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Short communication

Determination of total cefozopran concentrations in human peritoneal fluid by HPLC with cefepime as an internal standard: Comparative pharmacokinetics in the fluid and plasma

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ABSTRACT

A simple, rapid and precise HPLC method using ultrafiltration to remove protein was developed to determine total cefozopran concentrations in human peritoneal fluid in the same manner as in human plasma, irrespective of the amount of protein. The recovery of cefozopran after ultrafiltration in peritoneal fluid was higher than that in plasma, because the protein content in peritoneal fluid was lower than that in plasma. Furthermore, it was found that an internal standard with a similar protein-binding ratio to cefozopran could revise the cefozopran loss by ultrafiltration in plasma and peritoneal fluid samples irrespective of the amount of protein. Therefore, it was concluded that cefepime may be used as an internal standard. Cefozopran and cefepime were detected by measuring their ultraviolet absorbances at 235 nm. The calibration curve obtained for cefozopran in peritoneal fluid was linear from 0.2 to 200 μg/ml. The intraday and interday precisions were less than 5.77% (CV), and the accuracy was between 96.3% and 108% above 0.2 μg/ml. The lower limit of detection was 0.05 μg/ml in peritoneal fluid, which was the same as that in plasma. The assay has been applied to therapeutic drug monitoring of cefozopran in both plasma and peritoneal fluid and has contributed to peritoneal pharmacokinetic studies in patients.
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1. Introduction

Cefozopran (Fig. 1) is a parenteral cephalosporin that has been clinically available since the late 1990s, and possesses a broad antibacterial spectrum ranging from gram-positive to gram-negative aerobic and anaerobic bacteria [1]. Recently, cefozopran has often been used for antibacterial prophylaxis in abdominal surgery and for treatment of postoperative intra-abdominal infections [2]. Although the concentration of a drug in its target tissue (peritoneal fluid for cefozopran) is a key determinant of its efficacy, therapeutic drug monitoring of cefozopran has only been carried out using plasma. A major reason for this situation is the lack of a reliable determination method for peritoneal fluid. Therefore, it is important to develop a determination method for cefozopran in peritoneal fluid to monitor the concentrations of this drug.

Microbiological assays have traditionally been used for determining cephalosporin concentrations. However, it is known that microbiological assays require a long time and cannot differentiate cefozopran from other antibiotics. Although other excellent methods, such as micellar electrokinetic capillary chromatography, have been reported for the determination of cefozopran [3], capillary electrophoresis systems do not tend to be readily available. On the other hand, HPLC is a rapid and specific method, and HPLC systems are widely installed in clinical settings. However, there are no reports detailing a measurement method for cefozopran using HPLC except for that of Borner et al. [4,5]. Borner's method involves deproteinization of plasma by acetonitrile, followed by extraction and removal of the acetonitrile with methylene chloride because cefozopran is a hydrophilic drug. In a previous paper by Ikeda et al. [6], a simple one-step method using ultrafiltration to remove plasma proteins was reported and shown to be able to quantify cefozopran more easily and rapidly than Borner's method.

The purpose of the present study was to determine the cefozopran concentrations in both plasma and peritoneal fluid, although the protein contents of these samples differ (peritoneal fluid has a lower protein content). Therefore, the present method should be verified for practical use, in which an internal standard (IS) with a similar protein-binding ratio to cefozopran can revise the cefozopran loss by ultrafiltration in plasma and peritoneal fluid samples. Pharmacokinetic–pharmacodynamic studies for antibacterial prophylaxis in abdominal surgery and treatment of postoperative intra-abdominal infections were also performed.

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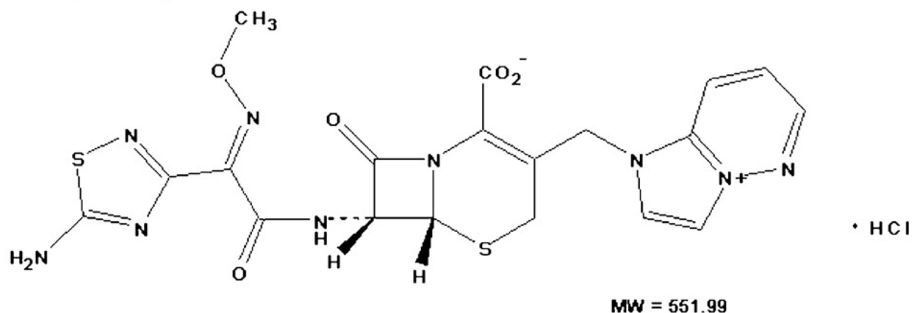

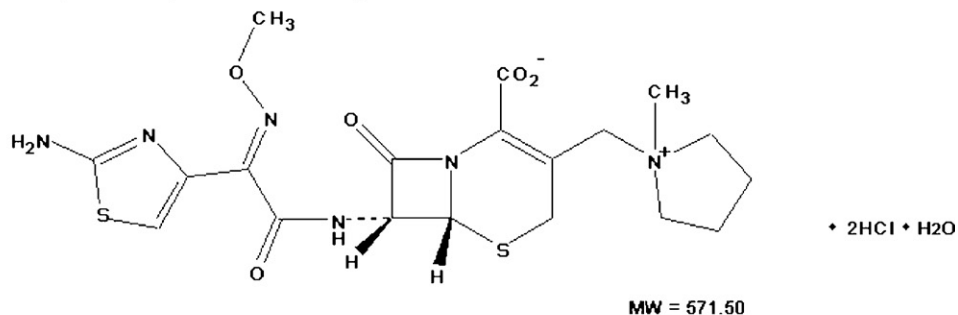

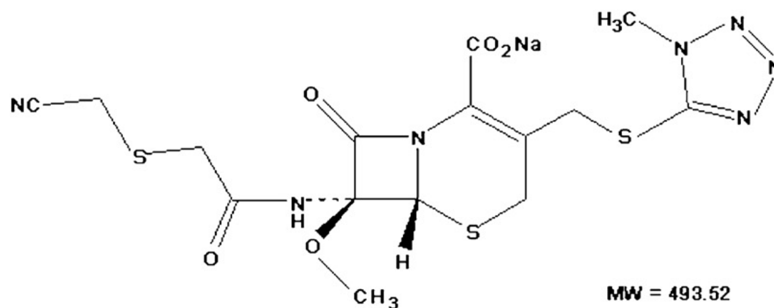

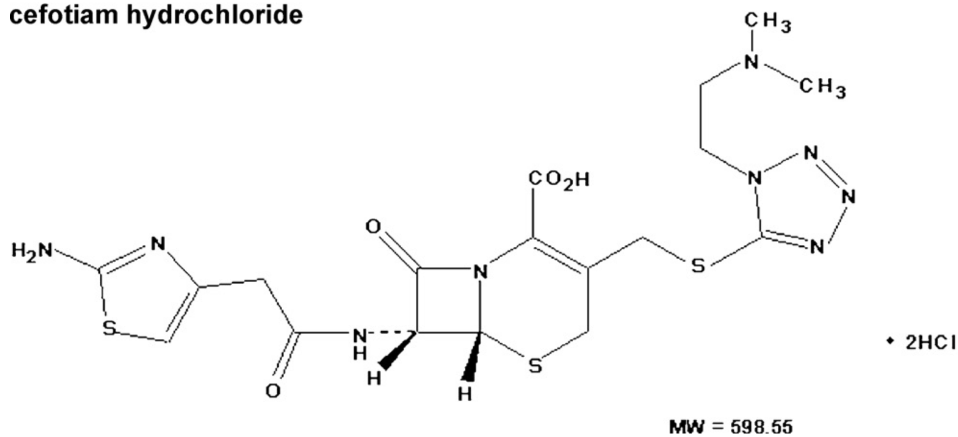

Fig. 1. Structures of cefozopran and IS.

2. Experimental

2.1. Reagents and materials

The cefozopran hydrochloride (titer, 932 μg/mg) used as a standard was provided by Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). Cefepime dihydrochloride hydrate (Fig. 1), which was used as an IS, was provided by Bristol-Myers Squibb K.K. (Tokyo, Japan). PIC B7 (Low UV), an ion-pairing reagent with a principal ingredient of heptanesulfonic acid, was purchased from Waters Corporation (Milford, MA, USA). Acetonitrile was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Nanosep 10K centrifugal filter devices were purchased from Pall Corporation (East Hills, NY, USA). All other chemicals were of analytical grade.

2.2. Equipment

The HPLC system consisted of a 600E system controller, a 700 Satellite WISP auto-sampler (Waters Corporation), an SPD-20A UV spectrophotometric detector (Shimadzu Corporation, Kyoto, Japan) and a Chromatocorder 21 (System Instruments Co. Ltd., Tokyo, Japan). The levels of albumin and total protein in plasma and peritoneal fluid were assayed using an S40 Clinical Analyzer (Hitachi Ltd., Tokyo, Japan), a desktop type clinical analysis instrument.

2.3. Chromatographic conditions

The samples were separated by chromatography using a Symmetry C18 5 μm (4.6 mm × 150 mm) column (Waters Corporation). The mobile phase was water–PIC B7–acetonitrile (953:2:45, v/v/v) and the pump flow rate was 1.0 ml/min. The auto-sampler was set to 4 °C. Each sample was diluted with an equal volume of 0.4% PIC B7 solution and the injection volume was 40 μl. The mobile phase was also used as a washing solution for the auto-sampler. The column temperature was room temperature (25 °C). The cefozopran and IS peaks were detected by ultraviolet absorbance at 235 nm. As an IS, three β-lactams (cefepime, cefmetazole and cefotiam) were checked for their abilities to revise the cefozopran loss by ultrafiltration.

2.4. Peritoneal fluid and plasma samples

Peritoneal fluid samples were stored at −40 °C until analysis. Control peritoneal fluid, a mixture of equal volumes of peritoneal fluid from six patients who were not given cefozopran, was also stored at −40 °C.

Blood samples were collected into heparinized vacuum tubes (Nipro Corporation, Osaka, Japan), and separated by centrifugation at 1000 × *g* for 10 min at 4 °C. Plasma samples were stored at −40 °C until analysis. Control human plasma, a mixture of equal volumes of plasma from six healthy volunteers, was also stored at −40 °C.

2.5. Analytical procedure

A stock solution of cefozopran was prepared daily. The control peritoneal fluid sample was spiked with cefozopran to final concentrations of 0.2, 0.5, 1.0, 5.0, 25.0, 50.0, 100, 150, and 200 μg/ml. Samples (100 μl) were mixed with 300 μl of 4.0 μg/ml IS (cefepime dihydrochloride hydrate) solution and transferred to Nanosep 10K centrifugal filter devices. The devices were centrifuged at 12,000 × *g* for 10 min at room temperature. The filtrates were diluted with an equal volume of 0.4% PIC B7 solution and aliquots (40 μl) were injected into the HPLC system for analysis. The plasma samples were processed in a similar manner to the peritoneal fluid samples.

Table 1
Accuracy and intraday and interday precision data for the measurement of total cefozopran in human peritoneal fluid.

Concentration added (μg/ml)	Total concentration found (mean ± S.D.) (μg/ml)	CV (%)	Accuracy (%)
Intraday assay (n = 6)			
0.2	0.216 ± 0.007	3.40	108
0.5	0.494 ± 0.004	0.85	98.9
1.0	0.989 ± 0.022	2.22	98.9
5.0	4.81 ± 0.02	0.50	96.3
25.0	24.6 ± 0.3	1.12	98.2
50.0	49.8 ± 0.3	0.68	99.6
100	99.6 ± 0.5	0.46	99.6
150	152 ± 1	0.74	101
200	199 ± 2	0.77	99.6
Interday assay (n = 6)			
0.2	0.201 ± 0.008	3.83	101
0.5	0.525 ± 0.030	5.77	105
1.0	0.989 ± 0.046	4.65	98.9
5.0	4.95 ± 0.13	2.62	99.0
25.0	24.5 ± 0.6	2.46	98.0
50.0	50.0 ± 0.7	1.33	100
100	99.8 ± 0.9	0.92	99.8
150	150 ± 1	0.50	100
200	200 ± 1	0.50	100

S.D.: standard deviation; CV (%): coefficient of variation.

2.6. Method validation

A calibration curve was created using the ratio of the observed peak areas of cefozopran and the IS (cefepime). A linear regression analysis of the calibration data was performed using the equation $y = mx + b$ with a weighting of $1/y$, where y is the peak area ratio, x is the concentration of cefozopran, and m and b are the slope and intercept, respectively.

The method was evaluated for linearity, specificity, accuracy and precision (expressed as the percent coefficient of variation, CV (%)). Peritoneal fluid standard samples (0.2, 0.5, 1.0, 5.0, 25.0, 50.0, 100, 150 and 200 μg/ml) were prepared using the control peritoneal fluid, and the intraday and interday assay precision and accuracy were determined. The limit of detection (LOD) of cefozopran was determined from the peak and standard deviation of the noise level, S_N. The LOD was defined as the sample concentration of cefozopran that resulted in peak areas threefold higher than the S_N. The limit of quantification (LOQ) of cefozopran was determined from the validation data shown in Table 1. The lower LOQ was chosen as the concentration that provided measurements with a precision and accuracy within the recommended ±20% from their nominal values, in accordance with the FDA guidelines [7].

2.7. Specificity

To evaluate the specificity of the present method, six blank peritoneal fluid samples from patients who were not given cefozopran were investigated for interference by endogenous matrix components. Specificity was also assessed in the presence of other β-lactams, namely biapenem, meropenem, doripenem, cefotiam and cefmetazole, at a concentration of 20.0 μg/ml.

2.8. Stability

Borner et al. [5] reported that cefozopran was stable in plasma at −20 °C for at least 4 months. In the present study, the stability of peritoneal fluid samples containing 5.0, 50.0 and 100 μg/ml of cefozopran was examined after storage at −40 °C. Control peritoneal fluid samples were spiked to contain 5.0, 50.0 and 100 μg/ml of cefozopran and stored at −40 °C. The cefozopran concentrations

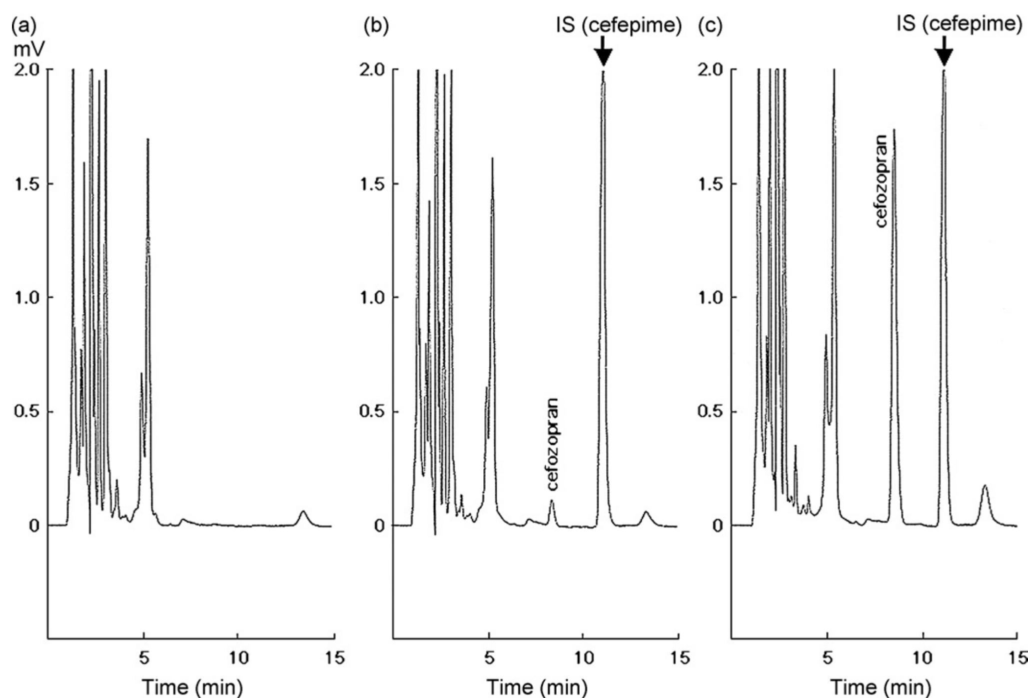

Fig. 2. Typical chromatograms: (a) Blank control peritoneal fluid. (b) Control peritoneal fluid spiked with 0.5 μg/ml cefozopran. (c) Patient peritoneal fluid sample (7.8 μg/ml, 6.5 h after administration to patient B in Fig. 4).

were determined at 0, 15, 30 and 60 days ($n = 3$). When determining the cefozopran concentration, an IS (cefepime) solution was added.

The freeze–thaw stabilities of peritoneal fluid samples containing 5.0, 50.0 and 100 μg/ml of cefozopran were also examined. The samples were stored at −40 °C for 24 h, completely thawed at room temperature (25 °C) and then refrozen at −40 °C for 12 h. The freeze–thaw cycle was repeated two more times, and the samples were analyzed after the third cycle ($n = 3$). When determining the cefozopran concentration, an IS (cefepime) solution was added.

2.9. Application to pharmacokinetic studies in patients

Two abdominal surgery patients (patients A and B) received a 0.5 h infusion of cefozopran (1 g) every 12 h. Patients A and B provided written informed consent to participate in this study. Their plasma and peritoneal fluid concentrations of cefozopran were measured at 0, 0.5, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 h after starting the infusion. Changes in the plasma and peritoneal fluid cefozopran concentrations were fitted to a three-compartment model and analyzed using a nonlinear least-squares computer program (MULTI-Win) [8]. The exposure time (T) during which the drug concentration remained at the minimum inhibitory concentration (MIC) for microorganisms, T > MIC (% of 24 h), for these patients was determined [9], because the efficiency of cefozopran is related to the T > MIC.

3. Results

3.1. Chromatographic conditions

Since the pH of the mobile phase is important for separation of ionized substances by reversed-phase HPLC, the optimal pH of the mobile phase for separation of the cefozopran peak from endogenous peaks in peritoneal fluid or plasma was examined using sodium phosphate solution (pH 3.0–7.0; 50 mM)–acetonitrile (94:6, v/v). However, between pH 3.0 and 7.0, the peak of cefozopran overlapped with endogenous compounds in plasma (data not shown). All the plasma samples from the six healthy volunteers had these overlapping endogenous compounds.

Subsequently, PIC B7 was used for separation of the peaks of cefozopran and the endogenous plasma components. PIC B7 is an ion-pairing reagent, and PIC B7 and cefozopran make an ion-pair. With a mobile phase of water–acetonitrile (955:45, v/v), the separation factor (α) of these peaks in plasma samples was 1.04 and their resolution factor (Rs) was 0.76. As the amount of PIC B7 in the mobile phase increased, α and Rs increased: $\alpha = 1.11$ and Rs = 1.94 with water–PIC B7–acetonitrile (953:2:45, v/v/v). Therefore, water–PIC B7–acetonitrile (953:2:45, v/v/v) was used as the mobile phase.

3.2. Typical chromatograms

Fig. 2 shows a biological matrix with blank control peritoneal fluid (Fig. 2a), blank control peritoneal fluid spiked with 0.5 μg/ml of cefozopran (Fig. 2b) and a 6.5 h peritoneal fluid sample from patient B with a concentration of 7.8 μg/ml (Fig. 2c). Interfering peaks were not evident and the retention time for cefozopran was 8.4 min.

3.3. Method validation

Table 1 summarizes the reproducibility and accuracy for each calibration standard from 0.2 to 200 μg/ml. For both the intraday and interday assays of cefozopran in peritoneal fluid, all CV values

Table 2
Recovery study in human peritoneal fluid.

Concentration of cefozopran (μg/ml)	Cefozopran recovery ($n = 6$, mean ± S.D.) (%)	IS (cefepime) recovery ($n = 6$, mean ± S.D.) (%)
1.0	84.6 ± 2.1	88.0 ± 0.4
5.0	87.2 ± 1.5	90.7 ± 1.3
50.0	89.9 ± 0.5	89.6 ± 0.5
100	89.5 ± 0.6	89.2 ± 0.4
200	88.2 ± 1.1	90.3 ± 1.2

were ≤5.77% and the accuracy values were 96.3–108%. These data were almost the same as those in plasma: all CV values were ≤7.34% and accuracy values were 96.6–104% [6]. The calibration curve of cefozopran in human peritoneal fluid was also linear from 0.2 to 200 μg/ml (r^2 > 0.999 (mean) ± 3.48 × 10^{-5} (S.D.), n = 6).

The total cefozopran concentrations were determined using cefozopran/cefepime. As shown in Tables 2 and 3, the recoveries of cefozopran and an IS (cefepime) were almost the same. Therefore, the cefepime protein-binding ratio was similar to that for cefozopran and could revise the cefozopran loss by ultrafiltration (Fig. 3a and b). Two other β-lactams (cefmetazole and cefotiam) were checked for their abilities to act as an IS. However, cefmetazole and cefotiam had relatively high protein-binding abilities compared with cefozopran, and could not be used as an IS (Fig. 3c and d). When the albumin content increased, the recovery of cefmetazole (or cefotiam) decreased and therefore the peak area ratio of cefozopran to cefmetazole (or cefotiam) increased (Fig. 3c and d). It was not clear why the peak area ratios of cefozopran to cefmetazole in peritoneal fluid samples differed from those in plasma samples. Taken together, these data indicate that cefepime can be used as an IS, whereas the other two β-lactams cannot.

Table 3 shows the recoveries of cefozopran from various plasma and peritoneal fluid samples in detail. When the recovery of cefozopran tended to increase at lower concentrations of plasma and peritoneal fluid (100% < 50% < 25% dilution), the recovery of cefepime with a similar protein-binding ratio to cefozopran increased at the same rate as cefozopran (Table 3). Therefore, the peak area ratios of cefozopran to cefepime were almost the same (Table 3, Fig. 3b). The total cefozopran concentration could be successfully determined using cefepime as an IS.

3.4. Limits of detection and quantification

The LOD of peritoneal fluid, defined as the concentration of cefozopran giving a signal-to-noise ratio of >3:1, was 0.05 μg/ml, using a 40 μl injection volume. A 40 μl injection volume corresponded to a 20 μl filtrate volume because the filtrate was diluted with an equal volume of 0.4% PIC B7 solution. Since all the precision and accuracy data were <10% (Table 1), the LOQ was determined to be the lowest concentration of standard used (0.2 μg/ml [CV: 3.40%, accuracy: 108%, intraday assay, n = 6]).

3.5. Specificity

Six peritoneal fluid samples were investigated for interference by endogenous matrix components, and no interference peaks were observed. Specificity was assessed in the presence of other β-lactams, namely biapenem, meropenem, doripenem, cefotiam and cefmetazole, at a concentration of 20.0 μg/ml. None of the chromatograms revealed any limitations for the assay.

3.6. Stability

The stability of peritoneal fluid samples containing 5.0, 50.0 and 100 μg/ml of cefozopran was examined after storage at −40 °C. The mean concentrations (±S.D.; n = 3 for each) of cefozopran after 60 days were 98.2 ± 0.6%, 98.3 ± 1.5% and 102 ± 0.3% of the initial concentrations of 5.0, 50.0 and 100 μg/ml, respectively.

The freeze–thaw stabilities of peritoneal fluid samples containing 5.0, 50.0 and 100 μg/ml of cefozopran were also examined. The mean concentrations (±S.D.; n = 3 for each) after three freeze–thaw cycles were 101 ± 0.4%, 96.4 ± 3.1% and 103 ± 1.8% of the initial concentrations of 5.0, 50.0 and 100 μg/ml, respectively.

Table 3
Recovery study to examine the influence of variations in the protein contents of frozen-thawed plasma and peritoneal fluid samples using cefepime as an IS.

Plasma or peritoneal fluid	Recovery (n = 6) of cefozopran (mean ± S.D.) (%)	Recovery (n = 6) of IS (cefepime) (mean ± S.D.) (%)	Peak area ratio of cefozopran to IS (cefepime) (mean ± S.D.)	Total concentration found (mean ± S.D.) (μg/ml)	Albumin (g/dl)	Total protein (g/dl)
Control plasma	85.4 ± 1.0	87.8 ± 1.6	5.22 ± 0.06	49.4 ± 0.5	4.6	7.5
Control plasma diluted by 50%	87.7 ± 0.6	89.6 ± 1.8	5.22 ± 0.11	49.3 ± 1.1	2.3	3.8
Control plasma diluted by 25%	94.0 ± 0.3	93.5 ± 1.1	5.40 ± 0.05	51.1 ± 0.5	1.2	1.9
Plasma A	87.2 ± 0.5	89.8 ± 1.1	5.22 ± 0.05	49.3 ± 0.5	5.0	7.6
Plasma B	88.4 ± 0.5	89.6 ± 1.4	5.30 ± 0.07	50.1 ± 0.7	4.5	7.1
Plasma C	88.5 ± 0.8	88.1 ± 1.1	5.40 ± 0.09	51.0 ± 0.8	4.2	7.3
Plasma D	86.3 ± 0.6	88.7 ± 1.0	5.23 ± 0.04	49.4 ± 0.4	4.4	7.4
Plasma E	86.5 ± 0.5	90.8 ± 1.5	5.12 ± 0.07	48.4 ± 0.6	4.4	7.2
Control peritoneal fluid	89.9 ± 0.5	89.6 ± 0.5	5.34 ± 0.04	49.9 ± 0.4	1.8	3.3
Control peritoneal fluid diluted by 50%	93.1 ± 0.2	92.4 ± 1.2	5.36 ± 0.06	50.2 ± 0.6	0.9	1.7
Control peritoneal fluid diluted by 25%	96.1 ± 0.4	96.7 ± 1.9	5.28 ± 0.10	49.4 ± 1.0	0.5	0.8
Peritoneal fluid F	92.3 ± 0.2	92.9 ± 1.5	5.27 ± 0.08	49.4 ± 0.8	1.0	2.1
Peritoneal fluid G	87.6 ± 0.6	88.7 ± 1.4	5.25 ± 0.05	49.1 ± 0.5	1.6	2.5
Peritoneal fluid H	90.8 ± 0.7	91.5 ± 0.8	5.27 ± 0.02	49.4 ± 0.2	2.2	4.1
Peritoneal fluid I	93.8 ± 0.7	92.8 ± 0.3	5.37 ± 0.05	50.3 ± 0.5	1.2	2.3
Peritoneal fluid J	88.6 ± 1.3	89.3 ± 1.9	5.27 ± 0.05	49.4 ± 0.5	3.1	5.3

The concentration of cefozopran was 50 μg/ml.

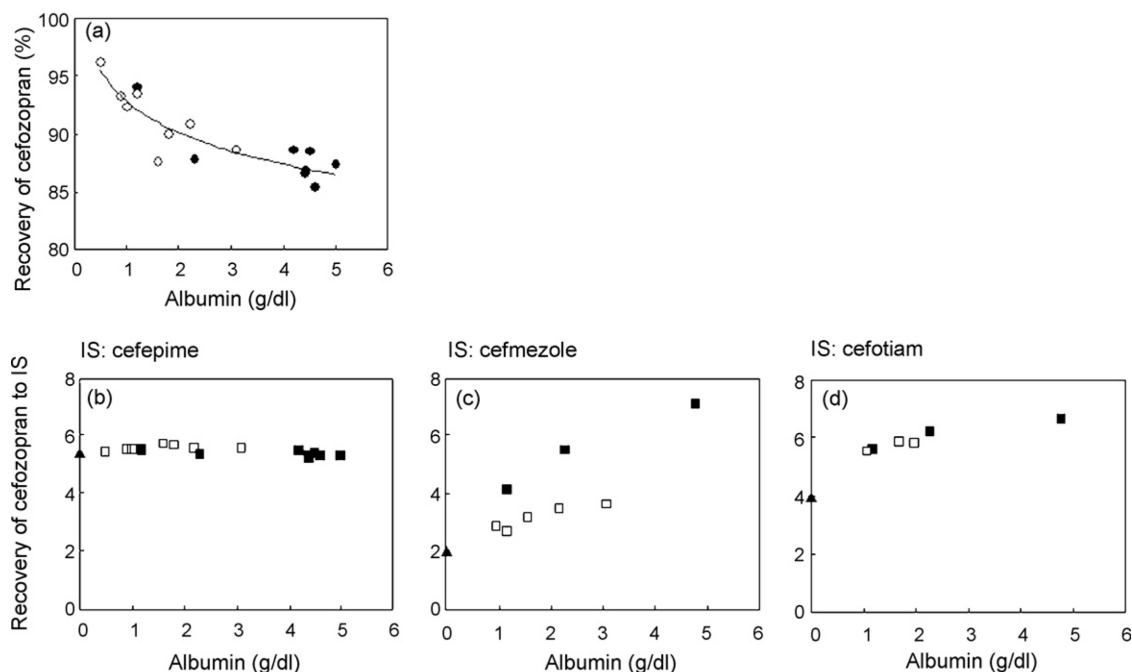

Fig. 3. Recoveries of cefozopran relative to the albumin content and peak area ratios of cefozopran to the IS relative to the albumin content. (a) Recoveries of cefozopran relative to the albumin content. Plasma: closed circles; peritoneal fluid: open circles (means, $n = 6$). (b)–(d) Peak area ratios of cefozopran to the IS relative to the albumin content. Plasma or peritoneal fluid samples (100 μl; cefozopran concentration, 50.0 μg/ml) were mixed with 300 μl of 4.0 μg/ml cefepime dihydrochloride hydrate (b), 20.0 μg/ml cefmetazole sodium (c) or 14.0 μg/ml cefotiam hydrochloride (d), and transferred to Nanosep 10K centrifugal filter devices. Standard solution (without ultrafiltration): closed triangles; plasma: closed squares; peritoneal fluid: open squares (cefepime dihydrochloride, means, $n = 6$; cefmetazole sodium and cefotiam hydrochloride, means, $n = 3$). The mobile phase in (d) was water–PIC B7–acetonitrile (923:2:75, v/v/v).

Table 4
Patient information and pharmacokinetic data of the patients administered cefozopran (1 g) over 0.5 h infusion.

Patient	Sex	Age (years)	Weight (kg)	C_{max} in plasma (μg/ml)	C_{max} in peritoneal fluid (μg/ml)	$T > MIC$ (%)[a]	
						Plasma MIC = 8 μg/ml	Peritoneal fluid MIC = 8 μg/ml
A	M	38	68	83.9	62.6	85	100
B	F	23	59	114	56.5	73	74

[a] Predicted $T > MIC$ (% of 24 h) when cefozopran (1 g) was administrated over 0.5 h infusion every 12 h.

3.7. Application to peritoneal pharmacokinetic studies in patients

Table 4 and Fig. 4 show the results for peritoneal pharmacokinetic studies in two patients. A three-compartment model (central, peripheral and peritoneal) was chosen as the basic pharmacokinetic model because it described the current data set better than a two-compartment model (central and peritoneal). The Akaike information criterion (AIC) values were 42.3 and 66.5 for patients A and B, respectively.

Although the simulated peak concentrations of cefozopran in plasma were 83.9 and 114 μg/ml for patients A and B, respectively, the corresponding peak concentrations of cefozopran in peritoneal fluid were 62.6 and 56.5 μg/ml, respectively (Table 4). However, the $T > MIC$ values in peritoneal fluid at the MIC of 8 μg/ml were equivalent to or somewhat larger than those in plasma. The peritoneal pharmacokinetic studies for these two patients revealed that intravenous cefozopran penetrated the peritoneal fluid rapidly and extensively, possibly because the relatively low protein-binding

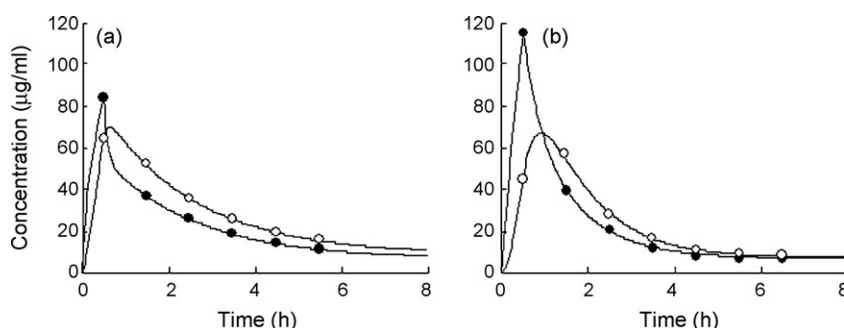

Fig. 4. Time courses of cefozopran concentrations in plasma and peritoneal fluid samples from two patients infused with 1 g of cefozopran for 0.5 h: (a) patient A and (b) patient B. Plasma: closed circles; peritoneal fluid: open circles. The lines show the changes in the plasma and peritoneal fluid cefozopran concentrations fitted to a three-compartment model.

ratio brought about high penetration. The drug-exposure times in peritoneal fluid were greater than or equal to those in plasma (Table 4), maintaining over 70% of the $T > \text{MIC}$ required for bactericidal effects of cefozopran. For cephalosporins, the $T > \text{MIC}$ target required is 60–70% of the dosing interval [10,11].

4. Discussion

Cefozopran bound to plasma or peritoneal fluid proteins cannot be separated by ultrafiltration. The present study showed that total cefozopran could be quantified using cefepime with a similar protein-binding ratio to cefozopran to revise the cefozopran loss by ultrafiltration. The influence of cefepime on cefozopran determinations in the presence of various plasma or peritoneal fluid proteins was confirmed. The recovery of cefozopran after ultrafiltration tended to increase at lower concentrations of plasma or peritoneal fluid when control plasma or peritoneal fluid samples diluted 1:2 and 1:4 with water were compared. However, the total cefozopran concentrations detected were not influenced by the albumin or total protein concentrations in the plasma or peritoneal fluid.

Borner et al. [5] determined the concentration of cefozopran and reported its recovery from spiked blank sera by solvent extraction. For serum, 10 concentrations ranging from 0.7 to 160 mg/l were examined. They reported that the overall recovery was 96.7% (range, 84.0–103%). The recoveries of the drug by solvent extraction were superior to those by ultrafiltration. However, cefepime could revise the cefozopran loss by ultrafiltration.

Both cefozopran and cefepime are fourth-generation cephem antibiotics. For practical applications, antibiotics of similar generations are not administered to patients simultaneously. Therefore, the fluid samples from patients did not contain both cefozopran and cefepime.

After centrifugation for 10 min, filtrates can be analyzed by HPLC, which requires only a few minutes and no special techniques. Since the data for patients A and B in this study were obtained within 1 h after the final sampling time in time-management analyses, subsequent medication could be changed according to the findings. It is concluded that the present method may become a very useful, simple and rapid technique in clinical settings as well as for pharmacokinetic–pharmacodynamic studies.

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