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Simultaneous determination of an active metabolite and open-ring metabolites by high performance liquid chromatography and pharmacokinetic studies of a penem antibiotic, FCE22891, in dogs

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Abstract

A sensitive high performance liquid chromatographic method for the simultaneous determination of an active metabolite (FCE22101) and open-ring metabolites (P1, P2) of a penem antibiotic, FCE22891, in dog plasma was developed. Plasma samples were pretreated only by ultrafiltration for the determination of the metabolites. The filtrates were directly analyzed by a reversed-phase high-performance liquid chromatographic system using a two-sided bracketing injection technique. The quantitation limits of FCE22101, P1 and P2 were 0.03, 0.1 and 0.15 μg ml^{-1}, respectively. Analysis of the spiked plasma samples demonstrated the good accuracy and precision of the method.

The proposed method was applied to the pharmacokinetic studies of an active metabolite and open-ring metabolites after oral administration of a penem antibiotic, FCE22891, in dogs. In addition, the plasma levels of unchanged FCE22891 and the possible changes of formaldehyde and acyl-L-carnitine levels in plasma, which will be generated from the ester group of FCE22891, were also investigated.

Keywords: Penem antibiotic; Open-ring metabolites; Pharmacokinetics; High performance liquid chromatography

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-carboxylate} and its acetoxymethyl ester, FCE22891, an orally absorbed prodrug of FCE22101, 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]. Then, FCE22101 undergoes renal metabolism [5] by dehydropeptidase

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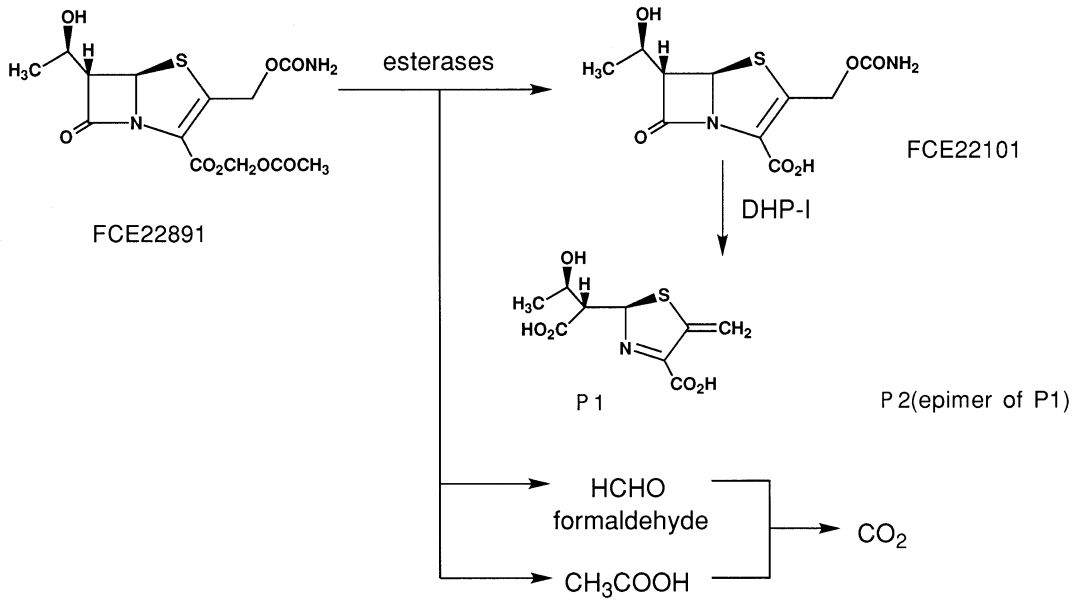

Fig. 1. Postulated metabolic pathways of FCE22891.

I (DHP-I), tubular dipeptidase, like other penem and carbapenem antibiotics [6,7]. The metabolites of FCE22101 by DHP-I catalysed hydrolysis or alkaline hydrolysis in vitro [8] have been certified to open-ring products, P1 and its epimer P2. Fig. 1 shows the postulated metabolic pathways of FCE22891.

We investigated the simultaneous determination method of an active metabolite and open-ring metabolites to evaluate the pharmacokinetics of FCE22891, quantitatively. The metabolites P1 and P2 have not been easily obtained and stored as pure anhydrous solids. However, this paper shows that aqueous solutions of P1 and P2 can be easily obtained quantitatively by alkaline hydrolysis of FCE22101 and these show enough quality to use as standards for determination. The plasma samples spiked with standard solutions of FCE22101, P1 and P2 were deproteinized by ultrafiltration, and analyzed by HPLC using a two-sided injection technique.

It was reported that no unchanged FCE22891 was detected in plasma after oral administration of ^{14}C-labelled FCE22891 to some animals [9], and Vincon et al. [10] analyzed unchanged FCE22891 in plasma samples by adding 100 μl of an aqueous solution of potassium fluoride (86 mM) to inhibit esterases. However, there are no reports for the determination method of unchanged FCE22891 which have sufficient analytical sensitivity and reproducibility avoiding the hydrolysis of unchanged FCE22891 by esterases in plasma. We investigated the sensitive determination method of unchanged FCE22891 in plasma by an extraction method with ethyl acetate for pharmacokinetic studies of FCE22891. It was found that the addition of stabilizer and potassium fluoride to the plasma samples was useful for the inhibition of hydrolysis of the ester chain of unchanged FCE22891.

The two proposed methods for the simultaneous determination of FCE22101 and open-ring metabolites (P1, P2) and for the determination of unchanged FCE22891 were highly sensitive, accurate, reproducible and suitable for the routine analysis of large numbers of clinical samples for the pharmacokinetic study of FCE22891. Because FCE22891 contains an ester group, the possibility was considered that it was metabolized to produce fatty acid and formaldehyde, and the fatty acid

was condensed with endogenous L-carnitine, and produced acyl-L-carnitine [11,12]. This may cause low L-carnitine disease in humans. Formaldehyde produced by the metabolism of the ester group should also be monitored to confirm drug safety, especially in long-term therapy. Therefore, this paper also described the plasma levels of acyl-carnitine and formaldehyde after dosing FCE22891 to dogs.

2. Experimental

2.1. Materials and reagents

FCE22891 and FCE22101 were provided by Farmitalia Carlo Erba (Milan, Italy). *m*-Acetamidobenzoic acid (IS), ethyl acetate, potassium dehydrogen phosphate, phosphoric acid, hydrochloric acid, sodium dodecyl sulfate (SDS), sodium hydroxide and potassium fluoride (KF) were purchased from Katayama (Osaka, Japan). Acetonitrile, 3-(morpholino) propanesulfonic acid (MOPS) and ethylene glycol (EG) were supplied by Wako (Osaka, Japan).

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

2.2. Instruments

The HPLC system (Shimadzu LC-10AD, Kyoto, Japan) was equipped with a variable-wavelength UV–visible detector (SPD-10A) and a computing integrator (C-R7A). The columns were of stainless steel, prepacked with TSK-GEL SUPER ODS (100 mm × 4.6 mm i.d., 2 μm) packing (TOSOH, Tokyo, Japan) and with Cosmosil 5C18 (150 mm × 4.6 mm i.d.) packing (Nacalai Tesque, Kyoto, Japan).

2.3. Preparation of standard solutions of FCE22101 and metabolites P1 and P2

Aqueous solutions containing known concentrations of FCE22101, P1 and P2 were prepared by quantitative alkaline hydrolysis [13] of FCE22101 for identification and calibration.

An aqueous solution containing 10 mg (potency) of FCE22101 was prepared by dissolving in 0.4 ml of acetonitrile and 0.6 ml of 0.1 M KH_2PO_4, and made up to 10 ml with distilled water (1 mg ml^{-1}; 3.3 mM). A 0.5-ml aliquot of this solution was hydrolyzed with 0.5 ml of 0.1 M NaOH at room temperature for 30 s. Then, 0.8 ml of 0.1 M KH_2PO_4 and 0.7 ml of distilled water were added and the final solution was analyzed by HPLC to determine the residual concentration of FCE22101 and the concentrations of P1 and P2 produced. This solution was immediately chilled and stored at 4°C and was stable for a week.

2.4. Internal standard solution and stabilizer

Internal standard (IS) solution was prepared by dissolving 10 mg of *m*-acetamidobenzoic acid in 10 ml of 40% acetonitrile–0.1 M phosphate buffer (pH 4.5). This solution was stored at 4°C and was stable for at least a month. The solution was diluted with distilled water to a suitable concentration.

The stabilizer solution consisted of MOPS solution and EG (1:1). The MOPS solution was prepared by dissolving 20.9 g of MOPS in 100 ml distilled water and was adjusted to pH 5.0 with 6 M potassium hydroxide. The MOPS solution was mixed with 100 ml of EG. The solution was stored at 4°C.

2.5. Chromatographic procedure

The chromatographic conditions are summarized in Table 1. The mobile phases were freshly prepared 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^{-1}. The detection wavelength was set at 320 nm for measurement of FCE22891, FCE22101, P1 and P2.

Plasma samples were injected by a two-sided bracketing technique [14] with 1 M phosphate buffer (pH 2.5) to focus the solution as a narrow band on the top of the column.

2.6. Plasma samples

Blood samples obtained from beagle dogs were collected in heparinized containers and centrifuged. The plasma obtained was added to an equal volume of the stabilizer solution and mixed. The plasma samples were stored at −80°C until analysis.

2.7. Sample preparation for FCE22101, P1, P2 and for unchanged FCE22891

For FCE22101, P1 and P2 analysis, plasma samples (0.5 ml) containing 0.25 ml of the stabilizer were added to 0.05 ml of distilled water or the calibration standard solution, and 0.1 ml each of the internal standard (IS; 0.25 mg ml^{-1}) and 40 mM SDS. After mixing for 15 s, the solutions were transferred to ultrafiltration tubes, and the tubes were centrifuged at $2000 \times g$ for 1 h at 15°C. A 0.05-ml aliquot of the filtrate was injected into the HPLC system by a two-sided bracketing technique.

For unchanged FCE22891 assay, plasma samples (1.0 ml) containing 0.5 ml of stabilizer were added to 0.05 ml of distilled water or calibration standard solution, 0.1 ml of KF solution (125 mg ml^{-1}) and 5 ml of ethyl acetate. The mixture was vigorously shaken for 10 min and centrifuged at $1500 \times g$ for 5 min. The organic layer was collected, and the extraction procedure was repeated with 3 ml of ethyl acetate. The organic layer collected was evaporated to dryness at 50°C under a stream of nitrogen gas. The residue was dissolved in 0.4 ml of 40% acetonitrile–0.05 M phosphate buffer (pH 2.7), and 0.1 ml of IS solution was added. A 0.05-ml aliquot of the sample solution was injected into the HPLC system by a two-sided bracketing technique.

Table 1
HPLC conditions for analysis of FCE22891 and FCE22101, P1 and P2

Parameter	FCE22891	FCE22101, P1 and P2
Column	Cosmosil 5C18 (150 mm × 4.6 mm i.d., 5 μm)	TSK-GEL SUPER ODS (100 mm × 4.6 mm i.d., 2 μm)
Guard column	Lichro Cart RP-18 (5 μm)	Lichro Cart RP-18 (5 μm)
Flow rate	1.0 ml min^{-1}	1.0 ml min^{-1}
Column temperature	40°C	40°C
Detection wavelength	320 nm	320 nm
Injection volume	50 μl	50 μl
Calculation	Peak area	Peak area
Internal standard	*m*-Acetamidobenzoic acid	*m*-Acetamidobenzoic acid
Mobile phase	(A) 0.05 M phosphate buffer (pH 2.7) (B) A:acetonitrile (3:2, v/v)	(A) 0.02 M phosphate buffer (pH 2.3) (B) A:acetonitrile (3:2, v/v)
Gradient profile	0 min B 0% 5 min B 0% 25 min B 50% 30 min B 100% 35 min B 100% 45 min B 0%	0 min B 0% 1 min B 0% 11 min B 15% 16 min B 5% 31 min B 0%

For unchanged FCE22891 assay, plasma samples (1.0 ml) containing 0.5 ml of stabilizer were added to 0.05 ml of distilled water or calibration standard solution, 0.1 ml of KF solution (125 mg ml^{-1}) and 5 ml of ethyl acetate. The mixture was vigorously shaken for 10 min and centrifuged at $1500 \times g$ for 5 min. The organic layer was collected, and the extraction procedure was repeated with 3 ml of ethyl acetate. The organic layer collected was evaporated to dryness at 50°C under a stream of nitrogen gas. The residue was dissolved in 0.4 ml of 40% acetonitrile–0.05 M phosphate buffer (pH 2.7), and 0.1 ml of IS solution was added. A 0.05-ml aliquot of the sample solution was injected into the HPLC system by a two-sided bracketing technique.

2.8. Calculation of pharmacokinetic parameters

The time elapsed to peak (Tmax) and the maximum concentration (Cmax) were taken directly from the observed value. The half-life ($t_{1/2}$) was calculated using the least squares method. The area under curves was computed by the linear trapezoidal rule from 0–5 h (AUC5). The value of $AUC\infty = AUC5 +$ [plasma concentration at 5 h after dosing/the elimination rate constant].

3. Results and discussion

3.1. Preparation of standard solution of open-ring metabolites P1 and P2

Penem antibiotic was known to be hydrolyzed by DHP-I or alkaline solutions to an open-lactam derivative in vitro [8] and FCE22101 was also hydrolyzed to a mixture of two open-lactam derivatives, designated 'P1' and 'P2' (epimer of P1). The determination of the active metabolite has already been established by HPLC with ultrafiltration [15] or liquid–liquid extraction meth-

ods [16]. The metabolites P1 and P2 are more polar than the active metabolite, and it was difficult to determine them simultaneously. Therefore, we quantitatively investigated the simultaneous determination method of the active metabolite and the open-ring metabolites for the evaluation of the pharmacokinetics of FCE22891. Although P1 and P2 were remarkably stable in dilute and slightly alkaline solutions, there was extensive degradation when attempts were made to isolate pure solid samples from a mixture of the two products [17]. Therefore, we investigated the following calculation method to determine their concentrations. The standard solution of FCE22101 and alkaline hydrolyzed solution containing FCE22101, P1 and P2 were analyzed by HPLC. On the chromatograms obtained from the hydrolyzed solution, only three peaks (FCE22101, P1 and P2) appeared; there were no other peaks detected at 320 nm under the HPLC conditions used, and at specific wavelengths of 210 and 254 nm. Visentin et al. [18] reported that the metabolite P2 was the epimer of P1. Thus, we confirmed the UV spectra of the fractions of P1 and P2 prepared by HPLC analysis. P1 and P2 exhibited the same UV spectra ranging from 190 ~ 400 nm. Therefore, it could be defined that the disparity in the concentrations of FCE22101 showed the total concentrations of P1 and P2, and the concentration of P1 and P2 could be proportional to each peak area. Each concentration of P1 and P2 can be calculated by the following equation. The concentration of P1 (M) = (peak area of P1)/(peak area of P1 + P2) × disparity in concentrations (M) of FCE22101 between standard and hydrolyzed solution. The solution of 50% of FCE22101 hydrolyzed was useful as a standard for calculation of concentrations of these compounds because the FCE22101 concentration could not be calculated in a completely hydrolyzed solution. So, we investigated the effect of hydrolysis time for the remaining concentration of FCE22101 and producing concentrations of P1 and P2 from the starting solution. Fig. 2 shows FCE22101 in hydrolyzed solution was completely converted to P1 and P2 after 5 min at room temperature. After 30 s, about 50% of FCE22101 was hydrolyzed to P1 and P2 compounds. The concentration ratio

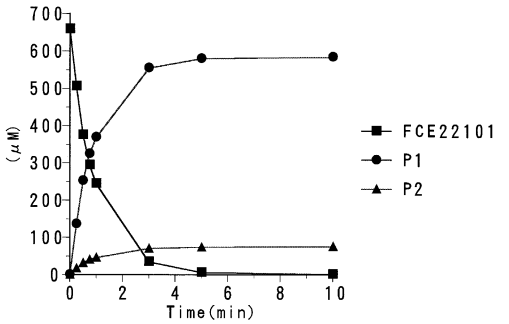

Fig. 2. Effect of time of hydrolysis for residual FCE22101 and productive P1 and P2.

P1:P2 (about 8:1) obtained from our examination was the same ratio observed upon enzymatic hydrolysis by DHP-I in vitro [18]. The resulting solution can be safely stored at 4°C for a week without appreciable degradation of P1 and P2. Typical chromatograms are shown in Fig. 3.

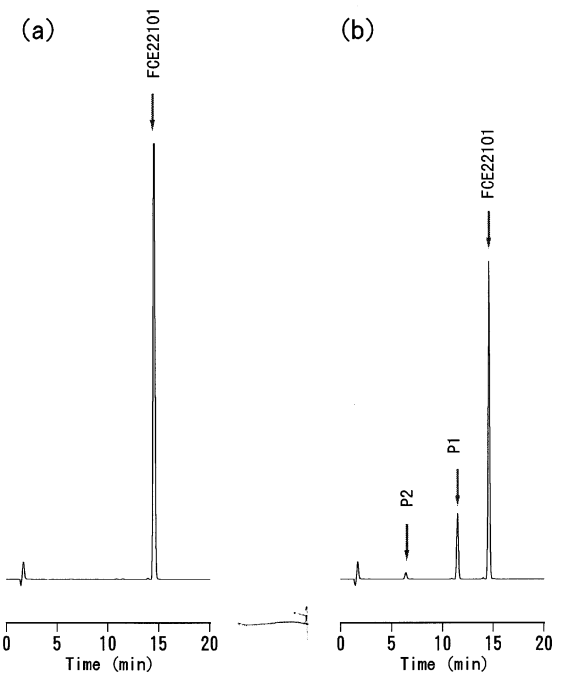

Fig. 3. Typical chromatograms of aqueous FCE22101 solution (a) before, (b) after hydrolysis for 30 s.

3.2. Chromatography

When the aliquots of the sample solutions were directly injected into the HPLC system, the peaks of FCE22101, P1 and P2 were observed to broaden due to the effect of EG which was a component of a stabilizer in the plasma samples. We investigated the HPLC method using a two-sided bracketing technique. Using the bracketing injection technique, the two-sided 'bracketing sample' with 1 M phosphate buffer (pH 2.5) reached the column as a narrow band. As a result, the shape of the FCE22101 peak injected using the two-sided bracketing technique was sharper and 1.2 times higher than using direct injection. Under the conditions used, FCE22101, P1 and P2 made good separation from endogenous substances in plasma.

The rapid and simple routine analysis conditions for a large number of samples of FCE22101, P1 and P2 were investigated using superior ODS-silica packing materials and a shorter length column. The shorter column packed with high-efficiency materials provided faster separations than using conventional columns (column length: 15 cm) at the same flow rates without adversely affecting resolution. So, a column of 10 cm length with a particle size of 2 microns for packing was selected for the sensitive and rapid determination of FCE22101, P1 and P2. Typical chromatograms of FCE22101, P1 and P2 in plasma samples are shown in Fig. 4.

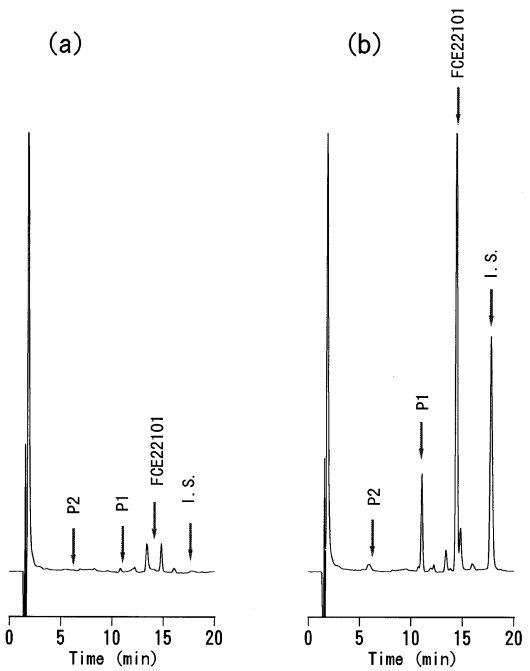

Fig. 4. Typical chromatograms of plasma samples; (a) blank plasma; (b) plasma spiked with FCE22101, P1 and P2 (5.9, 3.35 and 0.4 μg ml^{-1}).

3.3. Calibration curves, accuracy and precision of FCE22101, P1 and P2

We found a good relationship between the peak-area ratio and FCE22101, P1 and P2 concentrations in plasma samples in the standard ranges studies, which corresponds to the regressions: $y = 0.257x + 0.0009$ $(r = 0.9999)$ for FCE22101, $y = 0.1002x + 0.0009$ $(r = 1.0000)$ for P1 and $y = 0.1022x - 0.0005$ $(r = 0.9999)$ for P2.

The absolute recoveries of FCE22101, P1 and P2 were measured on the blank plasma spiked with FCE22101, P1 and P2 at the different concentrations (Table 2). The results show that the recoveries of FCE22101, P1 and P2 from plasma samples were 95.4–100.9%, 108.3–110.7% and 87.7–97.9%, respectively.

The accuracy and precision of the assay method developed for FCE22101, P1 and P2 in plasma were determined by adding known amounts of

Table 2
Recoveries of FCE22101, P1 and P2 from plasma

Concentration added (μg ml^{-1})	Recovery (%) (mean ± S.D., $n = 6$)
FCE22101	
0.0295	95.4 ± 9.1
0.236	100.9 ± 2.7
2.36	100.3 ± 0.5
5.90	98.9 ± 0.4
P1	
0.119	109.6 ± 8.7
1.19	108.3 ± 0.4
2.96	110.7 ± 0.7
P2	
0.146	87.7 ± 5.4
0.365	97.9 ± 5.0

Table 3
Accuracy and precision for the plasma samples spiked with FCE22101, P1 and P2

	Concentration (μg ml^{-1})		Accuracy*	Precision**
	Added	Found (mean ± S.D.)	(%)	(%)
Within-day ($n = 6$)				
FCE22101	0.0295	0.0335 ± 0.0013	113.7	4.0
	0.236	0.228 ± 0.0028	96.5	1.2
	2.36	2.29 ± 0.0053	96.8	0.2
	5.90	5.82 ± 0.0396	98.7	0.7
P1	0.119	0.101 ± 0.0157	85.0	15.6
	1.19	1.14 ± 0.0189	96.1	1.7
	2.96	2.90 ± 0.0302	97.7	1.0
P2	0.146	0.114 ± 0.0161	77.7	14.2
	0.365	0.366 ± 0.0113	100.2	3.1
Between-day ($n = 6$)				
FCE22101	0.0295	0.0341 ± 0.0006	115.4	1.9
	0.236	0.229 ± 0.0013	97.1	0.5
	2.36	2.29 ± 0.0084	97.1	0.4
	5.90	5.87 ± 0.0335	99.4	0.6
P1	0.119	0.0967 ± 0.0106	81.5	10.9
	1.19	1.14 ± 0.0259	96.5	2.3
	2.96	2.92 ± 0.0254	98.5	0.9
P2	0.146	0.115 ± 0.0174	78.8	15.1
	0.365	0.375 ± 0.0099	102.7	2.6

*Accuracy: (Found/added) × 100.
**Precision: Relative standard deviation (R.S.D.).

FCE22101, P1 and P2 to blank plasma. The within-day and between-day reproducibility were studied at four concentrations of FCE22101, three concentrations of P1 and two concentrations of P2. The results are shown in Table 3. The within-day and between-day relative standard deviations (R.S.D.) ranged from 0.2 to 15.6%; accuracy defined as (amount found/amount added) × 100 (%) was from 78.8 to 115.4% for all samples assayed. It was estimated that the limits of determination were 0.03, 0.1 and 0.15 μg ml^{-1}, respectively. From the results, it is evident that the proposed method was satisfactory in both accuracy and precision.

The concentration values analyzed using the ultrafiltration method showed total (protein bound and unbound) concentrations of FCE22101, P1 and P2 because of the addition of SDS to plasma samples. This was confirmed because absolute recoveries from plasma samples were about 100% and the concentrations of FCE22101 after oral administration of FCE22891 into dose measured by this ultrafiltration method showed a good relationship to those by the liquid–liquid extraction method [16]. Therefore, it was considered that the proposed method was suitable for pharmacokinetic and bioavailability studies.

3.4. Calibration curves, accuracy and precision of unchanged FCE22891

Battaglia et al. reported that 0.1 ml of 86 mM KF (0.5 mg) as an inhibitor of esterases was added to blood to avoid the hydrolysis of FCE22891, as soon as possible after blood was collected for analysis of the concentration of unchanged FCE22891 in plasma [9]. However, it was found that this amount of KF was insufficient to inhibit esterases. Therefore, we investigated the effect of inhibition to esterases with the concentration of KF and with addition of stabilizer to

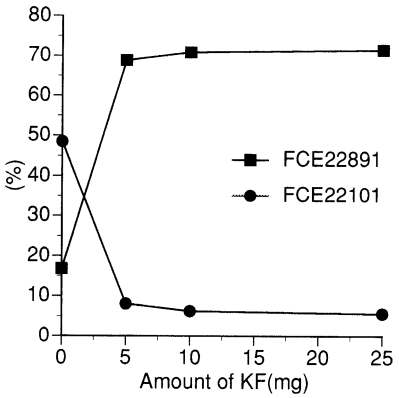

Fig. 5. Effect of KF on inhibition of plasma esterases.

plasma in vitro. As a result, the amount of KF required to inhibit esterases was above 25 mg in 1 ml of plasma (Fig. 5). Moreover, the addition of stabilizer was useful to confirm the stability of unchanged FCE22891 in plasma (Fig. 6). The proposed method demonstrated that the inhibition of hydrolysis of the ester was useful by addition of stabilizer and KF.

We found a good relationship between the peak-area ratio and FCE22891 concentrations in plasma samples in standard ranges studies, which corresponds to the regression: $y = 0.4082x + 0.0021$ ($r = 0.9997$) for FCE22891.

The absolute recoveries of FCE22891 were measured in blank plasma spiked with FCE22891 at three different concentrations (Table 4). The

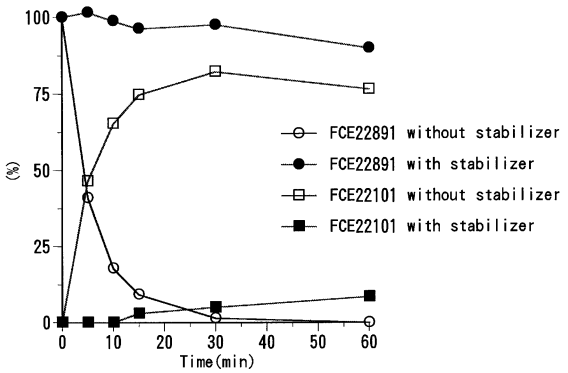

Fig. 6. Inhibitory effect of stabilizer on the enzymatic hydrolysis of FCE22891 in plasma (at 25°C).

Table 4
Recoveries of FCE22891 from plasma

Concentration added ($\mu g\ ml^{-1}$)	Recovery (%) (mean ± S.D., $n = 6$)
0.01	104.6 ± 2.9
0.10	95.7 ± 1.9
1.00	94.9 ± 1.5

results show that the recoveries were from 94.9 to 104.6% in plasma samples.

To evaluate the within-day and between-day reproducibility, three concentrations of FCE22891 were used. Within-day and between-day precision ranged from 0.8 to 10.9%; accuracy was from 96.0 to 101.9% for all samples assayed (Table 5). It was estimated that the limit of determination was 0.01 $\mu g\ ml^{-1}$. From the results, it is evident that the proposed method was satisfactory in both accuracy and precision.

Typical chromatograms of unchanged FCE22891 in plasma are shown in Fig. 7.

3.5. Pharmacokinetics of FCE22891 in dog

The two methods described were applied to the determination of FCE22101, P1, P2 and FCE22891 concentrations in plasma of dogs orally dosed with 3, 10 and 30 mg kg^{-1} of FCE22891 suspension.

The plasma concentrations and the pharmacokinetic parameters of FCE22101, P1 and P2 are shown in Fig. 8 and Table 6. Plasma levels of FCE22101 rose rapidly after a single oral administration of 3, 10 and 30 mg kg^{-1}, and the plasma concentrations of FCE22101 increased proportionally to the doses. The peak levels, reached at 0.5–0.56 h after administration, were 1.49, 3.16 and 9.42 $\mu g\ ml^{-1}$, respectively. The elimination half lives were from 0.5 to 0.54 h. The elimination half lives and Tmax of FCE22101 were almost constant regardless of dose administered, and the Cmax and AUC of FCE22101 were proportional to the dose, suggesting linearity of the pharmacokinetics of FCE22891. Plasma levels of P1 and P2 rose rather more slowly than those of FCE22101 to peak at 0.9–1.6 h after dosing, and

Table 5
Accuracy and precision for the plasma samples spiked with FCE22891

Concentration (μg ml^{-1})		Accuracy* (%)	Precision** (%)
Added	Found (mean ± S.D.)		
Within-day ($n = 6$)			
0.01	0.010 ± 0.0006	101.4	5.9
0.10	0.097 ± 0.0012	96.9	1.3
1.00	0.967 ± 0.0073	96.7	0.8
Between-day ($n = 6$)			
0.01	0.010 ± 0.0011	101.9	10.9
0.10	0.097 ± 0.0034	97.1	3.5
1.00	0.960 ± 0.0239	96.0	2.5

*Accuracy: (Found/added) × 100.
*Precision: Relative standard deviation (R.S.D.).

the elimination half lives of P1 and P2 were 1.2–2.2 h. Although it was difficult to determine where the P1 and P2 originated, it was considered according to the major metabolic pathways in Fig. 1, but the minor process would be possible from cleavage of the β-lactam ring of FCE22891, and de-esterification of this open ring product [19], either in the gastrointestinal tract or during absorption.

The plasma concentrations of unchanged FCE22891 after dosing were determined by the

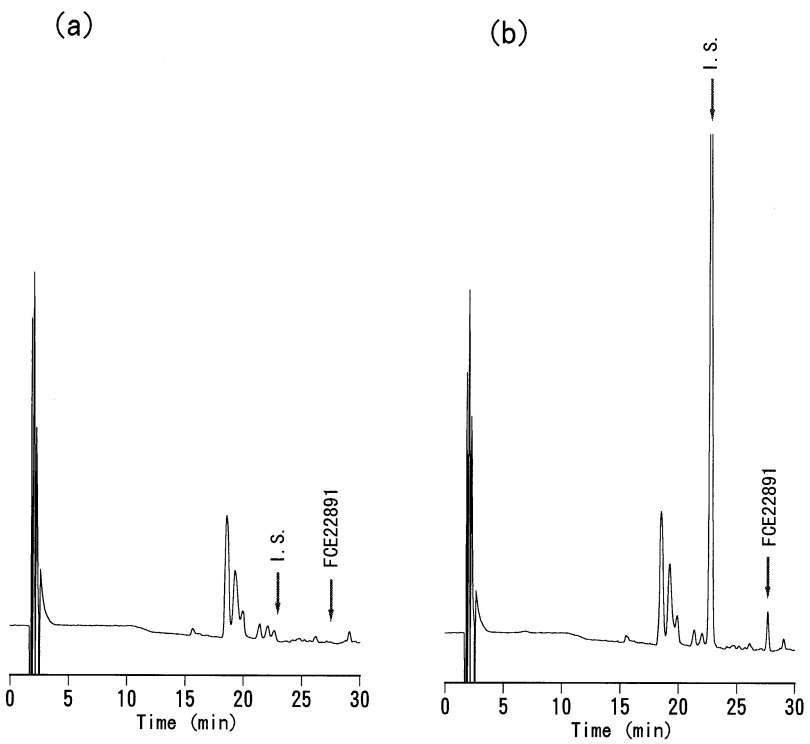

Fig. 7. Typical chromatograms of plasma samples; (a) blank plasma; (b) plasma spiked with FCE22891 (0.1 μg ml^{-1}).

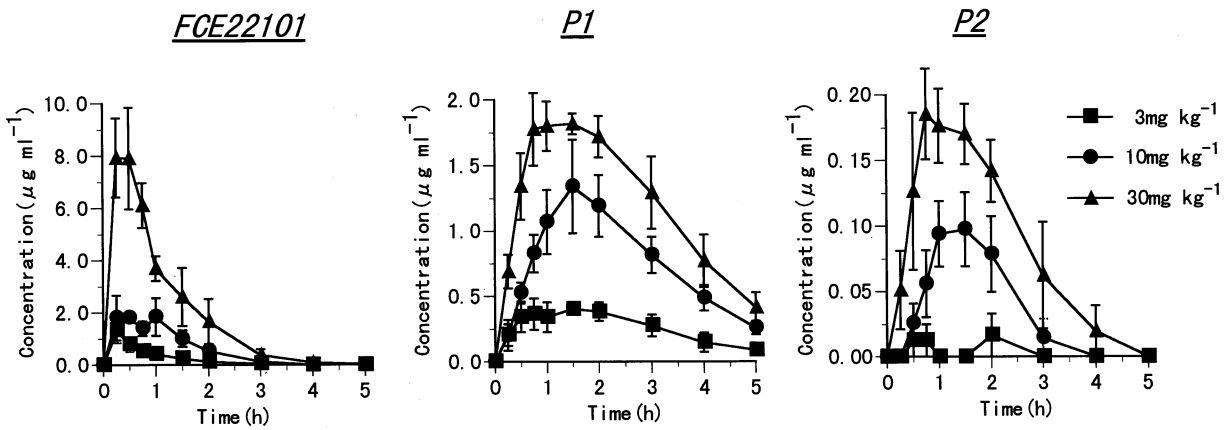

Fig. 8. Plasma concentrations of FCE22101, P1 and P2 after oral administration of FCE22891 at doses of 3, 10 and 30 mg kg^{-1} (mean ± S.E., $n = 4$).

proposed method. No unchanged FCE22891 was detected at any sampling points. The absence of unchanged FCE22891 in plasma even at the first sampling point, confirms that FCE22891 is rapidly hydrolyzed by blood or tissue esterases to the active metabolite, FCE22101.

3.6. Plasma levels of acetyl-L-carnitine and formaldehyde

It has been reported that the prodrug having the pivaloyloxymethyl ester group in this type of chemical structure was metabolized to produce pivalic acid. This fatty acid was condensed with endogenous L-carnitine and caused the low L-carnitine blood disease in human [20]; in general, acyl-L-carnitine might be generated from the fatty acid and L-carnitine [11,12]. It was thought that acetic acid given from the acetoxymethyl ester group of FCE22891 might combine with carnitine to produce acetyl-L-carnitine. Therefore, the plasma levels of acetyl-L-carnitine which might be generated from acetoxymethyl ester group after oral administration of FCE22891 were studied. The 1-aminoanthracene derivative method was used with HPLC by fluorimetric detection [21]. The plasma concentrations of L-carnitine and

Table 6
Pharmacokinetic parameters for FCE22101, P1 and P2 after oral administration of FCE22891

	Dose (mg kg^{-1})	Tmax (h)	Cmax (μg ml^{-1})	$t_{1/2}$ (h)	AUC5 (μg·h ml^{-1})	AUC∞ (μg·h ml^{-1})
FCE22101						
	3	0.56 ± 0.31	1.49 ± 0.35	0.54 ± 0.10	1.09 ± 0.09	1.09 ± 0.09
	10	0.56 ± 0.19	3.16 ± 0.38	0.52 ± 0.06	2.98 ± 0.38	2.98 ± 0.38
	30	0.50 ± 0.10	9.42 ± 1.57	0.50 ± 0.05	9.91 ± 1.10	9.92 ± 1.11
P1						
	3	0.94 ± 0.36	0.51 ± 0.02	1.22 ± 0.11	1.31 ± 0.13	1.48 ± 0.22
	10	1.63 ± 0.13	1.37 ± 0.35	1.19 ± 0.09	3.78 ± 0.67	4.24 ± 0.74
	30	1.31 ± 0.28	2.04 ± 0.16	1.18 ± 0.08	6.10 ± 0.51	6.84 ± 0.72
P2						
	10	1.38 ± 0.31	0.10 ± 0.03	2.19 ± 0.33	0.18 ± 0.04	0.18 ± 0.04
	30	0.88 ± 0.22	0.21 ± 0.03	1.69 ± 0.45	0.43 ± 0.07	0.43 ± 0.07

Values are mean ± S.E., $n = 4$.

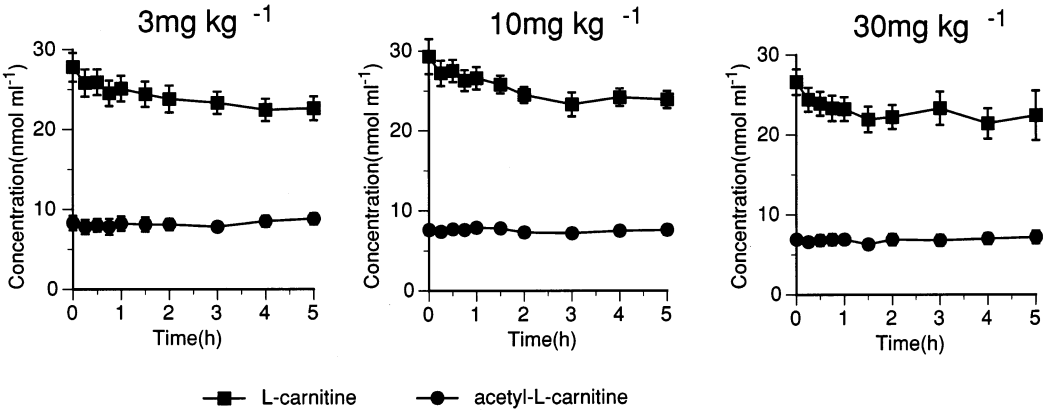

Fig. 9. Plasma levels of L-carnitine and acetyl-L-carnitine after oral administration of FCE22891 (mean ± S.E., $n = 4$).

acetyl-L-carnitine after administration of FCE22891 almost maintained endogenous levels (Fig. 9). It was considered that the acetic acid metabolized from the ester group of FCE22891 was not condensed with endogenous L-carnitine in biological fluids. A similar metabolic fate of FCE22891 has been reported in the study performed in rat using ^{14}C-labelled compound in the ester group [22]. The ester moiety of the antibiotic was metabolized to $^{14}CO_2$.

Moreover, the plasma concentration of formaldehyde which might be derived from the acetoxymethyl ester group of FCE22891 after oral administration was measured. The 1,3-cyclohexanedione derivative method was used with HPLC by fluorimetric detection [23]. The plasma concentrations of formaldehyde remained at endogenous levels, and it was not dependent on the plasma level of FCE22101 after oral administration of FCE22891. It was assumed that formaldehyde produced from the ester group of FCE22891 by esterase was metabolized to CO_2 [24,25].

4. Conclusions

The methods developed are simple, sensitive and reproducible for the simultaneous determination of FCE22101, P1 and P2, and for the determination of unchanged FCE22891 in the plasma of dog. Plasma levels of FCE22101 rose rapidly after single oral administration, and the peak levels were 1.49, 3.16 and 9.42 μg ml^{-1}, respectively. The pharmacokinetic property of FCE22891 was supposed to be linear after oral administration at doses of 3–30 mg kg^{-1}. Tmax of P1 and P2 were greater than those of FCE22101, and the elimination half lives of P1 and P2 were 1.2–2.2 h. No unchanged FCE22891 was detectable in any plasma samples after dosing. The plasma concentrations of L-carnitine and acetyl-L-carnitine or formaldehyde after dosing were maintained at endogenous levels.

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