Journal of Chromatography, 615 (1993) 174–179
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6771

Short Communication

Determination of a new cephalosporin, SCE-2787, in serum and urine by high-performance liquid chromatography

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(First received November 3rd, 1992; revised manuscript received February 1st, 1993)

ABSTRACT

A high-performance liquid chromatographic method for the determination of a new-broad spectrum cephalosporin (I, SCE-2787) has been developed. The analyte was extracted from serum by precipitation of serum proteins with acetonitrile. Acetonitrile was extracted from the protein-free supernatant by dichloromethane. Urine was simply diluted with mobile phase. Separation was performed by ion-pair chromatography on a reversed-phase column (Nucleosil $5C_{18}$; 125 mm × 4.0 mm I.D.; 5 μm average particle size). The guard column was Perisorb RP18 (30 mm × 4.0 mm I.D.; 30–40 μm particle size). The mobile phase was acetonitrile–buffer solution containing 15 m*M* heptanesulphonic acid (pH 3.2) (4.5:95.5, v/v). Detection was performed at 235 nm with a diode-array detector, which also served to record ultraviolet spectra. The assay was sensitive, precise, accurate and fast. Specificity was controlled by on-line recording the ultraviolet spectrum of I and also by enzymic degradation with β-lactamase. No interferences were observed during the analysis of serum and urine of healthy volunteers in pharmacokinetic studies.

INTRODUCTION

SCE-2787 (I) is a new injectable broad-spectrum cephalosporin. Its chemical name is (−)-1-[[(6*R*,7*R*)-7-[(*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-methoxyiminoacetamido]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-imidazole[1,1-2]pyradazinium hydrochloride (Fig. 1). Existing analytical methods for I in bi-

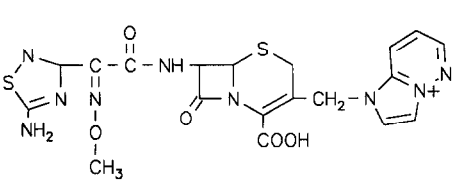

Fig. 1. Structure of I.

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0378-4347/93/$06.00

ological fluids are a microbiological assay and a high-performance liquid chromatographic (HPLC) method [1]. The latter involves column switching.

This paper describes a simple isocratic HPLC method using ion-pair chromatography on a reversed-phase column. The method was successfully applied in studies on human volunteers.

EXPERIMENTAL

Materials

Compound I (lot No. M433-533, purity of the hydrochloride 98.2%, potency 917 mg free base per g) was kindly provided by Takeda (Aachen, Germany). Analytical-grade $NaH_2PO_4 \cdot 2H_2O$, dichloromethane and acetonitrile were purchased from E. Merck (Darmstadt, Germany). Heptanesulphonic acid (PIC B7, low-UV grade) was purchased from Waters (Königstein, Germany). Double-distilled water was used in all experiments. β-Lactamase, type II, from *Bacillus cereus* was purchased from Sigma (Munich, Germany).

Solutions

A 5 m*M* stock solution of sodium phosphate buffer (pH 7.0) was made up from solution A (71.19 g of $Na_2HPO_4 \cdot 2H_2O$ in 1 l of distilled water) and solution B (62.39 g of $NaH_2PO_4 \cdot 2H_2O$ in 1 l of distilled water) as follows: 61.0 ml of solution A were mixed with 39.0 ml of solution B, and *ca.* 0.5 ml of 4 *M* NaOH were added to adjust the pH to 7.0. Distilled water was added to a volume of 800 ml.

To obtain the working solution, one volume of stock solution was diluted with nine volumes of distilled water.

A 5 m*M* stock solution of sodium phosphate buffer (pH 3.2) was made by dissolving 7.79 g of $NaH_2PO_4 \cdot 2\ H_2O$ in 990 ml of distilled water. The pH was adjusted to 3.2 with concentrated phosphoric acid, and distilled water was added to a volume of 1 l. To obtain the working solution, the stock solution was diluted 1:10 with distilled water.

The PIC B7 solution (15 *M* heptanesulphonic acid, pH 3.2) was made by diluting three bottles of heptanesulphonic acid (Waters) with distilled water to 1 l.

To make the mobile phase, 45 ml of acetonitrile and three bottless of heptanesulphonic acid were diluted with distilled water to 1 l. It was filtered through a 0.45-μm filter (type HVLP, Millipore, Eschborn, Germany), and could be recycled for 24 h.

Calibrators and control samples. An aqueous stock standard solution was made by dissolving 108 mg of reference material of I in 100 ml of sodium phosphate buffer (pH 7.0) to give a concentration of 1000 mg/l. It was stored at −80°C.

To make the serum calibrators, the aqueous stock standard solution was diluted with drug-free human serum to the following concentrations: 1, 2, 5, 10, 20, 30, 50, 100 and 200 mg/l.

To make the urine calibrators the aqueous stock standard solution was diluted with sodium phosphate buffer (pH 7.0) to final concentrations of 1, 2.5, 5, 10, 25, 50, 100, 200, 500 and 1000 mg/l.

Blank serum and urine samples from volunteers or drug-free serum were spiked in various concentrations. All calibrators and control materials were stored at −80°C.

Samples and storage

Serum and urine samples were stored at −80°C immediately after collection without addition of any stabilizers.

Instrumentation

The mobile phase delivery system (Model LC 2/2, Perkin-Elmer, Überlingen, Germany) was set at a flow-rate of 1.5 ml/min, yielding a pressure of 18 MPa. The sample tray of the autoinjector (Model ISS-101, Perkin Elmer) was kept at 18°C. The injection volume was 20 μl for serum and 5 μl for urine. A guard column filled with Perisorb RP18 (E. Merck) (30 mm × 4.0 mm I.D., particle size 30–40 μm) protected the main column. Separation was performed on a column of Nucleosil $5C_{18}$ (Macherey & Nagel, Düren, Germany, 125 mm × 4.0 mm I.D., particle size 5 μm) at room temperature (20–22°C). A diode-array spectrometer (Model LC 480, Perkin Elmer)

was used for detection. The absorbance of the eluate was continuously recorded at 235 nm. Spectral scans were performed from 200 to 300 nm. Data were collected by an integrator (Model C-R3A, Shimadzu, Duisburg, Germany). Concentrations were calculated from peak areas and by reference to external calibrators. The retention time of I was *ca.* 5.3 min.

Sample preparation

Serum. To 0.3 ml of serum or calibrator, 0.3 ml of 5 m*M* sodium phosphate buffer (pH 3.2) and 0.8 ml of acetonitrile were added. The mixture was shaken for 2 min in a mechanical agitator and subsequently centrifuged at 13 000 *g* for 5 min. To 1.4 ml of the supernatant, 2.0 ml of dichloromethane were added. After agitation for 1 min the mixture was centrifuged at 2000 *g* for 5 min. A 0.3-ml portion of the supernatant was diluted with 0.6 ml of the mobile phase. The final mixture was transferred to autosampler vessels and injected.

Urine. A 0.1-ml sample of urine was diluted with 0.9 ml of buffered heptanesulphonic acid solution. Urine samples with high concentrations (*e.g.* from the 0–3 h collection period) were prediluted with distilled water.

Stability

Serum and urine samples, and calibrator solutions, were stable at −20°C and −80°C, respectively for at least four months.

Enzymatic degradation with β-lactamase

β-Lactamase was dissolved in Sörensen's 10 m*M* phosphate buffer (pH 7.0) to a concentration of 3700 U/l. One volume of serum or urine was mixed with one volume of enzyme solution and incubated at 25°C for 24 h. In a blank assay the enzyme was omitted. The incubated mixture was deproteinized as described for serum.

Comparative microbiological assay

Serum and urine samples were also analysed by an agar plate diffusion assay [2]. The test organism was *Escherichia coli* NIHJ (IFO 14249). All assays were performed in triplicate.

Statistical methods

Results of the microbiological assay and determination by HPLC were compared by the method of bivariate regression analysis [3] and by the sign test for pair differences.

RESULTS AND DISCUSSION

Compound I is a typical cephalosporin antibiotic for intravenous use with a broad spectrum of antimicrobial activity. (It still does not have an international non-proprietary name.) For pharmacokinetic studies, a rapid and specific analytical procedure was required that could be used with automated equipment. The confidential SCE-2787 investigators' manual described a microbiological assay and an HPLC method, which used a column-switching system and a gradient elution of the mobile phase, and required run times of at least 30 min [1]. The present method uses a simplified, isocratic elution mode and required run times of less than 10 min. It is suitable for continuous operation, *e.g.* overnight.

Method development

Attempts to separate I by reversed-phase chromatography on a C_{18} column with a mobile phase consisting of acetonitrile and aqueous buffer resulted in low k' values and interferences from endogenous substances. Compound I is a rather polar substance with two pK values at −0.2 and +2.2 [4]. Addition of the ion-pair reagent heptanesulphonic acid led to satisfactory separations (Figs. 2 and 3). The absorbance maximum at 235 nm was chosen as the detection wavelength. Serum proteins were precipitated with acetonitrile. In order to lower the detection limit, acetonitrile was extracted with dichloromethane, which concentrated the analyte in the aqueous phase.

Validation of the method

The lower limit of detection was 0.6 mg/l for serum and 3.5 mg/l for urine. The present method was sensitive enough to determine I in serum 12 h after a single intravenous dose of 1.5 g. The plot of concentration *versus* peak area was linear in the following ranges: for serum, 1.0–200 mg/l (*r*

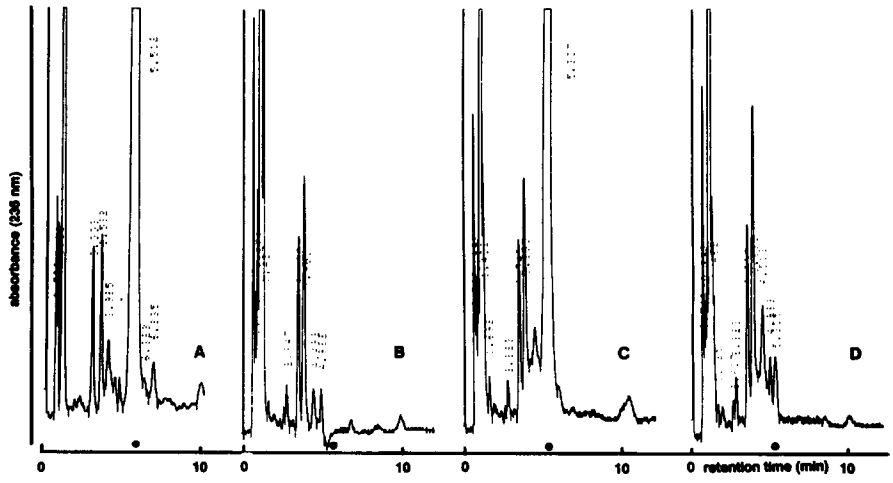

Fig. 2. Serum chromatograms. (A) Serum standard containing 100 mg/l I; (B) blank serum of a volunteer; (C) serum of a volunteer 30 min after intravenous injection of 1.5 g of I, containing 79.9 mg/l; (D) serum 12 h after intravenous injection of 1.5 g of I, containing 1.4 mg/l. ● corresponds to peak of I.

= 0.998); for urine, 5.0–1000 mg/l (r = 0.998).

The precision was assessed by analysis of pooled samples from volunteers. For serum, nine pools with concentrations ranging from 0.7 to 160.0 mg/l were examined to obtain a within-series value. The mean coefficient of variation (C.V.) was 3.2%, with a range of 0.7–7.7%. The between-series C.V. was 10.6 and 3.4% at concentrations of 3.5 and 75.0 mg/l, respectively. For urine, six pools with concentrations from 5.0 to 500.0 mg/l were examined. The mean within-series C.V. was 4.1% with a range of 1.5–10.7%. The between-series C.V. was 5.7, 3.4 and 4.3% at concentrations of 50.0, 150.0 and 300 mg/l, respectively.

The accuracy was assessed by measuring the

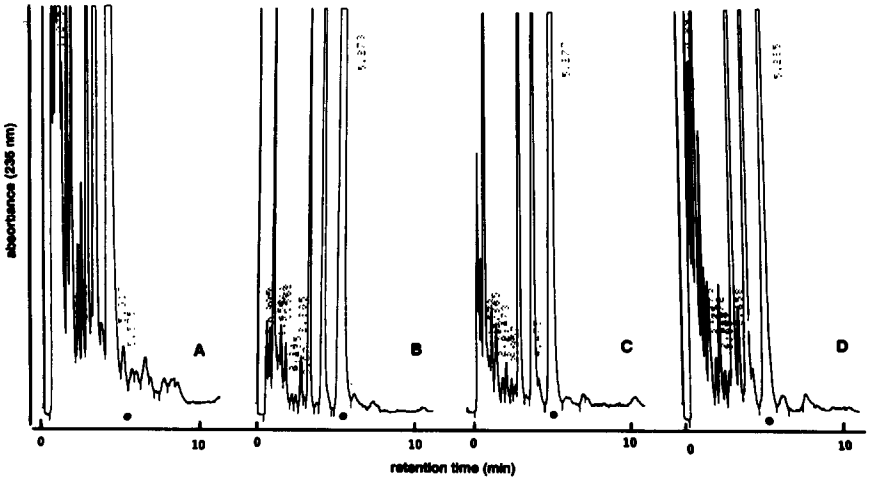

Fig. 3. Urine chromatograms. (A) Blank urine of a volunteer; (B) chromatogram of urine 0–3 h after administration of 1.5 g of I, concentration 661 mg/l; (C) collection period 3–6 h, concentration 329 mg/l; (D) collection period 6–12 h, concentration 220 mg/l.

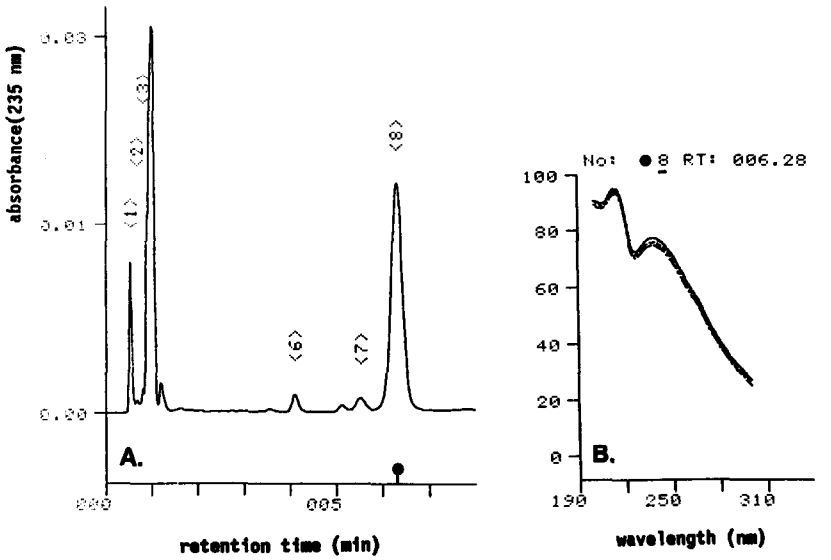

Fig. 4. Identification of I in a volunteer's serum by means of a diode-array spectrophotometer. (A) In the chromatogram, I has a retention time of 6.28 min (peak no. 8). (B) In the normalized spectrum of peak 8, the maxima are at 236 and 213 nm. After replacement of main column the retention time increased.

recovery from spiked blank sera. For serum, ten concentrations were examined from 0.7 to 160.0 mg/l. The overall recovery was 96.7% (range 84.0–103.0%). The recovery from urine was examined at eight concentrations ranging from 5.0 to 500.0 mg/l. The overall recovery was 98.6% (range 82.0–105.0%).

The specificity of the assay was confirmed by complete enzymic hydrolysis of the analyte resulting in complete disappearance of the chromatographic peak [5]. The UV spectrum of I recorded on-line was identical with the spectrum obtained from pure reference material (Fig. 4).

Results obtained by HPLC were compared with those from a microbiological assay by means of linear regression analysis and by the sign test of pair differences (Table I). The slope of both regression lines did not differ significantly from 1.000. The sign test of serum yielded no significant difference. The sign test of urine results showed a slight significant difference. This difference was not considered to be relevant for the performance of the HPLC method.

Chromatograms of samples from healthy volunteers and comparative results from the microbiological assay gave no evidence for an active biotransformation product in serum or urine.

TABLE I

METHOD COMPARISON: HPLC (x) VERSUS MICROBIOLOGICAL ASSAY (y) RESULTS

Parameter	Serum	Urine
Number of pairs	401	104
Slope, b	1.011	0.953
t-test of b	$p > 0.10$	$p > 0.05$
Intercept, a (mg/l)	−0.22	+4.1
Correlation coefficient, r	0.985	0.986
Sign test	$p > 0.05$	$p < 0.01$[a]

[a] Indicates a significant test result. Slope und intercept were calculated by bivariate regression analysis [3].

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