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High-performance liquid chromatographic determination of isepamicin in plasma, urine and dialysate

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SUMMARY

A high-performance liquid chromatographic method for the measurement of isepamicin, a new aminoglycoside, in plasma, urine and dialysate is reported. The assay utilizes a simple extraction of isepamicin in plasma using commercially available Cyano solid-phase cartridges and dilution of urine and dialysate samples. The separation is performed on a Hypersil C_{18} column (15 cm × 4 6 mm I.D., 5 μm particle size) and utilizes a mobile phase consisting of 10% methanol and 90% buffer solution containing 0.01 *M* sodium hexanesulfonate, 0.1 *M* sodium sulfate and 17 m*M* acetic acid. The flow-rate is 1.1 ml/min. Dibekacin is used as the internal standard. Isepamicin is derivatized post-column with *o*-phthalaldehyde for spectrofluorometric detection The method can also be used for the measurement of other aminoglycosides, i.e tobramycin, kanamycin, netilmicin and gentamicin. The assay is fast, accurate and has a quantitation limit of 100 ng/ml isepamicin in plasma and 50 ng/ml in urine and dialysate.

INTRODUCTION

A variety of high-performance liquid chromatographic (HPLC) methods have been developed for the analysis of aminoglycosides in biological fluids [1–

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8]. Due to the similarity of structures of the aminoglycosides, these methods are theoretically applicable to other members of the group. Separation techniques ranging from sample precipitation [1,2], aqueous extraction and column switching [3] to laboratory-prepared solid-phase extractions (silicic acid [4], Amberlite CG50 [5] or CM-Sephadex [6] have been utilized.

Isepamicin (SCH 21420, HAPA-B) is a new aminoglycoside antibiotic which in animal studies shows less potential for nephrotoxicity and ototoxicity than other aminoglycosides [9,10].

In this report we describe a new, fast and accurate HPLC assay for the measurement of isepamicin in plasma, urine and dialysate. The method utilizes a simple extraction of isepamicin in plasma on a commercially available Cyano (CN) solid-phase cartridge or dilution of urine and dialysate samples. The method uses post-column derivatization of the aminoglycoside with *o*-phthalaldehyde and spectrofluorometric detection. The method can also be used for the measurement of kanamycin and tobramycin in plasma, urine and dialysate without any modification. Furthermore, measurements of gentamicin components and netilmicin are possible using this method with minor modifications of the mobile phase.

EXPERIMENTAL

Materials

Isepamicin powder (Lot B.A. 10449-57I; potency 734 μg/mg), netilmicin and the internal standard (dibekacin) were kindly provided by the Department of Drug Metabolism, Schering (Bloomfield, NJ, U.S.A.). Gentamicin and kanamycin were purchased from Sigma (St. Louis, MO, U.S.A.). Tobramycin was obtained from Eli Lilly (Indianapolis, IN, U.S.A.).

The *o*-phthalaldehyde and β-mercaptoethanol were purchased from Eastman Kodak (Rochester, NY, U.S.A.). Sodium hexanesulfonate was obtained from Sigma. Methanol was HPLC grade from Fisher Scientific (Minneapolis, MN, U.S.A.). Solid-phase extraction cartridges (3 ml cyanopropylsilane, CN) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was purified by reverse osmosis. Dialysate was obtained from Renal Systems, Division of Minntech (Minneapolis, MN, U.S.A.) and contained a final concentration of dextrose of 2 g/l. All other chemicals were of reagent grade.

Instrumentation

The mobile phase solvent delivery system (Model LC-6A, Schimadzu Scientific Instruments, Columbia, MO, U.S.A.) was set at a flow-rate of 1.1 ml/min. The autoinjector (Model WISP 710B, Waters Chromatography, Division of Millipore, Milford, MA, U.S.A.) was set up with a needle wash solution of methanol–water (1:1). The solvent delivery system for the *o*-phthalaldehyde

derivatizing agent (Model 6000A, Waters Chromatography), set at 0.4 ml/min, was equipped with a pulse dampener.

Separation was performed on a Hypersil C_{18} column (15 cm×4.6 mm I.D., 5 μm particle size) equipped with a Hypersil C_{18} guard column (10 mm×4.6 mm I.D., 5 μm particle size) (Alltech Assoc., Applied Science Labs., Deerfield, IL, U.S.A.). The reaction coil was knitted TFE tubing (3 m×0.05 mm I.D.) from Supelco (Bellefonte, PA, U.S.A.). The column, mixing tee and reaction coil were housed in a CH30 column heater with TC-50 controller (Fiatron Laboratory Systems, Oconomowoc, WI, U.S.A.) set at 25°C.

A Spectroflow 980 fluorescence detector (Kratos Analytical, a division of Spectros, Ramsey, NJ, U.S.A.) was used for sample detection with the following setting: photomultiplier tube signal, 0.1 μA; range, 0.2 μA; high volt signal, −1150 V; rise time, 1 s. The excitation wavelength was set at 338 nm and the fluorescence emission was monitored using a 418-nm bandpass filter.

Computing integrator (Model SP4290, Spectra-Physics Autolab Division, San Jose, CA, U.S.A.) was used for data collection and recording. The peak-area ratio of isepamicin to dibekacin was used to estimate the drug concentration.

Preparation of derivatizing agent

Potassium borate buffer (1 *M*, pH 10.4) was prepared by mixing equimolar solutions of potassium hydroxide and boric acid. The derivatizing agent was prepared by dissolving 0.4 g of *o*-phthalaldehyde in 3 ml of methanol and adding the solution of 390 ml of potassium borate buffer in a 500-ml volumetric flask. β-Mercaptoethanol (2 ml) was then added and the solution was diluted to volume with water. The derivatizing agent was stored in brown glass flask in the refrigerator at 4°C and remained usable for one week. Once every two weeks the derivatizing agent solvent lines were flushed with methanol–water solution to clean the pumps and prevent discoloration of the reaction coil.

Preparation of mobile phase and extraction buffers

Buffer A was used to dilute plasma samples and as a wash step in the extraction procedure. In a 500-ml volumetric flask, 0.94 g of sodium hexanesulfonate was dissolved in 300 ml of water. Glacial acetic acid (0.5 ml) was then added and the solution was diluted to volume with water.

Buffer B was used for mobile phase preparation. Buffer B was prepared by dissolving 3.76 g of sodium hexanesulfonate and 28.4 g of sodium sulfate in 2 l of water. The solution was acidified with 2 ml of concentrated glacial acetic acid to pH 3.4. The final composition of buffer B was 0.01 *M* sodium hexanesulfonate, 0.1 *M* sodium sulfate and 17 m*M* acetic acid.

The mobile phase was a mixture of methanol–buffer B (10.90, v/v). This mixture was also used as an eluting solvent in the extraction procedure. Meth-

anol and buffer B were filtered separately through a 0.2-μm Nylon filter (Chrom Tech, Apple Valley, MN, U.S.A.) and degassed under vacuum after mixing.

Preparation of stock solutions

The stock solutions of isepamicin used to prepare the standard curve were prepared with water and stored in polypropylene test tubes. All standard solutions, plasma, urine and dialysate samples were stored in plastic containers and all sample transfers were performed with plastic tip pipetors.

A stock solution of 1 mg/ml isepamicin was prepared relative to potency. Solutions of 100, 10 and 1 μg/ml were prepared by diluting the original stock solution.

A stock solution of dibekacin (1 mg/ml) was prepared in water. The solution was further diluted with water to 0.1 mg/ml for the working solution.

Preparation of quality controls

A fresh stock solution of 1 mg/ml Isepamicin was prepared in water starting from powder. Three quality controls within the range of standard curve were prepared by adding appropriate volumes of isepamicin stock solution to plasma, urine and dialysate. The quality controls were aliquoted into sample size and stored in polypropylene tubes at −20°C.

Sample preparation

Plasma. The appropriate isepamicin stock solutions were used to prepare the standard curve at the following concentrations: 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μg/ml. All standards were diluted to a 1-ml volume with pooled human plasma. Aliquots of quality controls (1 ml) or patient samples (1 ml) were added to the appropriately labelled tubes. From this point on, all samples were treated the same. The internal standard (100 μl of 0.1 mg/ml dibekacin) was added to each sample, with the exception of the plasma blank, and vortex-mixed for 15 s. Samples were diluted with 1 ml of buffer A, vortex-mixed for 15 s and centrifuged for 7 min at 3100 *g* and 4°C. Prior to sample extraction, the CN cartridges were rinsed with 2-ml aliquots of the following solvents: methanol, water and buffer A. Plasma samples were then added to the cartridges. Buffer A (0.5 ml) was added as the first wash solvent. This was eluted to frit level, and 0.25 ml of the mobile phase was added as the second wash solvent. The columns were eluted to dryness. A second 0.25-ml portion of the mobile phase was added to the cartridges to elute the remaining fraction of isepamicin and dibekacin. The cartridges were then washed thoroughly for reuse with 2 ml each of buffer A, water and methanol.

Urine and dialysate. Isepamicin standard curves in urine and dialysate were prepared in the same way as for plasma. Standard curve samples were diluted to 1 ml with the appropriate volume of 1:100 diluted urine or dialysate solution. Quality controls and patient samples were diluted 1:100 with water. Di-

bekacin (100 μl of 0.1 mg/ml) was added to each standard, quality control and patient sample, with the exception of the blank. Each diluted sample was mixed well, and 100 μl were injected.

RESULTS

Chromatography

Chromatograms of plasma samples with and without isepamicin are shown in Fig. 1. The retention times of isepamicin and dibekacin were 6.7 and 17 min, respectively. Chromatograms of drug-free and spiked urine and dialysate samples are shown in Fig. 2.

Assay variability

Plasma. The within-day and between-day variabilities in the measurement of isepamicin in plasma are shown in Table I. The within-day variabilities in high (29 μg/ml), medium (11 μg/ml) and low (2.2 μg/ml) quality control plasma samples were 2.5, 1.8 and 0.5%, respectively. The between-day variability of the assay was determined by comparing the measured concentration of isepamicin in quality control plasma samples run daily on four consecutive

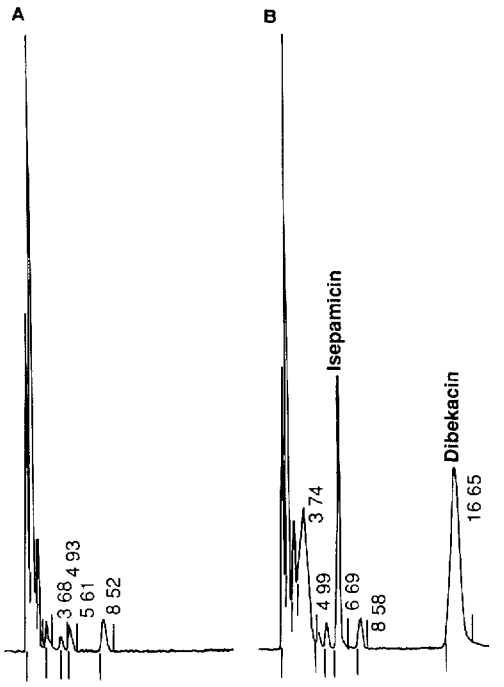

Fig. 1 Chromatograms of (A) blank plasma and (B) plasma spiked with 10 μg/ml isepamicin and 10 μg dibekacin.

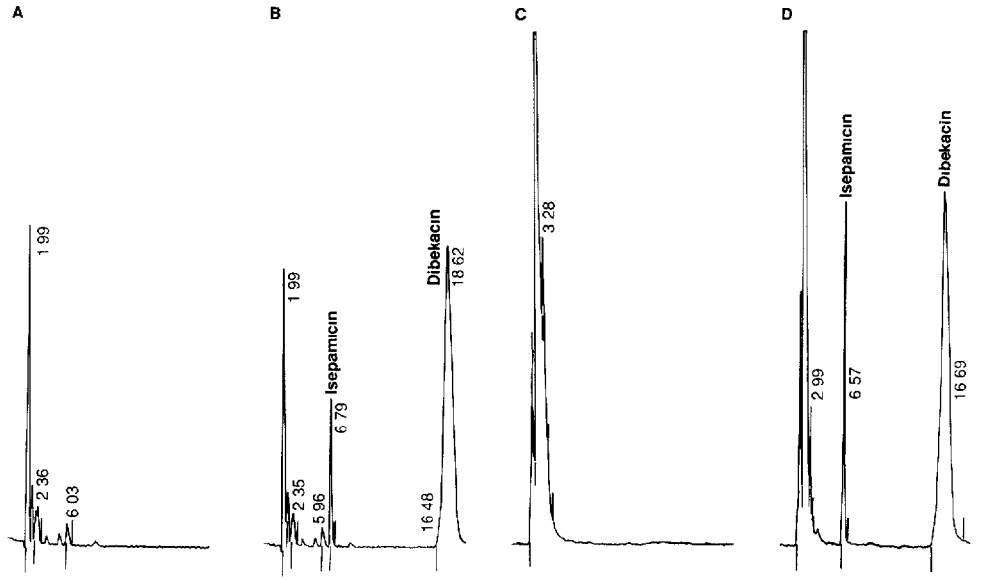

Fig. 2. Chromatogram of (A) blank urine diluted 1 100, (B) patient urine diluted 1 100 and spiked with 2.5 μg/ml isepamicin and 10 μg dibekacin, (C) blank dialysate diluted 1 100 and (D) patient dialysate diluted 1·100 and spiked with 1 μg/ml isepamicin and 10 μg dibekacin.

TABLE I

WITHIN- AND BETWEEN-DAY VARIABILITY IN MEASURED ISEPAMICIN CONCENTRATION IN PLASMA, URINE AND DIALYSATE

Sample	Added (μg/ml)	Measured (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Within-day (n=3)			
Plasma	2 2	2.2 ± 0 0	0 5
	11	9.5 ± 0 2	1.8
	29	25.0 ± 0 6	2.5
Urine	50	59.0 ± 2 1	3.4
	100	114.1 ± 1.2	1.0
	500	497.0 ± 32.2	6.5
Dialysate	50	50.3 ± 2.4	4.7
	100	112.4 ± 7.7	6.8
	500	520.4 ± 30.8	5.9
Between-day (n=4)			
Plasma	2.2	2.2 ± 0.1	3.1
	11	9.7 ± 0.5	5.5
	29	25.9 ± 0.7	2.6
Urine	50	55.0 ± 4.2	7.5
	100	110 1 ± 5.9	5.3
	500	508.0 ± 14.5	2.9
Dialysate	50	52.5 ± 3.1	5.9
	100	109.4 ± 4.7	4.3
	500	516.5 ± 14 1	2 7

TABLE II

MEASURED CONCENTRATIONS OF ISEPAMICIN IN PLASMA SAMPLES CONTAINING THE DRUG WHICH WERE PREPARED BY AN OUTSIDE LABORATORY

Added concentration (μg/ml)	Measured concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	n
45	45.44 ± 3.73	8.2	8
30	31 02 ± 2.28	7 4	8
19	19.41 ± 0.98	5.1	8
10	10 46 ± 0 88	8 4	8
5	5.07 ± 0.39	7.7	8
0.98	0.93 ± 0.039	4.2	8
0.65	0.75 ± 0 035	4 7	8
0.38	0.37 ± 0.031	8 4	8
0 24	0.24 ± 0.025	10.1	10
0	0 ± 0	-	10

days. The variabilities of the high, medium and low quality controls were 2.6, 5.5 and 3.1%, respectively. Further evidence of the accuracy and reproducibility of this method at a much wider concentration range is shown in Table II. Samples prepared by an outside laboratory were assayed as unknowns and the results were evaluated.

Urine and dialysate. The within-day and between-day variabilities in the measurement of isepamicin in diluted urine and dialysate samples were determined as for plasma (Table I). The within-day variabilities of quality control urine samples containing 50, 100 and 500 μg/ml isepamicin were 3.4, 1.0 and 6.5%, respectively. The quality control dialysate samples had within-day variabilities of 4.7, 6.8 and 5.9%, respectively, at similar concentrations.

Between-day variabilities for quality controls containing 50, 100 and 500 μg/ml isepamicin were 7.5, 5.3 and 2.9% in urine and 5.9, 4.3 and 2.7% in dialysate, respectively.

Extraction efficiency in plasma

The extraction efficiencies of isepamicin and dibekacin were determined by extracting samples containing 1 and 50 μg/ml in triplicate and comparing them to unextracted samples prepared by dilution with water. The extraction efficiency of isepamicin and dibekacin were 4.9 ± 0.1 and 7.9 ± 0.7%, respectively, and did not vary at the two concentration levels.

Detection limit

Plasma. The detection limit of isepamicin in plasma was determined by extending the standard curve to include 0.05, 0.1, 0.25, 0.5 and 1 μg/ml. Sam-

ples containing concentrations of 0.1 μg/ml isepamicin were measured accurately. Concentrations less than 0.1 μg/ml were chromatographed, but did not provide consistent and reproducible measurements. Therefore, the limit of quantitation of isepamicin in plasma is 100 ng/ml.

Urine and and dialysate. The detection limits of isepamicin in urine and dialysate were determined by extending the standard curve to include the following: 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 μg/ml. Isepamicin was accurately quantitated at 0.05 μg/ml in both urine and dialysate. The quantitation limit is 50 ng/ml.

Linearity

The standard curve of isepamicin was linear over the range 0–100 μg/ml in plasma, urine and dialysate. Initially, a non-linear response of isepamicin concentrations greater than 20 μg/ml was observed. This appeared to be due to the saturation of the photomultiplier tube signal and it was solved by decreasing the photomultiplier voltage by 10%.

Cartridge reusability

Plasma samples, ranging from 0 to 25 μg/ml were prepared twice daily and extracted on the same cartridges for a total of three days. The cartridges were reused indiscriminately among the samples, and the peak areas from each extraction were compared. Significant decrease in the peak areas of both isepamicin and dibekacin were observed after reusing the cartridges three times. However, the peak-area ratios of isepamicin to dibekacin did not change. Thus, the cartridges may be reused for up to three times.

Analysis of other aminoglycosides

The extraction procedure utilized in this report for the analysis of isepamicin may be applicable to other aminoglycosides. Solutions of gentamicin, kanamycin, netilmicin and tobramycin (1 mg/ml) were prepared in water. The solutions were used to prepare 100 μg/ml samples of each compound in plasma. The plasma samples were extracted by the same extraction procedures for isepamicin. The same mobile phase was also used.

Kanamycin and tobramycin retention times were 6 and 13 min, respectively. They appear to extract with the same or better efficiency as isepamicin. Fig. 3 shows chromatograms of isepamicin, kanamycin and tobramycin in plasma.

Both gentamicin and netilmicin were retained for a long time on the separation column using the same mobile phase for isepamicin. The peak retention times for the gentamicin components were 30, 48, 50 and 67 min, while netilmicin eluted at 58 min.

A change in the mobile phase from 10 to 15% methanol caused a decrease in the elution time of the gentamicin components to 9, 11, 12 and 14 min. By changing the mobile phase ion-pairing reagent to pentanesulfonate, the reten-

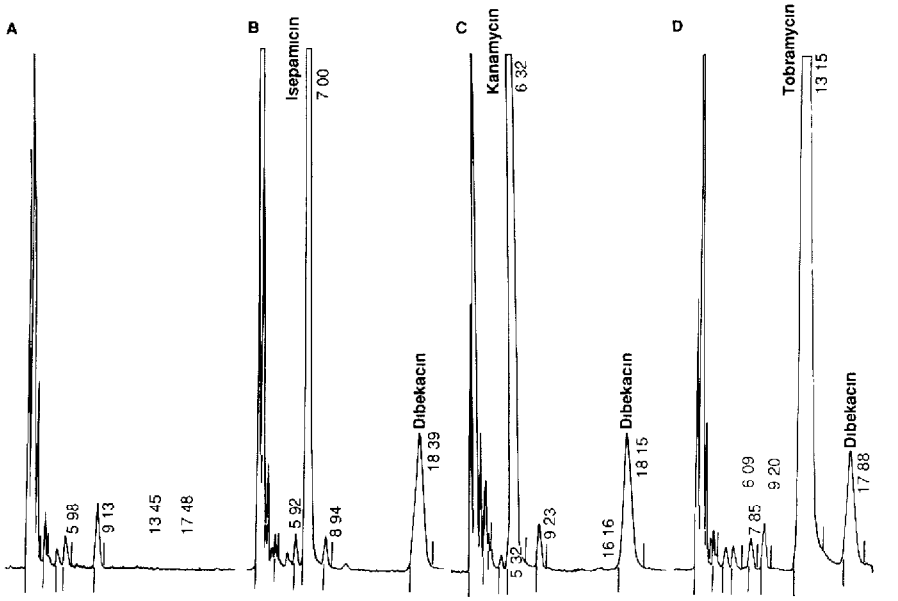

Fig. 3. Chromatograms of (A) blank plasma, (B) plasma spiked with 100 μg/ml isepamicin and 10 μg dibekacin, (C) plasma spiked with 1000 μg/ml kanamycin and 10 μg dibekacin and (D) plasma spiked with 100 μg/ml tobramycin and 10 μg dibekacin

tion times for gentamicin were 11, 17, 17.5 and 22 min, while netilmicin eluted at 16 min.

DISCUSSION

Other types of solid-phase sorbents have been utilized previously for the separation of other aminoglycosides [4–6]. Marples and Oates [5] utilized Amberlite resin with derivatization performed directly on the cartridge and elution of the derivatized products. Anhalt and Brown [6] developed an extraction procedure on CM-Sephadex 25 with a sensitivity limit of 1 μg/ml. However, in the present assay we used a commonly available solid-phase cyano cartridge for extraction. The method is fast compared to the combined use of an extraction step prior to further separation with column switching.

The extraction efficiencies of isepamicin and dibekacin in the present studies were low. Large portions are eluted off in the washes prior to the final step. This is necessary to remove plasma interferences that have similar affinities for the sorbent. While the extraction efficiency of isepamicin is only 5%, the assay quantitation limit is low (100 ng/ml). Other types of solid-phase materials were examined. Carboxylic acid sorbents were found to produce chroma-

tograms similar to those using the CN cartridges. Isepamicin and dibekacin were also retained on C_{18} cartridges, but plasma interferences increased.

Although the CN cartridges are costly, they may be reused for three extractions. Furthermore, the method reduces the time required for performing the extraction and eliminates the need for extra equipment, such as column switching. The present assay offers a new option for the solid-phase extraction of aminoglycosides. Extraction can be performed in 15 min and the sample chromatographed in 18–20 min. Detection limits of 100 ng/ml isepamicin in plasma and 50 ng/ml in urine and dialysate are achieved. Finally, the assay may be used for other aminoglycosides, with only minor changes necessary to optimize the extraction and chromatography.

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