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Short communication

Quantitative determination of unbound cefoperazone in rat bile using microdialysis and liquid chromatography

Yuh-Lih Chang [a], Shih-Hwa Chiou [b,c], Yueh-Ching Chou [a,d], Chih-Ju Yen [e], Tung-Hu Tsai [e,f,*]

[a] *Department of Pharmacy, Taipei Veterans General Hospital, Taipei, Taiwan*
[b] *Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan*
[c] *Institute of Clinical Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[d] *Institute of Pharmacy, Taipei Medical University, Taipei, Taiwan*
[e] *Institute of Traditional Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[f] *Department of Education and Research, Ren-Ai Branch, Taipei City Hospital, Taipei, Taiwan*

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Abstract

Cefoperazone is a third generation cephalosporin antibiotic with a broad spectrum against gram-positive and gram-negative bacteria. It is clinically effective in the treatment of the biliary tract infections. In the present study, we utilized microdialysis sampling technique with shunt linear probe for continuous monitoring levels of cefoperazone from rat biliary ducts. The effects of berberine (a potential P-glycoprotein enhancer) pretreatment were also evaluated. Analysis of cefoperazone in the dialysates was achieved using a reversed phase RP-18 column (250 mm × 4.6 mm i.d.; particle size 5 μm) maintained at ambient temperature. The mobile phase comprised 100 mM monosodium phosphate (pH 5.5)–methanol (70:30, v/v), and the flow rate of the mobile phase was 1 ml/min. The UV detector wavelength was set at 254 nm. The area under the concentration–time curve and elimination half-life of cefoperazone were about 242.3 ± 13.4 min mg/ml and 64.1 ± 28.2 min, respectively. No significant effect was showed on the pharmacokinetics of cefoperazone with berberine pretreatment. This study represents a successful application of biliary microdialysis sampling technique, which is feasible for pharmacokinetic and biliary drug excretion studies.

Keywords: Berberine; Biliary excretion; Cefoperazone; Microdialysis; Pharmacokinetics

1. Introduction

Cefoperazone (Fig. 1) is a third generation cephalosporin antibiotic with a board spectrum of activity against gram-positive and gram-negative bacteria [1]. It is clinically effective in the treatment of infections in the biliary tract [2,3]. The ideal drug for the treatment of biliary tract infection should have excellent effects against potential biliary pathogens and should easily penetrate and be concentrated in the biliary tree [4,5]. Cefoperazone is excreted primarily in the bile where it can achieve concentrations of more than 6000 μg/ml and can be many folds higher than the simultaneous serum level [2,6–9]. The unique biliary pharmacokinetics of cefoperazone combined with its excellent in vitro activity against most commonly isolated biliary pathogens makes it the ideal drug for the treatment of biliary tract infection.

The use of herbal products to treat a wide range of conditions is rising rapidly, leading to increased intake of phytochemicals. Berberine, an alkaloid isolated from the roots and bark of *Berberis aristata* and *Coptis chinensis*, extracts of which have been found to have antibacterial effects [10] and commonly used in traditional oriental medicine. It has been reported that berberine might up-regulate the multi-drug resistance (MDR) transporter expression and function in human and murine hepatoma cells [11]. Moreover, some previous studies indicated that plant amphipathic cations, like berberine alkaloids, were good MDR substrates [12,13]. Herbal medicines are usually used by an increasing number of patients who typically do not advise their clinicians of concomitant use [14]. Cefoperazone and berberine may be concurrently used in patients with infec-

* Corresponding author at: Institute of Traditional Medicine, School of Medicine, National Yang-Ming University, 155, Sec. 2, Li-Nong Street, Taipei 112, Taiwan. Tel.: +886 2 2826 7115; fax: +886 2 2822 5044.
E-mail address: thtsai@ym.edu.tw (T.-H. Tsai).

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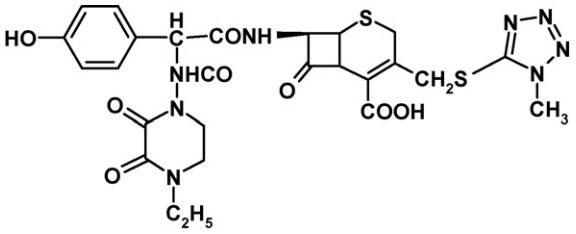

Fig. 1. Chemical structure of cefoperazone.

tions. Hence, it is of particular interest to examine the effects of berberine on the dispositions of cefoperazone in the biliary tracts.

The selection of an antibiotic for the treatment of infection is usually based on information given by the minimum inhibitory concentration (MIC) and the time versus serum concentration profile of drugs. Conversely, the data obtained from the serum may not be appropriate for evaluating infections of an extravascular compartment, such as a biliary tract or soft tissues [15,16]. In these cases the pharmacokinetic profiles in tissues rather than in serum determine the clinical outcome of antibiotic therapy [17]. However, knowledge about tissue concentrations of antibiotics is insufficient. In fact, only the unbound drug is considered active because it can spread to reach the target tissues. And only the free non-protein bound fraction of an antibiotic exerts antimicrobial activity. Microdialysis is a well-developed technique allowing the unbound drug fraction both in plasma and tissues of interest to be sampled. It can also provide a unique way to obtain near-complete concentration profiles of drugs in anatomically clearly defined tissues and organs. Cefoperazone levels in different tissues have been determined previously by microbiological methods [3,18] or HPLC [18–22]. Some of the HPLC assays with different extraction techniques have been extensively applied to pharmacokinetic studies [22,23].

To the best of our knowledge, this is the first study in which the disposition of cefoperazone in rat bile is examined by the technique of microdialysis. In this paper, we utilize a shunt linear probe [24] for continuous monitoring microdialysates of cefoperazone from the rat biliary ducts. In addition, the effects of berberine on the pharmacokinetics of cefoperazone were evaluated. Hence, to establish the biliary drug–drug interaction profile of cefoperazone, we use an in vivo on-line microdialysis sampling method coupled with the HPLC analytical system for measuring cefoperazone in rat biliary ducts.

2. Experimental

2.1. Chemicals and reagents

Cefoperazone was purchased from Pfizer (Roma, Italy). Berberine injection (5 mg/ml) was purchased from Kyorin (Taoyuan, Taiwan). Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triple de-ionized 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 urethane 0.8 g/ml and chloralose 0.08 g/ml (0.1 ml/kg, i.p.). Throughout the experimental period, anesthesia was maintained by administering one quarter of the initial dose at each hour. The experimental animals were kept warm with a heating pad throughout the experiments.

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). Cefoperazone dialysate was separated using a LiChrosorb RP-18 column (Merck, 250 mm × 4.6 mm i.d.; particle size 5 μm) maintained at ambient temperature. The mobile phase comprised 100 mM monosodium phosphoric acid (pH 5.5)–methanol (70:30, 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. Detecting UV wavelength was set at 254 nm. Output signal from the HPLC-UV was recorded using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.4. Method validation

All calibration curves of cefoperazone (external standards) were made prior to the experiments with correlation values of at least 0.995. The intra-day and inter-day variabilities for cefoperazone were assayed (six replicates) at concentrations of 0.5, 2, 10, 50, 250, 1000 and 4000 μg/ml on the same day and on six 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 (%) = $[(C_{nom} - C_{obs})/(C_{nom})] \times 100$. The relative standard deviation (R.S.D.) was calculated from the observed concentrations as follows: % R.S.D. = [standard deviation (S.D.)/C_{obs}] × 100. Accuracy (% Bias) and precision (% R.S.D.) values of within 15% covering the range of actual experimental concentrations were considered acceptable [25].

2.5. Microdialysis experiment

The bile duct microdialysis probes were manually made in-house based on the design originally described by Scott and Lunte [24]. The detailed construction of the flow-through microdialysis probe has been described in our previous reports [25,26]. In brief, a 7-cm piece of dialysis membrane (spectrum, 150 μm outer diameter with a cut-off at nominal molecular weight of

9000, 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 piece of 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. At least 24 h was allowed for the epoxy to dry. After bile duct cannulation, the probe was perfused with normal saline and the flow rate set at 2 μl/min. Outflows from the bile microdialysis probe were connected to an on-line injector and automatically injected every 10 min. After dialysate levels had stabilized (approximately 2 h), cefoperazone (30 mg/kg) was intravenously administered via the femoral vein. From each sample, 20 μl of dialysate was assayed using the HPLC system. In the pretreatment group, 30 mg/kg berberine was given intravenous 24 h before cefoperazone administration.

2.6. Recovery of microdialysate

For in vivo recovery, normal saline solution containing cefoperazone (50 or 100 μg/ml) were pumped through the probes at a constant flow rate (2 μl/min) using the infusion pump (CMA/100). After a stabilization period of 2 h, the inlet (C_{in}) and outlet (C_{out}) concentrations of cefoperazone were determined by HPLC. The in vivo recovery ratios were then calculated by the following equation [27]: $\text{Recovery}_{\text{in vivo}} = 1 - (C_{out}/C_{in})$.

2.7. Drug administration

After a 2-h post-surgical stabilization period, cefoperazone (30 mg/kg) was administered via femoral vein by i.v. bolus injection for each rat. Berberine (30 mg/kg) was pretreated 24 h before cefoperazone administration in the berberine group. Six animals were used in each group. All dialysates were collected every 10 min and then measured by a validated HPLC system.

2.8. Pharmacokinetic study

The concentrations of cefoperazone in the rat bile dialysates were determined from the calibration curves. Absolute concentrations in extracellular fluid were calculated from the concentrations in dialysates by the following equation: concentration = dialysate/recovery. Pharmacokinetic calculations were performed using the observed data. All data were subsequently processed by the computer pharmacokinetic program WinNonlin standard version 1.1 (Science Consulting Inc., Apex, NC, USA) for the calculation of pharmacokinetic parameters according to the non-compartmental model [28]. All data are presented as mean ± standard error. 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 [29]. The mean residence time and clearance was calculated as follows: MRT = AUMC/AUC, CL = Dose/AUC. The *t*-test was employed and the level of significance was set to $p < 0.05$.

Table 1
Intra-assay and inter-assay accuracy of cefoperazone

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	CV (%)	Accuracy (% bias)
Intra-assay			
0.5	0.48 ± 0.04	8.45	3.33
2	2.04 ± 0.06	2.90	−2.08
10	9.95 ± 0.10	0.99	0.48
50	50.03 ± 0.18	0.35	−0.07
250	249.93 ± 0.60	0.24	0.03
1000	998.15 ± 1.60	0.16	0.19
4000	4026.02 ± 48.40	1.20	−0.65
Inter-assay			
0.5	0.46 ± 0.05	10.00	7.33
2	2.04 ± 0.07	3.54	−1.75
10	9.97 ± 0.12	1.25	0.33
50	49.97 ± 0.05	0.10	0.07
250	250.45 ± 2.67	1.07	−0.18
1000	998.03 ± 9.33	0.94	0.20
4000	4000.40 ± 2.13	0.05	−0.01

3. Results and discussion

The chromatograms obtained evolving from the liquid chromatographic method shown in Fig. 2. Each analysis of the microdialysate was completed within 10 min. Separation of cefoperazone from endogenous chemicals in dialysates from biliary duct was achieved in an optimal mobile phase containing 70% of 100 mM monosodium phosphate (pH 5.5) and 30% of methanol. The retention time of cefoperazone was 6.0 min (Fig. 2). Peak areas of cefoperazone were linear ($r^2 > 0.995$) over a concentration range from 0.5 to 4000 μg/ml. Fig. 2A shows a typical chromatogram of a standard mixture containing cefoperazone (50 μg/ml). The blank sample (Fig. 2B) shows that the chromatographic conditions revealed no biological substances that would interfere significantly with the determination of cefoperazone. Fig. 2C depicts a chromatogram of microdialysate from rat biliary duct. The sample contains cefoperazone (27.8 μg/ml) collected from the microdialysates at 360 min following cefoperazone intravenous administration (30 mg/kg).

Intra-assay and inter-assay (Table 1) accuracy of cefoperazone levels fell well within the predefined limits of acceptability. All % bias and % CV values were within 10%. This method has a quantitative limit of 0.5 μg/ml. The in vivo recovery of cefoperazone is shown in Table 2. It can be seen that this method is sensitive enough to measure cefoperazone from microdialysates of rat biliary duct and to be applied for pharmacokinetic study. The concentration versus time curve of cefoperazone of rat biliary dialysate is shown in Fig. 3. As can be seen, cefoperazone appeared in the biliary microdialysates in the first sampling points and the average concentration got to 1100 μg/ml. The

Table 2
In vivo microdialysis recoveries (%) of cefoperazone in rat bile

Concentration (μg/ml)	Recovery (%)
50	0.58 ± 0.01
100	0.59 ± 0.01

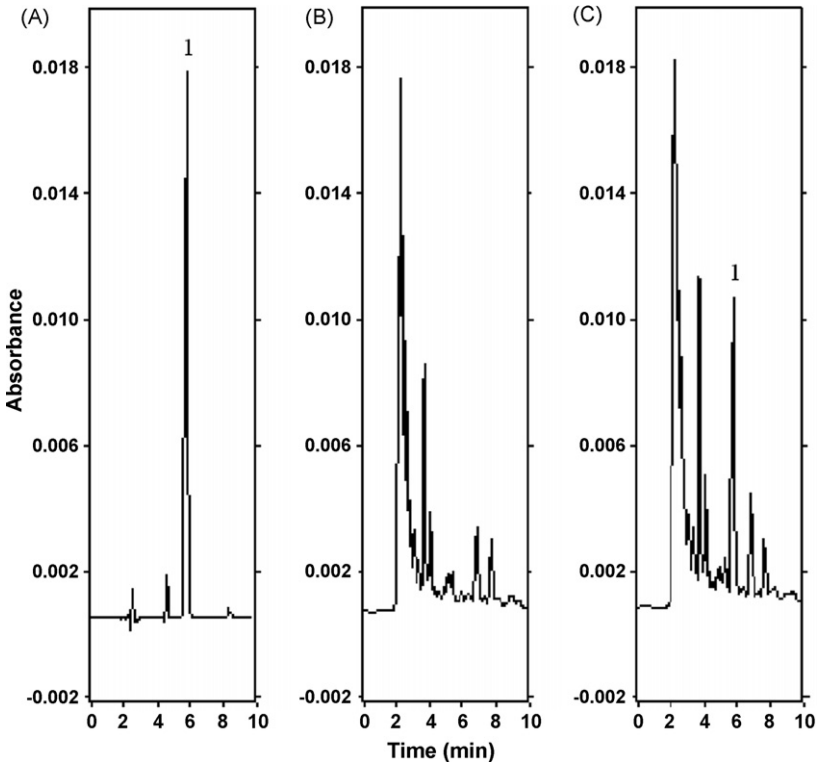

Fig. 2. Typical chromatograms for injection of (A) standard cefoperazone (50 μg/ml), (B) a blank bile dialysate, and (C) a bile dialysate sample containing cefoperazone (27.8 μg/ml) collected from rat bile microdialysate 360 min post cefoperazone administration (30 mg/ kg, i.v.). 1: cefoperazone.

drug levels reached the plateau within 20 min after drug administration and revealed a steady decline in the following 300 min. These facts suggested that cefoperazone is readily distributed into the hepatobiliary system after intravenous administration.

P-glycoprotein mediated transport in the liver has been shown to be responsible for the excretion of xenobiotics via the canalicular membrane of hepatocytes into bile, and this physiological function may be a control mechanism to accelerate the processes of hepatobiliary excretion [30]. Previous study reported that berberine might up-regulate the multidrug resistance transporter expression and function in human and murine hepatoma cells [11]. The treated concentrations of berberine were 32 and 320 μM (equal to 10 and 100 μg/ml). The previously published work by our lab [31] revealed that the biliary concentration of berberine sustained above 10 μg/ml for at least 120 min after i.v. administration of 30 mg/kg berberine. To ensure the time course needed for effective MDR regulation, we choose the berberine dose of 30 mg/kg i.v. for 24 h pretreatment in the current study. However, the results showed berberine pretreatment had no significant impact on the biliary disposition of cefoperazone. In Table 3, the $t1/2$, C_{max} and AUC also indicated insignificant difference in both treatment groups.

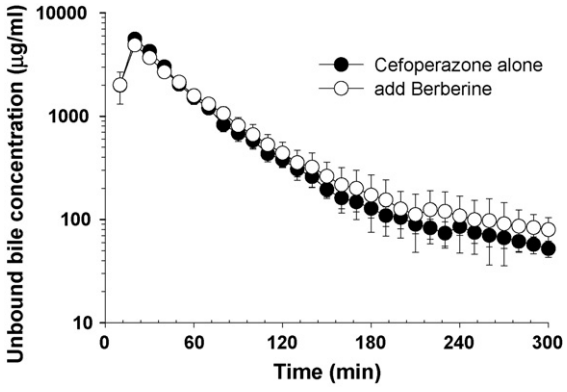

Fig. 3. Unbound concentration–time profile of cefoperazone in biliary duct after i.v. administration of 30 mg/kg or pretreatment with berberine 30 mg/kg. Each point is represented as means ± S.E.M.

The rationale for monitoring unbound drug concentrations is founded on the basic pharmacological principle. Even though it is suggested as the most reasonable approach for many drugs, monitoring of protein unbound drug concentrations is still a relatively new concept. Microdialysis represents a reliable tool for the measurement of unbound peripheral compartment concentrations [32]. However, only with the development of suitable analytic techniques has the routine measurement of unbound drug been possible [33], because the small sample volume and low concentrations obtained from this technique require a more sensitive and analytical system. Compare to previous published

Table 3
Estimated pharmacokinetic parameters of bile following cefoperazone administration (30 mg/kg, i.v.) or berberine pretreatment (30 mg/kg, i.v.)

Parameters	Cefoperazone alone	Add berberine
$t_{1/2}$ (min)	64.1 ± 28.2	62.5 ± 10.0
C_{max} (mg/ml)	5.4 ± 0.4	4.4 ± 0.8
AUC (min mg/ml)	242.3 ± 13.4	218.6 ± 41.0

Data are expressed as mean ± S.E.M. ($n = 6$); asterisks represents $p < 0.05$.

Table 4
Analytical methods used for determination of biliary concentrations of cefoperazone

Authors	Mobile phase	Column type	Limit of detection (μg/ml)	Linear range of analysis (μg/ml)	Reference
Current study	100 mM $NaH_2PO_4^-$ methanol = 70:30, v/v (pH 5.5)	LiChrosorb RP-18 column	0.1	0.5–4000	–
Muder et al.	0.005 M tetra-butyl-ammonium buffer: acetonitrile = 80:20	μBondapak phenyl	1	5–50	[34]
Haghgoo et al.	30 mM KH_2PO_4: Methanol = 80:20 (pH 5)	Cosmosil C18	0.2	0.2–200	[20]
Bawdon et al.	0.1 M $Na_3PO_4^-$ acetonitrile = 84:16 (pH 6)	C18μ Bondapak	2	2–100	[35]

papers concerned about the analytical procedures of cefoperazone assay for biliary samples, as can be seen in Table 4, the method of the current study provided the most sensitive detection with board range of linearity for analysis [20,34,35].

From these microdialysis sampling data, the pharmacokinetic parameters were calculated using the non-compartmental model (Table 3). These data have been corrected for in vivo recoveries. The current results were comparable with previously conducted studies, in which bile samples were obtained either during the liver transplantation [19] or from patient undergoing endoscopic retrograde cholangiopancreatography [36]. Conventional methods used to measure drug concentration in the bile use bile fluid collection. However, these methods require a relatively complicated clean-up process before samples can be analyzed. Furthermore, the methods of obtaining bile (T-tube drainage and duodenal tube aspiration) rarely result in a complete collection [2]. Thus, the attempts to determine drug concentrations continuously from the bile duct with no bile loss have had limited success. To overcome these shortcomings of traditional methods, we employed an automatic on-line flow-through microdialysis probe [24,37] for bile duct sampling coupled with an HPLC analytical system. It can provide a near real-time analysis of cefoperazone in bile dialysate. The application of microdialysis to monitor biliary drug concentrations did provide a dynamic profile, which characterizes the disposition of cefoperazone in the biliary tract. These approaches have been successfully applied in our previous studies [26,38–40].

In the current study we demonstrated an efficient and sensitive chromatographic method for the monitoring of cefoperazone in rat biliary tracts using the microdialysis technique. This method results in less tissue damage, fewer animal use, and minimal biological fluid loss. The pharmacokinetic profiles of cefoperazone did not show significant difference between control group and berberine-pretreatment group. This work represents a successful application of biliary microdialysis sampling technique, which is feasible for the pharmacokinetic and drug-interaction studies.

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