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

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Abbreviations: Ber, berberine; M1, berberrubine; M2, thalifendine; M3, demethyleneberberine; M4, jatrorrhizine; AUC, area under the plasma concentration-time curve; AUMC, area under the first moment curve; MTT, mean transit time; TIC, total ion chromatogram; SIM, selecting ion monitor; LOD, limit of detection; RIB, rat intestinal bacteria; HIB, human intestinal bacteria.

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ABSTRACT

Berberine (Ber) and its main metabolites were identified and quantified using liquid chromatography/electrospray ionization (ESI)-ion trap mass spectrometry. Rat plasma contained the main metabolites, berberrubine (M1), thalifendine (M2), demethyleneberberine (M3) and jatrorrhizine (M4) as free and glucuronide conjugates after oral 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 oral 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 determined in conventional and pseudo germ-free rats (treated with antibiotics) after oral administration with 40 $mg \cdot kg^{-1}$of Ber. The $AUC_{0\text{-limt}}$ and mean transit time (*MTT*) 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 Ber. In this sense, the liver and intestinal bacteria participate in the metabolism and disposition of Ber *in vivo*.

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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.. Berberine extracts and decoctions have significant antimicrobial activities. Recent pharmacological studies have demonstrated that Ber also possesses antitumor (Kettmann et al., 2004), anti-HIV (Gudima *et al*., 1994), antifungal (Vollekova *et al*., 2003), cardio-protective (Zheng *et al*., 2003), immunoregulative (Kim *et al*., 2003), antimalarial (Tran *et al*., 2003), anti-inflammatory (Kupeli *et al*., 2002), anti-oxidative (Rockova *et al*., 2004), cerebro-protective (Ma *et al*., 1999), antimutagenic (Cernakova *et al*., 2002), vasorelaxing (Ko *et al*., 2000), anxiolytic (Peng *et al*., 2004) and analgesic (Yesilada *et al*., 2002) effects. Berberine is generally administered as a chloride or sulfate for clinical applications.

The pharmacokinetics of Ber have been examined by several investigators using a tritium-labeled derivatives or 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 *et al*., 1995). Therefore, the remarkable variety of pharmacological effects exerted by Ber at blood concentrations below the effective dose required for activity *in vitro* have been regarded with considerable skepticism. Nevertheless, investigators have investigated the disposition process of Ber *in vivo*. Berberine metabolites have recently been studied in the urine of healthy volunteers using LC/MS after oral Ber administration

(Pan *et al*., 2002) and in rat bile after intravenous injection (Tsai and Tsai, 2004). However, the disposition and metabolites of Ber in the plasma of humans and other animals remains obscure.

The present study identifies and quantifies Ber and its main metabolites using LC/MS-MS with electrospray ionization (ESI) in rat plasma after oral 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.

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Materials and Methods

Chemicals and Reagents. Berberine chloride, oxytetracycline hydrochloride (terramycin), cefadroxil, erythromycin, β-glucuronidase (EC 3.2.2.31, type 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, U.S.A.). 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 (GAM) broth was purchased from Nissui Co. (Tokyo, Japan). Berberrubine chloride and demethyleneberberine chloride were synthesized, and jatrorrhizine chloride and thalifidine 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 (Das *et al*., 2002; Pan *et al*., 2001; Wu *et al*., 1976; Shamma *et al*., 1965).

Instruments. Compounds were analyzed by ^{1}H- and ^{13}C-NMR as well as 2D 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

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source. All LC/MS-MS data were acquired using Esquire Control software and analyzed using software from Bruker Daltonik.

Synthesis of berberrubine (M1). Berberine 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) 8-10 times. The combined organic layer was evaporated *in vacuo* to give a residue (32 mg), that was eluted with $CHCl_3$:MeOH (9:1) through a silica gel column (2 × 40 cm) to separate berberrubine chloride (yield: 67%) (Das *et al*., 2002).

Synthesis of demethyleneberberine (M3). Berberine chloride (100 mg) and phloroglucin (100 mg) were mixed with 60% H_2SO_4 (100 ml) and stirred at 100□ in an oil bath for 20 min. The mixture was cooled, concentrated and resuspended in H_2O – acetone (1:1), and then M3 was purified by silica gel chromatography (column: 2 × 40 cm) with $CHCl_3$:MeOH (20:1→ 9:1) (yield 42%) (Pan *et al*., 2001).

Isolation of thalifendine (M2) and jatrorrhizine (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 (Wu *et al.*, 1976; Shamma *et al*., 1965). Briefly, Ber chloride (500 mg) was dissolved in 1% NH_4OH (10 ml) and eluted through a polyamide C-100 column (4 × 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 × 40 cm), with $CHCl_3$:MeOH (100:1→ 20:1→ 9:1) to give M2

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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 orally administered with cefadroxil (100 $mg \cdot kg^{-1}$), terramycin (300 $mg \cdot kg^{-1}$) and erythromycin (300 $mg \cdot kg^{-1}$) for 3 d, 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 (Elmer *et al*., 1984).

Collection of urine, bile and plasma samples. Rats were fed with standard laboratory chow for one 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 light 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 oral 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 8000×g for 15 min to separate the plasma, and then all samples were stored at –20ºC 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 37ºC, dissolved in 3 volumes of MeOH, and then centrifuged at 8000×g 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

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half volume of thawed plasma and incubated for 16 h at 37ºC. Enzyme-digested and undigested plasma samples were passed through Solid Phase Extraction cartridges (Waters Co., Milford, U.S.A.) that had been washed with 3 ml of MeOH and equilibrated with 6 ml of water. The constituents were eluted with 2-3 ml of MeOH from the cartridge, then the eluate was evaporated under a stream of nitrogen at 35ºC 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 orally administered with 40 mg·kg^{-1} of 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 8000×g 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 DMSO (0.5 ml) and then incubated at 37ºC for 4 h. The mixture was extracted with 3 volumes of MeOH, concentrated and filtered.

Metabolism by intestinal bacteria. Mixtures of rat (RIB) or human (HIB) intestinal bacteria (5 g each) prepared as described (Xie *et al*., 2003), together with Ber or metabolites (5 mg) dissolved in DMSO (0.5 ml) were added to GAM broth (50 ml), and anaerobically incubated at 37ºC for 7 d. The incubation mixture was extracted with 3 volumes of MeOH, and then passed through a 0.45 μm 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

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major blood vessels were excluded from the ligatures. Berberine (0.02 mM) dissolved in 2.5 ml of saline containing 1 $mg \cdot ml^{-1}$ 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 μm; 4.6×150 mm i.d., Tosoh Co., Tokyo, Japan). Samples were eluted 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 $ml \cdot min^{-1}$ at 30ºC. The standard positive ion mode was selected under the following conditions: full scan range, 50-600 *m/z*; scan resolution, 13000 *m/z* /sec; nebulizer, 50.0 psi; dry gas, 10.0 l/min; dry temperature, 360ºC.

Linearity: Reference compounds were dissolved in MeOH to a final concentration of 1 $mg \cdot ml^{-1}$, 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 (LOD) of the method for each constituent was established when the signal to noise ratio

(S/N) was 5.

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

Recovery: Two standard concentrations were evaporated to dryness and mixed with rat plasma samples after the oral 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 refrigerator at 4ºC for 1, 3 and 5 d. The average peak areas of constituents in the samples and relative standard deviation (RSD) were calculated.

Data analysis. Pharmacokinetics were evaluated using a non-compartmental 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 to the mean transit time (*MTT*).

Results

Identification of Ber metabolites in rat plasma. Plasma collected from rats that had received oral Ber chloride was analyzed by LC/MS-MS. Full scanning in the region of *m/z* 50-600 assigned several peaks to Ber and metabolites (a-e) in the total ion chromatogram (TIC), when compared with those of a blank plasma sample (Fig. 1).

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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. 3-A), t_R and the second MS spectrum showed that the highest ion peak at retention time (t_R) = 7.0 min was derived from berberine ($C_{20}H_{18}NO_4^+$ = 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), demethyleneberberine (*m/z* 324), and jatorrhizine (*m/z* 338). 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. Since 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), demethyleneberberine 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.

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 d. However, the original drug, the four main metabolites and their glucuronide conjugates were detected in liver tissues 0.5 h after oral 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 (LOD) of Ber and its metabolites in rat plasma samples. Since 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 intra- and inter-day (n=5) variations of Ber and its metabolites in rat plasma samples. The coefficient of variation (CV) did not exceed 10%, and the accuracy rates were all within 90-110 %. Table 5 shows that the CV values from the recovery tests were below 15% at a low concentration, and below 10% at medium and high concentrations with recovery rates in the range of 85-110%. The stability test showed that relative standard deviation (RSD) remained within 5% under all conditions, so the samples were stable during the test. Thus the accuracy, recovery and stability tests met the criteria for quantitative determinations in biological samples.

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Pharmacokinetics of Ber and its metabolites in conventional and pesudo 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 oral administration of 40 $mg \cdot kg^{-1}$ 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 $AUC_{0\text{-limt}}$ values of M1-M4 were obviously reduced in the latter compared with the former. Metabolites of Ber eliminated very 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. Berberine 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.

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 oral Ber

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administration, and these were later identified as jatrorrhizine 3-sulfate, demethyleneberberine 2-sulfate and thalifendine 10-sulfate and the major metabolite, 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, on the basis of ion peaks at *m/z* 322 and *m/z* 498 in bile samples determined by LC/MS. The fragment ion at *m/z* 322 $[336 - CH_3 + H]^+$ might 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 might 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 an 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 (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 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 Coptisious

plant have antimicrobial, antioxidant and cytotoxic activities (Wu *et al*., 1976; Wright *et al*., 2003; and 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 the metabolites in the rat after oral administration indicates phase I and phase 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).

Berberine 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 oral administration are too low to detect using 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, so trace concentrations in biological samples are difficult to identify. Here, we validated the

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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 in comparison to 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 non-compartment models, of which the former is considered limited, since the influence of drug and metabolite distribution in the body on concentration-time curves is often neglected (Weiss, 1988). Therefore the non-compartment model is increasingly being applied to the pharmacokinetic evaluations of metabolites. Some parameters such as mean absorption time (*MAT*), formation clearance (*Clf*) and total formation clearance (*Clt*) 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 non-compartmental approach. The concentration- time course experiment (Fig. 5) showed that the concentration peak value of Ber reached 10 $ng \cdot ml^{-1}$ at 2 h after oral administration, was eliminated within 12 h, and then maintained a very low plasma concentration for 48 h. However, the $AUC_{0\text{-limt}}$ and concentration peak

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values of the metabolites were much higher than those of Ber. The $AUC_{0\text{-limt}}$ 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 $AUC_{0\text{-limt}}$ 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, so little 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 to unbound ratios of the four metabolites differed. Most M4 assumed the conjugate form whereas 50% of the M1-M3 existed as bound glucuronides in rat plasma.

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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 the 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 $mg \cdot kg^{-1}$ Ber were eliminated from the urine and bile after i.v. bolus administration, so 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*.

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452-454.

Footnote

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Legends for Figures

Fig. 1. *Total ion chromatograms (TIC) of rat plasma blank (A) and after oral administration of Ber (B).*

Fig. 2. *TIC peaks in rat plasma after oral 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).

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).*

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

Fig. 5. *Plasma concentration-time courses after oral administration of Ber (40 mg·kg^{-1}) in conventional (CON) and pseudo germ-free (GF) rats.*

Fig. 6. *Plasma concentration-time courses of free and total metabolites after oral administration of Ber (40 mg·kg^{-1}) in conventional (CON) and pseudo germ-free (GF) rats.*

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

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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 (demethyleneberberine)	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

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TABLE 2

Regression equations, correlation coefficients (γ), linearity ranges and LOD of Ber and its metabolites in rat plasma.

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

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TABLE 3

Intra-day (n=5) variations of Ber and its metabolites in rat plasma.

	Added ($ng \cdot ml^{-1}$)	Found (CV %) ($ng \cdot ml^{-1}$)	Accuracy (%)
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

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TABLE 4

Inter-day (n=5) variations of Ber and its metabolites in rat plasma.

	Added ($ng \cdot ml^{-1}$)	Found (CV %) ($ng \cdot ml^{-1}$)	Accuracy (%)
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

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TABLE 5

Recovery of Ber and its metabolites from rat plasma.

	Added ($ng \cdot ml^{-1}$)	Found ($ng \cdot ml^{-1}$)	Recovery (%)	CV (%)
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 means±S.D. (n = 3).

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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^{-1} oral Ber.

	Pharmacokinetic parameters	
	$AUC_{0\text{-limt}}$ (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

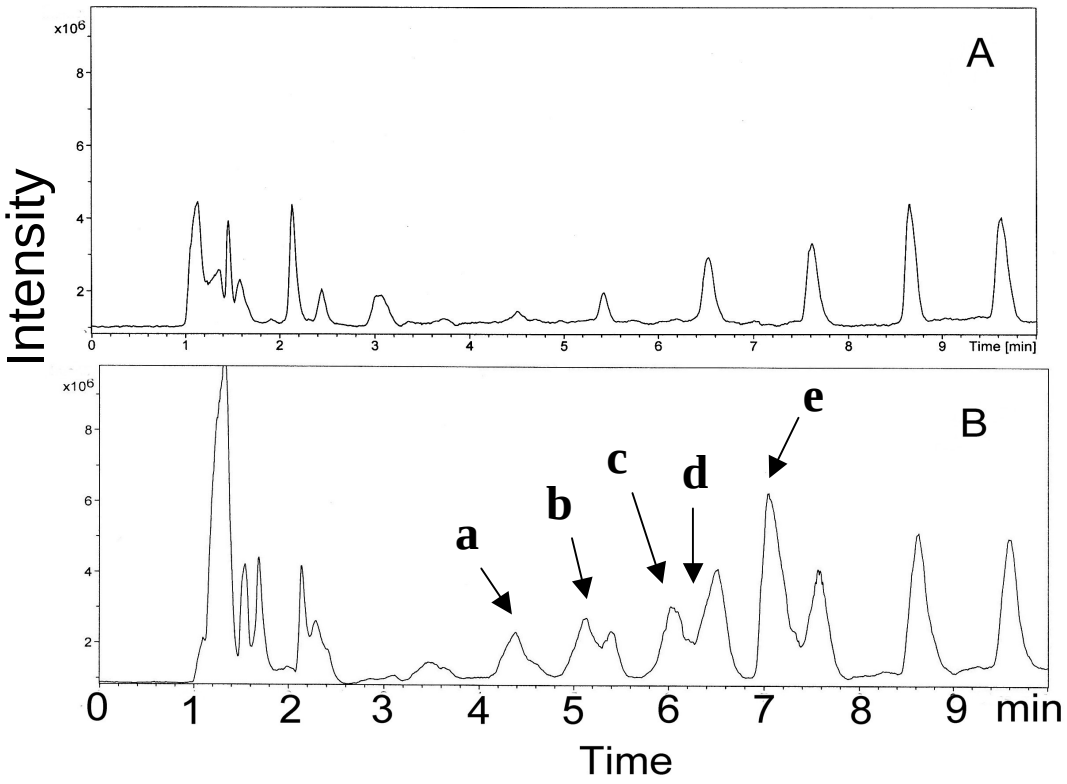

Fig. 1

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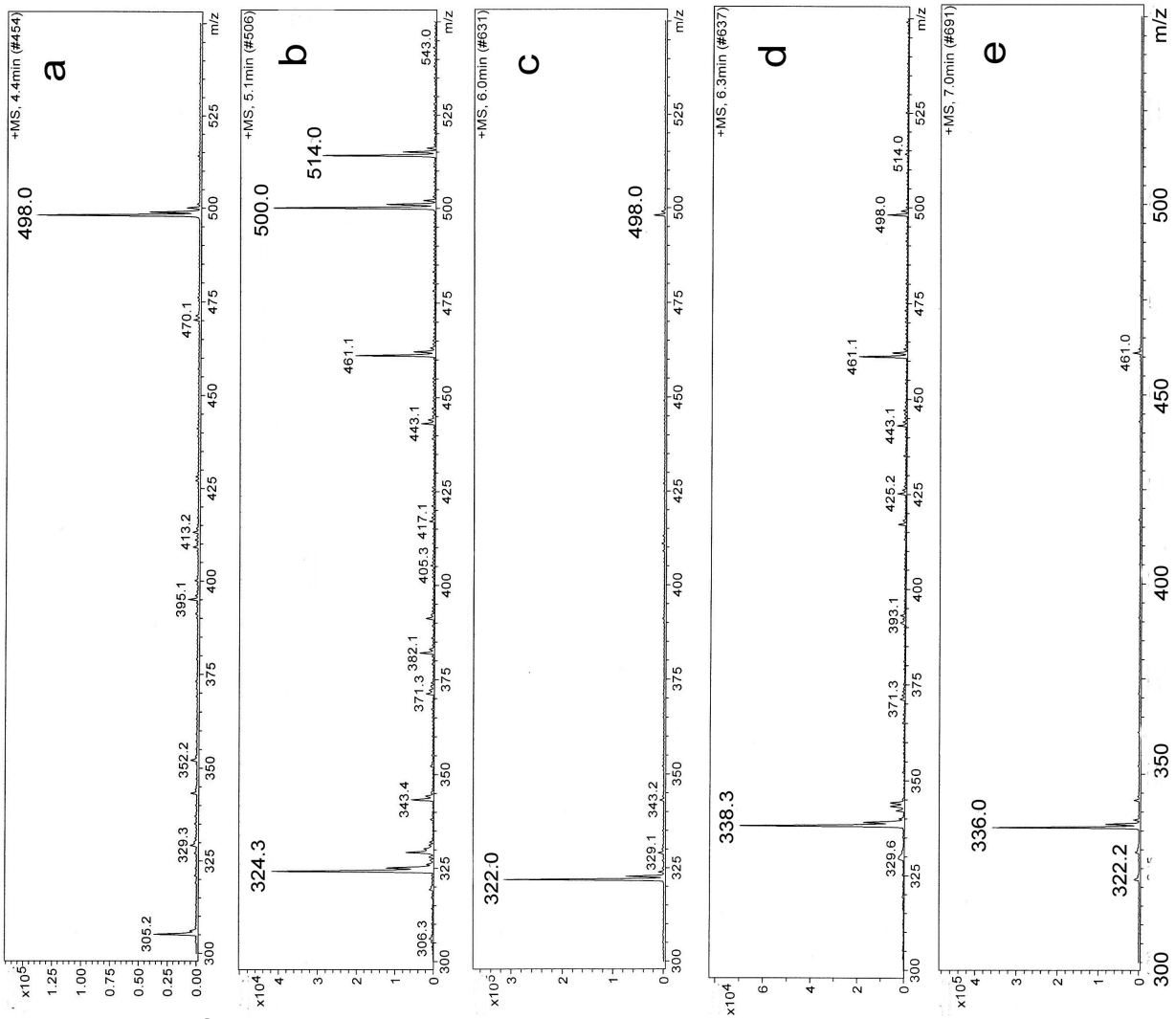

Fig. 2

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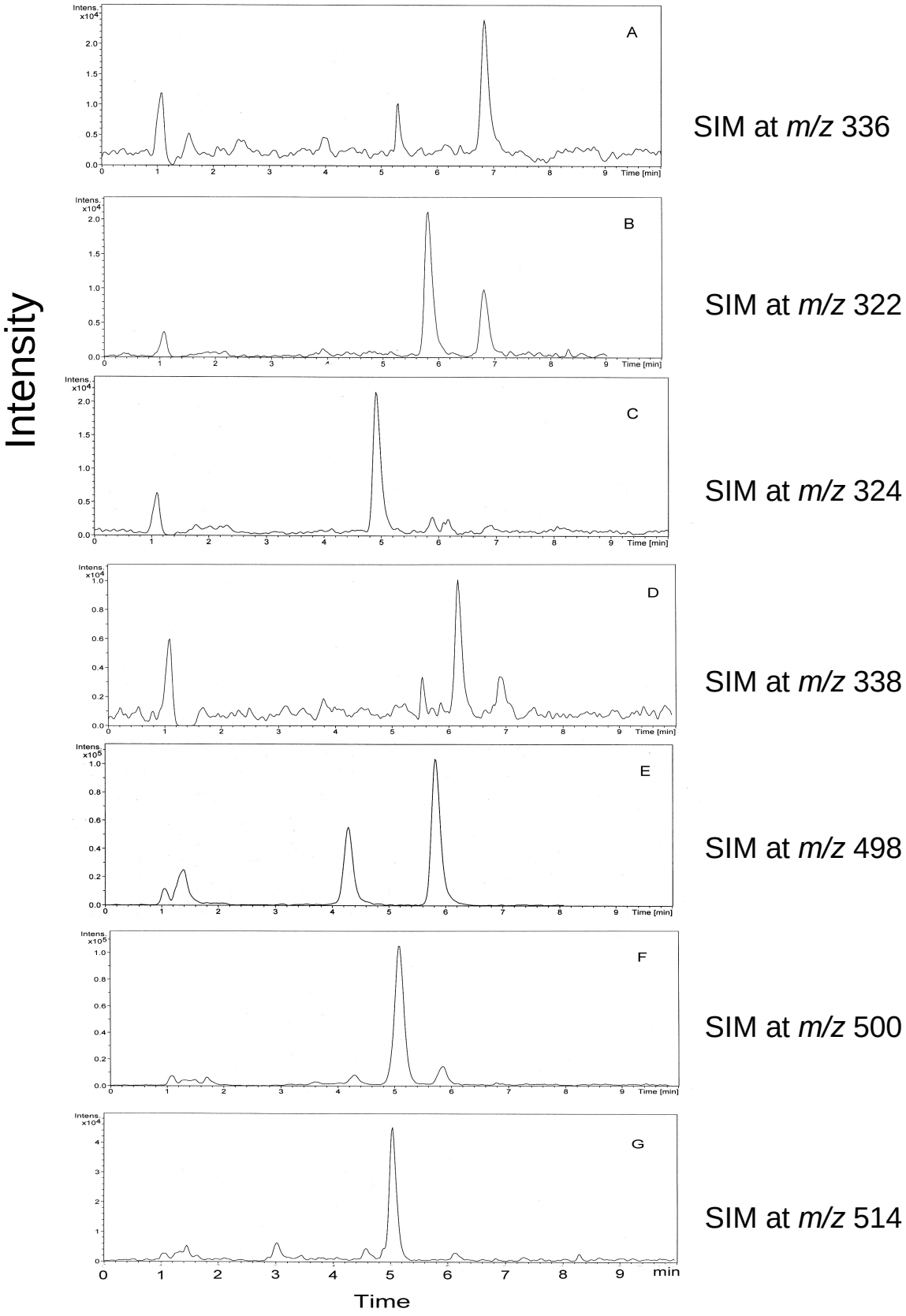

Fig. 3

Fig. 4

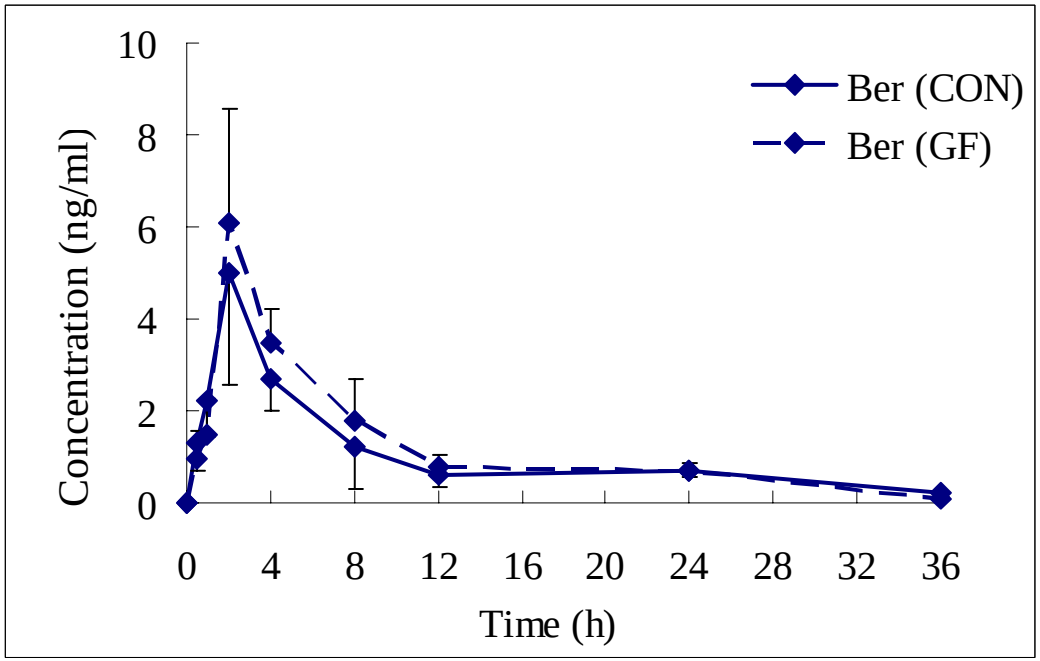

Fig. 5

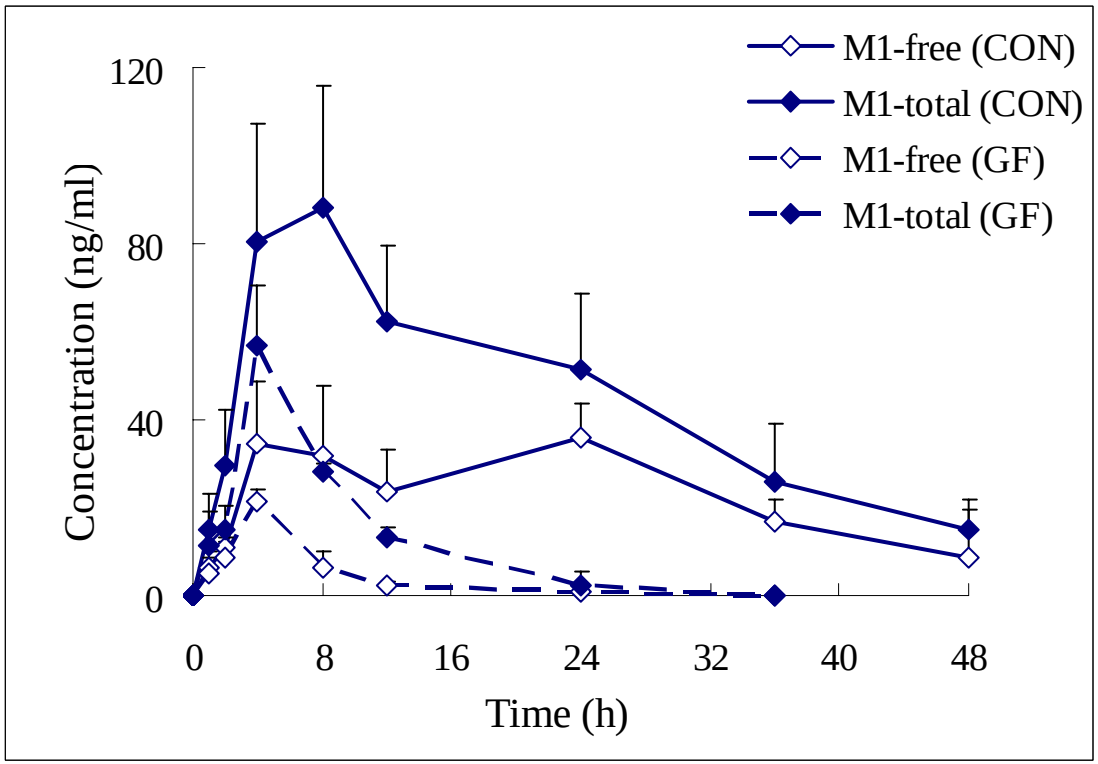

Fig. 6-A

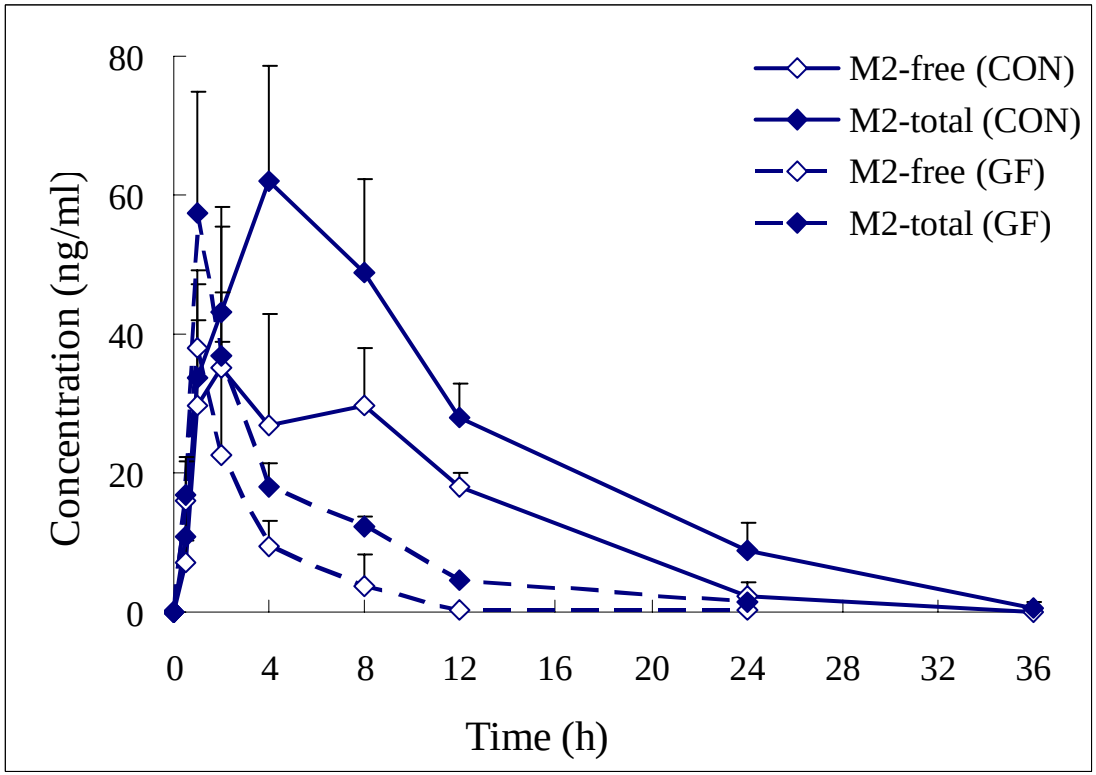

Fig. 6-B

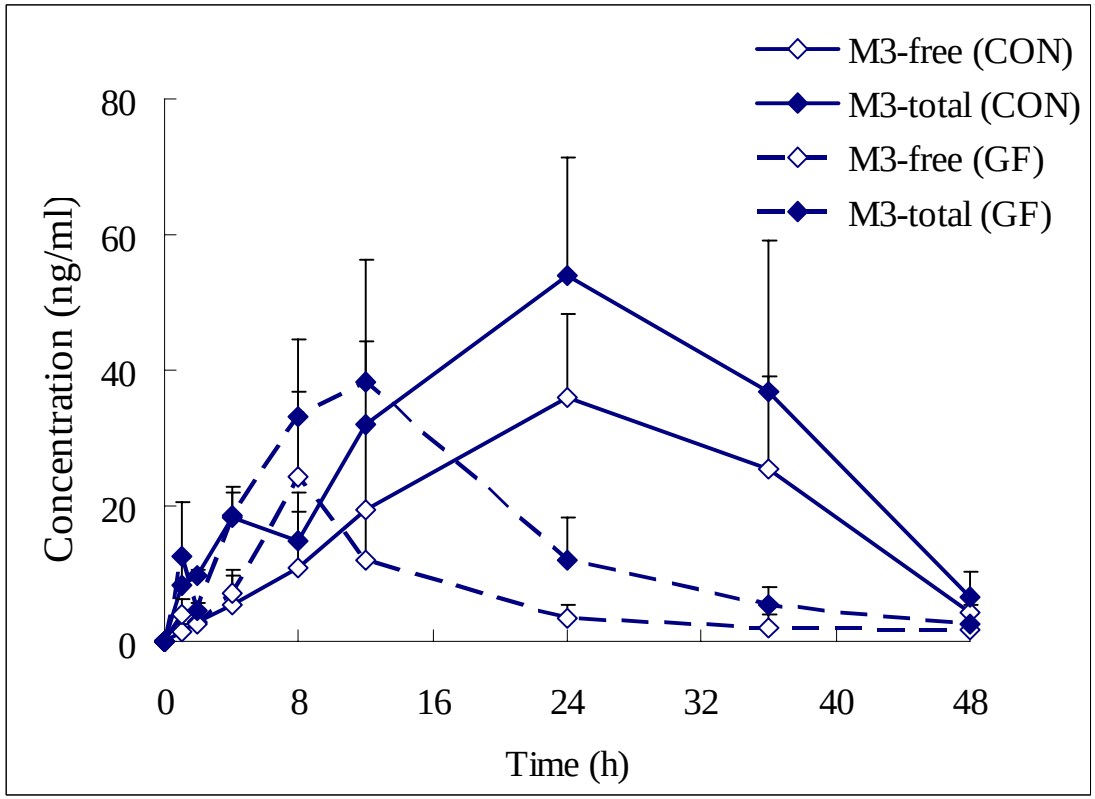

Fig. 6-C

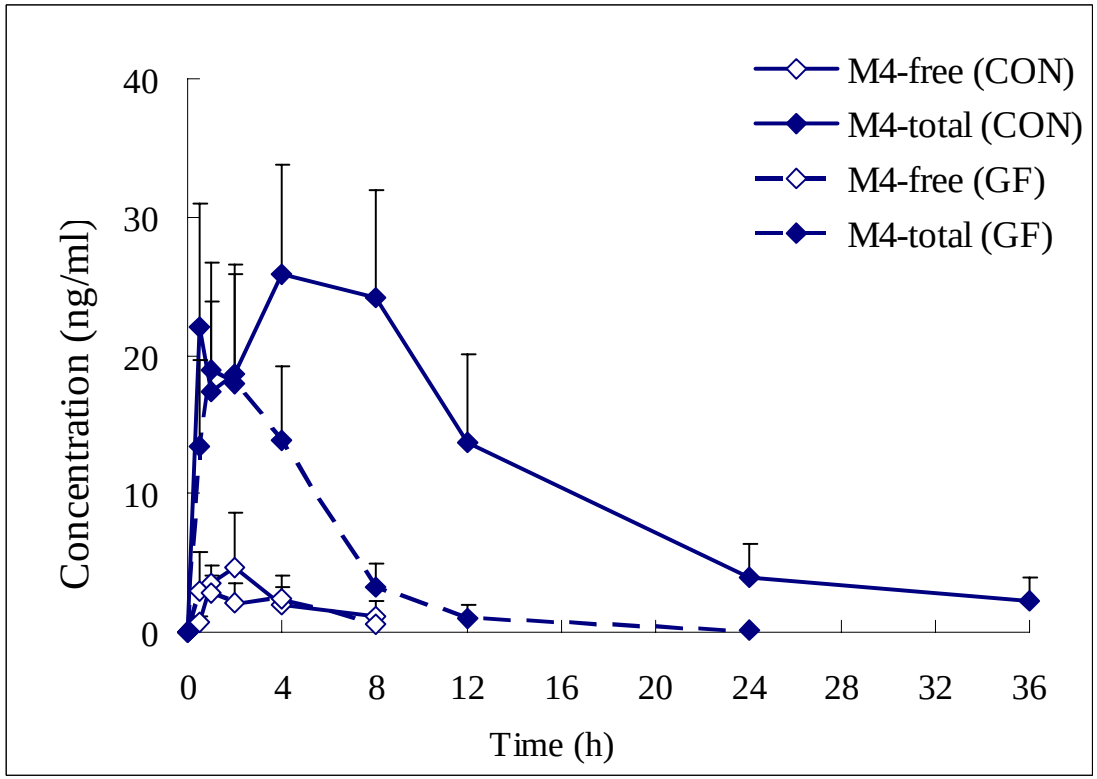

Fig. 6-D

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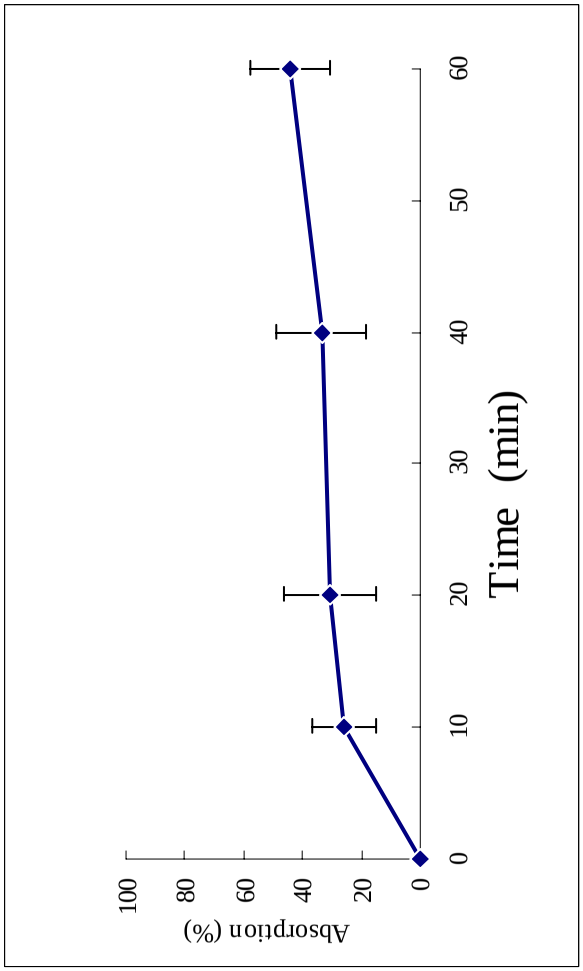

Fig. 7