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High-performance liquid chromatographic determination of cefepime in human plasma and in urine and dialysis fluid using a column-switching technique

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Abstract

A high-performance liquid chromatographic method with UV absorbance was developed for the analysis of cefepime in human plasma and urine, and in dialysis fluid. Detection was performed at 280 nm. The assay procedure for cefepime in plasma involves the addition of an internal standard (cefpirome) followed by treatment of the samples with trichloracetic acid, acetonitrile and dichloromethane. To quantify cefepime in diluted urine (1:20) and in the dialysis fluid, samples spiked with the internal standard (cefpirome) were analysed using a column-switching technique. The HPLC column, Nucleosil C_{18}, was equilibrated with an eluent mixture composed of acetonitrile–ammonium acetate (pH 4). Linear detector responses were observed for the calibration curve standards in the range 0.5 to 100 μg/ml, which spans what is currently thought to be the clinically relevant range for cefepime concentrations in body fluids. The limit of quantification was 0.5 μg/ml in the three matrices. Extraction recoveries proved to be more than 84%. Precision, expressed as %RSD, was in the range 1.5 to 9%. Accuracy ranged from 93 to 105%. This method was used to follow the time course of the concentration of cefepime in plasma, urine and dialysate outlet samples after a 10-min infusion period of 2 g of this drug in patients with acute renal failure undergoing hemodiafiltration.

Keywords: Cefepime

1. Introduction

Acute renal failure is a serious and common complication in critically ill patients. Kidneys are rarely the only organs that fail: there is often a multiple-organ dysfunction syndrome (MOF). Hemodiafiltration is usually performed in these patients for its good hemodynamic tolerance compared to dialysis [1]. Hemodiafiltration can be performed either intermittently or continuously. Intermittent venovenous hemodiafiltration (IVVHD) is a well-established tool in nephrologic therapy. Another problem with patients undergoing hemodiafiltration is to determine the best method for administering antibiotics. However, knowledge of the impact of hemodiafiltration on the pharmacokinetic profile of drugs is poorly documented. Thus, deciding on a

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drug-dosing regimen is often difficult. During hemodiafiltration, a substantial amount of the drug may be removed, and the patient may be exposed to subtherapeutic concentrations. Therefore, the monitoring of concentrations of the drug in plasma during hemodiafiltration and inter-hemodiafiltration periods should be performed in patients treated by IVVHD.

In septic patients, the principles of rational therapy include a combination therapy with a β-lactam antibiotic and an aminoglycoside or the use of monotherapy with either a carbapenem or fourth-generation cephalosporin, or selected third-generation cephalosporin [2].

Cefepime, 7-[α-(2-aminothiazol-4-yl)-α-(*z*)-methoxyiminoacetamido]-3-(1-methylpyrrolidino)-methyl-3-cephem-4-carboxylate, is a parenteral fourth-generation cephalosporin antibiotic with a broad spectrum of antimicrobial activity, and low affinity and good stability for extended-spectrum β-lactamase [3,4]. Cefepime differs from other cephalosporins by a quaternerized *N*-methyl-pyrrolidine substitution at the 3 position of the cephem nucleus, i.e. it is a zwitterion (Fig. 1) [5,6]. Its molecular mass is 480.6 Da.

Several HPLC methods have been reported for the determination of cefepime [7–12] in plasma, serum, urine, tissue or vitreous fluid. However, most of the published methods either did not report assay validation or reported assay validations which were incomplete. Moreover, some of them did not use an internal standard [8,9,11]. An HPLC assay for the determination of cefepime in human serum using an ultrafiltration extraction procedure has been published recently [13].

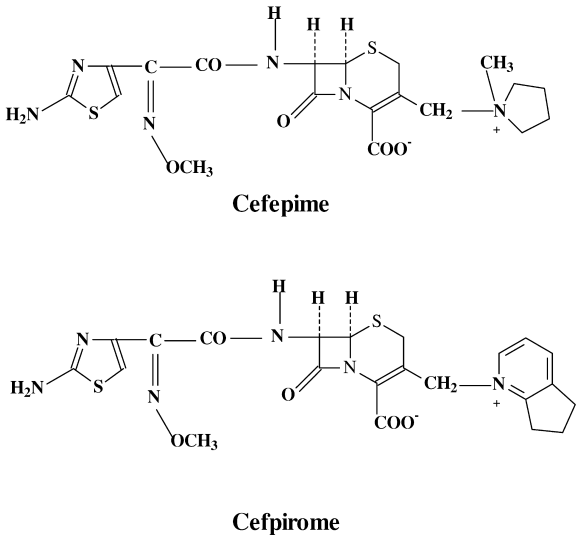

Fig. 1. Structural formulae of cefepime and cefpirome.

The purpose of this study was to develop reproducible, reliable, rapid and selective methods for the determination of cefepime in plasma, dialysate outlet and urine for therapeutic drug monitoring on patients with septic shock treated by IVVHD. Two different methods were developed, a liquid–liquid extraction to quantify cefepime in plasma and a solid-phase extraction (SPE) method based on column switching for urine and dialysis fluid assays of this drug. These methods have enhanced precision due to the use of an internal standard (another fourth-generation cephalosporin, cefpirome) with retention times very close to that of the analysed drug. These methods were validated with respect to accuracy, precision, selectivity, and limits of quantitation and of detection according to Good Laboratory Practice Guidelines [14–16].

2. Experimental

2.1. Materials and reagents

Cefepime and cefpirome were obtained from Bristol-Myers Squibb (Paris, France) and Hoechst Marion Roussel (Swindon, UK), respectively. The structural formulae of these compounds are shown in Fig. 1. Acetonitrile and dichloromethane were Chromasol grade (SDS, Peypin, France) and used without further purification. Ammonium acetate, disodium hydrogen phosphate, and trichloracetic, orthophosphoric and acetic acids were all analytical grade (Merck, Nogent sur Marne, France). Di-sodium hydrogen phosphate (0.01 *M*, pH 7) was prepared in purified water (Laboratoires Fandre, Ludres, France) and adjusted with orthophosphoric acid (10%). The buffer consisted of 1.54 g ammonium acetate in 1 l of purified water adjusted to pH 4.0 with acetic acid.

Stock solutions of cefepime (1 and 10 mg/ml) and internal standard (10 mg/ml) were prepared in purified water.

Drug-free human plasma and urine obtained from pooled samples collected from healthy volunteers as well as the dialysis fluid were used for the validation of the method. Blood was collected in heparinized tubes and then centrifuged at 2000 *g* for 10 min. The obtained drug-free plasma and drug-free urine were stored at −30°C before use.

2.2. Instrumentation

Analysis by HPLC was performed using a Gilson instrument (Paris, France) with a Rheodyne loading valve fitted with a 100-μl sample loop, an automatic sample injection system (Gilson 232), an oven (Jones Chromatography, Touzart Matignon, Paris, France), a stainless-steel column (100×4.6 mm I.D., ThermoQuest, Hypersil Division, Paris, France) packed with Nucleosil C_{18} (5 μm), and a guard column (15×4.6 mm I.D.; ThermoQuest) packed with hypersil ODS (5 μm) placed just before the inlet of the analytical column. All the chromatographic conditions were controlled using the GME 712 Gilson software.

For on-line SPE clean-up and pre-concentration of the samples (urine or dialysate outlet), the basic chromatographic apparatus was supplemented with a pre-column (30×4.6 mm), dry filled with Spheri 5 amino (5 μm; Brownlee, Touzart Matignon), a constant flow pump (Gilson) for pumping the necessary solvent for the clean-up and pre-concentration on the pre-column, a six-way high-pressure valve and a Gilson sample controller for the complete automation of the switching operations. A scheme of the chromatographic apparatus used for on-line clean-up of samples with column switching is shown Fig. 2. The column effluent was monitored with a variable-wavelength UV detector (Model SPD-6AV; Shimadzu Instruments, Touzart Matignon) operated at 280 nm.

2.3. Chromatographic conditions

The mobile phase, containing acetonitrile and ammonium acetate (pH 4) (10:90, v/v for plasma; 12:88, v/v for urine or dialysate outlet), was deaerated prior to use. The flow-rate was 1 ml/min, which corresponds to a pressure of ~90 bar (9 MPa). The oven temperature was set at 35°C.

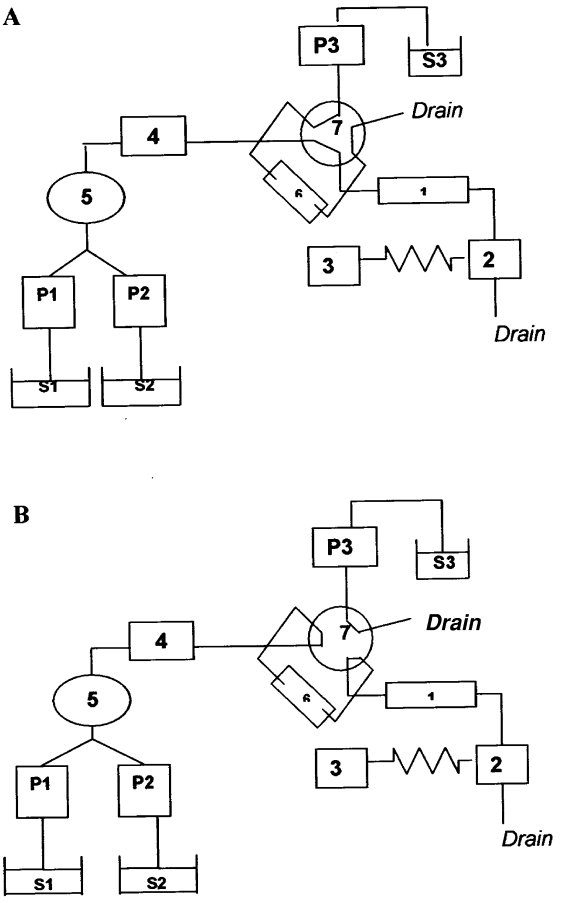

Fig. 2. Scheme of the HPLC system for quantitation of cefepime in urine and hemodiafiltrate. P1, P2 and P3, pumps; S1 and S2, solvents of the mobile phase; S3, washing solvent; 1, analytical column; 2, UV detector; 3, data processor; 4, automatic injection system with a Rheodyne loading valve fitted with a 100-μl sample loop; 5, mixer; 6, pre-column for on-line SPE; 7, Rheodyne loading valve. (A) Rheodyne valve is positioned for on-line SPE clean-up and pre-concentration. (B) Rheodyne valve is positioned to transport sample to the analytical column.

2.4. Analytical procedure

2.4.1. Calibration curves and quality control (QC) samples

Quantitation was based on the internal standard method. Stock solutions of cefepime were used to spike plasma (0.5 ml), diluted urine (1:20 in purified water, 0.5 ml) and dialysis fluid (0.5 ml) samples in order to obtain calibration curves at concentrations of 0.5, 1, 5, 10, 20, 50 and 100 μg/ml. For these three

matrices, inter-day repeatability was determined for calibration curves prepared on different days. Intra-day repeatability was determined for calibration curves prepared on the same day in plasma and urine. In each case, the number of replicates was seven.

QC samples, at high, middle, and low concentrations (0.8, 40 and 80 μg/ml), were prepared in plasma, diluted urine and dialysis fluid.

2.4.2. Extraction procedure

Plasma samples (0.5 ml) were spiked with internal standard (3 μl of cefpirome at 10 mg/ml) and homogenised. Trichloracetic acid (5% in purified water, 0.3 ml), acetonitrile (0.5 ml) and dichloromethane (1.5 ml) were added to all samples and the mixture Vortex-mixed for 10 s, then all vials were centrifuged at 2000 *g* for 10 min. An aliquot of 100 μl of the supernatant (aqueous phase) was injected onto the column.

To quantify cefepime in urine and dialysis fluid, diluted urine samples in purified water (1:20, 0.5 ml) or non-diluted dialysis fluid samples (0.5 ml) were spiked with internal standard (1 μl of cefpirome at 10 mg/ml) and homogenised. A 100 μl volume of this solution was injected. The sample was loaded on the pre-column, where the clean-up and pre-concentration took place; the pre-column, after sample injection, was flushed for 1 min with Na_2HPO_4 (pH 7.0) at a flow-rate of 0.25 ml/min in order to eliminate endogenous compounds. Then, after valve switching, the pre-column was connected to the analytical column where analytes were transferred by the HPLC mobile phase, the pre-column was disconnected after 7 min and then, while chromatography took place on the analytical column, it was re-equilibrated with Na_2HPO_4 for 2 min. The next sample was then injected.

2.5. Data analysis

Peak-height ratios of cefepime to internal standard were used to construct the standard curves. Unweighted least squares linear regression of the peak-height ratios as a function of the theoretical concentrations was applied to each standard curve (formula: $y = b + ax$, where x is concentration (μg/ml), y the peak-height ratio, a the slope and b the intercept). The equation parameters (slope and intercept) of each standard curve were used to obtain concentration values for that day QC samples and unknown samples.

The ''Lack of Fit'' test was used to confirm the linearity of the method. Moreover, the back-calculated concentrations (C_{TEST}) were compared with the theoretical concentrations (C_{REF}), and the bias (or mean predictor error) was computed as follows:

$$\text{Bias} = \frac{1}{n}\sum_{i=1}^{i=n}[C_{TEST}(i) - C_{REF}(i)]$$

In this expression the index i refers to the concentration number and n is the sample size.

The 95% confidence interval for bias was also computed.

2.6. Specificity

To evaluate the specificity of the method, 15 different batches of drug-free plasma and urine samples were passed through the assay procedure and the retention times of endogenous compounds were compared with those of cefepime and internal standard.

The interference from other drugs that could be co-administered was also studied. The following drugs were checked: amikacin, tobramycin, isepamicin, cimetidine, ranitidine, amphotericin B, heparin.

2.7. Precision and accuracy

Precision and accuracy were assessed by performing replicate analyses of QC samples in plasma, diluted urine and dialysis fluid against a calibration curve. The procedure was repeated on different days on the same spiked standards to determine inter-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate on the same day.

Accuracy is expressed as percent recovery [(mean back-calculated concentrations/theoretical concentrations)·100], while precision is given by the inter-day and intra-day percent relative standard deviations (RSDs).

2.8. Determination of the limits of quantitation (LOQ) and detection (LOD)

The LOQ was defined as the lowest drug concentration which can be determined with an accuracy of 80–120% and a precision of ≤20% on a day-to-day basis [13–15].

The LOD was defined as the simple concentration resulting in a peak area of three times the noise level.

2.9. Recovery

The extraction efficiency (recovery) was determined by comparing peak heights for drug-free plasma, urine and dialysis fluid spiked with known amounts of drugs (0.8, 40 and 80 μg/ml), assayed accordingly, versus peak heights of the same concentrations prepared in purified water injected directly onto the analytical column. Each sample was determined in triplicate.

The extraction efficiency was also determined for the internal standard.

2.10. Stability study

The stability of stock solutions was tested at ambient temperature (22–25°C) and at −30°C.

For stability studies in the three matrices, QC samples were used.

The stability of cefepime in plasma, urine and dialysis fluid was inspected during all the storage steps (i.e., at room temperature, at +4°C and at −30°C). Spiked samples were analysed, against a calibration curve, immediately after preparation (reference values) and after storage. Each determination was performed in triplicate.

The freeze–thaw stability was also determined. Spiked samples were analysed immediately after preparation and on a daily basis after repeated freezing–thawing cycles at −30°C on 3 consecutive days.

The stability of cefepime and cefpirome in the aqueous extracts (i.e., extracts originating from plasma spiked with these two drugs) was also investigated at 20°C.

Stability was defined as <10% loss of the initial drug concentration.

2.11. Pharmacokinetic study

The study was carried out on patients with acute renal failure undergoing intermittent hemodiafiltration (each 48 h) for septic shock. They received a 30-min intravenous infusion of 2 g of cefepime via an infusion pump each 12 h. Venous blood samples were collected in heparinized glass tubes before drug administration, at the end of infusion (30 min), and 45 min, 1 (start of hemodiafiltration), 1.5, 2, 4, 5, 6, 8 or 12 (end of hemodiafiltration), 16, 20, 24, 36 and 48 h after the start of infusion. Immediately after collection, blood samples were centrifuged (2000 *g* for 10 min); the plasma was placed in polypropylene tubes and immediately frozen (−30°C) until assay. Dialysate outlet was obtained every 2 h during the hemofiltration periods. When urine data were available, the total urine output was taken from an indwelling catheter every 4 h.

The protocol was approved by the institutional review board.

3. Results

3.1. Retention times and specificity

In plasma, observed retention times were 3.9±0.2 and 7.0±0.02 min for cefepime and internal standard, respectively. In urine and dialysis fluid, they were 3.5±0.04 and 6.4±0.07 min, respectively. The corresponding k' values were 2.77 and 5.36 in plasma, and 2.0 and 4.5 in urine and dialysis fluid. The time intervals where cefepime and the internal standard eluted were free of interferences in all of the drug-free plasma and urine samples tested (Figs. 3a and 4a). No interference was found with all drugs (and their metabolites) tested that could be co-administered.

Representative chromatograms are shown in Figs. 3 and 4.

3.2. Linearity

The determination coefficients (r^2) for calibration curves were ≥0.998. Peak-height ratios of cefepime over the internal standard varied linearly with con-

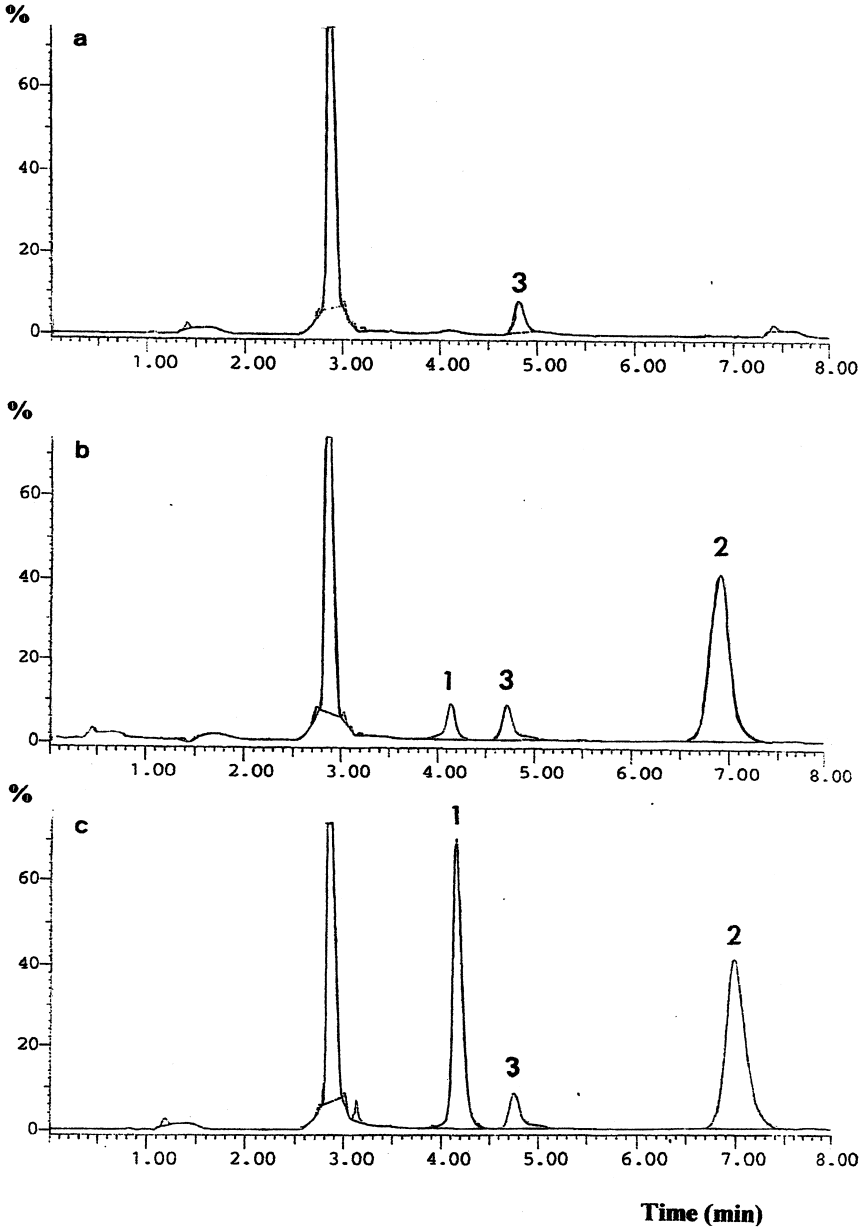

Fig. 3. Chromatograms of a blank plasma sample (a), plasma spiked with 1 μg/ml of cefepime and 60 μg/ml of cefpirome (internal standard) (b), and one subject's 8-h post-dose plasma sample after intravenous infusion of 2 g of cefepime (concentration, 46.6 μg/ml) (c). Peak 1 is cefepime, peak 2 is the internal standard and peak 3 is an endogenous compound. A.U.F.S.: 0.04.

centration over the range used. The ''Lack of Fit'' test showed no significant deviation from linearity.

For each point of the calibration standards, the concentrations were back-calculated from the equation of the linear regression curves and the percent RSD values were computed. A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept equal to 0 (Student's *t*-test). The distribution of the residuals (difference between nominal and back-

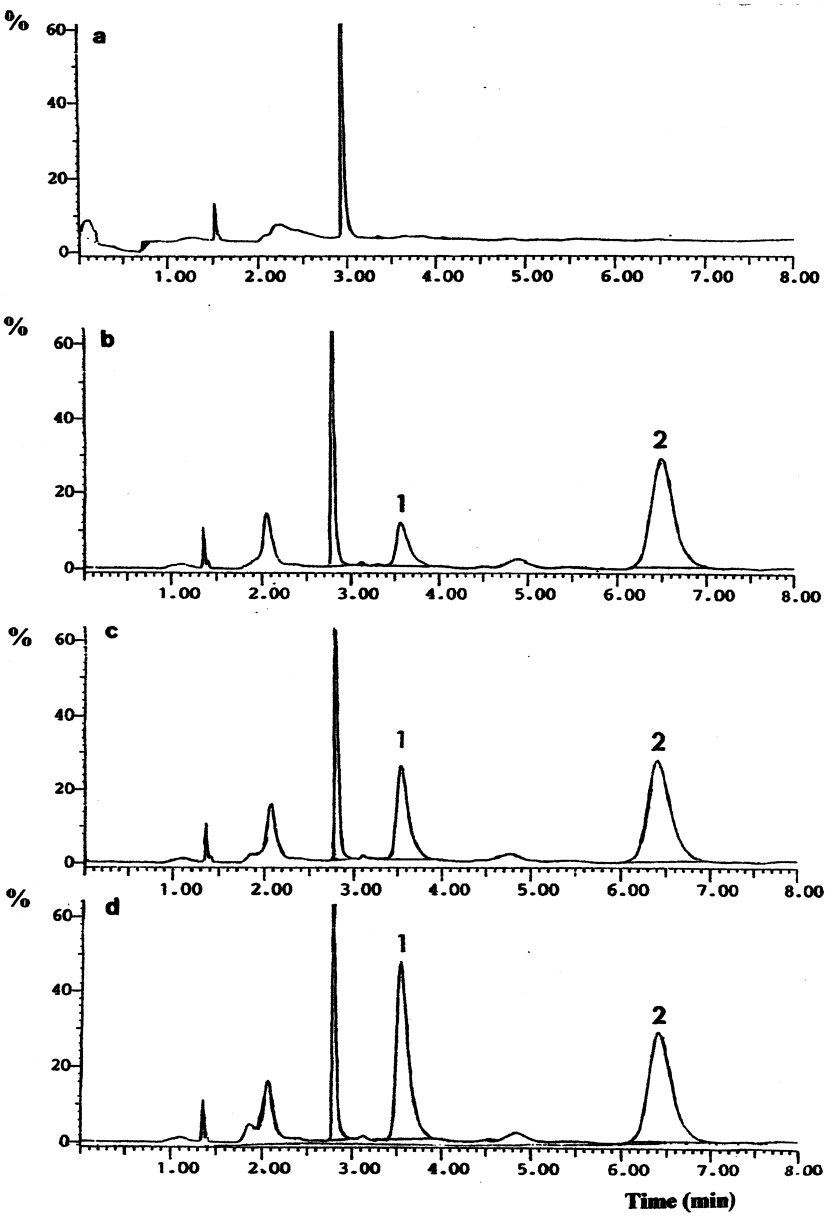

Fig. 4. Chromatograms of blank urine (a), urine spiked with 1 μg/ml of cefepime and 30 μg/ml of cefpirome (internal standard) (b), one subject's 6-h post-dose urine sample after intravenous infusion of 2 g of cefepime (concentration, 10.9 μg/ml in diluted sample) (c) and one subject's 6-h post-dose dialysate outlet sample after intravenous infusion of 2 g of cefepime (concentration, 38.9 μg/ml) (d). Peak 1 is cefepime, and peak 2 is the internal standard. A.U.F.S.: 0.04.

calculated concentrations) shows random variations, the number of positive and negative values being approximately equal. They were normally distributed and centered around zero. The bias values ($-1.78 \cdot 10^{-3}$ for plasma; $0.03 \cdot 10^{-4}$ for dialysis fluid; $-1.33 \cdot 10^{-4}$ for urine) were not statistically different from zero (Student's *t*-test) and the 95% confidence intervals included the zero value.

Table 1
Assay linearity for cefepime

Sample[a]	Correlation coefficient (r) of the linear[b] regression analysis (mean±SD)		Slope (a) (mean±SD)		Intercept (b) (mean±SD)	
	Intra-day repeatability	Inter-day repeatability	Intra-day repeatability	Inter-day repeatability	Intra-day repeatability	Inter-day repeatability
Human plasma	$0.999\pm5.0\cdot10^{-4}$	$0.999\pm1.4\cdot10^{-3}$	$0.0221\pm6.0\cdot10^{-4}$	$0.0218\pm1.2\cdot10^{-3}$	$-0.005\pm1.2\cdot10^{-2}$	$0.004\pm5.0\cdot10^{-3}$
Human urine	$0.999\pm2.0\cdot10^{-4}$	$0.999\pm3.0\cdot10^{-4}$	$0.0735\pm2.4\cdot10^{-3}$	$0.0748\pm2.5\cdot10^{-3}$	$0.042\pm1.1\cdot10^{-2}$	$0.023\pm1.5\cdot10^{-2}$

[a] $n = 7$.
[b] Linear unweighted regression, formula: $y = b + ax$.

Results obtained for plasma and urine are reported in Tables 1 and 2.

For dialysis fluid, the inter-day average slope of the fitted straight lines was 0.078 (coefficient of variation, 2%). For concentrations of calibration standards, the precision around the mean value ranged from 0.2 to 5%.

3.3. Precision, accuracy and extraction recovery

For plasma and urine, the results for accuracy, intra-day, and inter-day precision for QCs are presented in Table 2. For the dialysis fluid, the precision was below 5% and accuracy ranged from 98 to 104%.

Table 2
Intra- and inter-assay reproducibilities of the HPLC analysis of cefepime[a]

Theoretical concentration (μg/ml)	Intra-day repeatability			Inter-day repeatability		
	Calculated concentration (μg/ml)	RSD (%)	Mean recovery (%)	Calculated concentration (μg/ml)	RSD (%)	Mean recovery (%)
Human plasma						
0.5	0.488	3.3	97.7	0.497	6.4	99.4
0.8	0.835	5.7	104.0	0.790	8.9	98.7
1	0.999	6.5	99.9	1.06	10.1	106.4
5	5.28	10.0	105.6	5.11	9.1	102.3
10	10.1	5.3	101.4	10.2	8.1	102.0
20	18.9	2.7	94.8	19.9	6.6	99.9
40	37.2	5.1	93.1	42.1	5.2	105.0
50	49.9	3.2	99.9	49.9	7.2	99.8
80	83.0	6.2	104.0	79.9	7.2	99.8
100	100.2	0.70	100.2	99.9	1.3	99.9
Human urine						
0.5	0.507	6.7	101.5	0.526	2.9	105.3
0.8	0.790	8.8	98.7	0.810	8.4	101.0
1	0.980	7.8	97.6	1.02	7.6	101.9
5	4.95	5.9	99.1	4.92	5.6	98.5
10	10.4	5.7	103.9	10.5	3.4	105.5
20	20.9	1.8	104.3	20.2	2.4	101.0
40	38.9	2.0	97.3	40.1	4.4	100.0
50	49.5	1.1	98.9	49.3	2.3	98.6
80	81.6	1.5	102.0	79.5	4.3	99.4
100	100.1	0.3	100.1	100.2	0.5	100.2

[a] Calibration standards ($n = 7$): 0.5, 1, 5, 10, 20, 50 and 100 μg/ml. Quality control samples ($n = 6$): 0.8, 40 and 80 μg/ml.

For plasma, the mean recovery ($n = 9$) averaged 86.0±3.8% for cefepime and 85.0±3.9% ($n = 6$) for the internal standard. For urine and dialysis fluid, recoveries were 87.0±5.3 and 84.0±3.5% for the two analytes, respectively. The extraction efficiency was not statistically different over the range of concentrations studied.

3.4. Limit of quantitation and limit of detection

The limit of quantitation was 0.5 μg/ml in the three matrices studied. The limit of detection was 0.2 μg/ml.

3.5. Stability

Stock solutions of cefepime and internal standard in aqueous solution did not reveal any appreciable degradation after 1 month of storage at −30°C. At 20°C, they were stable for 8 h.

In plasma, after bench-top storage at room temperature, cefepime was stable for 4 h; the percent recovery ranged from 97 to 103%. A mean loss of 13% was observed after 6 h. At +4°C, cefepime was stable for at least 8 h. Frozen at −30°C, cefepime was stable for 45 days; a significant degradation averaging 15–22% was observed after 60 days.

In urine and dialysis fluid, cefepime was stable for at least 8 h at ambient temperature and at +4°C, and for at least 60 days at −30°C; for each time study, no statistical difference was found on comparison with the reference values.

At least three freeze–thaw cycles can be tolerated without losses of greater than 10%.

In aqueous extracts originating from plasma, after sample pretreatment, cefepime and cefpirome were stable for at least 2 h at 20°C.

3.6. Pharmacokinetic study

Fig. 5 shows plasma concentration versus time profiles obtained for a patient during three hemodiafiltration periods. The elimination half-life ranged from 3 to 7.2 h; these values are very close to those determined from the variation with time of the cefepime excretion rate in the dialysate outlet (3 to 6.8 h). The differences in the elimination rate were due to the differences in the flow-rates of the blood pump and of the ultrafiltration used during each hemodiafiltration period. Mean total clearance was 61 ml/min (46–75 ml/min). The fraction of cefepime removed by hemodiafiltration accounted for 20–24%; this patient still had some urine output, and 4 to 6% of the dose was excreted in urine. The

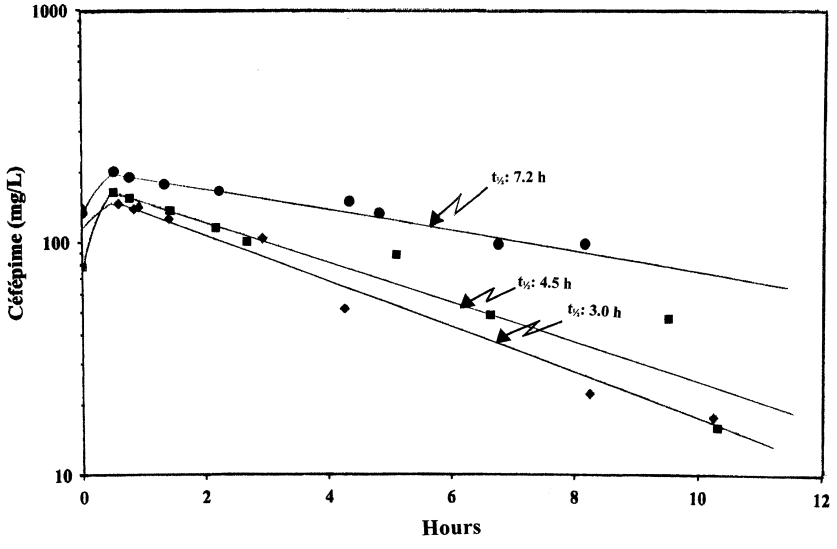

Fig. 5. Evolution of cefepime concentrations in plasma during hemodiafiltration periods [hemodiafiltration No. 1 (●), No. 2 (♦), No. 3 (■)].

hemodiafiltration clearance ranged from 11 to 17 ml/min. The renal clearance of 2 to 3 ml/min was very close to the creatinine clearance of this patient (2.4–3.6 ml/min). During the interhemodiafiltration period, the elimination half-life of the drug was 24 h.

4. Discussion and conclusion

The present HPLC method enables a rapid assay of cefepime in plasma, urine and dialysate outlet with a run time shorter than 10 min. The sample extraction and clean-up procedure consistently gave high recoveries with no significant interference. The methods described were found to be suitable for the analysis of all samples collected during pharmacokinetic investigations in humans. The column-switching method with a LOQ of 0.5 μg/ml in urine and dialysis fluid, which generally satisfies pharmacokinetic needs, is very quick since during the chromatographic run it performs automatic clean-up and pre-concentration of the samples.

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