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Short Communication

Automated high-performance liquid chromatographic method for the simultaneous determination of cefotiam and Δ^3-cefotiam in human plasma using column switching

Kenji Yamashita*, Michio Motohashi and Takatsuka Yashiki

Takeda Analytical Research Laboratories, Ltd., Juso-Honmachi, Yodogawa-ku, Osaka 532 (Japan)

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ABSTRACT

An automated high-performance liquid chromatographic method using column switching was established for the simultaneous determination of cefotiam (I) and Δ^3-cefotiam (II) in human plasma after oral administration of cefotiam hexetil dihydrochloride. The method allowed the determination of analytes in plasma by the direct injection of diluted specimen with phosphate buffer. The analytes were enriched onto the C_{18} short pretreatment column by 0.05 *M* phosphate buffer (pH 7.7), while proteins and endogenous hydrophilic substances in plasma were washed off to waste. The enriched analytes were then back-flushed onto the analytical C_{18} column, separated by a mixture of 0.05 *M* phosphate buffer (pH 7.7)–acetonitrile (88:12, v/v) and detected by the ultraviolet absorbance at 254 nm. Recoveries from spiked plasma were quantitative, and the coefficients of variation were below 4%. The lower detection limits in plasma were 10 ng/ml for both I and II. Concentrations of I and II in plasma determined by the present method were in good agreement with those obtained by the conventional deproteinization method.

INTRODUCTION

Cefotiam hexetil dihydrochloride (Fig. 1), an orally active cephalosporin, is a prodrug whose parent drug is cefotiam (I) [1]. Compound I, a second-generation cephalosporin, shows excellent antibacterial activity against Gram-positive and Gram-negative bacteria [2]. Cefotiam hexetil dihydrochloride is absorbed from the gastrointestinal tract after oral administration. The drug is hydrolysed to I in the intestinal membrane during absorption, and only I and a small amount of its Δ^3-isomer (II) are detected in plasma [3].

Several papers [4–6] have reported a method for the determination of I alone in biological fluids using high-performance liquid chromatography (HPLC). No microbiological method can determine the inactive metabolite II. We have previously described an HPLC method for the simultaneous determination of I and II in human plasma and urine, and have applied it to clinical studies of cefotiam hexetil dihydrochloride [7]. The method allows the direct injection of urine with only dilution by 0.1 *M* potassium dihydrogenphosphate solution. However, for plasma samples, although the clean-up procedure is not so laborious, a time-consuming evaporation step is required after deproteinization by acetonitrile. Recently, Kees *et al.* [8] reported an HPLC method for determining I and II in human plasma.

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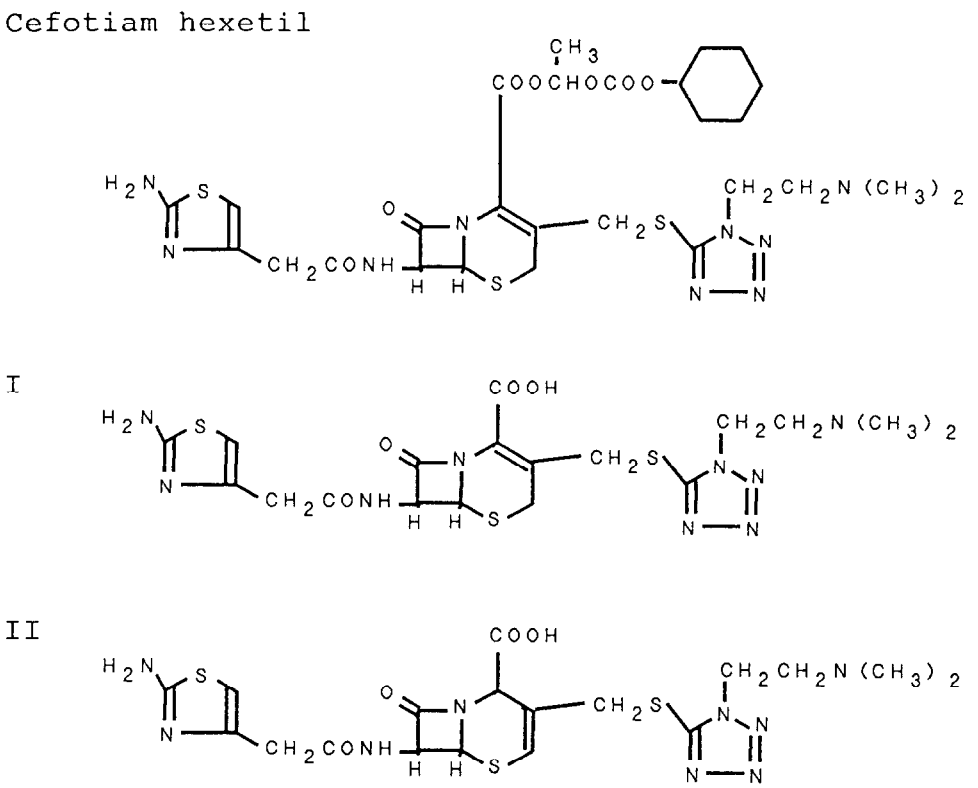

Fig. 1. Structures of cefotiam hexetil, cefotiam (I) and Δ^3-cefotiam (II).

Although the method contains no evaporation step, it still requires a washing step by dichloromethane after deproteinization by acetonitrile. Therefore we thought that it would be useful to develop a direct injection technique to determine I and II in plasma as a simpler method. As a direct injection method for biological fluids, the use of specially designed columns such as an internal surface reversed-phase (ISRP) column [9–11] or column-switching techniques [12–27] has been reported. However, to our knowledge, there have been no reports of direct injection techniques for I and II in biological fluids. This paper describes a direct injection method for the simultaneous determination of I and II in human plasma.

EXPERIMENTAL

Reagents and materials

Cefotiam hexetil dihydrochloride, I, II and cefmenoxime used as the internal standard (I.S.) for the deproteinization method were all synthesized in the Research and Development Division, Takeda Chemical Industries (Osaka, Japan). Acetonitrile was of HPLC grade (Wako, Osaka, Japan). All other reagents were of analytical reagent grade and were used without further purification.

Plasma pretreatment

Human plasma was diluted 1:5 with 0.05 *M* phosphate buffer (pH 7.7) and an aliquot of 500 μl was injected into the HPLC system.

Instruments

The HPLC system consisted of three LC-6A pumps, an SPD-6A ultraviolet (UV) detector, an FCV-2AH six-port switching valve and an SIL-6A autosampler, all of which were controlled by an SCL-6A controller (all from Shimadzu, Kyoto, Japan). The autosampler was

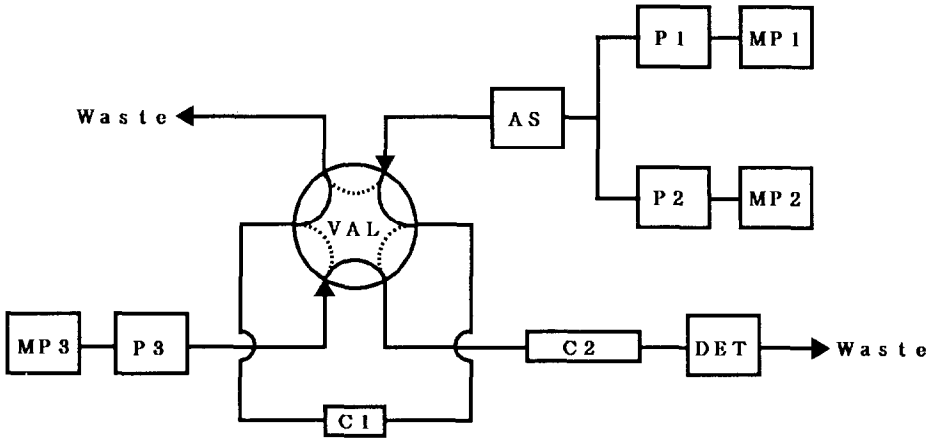

Fig. 2. Schematic diagram of the HPLC system. P1, P2 and P3 = pumps 1, 2 and 3; AS = autosampler; VAL = six-port valve; C1 and C2 = columns 1 and 2; DET = UV detector. The solid and dotted lines in the six-port valve indicate the valve positions 1 and 2, respectively.

thermostated at 4°C by a WIG-111A cooler (Ishido, Chiba, Japan). UV detection was carried out at 254 nm. The peak heights of analytes were measured by a C-R3A integrator (Shimadzu). The pretreatment column (C1) was Guard Pak μBondapak C_{18} (Waters, Milford, MA, USA). The analytical column (C2) was YMC ODS A-302 (5 μm particle size, 150 mm × 4.6 mm I.D.; Yamamura Chemical, Kyoto, Japan). The mobile phases (MP1, MP2 and MP3) were as follows: 0.05 *M* phosphate buffer (pH 7.7) for MP1, acetonitrile–water (6:4, v/v) for MP2 and 0.05 *M* phosphate buffer (pH 7.7)–acetonitrile (88:12, v/v) for MP3. The temperature and the flow-rate for both columns were 25°C and 1.0 ml/min, respectively.

Analytical system and procedure

A schematic diagram of the HPLC system is shown in Fig. 2. The timing of the column switching is shown in Table I. The analytes in the injected sample were enriched on the top of C1 with MP1, while proteins and endogenous hydrophilic substances were washed off to waste. At 10 min after injection, the enriched analytes were backflushed onto C2 with MP3. The analytes were then separated on C2 with MP3, while C1 was washed with MP2 for 10 min and switched back to MP1 for the next injection. The analysis of each sample was completed within 35 min.

Determination of I and II in plasma by the deproteinization method

The method was previously reported [7]. The outline of the procedure is as follows. To 400 μl of plasma were added 150 μl of 0.33 *M* hydrochloric acid and 1.5 ml of acetonitrile containing 0.6 μg of cefmenoxime (I.S.). The mixture was vortex-mixed for 30 s and centrifuged for 5 min at 2000 *g*. The decanted supernatant was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 300 μl of 0.1 *M* potassium dihydrogenphosphate solution and an aliquot of 100 μl was injected into the HPLC system. The HPLC conditions were as follows: column, YMC ODS A-302 (5 μm particle size, 150 mm × 4.6 mm I.D.); mobile phase, 0.05 *M*

TABLE I

TIMING OF THE COLUMN SWITCHING OF THE HPLC SYSTEM

	Operation time (min)				
	0	10	15	25	35
Valve position	1	2	1		
Mobile phase for C1	MP1	MP3	MP2	MP3	
Mobile phase for C2	MP3				

phosphate buffer (pH 7.7)–acetonitrile (88:12, v/v); detection, UV 254 nm; flow-rate, 1.0 ml/min; temperature, 25°C.

RESULTS AND DISCUSSION

The analytical conditions (C2, MP3, temperature and detection) were similar to those reported previously for the deproteinization method [7]. As the pretreatment column (C1), TSK precolumn BSA-ODS (Tosoh, Tokyo, Japan) and Guard Pak μBondapak C_{18} were tested. Although both columns showed similar retention behaviours for I and II, the second column was adopted for the present study because of its convenience for the replacement of the cartridge column. As the flushing eluent (MP1), water and 0.05 *M* phosphate buffer (pH 7.7) were investigated. The analytes were not sufficiently retained with water on C1. Phosphate buffer retained both I and II more strongly, and the compounds were not eluted from C1 with 20 ml of this solvent. The $pK_{a(3)}$ value of I is 7.0 [28] and this is considered to be why the analytes were retained more strongly with phosphate buffer (pH 7.7) on the reversed-phase pretreatment column. Furthermore, the baseline drift after column switching was smaller with the phosphate buffer, which is the same with the aqueous composition of the analytical mobile phase (MP3). Thus, 0.05 *M* phosphate buffer (pH 7.7) was adopted as MP1.

There are two methods eluting the enriched analytes from C1 to C2 with MP3: the straight-flush and back-flush methods. In order to investigate the peak broadening, the peak areas and heights after column switching with straight- or back-flush modes were compared with those obtained with C2 alone without column switching (Table II). The peak areas obtained were almost the same as those obtained without column switching, which shows that the recoveries of the analytes from C1 both with straight- and back-flush modes were satisfactory. The straight-flush mode showed lower peak heights, caused by peak broadening. However, the back-flush mode caused no peak broadening, as demonstrated by the fact that peak heights were similar to those obtained without column switching. The analytes were considered to be enriched on the top of C1 with MP1. Back-flush mode was finally adopted for the present study to minimize peak broadening. A washing step of C1 by 60% acetonitrile (MP2) was added to elute the retained hydrophobic substances from C1 after the analytes were transferred to C2. This extended the lifespan of C1 (data not shown). The precolumn was replaced approximately every 50 samples, although its lifespan was considered to be much longer.

TABLE II

COMPARISON OF PEAK HEIGHTS AND AREAS OF I AND II WITH AND WITHOUT COLUMN SWITCHING

RPH = relative peak height compared with that without column switching; RPA = relative peak area compared with that without column switching.

	I		II	
	RPH	RPA	RPH	RPA
Without column switching	(100)	(100)	(100)	(100)
With column witching				
Back-flush mode	98.5	99.3	98.1	97.8
Straight-flush mode	76.7	94.6	77.7	94.0

Fig. 3 shows the chromatograms of plasma samples obtained with the present method. Compounds I and II were satisfactorily separated from each other, and no interferences were observed at the retention times of both compounds. No memory effect was observed after the repeated injections of plasma sample.

The calibration graphs were obtained by analysing spiked plasma samples over the concentration ranges 10–8000 ng/ml for I and 10–500 ng/ml for II. The least-squares regression fit showed good linearity, passing through the origin for both analytes (Table III). Recoveries of the analytes from plasma, calculated by comparing the peak heights with those of standard aqueous solution treated similarly, were quantitative, with the coefficients of variation (C.V.) being below 4% for both compounds (Table IV). Although no internal standard was used in the present

TABLE III

REGRESSION DATA FOR CALIBRATION CURVES OF I AND II IN PLASMA

Equation: peak height = concentration (ng/ml) × a + b; n = number of points.

Compound	Concentration range (ng/ml)	n	Slope (a)	Intercept (b)	Correlation coefficient
I	10–8000	10	4.96	−45.2	0.999
II	10–500	6	5.12	− 3.3	0.999

method, satisfactory validation data were obtained. The detection limits in plasma were 10 ng/ml for both I and II at a signal-to-noise ratio of 3, which are the same as those obtained by the deproteinization method [7].

Concentrations obtained by the present method were compared with those obtained by the deproteinization method [7], using 1–2 h plasma samples after oral administration of cefotiam hexetil dihydrochloride (400 mg potency equivalent to cefotiam) in man. As shown in Table V, both concentrations were in good agreement.

In conclusion, an automated HPLC method using column switching was established for the simultaneous determination of I and II in human plasma. This method is simple and should be ap-

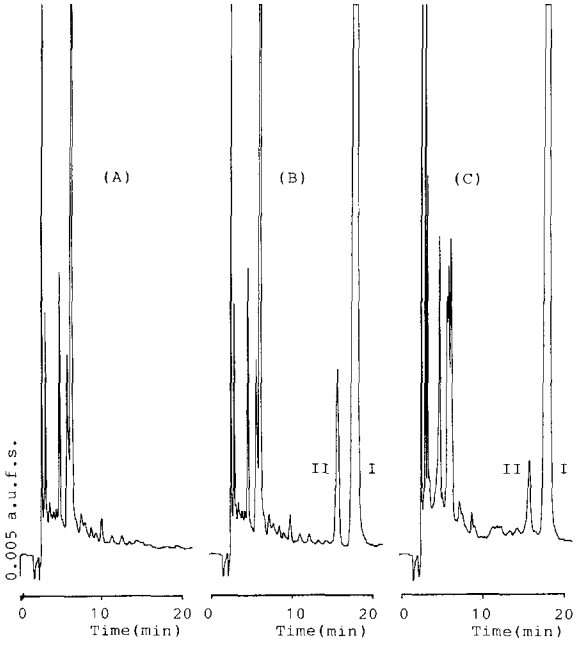

Fig. 3. Chromatograms after column switching. (A) Drug-free plasma; (B) plasma spiked with I (4 μg/ml) and II (250 ng/ml); (C) No. 10 plasma sample in Table V (I = 3.55 μg/ml, II = 107 ng/ml).

TABLE IV

REPRODUCIBILITY OF RECOVERY DATA FOR I AND II FROM SPIKED PLASMA

Compound	Concentration (ng/ml)	Recovery (%)	C.V. (%)	Number of experiments
I	4000	103.9	0.88	5
	1600	101.6	1.84	5
II	250	101.8	1.10	5
	100	100.0	3.08	5

TABLE V

COMPARISON OF CONCENTRATIONS OF I AND II DETERMINED BY THE PRESENT METHOD (A) AND BY THE DEPROTEINIZATION METHOD (B)

Plasma samples were obtained from human volunteers who received cefotiam hexetil dihydrochloride orally at a dose of 400 mg (equivalent to I).

Sample No.	Concentration of I (μg/ml)		Concentration of II (ng/ml)	
	Method A	Method B	Method A	Method B
1	2.94	2.71	87	69
2	4.45	4.17	155	139
3	4.38	4.06	118	110
4	2.38	2.06	89	88
5	3.87	3.67	97	89
6	3.41	3.26	113	99
7	2.71	2.82	72	70
8	1.84	1.89	59	58
9	4.62	5.05	153	155
10	3.55	3.80	107	111

plicable to plasma samples after oral administration of cefotiam hexetil dihydrochloride in man.

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