

Pharmacokinetics of Berberine and Its Main Metabolites in Conventional and Pseudo Germ-Free Rats Determined by Liquid Chromatography/Ion Trap Mass Spectrometry

Feng Zuo, Norio Nakamura, Teruaki Akao, and Masao Hattori

Institute of Natural Medicine (F.Z., N.N., M.H.) and Faculty of Pharmaceutical Sciences (T.A.), University of Toyama, Toyama, Japan

Received June 7, 2006; accepted September 5, 2006

ABSTRACT:

Berberine (Ber) and its main metabolites were identified and quantified using liquid chromatography/electrospray ionization/ion trap mass spectrometry. Rat plasma contained the main metabolites, berberrubine, thalifendine, demethyleneberberine, and jatrorrhizine, as free and glucuronide conjugates after p.o. Ber administration. Moreover, the original drug, the four main metabolites, and their glucuronide conjugates were all detected in liver tissues after 0.5 h and in bile samples 1 h after p.o. Ber administration. Therefore, the metabolic site seemed to be the liver, and the metabolites and conjugates were evidently excreted into the duodenum as bile. The pharmacokinetics of Ber and the four metabolites were deter-

mined in conventional and pseudo germ-free rats (treated with antibiotics) after p.o. administration with 40 mg/kg Ber. The AUC_{0-limt} and mean transit time values of the metabolites significantly differed between conventional and pseudo germ-free rats. The amounts of metabolites were remarkably reduced in the pseudo germ-free rats, whereas levels of Ber did not obviously differ between the two groups. The intestinal flora did not exert significant metabolic activity against Ber and its metabolites, but it played a significant role in the enterohepatic circulation of metabolites. In this sense, the liver and intestinal bacteria participate in the metabolism and disposition of Ber in vivo.

Berberine (Ber) is an isoquinoline alkaloid of the protoberberine type, with a long history of medicinal use in traditional eastern medicine. It is found in the root, rhizome, and stem bark of many plant species such as *Coptis chinensis* Franch., *Coptis japonica* Makino., *Berberis thunbergii* DC., *Hydrastis canadensis* L., and *Thalictrum lucidum* Ait. Ber extracts and decoctions have significant antimicrobial activities. Recent pharmacological studies have shown that Ber also possesses antitumor (Kettmann et al., 2004), anti-HIV (Gudima et al., 1994), antifungal (Vollekova et al., 2003), cardioprotective (Zheng et al., 2003), immunoregulative (Kim et al., 2003), antimalarial (Tran et al., 2003), anti-inflammatory (Kupeli et al., 2002), antioxidative (Rockova et al., 2004), cerebroprotective (Ma et al., 1999), antimutagenic (Cernakova et al., 2002), vasorelaxing (Ko et al., 2000), anxiolytic (Peng et al., 2004), and analgesic (Yesilada and Kupeli, 2002) effects. Ber is generally administered as a chloride or sulfate for clinical applications.

The pharmacokinetics of Ber have been examined by several investigators using tritium-labeled derivatives or high-performance

liquid chromatography (HPLC) (Xiong et al., 1989; Shen et al., 1993; Li et al., 1995). Because the plasma concentrations are very low, Ber was always thought to be poorly absorbed through the gut wall (Miyazaki et al., 1978; Chen and Chang, 1995). Therefore, the remarkable variety of pharmacological effects exerted by Ber at blood concentrations below the effective dose required for activity in vitro has been regarded with considerable skepticism. Nevertheless, investigators have investigated the disposition process of Ber in vivo. Ber metabolites have recently been studied in the urine of healthy volunteers using liquid chromatography/mass spectrometry (LC/MS) after p.o. Ber administration (Pan et al., 2002) and in rat bile after i.v. injection (Tsai and Tsai, 2004). However, the disposition and metabolites of Ber in the plasma of humans and other animals remain obscure.

The present study identifies and quantifies Ber and its main metabolites using liquid chromatography/tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI) in rat plasma after p.o. Ber administration. The procedure identified the major Ber metabolites as berberrubine (M1), thalifendine (M2), demethyleneberberine (M3), and jatrorrhizine (M4), together with their respective glucuronide conjugates.

Materials and Methods

Chemicals and Reagents. Ber chloride, oxytetracycline hydrochloride (Terramycin), cefadroxil, erythromycin, β -glucuronidase (EC 3.2.2.31, type

This study was partially supported by a 21st Century of Center of Excellence Program grant from the Japan Ministry of Education, Culture, Sports, Science and Technology (MEXT).

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.106.011361.

ABBREVIATIONS: Ber, berberine; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; LC/MS/MS, liquid chromatography/tandem mass spectrometry; ESI, electrospray ionization; M1, berberrubine; M2, thalifendine; M3, demethyleneberberine; M4, jatrorrhizine; TIC, total ion chromatogram; AUC, area under the plasma concentration-time curve; AUMC, area under the first moment curve; MTT, mean transit time; SIM, selective ion monitor.

B-1 from bovine liver), sulfatase (EC 3.1.6.1, type VIII from abalone entrails), and bovine serum albumin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Liquid chromatographic grade solvents, phloroglucin, sodium bisulfite, and polyamide C-100 and alumina for column chromatography were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). General anaerobic medium broth was purchased from Nissui Co. (Tokyo, Japan). Berberubine chloride and demethyleneberberine chloride were synthesized, and jatrorrhizine chloride and thalifendine chloride were isolated from a commercial Ber preparation in our laboratory. The structures of these alkaloids were determined by comparing their spectra with those reported (Shamma et al., 1965; Wu et al., 1976; Pan et al., 2001; Das and Srinivas, 2002).

Instruments. Compounds were analyzed by ^1H and ^{13}C NMR, as well as two-dimensional NMR, using a Unity Plus 500 (Varian) NMR spectrometer with tetramethylsilane as an internal standard, and chemical shifts are shown as δ values. Intestinal bacteria were anaerobically incubated using an EAN-140 (Tabai Co., Osaka, Japan). The HPLC instrument was an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) comprising an Agilent 1100 series binary pump with a photodiode array detector and a series 7725i injector with a 20- μl loop. Data were acquired and integrated using a ChemStation. The HPLC system was connected to an Esquire 3000^{plus} mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an ESI source. All the LC/MS/MS data were acquired using Esquire Control software and analyzed using software from Bruker Daltonik.

Synthesis of M1. Ber chloride (50 mg) was irradiated for 5 min in a flask placed in an alumina bath inside a commercial microwave oven. Water (50 ml) was added, and the mixture was extracted with chloroform (150 ml) 8 to 10 times. The combined organic layer was evaporated in vacuo to give a residue (32 mg) that was eluted with $\text{CHCl}_3/\text{MeOH}$ (9:1) through a silica gel column (2 \times 40 cm) to separate berberubine chloride (yield 67%) (Das and Srinivas, 2002).

Synthesis of M3. Ber chloride (100 mg) and phloroglucin (100 mg) were mixed with 60% H_2SO_4 (100 ml) and stirred at 100°C in an oil bath for 20 min. The mixture was cooled, concentrated, and resuspended in $\text{H}_2\text{O}/\text{acetone}$ (1:1), and then M3 was purified by silica gel chromatography (column: 2 \times 40 cm) with $\text{CHCl}_3/\text{MeOH}$ (20:1 \rightarrow 9:1) (yield 42%) (Pan et al., 2001).

Isolation of M2 and M4. Commercial Ber chloride (95% pure) contained two impurities with peaks at m/z 338 and 322 on LC/MS/MS. These compounds were isolated (Otsuka et al., 1981) by column chromatography and identified by comparing their spectral data with those reported for thalifendine

and jatrorrhizine (Shamma et al., 1965; Wu et al., 1976). In brief, Ber chloride (500 mg) was dissolved in 1% NH_4OH (10 ml) and eluted through a polyamide C-100 column (4 \times 40 cm) with 1% NH_4OH (300 ml), and then fractions containing compounds with quasi molecular ions at m/z 338 and 322 were combined, concentrated, and eluted through a column of silica gel (1 \times 40 cm), with $\text{CHCl}_3/\text{MeOH}$ (100:1 \rightarrow 20:1 \rightarrow 9:1) to give M2 and M4 in yields of 12 and 2 mg, respectively.

Preparation of Pseudo Germ-Free Rats. Male Wistar rats (8 weeks old) purchased from SLC Co. (Hamamatsu, Japan) were administered p.o. cefadroxil (100 mg/kg), Terramycin (300 mg/kg), and erythromycin (300 mg/kg) for 3 days, and then pharmacokinetic experiments proceeded 2 days later. On the 1st and 5th days of antibiotic administration, the cecal contents were collected from the rats, and the bacteria status was examined as described previously (Elmer and Rimmel, 1984).

Collection of Urine, Bile, and Plasma Samples. Rats were fed standard laboratory chow for 1 week, fasted overnight, and given free access to water before drug administration. Urine samples were collected while the rats remained isolated in metabolic cages. The animals were lightly anesthetized with diethyl ether during surgical procedures. Bile sample was collected by cannulating a polyethylene tube (PE-10) into the bile duct. At intervals of 0, 0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h after p.o. administration, the abdomen was exposed by a midline abdominal incision, and the blood sample was collected from the inferior vena cava using a heparinized injector. The blood samples were centrifuged at 8000g for 15 min to separate the plasma, and then all the samples were stored at -20°C for later analysis.

Sample Preparation for LC/MS/MS. Thawed urine and bile samples (0.5 ml) were incubated with β -glucuronidase (1000 units/ml) and sulfatase (500 units/ml) for 16 h at 37°C, dissolved in 3 volumes of MeOH, and then centrifuged at 8000g for 15 min. The supernatant was passed through a 0.45- μm Millipore syringe filter (Nihon Millipore, Tokyo, Japan) for LC/MS/MS analysis. β -Glucuronidase was mixed with a half volume of thawed plasma and incubated for 16 h at 37°C. Enzyme-digested and undigested plasma samples were passed through solid-phase extraction cartridges (Waters Co., Milford, MA) that had been washed with 3 ml of MeOH and equilibrated with 6 ml of water. The constituents were eluted with 2 or 3 ml of MeOH from the cartridge, and then the eluate was evaporated under a stream of nitrogen at 35°C to leave a residue that was dissolved in 100 μl of MeOH for LC/MS/MS analysis.

Metabolism of Ber by Liver in Vivo and in Vitro. Rats ($n = 4$) were

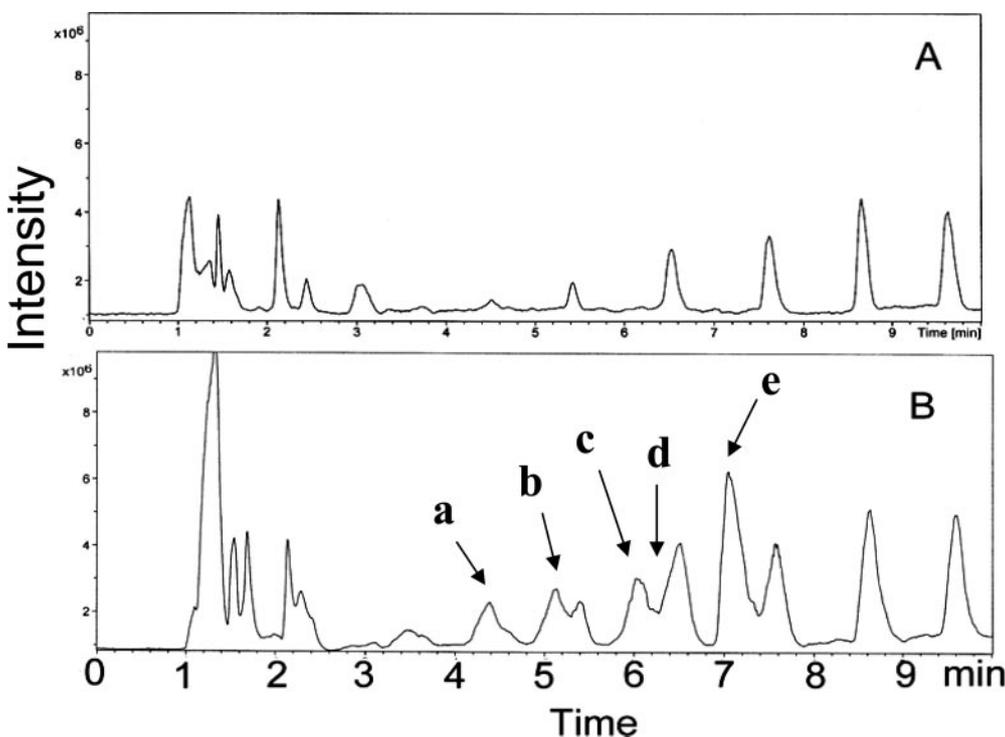


FIG. 1. TIC of rat plasma blank (A) and after p.o. administration of Ber (B).

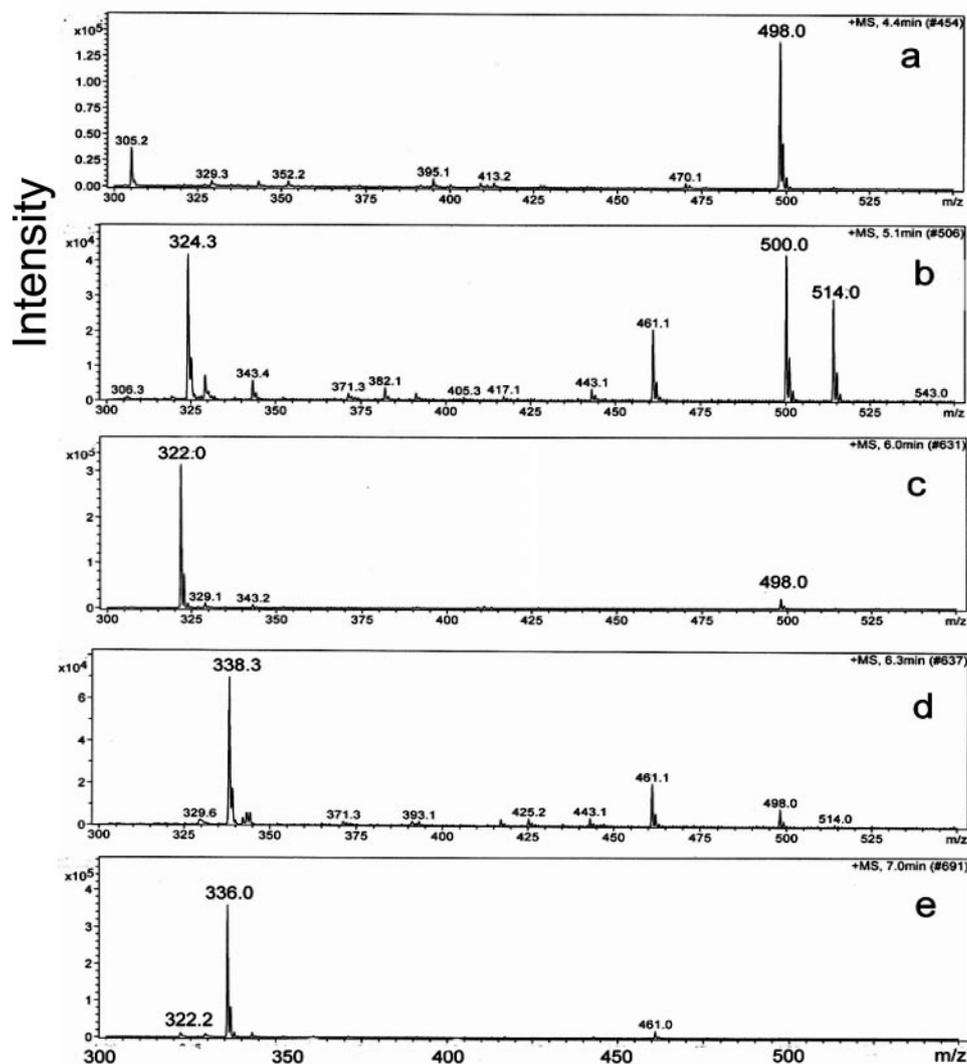


FIG. 2. TIC peaks in rat plasma after p.o. Ber administration. Ion peaks are located at t_R 4.4 (a), 5.1 (b), 6.0 (c), 6.3 (d), and 7.0 min (e).

administered p.o. 40 mg/kg Ber, and then the liver was removed 30, 60, and 120 min later, washed with normal saline, cut into pieces, and extracted with MeOH. The mixture was centrifuged at 8000g for 15 min, and the supernatant was concentrated and filtered for LC/MS/MS analysis. Fresh liver samples were homogenized on ice using a Wheaton homogenator with 4 volumes of phosphate buffer (pH 7.4), thoroughly mixed with Ber chloride (5 mg) in dimethyl sulfoxide (0.5 ml), and then incubated at 37°C for 4 h. The mixture was extracted with 3 volumes of MeOH, concentrated, and filtered.

Metabolism by Intestinal Bacteria. Mixtures of rat or human intestinal bacteria (5 g each), prepared as described previously (Xie et al., 2003), together with Ber or metabolites (5 mg) dissolved in dimethyl sulfoxide (0.5 ml) were added to general anaerobic medium broth (50 ml) and anaerobically incubated at 37°C for 7 days. The incubation mixture was extracted with 3 volumes of MeOH and then passed through a 0.45- μ m filter.

Absorption of Ber from the Small Intestine Measured Using an in Situ Loop. The small intestine of a Wistar rat that had been fasted overnight was exposed under general anesthesia. An 8-cm loop of empty, middle-tract jejunum was ligated at both ends, and major blood vessels were excluded from the ligatures. Ber (0.02 mM) dissolved in 2.5 ml of saline containing 1 mg/ml bovine serum albumin was injected into the jejunal loop through a cannulated polyethylene tube inserted at one end of the ligated jejunum. The organs were returned to the abdomen, and the incision was closed. Portions (0.1 ml) of the jejunal contents were withdrawn 10, 20, 40, and 60 min later. Absorption was calculated as the difference in the amount of the initial and final solutions (Wang et al., 1994; Akao et al., 2000).

Validation of an LC/MS/MS. The LC/MS/MS equipment comprised a

column containing TSK gel ODS-80 Ts (particle size, 5 μ m; 4.6 \times 150 mm i.d.) (Tosoh Co., Tokyo, Japan). Samples were separated through the column with a gradient of water/AcOH (100:1, v/v) and MeOH (0 min, 80:20; 10 min, 60:40; 20 min, 0:100) at a flow rate of 1 ml/min at 30°C. The standard positive ion mode was selected under the following conditions: full scan range, 50 to 600 m/z ; scan resolution, 13,000 m/z s; nebulizer, 50.0 psi; dry gas, 10.0 l/min; and dry temperature, 360°C.

Linearity. Reference compounds were dissolved in MeOH to a final concentration of 1 mg/ml, respectively. Seven dilutions of standard compounds were evaporated to dryness under a nitrogen stream, and then 1 ml of rat plasma (blank) was added to each dilution to prepare standards. The plasma was prepared as described above. Response linearity was determined for the seven concentrations after three injections for each level. Standard curves were constructed from the peak areas of compounds in the total ion chromatogram (TIC) under the second MS scan conditions. The limit of detection of the method for each constituent was established when the signal to noise ratio was 5.

Accuracy. Intra-assay and interassay variability was determined by analyzing high, medium, and low standard concentrations of rat plasma five times on the same day and continuously for 5 days, respectively.

Recovery. Two standard concentrations were evaporated to dryness and mixed with rat plasma samples after the p.o. administration of Ber with a known amount of constituent, and recovery rates of the added amounts were calculated.

Stability. Three concentrations of plasma samples that had been prepared for LC/MS/MS analysis were placed at room temperature for 12 h or in a

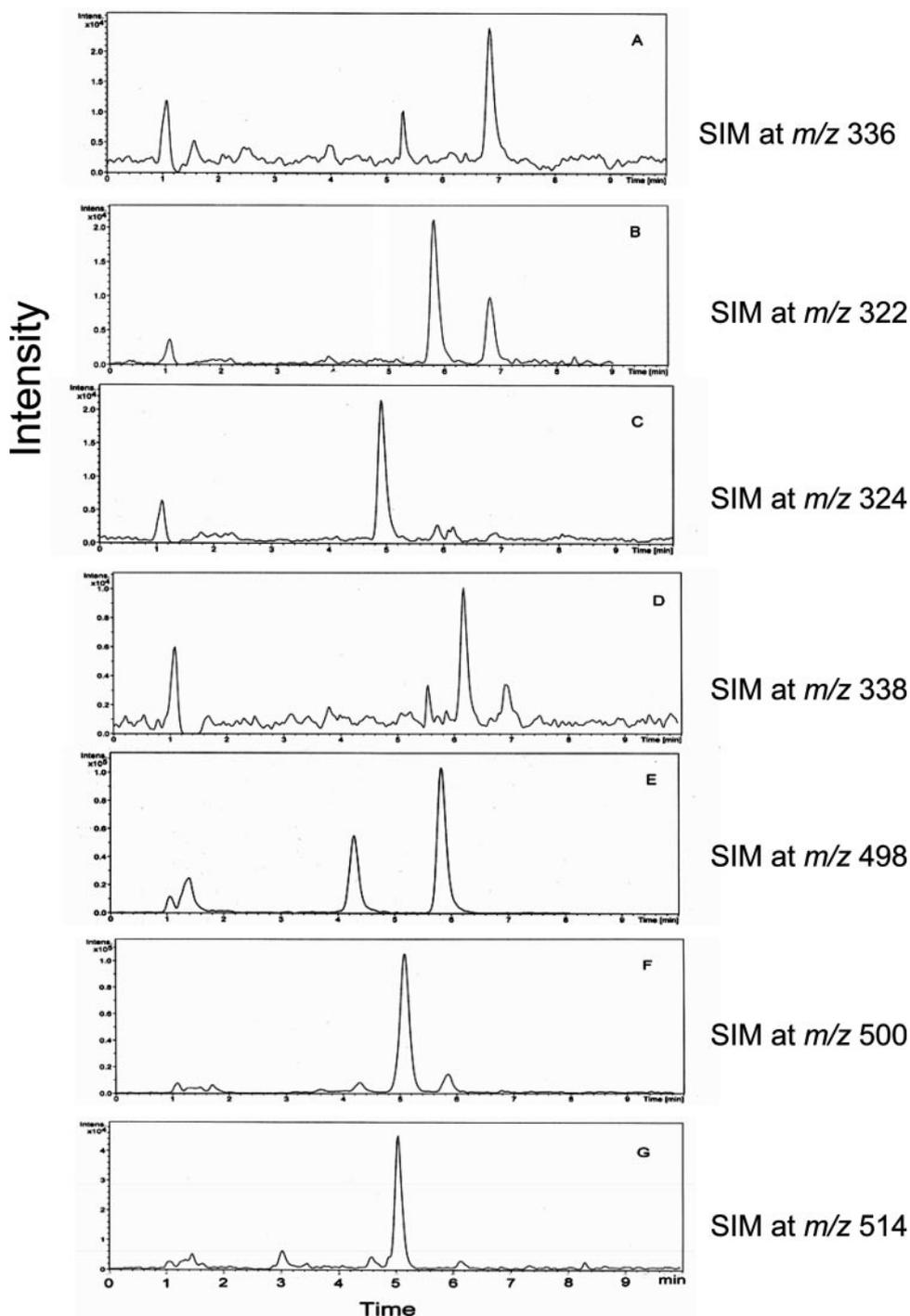


FIG. 3. SIM chromatograms of Ber at m/z 336 (A) and metabolites at m/z 322 (B), 324 (C), and 338 (D); glucuronide conjugates at m/z 498 (E), 500 (F), and 514 (G).

refrigerator at 4°C for 1, 3, and 5 days. The average peak areas of constituents in the samples and relative standard deviation were calculated.

Data Analysis. Pharmacokinetics were evaluated using a noncompartmental analysis of the plasma concentration-time data based on statistical moment theory. The mean concentration of the rats ($n = 4$) at each time point was used. The area under the plasma concentration-time curve (AUC) and area under first moment curve (AUMC) were calculated by linear trapezoidal rule; the ratio of AUMC to AUC was described as the mean transit time (MTT).

Results

Identification of Ber Metabolites in Rat Plasma. Plasma collected from rats that had received p.o. Berberine chloride was analyzed by LC/MS/MS. Full scanning in the region of m/z 50 to 600 assigned

several peaks to Ber and metabolites (a through e) in the TIC when compared with those of a blank plasma sample (Fig. 1). The MS spectra revealed intense ion peaks at m/z of 336, 322, 324, 338, 498, 500, and 514 (Fig. 2). Subsequently, regions at m/z 336, 322, 324, 338, 498, 500, and 514 (Fig. 3) were analyzed by selective ion monitoring (SIM), along with a second MS spectrum at the respective ion peaks. The SIM profile at m/z 336 (Fig. 3A), t_R , and the second MS spectrum showed that the highest ion peak at retention time (t_R) = 7.0 min was derived from Ber ($C_{20}H_{18}NO_4^+ = 336.12$). Similarly, the major ion peaks at $t_R = 6.9$ or 5.8 min, 4.9 and 6.2 min in the SIM profiles were derived from berberrubine or thalifendine (both m/z 322), demethylenberberine (m/z 324), and jatrorrhizine (m/z 338).

TABLE 1
Retention time (t_R) and MS spectra of Ber and its metabolites

	t_R (min)	MS Spectra
Ber	7.0	336, 321
M1 (berberrubine)	6.9	322, 307
M2 (thalifendine)	5.8	322, 307
M3 (demethylenerberberine)	4.9	324, 309
M4 (jatrorrhizine)	6.2	338, 323
M1, M2-glucuronide	4.3, 5.7	498, 322, 307
M3-glucuronide	5.1	500, 324, 309
M4-glucuronide	5.0	514, 338, 323

TABLE 2
Regression equations, correlation coefficients (γ), linearity ranges, and limit of detection (LOD) of Ber and its metabolites in rat plasma

Regression Equation	γ	Linearity Range $ng \cdot ml^{-1}$	LOD (Signal/Noise = 5) ng	
Ber	$Y = 1515.6X + 70.7$	0.9989	0.05–5.0	0.02
M1	$Y = 207.7X + 51.2$	0.9996	0.2–10.0	0.1
M3	$Y = 186.21X + 52.6$	0.9984	0.25–10.0	0.2
M4	$Y = 440.86X + 84.0$	0.9973	0.2–10.0	0.1

The ion peaks at m/z 498, 500, and 514 were considered to be 176 mass units higher than the molecular ions of free metabolites ($498 = 322 + 176$, $500 = 324 + 176$, and $514 = 338 + 176$). The fragmentation of an ion at m/z 498 yielded ion peaks at m/z 322 and 307. Similarly, ions at m/z 500 and 514 showed fragment ions m/z 324 and 309 and at m/z 338 and 323, respectively. Because these fragment ions included the molecular ion peaks of free metabolites, we concluded that the molecular ions at m/z 498, 500, and 514 were derived from berberrubine glucuronide (or thalifendine glucuronide), demethylenerberberine, and jatrorrhizine glucuronide, respectively. Moreover, after incubation with β -glucuronidase, the peaks that were considered to be the respective glucuronide conjugates disappeared from the TIC (data not shown). However, although the t_R values of some peaks in the TIC were similar, such as Ber (m/z 336) and M1 (m/z 322) at 7.0 and 6.9 min, respectively, and M4-glucuronide (m/z 514) and M3-glucuronide (m/z 500) at 5.0 and 5.1 min, respectively, the peaks in SIM chromatograms were adequately separated and easily identifiable. Table 1 shows the retention times and significant molecular ions of Ber and its metabolites.

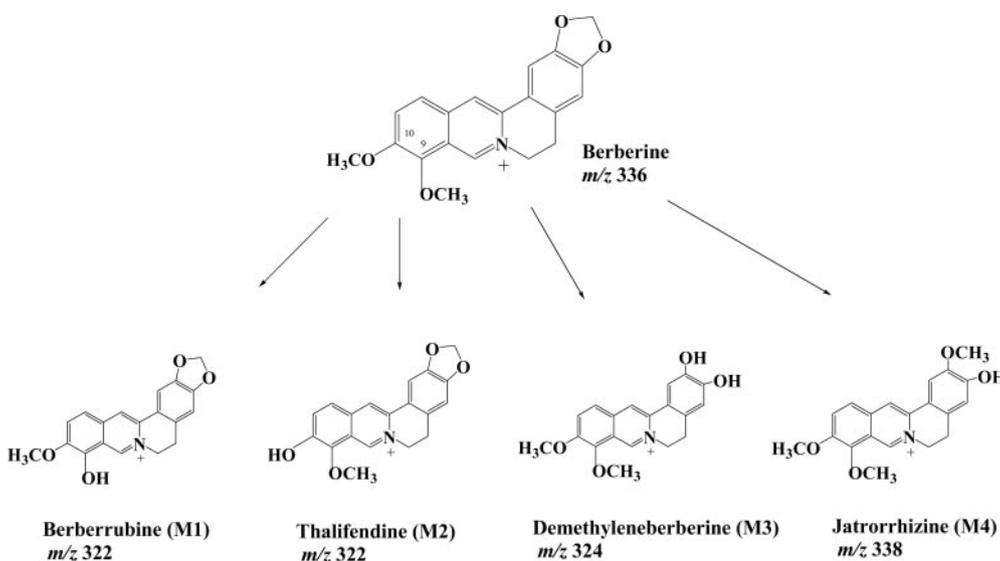


Fig. 4. Structure of Ber and its main metabolites in rat plasma.

Metabolism of Ber by Liver Tissues and Intestinal Bacteria. Neither Ber nor its metabolites M1, M3, and M4 (the amount of M2 was insufficient for analysis) were metabolized by human or rat intestinal bacteria flora even after incubation for 7 days. However, the original drug, the four main metabolites, and their glucuronide conjugates were detected in liver tissues 0.5 h after p.o. administration. In contrast, only the metabolites formed by the phase I metabolic enzymes (M1, M2, M3, and M4) were detected after incubating Ber with a rat liver homogenate, but the respective conjugates formed by phase II enzymes were undetectable.

Validation of LC/MS/MS Quantitation. Table 2 shows the regression equations, correlation coefficients (γ), linearity ranges, and limits of detection of Ber and its metabolites in rat plasma samples (Fig. 4). Because the amount of M2 (thalifendine) was insufficient to construct a calibration curve, the content is represented as a relative value to that of berberrubine. Tables 3 and 4 show the intraday and interday ($n = 5$) variations of Ber and its metabolites in rat plasma samples. The CV did not exceed 10%, and the accuracy rates were all within 90 to 110%. Table 5 shows that the CV values from the recovery tests were less than 15% at a low concentration and less than 10% at medium and high concentrations with recovery rates in the range of 85 to 110%. The stability test showed that relative standard deviation remained within 5% under all the conditions; therefore, the samples were stable during the test. Thus, the accuracy, recovery, and stability tests met the criteria for quantitative determinations in biological samples.

Pharmacokinetics of Ber and Its Metabolites in Conventional and Pseudo Germ-Free Rats. The concentrations of Ber and its main metabolites including free alkaloids and the total contents in the rat plasma samples were calculated after the p.o. administration of 40 mg/kg of Ber. Figures 5 and 6 show the concentration-time curves of Ber and its main metabolites in conventional and pseudo germ-free rats, and Table 6 shows the pharmacokinetic parameters. The amount of metabolites significantly differed between conventional and pseudo germ-free rats, and the AUC_{0-limt} values of M1 through M4 were obviously reduced in the latter compared with the former. Metabolites of Ber eliminated quickly in pseudo germ-free rats, and the MTT values of the metabolites were noticeably prolonged in conventional rats, but the Ber parameters did not differ between the two groups.

Absorption of Ber in the Small Intestine. Intestinal absorption of Ber in conventional rats was evaluated in situ using an intestinal loop.

TABLE 3

Intraday ($n = 5$) variations of Ber and its metabolites in rat plasma

	Added	Found (CV%)	Accuracy
	$ng \cdot ml^{-1}$	$ng \cdot ml^{-1}$	%
Ber	0.05	0.047 (8.5)	94.0
	0.5	0.491 (5.1)	98.2
	5	5.112 (2.6)	102.2
M1	0.2	0.184 (6.4)	92.0
	1.0	0.972 (3.9)	97.2
	10	9.861 (2.2)	98.6
M3	0.25	0.246 (6.2)	98.4
	1.0	1.023 (4.5)	102.3
	10	10.512 (4.3)	105.1
M4	0.2	0.189 (6.9)	94.5
	1.0	0.946 (3.2)	94.6
	10	10.323 (5.5)	103.2

TABLE 4

Interday ($n = 5$) variations of Ber and its metabolites in rat plasma

	Added	Found (CV%)	Accuracy
	$ng \cdot ml^{-1}$	$ng \cdot ml^{-1}$	%
Ber	0.05	0.048 (10.7)	96.0
	0.5	0.511 (3.7)	102.2
	5	4.825 (3.2)	96.5
M1	0.2	0.186 (7.6)	93.0
	1.0	1.062 (3.0)	106.2
	10	9.873 (3.8)	98.7
M3	0.25	0.228 (8.2)	91.2
	1.0	0.971 (3.7)	97.1
	10	10.324 (5.2)	103.2
M4	0.2	0.211 (4.8)	105.5
	1.0	0.947 (3.1)	94.7
	10	10.214 (4.2)	102.1

Ber that had been injected into the loop quickly disappeared in a time-dependent manner. The proportion of Ber absorbed by the jejunum was 19.1%, 26.5%, 26.8%, and 33.6% at 10, 20, 40, and 60 min, respectively (Fig. 7).

Discussion

During our studies on the metabolism of various natural products by human intestinal bacteria, we found that Ber and its reduction products tetrahydroberberine and dihydroberberine were not further metabolized. Yu et al. (2000) originally found four unknown metabolites in human urine samples using HPLC after p.o. Ber administration, and these were later identified as jatrorrhizine 3-sulfate, demethyleneberberine 2-sulfate, and thalifendine 10-sulfate; and the major metabolite was demethyleneberberine 2-sulfate (Pan et al., 2002). Tsai et al. (2004) subsequently reported that Ber was metabolized in the rat liver via phase I demethylation and phase II glucuronidation based on the ion peaks at m/z 322 and 498 in bile samples determined by LC/MS. The fragment ion at m/z 322 [$336 - CH_3 + H$]⁺ may be derived from demethylation of the methoxyl group at C-9 or C-10 of Ber. The fragment ion at m/z 498 [$322 + C_6H_8O_6$]⁺, which may be derived from a glucuronide, attached at C-9 or C-10. The present study identified molecular ion peaks at m/z 322, 324, 338, 498, 500, and 514 in the rat plasma, and the latter three were assigned to glucuronides because they disappeared after incubation with β -glucuronidase for 16 h. The molecular ions at m/z 322 were assigned to berberrubine ($t_R = 6.9$ min, M1) and thalifendine ($t_R = 5.8$ min, M2), and those at m/z 324 and 338 to demethyleneberberine (M3) and jatrorrhizine (M4), respectively, based on comparisons of their retention times, as well as the first and the second mass spectra with those of authentic samples and reported values. We discovered that M1

TABLE 5

Recovery of Ber and its metabolites from rat plasma

	Added	Found	Recovery	CV
	$ng \cdot ml^{-1}$	$ng \cdot ml^{-1}$	%	%
Ber	0.05	0.043 ± 0.006	86.0	13.9
	0.5	0.471 ± 0.025	94.2	5.3
M1	0.2	0.180 ± 0.016	90.0	8.9
	1.0	0.983 ± 0.038	98.3	3.9
M3	0.25	0.228 ± 0.018	91.2	7.9
	1.0	1.072 ± 0.053	107.2	4.9
M4	0.2	0.189 ± 0.010	94.5	5.3
	1.0	1.036 ± 0.045	103.6	4.3

Data are expressed as mean \pm S.D. ($n = 3$).

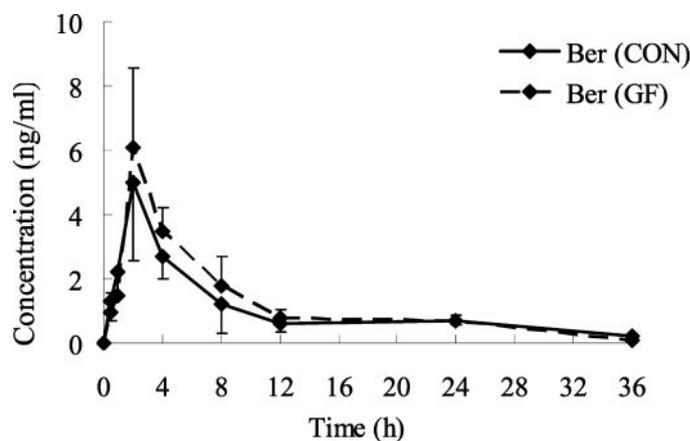


Fig. 5. Plasma concentration-time courses after p.o. administration of Ber (40 mg/kg) in conventional (CON) and pseudo germ-free (GF) rats.

(berberrubine), which is formed by demethylation of the C-9 of Ber, is a metabolite of Ber. Berberrubine reportedly has antimicrobial and antitumor activities. The antitumor activity of berberrubine is the most powerful of all the protoberberine alkaloids, whereas Ber and tetrahydroberberine have no such effects in vitro (Hoshi et al., 1976). Berberrubine is also the most potent inhibitor of topoisomerase II among several structurally related compounds (Kobayashi et al., 1995; Makhey et al., 1995). M2 (thalifendine) and M4 (jatrorrhizine) from the *Coptis* plant have antimicrobial, antioxidant, and cytotoxic activities (Wu et al., 1976; Wright et al., 2003; Rockova et al., 2004). Although its pharmacological activity has not been examined in detail, M3 (demethyleneberberine) is considered the most potent inhibitor of topoisomerase I in vitro, among several Ber analogs (Makhey et al., 1995). We simultaneously detected Ber, four metabolites, and their glucuronide conjugates in rat plasma, urine, bile, and liver tissue samples. The presence of free and conjugated metabolites in the rat after p.o. administration indicates phase I and II reactions against Ber in the liver. We found only phase I metabolites (M1, M2, M3, and M4) in liver homogenates, perhaps because the incubation conditions were not appropriate for glucuronidation (Teyssier and Siess, 2000).

Ber is not metabolized by human fecal bacterial mixtures in vitro, and the fate of the four metabolites and glucuronide conjugates excreted to the duodenum with bile and their enterohepatic circulation remains obscure. Intestinal flora are undoubtedly responsible for efficient deglucuronidation (Kobashi et al., 1992). We examined the disposition of Ber in vivo by performing a pharmacokinetic study of Ber and its main metabolites in conventional rats and in pseudo germ-free rats administered with antibiotics.

The pharmacokinetics of Ber are relatively obscure because plasma concentrations after p.o. administration are too low to detect using

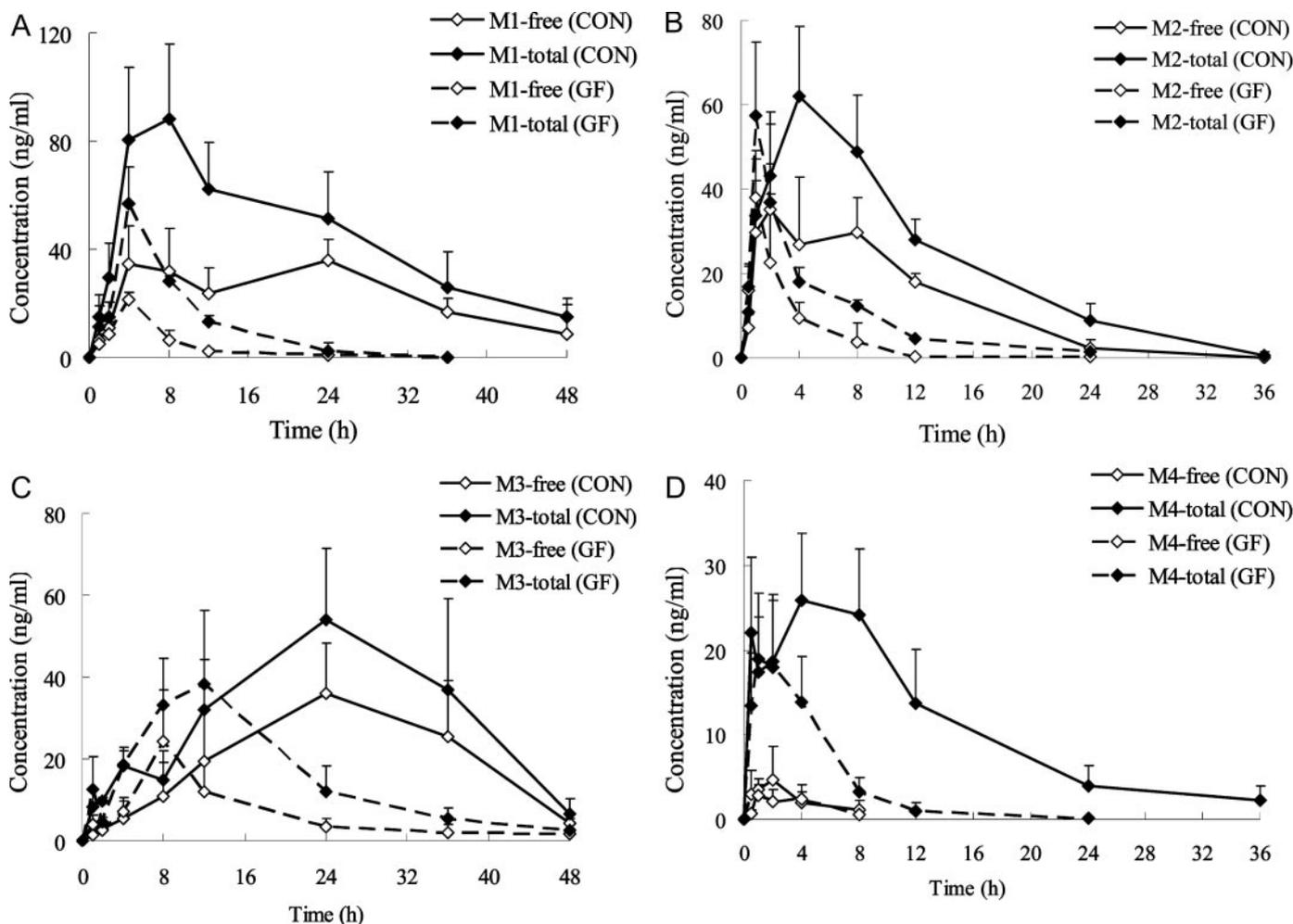


FIG. 6. Plasma concentration-time courses of free and total metabolites after p.o. administration of Ber (40 mg/kg) in conventional (CON) and pseudo germ-free (GF) rats.

TABLE 6

Pharmacokinetic parameters of Ber and its metabolites (total contents) in conventional (CON) and pseudo germ-free (GF) rats after administration of 40 mg/kg p.o. Ber

	Pharmacokinetic Parameters	
	AUC _{0-lim} ng · h · ml ⁻¹	MTT h
Ber (CON)	37.42	10.53
Ber (GF)	40.89	10.25
M1 (CON)	1879.64	18.32
M1 (GF)	437.29	7.45
M2 (CON)	811.05	7.62
M2 (GF)	287.85	4.84
M3 (CON)	1763.62	24.68
M3 (GF)	735.22	15.08
M4 (CON)	356.05	10.32
M4 (GF)	101.98	4.05

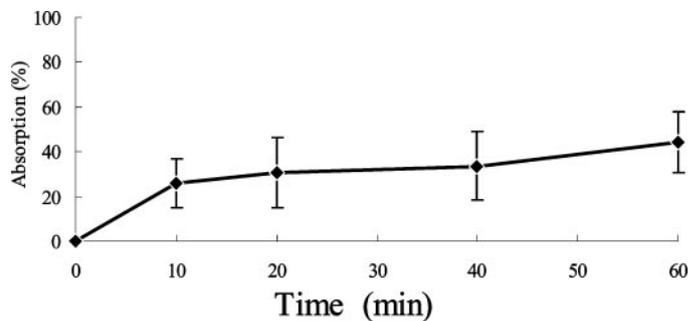


FIG. 7. Absorption of 0.02 mM Ber in rat jejunum determined in situ.

general analytical approaches such as HPLC. Hence, most researchers considered that the gastrointestinal tract absorbed very little Ber. Generally, the detection limits of HPLC are at nanogram levels; therefore, trace concentrations in biological samples are difficult to identify. Here, we validated the quantitation of Ber and its four main metabolites in plasma by LC/MS/MS and detected levels as low as 20 pg of Ber in rat plasma. Thus, the detection sensitivity was increased almost 100-fold compared with HPLC with a photodiode array detector. Compared with enzyme-label immunoassay, which is highly

sensitive and specific, LC/MS/MS had not only better sensitivity and specificity but also good accuracy and reproducibility. The primary application of LC/MS was to identify original compounds and their metabolites with ion peaks and fragments. However, this method is now being applied by many investigators to identify trace levels in biological fluids (Choo et al., 2005).

The basis of pharmacokinetic calculation consists of compartment and noncompartment models, of which the former is considered limited because the influence of drug and metabolite distribution in the body on concentration-time curves is often neglected (Weiss, 1988). Therefore, the noncompartment model is increasingly being applied to the pharmacokinetic evaluations of metabolites. Some parameters such as mean absorption time, formation clearance, and

total formation clearance of metabolites can clarify the metabolic process in vivo through concentration-time curves (Weiss, 1996; Bouwmeester et al., 2004). Here, we calculated the pharmacokinetic parameters of four metabolites against the original compound using a noncompartmental approach. The concentration-time course experiment (Fig. 5) showed that the concentration peak value of Ber reached $10 \text{ ng} \cdot \text{ml}^{-1}$ at 2 h after p.o. administration, was eliminated within 12 h, and then maintained a very low plasma concentration for 48 h. However, the $\text{AUC}_{0\text{-}12\text{h}}$ and concentration peak values of the metabolites were much higher than those of Ber. The $\text{AUC}_{0\text{-}12\text{h}}$ values of M1, M2, M3, and M4 were almost 50.2-, 21.7-, 47.1- and 9.5-fold greater than that of Ber in conventional rats. The time courses of Ber in conventional and pseudo germ-free rats were quite similar, whereas those of the metabolites obviously differed between the two groups. The amounts of metabolites were significantly reduced in pseudo germ-free rats. Free M1 comprised at least two concentration peaks in conventional rats but only one in pseudo germ-free rats, from which it was immediately eliminated. The two significant parameters of pharmacokinetics, namely $\text{AUC}_{0\text{-}12\text{h}}$ and MTT, were greater in the conventional than in the germ-free rats. These findings suggested that the metabolites, including their glucuronide conjugates, were excreted into the intestine by bile, where the conjugates were hydrolyzed to free metabolites for absorption via the enterohepatic circulation. Conjugate conversion to the free form was restricted in the pseudo germ-free rats treated with antibiotics; therefore, few free metabolites were reabsorbed from the gastrointestinal tract, which resulted in the rapid decrease in the metabolite concentration after the first peak. The intestinal flora had no significant metabolic activity against Ber and its metabolites but might play a significant role in the enterohepatic circulation of the metabolites. In this sense, the liver and intestinal bacteria all participate in the metabolism and disposition of Ber in vivo. In addition, the four main metabolites were all partly conjugated with glucuronide in the liver, but the bound/unbound ratios of the four metabolites differed. Most M4 assumed the conjugate form, whereas 50% of the M1 through M3 existed as bound glucuronides in rat plasma.

A common assumption has been that Ber, like quaternary alkaloids, is very difficult to absorb because of its relatively low plasma concentrations. However, we clarified that although the Ber concentration was quite low in plasma, its metabolites remained at much higher concentrations for a long time in the rat. Tsai and Tsai (2004) have indicated that Ber is rapidly transferred from the blood to the liver and bile through active transportation in rats. Chen and Chang (1995) found that only 4.93 and 0.5% of a dose of 2 mg/kg Ber were eliminated from the urine and bile after i.v. bolus administration; therefore, Ber might be extensively metabolized in the body. Pan et al. (2001) also proposed that the amounts of metabolites of Ber in the body should not be ignored. Finally, we carried out an absorption test of Ber using an intestinal in situ loop. The results indicated that the absorption ratio of Ber reached about 33.6% within 1 h. Hence, we deemed that the blood clearance of Ber was very fast and that its biotransformation in the liver was rapid and substantial, allowing for immediate circulation of the metabolites in the body, which would account for the trace plasma concentrations. These metabolites must be active components of Ber, as indicated by the range of pharmacological effects in vivo.

Acknowledgments. We thank Dr. Hong-Bo Qin, Faculty of Pharmaceutical Sciences of Tokyo University, for assistance with the synthesis for M1 (berberrubine).

References

- Akao T, Kawabata K, Yanagisawa E, Ishihara K, Mizuhara Y, Wakui Y, Sakashita Y, and Kobashi K (2000) Baicalin, the predominant flavone glucuronide of *Scutellariae Radix*, is absorbed from the rat gastrointestinal tract as the aglycone and restored to its original form. *J Pharm Pharmacol* **52**:1563–1568.
- Bouwmeester NJ, Anderson BJ, Tibboel D, and Holford NHG (2004) Developmental pharmacokinetics of morphine and its metabolites in neonates, infants and young children. *Br J Anaesth* **92**:208–217.
- Cernakova M, Kost D, Kettmann V, Plodova M, Toth J, and Drimal J (2002) Potential antimutagenic activity of berberine, a constituent of *Mahonia aquifolium*. *BMC Complement Altern Med* **2**:2.
- Chen CM and Chang HC (1995) Determination of berberine in plasma, urine and bile by high-performance liquid chromatography. *J Chromatogr B* **665**:117–123.
- Choo RE, Murphy CM, Jones HE, and Huestis MA (2005) Determination of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, 2-ethyl-5-methyl-3,3-diphenylpyrrolidine and methadol in meconium by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry. *J Chromatogr B* **814**:369–373.
- Das B and Srinivas K (2002) Conversion of berberine into berberrubine by selective demethylation under microwave irradiation. *Synthetic Communications* **32**:3027–3029.
- Elmer GW and Remmel RP (1984) Role of the intestinal microflora in clonazepam metabolism in the rat. *Xenobiotica* **14**:829–840.
- Gudima SO, Memelova LV, Borodulin VB, Pokholok DK, Mednikov BM, Tolkachev ON, and Kochetkov SN (1994) Kinetic analysis of interaction of human immunodeficiency virus reverse transcriptase with alkaloids *Mol Biol (Moscow)* **28**:1308–1314.
- Hoshi A, Ikekawa T, Ikeda Y, Shirakawa S, and Iigo M (1976) Antitumor activity of berberrubine derivatives. *Gann* **67**:321–325.
- Kettmann V, Kostalova D, Jantova S, Cernakova M, and Drimal J (2004) In vitro cytotoxicity of berberine against HeLa and L1210 cancer cell lines. *Pharmazie* **59**:548–551.
- Kim TS, Kang BY, Cho D, and Kim SH (2003) Induction of interleukin-12 production in mouse macrophages by berberine, a benzodioxolquinoline alkaloid, deviates CD4⁺ T cells from a Th2 to a Th1 response. *Immunology* **109**:407–414.
- Ko WH, Yao XQ, Lau CW, Law WI, Chen ZY, Kwok W, Ho K, and Huang Y (2000) Vasorelaxant and antiproliferative effects of berberine. *Eur J Pharmacol* **399**:187–196.
- Kobashi K, Akao T, Hattori M, and Namba T (1992) Metabolism of drugs by intestinal bacteria. *Bifidobacteria Microflora* **11**:9–23.
- Kobayashi Y, Tamashita Y, Fujii N, Takaboshi K, Kawakami T, Kawamura M, Mizukami T, and Nakano H (1995) Inhibitors of DNA topoisomerase I and II isolated from the *Coptis* rhizomes. *Planta Med* **61**:414–418.
- Kupeli E, Kosar M, Yesilada E, Husnu K, and Baser C (2002) A comparative study on the anti-inflammatory, antinociceptive and antipyretic effects of isoquinoline alkaloids from the roots of Turkish *Berberis* species. *Life Sci* **72**:645–657.
- Li BX, Zhang MS, and Bao LH (1995) Study on the pharmacokinetics of berberine after oral administration in human being. *J Haerbin Med Univ* **29**:382–385.
- Ma L, Xiao P, Guo B, Wu J, Liang F, and Dong S (1999) Cerebral protective effects of some compounds isolated from traditional Chinese herbs. *Zhongguo Zhong Yao Za Zhi* **24**:238–239.
- Makhey D, Gatto B, Yu C, Liu A, Liu LF, LaVoie E, and Edmond J (1995) Protoberberine alkaloids and related compounds as dual inhibitors of mammalian topoisomerase I and II. *Med Chem Res* **5**:1–12.
- Miyazaki H, Shirai E, Ishibashi M, and Niizima K (1978) Quantitative analysis of berberine in urine samples by chemical ionization mass fragmentography. *J Chromatogr* **152**:79–86.
- Otsuka H, Fujimura H, Sawada T, and Goto M (1981) Studies on anti-inflammatory agents. II. Anti-inflammatory constituents from Rhizome of *Coptis japonica* Makino. *Yakugaku Zasshi* **101**:883–890.
- Pan JF, Yu C, Zhu DY, Zhang H, and Ren JY (2001) Preparation of six isoquinoline alkaloid salt. *Faming Zhuanli Shenqing Gongkai Shoumingshu* CN 1314347, Sep 26.
- Pan JF, Yu C, Zhu DY, Zheng H, Zeng JF, Jiang SH, and Ren JY (2002) Identification of three sulfate-conjugated metabolites of berberine chloride in healthy volunteers' urine after oral administration. *Acta Pharmacol Sin* **23**:77–82.
- Peng WH, Wu CR, Chen CS, Chen CF, Leu ZC, and Hsieh MT (2004) Anxiolytic effect of berberine on exploratory activity of the mouse in two experimental anxiety models: interaction with drugs acting at 5-HT receptors. *Life Sci* **75**:2451–2462.
- Rockova L, Majekova M, Kost D, and Stefek M (2004) Antiradical and antioxidant activities of alkaloids isolated from *Mahonia aquifolium*. Structural aspects. *Bioorg Med Chem* **12**:4709–4715.
- Shamma M, Greenberg MA, and Dudock Bernard S (1965) Thalictrom alkaloids I. Thalifendlerine and thalifendine. *Tetrahedron Lett* **40**:3595–3598.
- Shen MP, Sun Q, and Wang H (1993) Studies on the intravenous pharmacokinetics and oral absorption of berberine HCL in beagle dogs. *Chin Pharmacol Bull* **9**:64–67.
- Teyssier C and Siess MH (2000) Metabolism of dipropyl disulfide by rat liver phase I and phase II enzymes and by isolated perfused rat liver. *Drug Metab Dispos* **28**:648–654.
- Tran QL, Tezuka Y, Ueda JY, Nguyen NT, Maruyama Y, Begum K, Kim HS, Wataya Y, Tran QK, and Kadota S (2003) In vitro antiparasitoid activity of antimalarial medicinal plants used in Vietnamese traditional medicine. *J Ethnopharmacol* **86**:249–252.
- Tsai PL, and Tsai TH (2004) Hepatobiliary excretion of berberine. *Drug Metab Dispos* **32**:405–412.
- Vollekova A, Kost D, Kettmann V, and Toth J (2003) Antifungal activity of *Mahonia aquifolium* extract and its major protoberberine alkaloids. *Phytother Res* **17**:834–837.
- Wang Z, Kurosaki Y, Nakayama T, and Kimura T (1994) Mechanism of gastrointestinal absorption of glycyrrhizin in rats. *Biol Pharm Bull* **17**:1399–1403.
- Weiss M (1988) A general model of metabolite kinetics following intravenous and oral administration of the parent drug. *Biopharm Drug Dispos* **9**:159–176.
- Weiss M (1996) A novel extravascular input function for the assessment of drug absorption in bioavailability studies. *Pharm Res (NY)* **13**:1547–1553.
- Wright CW, Marshall SJ, Russell PF, Anderson MM, Phillipson JD, Kirby GC, Warhurst DC, and Schiff PL (2003) In vitro antiplasmodial, antiamebic, and cytotoxic activities of some monomeric isoquinoline alkaloids. *J Nat Prod* **63**:1638–1640.
- Wu WN, Beal J, Clark GW, and Mitscher LA (1976) Antimicrobial agents from higher plants. Additional alkaloids and antimicrobial agents from *Thalictrum rugosum*. *Lloydia* **39**:65–75.
- Xie LH, Ahn EM, Akao T, Abdel-Hafez AAM, Nakamura N, and Hattori M (2003) Transfor-

- mation of arctiin to estrogenic and antiestrogenic substances by human intestinal bacteria. *Biol Pharm Bull* **51**:378–384.
- Xiong CY, Shi XB, Dai ZS, and Fang DC (1989) Pharmacokinetic study of ³H-berberine in rabbits and mouse. *Chin Pharmacol Bull* **5**:293–296.
- Yesilada E and Kupeli E (2002) Berberis crataegina DC. root exhibits potent anti-inflammatory, analgesic and febrifuge effects in mice and rats. *J Ethnopharmacol* **79**:237–248.
- Yu CH, Zhang H, Pan JF, Hong YC, Ren JY, Zhu DY, and Xu XR (2000) Determination and preliminary studies of metabolism of berberine in human urine after oral administration. *Chin J Clin Pharmacol* **16**:36–39.
- Zheng L, Zhou Z, Tao D, and Lan T (2003) Protective effect of berberine on cardiac myocyte injured by ischemia reperfusion. *Sichuan Da Xue Xue Bao Yi Xue Ban* **34**:452–454.

Address correspondence to: Masao Hattori, Department of Metabolic Engineering, Institute of Natural Medicines, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. E-mail: saibo421@ms.toyama-mpu.ac.jp
