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Determination of cefepime and cefpirome in human serum by high-performance liquid chromatography using an ultrafiltration for antibiotics serum extraction

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Abstract

The aim of this study was to describe a high-performance liquid chromatography (HPLC) assay for the determination of cefepime and cefpirome in human serum without changing chromatographic conditions. The assay consisted to measure cefepime and cefpirome which were unbound to proteins having a molecular mass of 10 000 or more by ultrafiltration followed by HPLC with a Supelcosil ABZ+ column and UV detection at a wavelength of 263 nm. The assay was been found to be linear and has been validated over the concentration range 200 to 0.50 μg/ml for both cefepime and cefpirome, from 200 μl serum, extracted. In future, the assay will support therapeutic drug monitoring for cefepime and cefpirome in neutropenic patients in correlation with microbiological parameters such as MIC_{90} (minimal inhibitory concentration of antibiotic which kills 90% of the initial bacterial inoculum) and clinical efficacy.

Keywords: Cefepime; Cepirome

1. Introduction

In empirical antimicrobial chemotherapy for febrile neutropenic patients, drug combinations are commonly used, and aminoglycosides are usually included for their excellent activity on Gram-negative organisms and for potential synergism. However, new β-lactams have at least as good a spectrum and in many ways better pharmacology [1,2]. Many clinical studies suggest that the importance of the antibiotic dosage schedule for therapeutic efficacy in severe infection and when host defences are impaired is related to the class of antibiotic [3]. The efficacy of β-lactams is mainly dependent on the maintenance of adequate antibiotic concentrations in serum during the entire treatment interval and not on high peak concentrations such as aminoglycosides [4]. Cephalosporins are one of the mainstays of antibiotic therapy, and third-generation such as ceftazidime and new-generation such as cefepime or cefpirome are first-line agents for the empirical antibiotic therapy in the febrile neutropenic patients. Combinations of third- or new-generations of cephalosporins and aminoglycosides have been standard therapy for suspected infections in neutropenic patients. But recent studies have shown that monotherapy of

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ceftazidime [5] or cefepime [6] was as effective overall as combination therapy for the empirical treatment of such infections.

The purpose of this study was to investigate a rapid, selective and sensitive high-performance liquid chromatographic method using an ultrafiltration extraction procedure for the determination of cefepime and cefpirome in human serum without changing the chromatographic conditions. This assay will be used for pharmacokinetic and pharmacodynamic studies and finally for the clinical evaluation of new-generation of cephalosporins for the treatment of febrile episodes in neutropenic patients in association with microbiological and pharmacokinetic parameters.

2. Experimental

2.1. Chemicals

Cefepime was obtained from Bristol Myers Squibb (Paris, France), cefpirome from Roussel (Paris, France) and ceftazidime, the internal standard, was obtained from Glaxo (Paris, France).

Potassium dihydrogenphosphate, Normapur quality, concentrated hydrochloric acid, Normapur quality, and concentrated orthophosphoric acid, Normapur quality, were from Prolabo (Nogent sur Marne, France). Tris(hydroxymethyl)methylamine (Tris) was from Aldrich (Saint Quentin Fallavier, France). Acetonitrile, Chromar HPLC quality, was purchased from Mallinckrodt (Deventer, The Netherlands).

Drug-free plasma was purchased from the Aquitaine transfusion establishment (Bordeaux, France).

2.2. Instrumentation

The Kontron Model 300 high-performance liquid chromatography (HPLC) system (Milan, Italy) consisted of a Model 525 pump, a Model 360 autosampler and a Model 332 UV detector connected to a Model D450 software for signal treatment.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–20 m*M* potassium dihydrogenphosphate buffer (6:94, v/v). The pH was adjusted to 2 with concentrated orthophosphoric acid and the mobile phase was filtered though a Millipore (Saint Quentin en Yveline, France) filter HPLV 0.45 μm. The elution conditions were isocratic, and the mobile phase flow-rate was set at 1 ml/min. The analytical column was a Supelcosil ABZ+ (5 μm; 150×4.6 mm) from Supelco (Saint Quentin Fallavier, France).

The sample injection volume was 20 μl. UV absorbance at a wavelength of 263 nm was used for detection and the range of the detector was set at 0.05 a.u.f.s. The chromatographic run time was 10 min.

2.4. Preparation of standards and seeded controls

Stock solutions of cefepime, cefpirome and ceftazidime were prepared by dissolving the appropriate amount of cefepime and cefpirome, accurately weighted, in distilled water to yield a final drug concentration of 2000 μg/ml for each drug. Working stock solutions of 2000, 500, 200, 100, 50, 20, 10, 5 μg/ml were prepared by appropriate dilution of the 2000 μg/ml standard solution. Aliquots of those solutions were stored at −20°C. Every day, working standards were prepared by 10-fold dilution of the stock solutions in drug-free plasma. The resulting standards ranged in concentration from 200 to 0.5 μg/ml for cefepim and for cefpirome. These ranges were based on human cefepime and cefpirome concentrations found in previous pharmacokinetic studies [7,8]. Quality control concentrations representing 5, 40 and 80 μg/ml of cefepime and cefpirome in serum were prepared by specific dilution of the 100 μg/ml stock solution in drug-free serum .

2.5. Sample treatment

Blood samples were collected in heparinized tubes and centrifuged (10 min, 1850 *g*, 20°C) as soon as possible before storing at −80°C.

2.6. Sample extraction procedure

The filters used were Microcon (10 000 M_r cut-off) from Amicon (Beverly, MA, USA). Daily, an aliquot of ceftazidime stock solution was thawed and then diluted 10-fold in Tris buffer. Tris buffer (100 m*M*) was prepared with Tris(hydroxymethyl)aminomethane (12.1 g/l) and adjusted to pH 7 with concentrated HCl^-. The sample (200 μl) and an equal volume of internal standard solution was dispensed on a filter. The mixture was stirred on a vortex and centrifuged at 10 000 *g* for 15 min.

The ultrafiltered sample was transferred to a microvial and 20 μl was injected into the column.

2.7. Calibration and calculation procedures

Daily calibration curves were constructed using the ratios of the observed peaks area of cefepime and cefpirome and the internal standard. Unknown concentrations were computed from the unweighted linear regression equation of the peak-area ratio against concentration for the calibration curve.

2.8. Accuracy, precision and recovery

The intra-day accuracy and precision of the method were estimated from the back-calculated standard concentrations. The overall mean precision was defined by the coefficient of variation (CV) with relative errors from six standards analyzed on the same day.

Inter-day variability was estimated from the triplicate analysis of three sample on six separate days. Recovery of cefepime, cefpirome and ceftazidime after the ultrafiltration was determined by comparing observed cefepime, cefpirome and ceftazidime concentrations in extracted plasma to those of non-processed standard solutions.

2.9. Specificity and selectivity

Specificity was assessed in the presence of clavulanic acid, tazobactam, sulbactam, amoxicillin, ampicillin, cefixime, cefotaxime, cloxacillin, imipenem, cefalotin, mezlocillin, latamoxef, penicillin G, piperacillin, ticarcillin, fusidic acid, ciprofloxacin, ofloxacin, pefloxacin, fosfomycin, vancomycin, erythromycin, rifampicin, amikacin, tobramycin, netilmicin, gentamicin, itraconazole and amphothericin B.

2.10. Limit of detection and quantitation

The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) is the minimum injected amount that gives precise measurements; chromatography, for example, typically requires peaks height 10–20-times higher than baseline noise. If the required precision of the method at the LOQ has been specified, the Eurachem [9] approach can be used. A number of samples with decreasing amounts of the analytes are injected six times. The calculated CV of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision (15%) is equal to the LOQ. The minimum quantifiable concentration (MQC) measuring the lowest concentration at which there is satisfactory a priori measures of accuracy and precision using the appropriate biological matrix was calculated.

3. Results

3.1. Chromatographic characteristics

Fig. 1 shows chromatograms of an extracted blank serum sample, an extracted serum sample containing 80 μg/ml of both cefepime and cefpirome, one subject's 2 h postdose serum sample after intravenous infusion of 2 g of cefepime and a second subject's 2 h postdose serum sample after intravenous infusion of 2 g of cefpirome. The extrapolated subject's 2 h postdose serum concentrations were respectively, 48 μg/ml for cefepime and 53 μg/ml for cefpirome.The calculated capacity factor (k') for cefepime, cefpirome and ceftazidime were 1.09, 3.47 and 5.04, respectively. Retention times were 2.81 min for cefepime, 6.37 min for cefpirome and 7.23

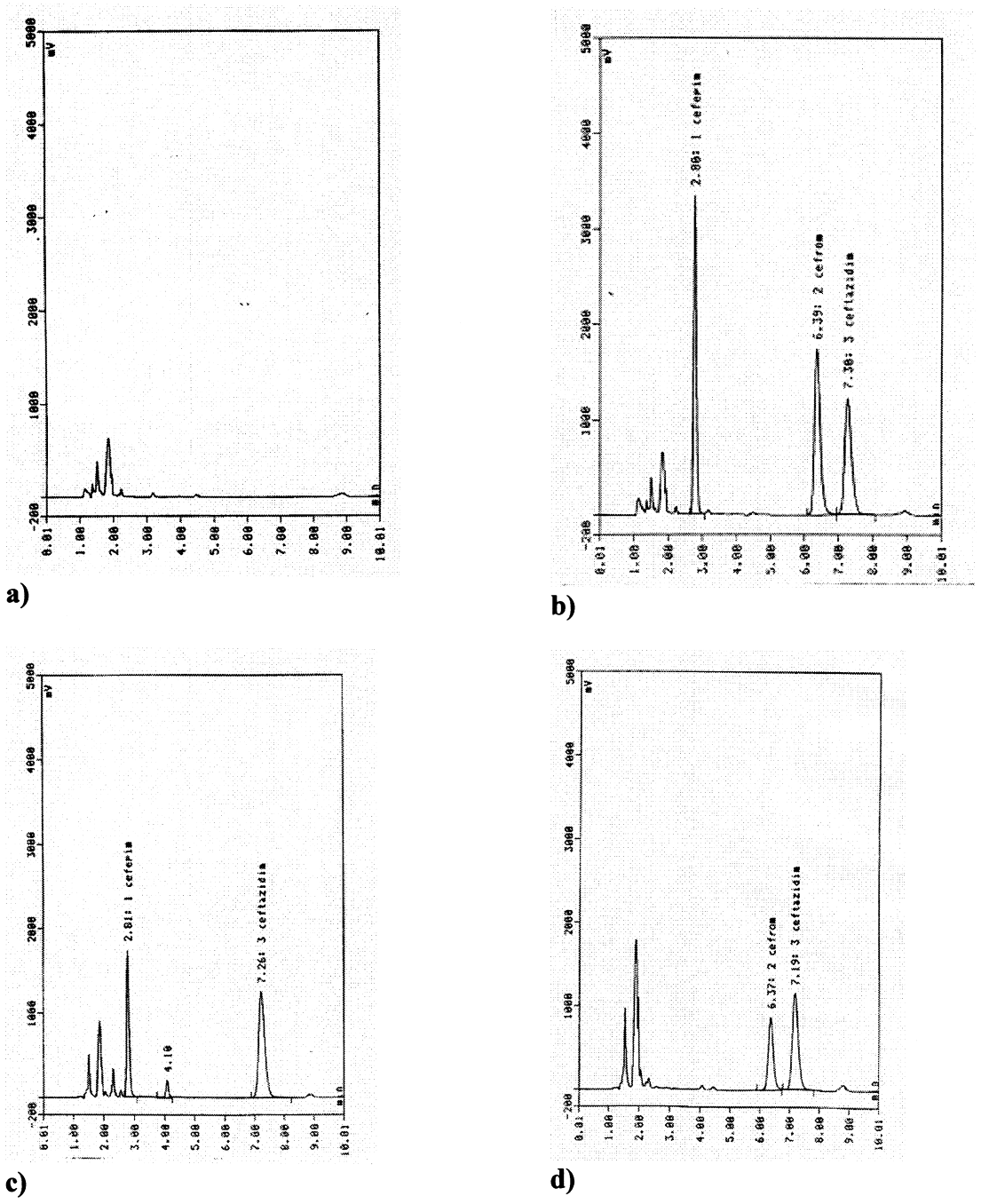

Fig. 1. Chromatograms of, an extracted blank serum sample (a), an extracted serum sample containing 80 μg/ml of both cefepime and cefpirome (b), one subject's 2 h post dose serum sample after intravenous infusion of 2 g of cefepime (c) and a second subject's 2 h post dose serum sample after intravenous infusion of 2 g of cefpirome (d).

Table 1
Intra- and inter-day inaccuracy and precision from the determination of cefepime in serum samples

Theoretical concentration (μg/ml)	Concentration found (mean±SD, μg/ml)	Inaccuracy (%)	CV (%)	Relative error (%)	*n*
Intra-day					
80	81.00±1.60	101.2	1.98	1.21	6
40	39.50±0.29	98.8	0.73	−1.20	6
5	5.05±0.26	101	5.15	1.00	6
Inter-day					
80	78.85±1.61	98.6	2.04	−1.44	18
40	39.50±2.67	98.8	6.76	−1.25	18
5	5.24±0.27	104.8	5.15	4.80	18

min for ceftazidime ($n=6$). Peak symmetry was 1.11 for cefepime and 1.09 for cefpirome, respectively.

3.2. Calibration curve

The analysis of cefepime and cefpirome in plasma exhibited excellent linearity ($r^2=0.9994$ and $r^2=0.9997$, respectively for cefepime and cefpirome) over the 0.5–200 μg/ml concentration range. Regression intercepts for the calibration curves were generally very small and were not statistically significant compared to zero. These daily calibration curves were used for calibration and calculation purposes. The results indicated that the assay of both cefepime and cefpirome had acceptable precision (<10% CV) and accuracy (relative error <5% for cefepime, <15% CV for cefpirome); the lower LOQ was 0.50 μg/ml for cefepime and 1 μg/ml for cefpirome with a 200-μl sample volume.

3.3. Accuracy, precision and recovery

The intra-day accuracy and precision of the method were estimated from the back-calculated standards. The overall mean precision as defined by the CV, ranged from 1.98 to 5.15% for cefepime and from 1.67 to 6.27% for cefpirome from six series standards analyzed within the same day (Table 1).

Inter-day variability, as estimated from the triplicate analysis of three samples on six separate days Table 2, was low, with CVs ranging from 2.04 to 6.76% for cefepime and from 2.50 to 4.68% for cefpirome, and with relative errors ranging from −1.44 to 4.80% for cefepime and from 1.82 to 10.00% for cefpirome over the concentration range.

Table 2
Intra- and inter-day inaccuracy and precision from the determination of cefpirome in serum samples

Theoretical concentration (μg/ml)	Concentration found (mean±SD, μg/ml)	Inaccuracy (%)	CV (%)	Relative error (%)	*n*
Intra-day					
80	89.44±1.33	99.3	1.67	−0.70	6
40	37.30±2.34	93.3	6.27	−6.75	6
5	5.75±0.19	115	3.30	15.00	6
Inter-day					
80	81.46±2.04	101.8	2.50	1.82	18
40	40.60±1.90	101.5	4.68	1.50	18
5	5.50±0.23	110	4.18	10.00	18

These results indicated that this assay was reliable and reproducible.

The extraction recoveries of cefepime and cefpirome from quality control samples are 86 and 89%, respectively. The recovery of internal standard, as evaluated at a concentration of 5 μg/ml, was 91%.

3.4. Specificity and selectivity

Blank plasma showed no interfering endogenous substances in the analysis of cefepime and cefpirome. Potentially coadministered drugs tested had retention times that were different from cefepime and cefpirome or were not detected.

3.5. LOD, LOQ and MQC

The LOD in plasma was 0.1 μg/ml for both drugs.

At 0.50 μg/ml for cefepime and 1 μg/ml cefpirome the percent deviation from the respective nominal concentrations and the relative standard deviation were both less than 12%. Thus, 0.50 μg/ml for cefepime and 1 μg/ml for cefpirome were defined to be the LOQs. The MQCs were 0.1 μg/ml for cefepime and 0.5 μg/ml for cefpirome.

4. Discussion and conclusion

In the method described by Barbhaiya et al. [7] and by Paradis et al. [10], the retention times of cefepime and cefpirome were 5.3 and 7.8 min, respectively. The plasma protein was precipitated with acetonitrile and trichloroacetic acid, followed by extraction of the acetonitrile into methylene chloride for delipidation. In the method described by Elkhaili et al. [11], the extraction procedure was simplified with an equal volume of acetonitrile; the remaining acetonitrile was then removed from the supernatant by methylene chloride, leading to an increased concentration of the antibiotic in the supernatant. These three methods were sensitive, efficient and precise but the mean liquid–liquid extraction time was 20 min without taking account the analytical run. We developed a specific, sensitive and rapid assay for the measurement of cefepime and cefpirome in plasma to facilitate pharmacokinetic studies and therapeutic drug monitoring. An advantage of this assay is that we measure the free levels of cefepime and cefpirome by using an ultrafiltration instead total levels measured with precipitation or liquid–liquid extraction [12,13]. In neutropenic patients, monitoring cefepime or cefpirome concentrations in steady-state may be imperative first to ensure optimal drug efficacy when the antibiotic agent was used in monotherapy and second to prevent the risk drug resistance. The use of the Supelcosil ABZ+ column provides all the benefits of silica-based reversed-phase HPLC columns; a polar group incorporated in the Supelcosil ABZ+ phase gave a high level of silanol deactivation and selectivity, markedly different from that of conventional or deactivated C_{18} reversed-phase columns [14].

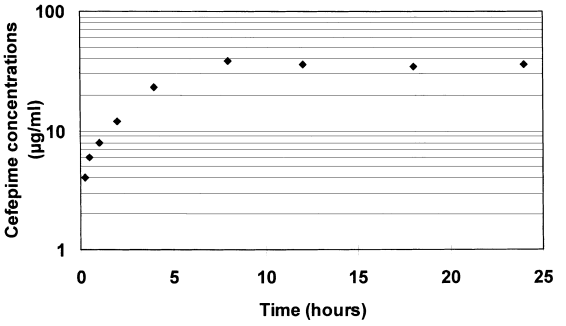

Fig. 2. Serum concentration–time curve following administration of 4 g cefepime by intravenous continuous infusion. Patient No. 25.

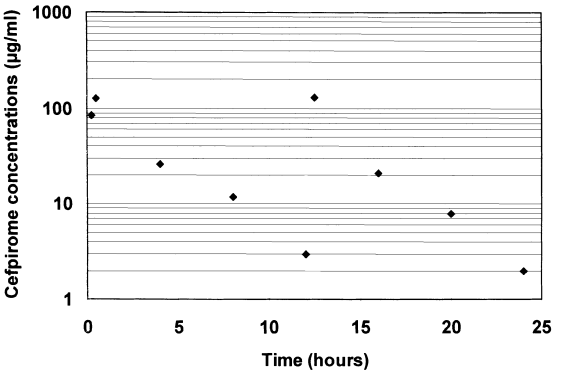

Fig. 3. Serum concentration–time curve following administration of 4 g cefpirome by intravenous intermittent infusion. Patient No. 42.

Supelcosil ABZ+ columns provide good peak shape and efficiency for polar compounds (such as cephalosporins). The HPLC run time for our assay is rapid and requires less than 10 min because extraction procedure has been simplified by using an ultrafiltration instead a liquid–liquid extraction for precipitate plasma proteins. Figs. 2 and 3 present the pharmacokinetic profiles of 4 g of cefepime in continuous infusion and 4 g of cefpirome in intermittent infusion. This new HPLC assay procedure permits the rapid, precise and accurate quantitation of cefepime and cefpirome in human serum. It seems to be much reliable than previous bioassays for both therapeutic drug monitoring and pharmacokinetic studies.

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