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Pharmacokinetics of metronidazole in rat blood, brain and bile studied by microdialysis coupled to microbore liquid chromatography

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Abstract

Metronidazole is a synthetic nitroimidazole-derived antibacterial and antiprotozoal agent used for the treatment of infections involving gram-negative anaerobes. The aim of this study is to develop an in vivo microdialysis with microbore high-performance liquid chromatographic system for the pharmacokinetic study of metronidazole in rat blood, brain and bile. In addition, to investigate the disposition mechanism of metronidazole, the P-glycoprotein modulator and cytochrome P450 inhibitor were concomitantly administered. Separation of metronidazole from various biological fluids was applied to a microbore reversed-phase ODS 5 μm (150×1 mm I.D.) column. Its mobile phase consists of an acetonitrile–50 m*M* monosodium phosphate buffer (pH 3.0) containing 0.1% triethylamine (10:90, v/v) with a flow-rate of 0.05 ml/min. The UV detector wavelength was set at 317 nm. The results suggest that metronidazole penetrates the blood–brain barrier (BBB) and goes through hepatobiliary excretion. However, these pathways of BBB penetration and hepatobiliary excretion of metronidazole may not be related to the P-glycoprotein.

Keywords: Microdialysis; Metronidazole

1. Introduction

Metronidazole is a well-known antimicrobial drug widely used for the treatment of bacterial vaginosis and *Trichomonas vaginalis*, and possesses a broad range of therapeutic uses. It is also used for the treatment of gastric *Helicobacter pylori* infections [1]. Metronidazole can be administered through a variety of routes, including oral, intravenous, rectal, and vaginal. For the oral route in healthy volunteers, the peak serum concentration occurred at 30 to 60 min, and the mean elimination half-life from the plasma was 14.4 h after oral administration. The human plasma protein binding of metronidazole was less than 15% [2] and can be distributed into the central nervous system [3]. Actually, metronidazole can penetrate well into the blood–cerebral spinal fluid (CSF) and blood–brain barrier (BBB) [4]. The drug has an oral bioavailability approaching 100%, but rectal and vaginal administration results in less drug absorption and lower serum concentrations [5]. The time to peak serum concentration after vaginal administration shows an inconsistency of about 8 to 12 h [5]. Metronidazole is converted in the liver to hydroxy metabolites, and further goes through glucuronidation. However, the metronidazole and hy-

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droxy-metronidazole areas under the plasma concentration curve from 0 to 24 h show no variation between the liver disease patients and healthy volunteers [6]. In vitro and in vivo studies indicate that metronidazole is not a substrate of liver enzyme CYP 3A4 [7]. Therefore, there is a need to develop a sensitive liquid chromatographic method for analyzing various biological fluid levels of metronidazole.

Several liquid chromatographic methods have been reported. They measure metronidazole in biological fluids using protein precipitation [8], liquid–liquid extraction [9], and solid–liquid extraction [10]. However, complicated biological sample clean-up steps prior to injection into liquid chromatography consume lots of time in pharmacokinetic studies. The samples of microdialysate are protein-free, making it possible for direct coupling of the microdialysis to the liquid chromatographic analysis with no sample clean-up required. Microdialysis technique has recently been shown to be a powerful tool for pharmacokinetic study on various tissues and fluid compartments [11–13].

In this study, we describe a rapid and sensitive microbore liquid chromatographic system for the determination and pharmacokinetic investigation of metronidazole in rat blood, brain and bile coupled to microdialysis sampling.

2. Experimental

2.1. Chemicals and reagents

Metronidazole was purchased from Rhone-Poulenc Rorer (Montrouge, France). Cyclosporin A (Sandimmun) was obtained from Novartis Pharma (Basle, Switzerland). Liquid chromatographic grade solvents and reagents were obtained from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Animals

All experimental protocols involving animals were reviewed and approved by the Institutional Animal Experimentation Committee of the National Research Institute of Chinese Medicine. Male specific pathogen-free Sprague–Dawley rats were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei, Taiwan. Before experimentation, animals were allowed a 1-week acclimation period at the animal quarters with air conditioning and an automatically controlled photoperiod of 12 h of light daily. Animals had free access to food (Laboratory rodent diet no. 5P14, PMI Feeds, Richmond, IN, USA) and water until 18 h prior to experimentation, at which time only food was removed. The rats were initially anesthetized with urethane 1 g/ml and α-chloralose 0.1 g/ml (1 ml/kg, i.p.), and remained anesthetized throughout the experimental period. The femoral vein was exposed for further drug administration. During the experiment, rat body temperature was maintained at 37 °C using a heating pad.

2.3. Chromatography

The 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 a UV–Vis detector (Varian, Walnut Creek, CA, USA). Samples obtained were separated using a microbore reversed-phase Inertsil-2 C_{18} column (150×1 mm; particle size 5 μm, GS Sciences, Tokyo, Japan). Chromatography was performed at ambient temperature. The mobile phase consisted of an acetonitrile–50 m*M* monosodium phosphate buffer (pH 3.0) containing 0.1% triethylamine (10:90, v/v). This mobile phase was filtered with a 0.22 μm Millipore membrane prior to being used for elution. The chromatographic pump flow-rate was set at 0.05 ml/min. The wavelength of metronidazole was determined to be 317 nm. The output signal from the HPLC UV–Vis detector was recorded using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.4. Microdialysis experiment

Blood, brain and bile microdialysis systems consisted of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and the appropriate microdialysis probes. The dialysis probe for blood (10

mm in length) [14], brain (3 mm in length) [15], and bile (7 cm in length) were made of silica glass capillary tubing arranged in a concentric design [16–18]. Their tips were covered by a dialysis membrane (150 μm outer diameter with a nominal molecular mass cut-off of 13,000, Spectrum, Laguna Hills, CA, USA) and all unions were cemented with epoxy. To allow adequate time for the epoxy to dry, the probes were made at least 24 h prior to use.

The blood microdialysis probes were positioned within the jugular vein/right atrium and then perfused with anticoagulant dextrose (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 using the CMA microinjection pump. The bile duct microdialysis probes were constructed in-house according to our previous studies [16–18]. For brain microdialysis, the rat was mounted on a stereotaxic frame and perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+; pH 7.0). After being washed with Ringer's solution at a flow-rate of 1 μl/min, the microdialysis probe was implanted in the right striatum (coordinates: AP 0.4 mm, ML −3.0 mm, DV −7.0 mm) according to the Paxinos and Watson atlas [19]. The positions of the probes were verified by standard histological procedure at the end of experiments.

2.5. Drug administration

After a 2-h post-implantation period, an intravenous dose of drug was administered via the femoral vein. Cyclosporin A, 20 mg/ml, was produced by diluting cyclosporin A injectable solution with a 5% aqueous dextrose solution. Metronidazole (10 mg/kg, $n=6$) was administered intravenously to the control group. For the cyclosporin A-treated group ($n=6$), cyclosporin A (20 mg/kg) was injected via the femoral vein 10 min prior to metronidazole administration. For the proadifen-treated groups, 10 mg/kg of proadifen was concomitantly injected via the left femoral vein 10 min prior to metronidazole injection. The volume of each injection was 1 ml/kg. The blood, brain, and bile dialysates were connected to an on-line injector (CMA 160) and a fraction collector (CMA/140). The sampling interval was 10 min for each probe. Blood, brain, and bile dialysates were measured by a microbore HPLC system on the same day as the experiment.

2.6. Recovery of microdialysate

For in vivo recovery, the blood, brain, and bile microdialysis probes were inserted into the jugular vein, striatum, and bile duct under anaesthesia with urethane. Perfusate solution containing metronidazole was passed through the microdialysis probe at a constant flow-rate (1 μl/min) using an infusion pump (CMA/100). Two hours after probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of metronidazole were determined by HPLC. The relative recovery (R_{dial}), in vivo, of metronidazole across the microdialysis probes inserted in the rat blood, brain, and bile were calculated according to the following equation, $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$.

2.7. Pharmacokinetic application

Metronidazole microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u = C_m/R_{dial}$. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA) by noncompartmental method [20]. The areas under the curve (AUC) from time zero to time infinity were calculated by the trapezoidal rule and extrapolated to time infinity by the addition of AUC_{t-inf}. The AUC values were thus given by adding the product of the measured concentrations and the collection time interval to the residual area, that is: $AUC = AUC_{0-t} + AUC_{t-inf}$.

2.8. Statistics

The results are represented as mean±standard error of the mean. The statistical analysis was performed with SPSS version 10.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for the comparison between the control (metronidazole treated alone) and various treated groups. All statistical tests were performed at the two-sided 5% level of significance.

3. Results and discussion

Typical chromatograms of metronidazole sampled from the biological fluids are shown in Fig. 1. Separation of metronidazole from endogenous chemicals in biological dialysates was achieved in an optimal mobile phase containing 90% of 50 m*M* monosodium phosphate buffer (pH 3.0) containing 0.1% triethylamine and 10% acetonitrile. Retention time of metronidazole was about 6.5 min. The calibration curve of metronidazole was linear over the investigated concentration ranges of 0.05–20 μg/ml. The relative standard error of the slope and intercept were 2.0% and 3.0%, respectively. The correlation coefficient was higher than 0.999, indicating good linearity. This chromatographic system has been validated for both within-day and between-day accuracy and the determined limits of this precision assay deemed acceptable (Table 1). The relative standard deviation (RSD) was less than 6.1% for the within-day and between-day assay.

Table 1
Within-day and between-day precision (RSD) and accuracy (bias) of the HPLC method for the determination of metronidazole

Nominal concentration (μg/ml)	Observed concentration[a] (μg/ml)	RSD (%)	Bias (%)
Within-day			
0.05	0.049±0.003	6.1	−2.0
0.10	0.097±0.002	2.1	−3.0
0.50	0.51±0.02	3.9	2.0
1.00	0.99±0.01	1.0	−1.0
5.00	4.71±0.19	4.0	−5.8
10.00	11.35±0.45	4.0	13.5
20.00	19.27±0.28	1.5	−3.7
Between-day			
0.05	0.047±0.001	2.1	−6.0
0.10	0.102±0.003	2.9	2.0
0.50	0.49±0.02	4.1	−2.0
1.00	1.03±0.04	3.9	3.0
5.00	4.88±0.20	4.1	−2.4
10.00	10.01±0.12	1.2	0.1
20.00	20.27±0.30	1.5	1.4

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

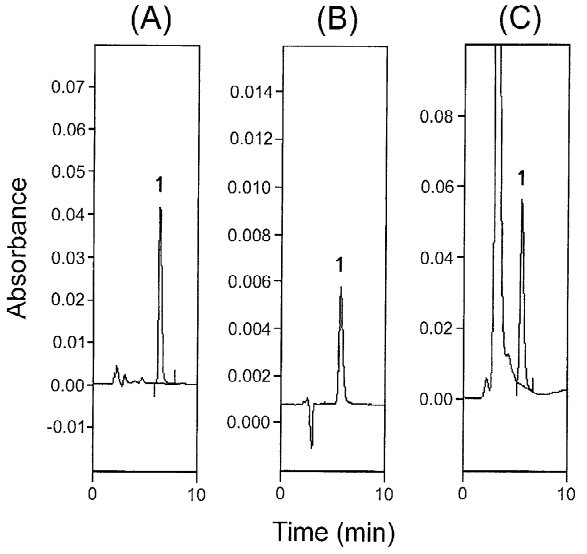

Fig. 1. Typical chromatograms of (A) blood dialysate sample containing metronidazole (5.21 μg/ml) collected from the rat blood microdialysate 30 min after metronidazole administration (10 mg/kg, i.v.), (B) brain dialysate sample containing metronidazole (0.89 μg/ml) collected from the rat brain microdialysate 30 min after metronidazole administration (10 mg/kg, i.v.), and (C) bile dialysate sample containing metronidazole (8.63 μg/ml) collected from the rat bile microdialysate 30 min after metronidazole administration (10 mg/kg, i.v.). 1, Metronidazole.

The limit of quantification (LOQ) was defined as the lowest concentration of the linear range which is 0.05 μg/ml. The limit of detection (LOD) was 0.01 μg/ml at signal-to-noise ratio of 3. The chromatograms of a blank blood, brain, and bile dialysate indicate that none of the observed peaks interfered with the analyte.

Fig. 1A shows the chromatogram of a blood dialysate sample containing metronidazole (5.21 μg/ml) collected 30 min after metronidazole administration (10 mg/kg, i.v.). Furthermore, none of the observed peaks interfered with the analyte in the chromatograms of brain and bile samples. Fig. 1B and C shows the chromatograms of brain and bile samples containing metronidazole 0.89 and 8.63 μg/ml collected from the brain and bile duct microdialysis probes, respectively, 30 min after metronidazole administration (10 mg/kg, i.v.).

In vivo recoveries of metronidazole (1 μg/ml) were 54.0±1.3% ($n=6$) for blood, 25.3±2.2% ($n=6$) for brain, and 77.3±2.6% ($n=6$) for bile. The in vivo recovery (or dialysis efficiency) can be affected

by certain factors, mostly physical in nature, such as temperature and perfusion rate. Also the materials used in the construction of the probe and the final dimensions of the probe can affect dialysis efficiency. Thus, drug concentration in the biological fluid must be corrected by the recovery.

The pharmacokinetic profiles of unbound metronidazole in rat blood, brain, and bile in control, cyclosporin A, and proadifen-treated groups are presented in Table 2. The results of the present study suggest that the metronidazole undergoes the pathway of hepatobiliary excretion. In addition, in the cyclosporin A concomitantly-treated group, the brain and bile concentrations of metronidazole were not significantly different, indicating that the mechanism of BBB penetration and hepatobiliary excretion may not be related to the P-glycoprotein (Table 2). The simultaneous multiple sites sampling in a single rat of blood, brain, and bile have been developed for the measurement of metronidazole in this study. Because there are no loss of biological fluids, microdialysis offers a number of advantages such as long period of time sampling, and higher temporal resolution for pharmacokinetic study. Traditional tissue homogenate techniques have long been used to determine drug distribution in the brain. However, only single concentration–time points can be provided in such tissue homogenization studies, as the experimental animals need to be sacrificed to obtain samples. The application of microdialysis has received the most attention and use in the neuroscience and pharmacokinetics study of the brain [21].

In conclusion, we have developed a specific and rapid microbore HPLC method for the determination of protein-unbound metronidazole in rat blood, brain, and bile. Current data obtained from rats show no significant impact of cyclosporin A on the pharmacokinetics of metronidazole in rat blood, brain, and bile when they are concomitantly injected.

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Table 2
Pharmacokinetic data of metronidazole (10 mg/kg) in rat blood, brain and bile, for the groups with and without cyclosporin A (20 mg/kg) or proadifen (10 mg/kg) treatment

Drug	Metronidazole (10 mg/kg)		
treatment	Alone	With cyclosporin A	With proadifen
Blood			
$t_{1/2}$ (min)	134±14	160±22	133±9
AUC (min μg/ml)	2587±109	1574±301*	1716±197*
Cl (ml/kg/min)	3.88±0.15	6.27±1.27*	6.15±1.13*
MRT (min)	189±12	211±26	180±12
Brain			
C_{max} (μg/ml)	2.84±0.27	1.87±0.16*	2.19±0.25
$t_{1/2}$ (min)	130±23	193±33	89±18
AUC (min μg/ml)	605±59	513±109	356±39*
MRT (min)	215±20	265±42	148±9
Bile			
C_{max} (μg/ml)	14.15±1.26	10.53±1.71	12.43±1.66
$t_{1/2}$ (min)	181±82	108±19	106±8
AUC (min μg/ml)	2442±589	1253±123*	1629±171
MRT (min)	256±91	175±28	166±14
AUC_{brain}/AUC_{blood}	0.23±0.02	0.33±0.07	0.20±0.02
AUC_{bile}/AUC_{blood}	0.94±0.23	0.79±0.08	0.95±0.10

Data are expressed as mean±SEM ($n=6$). *$P<0.05$, significantly different from the metronidazole alone group.

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