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Quantitative determination of cefepime in plasma and vitreous fluid by high-performance liquid chromatography

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Abstract

An isocratic reversed-phase HPLC method was developed to determine cefepime levels in plasma and vitreous fluid. Cefepime and the internal standard cefadroxil were separated on a Shandon Hypersil BDS C18 column by using a mobile phase of 25 m*M* sodium dihydrogen phosphate monohydrate (pH 3) and methanol (87:13, v/v). Ultraviolet detection was carried out at 270 nm. The retention times were 4.80 min for cefepime and 7.70 min for cefadroxil. This fast procedure which involves an efficient protein precipitation step (addition of $HClO_4$), allows a quantification limit of 2.52 μg ml^{-1} and a detection limit of 0.83 μg ml^{-1}. Recoveries and absolute recoveries of cefepime from plasma were 96.13–99.44% and 94–102.5% respectively. The intra-day and inter-day reproducibilities were less than 2% for cefepime at 10, 30, 50 μg ml^{-1} ($n=10$).The method was proved to be suitable for determining cefepime levels in human plasma and was modified to measure vitreous fluid samples.

Keywords: Plasma; Vitreous fluid; Cefepime

1. Introduction

Cefepime is a new injectable fourth-generation cephalosporin with a broad spectrum of activity against many gram-positive and gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It is more potent against some members of the Enterobacteriaceae family than other new broad-spectrum β-lactam antibiotics and is very effective against many β-lactamase-producing strains and has also high resistance to enzymatic hydrolysis [1–9]. However, there are no reports in literature to demonstrate that inhibitory aqueous and vitreous concentrations are attainable after intravenous administration of cefepime.

Cefepime differs from third-generation cephalosporins by having a positively charged quaternized *N*-methyl-pyrrolidine substitution at the 3 position of the cephem nucleus, which makes cefepime a zwitterion as shown in Fig. 1a. This property enhances the ability of cefepime to penetrate rapidly the outer cell membrane of gram-negative bacteria [10–13]. Cefadroxil (internal standard) is a typical semi-synthetic first generation cephalosporin, as shown in Fig. 1b.

Several methods have been reported for the determination of cefepime [14–17]. Second derivative spectroscopy [14] has been used for the determi-

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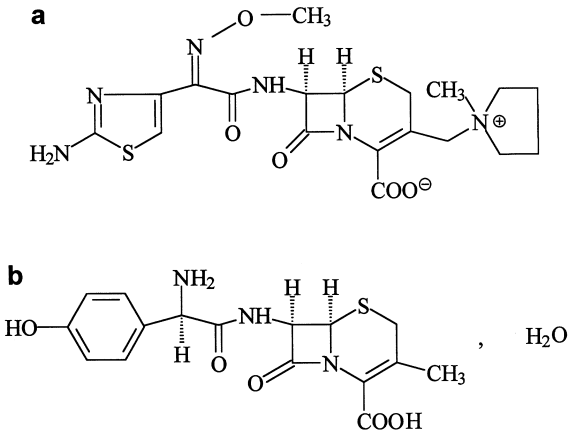

Fig. 1. (a) Structure of cefepime, 7-[α-(2-aminothiazol-4-yl)-α-(Z) - methoximino - acetamido] - 3 - [(1 - methyl - 1 - pyrrolidinio) - methyl]-3-cephem-4-carboxylate. (b) Structure of cefadroxil, 7-[D-(-)-α-amino-α-(4-hydroxyphenyl)acetamido]-3-methyl-3-cephem-4-carboxylic acid monohydrate.

nation of cefepime in various pharmaceutical forms, while a microbiological assay [15] and two HPLC techniques [16,17] have been used for the quantitation of cefepime in biological fluids.

In this work, we have tried to develop a sensitive, specific, rapid, easy and inexpensive HPLC method for the determination of cefepime in human plasma and vitreous fluid.

2. Experimental

2.1. Reagents and material

Cefepime and cefadroxil were kindly provided by Bristol–Myers Squibb SPA Sermoneta-Latina-Italy. Methanol and acetonitrile of HPLC grade were provided by Lab–Scan Analytical Sciences. Purified water was prepared with a Milli-Q Plus system (Millipore Co, USA). Perchloric acid and orthophosphoric acid were provided by Fluka and were of analytical grade. Sodium dihydrogen phosphate monohydrate is provided from Merck and was of analytical grade. Pooled plasma from healthy volunteers provided by a blood bank and vitreous fluid provided by a patient during an operation, were used for the calibration curves.

2.2. Standard solutions

A standard solution of 1 mg ml^{-1} cefepime in water was prepared and stored in the dark at 4°C. All working solutions were made by diluting this stock solution with water. Cefepime was stable in water for at least one month. All stock standards were prepared weekly and all working standards were prepared daily from the appropriate stock standard solution. Two solutions of 1 mg ml^{-1} for plasma samples and 50 μg ml^{-1} for vitreous fluid samples were prepared for cefadroxil to be used as the internal standard (I.S.).

2.3. Calibration procedure

Blank plasma and vitreous fluid were spiked with cefepime at concentrations within the studied range. I.S. solution (50 μl) was added to every calibration and unknown sample, and all tubes were vortex-mixed before the procedure.

2.4. Sample processing

To 0.2 ml plasma, 50 μl of I.S solution and 0.9 ml of water were added in a 5 ml screw-capped glass tubes. A 0.2 ml volume of $HClO_4$ 70% was also added for the precipitation of proteins. The tubes were vortex-mixed for 15 s, then centrifuged for 10 min at 2000 g. 0.2 ml of the supernatant was placed in a second test-tube and diluted with 0.8 ml of water. A 5 μl volume from this solution was injected into the column.

To 0.2 ml vitreous fluid, 50 μl of I.S. solution, 0.35 ml ACN and 0.1 ml of $HClO_4$ 70% were added. The tubes were vortex-mixed for 15 s, then centrifuged for 10 min at 2000 g. A 5 μl volume of the supernatant were injected into the column.

2.5. Chromatographic conditions

The HPLC system consisted of a Waters Model 501 solvent-delivery system with a Waters Model 486 variable-wavelength UV–Vis detector. A Shandon Hypersil BDS C18 column (250×4.6 mm I.D., 5 μm particle size) was used. A pre-column (10×4.0 mm) packed with the same packing material, was fitted just before the inlet junction of the analytical

column. The volume of the injection loop was 5 μl and the effluent was monitored at 270 nm. The mobile phase consisted of methanol 13% in a solution of sodium dihydrogen phosphate monohydrate 0.025 *M*, adjusted to pH=3 with orthophosphoric acid 25%. The mobile phase was degassed by filtering through a membrane filter (0.45 μm, Millipore) and delivered at a flow-rate of 1 ml min^{-1}. All separations were achieved at room temperature.

2.6. Precision

Precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. It is measured by analysing repeatedly ready-made samples and expressed as percentage relative standard deviation (R.S.D. %) of the results.

The intra-day precision (or repeatability) was evaluated by replicate analysis ($n=10$) of spiked plasma containing 10, 30, 50 μg ml^{-1} cefepime on five different times during a day.

The inter-day precision (or reproducibility) is defined as the long-term variability of the measurement process which here was determined from the same spiked plasma samples as above, analysed on five different days over a month period.

The resulting relative standard deviation (R.S.D.%) was indicated the intra-day repeatability and the inter-day reproducibility. The precision around the mean value should not exceed a R.S.D.% of 15%.

2.7. Accuracy/recovery

The analytical recovery (absolute recovery) was measured by spiking drug free plasma samples with known concentrations of cefepime and cefandroxil. After the extraction of the analytes from the matrix and injection onto the analytical column, their response was compared with the response of standard solutions of the drugs at the same concentration.

The accuracy of the method is defined as the degree of agreement of test results generated by the method to the true value. The recovery was used to assess the accuracy and it was calculated as the percentage ratio of measured/theoretical concentration of cefepime.

2.8. Specificity.

Specificity of an analytical method is its ability to measure accurately an analyte in the presence of interference that may be expected to be present in the sample matrix.

The specificity of the method was checked by testing samples from 22 patients receiving treatment with cefepime and other drugs.

2.9. In vivo study.

A 68 years old female patient, who was about to undergo vitrectomy received at 7.00 a.m an intravenous infusion of cefepime (Maxipime®) at a constant rate of 1 g h^{-1} for 6 h. Blood samples (5 ml) were withdrawn at various time intervals during the infusion period. A 0.5 ml volume of midcentral vitreous fluid was withdrawn at 12.30 p.m just before the start of the surgical procedure and before infusion of any vitrectomy solution. Blood samples were collected in sterile 7-cm tubes containing sodium

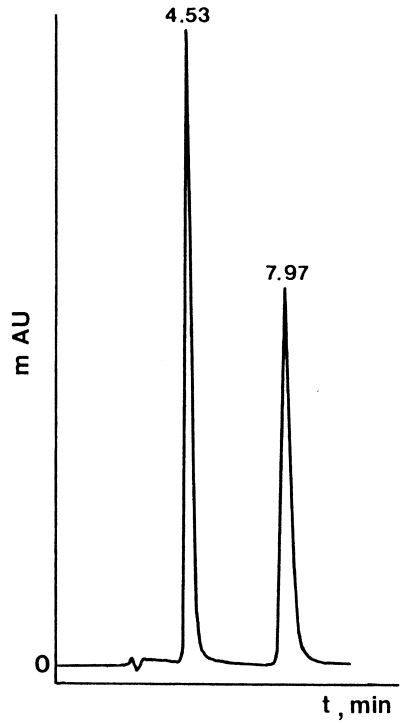

Fig. 2. Standard solution of cefepime (30 μg ml^{-1}) and cefadroxil (50 μg ml^{-1}).

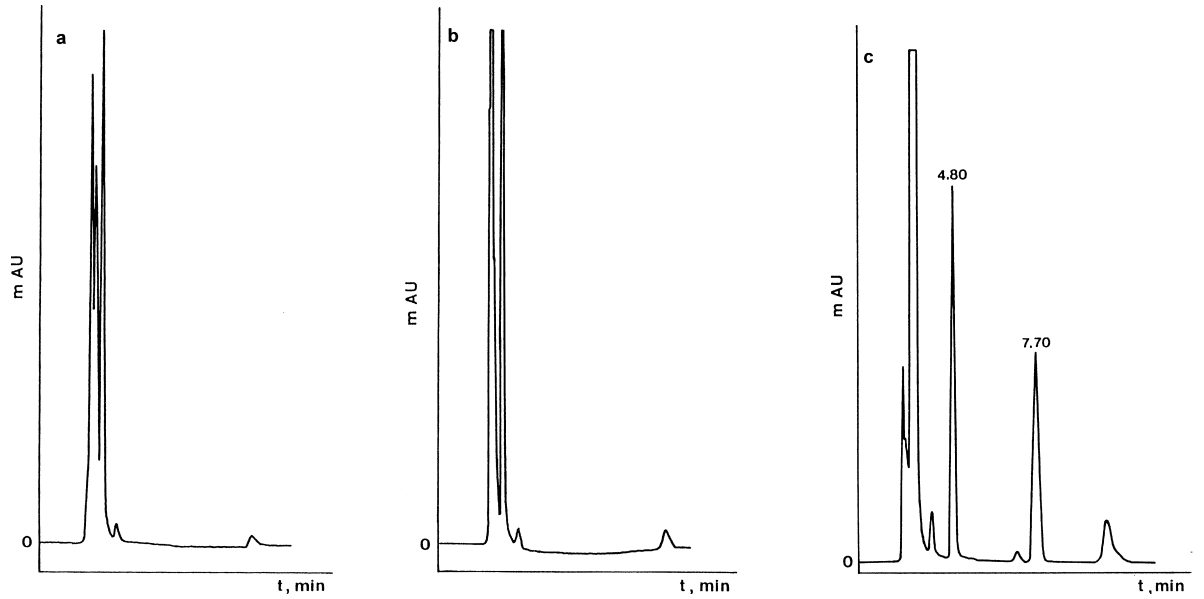

Fig. 3. Typical chromatograms of cefepime. (a) Blank plasma sample. (b) Blank vitreous fluid sample. (c) Spiked plasma sample containing 30 μg ml^{-1} cefepime (4.80 min) and 50 μg ml^{-1} cefadroxil (7.70 min).

heparin and then were centrifuged. All samples were frozen immediately at −20°C for the future analysis.

3. Results

Under the described chromatographic conditions, cefepime and cefadroxil are well separated. Fig. 2 shows the chromatographic separation of these compounds after injecting directly a standard solution containing 30 μg ml^{-1} cefepime and 50 μg ml^{-1} cefadroxil. The retention time is 4.53 and 7.97 min respectively. Typical chromatograms of blank plasma (a) and vitreous sample (b) as well as spiked plasma (c) are shown in Fig. 3. Plasma sample from a patient after intravenous administration of cefepime (a) and vitreous fluid sample from the same patient (b) are shown in Fig. 4.

The linearity for cefepime was checked in the concentration range 1–50 μg ml^{-1} (7 points) in plasma and 1–15 μg ml^{-1} (7 points) in vitreous fluid. Response ratio of peak areas between the corresponding compound and the internal standard versus theoretical concentrations was fitted by a least-squares linear regression to the equation: response ratio (y)=slope (b) ×concentration (C) +intercept (a). The calibration curves (Table 1) were

Table 1
Linear regression results of cefepime in plasma and vitreous fluid[a].

Plasma (n=5)				Vitreous fluid (n=5)[e]			
Slope±S.D.[b]	Intercept±S.D.[b]	$r^{2,c}$	SE[d]	Slope±S.D.[b]	Intercept±S.D.[b]	$r^{2,c}$	SE[d]
0.0345±0.0003	0.0118±0.0087	0.9996	0.014	0.135±0.0031	−0.0425±0.0279	0.9975	0.039

[a] The calibration curve was described as $y=bC+a$; y is the ratio of peak area of cefepime to internal standard; C is the concentration of cefepime in μg ml^{-1}.
[b] S.D., standard deviation.
[c] r^2, coefficient of determination.
[d] SE: standard error of estimate.
[e] n=5 determinations of each data point.

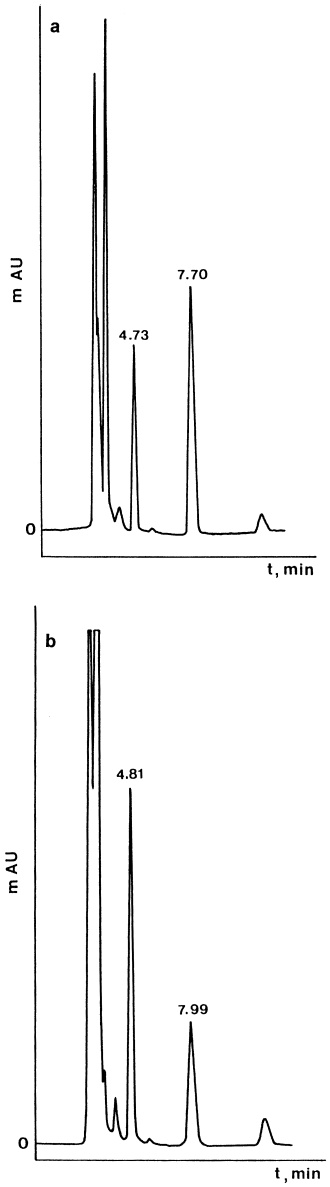

Fig. 4. Typical chromatograms of cefepime from a patient. (a) Plasma sample from a patient, after intravenous administration of cefepime [cefepime: 13 μg ml^{-1} (4.73 min) & cefadroxil: 50.0 μg ml^{-1} (7.70 min)]. (b) Vitreous fluid sample from the same patient [(cefepime: 13.33 μg ml^{-1} (4.81 min) & cefadroxil: 12.5 μg ml^{-1} (7.99 min)].

obtained from spiked plasma and vitreous fluid at seven different concentrations with $n=5$ determinations for each data point. The determination coefficient (r^2) of the calibration lines and the standard deviation of the slopes and the intercepts are also given.

A Student's *t*-test was performed to determine whether the experimental intercepts (α) of the above mentioned regression equations were different from the theoretical zero value. The test is based on the calculation of the quantities $t=\alpha/S_\alpha$, where α is the intercept of the regression equations and S_α is the standard deviation of α and their comparison with tabulated data of the *t*-distribution. The calculated *t*-value in plasma and in vitreous fluid are 1.35 and 1.52 respectively and these values do not exceed the 95% criterion of $t_p=2.571$ for $f=5$ df, which denotes that intercepts of the regression lines in plasma and vitreous fluid are not significantly different from zero.

The limit of detection (qualitative) LD and the limit of quantitation LQ of cefepime were obtained by use of the slope (*b*) and the standard deviation of the intercept (S.D.*a*) of the regression line as defined by IUPAC [18] and ICH Topic Q2B [19]. The limit of detection calculated from $y-a=3.3\times$S.D.*a* and $y-a=b\times$LD was 0.83 μg ml^{-1} in plasma and 0.68 μg ml^{-1} in vitreous fluid. The limit of quantitation, calculated from $y-a=10\times$S.D.*a* and $y-a=b\times$LQ was 2.52 μg ml^{-1} in plasma and 2.06 μg ml^{-1} in vitreous fluid.

The proposal HPLC method showed acceptable repeatability and reproducibility for cefepime. The intra-day and inter-day R.S.D. % values for human plasma samples at concentrations of cefepime 10, 30, 50 μg ml^{-1} are shown on Table 2.

The analytical recovery of cefepime in concentrations 10, 30, 50 μg ml^{-1} are 99.4%, 102.5%, 94.0% ($n=10$) respectively, while the analytical recovery of cefadroxil in concentration 50 μg ml^{-1} is 92%. The results of the recovery of the method from plasma spiked with cefepime at theoretical

Table 2
Precision of the determination

Concentration in plasma (μg ml^{-1})	R.S.D. (%)	
	Intra-day ($n=10$)	Inter-day ($n=10$)
10.0	1.45	1.81
30.0	1.95	1.06
50.0	1.95	0.43

concentrations 10, 30, 50 $\mu g\,ml^{-1}$ are shown on Table 3.

A number of commonly drugs were tested for possible interference with cefepime. The method was considered specific based on the fact that only two of the fifty selected drugs interfered with cefepime and cefadroxil as presented in Table 4.

The results of the in vivo study are shown in Fig. 5. The procedure was found to be appropriate for monitoring plasma levels and measuring the vitreous level of cefepime. An extensive study is presently carried out to assess the extent of the cefepime penetration into the vitreous fluid after intravenous administration.

4. Discussion.

The procedure described, uses a UV–HPLC chromatographic system for the quantitative determination of cefepime in plasma and vitreous fluid, with cefadroxil as an internal standard.

Compared with chromatographic methods, previously reported for cefepime determination, which involve extractions and back-extractions, this assay procedure is sensitive, specific, rapid, inexpensive and easier to perform, since the protein precipitation is achieved with perchloric acid 70% and the mobile phase consists of 13% methanol in a solution of sodium dihydrogen phosphate monohydrate 0.025 *M*

Table 3
Percentage recovery of cefepime in plasma

Theoretical concentration in plasma ($\mu g\,ml^{-1}$)	Measured concentration in plasma ($\mu g\,ml^{-1}$)	n[a]	Recovery (%)
10.0	9.60	10	96.13
30.0	29.80	10	99.44
50.0	48.15	10	96.34

[a] n = number of measurements

Table 4
Specificity of the method

Drug	t_R (min)	Drug	t_R (min)	Drug	t_R (min)
Acetazolamide	N.D[a]	diltiazem	N.D[a]	norfloxacin	N.D[a]
Acetylsalicylic acid	N.D[a]	dipyridamol	N.D[a]	nortriptiline[b]	11.03
Acyclovir[b]	2.09	enalapril	N.D[a]	ofloxacin	N.D[a]
Allopurinol	N.D[a]	furosemide	N.D[a]	paracetamol[c]	7.70
Alprazolame	N.D[a]	glibenclamide	N.D[a]	phenobarbital	N.D[a]
Amiloride	N.D[a]	gliclazide	N.D[a]	piracetam	N.D[a]
Amitriptiline[b]	12.09	hydrochlorothiazide	N.D[a]	piroxicam	N.D[a]
Amlodipine	N.D[a]	lidocaine[b]	2.91	pravastatin	N.D[a]
Amoxycillin[b]	5.92	lisinopril	N.D[a]	propranolol	N.D[a]
Ampicillin	N.D[a]	lovastatin	N.D[a]	salbutamol	N.D[a]
Betaxol	N.D[a]	mefenamic acid	N.D[a]	simvastatin	N.D[a]
Captopril	N.D[a]	metformin	N.D[a]	sulfemethoxazole	N.D[a]
Cephalexin	N.D[a]	metoprolol	N.D[a]	temazepame	N.D[a]
Ciprofloxacine[b]	12.04	naprocyn	N.D[a]	theophyline[c]	4.50
Clorazepate	N.D[a]	nifedipine	N.D[a]	timolol	N.D[a]
Diazepam	N.D[a]	nimesulid	N.D[a]	trimetazidine	N.D[a]
Dichlorofenamide	N.D[a]	norazepame	N.D[a]		

[a] N.D.: Not detected
[b] Detected but not interfered
[c] Detected but interfered

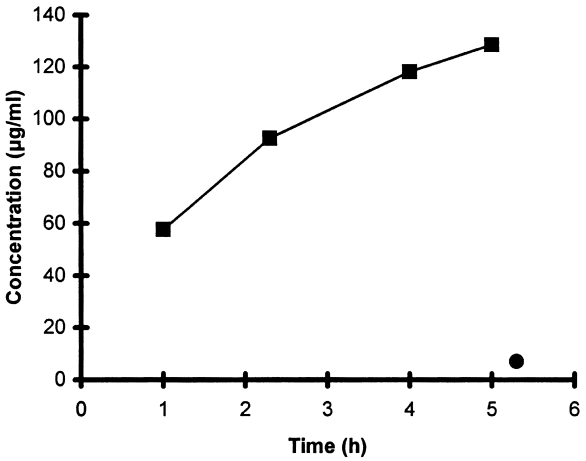

Fig. 5. Plasma concentration (■)-time profile for a patient receiving a constant intravenus infusion of 1 g h^{-1} cefepime for 6 h. The datum point (●) corresponds to 7,05 µg ml^{-1} cefepime in the vitreous fluid.

(pH 3). In the method described by Barbhaiya et al [16] (method applied in a pharmacokinetic study in rats) the plasma proteins were precipitated with acetonitrile and trichloroacetic acid, followed by extraction of the acetonitrile into dichloromethane, while the mobile phase consisted of acetonitrile and 0.005 *M* 1-octanosulfonic acid (12:88%, v/v). Furthermore, in the method described by Elkhaili et al [17] (method applied in a pharmacokinetic study in a micropig animal model), the extraction procedure was performed firstly with acetonitrile and secondly with methylene chloride, while the determination was achieved without the use of an internal standard.

In our study, two different sample extraction techniques were employed for plasma and vitreous fluid, based on the different physical and chemical properties of each biological fluid. These procedures minimize the interference of endogenous compounds and prolongs the working life of the HPLC column.

The sensitivity of our method is similar than that found by Elkhaili et all [17] and allows determination of concentrations as low as 0.83 µg ml^{-1} in plasma and 0.68 µg ml^{-1} in vitreous fluid in men, which are adequate for clinical and pharmacokinetic purposes.

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