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# Determination of paromomycin in human plasma and urine by reversed-phase high-performance liquid chromatography using 2,4-dinitrofluorobenzene derivatization

Jie Lu*, Michael Cwik, Thomas Kanyok

*Clinical Research Laboratory, College of Pharmacy, University of Illinois at Chicago, M/C 886, 833 S. Wood Street, Chicago, IL 60612, USA*

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**Abstract**

A sensitive high-performance liquid chromatographic method for the determination of paromomycin in human plasma and urine was developed. Paromomycin was quantitated following pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB). The chromatographic separation was carried out on a $C_{18}$ column at 50°C using a mobile phase consisting of 64% methanol in water adjusted to pH 3.0 with phosphoric acid. The eluents were monitored by UV detection at 350 nm. The linearity of response for paromomycin was demonstrated at concentrations from 0.5 to 50 μg/ml in plasma and 1 to 50 μg/ml in urine. The relative standard deviation of the assay procedure is less than 5%.

*Keywords:* Paromomycin; 2,4-Dinitrofluorobenzene

## 1. Introduction

Paromomycin (Pm, Fig. 1) is a broad spectrum aminoglycoside antibiotic first isolated in 1956 from the fermentation of streptomyces [1]. Currently, it is being developed for use against multi-drug resistant strains of tuberculosis [2] and for the treatment of intestinal cryptosporidiosis in AIDS patients [3,4]. Like other aminoglycoside antibiotics, paromomycin has serious dose-dependent side-effects such as nephrotoxicity and ototoxicity [5,6]. An understanding of the drug's pharmacokinetic properties, therefore, is needed to ensure its safe and effective use.

Few methods have been reported specifically for the determination of paromomycin. Microbiological assays are traditionally used for the determination of aminoglycoside antibiotics [7–10]. They are simple and inexpensive, but time consuming and non-specific. The sensitivity of this technique is 0.5–1.0 μg/ml [7–10]. A GC assay to determine the isomers of paromomycin in commercial preparations has been reported [11]. This method is not easily transferable to samples in serum or urine. A GC method for the assay of aminoglyciside antibiotics in serum uses paromomycin B as an internal standard [12]. This is a multi-step procedure requiring separate derivatization of the amino and hydroxyl groups. More recently, high-performance liquid chromatography (HPLC) has been used for the analysis of aminoglycosides such as neomycin, tobramycin, amikacin and kanamycin in biological fluids [13–18]. Because these antibiotics have little absorbance

*Corresponding author.


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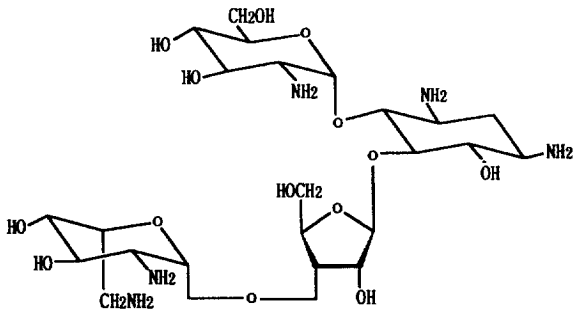
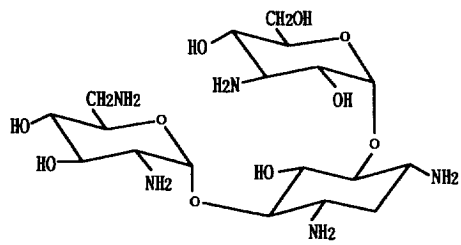


Paromomycin



Kanamycin B

Fig. 1. Structures of paromomycin and kanamycin B.

in the UV region, derivatization with 2,4-dinitrofluorobenzene (DNFB) [13,15,17,18], *o*-phthalaldehyde (OPA) [14], 2,4,6-trinitrobenzene sulfonic acid (TNBS) [16] or dansyl chloride [19] is necessary for detection. The most commonly used derivatizing agent for aminoglycosides is DNFB. It not only provides UV detection of the drugs but also improves their chromatographic characteristics by derivatizing the amino groups which cause peak tailing on silica based HPLC columns.

We report a sensitive, specific and accurate HPLC method for determining paromomycin in human plasma and urine.

## 2. Experimental

### 2.1. Reagents and materials

Paromomycin sulfate was obtained from the United States Pharmacopeial Convention (USPC, Rockville, MD, USA). Kanamycin B sulfate, Trizma-8.5 [tris (hydroxymethyl) aminoethane and hydrochloride] and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). The derivatizing agent, 2,4-dinitrofluorobenzene (DNFB), was from Aldrich (Milwaukee, WI, USA). Borosilicate glass disposable culture tubes 12×75 and 13×100 mm (Fisher Scientific, Pittsburgh, PA, USA) were used. Pooled human plasma and urine were collected from volunteers on site and stored at −20°C. Deionized water was obtained as needed from a Barnstead Nanopure II (Fisher Scientific).

Trizma buffer solution (1%) was prepared by dissolving 1 g of Trizma-8.5 in 100 ml 0.1 *M* phosphate buffer (80 ml 0.1 *M* sodium phosphate dibasic, 20 ml 0.1 *M* sodium phosphate monobasic) and adjusting pH to 7.8 with 6 *M* hydrochloric acid. 2,4-Dinitrofluorobenzene (2%) was prepared by transferring 2 ml of DNFB to a 100 ml volumetric flask and diluting to volume with ethanol.

### 2.2. Instrumentation

The HPLC System consisted of an M-510 pump, a WISP-712 autosampler and an M-490E programmable multi-wavelength detector (Waters Chromatography Division, Millipore, Milford, MA, USA). An HP-3359 laboratory automation system (Hewlett-Packard, Cupertino, CA, USA) was used for data acquisition.

### 2.3. Chromatographic conditions

A Zorbax SB-C18 column maintained at 50°C (5 μm, 250×4.6 mm I.D., Mac-Mod Analytical, Chadds Ford, PA, USA) was used for the chromatographic separation. The mobile phase consisted of 64% methanol in deionized water adjusted to pH 3.0 with concentrated phosphoric acid. The flow-rate was 2.0 ml/min. The run time was 30 min. Eluting peaks were monitored at 350 nm.

### 2.4. Extraction procedures

#### 2.4.1. Plasma

To 300 μl of plasma (blank, standard, control or patient sample) were added 30 μl of the internal

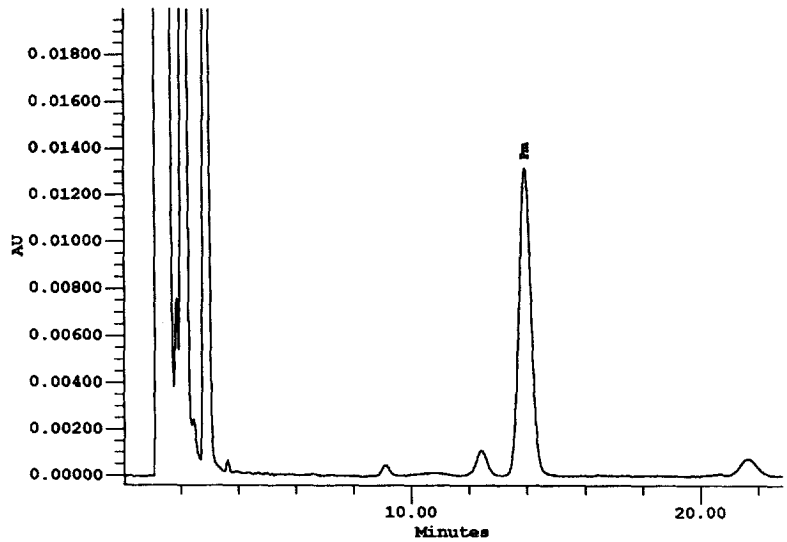



Fig. 2. Typical HPLC chromatogram of derivatized paromomycin obtained in an aqueous solution.

standard solution (Kanamycin B, 101.4 μg/ml in water) and 100 μl of 2 *M* perchloric acid to precipitate proteins. The samples were vortex mixed for 2–3 s and centrifuged at 1000 *g* for 5 min. The supernatant was transferred to a clean tube and neutralized with 1.5 *M* NaOH. 300 μl Trizma buffer, 400 μl DMSO and 100 μl DNFB were added. The samples were vortex mixed and incubated at 64°C for 30 min. Toluene (3 ml) was added and vortex mixed to remove excess reagents. The upper toluene layer was discarded after centrifugation. The derivatized Pm and I.S. were extracted by adding 3 ml acetonitrile–toluene (1:1, v/v) and vortex mixing for 5–10 s. The upper organic layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 30–40°C. The residue was reconsti-

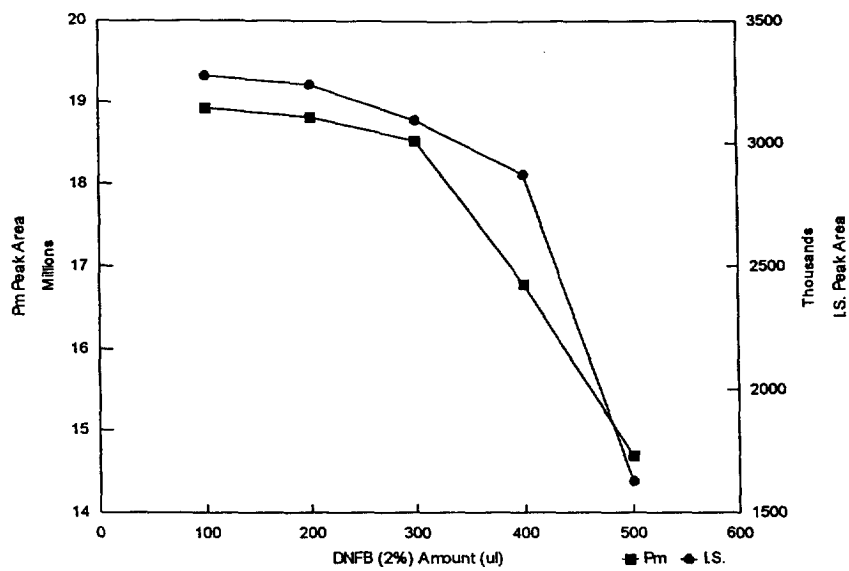



Fig. 3. Peak area of Pm and KmB derivatives as a function of DNFB (2%) amount. Pm concentration was 47 μg/ml in human plasma.

tuted in 1 ml acetonitrile–water (1:1, v/v) and 20 μl were injected onto the HPLC column.

*2.4.2. Urine*

Urine samples were diluted 1:100 with deionized water before analysis. To 300 μl of diluted urine were added 30 μl of I.S. solution, 300 μl Trizma buffer, 400 μl DMSO and 100 μl DNFB. The samples were vortex mixed and incubated at 64°C for 30 min. The samples were washed and extracted following the procedures for plasma. 10 μl of the reconstituted sample were injected onto the HPLC column.

## 3. Results and discussion

DNFB is commonly used for the derivatization of aminoglycosides for HPLC analysis. It reacts with both primary and secondary amines and also with

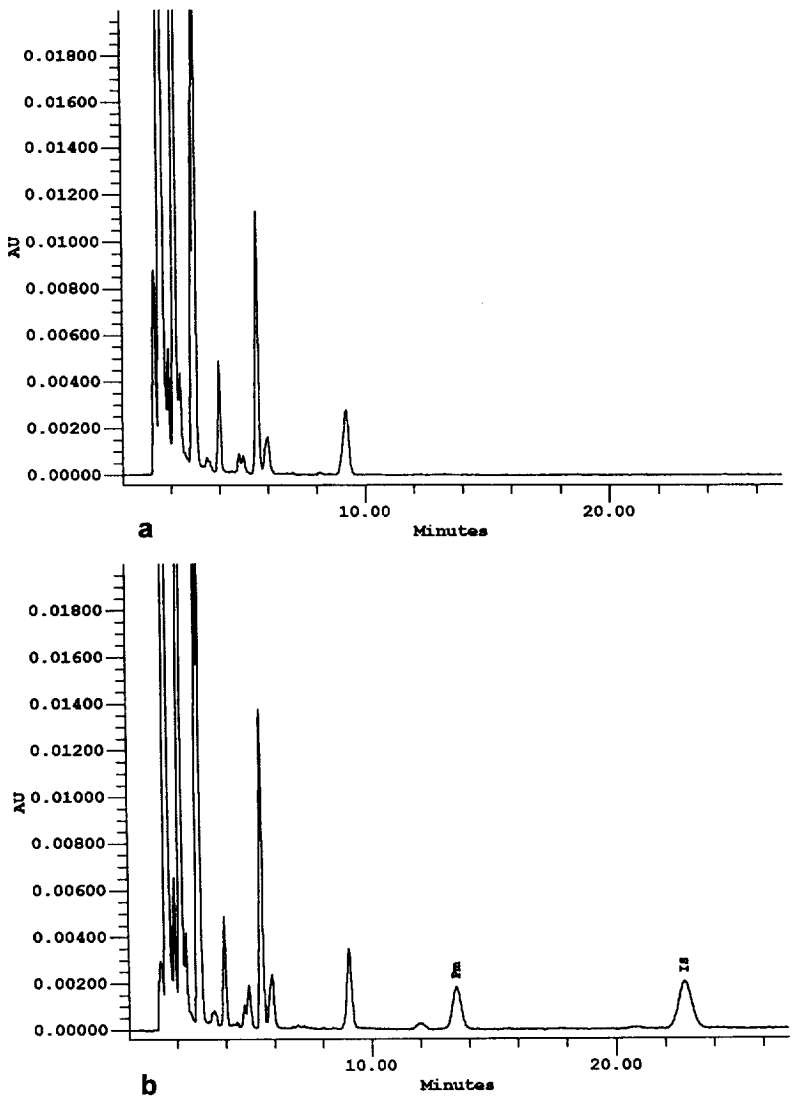



Fig. 4. (a) Chromatogram of extract of blank plasma. (b) Chromatogram of extract of a plasma sample from a normal volunteer 8 h after intra-muscular administration of 15 mg/kg paromomycin. Pm concentration was determined as 4.72 μg/ml.

aliphatic hydroxyl groups [20]. Barends et al. [15] reported that they could limit the reaction of DNFB with the hydroxyl groups of the aminoglycoside antibiotic tobramycin by controlling the pH of the reaction. They showed that under optimal conditions, the major product was derivatized at the amino groups only that this represented 75% of the products of derivatization. Fig. 2 shows a chromatogram of Pm reference standard in aqueous solution after derivatization with DNFB. The major peak accounts for 90% of the peak area related to Pm in this chromatogram.

Reaction conditions were investigated in order to optimize the yield of the major derivative. Several buffer systems (phosphate, bicarbonate, borate and Trizma), which had previously been reported for the derivatization of aminoglycosides with DNFB, were tested. A combination of Trizma (1%) and 0.1 *M* phosphate buffer at pH 7.8 was found to give the best yield. Lower pH (<7.5) decreased the yield of

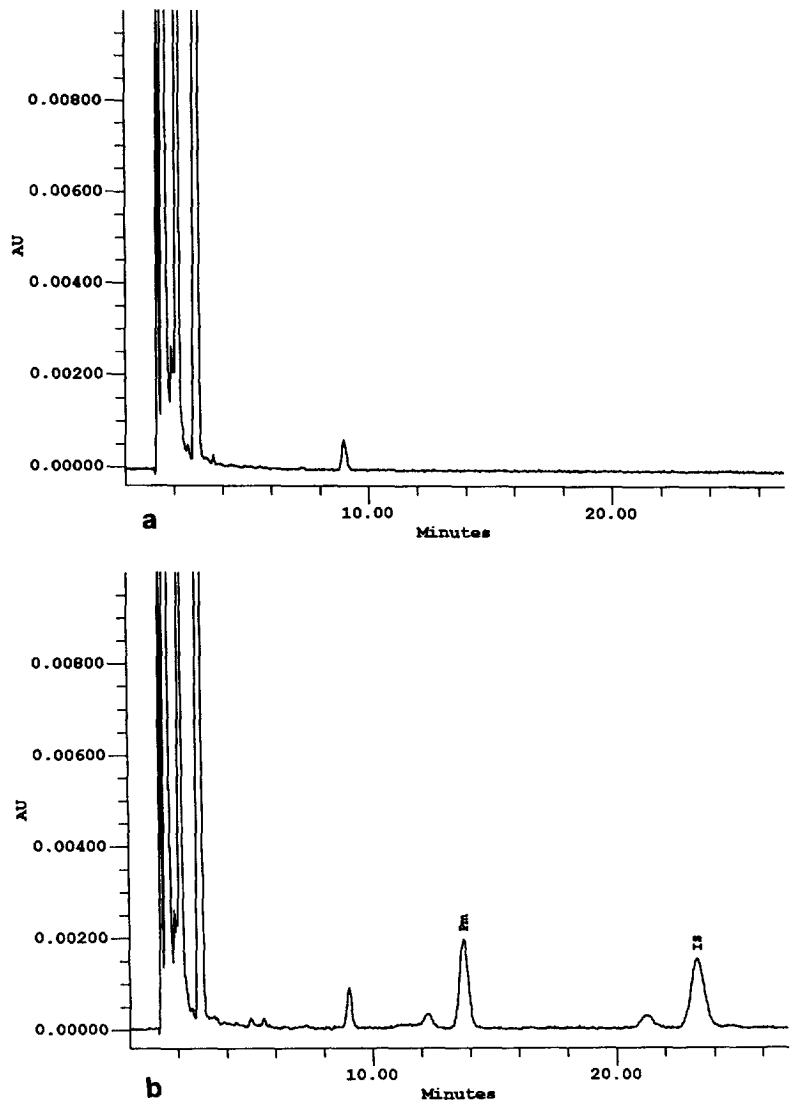



Fig. 5. (a) Chromatogram of extract of blank urine. (b) Chromatogram of extract of a urine sample from a normal volunteer 2 h after intra-muscular administration of 15 mg/kg paromomycin. Pm concentration was determined as 675 μg/ml.

Table 1
Accuracy and precision for the analysis of paromomycin in human plasma and urine

| | Concentration (μg/ml) | | | R.S.D. (%) | Accuracy (%) |
|---|---|---|---|---|---|
| | Nominal | Determined (Avg.) | S.D. | | |
| *Within-run* | | | | | |
| Plasma | 36.9 | 35.3 | 1.0 | 2.8 | 95.7 |
| ($n$=6) | 11.0 | 11.1 | 0.4 | 3.8 | 100.9 |
| | 2.21 | 2.25 | 0.1 | 5.7 | 101.8 |
| Urine | 36.6 | 36.6 | 0.6 | 1.5 | 100.0 |
| ($n$=7) | 14.6 | 14.9 | 0.1 | 0.9 | 102.1 |
| | 2.92 | 2.94 | 0.1 | 2.3 | 100.7 |
| *Between-run* | | | | | |
| Plasma | 36.9 | 36.5 | 1.1 | 2.9 | 98.9 |
| ($n$=7) | 11.0 | 11.4 | 0.3 | 3.1 | 103.6 |
| | 2.21 | 2.33 | 0.1 | 3.1 | 105.4 |
| Urine | 36.6 | 36.2 | 1.2 | 3.4 | 98.9 |
| ($n$=7) | 14.6 | 14.7 | 0.3 | 2.1 | 100.7 |
| | 2.92 | 2.88 | 0.1 | 2.3 | 98.6 |

the major Pm derivative. Higher pH (>8.0) gave greater yield but produced multiple products. Addition of 100 μl of 2% DNFB was found to give maximum yield for the major derivative. Fig. 3 shows the relationship between the amount of derivatizing agent and the peak area of Pm. The formation of Pm derivative was investigated from 50 to 100°C. No significant increase in the yield of major product was observed above 65°C. At this temperature, 20 min were sufficient to achieve full reaction. The toluene wash before extraction of the derivatives gave a cleaner chromatogram and extended column life by removing unreacted reagents from the injected sample. The relative recovery of Pm from plasma compared to aqueous solution, measured at 19 μg/ml, was 87%.

Representative chromatograms from blank plasma, a patient plasma sample, urine blank and a patient urine sample are shown in Figs. 4 and 5. The retention time was approximately 14.0 min for Pm

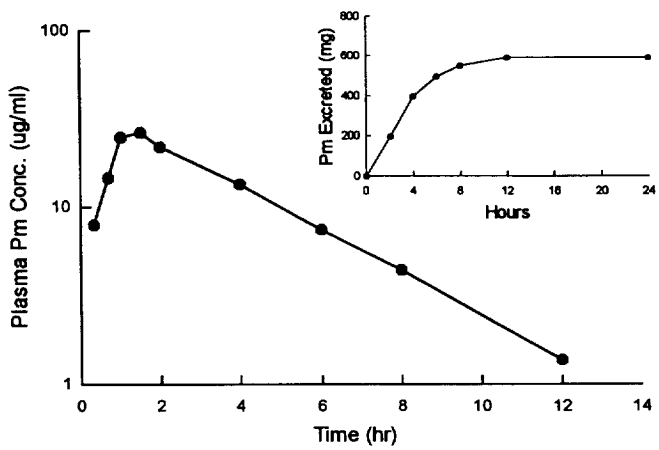



Fig. 6. Plasma concentration of paromomycin as a function of time obtained in a normal volunteer after intra-mascular administration of 12 mg/kg paromomycin. Inset: the cumulative urinary excretion of paromomycin in the same volunteer vs. time.

and 24.0 min for KmB (internal standard). No interferences to either Pm or KmB were found in blank plasma or urine. No interferences were observed when the following antituberculosis drugs were carried through the derivatization and extraction procedures: isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin, amikacin, ofloxacin and ciprofloxacin.

All quantitation for the current study is based on the peak area ratio of paromomycin and the internal standard. The detector response was linear over the range of 0.5–50 μg/ml in plasma and 1–50 μg/ml in urine. Correlation coefficients were all greater than 0.99 for the individual curves. The mean of slope ($n$=7) was 0.129 (S.D.±0.003) for plasma and 0.105 (S.D.±0.002) for urine. The $y$-intercepts were determined to be not significantly different from zero. The limit of quantitation (the lowest Pm concentration at which percent error and R.S.D. were <20%) was 0.5 μg/ml for plasma and 1.0 μg/ml for urine. The limit of detection ($S/N$>2) was determined as 0.2 μg/ml for plasma and 0.5 μg/ml for urine. The assay accuracy and precision results are presented in Table 1. No change in the amount of Pm was detected over three freeze–thaw cycles. Fig. 6 is a representative time profile of paromomycin in plasma and urine from a normal volunteer after intra-muscular administration of 12 mg/kg paromomycin.

In summary, this assay provides a sensitive, accurate and reproducible method for the determination of paromomycin concentration in plasma and urine.

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