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Liquid chromatographic analysis of penem antibiotic FCE22101 in biological fluids with a liquid–liquid extraction procedure

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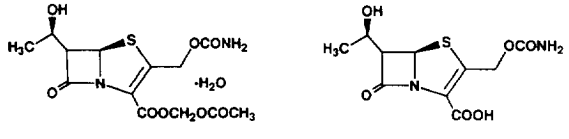
Abstract

A sensitive high-performance liquid chromatographic method has been developed for the determination of penem antibiotic FCE22101 in plasma and urine. FCE22101 was extracted from plasma and urine with ethyl acetate. After evaporating, the sample solutions were analyzed by a reversed-phase high-performance liquid chromatographic system using a two-sided bracketing injection technique. The determination limit of FCE22101 was 5 ng/ml in plasma. Analysis of the spiked plasma samples demonstrated the good accuracy and precision of the method. The proposed method improved from five- to ten-fold on the analytical sensitivity in comparison with the most commonly ultrafiltration method.

Keywords: Penem antibiotic

1. Introduction

The penem antibiotic FCE22101 {(+)-(5*R*,6*S*)-3-carbamoyloxylmethyl-6- [(1*R*)-1-hydroxyethyl] -7-oxo-4-thia-1-azabicyclo [3.2.0] hept-2-ene-2-caboxylate} and its acetoxymethyl ester, FCE22891, an orally absorbed prodrug (Fig. 1), are two penem antibiotics synthesized by Farmitalia Carlo Erba [1]. FCE22101 exhibits a broad spectrum of activity in vitro and in vivo against both Gram-positive and Gram-negative bacteria, remarkable stability to hydrolysis by various β-lactamases and good chemical stability in neutral and slightly acidic media [2,3]. Following oral administration, FCE22891 is rapidly absorbed and completely hydrolyzed to FCE22101 during transport across the intestinal membrane [4].



FCE22891 FCE22101

Fig. 1. Chemical structures of FCE22891 and FCE22101

The pharmacokinetics of FCE22891 in humans have been studied after single oral administration of the drug at a dose from 500 mg to 2 g [5–7], and after food intake [6]. Its absolute bioavailability as FCE22101 is about 30 to 40% [4–6].

FCE22891 is rapidly hydrolyzed to FCE22101 in vivo; no detectable unchanged FCE22891 is found in human plasma or urine, even when samples are collected over potassium fluoride to inhibit ex vivo hydrolysis [7].

The common methods for the determination of antibiotics in biological fluids are microbiological

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assay or high-performance liquid chromatography (HPLC). The microbiological assay procedure has been developed for the determination of FCE22101 in human plasma and urine [8]. The assay is inexpensive and simple, but may suffer from several deficiencies such as slow turnaround time, limited specificity because of interferences by other antimicrobial agents, and variable precision. Preparation procedures for the determination of antibiotics in biological fluids by HPLC are deproteinization [9] or ultrafiltration [10]. The deproteinization method may suffer from degradation of the antibiotics for the reason that the chemical stability of most antibiotics is not good under strong acidic conditions. The determination of FCE22101 has already been established using HPLC with ultrafiltration in biological fluids [11]. This method is very simple, but less sensitive, because with this method it is impossible to increase the concentration of the antibiotics in plasma and urine.

This paper describes the highly sensitive quantification of penem antibiotic FCE22101 in biological fluids with liquid–liquid extraction procedure by HPLC. Like other penem and carbapenem antibiotics, FCE22101 undergoes degradation under strong acidic and alkali conditions, however, no degradation of FCE22101 in plasma and urine was shown for the reason that the proposed method with liquid–liquid extraction was carried under mild acidic conditions.

The proposed method was highly sensitive, accurate, reproducible and suitable for the routine analysis of large numbers of clinical samples for the pharmacokinetic study of FCE22101.

2. Experimental

2.1. Materials and reagents

FCE22891 and FCE22101 were provided by Farmitalia Carlo Erba (Milan, Italy). Chloramphenicol was obtained from Aldrich (Milwaukee, WI, USA). Ethyl acetate (HPLC grade), potassium dihydrogen phosphate, ethylene glycol (EG), potassium hydroxide, phosphoric acid, hydrochloric acid, sodium dodecyl sulfate, and ammonium sulfate were purchased from Katayama Chemicals (Osaka, Japan). Acetonitrile (HPLC grade), methanol (HPLC grade) and 3-(morpholino)propanesulfonic acid (MOPS) were supplied by Wako Pure Chemical Industries (Osaka, Japan).

Ultrafiltration tubes (centrifree, MSP-3) were purchased from Amicom (Beverly, MA, USA).

2.2. Instruments

The HPLC system (Shimadzu LC-10AD, Kyoto, Japan) was equipped with a variable-wavelength UV-Vis detector (SPD-10A) and a computing integrator (C-R7A). The column (150×4.6 mm I.D.) was of stainless steel, prepacked with Cosmosil 5 C_{18} packing (Nacalai Tesque, Kyoto, Japan) and protected with a fitted guard column (LichroCART RP-18, Merck, Darmstadt, Germany).

2.3. FCE22101 standard solution

Stock solution of FCE22101 was prepared by dissolving 10 mg of the compound in 40% acetonitrile–60% 0.1 *M* phosphate buffer (pH 4.5) and made up to 10 ml. This stock solution was stored at 4°C for up to a week. The solution was diluted with distilled water to the suitable concentration.

2.4. Internal standard solution

Internal standard (I.S.) solution was prepared by dissolving 10 mg of chloramphenicol in methanol to make up 10 ml. The solution was diluted with distilled water to the concentration of 20 μg/ml. This solution was stored at 4°C.

2.5. Chromatographic procedure

The chromatographic conditions are summarized in Table 1. The mobile phases were prepared fresh on the day of analysis and were filtered and degassed by vacuum. All chromatographic operations were carried out at 25°C. The column was conditioned by passing the mobile phase through it for 2 h at a flow-rate of 1.0 ml/min. The detection wavelength was set at 320 nm for measurement of FCE22101.

Table 1
HPLC conditions for analysis of FCE22101

Column	Cosmosil 5 C_{18} (150×4.6 mm I.D.)	
Guard column	LiChroCart RP-18 (5 μm)	
Flow-rate	1.0 ml/min	
Column temperature	40°C	
Detection wavelength	320 nm	
Injection volume	200 μl	
Calculation	Peak area	
Mobile phase	A: 0.05 *M* phosphate buffer (pH 2.7)	
	B: A–acetonitrile (3:2)	
Gradient profile	Plasma	Urine
	0 min, 0% B	0 min, 0% B
	5 min, 0% B	15 min, 0% B
	25 min, 50% B	35 min, 50% B
	30 min, 100% B	40 min, 100% B
	35 min, 100% B	45 min, 100% B
	45 min, 0% B	55 min, 0% B

2.6. Injection techniques

Plasma samples were injected by a two-sided bracketing technique [12] with 1 *M* phosphate buffer (pH 2.5) to focus the solutes as a narrow band on the top of the column.

2.7. Stabilizer

The stabilizer solution was prepared by dissolving 20.9 g of MOPS in 100 ml distilled water. The pH of the solution was adjusted to 5.0 with 6 *M* potassium hydroxide. The MOPS solution was mixed to 100 ml of EG. The solution was stored at 4°C.

2.8. Plasma and urine samples

Blood samples obtained from healthy volunteers were collected in heparinized containers and centrifuged to obtain the plasma. The plasma was added to an equal volume of stabilizer and mixed. The plasma and urine samples were stored at −20°C until the time of analysis.

2.9. Sample preparation with liquid–liquid extraction method

For plasma samples, 0.1 ml of distilled water and 5 ml of ethyl acetate–isopropanol mixture (4:1, v/v) were added to 1.0 ml (containing 0.5 ml of stabilizer) of human plasma. The mixture was shaken vigorously for 10 min and centrifuged at 1500 *g* for 5 min. The organic layer was aspirated and discarded, 0.5 g of ammonium sulfate, 0.05 ml of 6 *M* hydrochloric acid and 5 ml of ethyl acetate were added to the aqueous layer. The mixture was shaken vigorously for 10 min and centrifuged at 1500 *g* for 5 min. The organic layer was taken, and the extraction procedure repeated with 5 ml of ethyl acetate again. The collected organic layer was evaporated to dryness at 50°C under a stream of nitrogen gas. The residue was dissolved in 0.2 ml of 0.05 *M* phosphate buffer (pH 2.7) and 0.1 ml of I.S. solution added. A 0.2-ml aliquot of the sample solution was injected into the HPLC system.

For urine samples, 1.0 ml of the urine samples containing 0.5 ml of the stabilizer was diluted with distilled water to make 50 ml, and 1.0 g of ammonium sulfate, 0.05 ml of 2 *M* hydrochloric acid and 5 ml of ethyl acetate were added to 1.0 ml of the diluted urine samples. The mixture was shaken vigorously for 10 min and centrifuged at 1500 *g* for 5 min. The organic layer was taken, and the extraction procedure repeated with 5 ml of ethyl acetate again. The collected organic layer was evaporated to dryness at 50°C under a stream of nitrogen gas. The residue was dissolved in 0.2 ml of 0.05 *M* phosphate buffer (pH 2.7) and 0.1 ml of I.S. solution added. A 0.2-ml aliquot of the sample solution was injected into the HPLC system.

2.10. Calibration curves, accuracy and precision

The plasma and urine calibration standard solution of FCE22101 were prepared at 11 different concentrations between 5 and 10 000 ng/ml for plasma, and between 0.5 and 1000 μg/ml for urine, and treated in the manner described above. Calibration curves of the chromatographic peak-area ratios (FCE22101/I.S.) versus FCE22101 concentration were constructed. FCE22101 concentrations in the unknown plasma and urine samples were calculated by interpolation from the calibration curves by a least-squares regression line treatment.

The accuracy and precision of the proposed method were determined by adding known amounts of FCE22101 to control plasma and urine.

3. Results and discussion

3.1. Sample preparation

To develop a highly sensitive and reproducible analytical method for biological samples, it is necessary to extract and separate the compounds of interest from a mixture. First, protein precipitation for isolating from biological matrices has been investigated: precipitation with perchloric acid was very inconvenient due to relevant degradation of FCE22101 under strongly acidic conditions. Thus, proteins in biological samples were precipitated by means of 60% (v/v) of acetonitrile, and the supernatants were analyzed by reversed-phase HPLC. But, this method led to lower assay precision and incomplete FCE22101 recovery.

Column switching techniques for the analysis of FCE22101 by direct injection of plasma samples into reversed-phase chromatographic columns are available. But, it was considered that they involved an inconvenient increase of the complexity of the liquid chromatography apparatus and showed an inadequate stability for the routine analysis of a very large number of samples.

The next selected approach, the ultrafiltration method was investigated. The method was observed to have a good precision and recovery using the proposed HPLC conditions, almost completely [11]. However, it was not satisfactory in relation to determination limit (0.05 μg/ml) for the determination of the pharmacokinetic study in human. Then, a highly sensitive and reproducible analytical method of FCE22101 with a liquid–liquid extraction procedure in biological fluids was investigated.

For the protection of FCE22101 from degradation, it is necessary to add stabilizer to FCE22101 in plasma and urine samples. Then, we investigated the method to discard EG in plasma samples without loss of FCE22101. As a result, extraction of EG with an ethyl acetate–isopropanol mixture was satisfactory without loss of FCE22101 in the aqueous layer under neutral pH conditions. In the next step, we investigated the extraction method of FCE22101 from aqueous layer.

FCE22101 is unstable under strong acidic and alkali solutions. However, when extracting FCE22101 from plasma, it is necessary to have acidic conditions in the aqueous solution as there is a carboxylic acid in the FCE22101 structure. Then, we investigated the extraction procedure of FCE22101 with no degradation of FCE22101 and with high recovery from biological fluids. As the result, the plasma solutions saturated with ammonium sulfate and acidified with a small amount of 6 *M* hydrochloric acid addition were kept at about pH 3, and FCE22101 in the aqueous layer was extracted with ethyl acetate, quantitatively and reproducibly.

3.2. Chromatography

When the aliquots of the sample solutions were injected into the HPLC system directly, the peaks of FCE22101 were observed to broaden due to the effect of a little residual EG from preparation with the proposed liquid–liquid extraction method. Then, we investigated the HPLC method using a two-sided bracketing technique. Using the bracketing injection technique, the two-sided "bracketed sample" with 1 *M* phosphate buffer (pH 2.5) reached the column as a narrow band. As the result, the shape of FCE22101 peak injected using the two-sided bracketing technique was sharper than that using direct injection with 1.2 fold the peak height. Under the conditions used, FCE22101 was well resolved from endogenous substances in plasma and urine. Typical chromatograms of FCE22101 in plasma and urine samples are shown in Fig. 2 and Fig. 3.

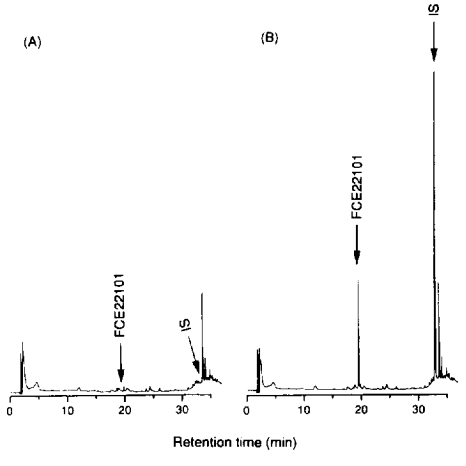

Fig. 2. Typical chromatograms of plasma samples: (A) blank plasma; (B) plasma spiked with FCE22101 (0.2 μg/ml).

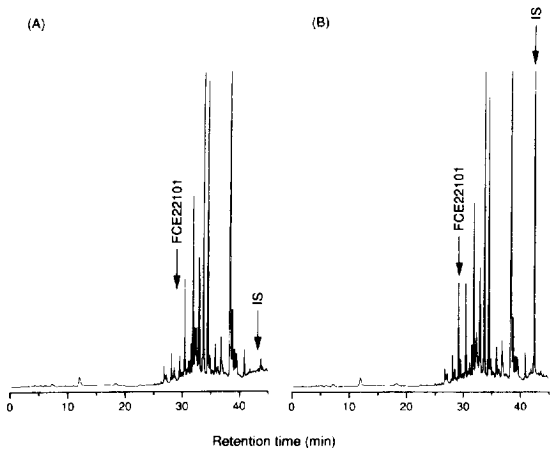

Fig. 3. Typical chromatograms of urine samples: (A) blank urine; (B) urine spiked with FCE22101 (10 μg/ml).

3.3. Calibration curves, accuracy and precision

We found a good linear relationship between the peak-area ratio and FCE22101 concentrations in plasma and urine samples in the standard ranges studied, which corresponds to the regression equations $y=0.8348x-0.0047$ ($r=1.0000$) for plasma samples and $y=2.001x+0.0111$ ($r=0.9989$) for urine samples. We estimated that the limits of determination were 5 ng/ml in plasma and 0.5 μg/ml in urine.

The recoveries of FCE22101 were measured on blank plasma and urine spiked with FCE22101 at three different concentrations (Table 2). The results show that the recoveries were from 75.6 to 84.8% in plasma samples and from 79.2 to 88.2% in urine samples, and therefore the drawback of ca. 20% loss of FCE22101 should be considered due to discard of the ethyl acetate–isopropanol mixture, poor extraction yields with ethyl acetate and the degradation of FCE22101 during the preparation procedure.

Table 2
Recoveries of FCE22101 from human plasma and urine

Sample	Concentration added (μg/ml)	Recovery (mean±S.D., $n=6$) (%)
Plasma	0.005	75.6±4.0
	0.200	82.4±2.3
	10.000	79.4±3.3
Urine	0.5	80.3±8.6
	25.0	79.2±3.5
	1000.0	88.2±1.0

The accuracy and precision of the assay developed for FCE22101 in plasma and urine were determined by adding known amounts of FCE22101 to blank plasma and urine. The within-day reproducibility was studied at three concentrations of FCE22101. To evaluate the between-day reproducibility, three concentrations of FCE22101 were used. The plasma and urine samples were analyzed by the HPLC procedure described. The results are shown in Table 3, where one can see that the coefficients of within-day and between-day variation (C.V.) ranged from 0.2 to 14.0%; accuracy, as defined as (amount found/amount added)×100(%), was from 71.3 to 91.1% for all samples assayed. From the results, it is evident that the proposed method was most satisfactory in both accuracy and precision. Therefore, it was considered that the proposed method was suitable for pharmacokinetic and bioavailability studies in human.

3.4. Applications

The described liquid–liquid extraction procedure and the ultrafiltration method [11] were applied to the determination of FCE22101 in plasma of the healthy volunteers orally dosed with 200 mg of FCE22891. The plasma concentrations of FCE22101 obtained by the proposed liquid–liquid extraction procedure were compared with those obtained by the ultrafiltration method. The correlation between the two methods, shown in Fig. 4, was satisfactory in cases where the plasma concentrations were more than 0.05 μg/ml by reason of the fact that the determination limit of the proposed method was much superior to that of the ultrafiltration method. The time course of plasma concentration as measured by the liquid–liquid extraction method is shown in Fig. 5. It was considered that the proposed method was adaptable enough in cases where the concentration of FCE22101 was below 0.05 μg/ml in plasma. Plasma levels of FCE22101 reached a

Table 3
Accuracy and precision for the plasma and urine samples spiked with FCE22101

Concentration added	Day 1			Day 2			Day 3			Day 4			Mean		
(μg/ml)	Found (μg/ml)	Accuracy* (%)	Precision** (%)	Found (μg/ml)	Accuracy* (%)	Precision** (%)	Found (μg/ml)	Accuracy* (%)	Precision** (%)	Found (μg/ml)	Accuracy* (%)	Precision** (%)	Found (μg/ml)	Accuracy* (%)	Precision** (%)
Plasma															
0.0050	0.0038	76.4	3.2	0.0043	86.7	14.0	0.0036	71.3	7.5	0.0040	79.9	5.3	0.0039	78.6	10.7
0.2000	0.1666	83.3	1.0	0.1582	79.1	0.7	0.1621	81.1	3.5	0.1673	83.6	1.1	0.1635	81.8	2.9
10.0000	8.1020	81.0	0.3	7.6867	76.9	2.6	7.9365	79.4	0.2	8.1425	81.5	0.4	7.9669	79.7	2.6
Urine															
0.50	0.41	82.8	3.6	0.43	85.5	7.7	0.39	78.1	6.0	0.37	74.3	8.1	0.40	80.2	7.9
25.00	22.06	88.2	3.2	22.65	90.6	2.7	22.54	90.2	0.8	20.90	83.6	4.4	22.04	88.1	4.2
1000.00	910.69	91.1	1.0	887.12	88.7	1.1	901.67	90.2	4.0	838.41	83.8	4.0	884.47	88.4	4.1

Measured data were expressed as means ($n=3$).
Accuracy*: (found/added) $\times$ 100.
Precision**: Coefficient of variation.

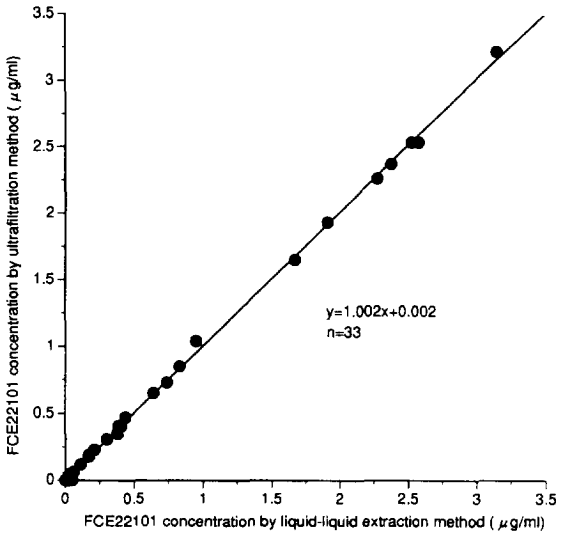

Fig. 4. Correlation between FCE22101 concentrations obtained by the liquid–liquid extraction method and the ultrafiltration method.

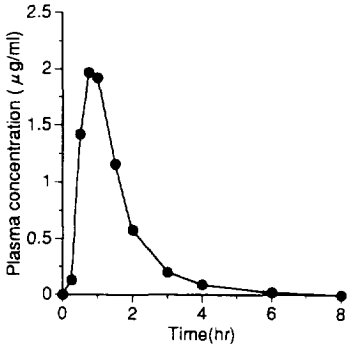

Fig. 5. Time course of plasma concentration of FCE22101 after oral administration of FCE22891 at 200 mg dose ($n=3$).

maximum at 1 h after administration, and thereafter the plasma level declined with an elimination half-life of ca. 0.5 h.

4. Conclusions

Through the present study, we have established a highly sensitive assay method for FCE22101 in human plasma and urine by HPLC with a liquid–liquid extraction procedure. The linearity, reproducibility and accuracy have been proved. The proposed method is useful for the evaluation of the pharmacokinetics of FCE22891 in humans and examination of the penetration of FCE22101 in human tissues.

Acknowledgments

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