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A chemical assessment and HPLC assay validation of bulk paromomycin sulfate

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Abstract

This paper describes the compositional analyses of four paromomycin sulfate samples and features a validated HPLC assay method that relies on the fluorescence detection of isoindole derivatives of paromomycin, formed by post-column reaction with *o*-phthalaldehyde and 2-mercaptoethanol.

Keywords: HPLC; Paromomycin sulfate; Post-column derivatization; Chemical composition

1. Introduction

Paromomycin (Fig. 1), an aminoglycoside or aminocyclitol antibiotic with a chemical structure similar to that of the more commonly-known neomycin, shows broad spectrum activity against both gram-positive and gram-negative bacteria. Binding of paromomycin to bacterial 30S ribosomal subunits causes the mistranslation of mRNA, followed by rapid atrophy and death of the bacterial cell. The chemistry of paromomycin, as well as that of other aminoglycoside antibiotics in the 4,5-disubstituted deoxystreptamine class, was reviewed in 1980 by Rinehart and Suami [1]. Recently, interest in paromomycin has renewed, particularly at the Walter Reed Army Institute of Research, due to the antibiotic's reported effectiveness against leishmaniasis and cryptosporidiosis [2,3].

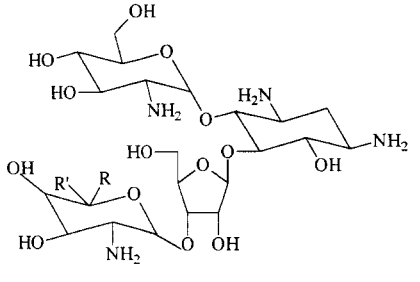

Fig. 1. The chemical structures of paromomycin I and II where for paromomycin I, R = H and R′ = -CH_2NH_2, and for paromomycin II, R = -CH_2NH_2 and R′ = H.

Critical to the development of effective formulations using paromomycin in combination with other active components (e.g. gentamicin) is the ability to identify and quantitate these active component(s) in the formulated drug products. The current compendial method [4] for determining

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paromomycin is based on mircobial assay. This compendial method uses a qualitative approach which lacks specificity, doesn't identify the active ingredient(s) and doesn't yield information on total chemical composition.

Like other aminoglycosides, paromomycin lacks a UV chromophore. Isoindole derivatives are commonly prepared to render primary amines detectable [5,6], and we have exploited this usage [7–9] in developing HPLC methods for quantitating paromomycin. Few chemical assay methods for paromomycin have appeared in the literature. Those reported include the use of capillary zone electrophoresis [10] and gas-liquid chromatography [11]. However, to our knowledge, none apply specifically to an HPLC analysis of paromomycin nor to a comprehensive compositional assessment by analytical means. Because paromomycin is very similar in structure to other aminoglycosides such as neomycin and gentamicin, it was reasonable to assume that HPLC methods reported for these antibiotics could be modified for paromomycin.

However, a quantitative HPLC method for paromomycin sulfate relies on a well-characterized paromomycin sulfate reference. Because no such reference exists, it was necessary to establish one.

This paper describes a procedure for the establishment of the chemical composition of a paromomycin sulfate reference using several independent analytical procedures. In addition, an HPLC assay method, based on fluorescence detection of isoindole derivatives of paromomycin, is described and validated.

2. Experimental

2.1. Reagents and materials

All chemicals and solvents were reagent or HPLC grade unless otherwise noted. Aqueous buffers and water were filtered through Millipore 0.22-micron polyvinylidene fluoride membrane filters before use. Four samples of paromomycin sulfate, fine, white crystalline, hygroscopic powders, were compared. Lot WR35928AV was received from the Walter Reed Army Institute of Research, lot 104H0434 was purchased from Sigma and lots F1 and F2 were purchased from the United States Pharmacopeial Convention. *P*-Hydroxybenzoic acid (Matheson, Coleman and Bell), methanol (Mallinckrodt, ChromAR HPLC grade) and sodium sulfate (Sigma lot 120H04632, 99.6% pure) were components in the ion chromatography mobile phase and the sulfate ion reference. Deuterium oxide (D_2O) (Aldrich) was the NMR solvent. The HPLC mobile phase reagents—sodium 1-heptanesulfonate, heptafluorobutyric acid, sodium sulfate and acetic acid—were purchased from Janssen Chimica, Aldrich, Sigma and Mallinckrodt. The HPLC derivatizing agents, *o*-phthalaldehyde and 2-mercaptoethanol, and pH-modifying agents, potassium hydroxide and boric acid were purchased from either Sigma or Pickering Laboratories. Sulfuric acid was purchased from Malinckrodt.

2.2. Establishment of chemical composition

2.2.1. NMR spectra

NMR spectra of the undried samples were obtained from ~ 10 mg ml^{-1} solutions in D_2O with a Varian Gemini 300 NMR spectrometer using standard ^1H parameters.

2.2.2. Determination of volatiles

Volatile content in the paromomycin sulfate samples, presumably primarily water, was determined by loss on drying to constant weight. Weighed sample portions between 250 and 600 mg were placed into clean, dry, pre-weighed vials. The vial openings were covered with filter paper and placed in an Abderhalden apparatus; the samples were dried under reduced pressure (< 5 mmHg) at 60°C (provided by refluxing $CHCl_3$). After a time interval, the vials were removed, equilibrated to room temperature in a desiccator and weighed; drying was repeated until a constant weight was observed.

2.2.3. Elemental analysis

Undried samples were analyzed for elemental carbon, nitrogen and hydrogen (UC Berkeley Microanalytical Laboratories). Percentages found

were compared with the percentages calculated for the paromomycin free base.

2.2.4. Determination of sulfate by ion chromatography

Ion chromatography was performed isocratically with aqueous 8 mM *p*-hydroxybenzoic acid, pH 8.2, containing 2.5% methanol and running at 1.0 ml min^{-1} through a Hamilton PRP-X100, 4.6 × 250 mm, anion exchange column using a Waters 510 solvent delivery system with a Rheodyne 7125 injector and a Shodex IC-CD-4 conductivity detector. Data were collected with an Intel 80486-based personal computer system using Hewlett Packard 3365 Series II Chemstation hardware and software.

Sulfate quantitations were based on a validated (validation for sulfate analysis was performed in house but the data are not included in this paper) external standard method. Four aqueous paromomycin sulfate solutions from each sample lot were prepared to concentrations of 5 mg ml^{-1}. Five aqueous sodium sulfate reference solutions were prepared to concentrations of 1 to mg ml^{-1}. A 10 μl aliquot from each sample and reference solution was chromatographed.

2.2.5. Determination of methanol and acetone content by GC

Methanol and acetone quantitations were performed with a Shimadzu Mini 2 gas chromatograph (GC) equipped with a 1.8 m × 2 mm i.d. glass column packed with 5% Carbowax 20 M on Carbopack BAW 80/100 mesh (for methanol) and a 10 m × 0.5 mm i.d. crosslinked polyethylene glycol-TPA AT-1000 column (for acetone). Methanol and acetone were detected by flame ionization at 200 and 240°C, respectively. The injector and column temperatures were set to 200 and 96°C for methanol and 125 and 40°C for acetone, respectively, and the carrier gas was N_2 at a flow rate of 40 ml min^{-1}.

Standard solutions of methanol and acetone in water were prepared. One μl aliquots were chromatographed. The peak heights or areas were recorded, and correlated against concentration to produce a standard curve. Sample solutions (10 mg ml^{-1} in water) were prepared and 1 μl aliquots were chromatographed. Sample methanol peak heights were compared with the standard curve, and data from the linear regression analysis of the reference peaks was used to quantitate the methanol in the WR35928AV sample, the only sample that showed the presence of methanol by NMR. The same approach was used to determine the amount of acetone in the Sigma sample.

2.3. HPLC-based assay method and method validation

2.3.1. HPLC systems

2.3.1.1. Fluorescence-detection system. The dual-pump HPLC system used was a modification of one used by Schering Corporation (private communication). One HPLC pump was used for delivering the mobile phase through the column and another for introducing the post-column derivatizing agent. A Rheodyne 7125 injector with a 20 μl loop was used to introduce the sample to the column. The mobile phase consisted of aqueous 0.2 M Na_2SO_4, 1.2 mM sodium 1-heptanesulfonate and 0.1% acetic acid and was delivered with a Waters M6000 pump at a flow rate of 1.0 ml min^{-1} through a 4.5 × 250 mm^2 Metachem Inertsil C8 stainless steel column. The mobile phase pump's plunger seals were continuously washed with water at a low flow rate (~ 30 μl min^{-1}) to prevent plunger and plunger seal damage in the presence of such high concentrations of sulfate in the mobile phase. The post-column derivatizing agent, consisting of aqueous pH 10 potassium borate buffer (prepared by adjusting the pH of 25 mg ml^{-1} boric acid with 25 mg ml^{-1} of aqueous KOH) containing 0.8 mg of *o*-phthalaldehyde per ml, 1% methanol and 0.1% 2-mercaptoethanol, was introduced through a tee at a flow rate of 1.0 ml min^{-1} with a Perkin Elmer (PE) Series 4 Solvent Delivery System. The combined derivatizing agent and the column effluent proceeded into a reaction coil, which was a 9 m long, 0.25 mm i.d., 1.6 mm o.d. stainless steel piece of tubing wrapped in 3–5 cm coils. Connected to the end of the coil was a Varian 9070 fluorescence detector, whose xenon lamp was pulsed at a rate of 20 Hz, and its excitation and

emission monochromators were set to wavelengths of 340 and 440 nm, respectively, with 20 nm bandwidths on both. Peak area data were collected with the Intel 80486-based PC with HP Chemstation hardware and software.

2.3.1.2. HPLC-mass spectrometry system. The post-column-derivatization, fluorescence-based detection scheme from system 1 was used to first optimize a compatible HPLC-MS mobile phase, which must be volatile for MS detection. A Waters μBondapak C18 column was used with a mobile phase consisting of MeOH:0.7% aqueous heptafluorobutyric acid (40:60, v/v) at a flow rate of 1.0 ml min^{-1}. After the mobile phase was optimized, an HPLC-coupled mass spectrometer operating in thermospray mode was used to collect molecular weight and fragmentation data from the two main paromomycin components.

2.3.2. Semi-quantitative HPLC comparisons

Solutions of each paromomycin sample were accurately prepared to concentrations of 2 mg per 50 ml of mobile phase 1. Using HPLC system 1, a 20 μl aliquot of each solution was chromatographed. Chromatographic profiles and peak area ratios from respective chromatograms were compared.

2.3.3. HPLC method validation

Using USP lot F-2, the fluorescence-based HPLC method (system 1) was validated for precision, accuracy, linearity, solution stability, sensitivity and specificity.

2.3.3.1. Precision. Five paromomycin sulfate solutions were prepared to concentrations of 0.10 mg ml^{-1} by weighing 2.5 mg portions of a dried sample (a 50 mg portion is dried for 4 h at 60°C and < 5 mmHg before use, in accordance with USP instructions) under anhydrous conditions into 25 ml volumetric flasks, dissolving the solids and diluting the solution to volume with 0.02% H_2SO_4. A 10 μl aliquot from each solution was chromatographed. To determine the precision of HPLC method 1, response factors (RFs) were calculated, by dividing the sum of the areas of the two major paromomycin peaks by sample weight, for each chromatographed solution.

2.3.3.2. Accuracy and linearity. Solutions of concentrations between 0.04 and 0.17 mg ml^{-1} were prepared and chromatographed analogously to the solutions used in the precision test procedure (Section 2.3.3.1). To determine the method's linearity, the sum of the areas of the two largest peaks was plotted against the sample weight for each chromatographed solution. To determine the method's accuracy, the sample weights were then calculated from the linear least-squares regression data, and the deviations from the actual weights were calculated.

2.3.3.3. Solution stability. Aliquots from a 68 μg paromomycin sulfate ml^{-1} solution in aqueous 0.02% H_2SO_4 at room temperature (21°C) were chromatographed at 41 min intervals for 24 h.

2.3.3.4. Sensitivity. The lower limit of detection, a measure of the method's sensitivity, is generally represented by the amount of injected analyte that corresponds to a peak height three times as intense as the average noise height. The lower limit of detection under these LC conditions was found by injecting successively dilute concentrations until the analyte peak height to noise ratio was near three.

2.3.3.5. Specificity. A portion of a paromomycin sulfate was heated in a vial for 1 min at 190°C. During this time, the sample's color changed from white to dark yellow. The decomposed sample was dissolved with H_2O, filtered and chromatographed.

3. Results and discussion

3.1. Chemical composition

Determining the composition of paromomycin sulfate is a matter of considering its chemical characteristics and knowing its history. Commonly, paromomycin is conjugated with an acid such as sulfuric acid. Thus, ideally, a paromomycin sulfate sample should contain only the paromomycin freebase and sulfuric acid. In reality, because of its hygroscopic nature, a sample

Table 1
Paromomycin sulfate compositional analyses

Sample	Amount of sulfuric acid (w/w%) by IC $n = 5$	Amount of water (w/w%) $n = 5$	Total found %C $n = 2$ or 3	Total found %N $n = 2$ or 3	Calculated % total freebase[a]	Other components[b] (w/w%) $n = 2$ or 3	Mass balance % (w/w%)[c]	Estimated paromomycin I and II freebase
WR35928AV	25.0 (RSD = 1.2)	6.0	30.38	7.49	66.2	1.5% methanol	98.7	64.1
Sigma	25.0 (RSD = 1.6)	6.4	30.81	7.81	68.3	0.4% acetone, 0.3% methanol	100.4	63.2
USP F1	24.3 (RSD = 0.7)	6.5	30.46	7.75	68.3	—	99.1	62.8
USP F2	24.3 (RSD = 0.1)	7.9	30.35	7.66	67.5	—	99.7	65.4
Paromomycin freebase (theory)	0	0	44.87	11.38	100	—		100

[a] The percentage listed here is calculated from the average percentages of C and N, corrected for methanol and acetone.
[b] Found by NMR and GC.
[c] Calculated from the addition of the amounts of sulfuric acid, water, total freebase and other components.

will probably contain water in addition to impurities that may have carried over from its isolation and purification steps. Because paramomycin is produced by fermentation, the sample could contain other aminoglycosides. The total sample composition can be ascertained by summing the weight percentages of the components found, which include aminoglycosides, sulfuric acid and water or other solvents.

Water and sulfuric acid content can be determined by well-established methods. However, determination of the paromomycin freebase is more difficult as it must be evaluated independently because no well-characterized references are available. The amount of freebase can be estimated from a mass balance of all the sample components found. Elemental carbon and nitrogen, elements that the freebase alone should contain, represent only paromomycin and semi-quantitative HPLC data will give the relative proportions of the amino-bearing constituents (paromomycin and any impurities of similar structure), assuming equal detector response per unit weight of all components.

The average percentages of water in the paromomycin samples found by loss on drying are shown in Table 1. These percentages can be fully attributed to water if no other volatile components are present in the sample.

A method of determining if volatile organic components are present is to examine the sample's NMR spectrum. The ^{1}H-NMR spectra of all samples (Fig. 2) are virtually superimposable, except for peak intensity differences in the WR35928AV sample spectrum near 3.4 ppm that indicated the presence of methanol, and an unidentified trace signal near 1.9 ppm. Spiking of the WR35928AV sample solution with methanol confirmed the presence of methanol (data not shown). GC analysis confirmed the presence of 1.5% methanol in the WR39528AV sample. Interestingly, this finding does not invalidate the percentages of volatiles found, because the methanol peaks persisted in the NMR spectrum of a WR35928AV sample dried at 60°C for 15 h under reduced pressure. The integrated methanol peaks in the NMR spectrum were quantitatively equivalent before and after drying. Apparently, the methanol is trapped

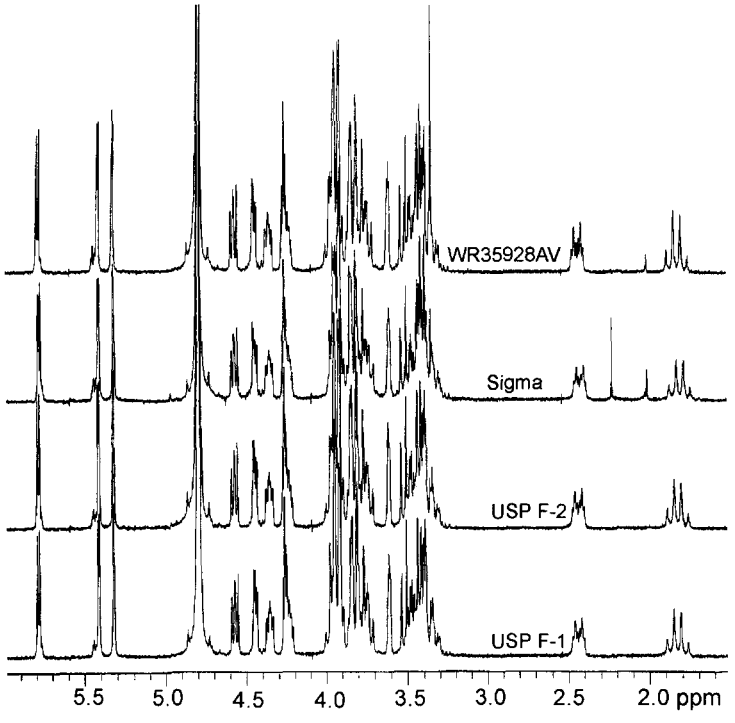

Fig. 2. ^{1}H-NMR spectra of the paromomycin samples. The presence of methanol in the WR35928AV sample at 3.4 ppm and acetone in the Sigma sample at 2.2 ppm is clearly visible in the spectra. In all other respects, the spectra are nearly identical.

in the sample matrix and is not available until the matrix is dissolved. Consequently, it was not removed under the loss-on-drying conditions.

Similarly, the Sigma sample was found to contain 0.4% acetone and 0.3% methanol.

The sulfate content in these paromomycin sulfate samples are likely to depends on the specific conditions of preparation. In general, the amount of sulfate can be determined by several techniques; one of which is by calculations from sample sulfur percentages. Although elemental sulfur is absolute, it lacks specificity. A more specific method is ion chromatography, which can distinguish sulfate species.

Ion chromatographic results indicate the WR35928AV sample, USP F1, USP F2 and Sigma samples contained 24.5, 23.8, 23.8 and 24.5% sulfate (w/w), respectively. When these sulfate percentages are calculated as sulfuric acid, the respective percentages are 25.0, 24.3, 24.3, and 25.0% sulfuric acid, respectively. These values are also listed in Table 1. The stoichiometry of paromomycin freebase to sulfate is approximately 1:2 and does not appear to vary significantly from source to source.

In establishing a reference standard for any compound, the sample composition (complexity) must be known. For a paromomycin sulfate sample, its appropriately obtained chromatographic profile is a key. If the profile is homogeneous, the sample's elemental percentages can provide a reliable estimate of sample purity, if the elements are relevant and the percentages are large relative to their errors of determination. If the sample is chromatographically heterogeneous, but the identities of the major components are known, sample purity estimation can still be based on elemental data, but the reliability of the estimate is lower.

Although the chromatographic profiles for these four samples of paromomycin are heterogeneous, they are similar. The identities of the two major components in each profile can be assumed to be paromomycin I and II on the basis of their molecular weights (data in the following HPLC-

MS section) and from published chemical information. Based on the preceding reasoning, sample purity estimates can be made from the sample elemental data. Because all four sample profiles also show minor chromatographic impurities, indicating that they are primary amine-containing and that they too are probably by-product aminoglycosides from the fermentation process, these impurities must be assumed to have the same empirical formula as paromomycin and react similarly with *o*-phthalaldehyde. This may or may not be the case. However, support for the validity of this assumption can be found in the C:N ratios.

All four samples contain water, which contributes to the H percentage; this contribution renders the percentage of elemental H less dependable than the percentages of C and N. Although this method of purity determination suffers some weaknesses, it should be acceptable until a chromatographically homogeneous sample is available.

Table 1 lists the percentages of C and N found in the WR35928AV, USP and Sigma samples, and also shows the expected percentages for the paromomycin freebase alone. The initial total 'freebase' percentages are derived by dividing the found percentages of carbon (corrected) and nitrogen by their calculated percentages in pure paromomycin freebase (last row) and averaging the resulting percentages. The total carbon must be first corrected for the methanol found or acetone present using the following calculation for total carbon.

$$C = (\text{weight\%freebase} \times C_{fb}) + (\text{weight\%methanol} \times C_m) + (\text{weight\%acetone} \times C_a)$$

where $C_{fb} = 44.87$, $C_m = 37.48$, and $C_a = 62.04$ the empirical percentages of carbon in paromomycin freebase, methanol, and acetone, respectively, and C is the found total percentage of carbon in the sample. The final averaged total percentage, based on an average of corrected C and N data, indicates the maximum possible percentage of paromomycin freebase in the sample; the actual percentage is lower if organic impurities other than methanol are present. This fraction of the paromomycins and the other amino-bearing components can be assessed from peak area ratios from semi-quantitative HPLC (Section 3.2).

Based on the available compositional data, the total percentage of the summed components is close to 100% in all cases, indicating good mass balances. These mass balances are strong evidence that there are no significant sample components unaccounted for.

3.2. Semi-quantitative HPLC

Fig. 3 shows the semi-quantitative chromatographic profiles of the four samples obtained from HPLC system 1. Although the chromatographic profiles of the WR35928AV, USP and Sigma samples are similar, respective individual peak areas among them differ somewhat. In all the chromatograms, the total peak area percentage is assumed to be equivalent to the percentage of 'freebase' calculated from elemental analysis. Individual peak area percentages are multiplied by the total freebase percentage to find the peak's weight percentage. Based on the chromatographic and elemental data, the final estimated percentages of paromomycin I and II in each sample are also listed in Table 1. The final percentages are the total 'freebase' percentages minus the percentages of impurities found by LC, assuming equal area responses.

In the USP F2 sample chromatogram, the two major peaks (Paromomycin I and II) constitute 6.4 and 90.5% ($s = 0.4\%$) of the total peak area, respectively; early-eluting trace components account for the other 3%. This chromatographic data indicates a total of 65.4% paromomycins in lot F2.

Lot F1 differs somewhat. The two major peaks constitute 6.5 and 85.5% and the minor components total 7.9%, indicating a total of 62.8% paromomycins. These early-eluting trace components are judged to be impurities by virtue of their shorter retention times when compared to the two paromomycin peaks. However, to be detected by the system, they must have at least one primary amine and probably have similar empirical formulae. Work is in progress to continue to characterize these minor peaks by HPLC-MS. For the

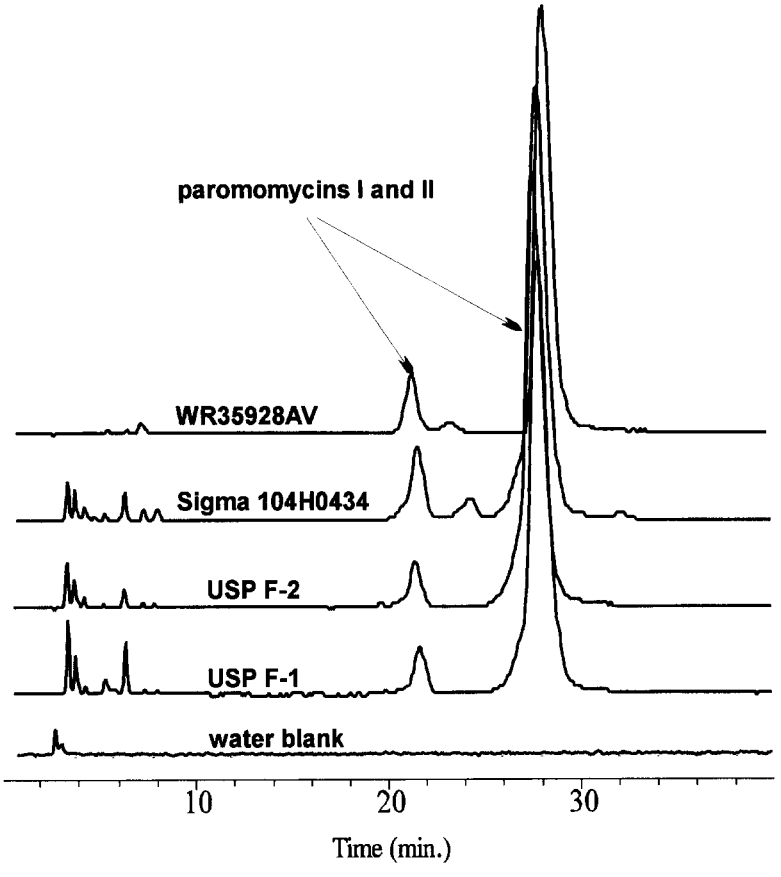

Fig. 3. Semi-quantitative HPLC chromatographic profiles of WR35928AV, USP and Sigma paromomycin samples from HPLC system 1.

moment, all peak areas are assumed to correlate to their weight percentages.

In the WR35928AV sample chromatogram, peaks 1 and 2 are 9.3 and 87.6% of the total peak area, respectively. The early-eluting components total 1.6% and a later impurity is 1.5%, indicating a total of 64.1% paromomycins.

In the Sigma sample chromatogram, peaks 1 and 2 are 10.3 and 82.3% of the total, with the minor peaks comprising a total of 7.3%, indicating a total of 63.2% paromomycins.

Differences in peak area ratios among the chromatograms probably indicate different paromomycin I and II ratios. This measurable difference in peak area ratios in paromomycin samples from different sources may be a means of correlating antibiotic activity with sample composition. Comparisons of chemical composition with antimicrobial activities are in progress.

Data from the alternative HPLC system, 2, strongly indicates that peaks 1 and 2 are paromomycin I and II because their molecular ions have the same, correct molecular weight, but cannot differentiate between the two isomers (Fig. 4). Though the chromatography is not identical to the fluorescence detection-based system due to the differences in mobile phases necessary for MS detection, the two peaks shown in Fig. 4 are presumed to correlate to the two major peaks shown in Fig. 3, on the basis of their similar area ratios.

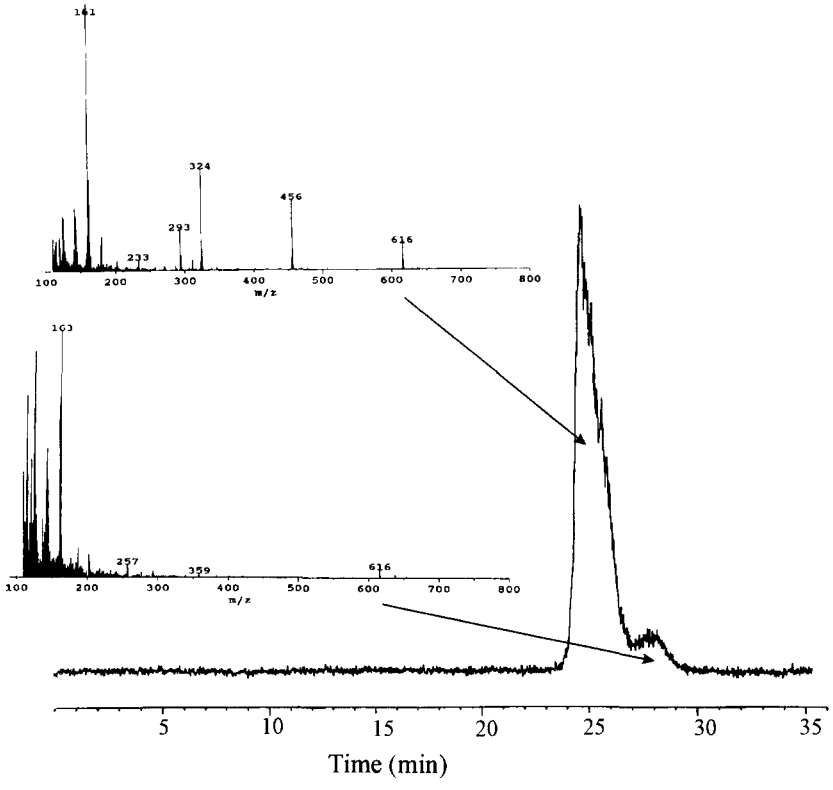

Fig. 4. HPLC-MS thermospray chromatographic profile of WR35928AV sample, overlaid with fractionation data. The differences seen in the two fractionation traces are due to the nature of thermospray mass spectrometry and the low signal of the second peak. The correct M + H ion of 616 appears in both traces.

3.3. HPLC method validation

The validity of the LC assay for paromomycin was demonstrated by the chromatographic data derived from the paromomycin solutions. The assay showed good precision; from peak area data from five injected paromomycin solutions, the RSD was 1.3% (Table 2). In addition, the LC assay was linear ($r = 0.9997$) and accurate (1.1% error). See Table 3 for this data. Note, however that although the linearity is good, the y-intercept is significantly higher than zero. Therefore, quantitations under these conditions should always be performed versus a standard curve rather than a single point reference. A plot of the percentage of peak area remaining (as compared to the time zero peak area) versus time indicates the paromomycin solution was stable for 24 h. This time period facilitates assay automation. The lower limit of detection of the assay, based on a 3:1 signal-to-noise ratio, is about 10 ng. Fermentation by-products and thermal decomposition products appear well-resolved from the two large paromomycin peaks, suggesting that the method is specific for paromomycin in the presence of its decomposition and by-products.

3.4. Technical notes

Because the mobile phase contains a high concentration of salts, it is very abrasive to the pump components, especially the pistons and seals. To reduce wear, it is recommended to continuously wash the pistons with a slow drip of water while the pump is in use. Some pumps facilitate this washing process (i.e. Waters) providing holes in the pump head for this purpose.

Table 2
Precision of LC assay for praomomycin

Sample	Sample weight (mg)	Sum of 2 major peak areas	Response factor
1	2.480	5855633	2361142
2	2.520	5918114	2348458
3	2.466	5783657	2345360
4	2.451	5873545	3296387
5	2.540	6135062	2415379
		Avg =	2373345
		s =	31021
		RSD =	1.3%

Column equilibration with the mobile phase is time consuming. Therefore, it is good practice to run the mobile phase slowly through the column overnight before running samples. Individual sample solutions should be chromatographed at 40 min intervals; shorter time periods inhibit column reequilibration and peaks begin to shift in retention times. One or two sacrificial equilibrating injections of sample solution are recommended to reduce retention time shifts before data collection begins.

The derivatizing agent degrades with time. Blanketing it with helium or making it up fresh daily is recommended.

As a solution in water, paromomycin tends to adsorb to glass surfaces, especially borosilicate glass, possibly due to the interaction of the free amines with the exposed silanol. As a solution in 2% sulfuric acid, paromomycin does not suffer from this problem. The acid has no apparent short-term adverse effect on stability.

4. Conclusion

The chemical composition of four paromomycin sulfate samples has been determined. The LC and LC-MS results indicate that paromomycin is a mixture of two isomers whose ratios vary from sample to sample.

The fluorescence-based HPLC method was shown to be specific, accurate, precise, linear and fairly sensitive. Paromomycin sulfate is stable at low concentrations in water for at least 24 h, a time period that is sufficient for most assays.

Table 3
Linearity and accuracy of LC assay for paromomycin

Sample	Actual sample weight (mg)	Sum of 2 major peak areas	Response factor[a]	Calculated sample weight (mg)[b]	% Difference (actual vs. calculated weight)
1	1.163	2655641	2283440	1.185	1.9
2	2.006	4503193	2244862	1.968	−1.9
3	2.550	5855972	2285460	2.542	−0.3
4	3.351	7848331	2342086	3.388	1.1
5	4.069	9423953	2316037	4.056	0.3
		Avg =	2296577		1.1
		s =	36390		
		RSD =	1.6%		

See text for LC conditions. Linear regression analysis of peak area vs. sample weight using $y = mx + b$ gave $y = (2.357 \times 10^6)x + 1.367 \times 10^5$, $r = 0.9997$.

[a] Response factor = peak area ÷ weight.

[b] Calculated weight is found by entering the peak area sum into the regression formula.

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