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Measurement and pharmacokinetic analysis of unbound ceftazidime in rat blood using microdialysis and microbore liquid chromatography

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Abstract

To evaluate the biodisposition of ceftazidime in rat blood, a rapid and simple microbore liquid chromatographic technique together with a microdialysis sampling technique were developed. This method involves an on-line design for blood dialysate directly injected into a microbore liquid chromatographic system. The chromatographic conditions consisted of a mobile phase of methanol–acetonitrile–100 m*M* monosodium phosphoric acid (pH 3.0) (10:10:80, v/v/v) pumped through a microbore reversed-phase column at a flow-rate of 0.05 ml/min. With the detection wavelength set at 254 nm, a good linear correlation was observed between the peak area and the ceftazidime concentration at 0.1 to 50 μg/ml ($r = 0.999$). Microdialysis probes, being custom-made, were screened for acceptable in vivo recovery while chromatographic resolution and detection were validated for response linearity, as well as intra-day and inter-day variabilities. This method was then applied to the pharmacokinetic profiling of ceftazidime in blood following intravenous 50 mg/kg administration to rats. The pharmacokinetics was calculated from the corrected data for dialysate concentrations of ceftazidime versus time. This method has been used to study ceftazidime pharmacokinetics in rats and has proven to be rapid and reproducible.

Keywords: Ceftazidimine

1. Introduction

Ceftazidime (Fig. 1), a beta-lactamase-stable third-generation cephalosporin with a broad spectrum of antimicrobial activity, has shown both a broad spectrum of in vitro antimicrobial activity and clini-

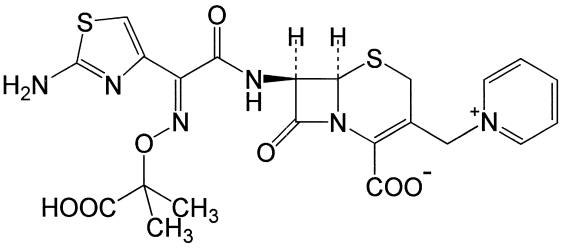

Fig. 1. Chemical structure of ceftazidime.

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cal utility for serious infections [1]. It has been effective as a monotherapy in the treatment of peritonitis, gynecologic infections, chronic bronchitis, and infections in patients with leukemia and granulocytopenia [2]. When a drug permeates a cell, it must traverse the cellular plasma membrane only if the protein-unbound drug is in equilibrium across the membrane. The present investigation was undertaken to determine the protein-unbound ceftazidime concentration in rat blood using a microdialysis technique for further pharmacokinetic studies. Various methods have previously been developed for determining ceftazidime in biological fluids, all of which involve various pretreatment procedures prior to high-performance liquid chromatography (HPLC) [3–6]. These pretreatment procedures include solid-phase extraction followed by ion-pairing reversed-phase liquid chromatography [3], the on-line solid-phase extraction method [4], ultrafiltration coupled to thermospray liquid chromatography–mass spectrometry [5] or methanol precipitation of serum and urine followed by reversed-phase chromatography [6] from biological samples. However, these techniques all suffer from the need for intensive sampling of blood, and time-consuming clean up procedures for small amounts of samples. Therefore, in this paper we describe the use of microdialysis to exclude protein-bound compounds from the microdialysis membrane in order to sample only the protein-unbound fraction of the drug [7–9]. This design was applied to the pharmacokinetic profiling of ceftazidime in blood. Coupling to microbore HPLC enhanced the resolution and reduced the required sample size, permitting the use of an on-line system for convenient and continuous monitoring.

2. Experimental

2.1. Reagents

Ceftazidime (Fig. 1) and chemical reagents were purchased from Sigma (St Louis, MO, USA). The chromatographic solvents were obtained from BDH (Poole, UK). Triple de-ionized water from Millipore (Bedford, MA, USA) was used for all preparations.

2.2. Liquid chromatography

The microbore liquid chromatographic system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), an on-line injector (CMA 160, Stockholm, Sweden) equipped with a 10 μl sample loop and an ultraviolet detector (Dynamax, Walnut Creek, CA, USA). Dialysates were separated using a reversed-phase C_{18} microbore column (150× 1 mm I.D.; particle size 5 μm, Bioanalytical Systems, West Lafayette, IN, USA) maintained at ambient temperature. The mobile phase consisted of methanol–acetonitrile–100 m*M* monosodium phosphoric acid (pH 3.0) (10:10:80, v/v/v) with a flow-rate of 0.05 ml/min. The mobile phase mixture was filtered through a 0.22 μm Millipore membrane, and degassed prior to use. The optimal UV detection for ceftazidime was at a wavelength of 254 nm. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.3. Animals

Adult male Sprague-Dawley rats (280–320 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 experimentation. The rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and remained anesthetized throughout the experimental period. The femoral vein was exposed for further drug administration, and the rat's body temperature was maintained at 37°C with a heating pad.

2.4. Method validation

All calibration curves for ceftazidime (external standards) were constructed prior to the experiments with linear correlation values of at least 0.995. The intra-day and inter-day variabilities of ceftazidime were assayed (six replicates) at concentrations of 0.1, 0.5, 1, 5, 10, 20 and 50 μg/ml on the same day and on six sequential days, respectively. The accuracy

(% Bias) was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentration (C_{obs}) as follows:

$$\text{Bias (\%)} = [(C_{obs} - C_{nom})/(C_{nom})] \times 100$$

The precision (relative standard deviation; RSD) was calculated from the observed concentrations as follows:

$$\text{\% RSD} = [\text{standard deviation (SD)}/C_{obs}] \times 100$$

Accuracy (% Bias) and precision (% RSD) values within ±15% covering the range of actual experimental concentrations were considered acceptable [10].

2.5. Blood and brain microdialysis

The microdialysis probes (active length 10 mm) were made of silica capillary in a concentric design and covered at the tips by dialysis membranes (Spectrum, 150 μm outer diameter with a cut-off at a nominal molecular weight of 13 000, Laguna Hills, CA, USA) [11,12]. The blood microdialysis probe was positioned in the jugular vein/right atrium (toward the heart) and then perfused with anticoagulant ACD solution (citric acid 3.5 m*M*; sodium citrate 7.5 m*M*; dextrose 13.6 m*M*) at a flow-rate of 1 μl/min. Following a 2 h post-surgical stabilization period, ceftazidime (50 mg/kg) was administered intravenously via the femoral vein. Then, blood dialysates were automatically injected into the microbore chromatographic system using an on-line injector (CMA/160) at 10 min intervals [13,14]. Aliquots of 10 μl of blood dialysates were assayed by microbore liquid chromatography.

2.6. Recovery of microdialysis probe

For in vivo recovery determinations, the microdialysis probes were inserted into the rat jugular vein under anesthesia with sodium pentobarbital. ACD solution containing ceftazidime (5 and 10 μg/ml) was perfused through the probes at a constant flow-rate (1 μl/min) using the infusion pump. After a 2 h stabilization period after the surgical procedure, inlet (C_{in}) and outlet (C_{out}) concentrations of ceftazidime were determined by HPLC. The in vivo recovery ($\text{Recovery}_{\text{in vivo}}$) of ceftazidime across the microdialysis probe was calculated by the following equation [15]:

$$\text{Recovery}_{\text{in vivo}} = [(C_{in} - C_{out})/C_{in}]$$

2.7. Pharmacokinetics

Ceftazidime concentrations were corrected by the in vivo recoveries of the respective microdialysis probes. Following a 2 h period of post-surgical stabilization, ceftazidime (50 mg/kg, i.v.) was administered. Blood dialysates were assayed every 10 min for an additional 210 min following ceftazidime administration. Pharmacokinetic parameters were obtained by the WinNonlin software program (version 1.1, Scientific Consulting, Apex, NC, USA) following the non-compartmental model [16,17]. The areas under the concentration curves (AUC) and the area under the moment versus time curve (AUMC) were calculated using the linear trapezoid method:

$$\text{AUC} = \text{AUC}_{last} + C_{last}/\lambda_z$$

$$\text{AUMC} = \text{AUMC}_{last} + (t_{last} \cdot C_{last}/\lambda_z) + C_{last}/(\lambda_z)^2$$

where C_{last} and t_{last} are the last observed concentration and time, respectively, and λ_z is the terminal slope, which was estimated by linear regression of the logarithmic value of the last observed data. The clearance (Cl) and the mean residence time (MRT) were estimated as follows:

$$\text{Cl} = \text{dose}/\text{AUC}$$

$$\text{MRT} = \text{AUMC}/\text{AUC}$$

The volume of the distribution at steady state (V_{ss}) was estimated from $V_{ss} = \text{Cl} \cdot \text{MRT}$ [18].

3. Results and discussion

Ceftazidime in blood dialysate was adequately resolved using microbore LC at a relatively short retention time of 4.8 min (Fig. 2). Fig. 2A shows typical chromatograms of ceftazidime at concentrations of 20 μg/ml. Fig. 2B shows a blank blood dialysate. Although a number of peaks appear in the

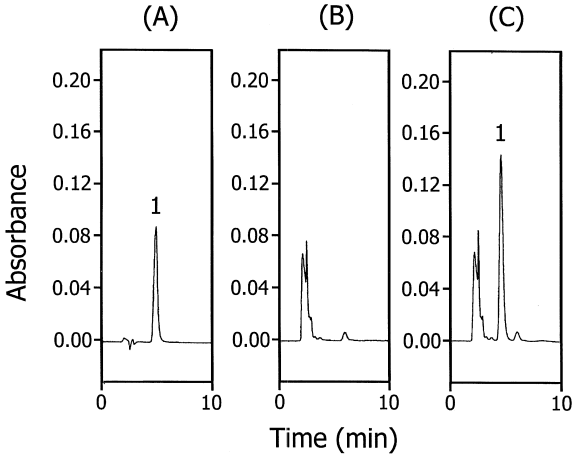

Fig. 2. Typical chromatograms of (A) standard ceftazidime (20 μg/ml), (B) blank blood dialysate from the microdialysis probe prior to drug administration, and (C) a blood dialysate sample containing ceftazidime (33.5 μg/ml) collected from rat blood microdialysate 20 min post-ceftazidime administration (50 mg/kg, i.v.) (1 = ceftazidime).

Table 1
Intra-day and inter-day accuracy and precision of the HPLC method for the determination of ceftazidime

Nominal conc. (μg/ml)	Observed conc. (μg/ml)[a]	RSD (%)	Accuracy (% bias)
Intra-assay (n = 6)			
0.10	0.103±0.005	4.8	3.0
0.50	0.49±0.01	2.0	−2.0
1.00	1.01±0.01	1.0	1.0
5.00	5.01±0.04	0.8	0.2
10.00	9.97±0.26	2.6	−0.3
20.00	20.39±0.62	3.0	1.9
50.00	49.86±0.19	0.4	−0.3
Inter-assay (n = 6)			
0.10	0.104±0.009	8.6	4.0
0.50	0.49±0.01	2.0	−2.0
1.00	0.99±0.03	3.0	−0.1
5.00	5.01±0.03	0.6	0.2
10.00	10.04±0.12	1.2	0.4
20.00	20.06±0.63	3.1	0.3
50.00	49.96±0.20	0.4	−0.1

[a] Observed concentration data are expressed as mean±SD.

chromatogram, none of them interfere with the analysis of ceftazidime. Fig. 2C shows the chromatogram of a blood dialysate sample containing ceftazidime at 33.5 μg/ml collected from rat blood 20 min following ceftazidime administration (50 mg/kg, i.v.).

The concentration–response relationship of the present method indicated linearity ($r^2 > 0.995$) over the concentration range 0.1–50 μg/ml for ceftazidime. Intra-day and inter-day precision (% RSD) and accuracy (% Bias) of ceftazidime fell well within the predefined limits of acceptability (<15%) (Table 1). The limit of detection was 0.05 μg/ml and the limit of quantification was 0.1 μg/ml. The average in vivo microdialysate recovery (%) of ceftazidime in rat blood was 17.8±3.5% (Table 2). Although ceftazidime is an electroactive cephalosporin, the limit of quantification for HPLC–electrochemical detection is low (0.75 μg/ml) [19]. In addition, HPLC–UV is the most common detection method for the analysis of ceftazidime. Granero et al. [20] reported that the detection limit of ceftazidime was 0.1 μg/ml using conventional HPLC–UV detection [20]. Our data show that the limit of detection of ceftazidime was 0.05 μg/ml measured by microbore HPLC–UV. Compared with other methods, microbore HPLC–UV appears to provide the best sensitivity for ceftazidime measurement. The HPLC–UV method described here was sufficient to separate and detect ceftazidime from the blood dialysate in less than 10 min.

In hospital therapeutical drug monitoring (TDM) or wildlife infection application [21], plasma samples are added to an equal volume of acid for plasma protein precipitation. Guitton et al. [19] reported that ceftazidime determination was based on the precipitation of plasma protein with 30% trichloroacetic acid [18]. This method obtains the total concentration (protein-bound and unbound forms) of ceftazidime. However, the protein-bound form of the drug cannot contribute to its pharmacological action. The microdialysis technique excludes the protein-

Table 2
In vivo microdialysis recoveries of ceftazidime in rat blood[a]

Conc. (μg/ml)	Recovery (%)
5	16.2±1.2
10	19.5±3.7

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

bound form of the drug and provides the protein-unbound form of ceftazidime from biological fluid. Under normal conditions, protein binding is constant in biological fluids.

Dialysate samples collected over the first 2 h were discarded to allow recovery from acute effects of the surgical procedures. Then, microdialysis-microbore LC was applied to determine the pharmacokinetic characterization of ceftazidime in rats. Fig. 3 shows the concentration profile of unbound ceftazidime in rat blood corrected by in vivo recovery, after ceftazidime (50 mg/kg, i.v.) administration. The pharmacokinetic data calculated using the non-compartmental model [17,18], the clearance (Cl), volume of distribution (V_{ss}) and mean residence time (MRT), were 7.08±0.57 ml/min/kg, 0.22±0.01 l/kg and 29.72±1.86 min, respectively. The pharmacokinetic parameters of ceftazidime in blood are presented in Table 3.

In summary, on-line microdialysis sampling coupled with microbore LC and ultraviolet detection permitted automated and continuous monitoring, and required no clean-up preparations or consumption of body fluids. This method exhibits no endogenous interference, and has sufficient sensitivity in blood dialysates. In addition, this method is applicable to further pharmacokinetic studies of protein-unbound drugs in rats.

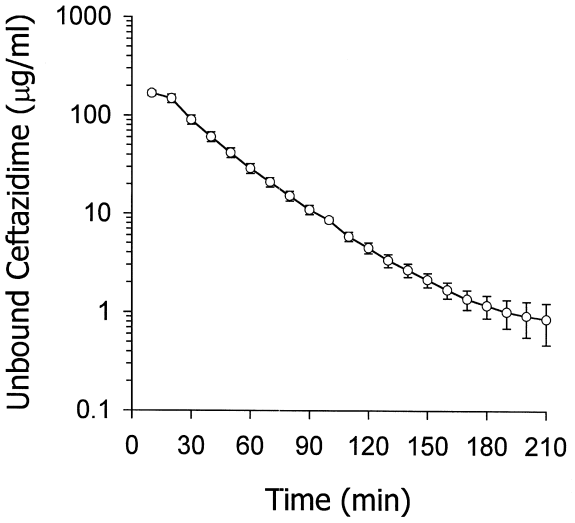

Fig. 3. Unbound ceftazidime concentrations in rat blood following 50 mg/kg intravenous administration. Data are presented as mean±S.E.M. ($n = 5$).

Table 3
Estimated pharmacokinetic parameters in rat blood after ceftazidime administration (50 mg/kg, i.v.)[a]

Parameter	Estimate
$t_{1/2,z}$ (min)	41.55±8.13
AUC (min μg/ml)	7226.35±593.41
Cl (ml/kg/min)	7.08±0.57
MRT (min)	29.72±1.86
V_{ss} (l/kg)	0.22±0.01

[a] Data are expressed as mean±S.E.M. ($n = 5$).

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