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Note

High-performance liquid chromatographic analysis of indapamide (RHC 2555) in urine, plasma and blood

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Indapamide, RHC 2555 [4-chloro-N-(2-methyl-1-indolinyl)-3-sulfamoyl benzamide], is an effective antihypertensive agent at single daily doses of 2.5 mg in patients with mild to moderate hypertension. A previously described fluorescence assay has been used to measure the drug's concentration in urine, plasma, and whole blood [1, 2]. A procedure for whole blood was necessary since indapamide is taken up by the red cells in vitro within 5 min of incubation at a ratio to plasma of approximately 9:1 [3]. The fluorescence procedure was shown to be linear and sufficiently sensitive to measure indapamide concentrations after therapeutic doses. However, this technique had the disadvantages of using an external rather than an internal standard and of not being readily installed in different laboratories.

Due to the present popularity of high-performance liquid chromatography (HPLC) equipment and procedures, an HPLC procedure for indapamide in biological fluids was developed. This procedure has now been successfully used at several laboratories to measure the concentrations of indapamide after therapeutic doses.

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EXPERIMENTAL

Apparatus

A Spectra-Physics Model 8000B liquid chromatograph was used in the assay of plasma samples of indapamide, while an Altex Model 110A liquid chromatograph was used in the assay of blood and urine samples. Both chromatographs were equipped with a Schoeffel Model 770 variable-wavelength UV detector. Reversed-phase columns housed in a temperature-controlled oven were used. For plasma samples, a Zorbax® ODS column, 25 cm × 4.6 mm, 5 μm particle size, from Dupont (Wilmington, DE, U.S.A.) was used, and for blood and urine samples, a LiChrosorb® C-18 column, 25 cm × 3.2 mm, 10 μm particle size, from Altex Scientific (Berkeley, CA, U.S.A.) was used. The two different systems were used since the work was performed in two different laboratories.

Reagents

Indapamide (RHC 2555) was supplied by Revlon Health Care Group (Tuckahoe, NY, U.S.A.) and the internal standard, sulfanilanilide (reagent grade) was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Acetonitrile was HPLC grade, and the water was deionized and distilled. All other reagents were analytical grade. The anhydrous diethyl ether used was extracted with 0.1 *N* sodium hydroxide daily prior to use.

Chromatography

The mobile phase for blood and urine samples consisted of acetonitrile—0.1 *M* sodium acetate buffer, pH 3.6 (35:65, v/v), with a flow-rate of 1.5 ml/min. For plasma samples, the mobile phase was acetonitrile—0.1 *M* sodium acetate buffer, pH 3.6 (43:57, v/v), with a flow-rate of 1.0 ml/min. The oven temperature was set at 54°C for all samples and the wavelength of detection was 241 nm.

Preparation of standards

Indapamide standards, ranging from 50—400 ng/ml, were prepared by adding appropriate aliquots of a 20 μg/ml solution of indapamide in acetonitrile to 100-ml volumetric flasks. Each flask was filled to volume with oxalated blood and 3-ml aliquots were stored at −20°C until needed. Urine and plasma standards were prepared similarly.

Extraction

Blood standards and samples (in their original tubes) were thawed and homogenized (Tissuemizer® Model SLT with 100 NE probe from Tekmar, Cincinnati, OH, U.S.A.).

One milliliter of each homogenized blood sample was pipetted into separate glass culture tubes (16 × 100 mm) with Teflon®-lined screw caps and 50 μl of the internal standard (10 μg/ml sulfanilanilide in acetonitrile) were added to each tube. Four milliliters of diethyl ether were added to each tube, which was then vortexed vigorously for 2 min. The phases were separated by centrifuga-

tion at 4°C and the ether layers were transferred to fresh tubes. Four milliliters of diethyl ether were again added to the aqueous layer, which was then vortexed for 2 min and centrifuged as described above. To the combined ether extracts, 0.5 ml of 0.01 *N* sodium hydroxide was added. The tubes were vortexed and centrifuged, and the ether layer was discarded. The aqueous layer was neutralized by the addition of 0.5 ml of 0.01 *N* hydrochloric acid and 0.25 ml of 0.05 *M* sodium phosphate (pH 7.4), and then extracted with 4 ml of diethyl ether, as described above. The ether extracts were transferred to clean tubes, evaporated to dryness, and stored at 4°C.

The extraction procedure for urine samples (1 ml) was similar to that used for blood, except that 0.5 ml of 0.05 *N* sodium hydroxide was added to the combined ether layers, and subsequently 0.5 ml of 0.05 *N* hydrochloric acid and 0.25 ml of 0.05 *M* sodium phosphate (pH 7.4) were added.

For plasma, 2 ml of the samples were pipetted into tubes that contained 100 μl of the 10 μg/ml sulfanilanilide solution. The tubes were extracted with 8.0 ml of anhydrous diethyl ether. The ether extracts were transferred to fresh screw-cap tubes and back-extracted into base by adding 1.0 ml of 0.1 *N* sodium hydroxide. The aqueous solution was neutralized by the addition of 1.0 ml of 0.1 *N* hydrochloric acid and 0.5 ml of 0.05 *M* sodium phosphate (pH 7.4), and then extracted as above, with 8.0 ml of anhydrous diethyl ether. The ether extracts were evaporated to dryness, and stored at 4°C.

For all biological fluids, the residue in each tube, after evaporation of the ether, was reconstituted in 200 μl of the mobile phase and a 50-μl aliquot was injected onto the column. Throughout the extraction procedures, the tubes were kept in crushed ice except when being processed.

Calculation of results

The heights of the indapamide and sulfanilanilide peaks were measured, and the peak height ratio of indapamide/sulfanilanilide was calculated. Calibration curves were constructed by plotting the peak height ratio versus the indapamide concentration for the standards and determining the linear regression line. Sample indapamide concentrations were calculated from their peak height ratio using the calibration curve.

RESULTS AND DISCUSSION

Chromatographic properties

Chromatograms of extracted blanks and standards in blood, urine, and plasma are depicted in Figs. 1, 2, and 3, respectively. Chromatograms of extracted human blood samples before and after the administration of indapamide are presented in Fig. 4. For both blood and urine samples, the retention times of sulfanilanilide and indapamide were 2.5 and 3.1 min, respectively. Using the slightly different chromatographic system, plasma samples gave retention times of 5.3 and 6.3 min for sulfanilanilide and indapamide, respectively. In each system the retention times of indapamide and sulfanilanilide for the extracted samples were identical to those obtained from solutions of the pure compounds injected directly. There were no interfering peaks from the extracted biological fluid with retention times the same as indapamide or the internal standard.

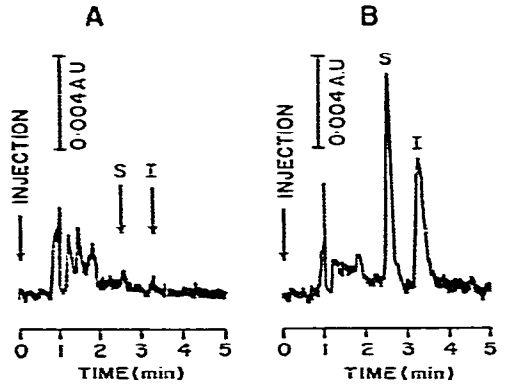

Fig. 1. (A) Chromatogram of an extract of blank blood (S and I indicate expected retention times of sulfanilanilide and indapamide). (B) Chromatogram of an extract of blood containing 500 ng/ml sulfanilanilide (S) and 200 ng/ml indapamide (I). Chromatographic conditions: LiChrosorb C_{18} column, 10 μm particle size, 25 cm × 3.2 mm with a mobile phase of acetonitrile—acetate buffer (35:65) at a flow-rate of 1.5 ml/min.

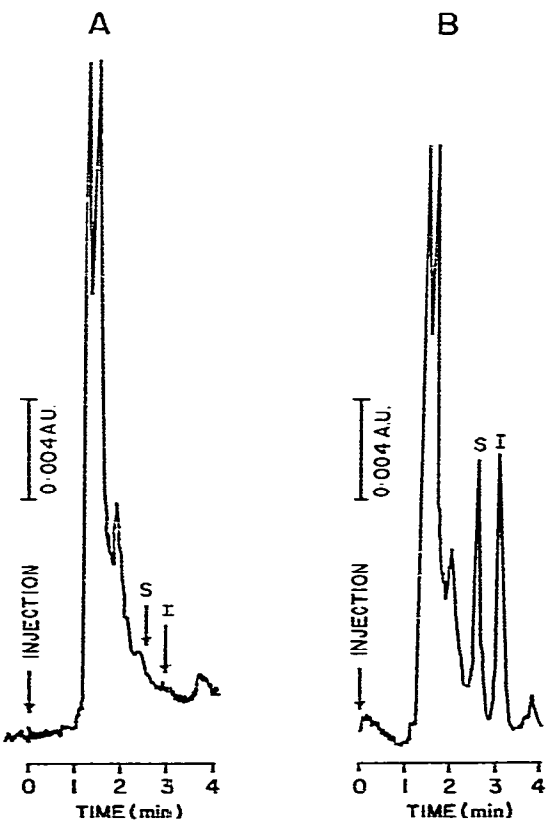

Fig. 2. (A) Chromatogram of an extract of blank urine (S and I indicate expected retention times of sulfanilanilide and indapamide). (B) Chromatogram of an extract of urine containing 500 ng/ml sulfanilanilide (S) and 200 ng/ml indapamide (I). Chromatographic conditions were as described in Fig. 1.

Extraction efficiency

By comparing the peak heights of standards extracted from the biological matrix with those of standards dissolved in mobile phase and chromatographed directly, the recoveries of indapamide and sulfanilanilide were determined. The recovery of indapamide from blood and urine, over the concentration range of 50—400 ng/ml, averaged 72 and 87%, respectively. The recovery of indapamide from plasma, over the concentration range of 25—200 ng/ml, averaged 96%. Recovery of indapamide was independent of concentration. Sulfanilanilide, at a concentration of 500 ng/ml, had extraction efficiencies of 79% in blood, 55% in urine, and 76% in plasma.

Suitability of internal standard

Sulfanilanilide was suitable for use as the internal standard for assay of indapamide, which is also a sulfonamide compound. It gave UV absorbance at

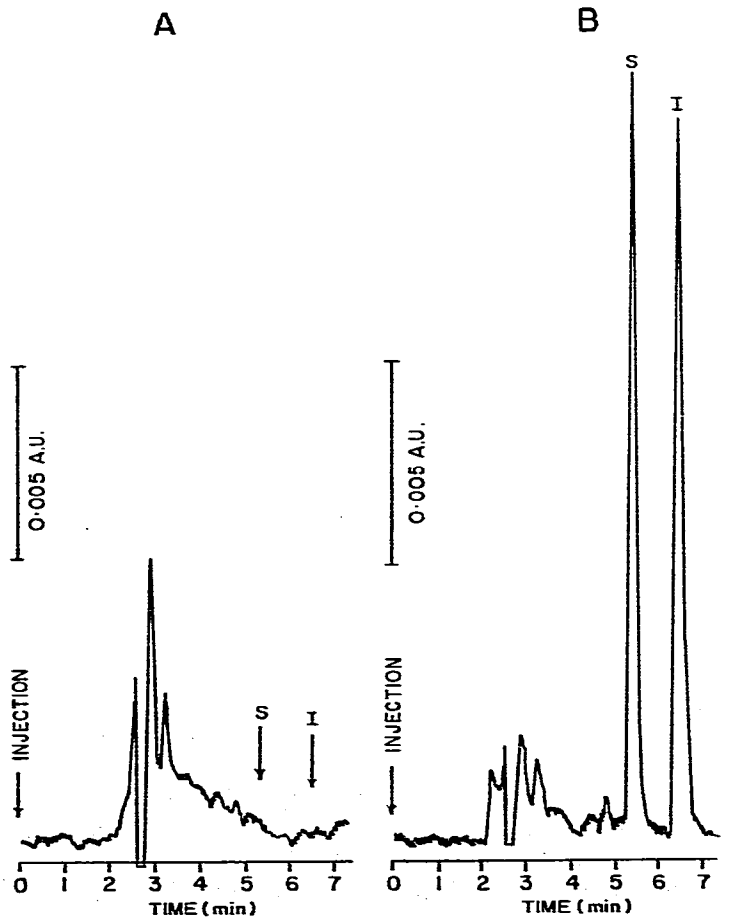

Fig. 3. (A) Chromatogram of an extract of blank plasma (S and I indicate expected retention times of sulfanilanilide and indapamide). (B) Chromatogram of an extract of plasma containing 500 ng/ml sulfanilanilide (S) and 200 ng/ml indapamide (I). Chromatographic conditions: Zorbax ODS column, 5 μm particle size, 25 cm × 4.6 mm with a mobile phase of acetonitrile—acetate buffer (43:57) at a flow-rate of 1.0 ml/min.

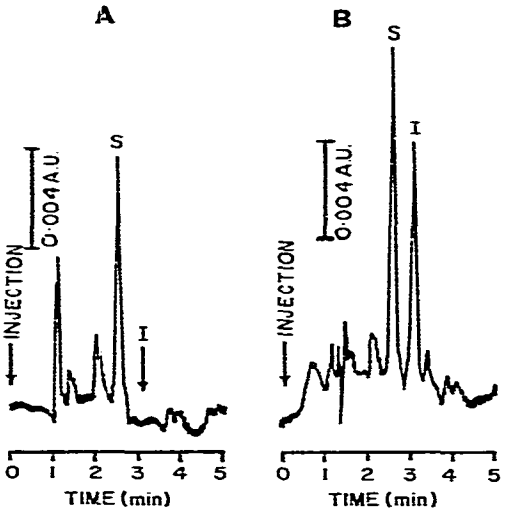

Fig. 4. Chromatograms of extracts of human blood samples before (A) and after (B) administration of indapamide. S indicates location of sulfanilanilide (500 ng/ml) internal standard. I indicates location of indapamide, which was not present in A and was determined to be 198 ng/ml in B. Chromatographic conditions were as described in Fig. 1.

the wavelength of 241 nm used for detection of indapamide. Also, the extraction efficiency of sulfanilanilide was reasonably similar to that of indapamide for blood and plasma; less so for urine. These factors made the use of sulfanilanilide favorable as an internal standard in this assay.

Precision and reproducibility

Standards, of various concentrations, were assayed in at least duplicate on either three of four different occasions to determine the precision and reproducibility of the assays. The composite relative standard deviation of peak height ratios for blood was 13.9% at 50 ng/ml (n = 8) and 8.1% at 400 ng/ml (n = 8); for urine the values were 8.8% at 50 ng/ml (n = 6) and 4.0% at 400 ng/ml (n = 6); for plasma the values were 12.8% at 25 ng/ml (n = 4) and 4.4% at 200 ng/ml (n = 4).

Linearity and sensitivity

Calibration curves obtained by plotting the ratio of the peak height of indapamide to that of sulfanilanilide versus the concentration of indapamide were linear over the concentration ranges studied (25—200 ng/ml for plasma, 50—400 ng/ml for blood and urine). For all three biological fluids, the correlation coefficient for the composite calibration curves, obtained as described above, were equal to or better than 0.986. The limits of sensitivity of the assays were considered to be 50 ng/ml for blood and urine, and 25 ng/ml for plasma.

Selectivity

Eleven blood samples from human clinical studies were assayed by the HPLC procedure and the previously described fluorescence procedure [2]. There were no significant differences between the indapamide concentrations determined

by the two procedures and the correlation coefficient was 0.97. No metabolites of indapamide have been shown to interfere with the HPLC procedure.

Human samples

The described HPLC procedures have been successfully used in different laboratories for the assay of human clinical samples from subjects who received various doses of indapamide. Fig. 5 shows typical blood concentrations of indapamide, as determined by the HPLC procedure, in a human subject who received a 5.0-mg dose. The assay procedures for blood, urine and plasma indapamide samples have demonstrated the linearity, precision and sensitivity needed for pharmacokinetic studies of this new antihypertensive agent.

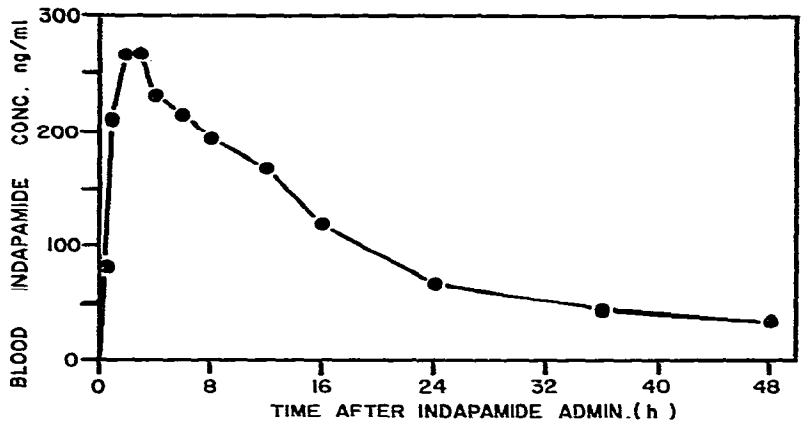

Fig. 5. Blood concentrations of indapamide in a human subject who received a 5.0-mg oral dose. The concentrations were determined by the described HPLC procedure.

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