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High-performance liquid chromatographic assay for cefepime in serum

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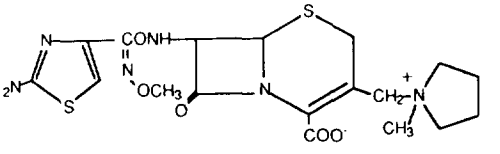
Abstract

A simple, rapid, specific and sensitive high-performance liquid chromatographic method was developed for the determination of cefepime 1-[[(6*R*, 7*R*)-7-[2-(2-amino-4-thiazolyl)glyoxylamido]-2-carboxy-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-en-3-yl]methyl]-1-methylpyrrolidinium hydroxide, inner salt, 7^2-(*Z*)-(O-methyloxime) in human serum. Separation was achieved on a reversed-phase Ultrasphere XL-ODS column (75×4.6 mm I.D.). The mobile phase was 7% acetonitrile in 20 m*M* ammonium acetate (pH 4). Cefepime eluted in the range of 1.8–2.2 min. Detection was by UV absorbance at 254 nm. The lower limit of quantitation of cefepime in plasma was 0.5 μg/ml. The average absolute recovery was 106.2±2.1%. The linear range was from 0.1 to 50 μg/ml, with a correlation coefficient greater than 0.999. The within-day C.V.s for human samples were 4.9 and 2.3% for 1 and 50 μg/ml, respectively. The between-day C.V.s for human serum samples were 14.5, 7.4 and 6.7 for 1, 25 and 50 μg/ml, respectively. Cefepime was found to be unstable in serum at room temperature. For delayed assay, samples must be stored at −80°C.

Keywords: Cefepime

1. Introduction

Cefepime, 1-[[(6*R*, 7*R*)-7-[2-(2-amino-4-thiazolyl)-glyoxylamido] -2-carboxy-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-en-3-yl]methyl]-1-methylpyrrolidinium hydroxide, inner salt, 7^2-(*Z*)-(O-methyloxime) in human serum [5] (Fig. 1) is a new parenteral cephalosporin that has been described as a fourth-generation, broad spectrum cephalosporin [1,2]. Cefepime is structurally similar to other amino-thiazolyl methoxyimino third-generation cephalosporins, such as cefotaxime and ceftazidime [3]. However, it differs from the third generation cephalosporins by a positively charged quaternized N-methylpyrrolidine substitution at the 3 position of the cephem nucleus, making cefepime a zwitterion. This property enhances the ability of cefepime to rapidly penetrate the outer cell membrane porins of Gram-negative bacteria [4]. With its quaternary nitrogen-containing substituents at the 3 position, cefepime



Cefepime

Fig. 1. Structure of cefepime.

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has much lower affinity for type I β-lactamase enzymes than other cephalosporins [6,7].

High-performance liquid chromatographic [8,9], microbiological [10,11] and simple spectrophotometric [12] assays have been used to determine cefepime concentrations in biological fluids. For new agents, HPLC is generally preferred to the microbiological assay. Traditionally, bioassays lack specificity, often cannot be used when multiple antibiotics are present in samples and do not distinguish biologically active metabolites from the parent compound. We have developed a simple, rapid, accurate and sensitive HPLC method for the determination of cefepime in serum, which could be used for pharmacokinetic and pharmacodynamic studies in animals and humans.

2. Experimental

2.1. Reagents and chemicals

Cefepime (as a pure titrated powder) was kindly provided by Bristol Myers Squibb (Paris, France). Stock solutions (200 mg/ml) were prepared in water and stored at −80°C. Acetonitrile, methylene chloride, ammonium acetate and acetic acid were all of analytical-reagent grade (E. Merck, Darmstadt, Germany). Water was obtained daily from a Milli-Ro-Milli-Q system (Millipore, Molsheim, France).

2.2. Chromatographic conditions

The isocratic liquid chromatograph consisted of a 126-solvent delivery module (Beckman, Fullerton, CA, USA), a Model 210 sample injection valve with a variable loop (Beckman) and a Model 166 variable-wavelength detector (Beckman). Chromatograms were processed by a Model 740 recording data processor (Millipore, Waters Division, Milford, MA, USA).

Chromatography was performed on a high-speed analytical column (75×4.6 mm I.D.) packed with 3-mm diameter particles (Ultrasphere XL-ODS, Beckman). The mobile phase was 7% acetonitrile in 20 m*M* ammonium acetate, adjusted to pH 4 with glacial acetic acid. The flow-rate was set at 1.0 ml/min and the eluent was monitored at 254 nm. The range setting of the spectrophotometer depended on the concentration of cefepime measured.

2.3. Extraction procedure for serum

As described previously [13,14], an aliquot of serum (0.5 ml) was mixed with an equal volume of acetonitrile in a 5-ml screw-capped glass tube on a Vortex-mixer (Vortex, Cleveland, OH, USA). The tube was gently shaken by rotation for 10 min (20 rpm). The resulting mixture was centrifuged 10 min at 1000 *g*. The supernatant was transferred with a Pasteur pipette to another screw-capped glass tube and 3.2 ml of methylene chloride were added. After shaking by rotation (20 rpm) for 10 min and centrifugation at 1000 *g* for 10 min, a 5-μl aliquot of the upper aqueous layer was injected into the column.

2.4. Recovery study

Five samples of serum were spiked with 1 and 50 μg/ml of cefepime. They were assayed and the resulting peak areas were compared with those resulting from aqueous solutions at the same concentrations.

2.5. Accuracy and limit of quantitation

Quantitation was based on peak areas measured by the integrator. The limit of quantitation (LOQ) was defined as the lowest antibiotic concentration that could be determined with confidence on a day-to-day basis. A standard curve was prepared for cefepime by spiking normal sera with increasing amounts of antibiotic.

The linearity of the method was assessed with antibiotic concentrations ranging from 0.1 to 50 μg/ml. Each concentration was measured three times.

Accuracy was evaluated by calculating the mean percent differences between theoretical values and measured values. The mean value should be within ±15% of the actual value, except at the LOQ, where it should not deviate by more than ±20% [15].

2.6. Precision

Both within- and between-day reproducibilities in serum were tested. Three concentrations of antibiotic were included in this study: the first, high (50 μg/ml); the second, medium (25 μg/ml) and the third, low (1 μg/ml). Nine aliquots of each sample were tested on the same day and the resulting coefficient of variation (C.V.) indicated the within-day reproducibility. Aliquots of the same sample were tested once a day over nine days and the resulting C.Vs indicated the between-day reproducibilities. The intra-assay C.V.s of working solutions of cefepime were investigated by analysing nine samples at concentrations of 0.5, 5 and 50 μg/ml, respectively. The precision around the mean value should not exceed a C.V. of 15%, except for LOQ, where it should not exceed 20% [15].

2.7. Specificity

Specificity was assessed in the presence of most β-lactam antibiotics (e.g., clavulanic acid, tazobactam, sulbactam, amoxicillin, ampicillin, cefixime, cefotaxime, ceftazidime, cloxacillin, imipenem, cefalotin, mezlocillin, latamoxef, penicillin G, piperacillin, ticarcillin) as possible interfering compounds with this assay. Other antibiotics were included in this study (fusidic acid, ciprofloxacin, fosfomycin, vancomycin, itraconazole, and hydroxyitraconazole, ofloxacin, pefloxacin, pristinamycin, rifampicin, roxithromycin, erythromycin, lincomycin, sulfamethoxazol, teicoplanin, tetracyclin, trimethoprim, amikacin, kanamycin, gentamicin, tobramycin, netilmicin, neomycin and calcium folinate).

2.8. Working solution stability

The working solution (20 μg/ml) of cefepime was repeatedly ($n=3$) injected into the chromatograph immediately after preparation (time 0) and after 1, 2, 3, 4 and 24 h of bench-top storage at room temperature (22°C) with light exposure and also at +4°C. This injection protocol was repeated after storage of these solutions at −80°C for 1, 3, 4, 15, 30, 60 and 90 days, respectively.

2.9. Bench-top stability of cefepime after serum preparation

Quality control (QC) concentrations representing 1 and 50 μg/ml of cefepime in serum were analysed in triplicate (each time three aliquots of the sample were extracted and injected at the indicated times) at the following times: 0, 1, 2, 3, 4 and 24 h of bench-top storage at +4°C. This test indicates the stability of cefepime in the supernatant and is more representative of what really occurs in routine handling of samples or during pharmacokinetic studies.

2.10. Short- and long-term freezer stability of cefepime in serum

QC concentrations of 1 and 50 μg/ml of cefepime in serum were analyzed in triplicate after preparation (time 0) and at 2, 4, 6 and 24 h. These samples were stored at 22°C with light exposure.

The injection protocol was repeated after 1, 7, 15, 30, 60 and 90 days storage of these solutions at −80°C. The drug was considered stable if more than 90% of the intact drug was found at the end of the study period.

3. Results

Typical chromatograms for high (50 μg/ml) and low (1 μg/ml) concentrations of cefepime in serum are shown in Fig. 2. The retention time of cefepime was in the range 1.8–2.2 min and each chromatographic run required approximately 5 min. Fig. 3 presents chromatograms obtained from analysis of (A) a blank serum and (B) a serum obtained from a micropig animal model treated with cefepime that had been administered intravenously at the standard dosage (1 g). Extraction and chromatographic analysis of five separate blank serum samples confirmed that there were no endogenous peaks that coeluted with cefepime. For exogenous compounds, both ceftazidime and sulbactam coeluted at 4 min retention time and they did not interfere with the antibiotic. Thus, the method was considered specific based on the fact that the presence of several commonly used antibiotics at concentrations achieved in plasma did not interfere with the quanti-

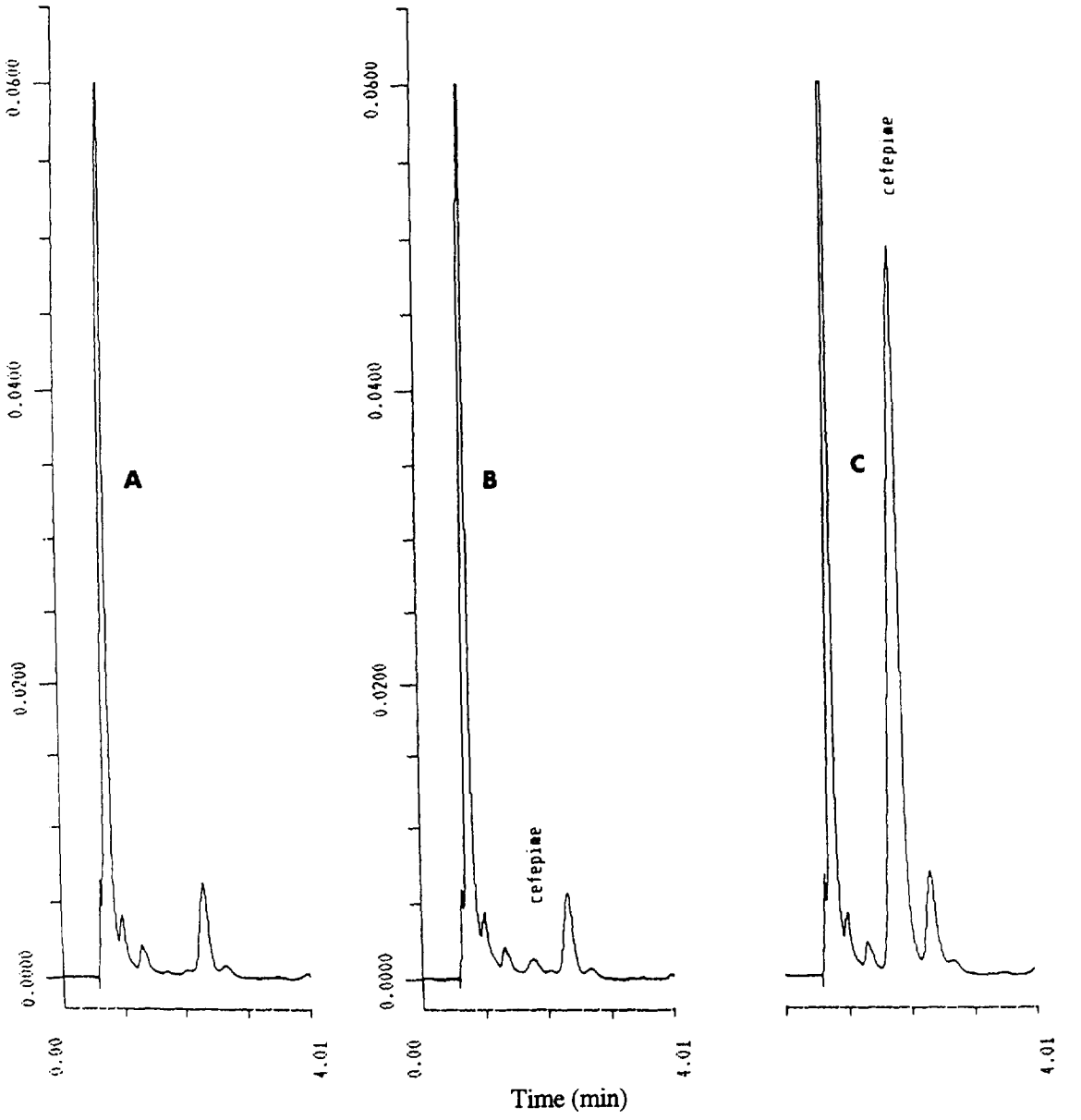

Fig. 2. Chromatograms of (A) cefepime-free normal serum, (B) serum spiked with 1 μg/ml and (C) 50 μg/ml of cefepime. Detection wavelength, 254 nm.

tation of cefepime. Linear regression analysis determined by plotting drug concentrations (x) against peak areas (y) gave a mean correlation coefficient of 0.9998 ± 0.0002 for cefepime for concentrations ranging from 0.1 to 50 μg/ml. Standard curves obtained from different calibration lines performed on different days were $y = 3.1992x + 0.0146$. The lowest detectable concentration was 0.1 μg/ml and the LOQ was set at 0.5 μg/ml of cefepime in serum. The extraction recovery from serum spiked with cefepime was $106.2 \pm 2.1\%$ (Table 1). The precision values in aqueous solution were 2.2, 2.5 and 3.6% (relative standard deviation) as determined by injection ($n = 9$) of 0.5, 5.0 and 50 μg/ml, respectively.

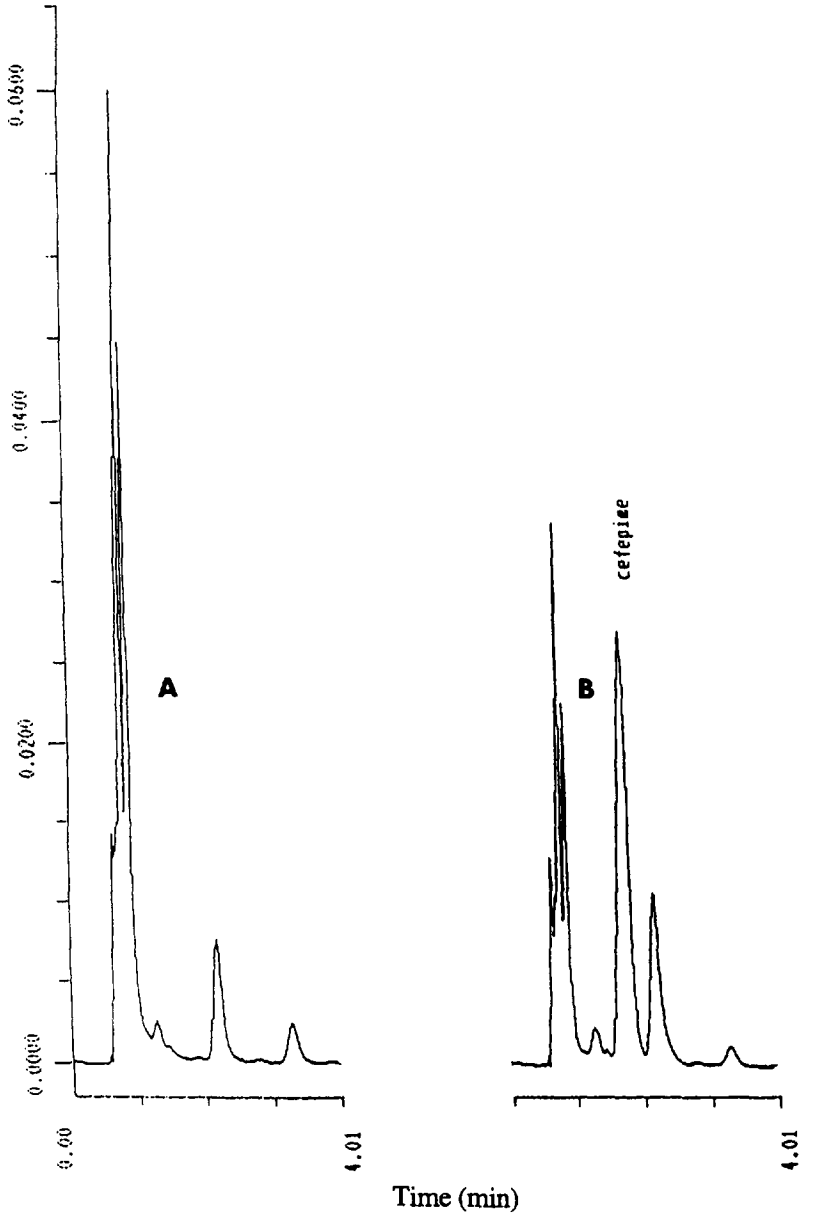

Fig. 3. Chromatograms of (A) micropig blank serum and (B) serum obtained from the animal 2 h after intravenous injection of cefepime (1 g).

Table 1
Extraction recoveries

Concentration (μg/ml)	Recovery (n=5) (%)
1	104.7±8.4
50	107.7±7.7

The within-day C.V.s for human serum samples were 4.9 and 2.3%, respectively, for 1 and 50 μg/ml. The between-day C.V. values at concentrations of cefepime in serum of 1, 25 and 50 μg/ml were 14.5, 7.4 and 6.7, respectively. Accuracy of the assay was evaluated by preparing triplicate samples (n=3 for

Table 2
Accuracy of cefepime assay in serum

Theoretical value (μg/ml)	Measured value (μg/ml)	Percentage difference from the theoretical value
0.1	0.044	56.0
0.5	0.426	14.80
1	0.922	7.8
5	4.995	0.10
10	10.5	5.0
25	24.637	1.45
50	50.076	0.15

serum) at cefepime concentrations ranging from 0.1 to 50 μg/ml (Table 2). The working solutions of cefepime were stable for at least 24 h when stored both at room temperature and at 4°C. The storage of extracted samples at 4°C had little effect on the accuracy of the control results. The stability of cefepime in serum was good after 6 h storage at 4°C, whereas an important degradation occurs after 24 h of storage at room temperature (Fig. 4). Cefepime was stable in human serum and working solutions were stable for up three months at −80°C.

4. Discussion

HPLC is an analytical approach for quantitating cephalosporin antibiotics in biological fluids that is becoming increasingly popular in clinical laboratories that do not have the capability of performing microbiological assays. The HPLC run time for this assay is rapid and requires less than 3 min. The retention times of cefepime in serum described by other authors were 5.3 and 7.5 min [8,9]. In the method described by Barbhaiya et al. [8] the plasma protein was precipitated with acetonitrile and trichloroacetic acid, followed by extraction of the acetonitrile into methylene chloride. We propose here a simplified extraction procedure with an equal volume of acetonitrile; the remaining acetonitrile is then removed from the supernatant by methylene chloride, leading to an increased concentration of the antibiotic in the supernatant. Furthermore, the methods using trichloroacetic acid or methanol for protein precipitation did not eliminate lipids [9,16]. The LOQ determined here (0.5 μg/ml) was at least similar, or better, than that found by others [8,9].

None of the antibiotics likely to be co-administered to patients receiving cefepime appeared to

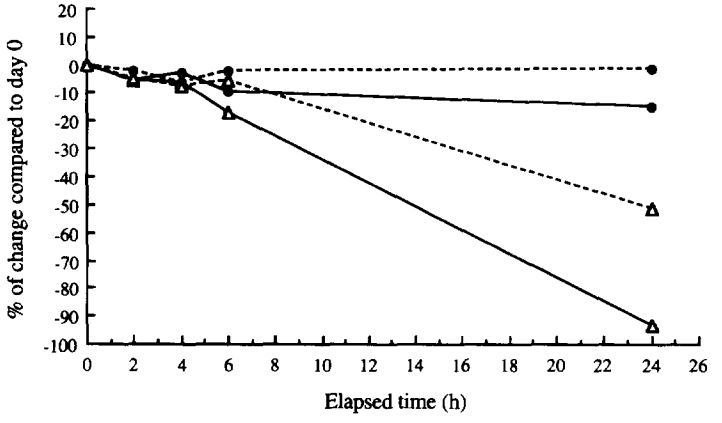

Fig. 4. Assay of the stability of cefepime after storage in serum at room temperature (22°C) (△) and 4°C (●). Concentrations tested were 1 (—) and 50 (- - -) μg/ml.

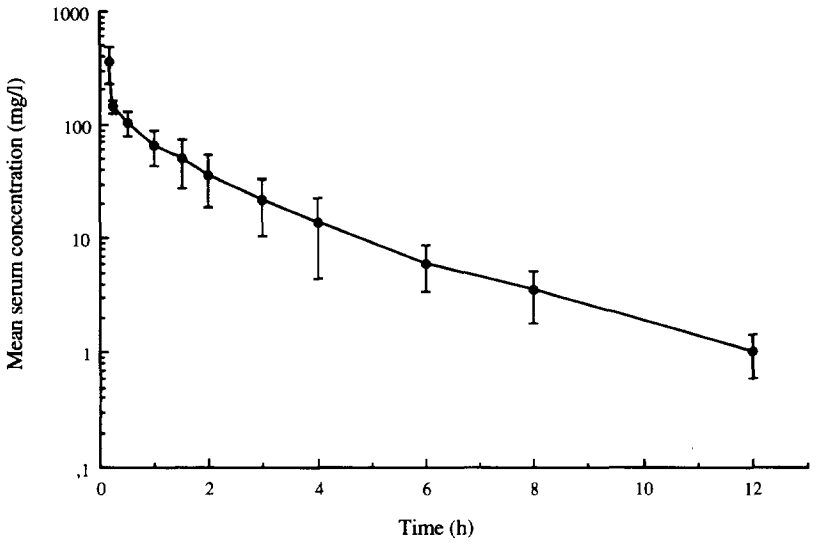

Fig. 5. Mean (±S.D.) serum concentration–time curve following administration of cefepime (1 g) to micropig ($n=3$) by intravenous injection.

interfere with this technique. Cefepime is an unstable drug. Thus, after drawing blood, samples must be provided immediately to the laboratory so that they can be handled quickly. For delayed dosage, the samples must be stored at −80°C. The microbiological methods cannot be recommended because the obligatory incubation at 37°C could certainly degrade a part of cefepime.

Our data concerning stability at −80°C are similar to those published by Barbhaiya et al. [8], who found that cefepime was stable in human plasma for up to 51 days at −20°C and for 138 days at −70°C. Surprisingly, cefepime was stable in working solutions over a 24-h period at room temperature. The method described here allows us to conduct pharmacokinetic and pharmacodynamic studies on cefepime in an interesting micropig model for the development of optimal dosage regimens (Table 3) [17]. Fig. 5 presents the mean pharmacokinetic profile of cefepime from the study.

Table 3
Pharmacokinetic parameters of cefepime in serum after intravenous administration to micropig and man[a]

	Species	
	Micropig	Man
$t_{1/2}$ (h)	1.57±0.26	1.8±0.2
Clp (ml min^{-1} kg^{-1})	1.97±0.79	1.82±0.22

[a] $t_{1/2}$, half-life; AUC, area under concentration–time curve for a 12-h dosing period; Clp, total body clearance.

This new HPLC assay procedure permits the rapid, precise and accurate quantitation of cefepime in human and micropig sera. It seems to be much more reliable than bioassays for both therapeutic drug monitoring and for pharmacokinetic studies.

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