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Short communication

Real-time therapeutic drug monitoring of cefozopran in plasma using high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple, rapid, and precise HPLC method using ultrafiltration to remove plasma protein was developed to determine cefozopran concentrations in human plasma for real-time therapeutic drug monitoring. Plasma was separated by centrifugation at 4 °C from blood collected in heparinized vacuum tubes. Cefozopran and an internal standard were detected by ultraviolet absorbances at 235 nm with no interfering plasma peak. The calibration curve of cefozopran in human plasma was linear from 0.2 to 200 μg/ml. The limit of detection was 0.05 μg/ml. The assay was applied to febrile neutropenia patients in a clinical setting.

Keywords: Cefozopran; Ultrafiltration; HPLC; Therapeutic drug monitoring; Febrile neutropenia

1. Introduction

Bacteria constitute an important cause of infection in neutropenic patients with cancer and other underlying myelosuppressive diseases, and febrile neutropenia is an important cause of morbidity [1]. Cefozopran (Fig. 1) is a parenteral cephalosporin which possesses a broad antibacterial spectrum ranging from Gram-positive to Gram-negative aerobic and anaerobic bacteria [2]. In Japan, cefozopran is the first-line antimicrobial agent for febrile neutropenia [3].

It has been reported that cephalosporins exhibit time-dependent in vitro and in vivo activity, and that the clinical outcome closely correlates with the duration of time that the drug concentration remains at the minimum inhibitory concentration (MIC) for the microorganisms (time above MIC: $T>$MIC). For cephalosporins, the $T>$MIC target required is the 60–70% of the dosing interval [4,5]. To confirm $T>$MIC meets this requirement, therapeutic drug monitoring (TDM) for febrile neutropenia patients is necessary but is not often undertaken. Real-time TDM of cefozopran is a desirable procedure for lifesaving of febrile neutropenia patients.

Traditionally, microbiological assay has been used for determinations of cephalosporins. But microbiological assay needs time and its specificity is low. HPLC is a rapid and specific method, and HPLC systems have been widely installed in clinical settings. Although other excellent methods such as micellar electrokinetic capillary chromatography have been reported for determination of cefozopran [6], unfortunately capillary electrophoresis systems are usually not available in clinical settings. Apart from that of Borner et al. [7,8], there are no reports detailing a measurement method for cefozopran using HPLC. Borners' method was that after deproteinization of plasma by acetonitrile, the acetonitrile was extracted and removed with methylene chloride because cefozopran is a hydrophilic drug. The procedure was therefore a little complex and needed time, which might be one of the reasons that TDM is hardly

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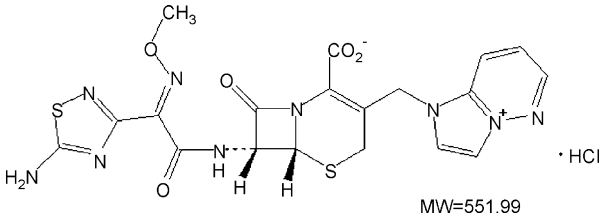

Fig. 1. Structure of cefozopran.

undertaken. In a clinical setting, especially for real-time TDM, it is desirable that the determination method is both simple and rapid. In many cases there are no special mechanical devices and little time and/or no highly skilled personnel. Thus, a simple one step method using ultrafiltration to remove plasma protein was developed to enable cefozopran levels to be more easily and rapidly determined in human plasma than by conventional methods.

2. Experimental

2.1. Reagents and materials

Standard cefozopran was provided by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Cefepime dihydrochloride which was used as the internal standard (IS) was provided by Bristol-Myers Squibb K.K. (Tokyo, Japan). PIC B7 (low UV) was purchased from Waters Corporation (Milford, MA, USA). Acetonitrile was purchased from Sigma–Aldrich Japan (Tokyo, Japan). The Nanosep 10 K centrifugal filter device was purchased from the PALL Corporation (NY, USA).

2.2. Equipment

The HPLC system comprised auto-sampler SIL-10ADVP, HPLC pump LC-6A, UV spectrophotometric detector SPD-6A (Shimadzu Corporation, Kyoto, Japan), and Chromatocorder 21 (System Instruments Co., Ltd., Tokyo, Japan).

2.3. Chromatographic conditions

The samples were separated by chromatography on a symmetry C_{18} 5 μm (4.6 mm × 150 mm) column (Waters Corporation, Milford, MA, USA). 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, and the injection volume was 20 μl. The mobile phase was also used as a washing solution of auto sampler. The column temperature was at room temperature (25 °C). The cefozopran and IS peaks were detected by ultraviolet absorbance at 235 nm.

2.4. Plasma samples

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 was a mixture of an equal volume of plasma from six healthy volunteers and stored at −40 °C.

2.5. Analytical procedures

A working stock solution of cefozopran was prepared daily at a concentration of 1 mg/ml in water. Control plasma was spiked with cefozopran with the final concentrations corresponded to 0.2, 0.5, 1.0, 5.0, 25.0 50.0, 100.0, 150.0, and 200.0 μg/ml. Samples (100 μl) were then mixed with 300 μl of 4 μg/ml IS solution and transferred to a Nanosep 10 K centrifugal filter device. Clinical plasma samples (100 μl) were mixed with 300 μl of 4 μg/ml IS solution and transferred to a Nanosep 10 K centrifugal filter device. The devices were centrifuged at 12,000 × *g* for 10 min at room temperature. Filtrate (20 μl) was injected into the HPLC system for analysis.

2.6. Method validation

A calibration curve was made using the ratio of the observed peak areas of cefozopran and IS. Linear regression analysis of the calibration data was performed using the equation $y = mx + b$ with a weighting of $1/y$ where y was the peak area ratio, x the concentration of cefozopran, m and b two constant.

This method was evaluated for linearity, accuracy and precision (expressed as the percent coefficient of variation [C.V. (%)]). 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 heights of 3-fold the S_N. The limit of quantitation (LOQ) of cefozopran was determined from validation data.

The recovery of cefozopran with this method was determined by comparing the peak areas from plasma standards with those from cefozopran standards that were similarly prepared except that cefozopran aqueous solution replaced spiked control plasma, and they were not filtered. Moreover, to examine the influence of different individual plasmas on recovery, cefozopran was spiked into each plasma from six different individuals at 50.0 μg/ml.

On the other hand, to investigate the influence of the dilution of plasma on recovery, plasma spiked with cefozopran was diluted to several times with water and applied to Nanocep 10 K without further dilution.

To evaluate for specificity, six blank plasma samples from healthy volunteers were investigated for interference of endogenous matrix components.

2.7. Application to real-time TDM for neutropenia patients

Neutropenia patients were infused with 1 g of cefozopran (FIRSTCIN; Takeda Pharmaceutical Co., Ltd.) over 1 h infusion. Plasma concentrations of cefozopran were measured at 1, 2, 4, and 6 h after starting the infusion. Changes in the plasma cefozopran concentration were fitted to a two-compartment model and analyzed with the nonlinear least-squares computer program (MULTI-Win) [9]. $T > \mathrm{MIC}$ (% of 24 h) for these patients were

determined according to the method for calculation of $T > MIC$ [10]. The study reported here was approved by the Ethics Committee at Kyoto Prefectural University of Medicine.

3. Results

3.1. Typical chromatograms

Fig. 2 illustrates biological matrix with blank control plasma (Fig. 2a) and control plasma spiked with 5.0 μg/ml of cefozopran (Fig. 2b), as well as a plasma sample from a patient with a concentration of 5.7 μg/ml (Fig. 2c). Fig. 2c is the chromatogram 6 h after administration to patient C in Fig. 4 and was the lowest level of cefozopran, because the dose of cefozopran in this regimen was higher than that usually used. Interfering peaks did not overlap with the peak of cefozopran and the retention time for cefozopran was 8.4 min.

There was an endogenous peak at about 7 min in most plasma (Fig. 2), and the amount of the peak was different between individuals. When the mobile phase was 50 mM sodium phosphate buffer (pH 7.0)–acetonitrile (96:4, v/v), 50 mM sodium phosphate buffer (pH 3.0)–acetonitrile (96:4, v/v), or water–acetonitrile (955:45, v/v), this endogenous plasma peak overlapped with the peak of cefozopran. PIC B7 was used for separation between the peaks of cefozopran and the endogenous plasma component. PIC B7 is an ion-pairing reagent with the principal ingredient being heptane sulfonic acid. With the mobile phase of water–acetonitrile (955:45, v/v), separation factor (α) of these peaks was 1.04 and their resolution factor (Rs) was 0.8. As the amount of PIC B7 of the mobile phase was increased, α and Rs increased: $\alpha = 1.06$ and Rs = 1.1 with water–PIC B7–acetonitrile (954:1:45, v/v/v) and $\alpha = 1.11$ and Rs = 1.9 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.

With high sensitivity, most plasma has peaks of endogenous components at around 13 min (Fig. 2). These peaks might come in succession in the following chromatogram with a short analysis time. A 15-min analysis time was adapted to avoid this in Fig. 2. But it can be analyzed with an around 12-min analysis time only to avoid overlapping these peaks with the cefozopran peak. In this way, only avoiding overlapping peaks resulted in appropriate chromatograms for determinations. Column washing was not needed during the 1-day analysis. After the analysis was finished, the column was washed with methanol of 30–60 volumes of the column.

3.2. Limits of detection and quantitation

The LOQ was determined as the lowest concentration of the standard (0.2 μg/ml (C.V.: 4.55%, accuracy: 96.6%, intra-day assay, $n = 6$)) and the LOD, defined in Section 2.6, was 0.05 μg/ml using a 20 μl injection volume.

3.3. Method validation

The linearity of the ultrafiltration method was good between 0.2 and 200 μg/ml ($r^2 = 0.9999$ (mean) $\pm 2.0 \times 10^{-5}$ (S.D.) ($n = 6$)). Table 1 summarizes the reproducibility and accuracy at each calibration standard.

Table 2 shows the recovery of cefozopran. It was around 90% at 1.0, 5.0, 50.0, 100.0, and 200.0 μg/ml of cefozopran. On the other hand, when plasma spiked with cefozopran was diluted several times with water, the recovery of cefozopran was determined without further dilution and is shown in Table 3. Moreover, recovery was hardly influenced by each of the dif-

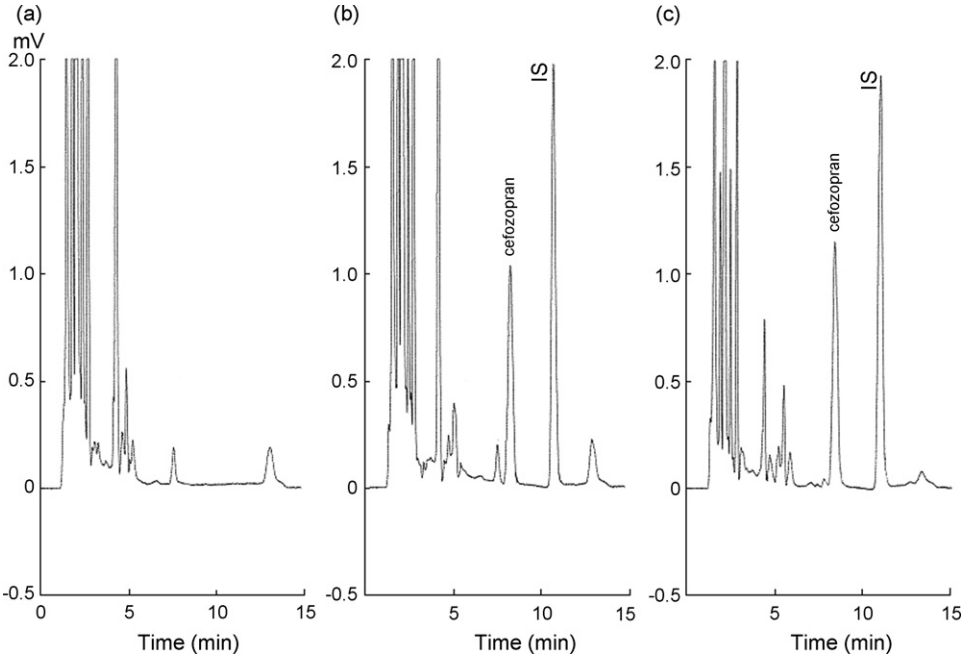

Fig. 2. Typical chromatograms. (a) Blank control plasma. (b) Control plasma spiked with 5.0 μg/ml cefozopran. (c) Patient plasma sample (5.7 μg/ml, 6 h after administration to patient C in Fig. 4).

Table 1
Accuracy and intra- and interday precision data for measurement of cefozopran in human plasma

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
Intraday assay ($n=6$)			
0.2	0.193 ± 0.009	4.55	96.6
0.5	0.514 ± 0.009	1.73	102.8
1.0	0.992 ± 0.044	4.47	99.2
5.0	5.183 ± 0.130	2.51	103.7
25.0	24.56 ± 0.956	3.89	98.2
50.0	49.86 ± 1.195	2.40	99.7
100.0	98.64 ± 1.253	1.27	98.6
150.0	153.07 ± 3.705	2.42	102.0
200.0	198.80 ± 2.505	1.26	99.4
Interday assay ($n=6$)			
0.2	0.200 ± 0.015	7.34	99.8
0.5	0.501 ± 0.012	2.48	100.3
1.0	0.998 ± 0.033	3.32	99.8
5.0	4.953 ± 0.155	3.12	99.1
25.0	24.95 ± 0.453	1.82	99.8
50.0	50.86 ± 0.926	1.82	101.7
100.0	100.88 ± 2.227	2.21	100.9
150.0	149.47 ± 2.359	1.58	99.6
200.0	199.01 ± 1.311	0.66	99.5

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

Table 2
Recovery study

Concentration of cefozopran (μg/ml)	Recovery ($n=6$) (mean ± S.D.) (%)
1.0	89.9 ± 3.7
5.0	87.0 ± 1.4
50.0	89.6 ± 1.3
100.0	90.6 ± 1.4
200.0	93.2 ± 0.9

ferent individual plasma samples. The recovery of cefozopran from each plasma of the six different individuals at 50.0 μg/ml was 89.3% (mean) ± 1.7 (S.D.) (C.V. = 1.52) ($n=6$), and was equivalent to the data from control plasma.

To evaluate for specificity, six blank plasma samples from different individuals were investigated for interference of endogenous matrix components, and no interference peak was observed. Specificity was assessed in the presence of other β-lactams: biapenem, meropenem, doripenem, cefotiam, or cefmetazol, at a concentration of 20 μg/ml. None of the chromatograms revealed any limitations for the assay (Fig. 3).

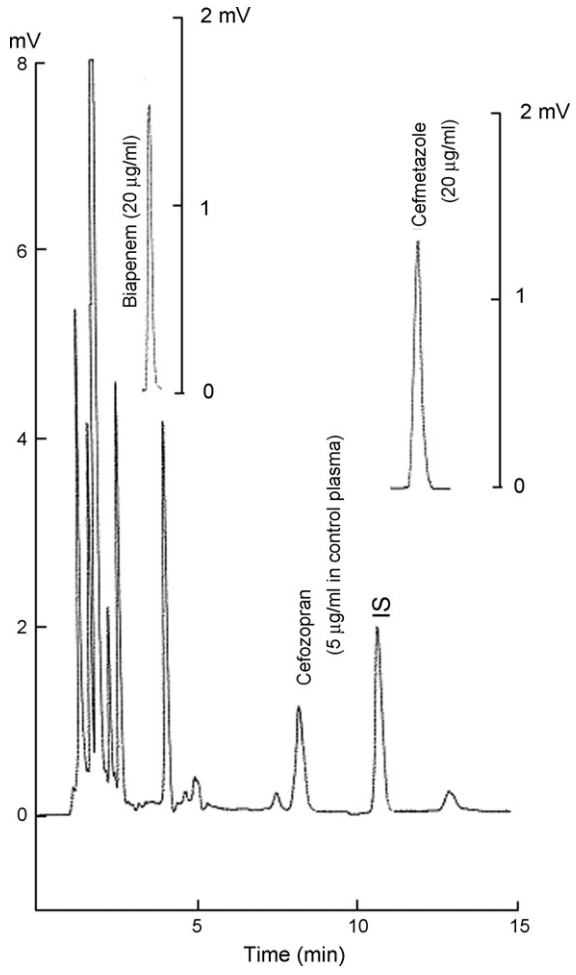

Fig. 3. Specificity in the presence of other β-lactams (biapenem, meropenem, doripenem, cefotiam, or cefmetazol, at a concentration of 20 μg/ml). Meropenem, doripenem, and cefotiam were not eluted in this chromatogram.

3.4. Real-time TDM

Fig. 4 shows time course of plasma cefozopran concentrations in five patients infused with 1 g cefozopran for 1 h with MULTI-Win analysis. The pharmacokinetic parameters obtained in this study are shown in Table 4. $T>$MIC (% of 24 h) for these patients on several regimens (infusion time: 1 h) are shown in Table 5, and "effective" is shown in italics. According to the FIRSTCIN package insert, it is usually administrated at 0.5 g every 12 h (0.5 g q12 h) or 1 g q12 h and 2 g q12 h or 1 g q6 h for obstinate or severe infections. A regimen administering 2 g (6:00), 1 g (14:00), and 1 g (22:00) of cefozopran with 1 h infusion time was used in this study for febrile neutrope-

Table 3
Influence of dilution on recovery

Concentration of cefozopran (μg/ml)	Dilution ratio				
	1 (without dilution)	2	3	4	5
5.0	77.8 ± 1.1[a]	85.4 ± 1.2	87.0 ± 0.8	87.0 ± 1.4	89.2 ± 1.0
50.0	76.5 ± 1.1	82.9 ± 1.0	86.2 ± 0.5	89.6 ± 1.3	87.2 ± 1.1

[a] Recovery ($n=6$) (mean ± S.D.) (%).

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

Patient	Age (year)	Weight (kg)	Serum creatinine (mg/dl)	V_d (l)	C_{max} (μg/ml)	$AUC_{0–24}$ (μg h/ml)
A	66	51.2	0.68	16.2	70.2	959.8
B	53	67.0	0.75	14.0	65.7	749.6
C	23	73.6	0.57	12.0	58.0	571.3
D	73	42.5	0.56	10.3	80.2	706.6
E	60	50.0	0.56	8.1	98.0	930.3

V_d: distribution volume; C_{max}: peak plasma cefozopran concentration; AUC: area under concentration time curve; $AUC_{0–24}$ was calculated to the regimen of 2 g (6:00), 2 g (14:00), and 1 g (22:00) with 1 h infusion time.

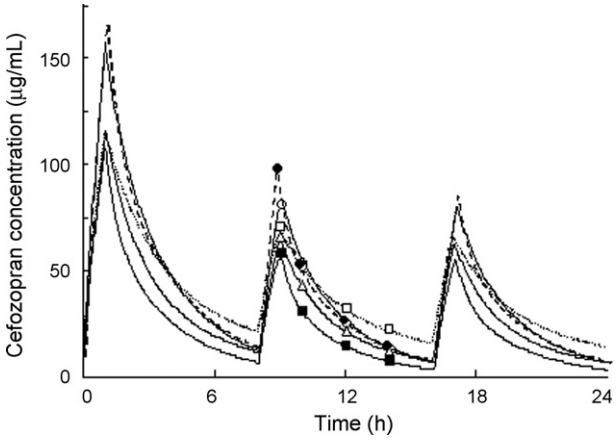

Fig. 4. Time course of plasma cefozopran concentrations in five patients infused with 1 g cefozopran for 1 h. Symbols, measured data; lines, change in plasma cefozopran concentration fitted to two-compartment model. Patient A: open square, small dotted line, B: open triangle, solid line, C: closed square, solid line, D: open circle, solid line and, E: closed circle, dashed line.

nia patients. Although the administration dose for this regimen in a day was the same as that of a 2 g q12 h regimen, $T > MIC$ was somewhat higher. The regimen here was effective for all five febrile neutropenia patients at an MIC of 8 μg/ml, and was effective for four patients at an MIC of 16 μg/ml.

4. Discussion

The LOD and LOQ of this method were 0.05 μg/ml and 0.2 μg/ml, respectively. With the method of Borner et al., LOD for human plasma was 0.6 μg/ml [8]. It was considered that the comparatively high sensitivity was because of the high recovery of cefozopran, though it dilutes with water. Another reason for the comparatively high sensitivity was considered to be the low baseline noise. Cefozopran was detected at a relatively shorter wavelength: 235 nm. With this method PIC B7 was used in the mobile phase: however, the content was very low (0.52 mM) compared to the method of Borner et al. (15 mM) [8]. Thus, it was considered that the low content of PIC B7 and nonuse of other reagents for plasma deproteinization brought about a low baseline noise level, and that this result in the high sensitivity. The method here is better than the previous one in respect of cost, because the PIC B7 reagent is expensive. However, PIC B7 could not be completely excluded from the mobile phase for the separation of the peak in plasma and the peak of cefozopran. The mobile phase was used as a washing solution of the 200 μl-sample valve of the auto sampler to make the baseline of the chromatogram stable especially for high sensitivity.

Two thousand or more samples could be analyzed in one column with this method without using a guard column. Molecules for which molecular weights are over 30 kDa were excluded: thus, the column could be used longer than when used with liquid extraction sample preparation. The column only needed to be washed with methanol after 1 day's analysis to avoid the plasma components with high lipid solubility being accumulated on the column.

With this method, cefozopran bound plasma protein cannot be separated by ultrafiltration and cannot be recovered. Since the protein binding of cefozopran is reported as 6.2% (FIRSTCIN package insert), plasma spiked with cefozopran was diluted with water and the recovery investigated. As shown, recovery increased with up to 4-times dilution (Table 3), therefore, samples were diluted 4-times with water. The recovery of cefozopran in spiked plasma samples was then around 90% (Table 2) and was unaffected by the content of plasma protein. Under clinical conditions, the protein content of plasma might fall to about

Table 5
Predicted $T > MIC$ (%) on each regimen and MIC

Patient	Regimen					
	1 g every 12 h		2 g every 12 h		2 g (6:00), 1 g (14:00), 1 g (22:00)	
	MIC = 8 (μg/ml)	MIC = 16 (μg/ml)	MIC = 8 (μg/ml)	MIC = 16 (μg/ml)	MIC = 8 (μg/ml)	MIC = 16 (μg/ml)
A	*71*	40	*100*	*71*	*100*	*97*
B	55	33	*80*	55	*97*	*68*
C	44	26	*62*	44	*78*	51
D	59	41	*76*	59	*93*	*70*
E	*60*	40	*79*	*60*	*96*	*74*

"Effective" is shown in italics.

50% at most. There was no significant difference between the recovery in control plasma and that in control plasma diluted 1:2 when they were diluted 4-times with water in the procedure (not shown in detail). Moreover, as described in Section 3.3, using this method, the recovery of cefozopran in six individual plasmas was determined and no significant difference was found. Some carbapenems (imipenem, biapenem, and meropenem) for which the protein binding ratios are low, have been determined as the total concentration in plasma using ultrafiltration for deproteinization [11–14]. A calibration curve was made using control plasma; the recovery of cefozopran was found to be high and moreover as an internal standard was used, it could therefore be considered that the total cefozopran concentration was determined.

Borner et al. [8] reported that cefozopran was stable in plasma at −20 °C for at least 4 months. In this study, the samples were measured in real-time, and had not been preserved for a long time; therefore, a stability test was not required for the longer period.

For real-time TDM of patients; these results were obtained within 1 h after the final sampling of blood with time-management analysis and a discussion of the regimen was presented. This method could provide tailor-made medical treatment in real-time.

5. Conclusion

A simple method using ultrafiltration to remove plasma protein was developed so that cefozopran levels in human plasma could be determined more easily and rapidly than by conventional ones. After a 10 min centrifugation, filtrates could be applied to HPLC, which required only a few minutes and no special techniques. Predicted $T > \mathrm{MIC}$ could be obtained within 1 h after the final sampling of blood, thus the next administration could be changed in accord with the TDM result. This method should be very useful in clinical settings and should contribute to lifesaving of patients.

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