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Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 131–137

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Development and validation of a method to extract and quantitate paromomycin and gentamicin from an Aquaphilic® cream formulation

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Abstract

Butanol and dilute sulfuric acid were used to extract paromomycin and gentamicin from Aquaphilic®-based formulated creams. The extraction procedure was validated over different antibiotic concentration ranges for linearity, precision, accuracy, limited specificity, sensitivity and solution stability. © 1997 Elsevier Science B.V.

Keywords: Paromomycin; Gentamicin; Extraction from cream formulation; HPLC; Post-column derivatization; Cutaneous leishmaniasis

1. Introduction

Few drugs are effective against leishmaniasis, an infection caused by hemoflagellate protozoa *Leishmania*, which are transmitted by the bite of the sandfly. In the past, the treatment of choice has been complexed pentavalent antimony [1,2], which is also toxic to the heart, liver, kidneys and pancreas. Because the Walter Reed Army Institute of Research (WRAIR) has found that formulations containing mixtures of paromomycin and gentamicin are effective agents against cutaneous leishmaniasis, it is critical to identify and quantitate the active component(s) in the formulated drug products.

The current USP compendial method [3] for assaying paromomycin is based on microbial assay. This compendial method uses a qualitative approach that lacks specificity, does not identify the active ingredient(s) and does not yield information on total chemical composition. Few chemical assay methods for paromomycin have appeared in the literature. Those reported have used capillary zone electrophoresis [4] and gas-liquid chromatography [5]. However, to our knowledge, none applies specifically to a paromomycin extraction from a cream formulation nor its subsequent HPLC analysis. Additionally, although a compendial method exists for extracting gentamicin from creams and ointments, the subsequent assay is also based on microbial assay. To our knowledge, there are no references in the literature concerning mixtures of paromomycin and gentamicin in creams.

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PII S0731-7085(96)02044-4

Table 1
Formulation compositions

Formulation	Paromomycin sulfate (%)	Gentamicin sulfate (%)	AWC (%)	Water (%)
232	15.0	0.5	67.8	16.7
304	5.0	0.3	91.4	3.3
305	10.0	1.0	84.0	5.0
306	15.0	0.5	77.0	7.5

Like other aminoglycosides, paromomycin and gentamicin lack UV chromophores. To make up for the lack of a chromophore, isoindole derivatives are commonly prepared to render primary amines detectable [6–10]. We have recently used this derivatization technique in an HPLC method for quantitating bulk paromomycin sulfate [11]. This current paper expands on that earlier work by describing a validated method for the extraction of paromomycin and gentamicin from Aquaphilic®-based cream formulations in order to quantitate them.

2. Experimental

2.1. Reagents

The formulating cream base, Aquaphilic® with carbamide 10% ointment (AWC), is produced by Medco Lab (Sioux City, Iowa, USA). AWC's protocol composition is 4% sorbitol, 6% propylene glycol, 39.85% water, 10% urea, 0.5% lactic acid, 0.75% sodium lauryl sulfate, 0.5% isopropyl palmitate, 19% stearyl alcohol, 19% white petrolatum, 0.15% propyl paraben, and 0.25% methyl paraben. Bulk samples of paromomycin sulfate (WRAIR sample WR35928AV, containing 64.1% paromomycin base) and gentamicin sulfate (WRAIR sample WR73633AE, containing 62.5% gentamicin base), and several lots of formulation 232 (also known as WR279396), labeled to contain the protocol amounts of 15% paromomycin sulfate, 0.5% gentamicin sulfate in 16.7% water and 67.8% AWC, were received from the WRAIR. All solvents were HPLC grade and all other chemicals were reagent grade. Aqueous buffers and deionized water were filtered through Millipore 0.22-μm polyvinylidene fluoride membrane filters before use. Sodium 1-heptanesulfonate (Janssen Chimica or Aldrich), sodium sulfate (Sigma) and glacial acetic acid (Mallinckrodt) were used to prepare the mobile phases. Potassium hydroxide (Mallinckrodt), boric acid (Sigma), 2-mercaptoethanol (Sigma) and *o*-phthalaldehyde (Sigma or Pickering Labs) were used to prepare the derivatizing agent. Sulfuric acid was purchased from Mallinckrodt.

2.2. Formulation preparation

Batches (2 g) of protocol formulation 232 were individually prepared in our lab by the following procedure. A 30-ml separatory funnel was tared on a top-loading balance, and the following ingredients were weighed into the funnel: 1.35 g AWC (66.1%), 0.33 g water (16.2%), 0.35 g of paromomycin sulfate (17.1%) and 0.011 g of gentamicin sulfate (0.5%). The side of the funnel containing the ingredients was dipped into a 70°C water bath to melt the cream base. The funnel was then shaken to emulsify the ingredients. Heating and shaking were repeated until the bulk antibiotics were blended into the cream.

Three other formulations, containing varied concentrations of ingredients, were prepared analogously. The various formulation compositions are listed in Table 1.

Samplings (2 g) of each lot of the pre-prepared formulation samples were also transferred to separatory funnels.

2.3. Extraction procedure

n-Butanol (3 ml) was added to each separatory funnel containing formulation, followed by 5 ml

of aqueous 2% H_2SO_4. The contents were thoroughly mixed after each addition. After the phases had separated, the lower, aqueous layer was drained into a 100-ml volumetric flask. Another 5 ml of 2% H_2SO_4 was added to the remaining, butanol layer, which was extracted a second time. The second aqueous layer was added to the volumetric flask, which was then filled to the mark with water. A portion of the diluted extract was passed through a 0.45-μm Nylon 66 syringe filter.

For gentamicin analysis, 10 μl of the filtrate was chromatographed without dilution. For paromomycin analysis, the filtrate was appropriately diluted with water to produce an on-scale chromatogram. A 10-μl aliquot of the resulting diluted solution was chromatographed.

2.4. Antibiotic reference solution preparation

Paromomycin sulfate (100 mg) was placed into a 100-ml volumetric flask, followed first by 25 ml of water and then by 3 ml of aqueous 2% H_2SO_4. The mixture was shaken to dissolve the antibiotic; the volume was then diluted to the mark with water. Aliquots of 4, 6, 8 and 10 ml of this solution were each diluted to 100 ml with water. Then 10 μl of each diluted solution was chromatographed.

Four 10-mg portions of gentamicin sulfate were each weighed into separate 100-ml volumetric flasks, 25 ml of water and 4 ml of aqueous 2% H_2SO_4 were added to dissolve the antibiotics, and the volume was then diluted to the mark with water. Then 10 μl of each solution was chromatographed.

2.5. HPLC analysis of extracted antibiotics

Because the chromatographic properties of paromomycin and gentamicin widely differ, separate mobile phases were used to determine each of the antibiotics. These mobile phases consisted of (1) aqueous 0.2 M Na_2SO_4, 1.2 mM sodium 1-heptanesulfonate and 0.1% acetic acid for paromomycin analysis and (2) aqueous 0.2 M Na_2SO_4, 0.3 mM sodium 1-heptanesulfonate and 0.1% acetic acid for gentamicin analysis. The mobile phases were delivered with a Waters M6000 pump at a flow rate of 1.0 ml min^{-1} through a 4.6 × 250 mm Metachem Inertsil C8 stainless steel column. The pump plunger seals were continuously washed with water at a low flow rate (~30 μl min^{-1}) to reduce plunger and seal wear.

A Rheodyne 7125 injector with a 20-μl loop was used to introduce the samples to the column.

The derivatizing agent was prepared by dissolving 0.8 g of *o*-phthalaldehyde and 1 ml of 2-mercaptoethanol in 10 ml of methanol and then diluting to 1000 ml with aqueous 2.5% boric acid that had been adjusted to pH 10 with aqueous 2.5% KOH. The derivatizing agent was introduced through a post-column 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 column effluent proceeded into a 9-m long, 0.25-mm i.d., 1.6 mm o.d. stainless-steel reaction coil, wrapped in 3- to 5-cm coils.

A Varian 9070 fluorescence detector was connected to the effluent end of the coil; the xenon lamp pulsed at 20 Hz, and the excitation and emission monochromators, each with a 20-nm bandwidth, were set to wavelengths of 340 and 440 nm, respectively. Peak area data was collected with an Intel 80486-based PC, interfaced with HP Chemstation hardware and software.

The percentages of the two antibiotics recovered from each formulation were determined separately by comparisons to the applicable reference solutions having approximately the same concentrations as the extracts. Typically, chromatograms of gentamicin extracts show at least four principal components (Fig. 1a); however only one of these components is baseline-resolved. A tailing urea peak in the gentamicin chromatograms of the extracts contributed to the interference of all but the last gentamicin peak. For these reasons, only the area of the last gentamicin peak was used for quantifying gentamicin. Chromatograms of paromomycin show two principal components (Fig. 1b), and the sum of these two peak areas is used to quantify this antibiotic.

2.6. Extraction method validation

The HPLC assay used to validate this extraction procedure has been previously validated for assays of bulk paromomycin sulfate [11] and gentamicin sulfate (data not shown). Only the extraction validation data is included here.

2.6.1. Limited specificity

A portion of formulation 232 was heated to 200°C for 20 s. The heated formulation was then extracted and assayed by HPLC to determine whether or not the thermal degradation products are chromatographically resolved from the peaks that are used to quantitate the antibiotics.

2.6.2. Precision

Several 2 g portions of several batches of formulation 232, either prepared individually in our labs or obtained from the WRAIR, were extracted and the antibiotics were assayed by HPLC. The results obtained were treated statistically to obtain extraction precision data.

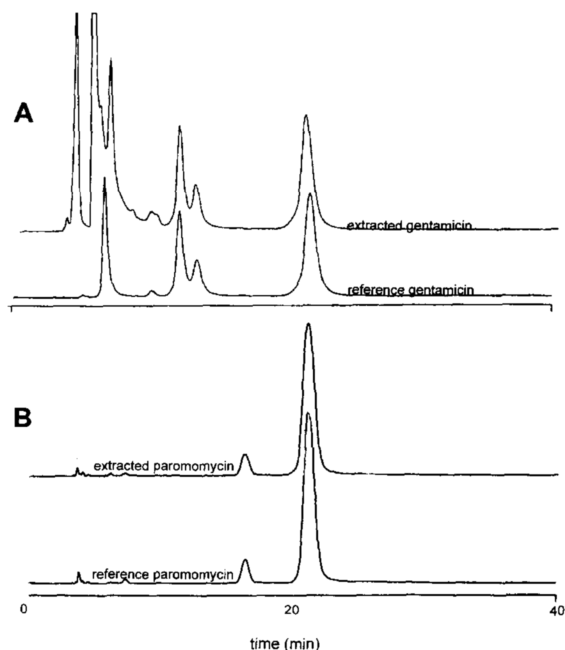


Fig. 1. (A) Typical chromatograms of extracted and reference gentamicin and (B) typical chromatograms of extracted and reference paromomycin.

2.6.3. Accuracy and linearity

Portions (2 g) of individually prepared formulations 232, 304, 305 and 306 (formulation compositions are defined in Table 1) were each extracted and assayed. The amounts of each antibiotic recovered from each formulation were determined by comparisons to the appropriate reference solutions, and compared with the amounts of antibiotic that had been weighed. The results were used to determine how accurate and linear the extraction procedure is over a concentration range, which is between 5 and 15% paromomycin and between 0.25 and 1% gentamicin (w/w) in the formulations.

2.6.4. Solution stability

The room temperature solution stabilities of the extracted paromomycin and gentamicin from formulation 232 were determined by comparisons of chromatographic results obtained over 24 h versus the respective reference values.

2.6.5. Sensitivity

To determine the limit of detection of the antibiotics in the formulations, 10 μg of paromomycin sulfate (which contains 6.4 μg of paromomycin base) and 5 μg of gentamicin sulfate (which contains 3.1 μg of gentamicin base) were combined with 1.36 g of AWC, which is the proportion of AWC that would be contained in 2 g of formulation 232. The mixture was extracted with n-butanol and 2% H_2SO_4, and the aqueous layer was transferred to a 10-ml volumetric flask and brought to the mark with water. The content of the flask was filtered with a 0.45-μm Nylon 66 syringe filter. Of the filtrate, 10 μl was chromatographed for gentamicin and paromomycin under each of the chromatographic assay conditions.

3. Results and discussion

3.1. Extraction of antibiotics

The purpose of this study was to completely remove the antibiotics from an Aquaphilic® matrix so that their quantitations could be performed.

Quantitative recoveries of the antibiotics requires complete dissolution of the formulation in one or more extraction solvents. The achievement of this goal presented some major difficulties. Because components in this particular formulation have vastly different polarities, no one solvent or mixture of miscible solvents could be found that would produce a homogeneous solution. Miscible solvents that were tried were methanol-water, hexane-chloroform, and methanol-chloroform. For this reason, a liquid-liquid extraction solvent system was sought to partition the hydrophobic components from the hydrophilic ones.

Unfortunately, solvent systems that were studied tended to form emulsions that limited the recovery of the antibiotics, a problem compounded by the presence of sodium lauryl sulfate in the formulation. Solvent systems that were explored that exhibited this emulsion problem included various ratios of chloroform-water, chloroform-water-methanol, hexane-water, hexane-chloroform-water and octanol-water. Solvent systems of butanol mixed with an aqueous solution of a basic salt, used to facilitate partitioning, have been reported to be useful for extraction of surfactants such as sodium lauryl sulfate from aqueous media [12]. In the case of formulation 232, the use of *n*-butanol-2% aqueous sulfuric acid (3:5, v/v) proved satisfactory. The acid serves to ionize the aminoglycosides and adds electrolyte to aid partitioning.

Acidification of the reference solutions is absolutely necessary as well. When no acid was used in the reference solutions, antibiotic recoveries apparently exceeded 110% and varied widely (10%). But, when sulfuric acid was included in the reference solutions in the same proportions as in the extract solutions, antibiotic recoveries from the extracts were essentially 100%, with R.S.D.s of 1% or less.

3.2. Extraction method validation

The quantitated peaks in the chromatograms of the samples are all well resolved in the presence of residual cream matrix components, although urea dominates early retained peaks in gentamicin chromatograms. This information suggests that the HPLC assay is specific with respect to matrix components, although the extraction method itself is not completely specific for the antibiotics alone, as evidenced by the presence of urea in the extract. We have experimentally demonstrated (data not included) that 20 s at 200°C is sufficient time to force-degrade samples of paromomycin and gentamicin in creams; longer heating periods completely destroy the active component, rendering the specificity determination fruitless. Antibiotic fermentation by-products and thermal decomposition products appear well-resolved from the two large paromomycin peaks and the gentamicin peaks, suggesting that the method is specific for paromomycin in the presence of its decomposition and by-products. However, detection is based on the response of a derivatized antibiotic. Any amine present in the matrix, including thermal decomposition products and urea, is derivatized, and will give a similar spectral profile. Absolute chemical homogeneity of the peaks of interest has therefore not been demonstrated, a factor that is an inherent weakness of the method.

Table 2 shows the precision data from the antibiotics recovered from several batches of formulation 232. The precisions averaged 1.6% for paromomycin and 1.5% for gentamicin.

Table 2
Precision: Recoveries of paromomycin and gentamicin from formulation 232

Sample	Paromomycin recovered[a] (%)	Gentamicin recovered[a] (%)
SRI form. 232	100.4 ($n=3$, R.S.D. = 0.3%)	97.8 ($n=3$, R.S.D. = 1.0%)
WR279396AB	100.1 ($n=5$, R.S.D. = 2.8%)	92.5 ($n=5$, R.S.D. = 1.7%)
WR279396AD	101.4 ($n=5$, R.S.D. = 2.9%)	99.2 ($n=5$, R.S.D. = 3.2%)
WR279396AF	100.4 ($n=3$, R.S.D. = 1.9%)	110.3 ($n=3$, R.S.D. = 0.4%)
WR279396AH	97.7 ($n=4$, R.S.D. = 0.3%)	104.2 ($n=2$, R.S.D. = 1.2%)

[a] Percentages of recovered paromomycin and gentamicin from the WR279396 formulations are reported as percentages of the labeled amounts.

Table 3
Accuracy: recoveries of paromomycin and gentamicin from all formulations

SRI formulation	Paromomycin recovered (%)	Gentamicin recovered (%)
232	100.4 ($n = 3$, R.S.D. = 0.3%)	97.8 ($n = 3$, R.S.D. = 1.0%)
304	97.0	95.0
305	98.0	98.0
306	101.0	98.0

The results from the accuracy and linearity study are shown in Table 3 and Table 4. The percentages of antibiotics recovered from individually prepared formulations over an antibiotic concentration range averaged 99.1%, R.S.D. = 1.9%, for paromomycin and 97.2%, R.S.D. = 1.5%, for gentamicin. Some deviation in linearity occurs when concentrations of gentamicin are very low (i.e. gentamicin from formulation 304).

The results from the solution stability study (data not shown) indicate that both antibiotics in the extract solutions are stable for at least 24 h.

The results from the sensitivity study show that the lower detection limit for paromomycin base in the cream formulations is 8 ng, which equates to 4 μg of paromomycin sulfate in 2 g of formulation (2 ppm). The lower detection limit for gentamicin base under these same conditions is 10 ng, which equates to 5 μg of gentamicin sulfate in 2 g of formulation (2.5 ppm).

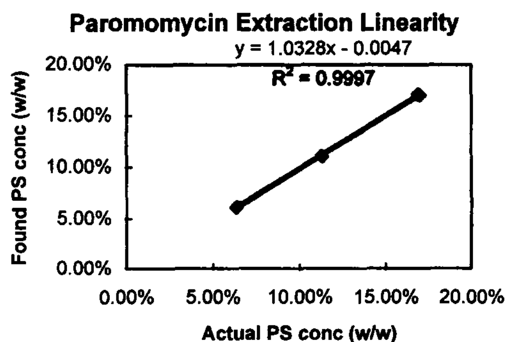
3.3. Technical notes

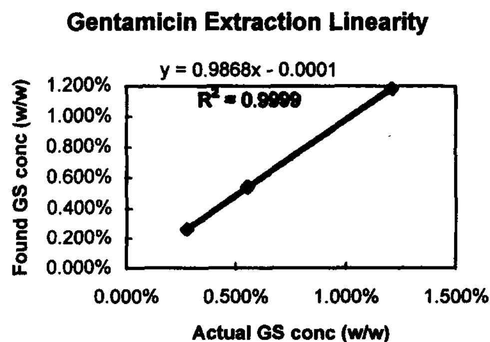
Because the mobile phase contains a high concentration of salts, it is very abrasive to the pump components, especially the pistons and seals. To reduce wear, it is recommended to continuously wash the pistons with a slow drip of water while the pump is in use. Some pumps facilitate this washing process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

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

Table 4
Linearity

Formulation	Actual paromomycin sulfate (%)	Actual gentamicin sulfate (%)	Found paromomycin sulfate (%)	Found gentamicin sulfate (%)
232	16.97	0.553	17.04	0.541
304	6.35	0.277	6.16	0.263
305	11.30	1.210	11.07	1.186
306	16.90	0.553	17.07	0.542





sample solutions should be chromatographed at 40-min intervals; shorter time periods inhibit column reequilibration and retention times begin to shift. 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, so blanketing with helium or making it up fresh daily is recommended.

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

4. Conclusion

A suitable method has been developed to extract paromomycin and gentamicin from AWC-based cream formulations. Extraction recoveries are close to 100% for both antibiotics. The extraction method has been shown to be precise, accurate, linear and provisionally specific. The lower limit of detection for gentamicin and paromomycin in the formulation creams was 2.5 and 2 ppm, respectively. The antibiotics are stable in extract solutions for at least 24 h at room temperature.

Acknowledgements

This work was supported entirely by the U.S. Army Medical Research and Development Command under contract No. DAMD17-91-C-1135. The views, opinions and/or findings are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless designated by other documentation.

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