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DETERMINATION OF A NEW ORALLY ACTIVE CEPHALOSPORIN IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN SWITCHING

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SUMMARY

A sensitive method for the determination of a new cephalosporin in human serum and urine is described. The sensitivity of the procedure is derived from a high-performance liquid chromatographic separation which utilizes the different selectivities of two columns. Partial separation of the agent from deproteinized serum or diluted urine is achieved by an anion-exchange column. To concentrate the large volume of the eluent fraction containing the compound from the anion-exchange column, a reversed-phase short column is placed between the anion-exchange column and a reversed-phase analytical column. The separation is completed by switching the eluent fraction containing the compound from the second column to the analytical column. The compound is detected by ultraviolet absorption at 295 nm. Quantitation is possible down to 0.05 μg/ml using 300 μl of serum and down to 0.5 μg/ml using 50 μl of urine. The coefficients of variation of the method are 6.8% and 0.6% in serum when spiked at the 0.05 μg/ml and 1.0 μg/ml level, respectively. One assay can be completed in 16 min. Serum levels and urinary excretion data obtained with this method are given for three healthy volunteers who had received a 100-mg oral dose of the compound.

INTRODUCTION

(6*R*,7*R*)-7-[(*Z*)-2-(2-Amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo-(4,2,0)-oct-2-ene-2-carboxylic acid* (hereinafter abbreviated as I) is a new orally active semisynthetic cephalosporin (Fig. 1). The compound differs in structure from commercially available materials which are cephalexin-type analogues. The antibacterial activity of I

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against the most commonly isolated gram-negative bacteria is higher than that of other orally active β-lactam antibiotics such as cephalexin, cefaclor and amoxicillin [1].

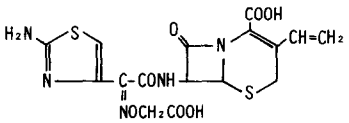

Fig. 1. Chemical structure of compound I.

In recent years, many high-performance liquid chromatographic (HPLC) methods have been reported for the determination of different cephalosporins in biological fluids. The reversed-phase mode using ultraviolet (UV) detection is most commonly used, with precipitation of serum proteins prior to injection [2—10]. These procedures were tried for the determination of I in serum, but the sensitivity was limited to 0.5 μg/ml which was insufficient to study the pharmacokinetics of the compound after oral administration of the usual clinical dose in man. Compound I, a highly polar and water-soluble compound, cannot be extracted from biological fluids by techniques such as conventional two-phase liquid—liquid extraction which works with cefazolin, cephalothin, cefamandole, cefoxitin, cefuroxime, cefotaxime and cefoperazone [11], or by ion-pair extraction with quaternary ammonium salts which works with cephalothin [12]. In addition, I lacks a primary amino function for reacting with fluorogenic reagents such as *o*-phthaldialdehyde and fluorescamine [13, 14].

This paper describes a sensitive method for the determination of I in human serum and urine. The method is based on HPLC column switching using an anion-exchange column and a reversed-phase column.

EXPERIMENTAL

Reagents and materials

Compound I was prepared by Fujisawa Pharmaceutical (Osaka, Japan). Methanol and acetonitrile of UV grade were used. Water used for all solutions and mobile phases was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). All other solvents and reagents were of analytical-reagent grade.

Standard solutions of I were prepared by dissolving the agent in 1/15 *M* phosphate buffer (pH 7.0) and diluting to appropriate concentrations.

Apparatus

All analyses were performed using a liquid chromatograph equipped with two solvent delivery pumps (Model 6000A; Waters Assoc., Milford, MA, U.S.A.), an automatic liquid sampler (WISP710B; Waters Assoc.), a variable wavelength UV detector (at 295 nm, UVIDEC 100-III; Japan Spectroscopic, Tokyo, Japan), and an integrator (Data Module; Waters Assoc.) or a 10-mV recorder. The three columns used were an anion-exchange column 1 cm × 4 mm I.D. (TSK-IEX540 DEAE, 5 μm; Toyo Soda, Tokyo, Japan) and two

reversed-phase columns 1 cm × 4 mm I.D. and 15 cm × 4.6 mm I.D. (TSK-LS410 ODS, 5 μm; Toyo Soda). Three-port and six-port switching valves were placed in line, and operated by a digital programmer (Toyo Soda). Fig. 2 shows the arrangement of the apparatus.

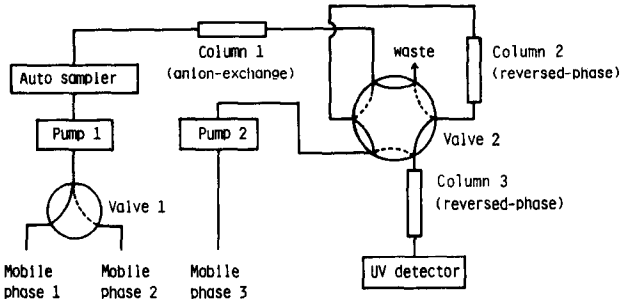

Fig. 2. Flow diagram of automated column switching for analysing I in human serum and urine. Valves 1 and 2 are three-port and six-port switching valves, respectively, and are operated by a digital programmer which is not shown in order to simplify the drawing. The solid and dotted lines in the valves show the off and on modes, respectively.

Mobile phase

Mobile phase 1 consisted of 0.03 *M* ammonium dihydrogen phosphate—phosphoric acid solution (pH 3.5). The solution was prepared from 0.03 *M* ammonium dihydrogen phosphate adjusted to pH 3.5 with dilute phosphoric acid. Mobile phase 2 consisted of 0.5 *M* sodium chloride. Mobile phase 3 consisted of 73% 0.03 *M* ammonium dihydrogen phosphate—phosphoric acid solution (pH 2.5) in methanol for analysis of serum. Mobile phase 3 for analysis of urine was prepared by mixing 150 ml of acetonitrile, 850 ml of water, and 2 ml of 1.8 *M* sulphuric acid. The final composition was acetonitrile—water—1.8 *M* sulphuric acid (15:85:0.2). The mobile phase was deaerated under vacuum before use. The flow-rate of both mobile phase 1 and mobile phase 2 was 2.0 ml/min and that of mobile phase 3 was 1.0 ml/min.

Sample preparation

To a 1.5-ml polypropylene tube (Eppendorf, Hamburg, F.R.G.) containing 300 μl of serum were added 30 μl of 1/15 *M* phosphate buffer (pH 7.0) and 600 μl of ethanol. The mixture was shaken in a vortex mixer, allowed to stand for 5 min, and centrifuged with an Eppendorf centrifuge (Model 5412; Eppendorf) for 1 min. An 80-μl aliquot of the supernatant was injected into the liquid chromatograph.

To a 1.5-ml polypropylene tube containing 1 ml of urine diluted twenty-fold or more, were added 100 μl of 1/15 *M* phosphate buffer (pH 7.0). The mixture was shaken in the vortex mixer and centrifuged with the Eppendorf centrifuge for 1 min; 40 μl of the supernatant were injected into the liquid chromatograph.

Timing of column switching

The timing of automated column switching for determination of I in the serum and urine is shown in Fig. 3. At 0 min, valves 1 and 2 were

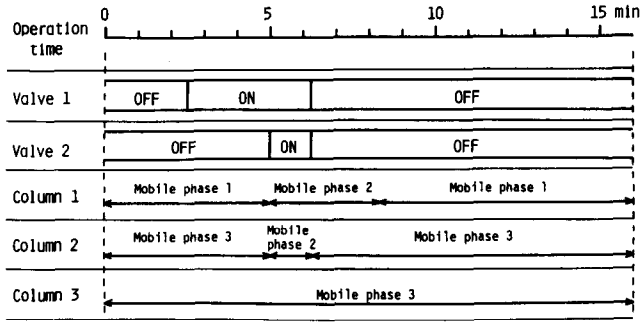

Fig. 3. Timing of automated column switching for analysing I in human serum and urine. Time lag between switching of valve 1 and switching of mobile phase in column 1 arises from a dead space between valve 1 and pump 1 (see Fig. 2.).

positioned (off) so that mobile phase 1 flowed through the anion-exchange column (column 1) to waste and mobile phase 3 flowed through the reversed-phase short column (column 2) and reversed-phase analytical column (column 3) to the UV detector. The sample was injected onto column 1 on which partial separation of I and other substances in the biological fluids was achieved. Valve 1 was switched on at 2.5 min after injection, but column 1 was still eluted with the mobile phase 1 remaining in the dead space between valve 1 and pump 1. At 5 min after injection when I began to desorb from column 1, valve 2 was switched on so that the effluent from column 1 flowed through column 2 to waste. Mobile phase 3 then flowed through column 3 to the UV detector. I was concentrated at the head of column 2 by aqueous mobile phase 2. At 6.25 min after injection, valves 1 and 2 were turned back to the original position (off), and mobile phase 3 then flowed through columns 2 and 3 where further separation of I and other substances in the biological fluid was achieved. Column 1 was then eluted with mobile phase 2 to wash out the substances remaining in column 1. At 8.25 min after injection, mobile phase 1 flowed through column 1 to give equilibration for the next analysis. The injection interval for this procedure was 16 min.

Quantitation

The procedure was standardized by analysing blank serum or urine samples to which had been added 30 or 100 μl of I standard solution instead of 30 or 100 μl of 1/15 *M* phosphate buffer (pH 7.0) as in the sample preparation. The peak height of I was used to establish the calibration graph for the serum and urine samples. The calibration graph was fitted to a $Y = aX + b$ equation by the least-squares method, and the concentrations in the unknown samples were calculated using the calibration graph.

Clinical study

A clinical study was performed on three healthy volunteers given an oral dose of a 100-mg capsule of I. Serum samples were obtained from blood collected by venipuncture at designated intervals and stored at −20°C until analysed. The total urine output was collected at intervals of 0—2, 2—4, 4—6,

6—8, 10—12, 12—23, and 23—24 h. The urine volumes were measured, and aliquots were kept at —20° C until analysed.

RESULTS

Separation

Typical chromatograms obtained from the human serum and urine samples are shown in Figs. 4 and 5. As shown in Figs. 4A and 5A, the background peaks of blank human serum and urine were few and almost completely separated from those of I. Figs. 4C and 5C show typical chromatograms of the serum and urine samples from a healthy volunteer after an oral dose of 100 mg of I. In these chromatograms there were no interferences at the retention times of I.

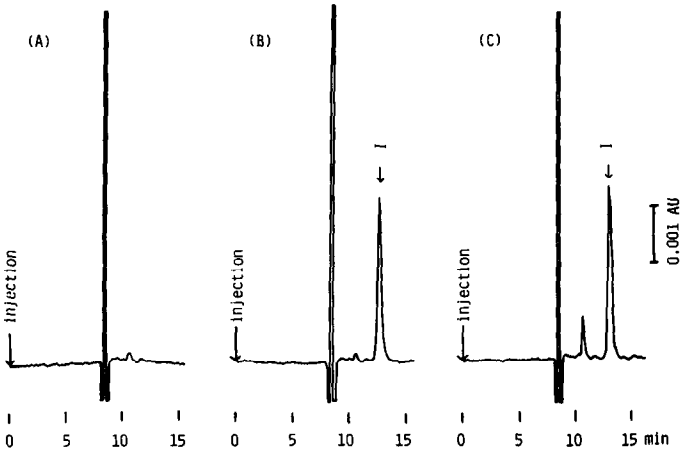

Fig. 4. Chromatograms of (A) blank human serum, (B) human serum containing 1 μg/ml of I, and (C) serum collected from a healthy volunteer after an oral dose of a 100-mg capsule of I (calculated concentration of I was 1.147 μg/ml).

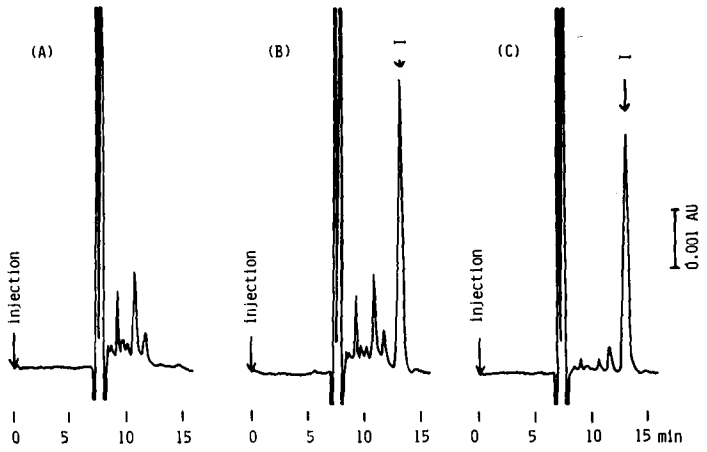

Fig. 5. Human urine (twenty-fold dilution) chromatograms: (A) blank urine, (B) urine containing 25 μg/ml of I, and (C) urine collected from healthy volunteer after an oral dose of a 100-mg capsule of I (calculated concentration of I was 23.3 μg/ml).

Recovery

Sample recovery of I from spiked solutions prepared with biological fluids was compared with that of 1/15 *M* phosphate buffer (pH 7.0). The values obtained from five replicate analyses of spiked samples were 91.8 ± 0.6% (mean ± S.D.) for serum (1 μg/ml of I) and 101.4 ± 0.4% for urine (25 μg/ml of I).

Calibration graph

Typical calibration graphs for human serum and urine are shown in Table I. All calibration graphs show good linearity in each range. The lower limits of sensitivity were 0.05 μg/ml for serum and 0.5 μg/ml for urine, with a signal-to-noise ratio of 3.

TABLE I

TYPICAL REGRESSION DATA FOR CALIBRATION CURVES IN HUMAN SERUM AND URINE

Sample	Concentration range (μg/ml)	Slope	Intercept	Correlation coefficient
Serum	0.05—10.0	7.0568	0.0364	0.9999
Urine	0.50—250	0.5029	0.0312	0.9999

Reproducibility

Reproducibility was evaluated by performing five replicate analyses of spiked serum and urine samples. The results are given in Table II. The coefficients of variation were 0.6% and 0.4% when 1.0 μg/ml and 25.0 μg/ml were spiked in the serum and urine, respectively. The respective values were 6.8% and 2.3% even when 0.05 μg/ml and 1.0 μg/ml were spiked.The actual concentration of I measured by HPLC ranged from 99% to 101% in the ten samples when spiked at higher concentrations in the serum and urine, and from 92% to 108% in the ten samples even when spiked at lower concentrations. This HPLC method for

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF I IN HUMAN SERUM AND URINE

Parameter	Serum	Serum	Urine	Urine
Actual concentration (μg/ml)	0.050	1.000	1.00	25.0
Number of analyses	5	5	5	5
Mean analysed concentration (μg/ml)	0.050	1.002	1.04	25.0
Percentage of actual concentration	100	100.2	104	100
Range (μg/ml)	0.046—0.054	0.994—1.009	1.01—1.06	24.9—25.1
S.D. (μg/ml)	0.003	0.006	0.02	0.1
Coefficient of variation (%)	6.8	0.6	2.3	0.4

the determination of I in human serum and urine thus provides good accuracy and precision even around the lower limit of sensitivity.

Serum levels and urinary excretion of I in man

Serum levels of I after an oral dose of a 100-mg capsule to healthy volunteers are shown in Fig. 6. The drug peaked in the serum (1.023—1.364 μg/ml) within 4—5 h after dosing, and thereafter decreased slowly to a mean of 0.264 μg/ml (0.191—0.305 μg/ml) at 12 h; 19.7—30.1% of the dose was excreted as the unchanged drug in the 0—24 h urine.

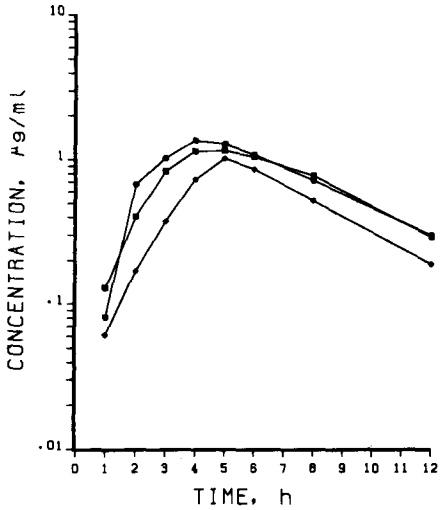

Fig. 6. Serum levels of I in healthy volunteers after an oral dose of a 100-mg capsule of I. (○) Subject A, (□) subject B, (◇) subject C.

DISCUSSION

Early studies on methods for determining I in the serum were carried out by direct injection of deproteinized biological fluids into a reversed-phase HPLC system with UV detection. However, these methods did not separate the background peaks of the biological fluids from that of I. In recent years, HPLC column switching has been found to be a useful technique for the determination of water-soluble materials in biological fluids [15—25]. In our present study, complete separation of the background peaks of biological fluids from that of I (Figs. 4 and 5) was achieved by HPLC column switching using an anion-exchange column and a reversed-phase column. In this system, a reversed-phase short column was placed between the anion-exchange column and the reversed-phase analytical column (Fig. 2) to concentrate the eluent fraction containing I from the anion-exchange column. The use of this short column made it possible to concentrate a large volume (2.5 ml) of the eluent fraction without disturbing the chromatogram on the analytical column (Figs. 4 and 5). The concentration of the large volume of eluent on the short column enhanced the sensitivity and reproducibility of the determination. Only the anion-exchange column has to be changed about every 150 samples. This column can be

easily packed in our laboratory with a small amount of packing material.

The assay was shown to be sufficiently sensitive to quantify I in human serum and urine after oral administration of the clinical dose (Fig. 6). The analysis time of 16 min seems to be acceptable since the method can be used overnight.

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