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Journal of Chromatography A, 914 (2001) 77–82

JOURNAL OF CHROMATOGRAPHY A

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Determination and pharmacokinetic study of unbound cefepime in rat bile by liquid chromatography with on-line microdialysis

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Abstract

Biliary excretion and intestinal reabsorption in enterohepatic circulation play major dispositional roles for some drugs. To investigate biliary excretion of drug, we inserted a microdialysis probe into the bile common duct of rat between the liver and the duodenum. In order to avoid the obstruction of bile fluid or bile salt waste, a shunt linear microdialysis probe was used for simultaneous and continuous sampling following intravenous administration of cefepime (50 mg/kg, i.v.). Separation and quantitation of cefepime in the dialysates were achieved using a LiChrosorb RP-18 column (Merck; 250×4.6 mm I.D., particle size 5 μm) maintained at ambient temperature. Samples were eluted with a mobile phase containing 100 m*M* monosodium phosphoric acid (pH 3.0)–methanol (87:13, v/v). The UV detector wavelength was set at 270 nm. The result indicates that the elimination half-life of cefepime in bile was 64.01±9.32 min. This study also served as an example for the microdialysis application in the biliary excretion study of drug.

Keywords: Microdialysis; Kinetic studies; Pharmaceutical analysis; Cefepime; Cephalosporins; Antibiotics

1. Introduction

Cefepime, a fourth-generation cephalosporin, has a broad spectrum of activity against a wide range of gram-positive and gram-negative bacteria, including most strains resistant to aminoglycosides or third-generation cephalosporins as well as those highly resistant to hydrolysis by most beta lactamases [1]. Different from the third-generation cephalosporins, cefepime has a quaternary nitrogen that is positively charged at the 3-position of the cephem nucleus, providing the properties of a zwitterion [2]. This zwitterion structure may enable cefepime to easily cross the lipid layer. Cefepime levels in biological fluids have previously been determined by microbiological [3,4], and spectrophotometric assay [5] as well as high-performance liquid chromatography (HPLC) using different extractive techniques, such as deproteination by methylene chloride [6,7], perchloric acid [8], or acetonitrile with trichloroacetic acid [9]. These procedures involved contributed to the measurement of protein-bound drug concentration. Although the ultrafiltration method [10] can be used to obtain the protein-free analytes, it requires a more complicated procedure for sample clean-up. In this paper, we use a microdialysis method for excluding the protein-bound compounds from the microdialysis membrane in order to sample only the protein-unbound fraction of the drug [11,12]. In

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0021-9673/01/$ – see front matter
PII: S0021-9673(00)01207-3

addition, to minimize the degradation of cefepime in the physiological environment, an automatic sampling system and a stable analytical condition are required. Therefore, in this study we use an in vivo on-line microdialysis sampling method coupled with the HPLC analytical system for measuring unbound cefepime in rat bile.

Conventional methods for measuring drug concentration in bile fluid for the pharmacokinetic study include cannulation the bile duct for biliary sampling. To minimize the bile fluid loss, we constructed an automatic on-line flow-through microdialysis system [13,14] for bile fluid sampling coupled with a HPLC system, thus providing near real-time analysis of cefepime in bile dialysate samples after drug administration.

2. Experimental

2.1. Chemicals and reagents

Cefepime (Fig. 1) was purchased from Bristol-Myers Squibb (Sermoneta Latina, Italy). Liquid chromatographic-grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Animals

Adult, male Sprague–Dawley rats (280–350 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and were allowed to acclimate to their environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least 5 days before the experiments began. At the start of experiments, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). When needed, anesthesia was maintained throughout the experimental period by administering one quarter of the initiation dose. The experimental animals were kept warm with a heating pad throughout the experiments.

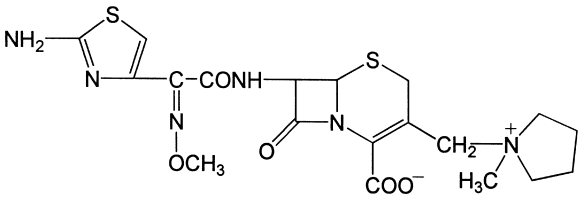

Fig. 1. Chemical structure of cefepime.

2.3. Chromatography

The HPLC system consisted of a chromatographic pump (BAS PM-80; Bioanalytical System, West Lafayette, IN, USA), an on-line injector (CMA/160, Stockholm, Sweden) equipped with a 20-μl sample loop and a UV detector (Soma S-3702, Tokyo, Japan). Cefepime dialysate was separated using a LiChrosorb RP-18 column (Merck; 250×4.6 mm I.D., particle size 5 μm) maintained at ambient temperature. The mobile phase comprised 100 m*M* monosodium phosphoric acid (pH 3.0)–methanol (87:13, v/v), and the flow-rate of the mobile phase was 1 ml/min. The buffer was filtered through a Millipore 0.45-μm filter and degassed prior to use. The UV detection wavelength was set at 270 nm. Output signal from the HPLC–UV system was recorded using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.4. Method validation

All calibration curves of cefepime (external standards) were made prior to the experiments with correlation values of at least 0.995. The intra- and inter-day variabilities for cefepime were assayed (six replicates) at concentrations of 0.05, 0.1, 0.5, 1, 2, 5, 10 and 50 μg/ml on the same day and on 6 consecutive days, respectively. The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: bias (%)=[($C_{nom}-C_{obs}$)/(C_{nom})]·100. The precision relative standard deviation (RSD) was calculated from the observed concentrations as follows: % RSD=[standard deviation (SD)/C_{obs}]·100. Accuracy (% bias) and precision (RSD) values of within ±15% covering the range of actual experimental concentrations were considered acceptable [15].

2.5. Microdialysis experiment

The bile duct microdialysis probes were constructed in our own laboratory based on the design originally described by Scott and Lunte [14]. In brief, a 7-cm piece of dialysis membrane (150 μm outer diameter with a cut-off at nominal molecular mass of 13 000; Spectrum, Laguna Hills, CA, USA) was inserted into a section of the polyethylene tubing (PE-60; 0.76 mm I.D.×1.22 mm O.D.), with the ends of the dialysis membrane connected to a silica tubing (40 μm I.D.×140 μm O.D.; SGE, Australia). A piece of PE-10 tubing (0.28 mm I.D.×0.61 mm O.D.) was then attached to both ends of the PE-60 tubing and all unions were cemented with epoxy (Fig. 2). The detailed construction of the flow-through microdialysis probe has been described in our previous reports [16,17]. After microdialysis probe was inserted into the bile common duct, then the probe was perfused with normal saline. Following a period of post-surgical stabilization (approximately 2 h), drug-free control samples were collected and then cefepime (50 mg/kg) was intravenously administered via a femoral vein. Each dialysate sample (20 μl) was assayed immediately using an on-line injector HPLC system.

2.6. Recovery of microdialysate

For in vivo recovery, normal saline solution containing cefepime (2 or 5 μg/ml) was pumped through the probes at a constant flow-rate (2 μl/min) using the infusion pump (CMA/100). After a post-surgical stabilization period of 2 h, the inlet (C_{in}) and outlet (C_{out}) concentrations of cefepime were determined by HPLC. The in vivo recovery ratios were then calculated by the following equation [18]: $\text{recovery}_{\text{in vivo}} = 1 - (C_{out}/C_{in})$.

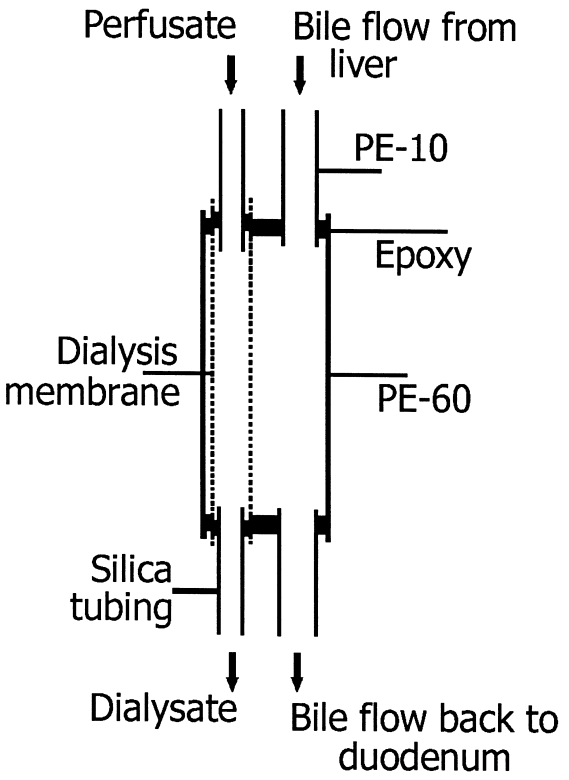

Fig. 2. Detailed description of a flow-through microdialysis probe.

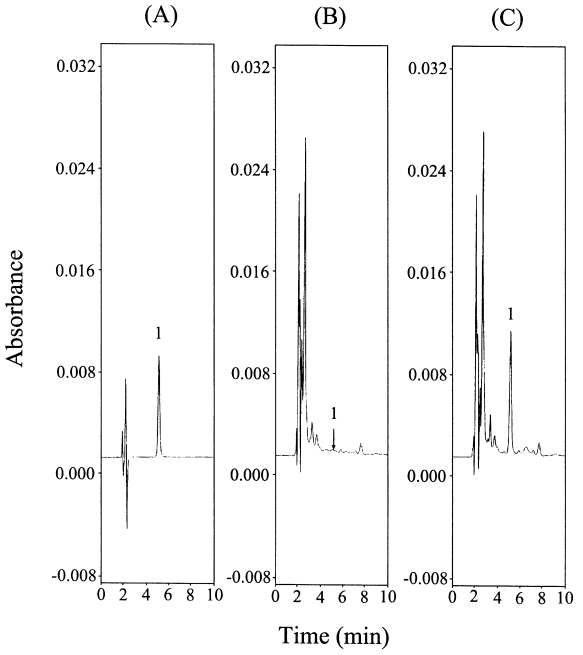

Fig. 3. Typical chromatogram for injection of (A) standard cefepime (2 μg/ml), (B) a blank bile dialysate, and (C) a bile dialysate sample containing cefepime (2.73 μg/ml) collected from bile fluid at 10 min after cefepime administration (50 mg/kg, i.v.). Peak 1=cefepime.

Table 1
Intra- and inter-day accuracy of cefepime

Nominal concentration (μg/ml)	Observed concentration[a] (μg/ml)	RSD (%)	Accuracy (% bias)
Intra-day (n=6)			
0.05	0.053±0.001	1.9	6.0
0.10	0.11±0.002	1.8	10.0
0.20	0.19±0.01	5.3	−5.0
0.50	0.48±0.02	4.2	−4.0
1.00	1.01±0.05	5.0	1.0
2.00	1.99±0.02	0.5	−0.5
5.00	4.97±0.02	0.4	−0.6
10.00	10.02±0.03	0.3	0.2
25.00	25.06±0.08	0.3	0.2
50.00	49.97±0.04	0.1	−0.1
Inter-day (n=6)			
0.05	0.054±0.004	7.8	8.0
0.10	0.09±0.006	6.7	−10.0
0.20	0.19±0.003	1.6	−5.0
0.50	0.51±0.01	2.0	2.0
1.00	1.01±0.01	1.0	1.0
2.00	2.02±0.04	2.0	1.0
5.00	4.96±0.17	3.4	−0.8
10.00	9.98±0.14	1.4	−0.2
25.00	24.91±0.70	2.8	−0.4
50.00	50.03±0.31	0.6	0.1

[a] Observed concentration data are expressed as means±SD (n=6).

2.7. Pharmacokinetic study

Drug concentrations in extracellular fluid were calculated from the concentrations in dialysates by the following equation: drug concentration=dialysate concentration/recovery. All data were subsequently processed by the pharmacokinetic computer program, WinNonlin standard version 1.1 (Science Consulting, Apex, NC, USA), for the calculation of pharmacokinetic parameters according to the non-compartmental model [19]. All data are presented as mean±standard errors. The area under the concentration curves (AUC), the area under the first moment curve (AUMC) and the mean residence time (MRT) were calculated by using statistical moments [20]. The mean residence time was calculated as follows: MRT=AUMC/AUC.

Table 2
In vivo microdialysis recoveries of cefepime in rat bile

Concentration (μg/ml)	Recovery[a] (%)
2	67.8±1.8
5	69.3±1.9

[a] Data are expressed as mean±SD (n=6).

3. Results and discussion

The chromatograms obtained using the liquid chromatographic system are shown in Fig. 3. Each

Table 3
Estimated pharmacokinetic parameters of bile following cefepime administration (50 mg/kg, i.v.)

Parameter	Estimated[a]
$t_{1/2}$ (min)	62.5±8.3
AUC (min mg/ml)	1.8±0.5
AUMC (min^2 mg/ml)	194.2±6.6
MRT (min)	102.3±12.1

[a] Data are expressed as mean±S.E.M. (n=6).

analysis was completed within 10 min. Separation of cefepime from endogenous chemicals in bile dialysate was achieved in an optimal mobile phase containing 100 m*M* monosodium phosphate (pH 3.0)–methanol (87:13). The retention time of cefepime in the chromatographic system was 5.6 min (Fig. 3). Peak areas of cefepime were linear ($r^2 >$ 0.995) over a concentration range of 0.05–50 μg/ml. Compared with other liquid chromatographic methods [7–10], the automatic hyphenation of microdialysis and HPLC proves to take less time in the complex sample clean-up procedures and consume less solvents.

Fig. 3A shows a typical chromatogram of a standard mixture containing cefepime (2 μg/ml). The blank sample (Fig. 3B) shows that the chromatographic conditions revealed no biological substances that would interfere significantly with the accurate determination of the drug. Fig. 3C depicts a chromatogram of actual unbound cefepime in rat bile. The dialysate sample contains cefepime (2.73 μg/ml) collected from the bile fluid at 10 min following cefepime administration (50 mg/kg, i.v.).

Intra- and inter-day (Table 1) accuracy of cefepime levels fell well within predefined limits of acceptability. All bias and RSD values were within ±15%. This method has quantitative limits of 50 ng/ml for cefepime. The in vivo recovery of cefepime is shown in Table 2. It can be seen that this method is sensitive enough to measure unbound cefepime in rat bile for pharmacokinetic study.

The average concentration of unbound cefepime in the bile increased during the first 10 min following drug administration. From these microdialysis sampling data, the pharmacokinetics of unbound cefepime in the rat bile was calculated according to the non-compartmental model. These data have been corrected for in vivo recovery. The pharmacokinetic parameters are shown in Table 3. The current results are in good agreement with those of Okamoto et al., who found that high concentrations of cefepime were excreted from bile fluid in the treatment of acute cholecystitis patient. Okamoto et al. reported that the average concentration ratio of cefepime in bile fluid/plasma was 14.44 in 33 patients undergoing elective cholecystectomy [21].

In summary, we described a rapid and sensitive chromatographic method for the determination of cefepime in rat bile fluid using in vivo microdialysis with HPLC–UV. This method results in less tissue damage, no biological fluid loss and exhibits no endogenous interference with sufficient sensitivity in bile dialysates. Examination of the disposition of cefepime in bile fluid reveals that it may undergo enterohepatic re-circulation.

Acknowledgements

This study was supported in part by research grants (NSC89-2113-M-077-009; NSC89-2320-B-077-013) from the National Science Council, Taiwan.

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