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Application Note 186

Analysis of Paromomycin by HPAE-IPAD

INTRODUCTION

Paromomycin (Figure 1) is an aminoglycoside antibiotic produced by *Streptomyces rimosus var. paromomycinus.*[1] The antibacterial spectrum of paromomycin is similar to other aminoglycosides that demonstrate broad spectrum activity against some gram-positive and many gram-negative bacteria.[2] Paromomycin has been widely used in human and veterinary medicine for the treatment of various bacterial infections. In humans, paromomycin has been used to treat leishmaniasis, cryptosporidiosis, and amebiasis.[3-6] Leishmaniasis is a parasitic disease that is transmitted from the bite of a sandfly, and is primarily concentrated in India, Bangladesh, Sudan, and Brazil.[3] Previous treatments for this parasitic disease used antimony, which can be toxic to the heart, liver, kidneys, and pancreas.[7] More recently, paromomycin has resurfaced as treatment for leishmaniasis, due to its effectiveness against the disease, low toxicity, and low cost relative to other available antibiotics.[8] Due to the work of the Institute for OneWorld Health, paromomycin was granted orphan drug status for the treatment of leishmaniasis. The Orphan Drug Act of 1983 encourages the development of drugs that are necessary but would be unprofitable to produce under normal circumstances.[9]

Determination of the active component(s) of a drug is critical to ensure a safe and effective formulation before release to the market. The current U.S. Pharmacopeia (USP) compendial method for assaying paromomycin uses a microbial assay.[10] This assay is qualitative and it lacks specificity, accuracy, and is time-consuming. In addition, microbial assays neither identify active ingredient(s) nor yield information on the total composition of the antibiotic formulation.

HPLC with UV or fluorescence detection has also been used for the determination of paromomycin.[8,12,13] Although these methods permit determination of the paromomycin isomers, pre- or postcolumn derivatization is required to achieve adequate sensitivity due to the lack of a suitable chromophore. Aminoglycoside compounds contain oxidizable groups (e.g., amines and hydroxyls) and can therefore be detected electrochemically. Electrochemical detection has advantages relative to other techniques in that an oxidation potential can be selected

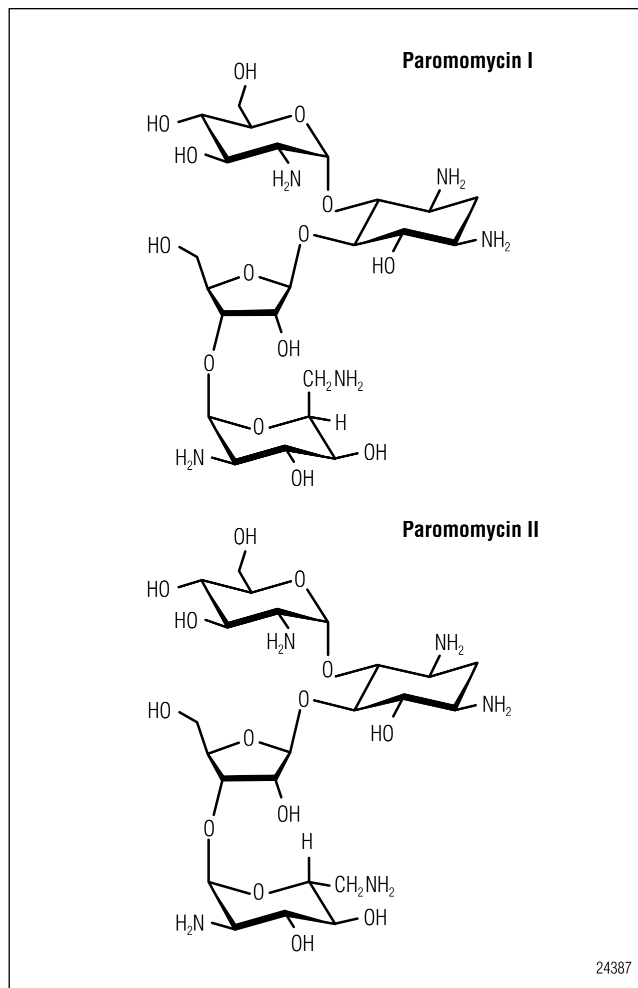

Figure 1. Paromomycin I and II.

for specific analytes while other compounds remain undetected, and derivatization is not required for detection, which simplifies the analysis. Integrated pulsed amperometric detection (IPAD) and PAD have been used succesfully to determine aminoglycosides, such as neomycin and tobramycin.[13,14] However, as previously reported, a six-potential IPAD waveform provides better sensitivity for aminoglycosides than a three or four-potential PAD waveform.[14,15] Therefore, the work presented in this application used a *AAA-Direct*™ waveform for the detection of paromomycin.

This Application Note demonstrates the use of an electrolytically generated potassium hydroxide eluent combined with the CarboPac® PA1 anion-exchange column and IPAD using a disposable *AAA* Au working electrode for the determination of paromomycin in a bulk pharmaceutical formulation. Similar to neomycin, separation of paromomycin with the CarboPac PA1 requires a weak hydroxide eluent (1.80 mM) making it difficult to use manually prepared eluents. Manually prepared NaOH may contain elevated concentrations of carbonate, which can impact retention time precision and chromatographic efficiency. An eluent generator (EG) prepares KOH eluent that is essentially carbonate-free, at accurate, precise concentrations. Carbonate that is present in the deionized water source used to supply the EG is removed from the system using a Continuously Regenerated Anion Trap Column (CR-ATC), which is installed after the eluent generator cartridge. This method accurately determines paromomycin without the need for pre- or postcolumn derivatization and meets the current USP performance requirements.

EQUIPMENT

Dionex ICS-3000 system consisting of:

- SP Single Pump or DP Gradient Pump with in-line degas option
- DC Detector Compartment (single or dual temperature zones) with electrochemical cell consisting of a pH/Ag/AgCl reference electrode (P/N 061879) and *AAA-Direct*™ Certified Au disposable working electrode (P/N 060082, package of 6; P/N 060140,package of 24)
- EG Eluent Generator module
- EluGen EGC II KOH cartridge (P/N 058900)
- EG Vacuum Degas Conversion Kit (P/N 063353)
- Continuously Regenerated Anion Trap Column, CR-ATC (P/N 060477)
- AS Autosampler with 20 µL injection loop
- Chromeleon® Chromatography Workstation
- 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

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

Paromomycin sulfate (USP, Catalog # 1500003 Lot G was used in this study)

Paromomycin sulfate (Sigma-Aldrich, P9297)

Sample

Humatin® (Paromomycin sulfate capsules, USP)

CONDITIONS

Columns:	CarboPac PA1 Analytical, 4 × 250 mm (P/N 035391) CarboPac PA1 Guard, 4 × 50 mm (P/N 043096)
Eluent:	1.8 mM KOH
Eluent Source:	EGC II KOH with CR-ATC
Flow Rate:	0.50 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C (lower compartment) 30 °C (upper compartment)
Detection:	Integrated pulsed amperometry, *AAA-Direct* Certified Disposable Electrodes (P/N 060082)
Background:	40-55 nC
System Backpressure:	~2600 psi

Waveform:

Time (s)	Potential (V vs. pH)	Gain Region	Ramp	Integration
0.00	+0.13	Off	On	Off
0.04	+0.13	Off	On	Off
0.05	+0.33	Off	On	Off
0.21	+0.33	On	On	On
0.22	+0.55	On	On	On
0.46	+0.55	On	On	On
0.47	+0.33	On	On	On
0.56	+0.33	Off	On	Off
0.57	-1.67	Off	On	Off
0.58	-1.67	Off	On	Off
0.59	+0.93	Off	On	Off
0.60	+0.13	Off	On	Off

PREPARATION OF SOLUTIONS AND REAGENTS

The use of electrolytically generated potassium hydroxide eluent is critical in order to maintain the retention time and peak area precision of paromomycin as described here; conditions in this method cannot be successfully duplicated using manually prepared hydroxide eluents. It is essential to use high quality deionized water with a resistivity of 18 MΩ-cm or better, with a low concentration of dissolved carbon dioxide. Eluents should be kept under a blanket of helium (~5-8 psi) at all times to minimize the introduction of atmospheric carbon dioxide.

USP Reference Standard Solutions

An official USP paromomycin sulfate reference standard (~120 mg) was placed in a pre-weighed 1.5 mL polypropylene microcentrifuge tube with screw cap, and the exact weight of the undried solid was determined. The vial (without cap) containing solid paromomycin sulfate was placed in a SpeedVac® Evaporator heated to 50 °C for 24 h at <0.7 mm Hg. The vial, cap, and dried paromomycin sulfate were reweighed together to determine the dried weight and the percent moisture content (for information only). The dried solid was dissolved in a volume of deionized water to make a 100 mg/mL concentration. The assay results stated by the USP were used to calculate the concentration for the paromomycin free base, which subtracts the mass of sulfate. USP lot G of paromomycin sulfate standard used for this application contained 730 µg paromomycin per mg solid (free base, dry basis). The calculated mg/mL concentration of paromomycin was converted to mM concentration using the paromomycin free base molecular weight of 615.6 daltons (Da) and was labeled as the “Stock Standard Concentrate Solution.”

A 1 mM stock standard intermediate solution was prepared by adding 123.1 µL of the 100 mg/mL stock concentrate solution to a 20 mL scintillation vial with deionized water added to bring the total volume to 20 mL. The 1 mM paromomycin stock intermediate solution was diluted to 100 µM paromomycin (2 mL of 1 mM solution in 18 mL of deionized water) and labeled “Stock Standard Solution.” Stock standard intermediate solution and stock standard solutions were prepared fresh daily. All solutions were stored at -40 °C until needed.

Stock Drug Substance Solutions

The drug substance was obtained from Sigma-Aldrich. The same procedure described above for the preparation of the USP reference standard solutions was used to prepare the Sigma drug solutions. The stock drug concentrate solution, the stock drug intermediate solution, and the stock standard solution were prepared fresh daily.

Working Standard Solutions

Prepare working standard solutions at lower concentrations by adding the appropriate amount of the 100 µM stock standard solutions and diluting with deionized water. For this Application Note, USP paromomycin reference standards were prepared at 1.25, 2.50, 3.50, 4.50, 5.00, 5.50, 6.00, 6.50, 7.50, 8.50 and 10.00 µM paromomycin as calibration standards. Once linearity was established, working drug substance solutions and working product solutions were prepared at the same concentrations as the USP reference standard solutions from their respective 100 µM stock standard.

SAMPLE PREPARATION

A paromomycin sulfate capsule (containing the equivalent of 250 mg paromomycin) was weighed on an analytical balance and the mass recorded. The capsule was carefully dissembled to expose the solid material and the entire capsule with solid was placed in a pre-weighed 120 mL HDPE bottle containing 100.0 ± 0.1 g of deionized water to dissolve the solid material. Duplicate 1.0 mL volumes of the dissolved solution

were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 10 min at 16,000 rpm, after which 0.50 mL of supernatant from each microcentrifuge tube was transferred to separate microcentrifuge tubes. Based on the label concentration, the mg/mL concentration of the solution was calculated using the following equation:

$$\text{mg/mL paromomycin free base} = \frac{250\text{mg}}{\text{tablet}} \times \frac{\text{tablet}}{100.0\text{mL}} = 2.50 \text{ mg/mL}$$

This solution was diluted to 100 µM paromomycin base by adding 0.493 mL of 2.50 mg/mL stock product solution to a 20 mL volumetric flask and bringing to volume. The 100 µM paromomycin sample was labeled as the stock product solution. The sample was prepared fresh daily.

SYSTEM PREPARATION AND SETUP

Determination of paromomycin using eluent generation (EG) requires installation of the ICS-3000 EG Vacuum Degas Conversion Kit (P/N 063353) to allow sufficient removal of the hydrogen gas formed with the potassium hydroxide eluent. Because installation of the kit requires access to the DP-3000 electronics compartment, the degas conversion kit must be installed by a Dionex Support Technical Representative or other authorized person. After installation of the degas conversion kit is complete, install an EGC II KOH cartridge in the EG-3000 and configure the setup of the cartridge with the Chromeleon server configuration. Connect the cartridge to the EG degas assembly and install backpressure tubing (~91.4 cm of 0.003" i.d.) in place of the column set to produce a system pressure of ~2000 psi at 1 mL/min. Condition the cartridge with 50 mM KOH for 30 min at 1 mL/min. After completing the conditioning process, disconnect the backpressure tubing temporarily installed in place of the column set. Install a CR-ATC between the EGC II KOH cartridge and the EGC degas. Hydrate the CR-ATC prior to use by following the instructions outlined in the EluGen Cartridge Quickstart Guide (Document No. 065037-02).

Install a 4 × 50 mm CarboPac PA1 guard and 4 × 250 mm CarboPac PA1 analytical column set. Ensure system backpressure is at an optimal pressure of 2400 ± 200 psi when 1.8 mM KOH is delivered at 0.5 mL/min. Install additional backpressure tubing between the EG degas and injection valve as necessary to achieve an optimal pressure reading. Calibrate the pH electrode according to the instructions provided in the Chromeleon software. Install a disposable *AAA* Au working electrode in the electrochemical cell, then install a short piece (~25 cm) of black tubing (0.010" i.d.) on the cell outlet.

The CarboPac PA1 column is stored in 200 mM NaOH. Upon installation, rinse the column set with 100 mM KOH for at least 1 h prior to connecting to the cell inlet. After completing the rinse step, equilibrate the column with 1.8 mM KOH for 24 h to obtain optimum retention time precision. Select the "Amino Acids (pH, Ag, AgCl reference)" waveform in Chromeleon. Set the waveform mode and reference electrode to "IntAmp" and "pH" respectively. Note: While the use of the carbohydrate waveform promotes a longer lifetime of the disposable Au electrode, the *AAA* waveform provides better sensitivity and was therefore used in this study.[15] Dionex specifies a lifetime of one week for the disposable Au electrode when the *AAA* waveform is used. However, actual lifetime may vary, depending on conditions. For more information, refer to the product manual for Disposable Gold Electrodes (Document No. 065040-03). After selecting the waveform, confirm flow is passing through the cell and turn the cell voltage to the ON position. The pH recorded by the reference electrode in the electrochemical cell should be between 11.2-11.5 once the column has been equilibrated with 1.8 mM KOH at 0.5 mL/min. Significant deviation from this range may be an indication of an excessive potential shift, and may require replacement of the reference electrode (typically every 6-12 months for the ICS-3000 cell). The electrochemical background recorded in this series of experiments was 44.5 ± 1.0 nC over a three week period. Generally, the background should be within 40-50 nC when operating under the specified method parameters. A significantly higher or lower background may be an indication of electrode malfunction or contamination within the system.

RESULTS AND DISCUSSION

Separation

Figure 2 shows separation of 5 µM USP grade paromomycin on the CarboPac PA1 column. The paromomycin isomers (paromomycin I and II) are represented by the two largest peaks eluting at approximately 5 and 8 min, respectively. Two baseline dips are observed in

the chromatogram using 1.80 mM KOH eluent. The first baseline dip (~15 min) may be caused by the presence of trace organic impurities present in the standard or sample injected, resulting in a negative response due to the exclusion of electrochemically active ions in the eluent. The second baseline dip (~30 min) is due to the presence of oxygen (also called the “oxygen dip”) in the standard or sample and appears as a function of the gas permeation volume of the column. The retention times of the baseline dips vary slightly from column to column, but are affected by flow rate, not eluent strength. The elution of the oxygen dip can be timed to occur at the end of the following injection to avoid interference with the target analytes. In this Application Note, the run time was reduced from 32 to 16 min to increase sample throughput.

Minor changes in the hydroxide eluent concentration

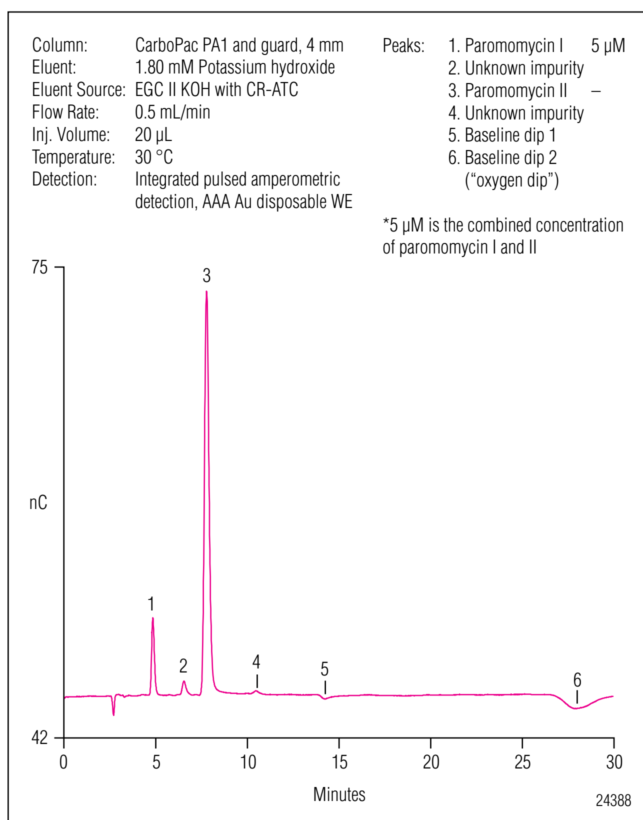

Figure 2. Determination of paromomycin (5 µM) using the CarboPac PA1 column with eluent generation.

can produce significant changes in the retention times of paromomycin I and II. For example, a concentration of 1.6 mM KOH eluent produced retention times of 6.2 min and 11.1 min for paromomycin I and II, respectively, compared to retention times of 5.1 min and 8.3 min using 1.8 mM KOH eluent. Reducing the eluent concentration increases the retention time of paromomycin, reduces sample throughput, and increases peak tailing. However, the resolution of some sample impurities will improve at lower hydroxide concentrations. A concentration of 1.8 mM KOH eluent was shown to provide good resolution between paromomycin II and an unknown impurity (Figure 2, peak 4) with a run time that allows for optimum sample thoughput.

LINEARITY, LIMIT OF QUANTITATION, AND LIMIT OF DETECTION

Linear range was determined by injecting paromomycin over a broad concentration range (0.005 to 20 µM) and plotting the sum of the peak areas of the two largest peaks (paromomycin I and II) against the injected concentration. Optimum linear range was considered to be where the response factor (ratio of paromomycin I + II peak areas/concentration injected) remained within 10% of the mean (average response factor for 2, 4, 5, and 6 µM paromomycin). The plot of the response factor versus the injected concentration showed a typical plateau region that represents an optimum level for operation (data not shown). The results demonstrated the optimum linearity for paromomycin was between 1.25 µM and 10 µM. The USP reference standard, Sigma drug substance, and Humatin sample were each prepared within the specified calibration range on three separate days. A summary of the calibration data is shown in Table 1. Each calibration was found to be linear using a least squares regression curve with correlation coefficients (r^2) of 0.9991 or better.

The USP method for validation specifies a signal-to-noise (S/N) ratio of 10 for the determination of the limit of quantitation (LOQ). Baseline noise was determined over a one minute time period during an analysis of a blank. The baseline noise ranged from 19 to 90 pC with an average noise of 46 ± 17 pC (n = 71 one-minute segments) measured over three weeks using three different *AAA* Au disposable electrodes. The LOQ for paromomycin based on the ratio of the sum of the peak heights to the average baseline noise was determined to be 0.10 µM (S/N = 10). The limit of detection (LOD) was estimated to be 0.030 µM (S/N = 3) for paromomycin (by extrapolation).

Table 1. Summary of Calibration Data for Paromomycin (Three Day Study)							
Day	Analyte	Source	Range (µM)	Linearity (r^2)	RSD	Intercept	Slope
1	Paromomycin	USP RS	1.25-10	0.9991	1.88	0.341	1.918
	Paromomycin	Sigma	1.25-10	0.9994	1.95	0.287	1.759
	Paromomycin	Humatin	1.25-10	0.9994	1.57	0.242	2.091
2	Paromomycin	USP RS	1.25-10	0.9993	2.08	0.203	1.895
	Paromomycin	Sigma	1.25-10	0.9992	2.41	0.069	1.886
	Paromomycin	Humatin	1.25-10	0.9995	1.41	0.347	2.173
3	Paromomycin	USP RS	1.25-10	0.9995	2.01	0.008	1.969
	Paromomycin	Sigma	1.25-10	0.9995	1.74	0.197	1.873
	Paromomycin	Humatin	1.25-10	0.9992	1.34	0.495	2.121

METHOD PERFORMANCE

Method performance was measured in terms of precision of replicate injections of paromomycin and recovery of spiked samples. The relative standard deviations (RSDs) were calculated for the sum of the paromomycin peak areas from a 5 µM standard. The intraday precision (i.e., a sequence of consecutive injections, n = 3) was <2% for USP grade paromomycin, ≤1% for paromomycin prepared from the Sigma drug substance, and <1% for paromomycin prepared from the Humatin sample based on independently prepared solutions analyzed on three separate days. The between-day precision for a three day period (i.e., day-to-day, n = 9) was <2% for paromomycin prepared from the USP reference standard, the Sigma drug substance, and the Humatin sample.

Ruggedness of an analytical method is defined by the USP as a measure of the degree of reproducibility for the same samples under a variety of conditions.[16] This is typically expressed as the lack of influence on the assay results under different conditions that would normally be expected from laboratory to laboratory and from analyst to analyst when operating under the defined method parameters. The ruggedness of the paromomycin assay was evaluated based on results from different analysts, instruments, lots of the column, and eluent generator cartridges. Each analyst used a USP reference standard solution containing 5 µM paromomycin, and the Sigma drug substance and Humatin product prepared at 100% of the target concentration (5 µM paromomycin) using different instruments, two different lots of the CarboPac PA1 column, and two different KOH eluent generator cartridges. Table 2 shows the overall procedure RSD and the RSD from two different eluent generator cartridges. Evaluation by single factor analysis of variance (ANOVA) test demonstrated that results obtained using different eluent generator cartridges or different CarboPac PA1 columns were not significantly different with a 95% confidence interval. The method was found to be rugged with respect to the variables evaluated in this study.

Humatin is a broad spectrum antibiotic that is supplied as a water-soluble capsule containing the equivalent of 250 mg paromomycin. This sample was analyzed over three days using independently prepared standards and diluted dosage solutions. The average paromomycin concentration was determined to be 279 ± 10 mg (3.7% RSD) over three trials performed on three separate days. The label states the product contains 250 mg paromomycin, however, our results indicate an average measured concentration that is 11.6% above the expected value. The USP specifies that the paromomycin sulfate capsules can contain the equivalent of not less than 90% and not more than 125% of the labeled amount of paromomycin. Our results demonstrate that this product concentration falls within these specifications. The higher measurement values found in this formulation may be designed to ensure longer shelf life. The accuracy of the procedure was evaluated by spiking the samples with known amounts of paromomycin. For samples spiked with 0.5, 1.0, and 2.5 µM paromomycin, recoveries were in the range of 96-106%, 98-107%, and 95-103%, respectively.

Table 2. Results of Ruggedness Study[a]						
Analyte	Source	Eluent Generator Cartridge C		Eluent Generator Cartridge D		Overall Precision
		Average (µM)	RSD	Average (µM)	RSD	RSD
Paromomycin	Sigma	4.80	2.03	4.84	1.00	1.12
		Average (mg/capsule)	RSD	Average (mg/capsule)	RSD	RSD
Paromomycin	Humatin	265	3.56	268	3.42	3.29

[a]*Average concentrations based on combined data from chemists A and B and columns E and F, n = 12.*

Table 3. Average Recoveries of Paromomycin Spiked Into a Bulk Pharmaceutical Formulation						
Sample	Analyte	Amount Added (µM)	Day 1 Average Recovery (%)	Day 2 Average Recovery (%)	Day 3 Average Recovery (%)	Overall Recovery[b] (%)
Humatin	Paromomycin	0.5	102.0 ± 5.2	100.2 ± 1.3	99.4 ± 3.3	100.5 ± 2.2
Humatin	Paromomycin	1.0	102.2 ± 4.0	100.2 ± 0.5	101.2 ± 3.8	101.2 ± 2.8
Humatin	Paromomycin	2.5	97.7 ± 1.7	99.4 ± 0.3	98.6 ± 4.0	98.6 ± 1.2

[a]*Average recoveries based on three independently prepared solutions, n = 3.*
[b]*Combined average recoveries over three days from independently prepared solutions, n = 9.*

Figure 3 compares a chromatogram of paromomycin detected in the Humatin sample to the same sample spiked with 1.0 µM paromomycin. Table 3 summarizes the average recoveries of known concentrations of paromomycin spiked into the Humatin sample over three days.

CONCLUSION

This Application Note demonstrates the use of HPAE-IPAD combined with electrolytic generation of potassium hydroxide eluent for the determination of paromomycin in a bulk pharmaceutical product. The data suggests that the method is linear, precise, and accurate for determintion of paromomycin and therefore meets current USP performance requirements. The use of automated eluent generation improves the consistency in producing a low potassium hydroxide concentration, making the method reproducible and rugged with respect to retention time and peak area precision. The use of disposable *AAA* Au electrodes further simplifies the method, providing good electrode-to-electrode reproducibility and assuring greater accuracy between instruments as well as between laboratories.

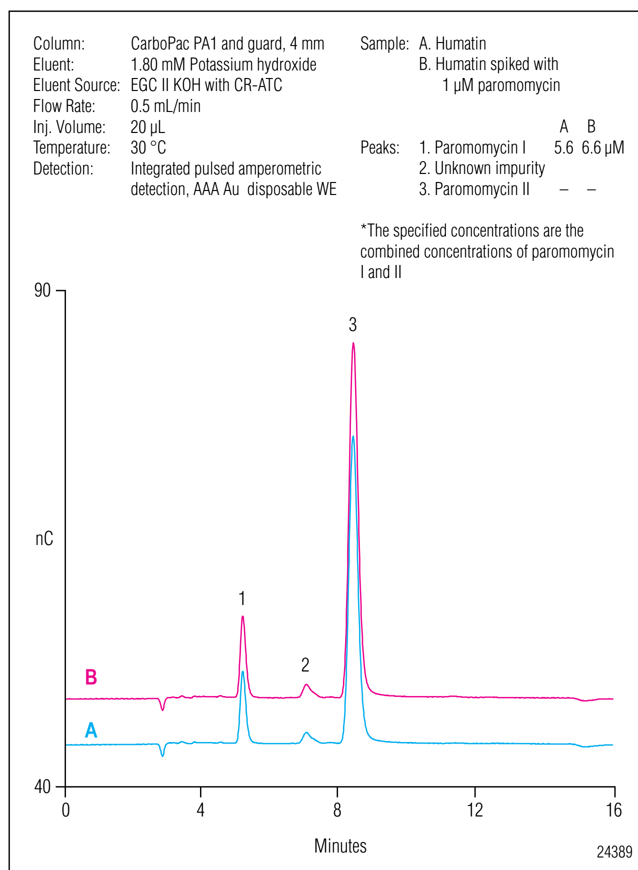

Figure 3. Comparison of A) unspiked and B) spiked Humatin sample containing paromomycin.

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