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Determination of ceftazidime and cefepime in plasma and dialysate-ultrafiltrate from patients undergoing continuous veno-venous hemodiafiltration by HPLC

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Abstract

We have developed and validated a new, rapid and reproducible HPLC method for the determination of cefepime and ceftazidime in plasma and dialysate-ultrafiltrate samples obtained from intensive care unit (ICU) patients undergoing continuous veno-venous hemodiafiltration (CVVHDF). The method for plasma samples involved protein precipitation with acetonitrile, followed by washing with dichloromethane to remove apolar lipophilic compounds. Dialysate-ultrafiltrate samples did not require any preparation. Separation was performed on a μBondapak C18 (30 cm × 3.9 mm × 10 μm) with UV detection. The mobile phase contained acetate buffer: ACN and was delivered at 2 ml/min. The coefficients of determination of the calibration curves were always ≥0.998 and R.S.D.% of the response factors <10%. The intra and inter-assay precision and accuracy of the quality controls (QC) and limit of quantification (LOQ) were satisfactory in all cases. Plasma and dialysate-ultrafiltrate samples were stable at −20 and −80 °C for 2 months and also after three freeze/thaw cycles. Dialysate-ultrafiltrate samples were stable in the chromatographic rack for 24 h at room temperature, but we recommend storing processed plasma samples at 4 °C until the analysis. The described method has proved to be useful to give accurate measurements of ceftazidime and cefepime in samples obtained from patients undergoing CVVHDF.

Keywords: Ceftazidime; Cefepime; HPLC; Continuous hemodiafiltration; Stability

1. Introduction

Ceftazidime and cefepime are third and fourth generation cephalosporins, respectively, with a broad spectrum of antimicrobial activity against gram-positive and gram-negative microorganims including *Pseudomonas aeruginosa* [1–4]. Their high degree of activity and their tolerability profile make them a useful option for the treatment of infections in critically ill patients in intensive care [4–8].

Ceftazidime and cefepime are excreted primarily by the kidney functions. Thus, ceftazidime is eliminated almost exclusively by glomerular filtration, with about 90% of the dose being excreted in the urine within 24 h of administration [3,6,9]. Cefepime clearance also occurs by glomerular filtration, with negligible tubular secretion, and more than 80% of the administered dose is recovered as unchanged cefepime in urine [10–12]. Consequently, in patients with impaired renal function their clearance becomes smaller and the elimination half-life increases significantly in correlation with the severity of the renal failure.

As low molecular weight molecules with low protein binding (<20%), cefepime and ceftazidime are susceptible to be eliminated by continuous veno-venous hemodiafiltra-

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tion (CVVHDF), so pharmacokinetic analysis is necessary in order to establish rational dosage regimens for the treatment of critically ill patients undergoing these techniques. Nowadays, there is relatively little clinical data on the removal of specific drugs by continuous renal replacement therapies (CRRT).

Before carrying out a pharmacokinetic study, it is necessary to develop a properly validated analytical methodology. The analysis of biological samples for drug testing is usually not performed immediately after sample collection and, therefore, it is very important to use optimal conditions for which the drug has been demonstrated to be stable during storage time [13,14]. Stability may be defined as the ability of a material to maintain a stated property (e.g. concentration) within the specified limits for a specified period of time when stored under specified conditions. The objective of stability testing is to identify and evaluate any significant degradation of the analytes when subjected to storage over time at different conditions.

In order to obtain this information and gather data about ceftazidime and cefepime dosing in critically ill patients receiving CRRT, the first step is the development of the technique for the determination of ceftazidime and cefepime in plasma and dialysate-ultrafiltrate. Thus, the aim of this study was to develop a rapid and reproducible HPLC method for the determination of cefepime and ceftazidime in plasma and dialysate-ultrafiltrate samples obtained from intensive care unit (ICU) patients undergoing CVVHDF. The method was adequately validated following present guidelines [15,16] and a complete stability study was also included.

2. Materials and methods

2.1. Chemicals

Ceftazidime 85% (EPC0690500) was supplied by LGC Promochem (Barcelona, Spain), and cefepime $2HCl{\cdot}H_2O$ (BMY-28142) was kindly supplied by Bristol–Myers Squibb (Madrid, Spain). Ammonium acetate, glacial acetic acid and dichloromethane (DMC) were purchased from Panreac Química (Barcelona, Spain), and acetonitrile (ACN) from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® Plus apparatus (Millipore). Saline solution (NaCl 0.9%) was provided by Biomendi S.A. (Bernedo, Álava, Spain) and plasma was obtained from Centro Vasco de Transfusiones (Galdakao, Spain).

2.2. Chromatographic system and procedure

The chromatographic system consisted of a Waters™ 616 (Waters, Milford, Massachusetts, USA) pump connected to a Waters™ 600S controller, a Waters™ in-line degasser, a thermostatted autosampler and a Waters™ 996 Photodiode array detector. The Empower™ software (Waters) was used to pilot the HPLC instrument and to process the data. The assay was performed on a μBondapak™ C18 (30 cm × 3.9 mm × 10 μm) HPLC column (Waters).

The mobile phase consisted in acetate buffer:ACN (90:10 for ceftazidime and 95:5 for cefepime, v/v). The buffered acetate solution was prepared by dissolving 1.54 g of ammonium acetate in 1000 ml of ultrapure water and the pH was adjusted to 4 with glacial acetic acid. The mobile phase was degassed in an ultrasonic bath Ultrasons (Selecta, Barcelona, Spain) and was delivered at 2 ml/min. The injection volume was 50 μl. The wavelength selected to detect ceftazidime was 257 nm and cefepime samples were detected at 280 nm. The chromatography was performed at room temperature (RT).

2.3. Standard solution and quality controls (QC)

2.3.1. Calibration and control standards in plasma

A ceftazidime standard solution was prepared every day by dissolving ceftazidime in water to obtain a 1000 μg/ml sample. The purity of the standard was taken into account for solution preparation. Plasma calibration standards were prepared at concentrations of 3, 5, 10, 20, 50, 100, 200 μg/ml, along with plasma QC at 7, 40 and 150 μg/ml were prepared.

Cefepime $2HCl{\cdot}H_2O$ was dissolved in water in order to obtain standard solutions with 1000 μg/ml concentration. Plasma calibration standards were prepared at concentrations of 1, 5, 10, 50, 75, 100, 200 μg/ml, along with plasma QC at 3, 25 and 150 μg/ml were prepared.

2.3.2. Calibration and control standard in dialysate-ultrafiltrate

Standard solutions for both ceftazidime and cefepime were prepared every day in saline solution to obtain 1000 μg/ml concentration, taking into account the purity of the standards. Calibration standards were prepared at concentrations of 3, 5, 10, 20, 50, 100, 200 μg/ml for ceftazidime and at concentrations of 1, 5, 10, 25, 50, 75 100 μg/ml for cefepime, along with QC at 7, 40 and 150 μg/ml for ceftazidime and 3, 40 and 80 μg/ml for cefepime were prepared.

2.4. Plasma and dialysate-ultrafiltrate collection

Blood and dialysate-ultrafiltrate samples were obtained from patients undergoing CVVHDF and treated in the ICU with ceftazidime or cefepime. The study protocol was approved by the Medical Ethical Committee of the Santiago Apóstol Hospital (Vitoria-Gasteiz, Spain). All patients or guardians provided written informed consent. Complete medical histories were obtained for all patients, and complete physical examinations and laboratory review of serum chemistry and hematology profiles were performed and reviewed before collection of samples for pharmacokinetic analysis.

Vascular access was obtained with 13.5FG dual lumen catheters (Niagara, Bard Canada, Inc., Mississauga, Ont., Canada). A hemodialfiltration machine (PRISMA, Hospal, Lyon, France) was used with an AN69 HF 0.9 m^2 poly-

acrylonitrile filter (PRISMA M100 Hospal). Both prefilter and posfilter blood samples (5 ml) were collected from the hemodiafiltration device at 0, 0.33, 0.5, 0.75, 1, 3, 6 and 8 h after the administration of each antibiotic. Blood specimens were obtained using lithium heparin as anticoagulant and were centrifuged within 1 h for 10 min at 1000 × g. The plasma was immediately frozen at −20 °C at the hospital. Dialysate-ultrafiltrate samples (4 ml) were collected also at the same sampling times from the hemodiafiltration device and frozen within 1 h at −20 °C. Within the following week plasma and dialysate-ultrafiltrate samples were stored at −80 °C. Samples were analysed within 1 month.

2.5. Sample preparation

A 500 μl of plasma samples were mixed for 30 s with 500 μl of ACN and centrifuged for 5 min at 3000 × g. Five milliliters dichloromethane were added to 800 μl of the upper layer phase, shaken for 5 min, centrifuged for 5 min at 3000 rpm, and the upper aqueous phase was introduced into 150 μl microvials (Waters). Dialysate-ultrafiltrate samples did not require any treatment; they were introduced directly into the microvials. A volume of 50 μl was used for HPLC analysis.

2.6. Method validation

Calibration curves were obtained by weighted least-squares linear regression analysis. The weighted factor was 1/concentration. Linearity was determined in three correlative days, and coefficients of determination (r^2) and relative standard deviation (R.S.D.%) of the response factors of each standard were calculated each day.

Selectivity was determined using blank samples: plasma from six different donors and six different saline solution batches without the addition of any antibiotic.

Precision and accuracy of the method were determined using QC samples. During 3 days six QC were analysed at the three concentration levels (7, 40 and 150 μg/ml for ceftazidime in plasma and dialysate-ultrafiltrate, 3, 25 and 150 μg/ml for cefepime in plasma and 3, 40 and 80 μg/ml for cefepime in dialysate-ultrafiltrate). Intra- and inter-assay precision was calculated as the R.S.D.% within a single run and between three assays respectively, and intra- and inter-assay accuracy as the percentage of deviation between nominal and calculated concentrations with the established calibration curves.

Intra- and inter-assay precision and accuracy of the limits of quantification (LOQ), which were considered the lowest levels included in the calibration curves, were calculated as well.

2.7. Stability

The stability of both antimicrobials in storage conditions was evaluated. QC samples at the same concentration levels that the QC used in accuracy and precision study were prepared in plasma and in saline solution, aliquoted (1 ml) and stored at −20 and −80 °C until the day of the assay, when three QC of each concentration were analysed. Measurements were carried out at 1 week, 2 weeks, 1 month and 2 months.

Analytes stability was also determined after three freeze and thaw cycles. Three samples of low, medium and high QC underwent three freeze and thaw cycles and were analyzed on the third cycle. Samples were thawed at room temperature.

Stability of processed samples in the autosampler was also determined, injecting three QC of each concentration at 4, 8, 12, 16, 20 and 24 h after having been prepared. The assay was carried out at room temperature (RT) and at 4 °C.

Samples were considered stable when R.S.D.% and the deviation (%) of the QC did not exceed the 15%.

2.8. Clinical applications of the HPLC method

This study was developed with the aim to determine the pharmacokinetics of ceftazidime and cefepime in critically ill patients undergoing CVVHDF by analysing plasma and dialysate-ultrafiltrate samples with an appropriate analytical technique. Thus, the HPLC method described in this article was used to determine ceftazidime and cefepime levels in blood from prefilter and posfilter lines, and dialysate-ultrafiltrate samples taken from critically ill patients. In every analytical run, a blank sample, the calibration curve samples, six QC samples and the samples from the patients were included.

After measuring ceftazidime or cefepime concentration in plasma and ultrafiltrate, a pharmacokinetic analysis was carried out and individual pharmacokinetic parameters were determined according to a non-compartmental analysis by using the WinNonlin version 1.1 (Pharsight Corporation, Mountain View, CA, USA).

3. Results

The proposed method was evaluated with respect to selectivity, linearity, precision, accuracy and LOQ. Stability was also determined both in stored samples and in the chromatographic rack.

3.1. Chromatograms and selectivity

Fig. 1(A) shows the ceftazidime's chromatographic profiles of the blank sample, the LOQ (3 μg/ml) and a saline solution sample corresponding to a concentration of 100 μg/ml. Fig. 1(B) shows the profiles of plasma samples (blank, LOQ and a 100 μg/ml sample). The retention time for ceftazidime was 5.25 min. No interfering peaks were observed.

Fig. 1(C) and (D) shows cefepime's chromatograms corresponding to saline solution and plasma blanks, LOQ

(1 μg/ml) and 100 μg/ml samples. The retention time was 6.85 min. No interfering peak was observed either.

Fig. 1(E) shows the chromatogram corresponding to a blank dialysate-ultrafiltrate obtained from one patient undergoing CVVHDF who had not received ceftazidime. In the same way, a blank dialysate-ultrafiltrate from a patient undergoing CVVHDF who had not received cefepime is also shown in Fig. 1(F). No interfering peaks were observed at the retention times of ceftazidime or cefepime.

3.2. Calibration curves: linearity

The standard curves for ceftazidime and cefepime in plasma and saline solution were adequately described by 1/concentration weighed linear regression analysis over the studied ranges. Table 1 shows the parameters of the three calibration curves used for the linearity study. The coefficients of determination were always ≥0.998 and % deviation of each standard was <10%. The residuals (difference between

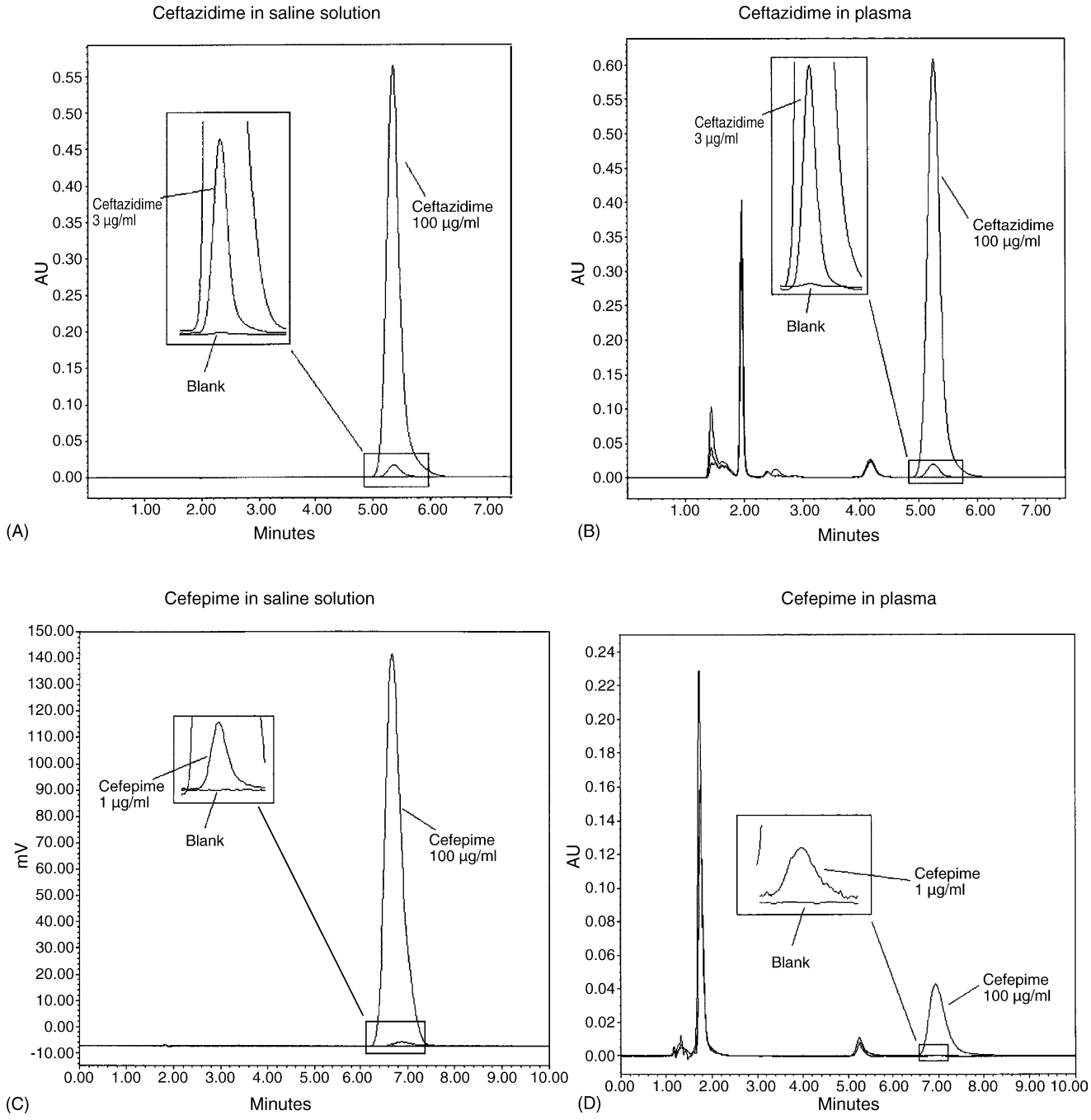

Fig. 1. Chromatographic profiles of ceftazidime and cefepime in saline solution (A and C) and in plasma (B and D). Chromatographic profiles of dilaysate-ultrafiltrate samples from patients to whom ceftazidime (E) or cefepime (F) were not administered.

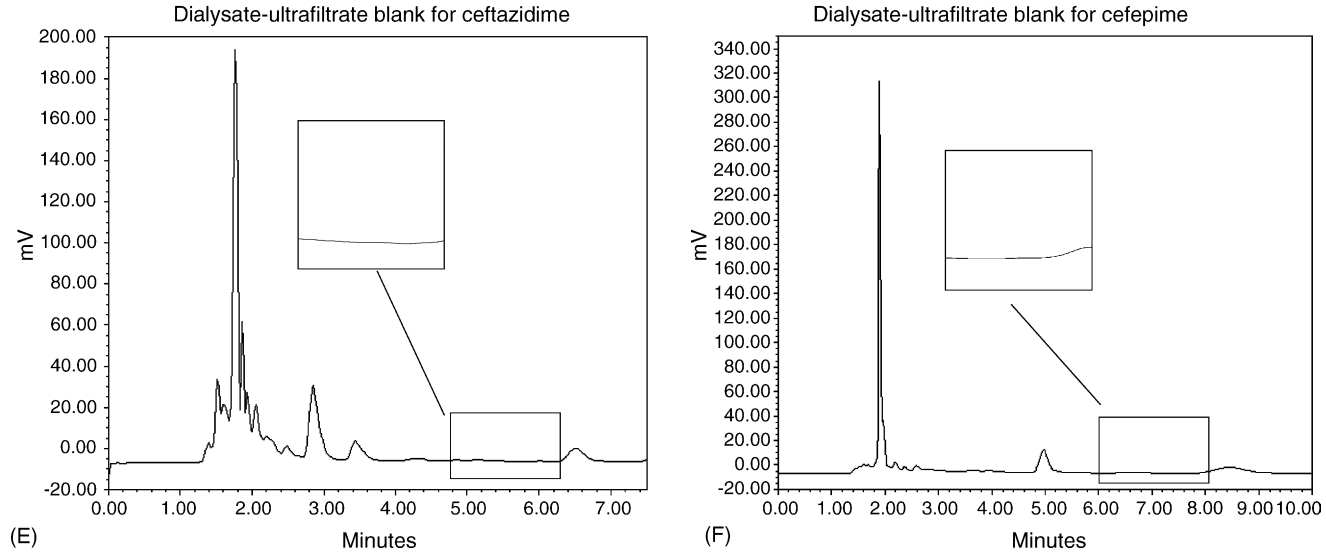

Fig. 1. (*Continued*).

observation and prediction) were normally distributed and centred around zero.

3.3. Validation of the HPLC method: precision, accuracy and LOQ

Table 2 describes the precision and accuracy of the QC and LOQ for ceftazidime in plasma and saline solution. The intra-assay ($n = 6$) precision was always <5% for both plasma and saline solution. The values obtained for inter-assay ($n = 18$) precision were <7% in plasma and <5% in saline solution. LOQ intra- and inter-assay precision was 4.95% and 5.63% for plasma and 4.94% and 5.47% for saline solution. The accuracy was in agreement with the FDA acceptance criteria ($\leq$15%) [16] for both matrixes, obtaining deviation values from the nominal concentrations lower than 8% at all times.

Table 3 features the precision and accuracy of the QC and LOQ for cefepime in plasma and saline solution. The plasma intra-assay ($n = 6$) precision was <6% and inter-assay

Table 1
Mean parameters of the calibration curves for ceftazidime and cefepime in plasma and saline solution

$y = bx + a$	r_1	r_2	r_3
Ceftazidime plasma			
a	−39615.79	−36474.77	−30702.99
b	62305.82	66115.65	57052.65
r^2	0.9994	0.9997	0.9987
Response factor R.S.D.%	8.59	7.45	8.73
Ceftazidime saline			
a	31863.61	22521.16	26897.54
b	94549.17	93919.77	90322.68
r^2	0.99995	0.99970	0.99987
Response factor R.S.D.%	4.09	2.83	3.92
Cefepime plasma			
a	4852.19	3146.67	3468.56
b	22703.91	34695.57	29874.85
r^2	0.99852	0.99998	0.99825
Response factor R.S.D.%	5.83	3.11	4.97
Cefepime saline			
a	486.52	666.55	673.95
b	25066.90	31861.04	35630.77
r^2	0.9949	0.9919	0.9998
Response factor R.S.D.%	0.59	0.76	1.68

Table 2
Precision and accuracy of the HPLC assay for ceftazidime in plasma and saline solution (precision and accuracy of the LOQ)

Nominal concentration (μg/ml)	Calculated concentration (μg/ml)	Precision R.S.D.%	Accuracy deviation (%)
Ceftazidime plasma			
Intra-assay ($n = 6$)			
3 (LOQ)	3.2 ± 0.2	4.95	7.77
7	6.8 ± 0.3	3.71	3.45
40	39.6 ± 1.0	2.42	1.12
150	150.5 ± 4.2	2.81	0.32
Inter-assay ($n = 18$)			
3 (LOQ)	3.1 ± 0.2	5.63	3.82
7	6.6 ± 0.2	3.44	5.77
40	37.1 ± 1.9	5.07	7.15
150	139.1 ± 8.8	6.31	7.24
Ceftazidime saline			
Intra-assay ($n = 6$)			
3 (LOQ)	3.0 ± 0.2	4.94	0.96
7	6.6 ± 0.3	4.78	5.45
40	38.6 ± 1.3	3.38	3.48
150	145.9 ± 0.6	0.42	2.74
Inter-assay ($n = 18$)			
3 (LOQ)	3.0 ± 0.2	5.47	0.84
7	6.8 ± 0.3	3.62	3.57
40	40.0 ± 1.3	3.34	0.09
150	149.1 ± 6.7	4.46	0.61

Table 3
Precision and accuracy of the HPLC assay for cefepime in plasma and saline solution (precision and accuracy of the LOQ)

Nominal concentration (μg/ml)	Calculated concentration (μg/ml)	Precision R.S.D.%	Accuracy deviation (%)
Cefepime plasma			
Intra-assay ($n = 6$)			
1 (LOQ)	1.1 ± 0.1	4.92	8.05
3	2.8 ± 0.1	4.77	7.49
25	25.6 ± 1.2	4.48	2.49
150	150.9 ± 8.4	5.53	0.58
Inter-assay ($n = 18$)			
1 (LOQ)	1.0 ± 0.1	11.41	1.83
3	2.9 ± 0.2	7.78	1.97
25	25.0 ± 1.4	5.45	0.04
150	147.4 ± 8.3	5.65	1.76
Cefepime saline			
Intra-assay ($n = 6$)			
1 (LOQ)	1.0 ± 0.0	3.35	2.88
3	3.0 ± 0.2	5.11	1.47
40	40.2 ± 0.5	1.31	0.43
80	76.5 ± 0.7	0.86	4.42
Inter-assay ($n = 18$)			
1 (LOQ)	1.1 ± 0.1	5.27	4.54
3	2.9 ± 0.2	5.28	2.59
40	39.8 ± 1.2	3.00	0.53
80	77.1 ± 1.2	1.59	3.62

($n = 18$), <8%. In saline solution intra- and inter-assay precision were <6%. The intra- and inter-assay precision for LOQ was 4.92% and 11.41% in plasma and 3.35% and 5.27% in saline solution. The accuracy was in concordance with FDA guidelines (≤15%) [16] in all cases for cefepime, with experimental values never departing more than 9% from nominal concentration.

3.4. Samples stability

Although samples stability in storage conditions was analyzed at 1 week, 2 weeks, 1 month and 2 months, Table 4 only shows the stability data corresponding to 2 months, because all the results were satisfactory and those were the latest ones. The accuracy deviation values were always <15%, as FDA [16] recommends for the QC.

Table 4 also shows results of the freeze/thaw stability study. No sample deviated more than 10% from the nominal concentration.

Post-preparative stability assays were carried out for both molecules. Figs. 2 and 3 describe the ceftazidime and cefepime stability in processed plasma and in saline solution samples during 24 h in the chromatographic rack at room temperature. Samples were stable in saline solution at least 24 h. Processed plasma samples of ceftazidime and cefepime were not stable, with deviations higher than 15% after 4 h at room temperature, as can be seen in Figs. 2 and 3. Table 4, therefore, only features data for 4 h. Due to the low stability at room temperature, other analyses were developed at 4 °C. Post-preparative plasma samples of ceftazidime were stable at 4 °C for 24 h, but cefepime plasma samples were stable for only 8 h. Data corresponding to these assays appear in Table 4.

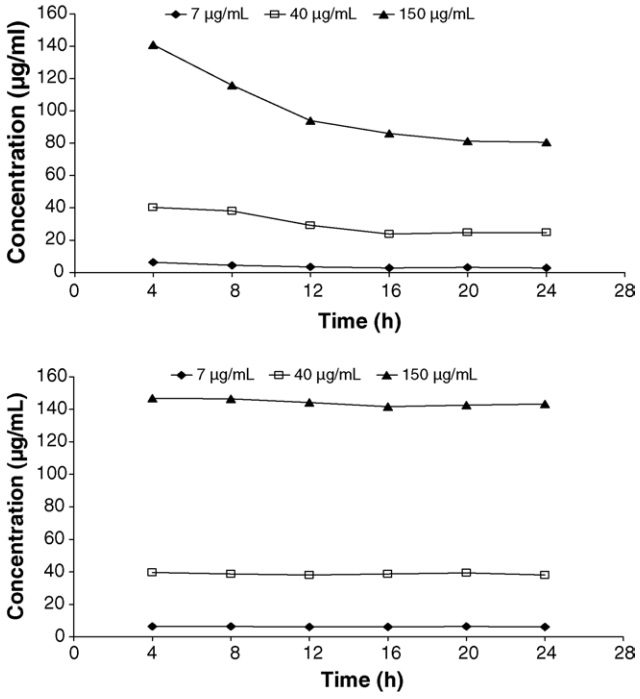

Fig. 2. Ceftazidime degradation in plasma (above) and in saline solution (below) at room temperature.

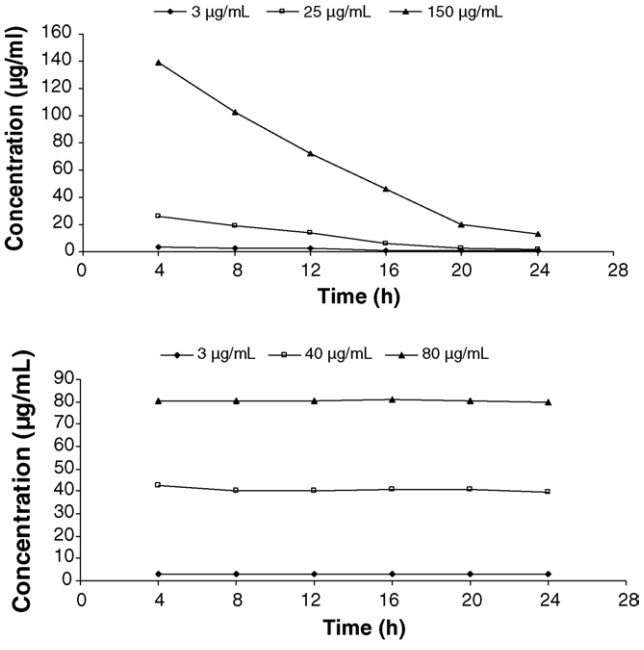

Fig. 3. Cefepime degradation in plasma (above) and in saline solution (below) at room temperature.

Table 4
Stability of ceftazidime and cefepime in plasma and saline solution samples under different conditions

Nominal concentration (μg/ml)	Low QC ($n=3$)			Medium QC ($n=3$)			High QC ($n=3$)		
	Mean ± S.D.	R.S.D.%	*E*%	Mean ± S.D.	R.S.D.%	*E*%	Mean ± S.D.	R.S.D.%	*E*%
Ceftazidime plasma									
−20 °C[a]	6.2 ± 0.3	5.14	11.40	35.1 ± 0.6	1.81	12.36	132.6 ± 1.7	1.27	11.63
−80 °C[b]	6.1 ± 0.1	1.60	13.44	36.4 ± 1.9	5.15	8.96	146.7 ± 7.1	2.23	2.23
Freeze/thaw[c]	6.5 ± 0.2	2.94	7.40	37.2 ± 1.6	4.38	7.06	138.4 ± 2.0	1.41	7.72
RT[d] (4 h)	6.3 ± 0.2	2.52	10.56	40.2 ± 0.3	0.81	0.52	140.9 ± 13.0	9.22	6.05
4 °C[e] (24 h)	6.4 ± 0.1	1.81	7.93	39.1 ± 0.2	0.48	2.34	132.0 ± 0.8	0.60	11.98
Ceftazidime saline									
−20 °C[a]	6.8 ± 0.1	1.54	3.58	43.3 ± 0.5	1.18	8.32	158.1 ± 6.4	4.02	5.39
−80 °C[b]	6.8 ± 0.1	1.41	2.43	41.6 ± 0.1	0.29	4.07	162.4 ± 6.5	3.99	8.28
Freeze/thaw[c]	6.9 ± 0.3	4.64	1.37	39.2 ± 0.6	1.49	2.10	149.2 ± 2.0	1.31	0.52
RT[d] (24 h)	6.3 ± 0.0	0.20	10.74	37.9 ± 0.7	1.93	5.20	143.3 ± 0.5	0.32	4.46
4 °C[e] (24 h)	6.4 ± 0.0	0.36	9.35	38.8 ± 0.1	0.19	2.90	145.2 ± 0.2	0.11	3.17
Cefepime plasma									
−20 °C[a]	3.1 ± 0.1	4.36	4.67	22.9 ± 1.2	5.05	8.29	149.0 ± 2.8	1.85	1.19
−80 °C[b]	3.0 ± 0.1	2.33	1.82	24.3 ± 0.5	2.18	2.94	143.6 ± 3.0	2.10	4.65
Freeze/thaw[c]	2.9 ± 0.0	0.68	2.44	22.9 ± 0.6	2.52	8.50	135.5 ± 3.8	2.76	9.66
RT[d] (4 h)	3.3 ± 0.2	4.52	9.61	26.2 ± 0.4	1.59	4.76	139.4 ± 1.0	0.72	7.08
4 °C[e] (8 h)	3.3 ± 0.1	1.39	10.05	22.0 ± 0.3	1.28	11.88	137.4 ± 1.0	0.69	8.41
Cefepime saline									
−20 °C[a]	2.7 ± 0.2	7.85	10.60	40.8 ± 1.8	4.38	1.92	75.1 ± 8.0	10.63	6.10
−80 °C[b]	2.7 ± 0.1	5.02	8.63	38.2 ± 1.6	4.17	4.60	75.7 ± 0.2	0.31	5.40
Freeze/thaw[c]	2.8 ± 0.0	1.38	5.61	39.5 ± 1.6	3.95	1.25	73.3 ± 0.6	0.82	8.34
RT[d] (24 h)	2.9 ± 0.1	2.22	2.43	39.5 ± 0.5	1.13	1.18	80.0 ± 0.5	0.65	0.02
4 °C[e] (24 h)	2.9 ± 0.1	2.94	3.91	40.5 ± 0.1	0.12	1.19	80.3 ± 0.6	0.74	1.16

QC: quality control, RT: room temperature, *E*%: accuracy deviation.
[a] Concentration measured after storing samples at −20 °C for 2 months.
[b] Concentration measured after storing samples at −80 °C for 2 months.
[c] Concentration measured after three freeze/thaw cycles.
[d] Concentration measured after keeping processed samples in the chromatographic system at room temperature during 24 h.
[e] Concentration measured after keeping processed samples in the chromatographic system at 4 °C during 24 h.

3.5. Clinical applications

The described HPLC method was used to analyse plasma and dialysate-ultrafiltrate samples from critically ill patients undergoing CVVHDF. The basis of accepting or rejecting the run was provided by the QC samples. At least four out of six of the QC samples were within the 15% of their respective nominal values. The detailed pharmacokinetic assessment will be reported elsewhere [17,18]. Fig. 4(A) shows the mean ceftazidime concentrations in plasma prefilter, posfilter and dialysate-ultrafiltrate from two critically ill anuric patients undergoing CRRT who received 1000 mg every 6 h by intravenous perfusion of 20 min duration and Fig. 4(B) shows mean concentration–time curves from two critically ill non-anuric patients who received 2000 mg every 6 h. The pharmacokinetic parameters of ceftazidime were studied and are shown in Table 5.

Mean concentrations of cefepime in plasma and in dialysate-ultrafiltrate from four patients to whom 2000 mg tid (every 8 h) were administered are also shown in Fig. 4(C). Table 5 shows the mean pharmacokinetic parameters of cefepime in those patients.

Table 5
Mean ± S.D. values of main pharmacokinetic parameters of ceftazidime ($n=4$) and cefepime ($n=4$) in critically ill patients undergoing CRRT after receiving 1000 or 2000 mg every 6 h of ceftazidime or 2000 mg every 8 h of cefepime

	Dose (mg)	C_{max} (μg/ml)	C_{min} (μg/ml)	Cl_T (ml/min)	X_{CRRT} (%)	Sc	fu	$t_{1/2}$ (h)	V (l)
Ceftazidime	1000	85.6 ± 38.6	48.5 ± 24.8	53.8 ± 25.9	52.1 ± 12.5	0.93 ± 0.06	0.86 ± 0.08	7.2 ± 3.4	65.9 ± 34.1
	2000	53.8 ± 0.1	16.2 ± 12.5	244.1 ± 126.9	7.3 ± 3.1				
Cefepime	2000	100.5 ± 40.9	20.1 ± 8.6	111.5 ± 43.1	27.4 ± 17.6	0.76 ± 0.21	0.79 ± 0.09	4.6 ± 0.9	46.0 ± 21.7

Cl_T: total body clearance; X_{CRRT}: total amount of drug eliminated by CRRT as the percentage of administered dose; Sc: sieving coefficient; fu: fraction of drug not bound to proteins; $t_{1/2}$: elimination half-life; V: volume of distribution.

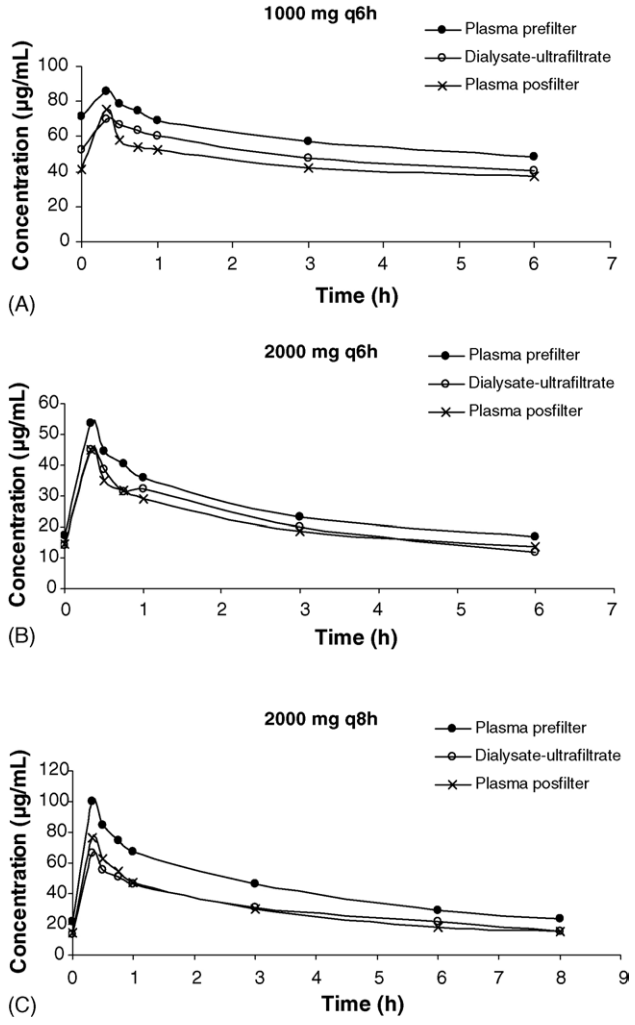

Fig. 4. (A) Ceftazidime plasma prefilter, plasma posfilter and dialysate-ultrafiltrate mean concentrations in two critically ill anuric patients who received 1000 mg every 6 h by intravenous perfusion; (B) ceftazidime mean concentrations in two critically ill non-anuric patients who received 2000 mg every 6 h by intravenous perfusion; (C) cefepime prefilter, posfilter and dialysate-ultrafiltrate mean concentrations in four critically ill patients who received 2000 mg every 8 h by intravenous perfusion.

4. Discussion and conclusions

This paper describes rapid and reproducible methods which enable the determination of ceftazidime and cefepime in plasma and in saline solution. These methods are applicable in pharmacokinetic studies in patients undergoing CRRT, but involve different solvent systems and detection wavelengths for ceftazidime and cefepime, thus separate analytical run are necessary for the analyse of each molecule. However, both antibiotics will not be probably found simultaneously in patients' samples, since it is not common to establish an antimicrobial treatment with both ceftazidime and cefepime.

About selectivity, the absence of interfering peak has been demonstrated in plasma samples, in saline solution samples and in dialysate-ultrafiltrate samples obtained from patients. As Robatel et al. [19] explained, there was some concern that the calibration samples prepared with plasma collected from healthy volunteers might not fully reflect the complexity of the plasma matrix from CVVHDF patients. But getting blood from such patients for calibration samples preparation would not be ethically acceptable. Moreover, plasma samples obtained from our patients presented a large inter-individual variability in the appearance (colour, turbidity). Consequently, plasma from healthy volunteers as a suitable source for the preparation of calibration samples was used as an adequate solution due to the difficulty to select a suitable matrix representative for all plasma samples. In the case of calibration curves prepared to measure dialysate-ultrafiltrate samples saline solution was used. In order to determine meropenem, other authors [19], used a pool blank dialysate obtained from one patient who was allergic to β-lactam antibiotics and, therefore, had neither received meropenem nor structurally related drugs. We decided not to proceed that way taking into account some issues: (i) it was checked that interfering peaks did not appeared in neither saline solution samples nor in dialysate-ultrafiltrate samples obtained from patients. Considering the difficulties to obtain dialysate-ultrafiltrate samples from other patients, it was decided to use saline solution to prepare calibration curves for sample analysis. (ii) The composition of dialysate-ultrafiltrate will be very different in critically ill patients considering the large inter-variability in their clinical situation and their pharmacological treatment, so in this case it would also be very difficult to obtain a suitable matrix representative for all samples.

During our study about 200 clinical samples from CVVHDF patients were analysed and no interfering peaks were detected at the retention time of ceftazidime or cefepime.

Although other HPLC methods have been published in the literature for ceftazidime [20–23] and cefepime [24–27] determination in different fluids, only a few of the works mentioned above provide data about drugs' stability [22,25,26,28]. Our work includes a complete stability study because stability assessment is considered a fundamental parameter for the validation of bioanalytical methods [16,29], and is critical for proper interpretation of analytical results [13].

Stability of ceftazidime and cefepime was evaluated at −20 and −80 °C (Table 4) because patients' samples were stored in the hospitals at −20 °C and within a week they were moved to an −80 °C freezer. We found that ceftazidime and cefepime were stable in plasma and in saline solution for at least two months in storage conditions (−20 and −80 °C). Humbert et al. [22] had previously found ceftazidime stable during at least 84 days at −196 °C, but this temperature condition is not usual in analytical laboratories. Elkhaïlï et al. [26] also described the stability of cefepime at −80 °C for up to three months, but they did not evaluated stability at −20 °C. Barbhaya et al. [30] found cefepime stable in human plasma at −20 °C for up to 51 days. Cherti et al. [25] described that cefepime was stable at −30 °C for at least 60 days in dialysis

fluid and described a degradation averaging 15–22% after 60 days in plasma. In our study, samples collected from patients were stored no more than a month until their analysis, thus molecules degradation might not be expected to be found before they were analysed.

In case any sample had to be re-analysed, it had to be thawed more than once. Thus, antibiotic stability was evaluated after three freeze/thaw cycles at three concentrations in triplicate (Table 4). Both ceftazidime and cefepime tolerate at least three freeze-thaw cycles in plasma and saline solution without losses of greater than 10%. Cherti et al. [25] described similar values for cefepime, but no other data is available in literature about ceftazidime. In no case did patient's samples undergo more than two thaw processes.

The stability of the processed samples in the autosampler is an important feature that limits the analytical sequence length. In our study (Table 4), saline solution samples of ceftazidime and cefepime were stable for 24 h and post-preparative plasma samples were only stable for 4 h at room temperature. Therefore, precautions should be taken to prevent ceftazidime and cefepime decomposition in processed plasma samples left at room temperature in the autosampler rack, and we would recommend to store them at 4 °C. This is the first work that includes a complete study about ceftazidime stability in processed plasma samples and in saline solution. Although other papers mentioned above provide information about stability, frequently no data about processed samples are shown. However, this information is very important because it conditions the analytical batches. Taking into account the data presented here about the short period of time the samples are stable in the chromatographic system, when a paper about pharmacokinetics studies of ceftazidime or cefepime is published, more details about the storage conditions of the processed samples should be provided. Cherti et al. [25] and Elkhaïlï et al. [26] described important degradation of aqueous extracts originating from plasma after sample pre-treatment in cefepime. Bugnon et al. [28] also showed that cefepime degradation in plasma samples at room temperature occurred in spite of prior plasma deproteinization and recommended to maintain samples at 4 °C. The stability of samples used for pharmacokinetic analyses is guaranteed with our HPLC method for the period of time described above.

The stability of stock solutions of ceftazidime and cefepime is not specifically reported in this work because it has been demonstrated that both molecules were stable at room temperature in saline solution samples (they did not require any treatment) for up to 24 h.

When analysing samples obtained from patients, plasma ceftazidime concentrations ranged from 48.5 ± 24.8 to 85.6 ± 38.6 μg/ml in anuric patients who received 1000 mg every 6 h, and from 16.2 ± 12.5 to 53.8 ± 0.1 μg/ml in patients who received 2000 mg every 6 h. The mean±S.D. sieving coefficient was 0.93 ± 0.06. Cefepime minimum and maximum concentrations were 20.1 ± 8.6 and 100.5 ± 40.9 μg/ml, respectively, and the sieving coefficient was 0.76 ± 0.09. Sieving coefficient of ceftazidime and cefepime correlated well with the free fraction of the drug, not bound to proteins. Although the stability of both molecules was well established, three controls were analysed after each patient's analyses. All controls were in the acceptance range.

In conclusion, the described method, which follows the main methodology described in FDA guidelines for bioanalytical method validation [16], is useful to provide accurate measurements of ceftazidime and cefepime in plasma and dialysate-ultrafiltrate samples obtained from critically ill patients undergoing CVVHDF. Moreover, very useful information about stability has also been provided.

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