis results*: loss on drying: 3.6%, sulfate: 29.1%, residue on ignition: 0.2%.

2.3. Drug products

These four drug products were kindly provided by the USP:

Neomycin Sulfate USP, Micronized, Neo-Rx: 675 mg/g solid (labeled assay potency) as free base concentration; NDC 39822-0300-1 (Xgen Pharmaceuticals, Inc., Northport, NY, USA). Certificate of Analysis Results: assayed at 684 μg per mg, moisture: 0.2%.
Neosporin G.U. Irrigant: 40 mg neomycin base per 1 mL ampoule; NDC 61570-047-10 (Monarch Pharmaceuticals/King Pharmaceuticals, Bristol, TN, USA).
Cortisporin® Ophthalmic Suspension Sterile: 3.5 mg neomycin base per 1 mL of suspension; NDC 61570-036-75 (Pfizer Consumer Healthcare, Morris Plains, NJ, USA).
Original Neosporin® Neomycin and Polymyxin B and Bacitracin Zinc First Aid Ointment: 3.5 mg neomycin base per 1 g of ointment; NDC 0081-0730-88 (Pfizer Consumer Healthcare, Morris Plains, NJ, USA).

2.4. Apparatus

Both chromatography systems consisted of an ICS-3000 gradient pump with degas option and GM-4 gradient mixer, EG Eluent Generator with EGC II KOH eluent generator cartridge (EluGen® II Hydroxide) and CR-ATC, vacuum degas conversion kit, DC Detector Compartment, AS Autosampler, and Chromeleon® chromatography workstation (Dionex Corporation, Sunnyvale, CA, USA). Mobile phase (2.40 mM KOH) was automatically prepared by the eluent generator equipped with an EluGen Hydroxide cartridge and supplied deionized water. Neomycin, impurities, and ingredients of product formulations 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). 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 to 0.46 s, +0.33 V from 0.47 to 0.56 s, −1.67 V from 0.57 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 with their specified gaskets. Solid formulations, and neomycin sulfate standards and drug substances were dried for >20 h at 0.3–0.5 Torr at 60 °C in microcentrifuge tubes with detachable caps (plastic, 1.5 mL, Sarstedt, P/N 163/204; or equivalent) using a SpeedVac Evaporator system (ThermoQuest

Savant E/C Division) consisting of SpeedVac model SVC100, Refrigerator Vapor Trap model RVT400, Vacuum Gauge model VG-5.

2.5. Procedure

Analysis was performed with a flow rate of 0.50 mL/min and a column temperature of 30 °C, using 20-μL injections and 15 min run times. The column set was washed before each test day with 100 mM KOH for 60 min and re-equilibrated for at least 2 h prior to use. Once a week, the column set was washed with 100 mM KOH for 60 min and re-equilibrated overnight to 2.40 mM to restore retention times to their initial values.

2.5.1. Instrument calibration

Instrument calibration checks were performed before and after the study. Pumps were calibrated for flow accuracy, with error <0.5%. The column and detector thermal compartment was accurate, with error <2 °C. Injection loops were calibrated gravimetrically, with error <0.5 μL.

2.5.2. Assumptions used in this study to calculate results

During the gravimetric preparation of concentrate, stock, and working solutions of neomycin sulfate (reference standard, drug substance, and products), and their respective dilutions, water density was assumed to be 1.000 g/mL. Liquid and suspension form of products were also assumed to have a density of 1.000 g/mL.

During the preparation of neomycin sulfate (reference standard, drug substance, and product), the labeled neomycin free base is assumed to be 100% neomycin B free base.

The accuracy of measured concentrations of neomycin free base in drug products was evaluated by comparison to the label concentration of neomycin free base specified for each product.

Except for the solid form drug product tested in this study, which reported a label concentration with three significant figures, all other products specified only two significant figures for label concentration. In this study, we assumed all label concentrations had three significant figures as we believe at least some manufacturers which would use this method have an unreported third significant figure, and thus we reported percent of label concentration values with three significant figures.

We assumed "neomycin" and "neomycin sulfate" on *Certificates of analysis* (CoA) or product labels referred to the free base form or to the salt forms, respectively. When the CoA did not match the product label, we used the CoA information.

2.5.3. Sample preparation

In a previous study (results not currently published) we determined that some aminoglycoside antibiotics are very hygroscopic, and that anhydrous materials are either not fully dried during their manufacture or that the moisture content increases during storage. To assure the highest accuracy possible, the solid reference standard, drug substance, and solid drug products were dried under vacuum prior to use. The un-dried solid neomycin sulfate (110–160 mg) was placed in a pre-weighed 1.5 mL polypropylene microcentrifuge tube with screw cap, and weighed. After removing the cap, the vial was placed into a SpeedVac Evaporator system, heated to 50 °C, and the solid material dried for 20–24 h at ≤0.65 mm Hg. After drying, the vials were quickly removed from the SpeedVac and tightly re-sealed using the same cap, and then reweighed. After reweighing, the moisture content was calculated by subtracting the dry weight from the wet weight, and dividing this factor by the dry weight to determine a percentage. In this study, we measured the moisture content of a new previously unopened container of neomycin sulfate to be 3.6% for the USP reference standard, and 7.2–7.4% for the drug substance. Without the additional drying in our lab, we would have been analyzing less than expected neomycin, leading to an over-estimation in the products. We measured up to 23% moisture content in some previously opened containers of neomycin sulfate.

2.5.3.1. Neomycin sulfate reference standard and drug substance.

2.5.3.1.1. Stock solutions. The dried solid was dissolved in the appropriate weight of deionized water to make a 100 mg/mL concentration. The manufacturer's CoA was used to calculate a concentration for the neomycin B free base. This solution was diluted in water to yield ~18 mL (the weight of the water was measured) of a 0.615 mg/mL (1.00 mM) neomycin B free base concentration, with a weighing accuracy of three significant figures or better. This solution was further diluted to 61.5 μg/mL (100 μM) neomycin B (2.0 mL in 18.0 mL water) and served as the "Stock Standard Solution". The drug substance was prepared using the same method described above for the reference standard solutions. All the required dilutions were made gravimetrically to calculate an accurate concentration with minimal dilution errors to 3 or 4 significant figures. These solutions were maintained frozen at −40 °C until needed.

2.5.3.1.2. Working solutions. The 61.5 μg/mL (100 μM) Stock Standard Solution is diluted with water to prepare Working Standard Solutions of concentrations 0.1, 1, 5, 25, 50, 70, 80, 90, 100 (in triplicate), 110, 120, 130, and 150% of the target free base concentration of 3.07 μg/mL (5 μM). The 61.5 μg/mL (100 μM) Stock Neomycin B Drug Substance Solutions were diluted with water to prepare Working Drug Substance Solutions of concentrations 70, 80, 90, 100 (in triplicate), 110, 120, 130, and 150% of the target concentration.

2.5.3.2. Four drug products.

2.5.3.2.1. Solid product preparation procedure. The solid neomycin sulfate product in powder or crystalline form was prepared in the same way as the reference standard and drug substance. The 61.5 μg/mL (100 μM) Stock Solution was diluted with water to prepare Working Product Solutions with concentrations of 70, 80, 90, 100 (triplicate), 110, 120, 130, and 150% of the target 3.07 μg/mL (5 μM). The weights for all volumes of product, diluted product, and water used for dilution were recorded, and the expected concentration corrected for dilution errors.

2.5.3.2.2. Liquid product preparation procedure. Based on the label concentration, we diluted the aqueous product solution in water to 61.5 μg/mL, equivalent to 100 μM neomycin B free base concentrations. The 70–150% of the target Working Product Solutions were prepared as described above for the solid product.

2.5.3.2.3. Suspension product preparation procedure. The suspension had a label concentration of 3.5 mg neomycin (free base) per g or per mL of product. The suspension was diluted to a target concentration of 61.5 μg/mL calculated from the following equation letting 1 g of liquid equal 1 mL to derive the concentration:

$$\text{Neomycin B free base conc.(in mg/mL)} = \frac{(\text{g weight of suspension})}{(\text{g weight of suspension}) + (\text{g of water})} \times \frac{3.5\,\text{mg}}{\text{g}}$$

The target concentration of 61.5 μg/mL is equivalent to 100 μM neomycin B base. The diluted suspension was centrifuged at 16,000 × *g* in a microcentrifuge for 10 min, and the supernatant was transferred to another vial. The weights of the supernatant and the water used for dilution were recorded to adjust resulting concentrations for pipetting errors. The solution was further diluted to prepare the 70–150% of the target Working Product Solutions as described above for the solid product.

2.5.3.2.4. Ointment, cream, and gel product preparation procedure. The ointment tested in this study had a label concentration of 3.5 mg neomycin (free base) per gram of product. Ointment

(18–32 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 weighed with three significant figures or better on an analytical balance. The sealed vial was placed in an 80 °C heating block for 5 min to melt the lipid component. The tube was vortexed for 10 s (high setting) halfway through the heating. After 5 min, the tube was vortexed (high setting) continuously for 5 min, and then placed in the refrigerator for >1 h to re-solidify the lipid components. The chilled extract was centrifuged at 16,000 × *g* in a microcentrifuge for 10 min, and the supernatant was separated from an upper lipid layer, where 0.650 mL was transferred to another vial. The concentration of neomycin base expected in the extracted supernatant in mg/mL was calculated from the following equation letting 1 g of liquid equal 1 mL:

$$\text{Neomycin B free base conc. (in mg/mL)} = \frac{(\text{mg weight of ointment or cream})}{(\text{g of water})} \times \frac{1\,\text{g}}{1000\,\text{g}} \times \frac{3.5\,\text{mg}}{\text{g}}$$

The Stock Product Extract Solution was then diluted to 61.5 μg/mL, equivalent to 100 μM neomycin B free base. The 70–150% of the target Working Product Solutions were prepared as described above for the solid product.

2.5.4. Study design

The study was designed to test repeatability at different concentrations for four different drug products, 3 different days, and two separate chromatography systems. For this study, a single reference standard and drug substance concentrate solution was prepared after drying. A stock solution was prepared from dilution of these concentrates. The concentrates and stock solutions were maintained frozen at −40 °C during the term of the study. Reference standard working solutions (calibration standards) and drug substance working solutions were prepared from the stock solutions on 3 separate days (labeled Days 1, 2, and 3). Exactly the same reference standard working solutions and drug substance working solutions for each day were tested on two separate chromatographic systems (i.e., different pump, column, and detector). The reference and drug substance working solutions were maintained frozen at −40 °C during the term of the study, and each "*Day*" was used with the corresponding day each drug product was tested (e.g., "*Day 1*" Reference and Drug Substance solutions with freshly prepared "*Day 1*" drug product solutions).

Each of the four drug products was prepared as three separate concentrates on separate days. For the solid drug product formulation, these concentrates were prepared after its drying. Each concentrate was designated "*Day 1*, *2*, and *3*". A fresh drug product stock solution was prepared from dilution of each concentrate, as three separate respective "*Day 1*, *2*, and *3*" preparations of solutions. Drug product working solutions were prepared fresh from each respective stock solution. Exactly the same drug product working solutions were tested on each of the two separate chromatographic systems (i.e., different pump, column, and detector) for each day. Drug products were spiked on each day at three different concentrations (70, 100, and 150% of target concentration) to evaluate accuracy of the method. The drug substance was included in this study to serve as a check of the calibration accuracy for different days and different systems.

3. Results and discussion

We previously published deliberate variations in the HPAE-IPAD neomycin analysis procedure, altering the flow rate, eluent concentration, chromatography column, column temperature, sample salt concentration, and sample preparation conditions to evaluate robustness [15,17]. During the development of the HPAE-IPAD method, first for tobramycin purity analysis, we also tested eluent generation cartridges and electrodes [14,16]. While the method was sufficiently robust for neomycin and tobramycin determinations, we found that the quality and reproducibility of the aminoglycoside antibiotic separations was poor using manually prepared hydroxide eluents compared to hydroxide eluents made using eluent generation.

We previously developed a sample preparation method to extract neomycin into water from ointments, gels, and creams and tested its robustness. This sample preparation procedure was not necessary, nor appropriate for the other three formulation types (i.e., solids, suspensions, and liquids). Ointment, gels, and creams contain significant volumes of water-insoluble material (e.g., lipids) that must be removed prior to analysis. For solid formulations, no extractions are needed. The solid formulation is dissolved in a defined volume of water, and any insoluble material, if present, removed by centrifugation. Liquid formulations are already in the desired state for analysis, and require no sample preparation other than dilution. Suspensions contain insoluble material that constitutes an insignificant volume of the total formulation, and therefore require only centrifugation to separate the insoluble components from the aqueous fraction that is analyzed; concentrations described on the label are directly used for calculating the needed dilution.

A target concentration of 3.07 μg/mL (5 μM) was chosen for this assay based on our previous published study that showed this concentration to match the near mid-point for the most linear region of the calibration range. The calibration range was from 70 to 150% of the target to match the percentages of label concentrations allowed in the 65 USP Monographs for the formulations tested, and other formulations not tested in this study. Drug products were diluted from their respective label concentration to the target concentration. In this study, we found the lower limit of quantification (LOQ) was 0.019 ± 0.012 μg/mL (0.031 ± 0.020 μM; 20 μL injections) for the 24 different calibrations performed supporting 4 drug products on 3 days and 2 systems. This was equivalent to 0.62% of the target concentration. We reported the LOQ to be 0.72 pmol for a 20-μL injection, equivalent to 0.022 μg/mL (0.036 μM; 0.72% of target) in our previous publication using an older model chromatography system [17]. The LOQ is well below the 70% of target, the lower end of the linear range selected for this study. The slopes for the 24 experiments with calibration between 50 and 150% of target concentration ranged from 0.099 to 0.145 area units (nC* min) per ng injected (0.61–0.89 area units per pmol), with mean (±SD) of 0.124 (±0.013) area units per ng. The average r^2 value was 0.970 ± 0.051. Although there was some variation of detector response from day-to-day and from system-to-system, it did not appear to significantly impact the accuracy of neomycin B measurement in the four drug products, as shown in the remainder of this paper.

3.1. Specificity

The high specificity of this method was evaluated from the chromatographic separations of drug products containing a variety of active and inactive ingredients present in each formulation. Table 1 lists the known ingredients for each of the four drug products tested in this study. Fig. 1 shows the chromatograms for the reference standard, drug substance, and each product. The chromatograms for the standard and drug substance show the separation of impurities from neomycin B, as previously published [15,17]. The chromatograms for each drug product show the absence of any interference to the neomycin B peak from other ingredients in the formulation. Though there is some variation in retention time (4% RSD for each system on all days and all drugs, 6% RSD for both systems on all days and all drugs), the standard

Table 1
The representative drug products used in this study with their ingredients and label concentrations.

Drug product	Manufacturer	USP Monograph formulation	Inactive ingredients	Label conc.
Neo-Rx neomycin sulfate USP	XGEN Pharmaceuticals	Micronized, USP	None	675 mg/g (≥600 mg/g)
Neosporin G.U. Irrigant, Sterile	Monarch Pharmaceuticals, Inc.	Neomycin sulfate–Polymyxin B sulfate solution for irrigation, USP	Methylparaben (0.1%)	40 mg/mL (90.0–130.0%)
Cortisporin® Ophthalmic Suspension, Sterile	Monarch Pharmaceuticals, Inc.	Neomycin and Polymyxin B sulfates, and hydrocortisone ophthalmic suspension, USP	Thimerosal (0.001%), cetyl alcohol, glyceryl monostearate, mineral oil, polyoxyl 40 stearate, propylene glycol, sulfuric acid.	3.5 mg/mL (90.0–130.0%)
Original Neosporin®	Pfizer Consumer Healthcare	Neomycin and Polymyxin B sulfates and Bacitracin Zinc ointment, USP	Cocoa butter, cottonseed oil, olive oil, sodium pyruvate, vitamin E, white petrolatum	3.5 mg/g (90.0–130.0%)

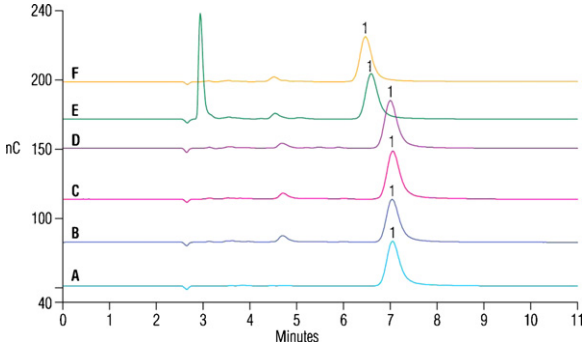

Fig. 1. HPAE-IPAD chromatograms of 5 μM neomycin B (peak 1) in (A) reference standard; (B) drug substance; (C) Neo-Rx (Micronized) solid drug product; (D) Sterile Neosporin G.U. Irrigant liquid drug product; (E) Sterile Cortisporin® Ophthalmic Suspension drug product; (F) original Neosporin® drug product. Chromatograms A, B, and C were run on the same day, chromatograms D, E, and F and separate days.

and samples run at the same time had the same retention time, and the results were consistent with results from other days. The high specificity of this method is attributed to the combined use of IPAD, which limits detection to substances capable of oxidation or reduction under the conditions used in the electrochemical program, and to the separation of neomycin B, its impurities, and other product ingredients by ion-exchange chromatography.

3.2. Accuracy

Accuracy of the HPAE-IPAD method was assessed by comparing the measured concentrations for the drug products to their labeled concentrations. Neomycin B concentration in the products is measured by putting the peak area for the product's neomycin B peak into the calibration curve created by analyzing a range of concentrations of neomycin B in the neomycin sulfate reference standard. Table 2 lists the concentrations measured for each drug, on each of 3 days, and for each of the two systems tested. The accuracy of the measured concentrations relative to the label concentrations ranged from 85 to 131% for all concentrations, drugs, days, and systems tested. No concentration-dependent relationship was observed between the accuracy of the measured neomycin concentration in the products to the label concentration within the 70–150% target concentration studied. The four drug products studied in this paper required dosages to be within 90–130% of the products' label concentration.

Method accuracy was also evaluated by the spike recovery of the reference standard into each drug product. Table 3 lists the measured recoveries for each drug, on each of 3 days, and for each of the two systems tested. Spike recoveries ranged from 73 to 110% for all concentrations, drugs, days, and systems tested. Similarly, no concentration-dependent relationship was observed with measured spike recovery of neomycin B from the drug products within the 70–150% target concentration range. The consistency of these results with the labeled concentration (derived using bioassay methods during manufacture) suggests that the HPAE-IPAD measurement of the neomycin B peak is comparable to the bioassay results. Less pure preparations of neomycin sulfate could contain less active forms of neomycin (e.g., neamine) that would respond to the bioassay but not be measured by HPAE-IPAD, which is only measuring the neomycin B peak. The chromatography of the four products and the results in Table 2 suggest that the less active forms of neomycin do not complicate the comparison of the HPAE-IPAD assay results to the bioassay results. We did not perform bioassays to confirm this point, but would recommend that a lab equipped for this procedure test our prediction that drug substances containing high levels of impurities would have a higher measured concentration than that obtained using HPAE-IPAD.

The percent error for the measured concentration of neomycin B drug substance relative to the neomycin reference standard, evaluated at varying concentrations within the range of 70–150% of target concentration, did not show any concentration-dependent effect on method accuracy (Table 4). There is a consistent negative bias of 5–9% which may reflect the lower purity of the drug substance compared to the reference standard. Our previous publication measuring the impurity content of neomycin sulfate, obtained from the same commercial source used for the drug substance in this study, reported a total impurity content of 11.4%, with 1.2% due to neamine (neomycin A) and 5.1% due to neomycin C [17]. Both neamine and neomycin C have known antibiotic activity [1,3], and would contribute to the measured concentration of neomycin using a microbial bioassay.

The peak area responses varied from day-to-day, and system-to-system (Table 5), in part due to the experimental design which included installation of new electrodes each day. Despite these variations, the responses for the calibration standards varied in the same proportion as for the samples, and there was no evidence that accuracy was compromised.

3.3. Precision

The retention time and peak area precision for replicate injections of the same solution of each drug product and replicate solutions of each drug product at its target concentration is

Table 2
Accuracy: percent measured concentration of neomycin B in drug products relative to their label concentration.

Sample	System A			System B		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Neomycin Sulfate USP, Micronized; Neo-Rx						
Each day						
Mean	99.7%	101%	101%	98.2%	106%	104%
RSD	2%	5%	1%	2%	4%	1%
N	10	10	10	10	10	10
Each system						
Mean		100%			103%	
RSD		3%			4%	
N		30			30	
Neosporin G.U. Irrigant						
Each day						
Mean	104%	109%	100%	113%	118%	90.3%
RSD	2%	5%	2%	2%	4%	4%
N	10	10	10	10	10	10
Each system						
Mean		104%			107%	
RSD		5%			12%	
N		30			30	
Cortisporin® Ophthalmic Suspension, Sterile						
Each day						
Mean	109%	109%	108%	112%	112%	114%
RSD	2%	5%	1%	1%	5%	5%
N	10	10	10	10	10	10
Each system						
Mean		109%			113%	
RSD		3%			4%	
N		30			30	
Original Neosporin® Neomycin and Polymyxin B and Bacitracin Zinc First Aid Ointment						
Each day						
Mean	116%	105%	107%	116%	103%	106%
RSD	4%	4%	1%	3%	3%	1%
N	10	10	10	10	10	10
Each system						
Mean		109%			108%	
RSD		6%			6%	
N		30			30	

Results describe the percent of our measured concentrations of neomycin B using HPAE-IPAD in each product relative to the theoretical label concentrations provided by the manufacturers. The statistics for each day include combined results for the measured drug concentration in each product when the drug concentrations were measured at 70–150% of target (single injections each concentration, 10 concentrations; $N = 10$). The statistics for each system include the combined results for 3 days within each system (single injections each concentration, 10 concentrations, 3 days; $N = 30$).

Table 3
Spike recovery of neomycin B from drug products.

Sample	System A			System B		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Neomycin Sulfate USP, Micronized; Neo-Rx						
Each day						
Mean	97.8%	101%	99.0%	104%	94.4%	97.5%
RSD	1%	1%	2%	2%	0.5%	2%
N	9	9	9	9	9	9
Each system						
Mean		99.4%			98.6%	
RSD		2%			4%	
N		27			27	
Neosporin G.U. Irrigant						
Each day						
Mean	98.9%	93.2%	103%	90.9%	85.4%	107%
RSD	2%	1%	2%	3%	2%	4%
N	9	9	9	9	9	9
Each system						
Mean		98.2%			94.3%	
RSD		4%			10%	
N		27			27	
Cortisporin® Ophthalmic Suspension, Sterile						
Each day						
Mean	95.3%	92.8%	93.3%	93.1%	82.8%	80.8%
RSD	6%	1%	2%	4%	5%	7%
N	8	9	9	8	9	9
Each system						
Mean		93.7%			85.3%	
RSD		3%			7%	
N		26			26	
Original Neosporin® Neomycin and Polymyxin B and Bacitracin Zinc First Aid Ointment						
Each day						
Mean	91.6%	95.1%	94.4%	89.6%	96.7%	93.4%
RSD	5%	2%	2%	3%	2%	2%
N	9	9	9	9	9	9
Each system						
Mean		93.7%			93.3%	
RSD		4%			4%	
N		27			27	

Results describe the amount of neomycin B measured using HPAE-IPAD after addition (spike) into each drug product relative to the theoretical amount added, expressed as a percentage. The statistics for each day include combined results for the measured concentration of neomycin B spike recovered from drug product when the drug concentrations were measured at 70, 100, and 150% of target, and the spike concentration was 20% of target (single injections each concentration, 3 concentrations, 3 replicate samples per concentration; $N = 9$). The statistics for each system include the combined results for 3 days within each system (single injections each concentration, 3 concentrations, 3 replicate samples per concentration, 3 days; $N = 27$). An outlying data point was rejected from the data set for Cortisporin on day 1 System A, and day 1 System B, thus $N = 26$ injections instead of 27.

presented in Table 5 for 24 different experiments using two chromatography systems. Retention time RSD within a single day ranged from 0.074 to 2.8%, and peak area RSD ranged from 0.53 to 14%. The high RSD of 14% is atypical for this method, and resulted from a single measure that was out of the normal range. Statistical tests confirmed this was an outlier. When we removed it from the data set, the peak area RSDs for within 1 day (16.5 h period) ranged from 0.53 to 7.6% for the 24 different experiments included in this study. We found intra-day precision to typically range from 1 to 4% RSD. The variance for both retention time and peak area for replicate injections of the same solution analyzed over 16.5 h tended to be slightly higher than the variance for different solutions analyzed consecutively over a period of only 0.6 h. Higher variance was observed when data included different days, and different systems.

Table 4
Percent error for the drug substance throughout the calibration range of 70–150% the target concentration.

Target %	Conc. μg/mL	Conc. μM	Percent error from expected			
			System A		System B	
			Mean	SD	Mean	SD
70%	2.2	3.5	−8.5	3.2	−7.5	2.4
80%	2.5	4.0	−8.2	2.6	−8.2	3.4
90%	2.8	4.5	−7.3	2.4	−6.7	2.0
100%	3.1	5.0	−7.2	2.3	−6.2	1.5
110%	3.4	5.5	−7.6	1.8	−6.1	1.6
120%	3.7	6.0	−7.0	2.6	−5.6	1.4
130%	4.0	6.5	−6.8	2.3	−4.7	2.9
150%	4.6	7.5	−7.4	1.9	−6.2	1.7

Based on a reference standard calibration curve 50–150% of target concentration. $N = 12$ separate days.

Table 5
Precision.

	Retention time (min)						Peak area (nC* min)					
	System A			System B			System A			System B		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Injection-to-injection variability (same sample; $N = 7$ injections)												
Neomycin Sulfate USP, Micronized; Neo-Rx												
Mean	7.12	7.03	7.01	7.33	7.03		10.59	11.56	9.77	7.95	8.74	7.91
SD	0.02	0.01	0.01	0.01	0.05	0.04	0.55	0.17	0.05	0.15	0.17	0.05
Neosporin G.U. Irrigant												
Mean	7.00	6.97	6.94	7.17	7.17	7.15	11.84	11.44	11.29	9.05	9.32	7.89
SD	0.01	0.02	0.03	0.01	0.01	0.02	0.49	0.26	0.59	0.22	0.16	1.14
Cortisporin® Ophthalmic Suspension, Sterile												
Mean	6.61	6.58	6.58	7.22	7.39	7.39	11.99	10.39	10.80	7.69	7.42	7.83
SD	0.01	0.01	0.01	0.03	0.01	0.01	0.31	0.24	0.14	0.23	0.37	0.59
Original Neosporin® Neomycin and Polymyxin B and Bacitracin Zinc First Aid Ointment												
Mean	6.42	6.44	6.33	7.79	7.83	7.72	11.41	10.40	10.58	9.52	8.67	8.98
SD	0.05	0.02	0.18	0.05	0.01	0.15	0.10	0.14	0.16	0.18	0.15	0.19
Sample-to-sample variability (different samples, $N = 3$ samples)												
Neomycin Sulfate USP, Micronized; Neo-Rx												
Mean	7.10	7.02	7.02	7.01	7.35	7.05	10.31	11.77	9.85	7.76	8.76	8.03
SD	0.00	0.01	0.01	0.00	0.01	0.01	0.03	0.14	0.14	0.19	0.10	0.09
Neosporin G.U. Irrigant												
Mean	6.99	6.95	6.92	7.18	7.15	7.13	11.34	11.11	10.79	8.97	9.26	6.80
SD	0.01	0.01	0.01	0.01	0.01	0.01	0.19	0.03	0.05	0.19	0.02	0.25
Cortisporin® Ophthalmic Suspension, Sterile												
Mean	6.62	6.57	6.57	7.21	7.39	7.39	11.51	10.26	10.64	7.59	7.22	7.60
SD	0.00	0.01	0.01	0.01	0.01	0.01	0.15	0.16	0.08	0.15	0.19	0.14
Original Neosporin® Neomycin and Polymyxin B and Bacitracin Zinc First Aid Ointment												
Mean	6.44	6.43	6.40	7.80	7.82	7.76	11.71	10.41	10.61	9.76	8.75	9.01
SD	0.01	0.01	0.01	0.00	0.01	0.01	0.35	0.20	0.26	0.37	0.22	0.19

The total time each day that injection-to-injection data was collected was 16.5 h. The total time that sample-to-sample injection data was collected was 0.6 h. Table reflects 24 experiments: 4 drugs × 3 days × 2 chromatography systems.

4. Conclusion

The results show high precision and accuracy for the HPAE-IPAD assay of neomycin B in drug products. The method is also amenable for neomycin identification and purity analysis. We believe this is a suitable neomycin assay for the 65 neomycin sulfate-containing formulations in the USP. We did not compare this method to the current microbial bioassay, but invite interested labs to compare this method with the microbial method for analytical performance, ruggedness, and total cost.

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