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Title page

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Pharmacokinetics and Metabolism of the Flavonoid Scutellarin in Humans after a Single Oral Administration

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Running title page

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Pharmacokinetics and Metabolism of Scutellarin in Humans

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Abbreviations used

AUC, area under the plasma concentration-time curve; C_{max}, peak plasma concentration of the drug; T_{max}, the time to reach C_{max}; $T_{1/2}$, elimination half-life; LC/MS^n, liquid chromatography/ion trap mass spectrometry; LC/MS/MS, liquid chromatography/tandem mass spectrometry; UDP, uridine diphosphate.

Abstract

Scutellarin is widely used in treating various cardiovascular diseases. Few data are available regarding its metabolism and pharmacokinetics in humans. The objectives of this study were to develop methods to identify major metabolites of scutellarin in human urine and plasma, and determine simultaneously the parent drug and its major metabolites in human plasma for pharmacokinetic studies. Four metabolites were detected in urine samples by liquid chromatography coupled with electrospray multi-stage mass spectrometry, but only one of them was found in plasma. Its structure was confirmed as scutellarein 6-*O*-β-D-glucuronide by MS, NMR and UV absorbance spectra. The plasma concentrations of scutellarin and the major metabolite were simultaneously determined using liquid chromatography-tandem mass spectrometry. After a single oral administration of 60 mg scutellarin to 20 healthy subjects, the plasma concentrations of scutellarin were very low and its plasma concentration–time curve was also anomalous. Plasma concentration of the major metabolite was comparatively high and the peak plasma concentration was 87.0 ± 29.1 ng/ml. The T_{max} was late (7.85 ± 1.62 h) and part of individual pharmacokinetic profiles showed double peaks, which indicated scutellarin could be absorbed into the intestine after hydrolysis to its aglycone by bacterial enzymes. This was followed by reconjugation in the intestinal cell and/or liver with glucuronic acid catalyzed by the phase II enzyme, which showed regioselectivity and species difference. The regioselectivity of glucurono-conjugation for scutellarin might be of importance for pharmacological activity. Plasma concentration of iso-scutellarin can be used as a biomarker of scutellarin intake.

Flavonoids are among the most ubiquitous groups of plant secondary metabolites distributed in various foods and medicinal plants. They have a variety of biological effects in numerous mammalian systems *in vitro* and *in vivo*: free radical scavengers, antioxidants, anti-inflammatory, anti-mutagens or purgative effects. Recently, an increasing number of publications on the health beneficial effects of flavonoids, such as in cancer, coronary heart and stroke diseases, have appeared (Le Marchand, 2002; Nestel, 2003). These effects could be explained by their abilities to inhibit the cell cycles, cell proliferation, or oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis and stimulate the immune system.

Scutellarin (Fig. 1), scutellarein 7-*O*-β-D-glucuronide, is one of the major bioactive flavonoid glucuronide isolated from a Chinese herb *Erigeron breviscapus* (Vant). This compound is also found in *Teucridium parvifolium*, *Tripora divaricata* (Grayer, et al., 2002) and *Scutelaria lateriflora* (Gafner, et al., 2003). It has been reported that scutellarin was useful in the therapy of various ailments such as cardiovascular diseases, sleep disorders, depression, migraine, pain, or memory impairment (Pouzet, et al, 2002; Gafner, et al., 2003; Goh, et al, 2005). Today, it is commonly used in China as a remedy in dilating blood vessels, improving microcirculation, decreasing the viscosity of blood, reducing the blood platelet count and inhibiting platelet aggregation activity, etc (Hong and Liu, 2004; Liu, et al., 2005).

With the increasing significance of a potential beneficial role of scutellarin in human health, there is a growing demand for research on its absorption, metabolism and excretion. Until now, reports on pharmacokinetics and metabolism of scutellarin only focused on animals (Liu, et al., 2003; Jiang, et al., 2003; Zhang, et al., 2003; Huang, et al., 2005). Shen and Feng (2006) reported a liquid chromatography–tandem mass spectrometry (LC/MS/MS) method to directly determine scutellarin in human plasma and described its pharmacokinetic curve with the mean C_{max} value of c.a. 12.0 ng/ml after an oral dose of 60 mg scutellarin. A similar method was developed in our laboratory, but we found that the plasma concentrations of scutellarin in humans after an oral administration were very low and its plasma concentration–time curve was anomalous, which implied that scutellarin might undergo extensive first-pass metabolism in humans. As a result, determination of parent drug in plasma may not reflect its pharmacokinetic process in humans. Therefore, it is necessary to identify the major metabolites of scutellarin in human plasma and further investigate its

pharmacokinetics.

Over the past 2 decades, liquid chromatography coupled with mass spectrometry (LC/MS) has proved to be one of the most effective techniques in biomedical research, particularly for the analysis of complex mixtures in biological samples because of its excellent specificity, speed, and sensitivity. Now, LC/MS methods have been widely applied to identify and quantify flavonoid glycosides and aglycone in biological fluids (Prasain, et al., 2004).

In the present study, we firstly identified scutellarin metabolites in humans with liquid chromatography–ion trap mass spectrometry. On this basis, we developed a rapid, selective and sufficiently sensitive LC/MS/MS method to simultaneously determine scutellarin and its major metabolite in human plasma and to characterize the absorption and the plasma pharmacokinetics after an oral administration of 60 mg scutellarin.

Materials and Methods

Chemicals. Scutellarin (96.5% purity) and baicalin (internal standard, IS, 98.0% purity) were supplied by Liaoning Institute for the Control of Pharmaceutical Products (Shenyang, China). Iso-scutellarin (94.1% purity by HPLC), a major metabolite of scutellarin, was isolated and prepared from human urine in our laboratory.

Subjects. Twenty healthy male Chinese volunteers participated in the pharmacokinetic study after giving written informed consent. Their ages ranged from 20 to 28 years (mean 23 years) and weights from 58-75 kg (mean 64 kg). The subjects were all apparently healthy with no history of disease of the gastrointestinal tract, such as lactose intolerance or irritable bowel syndrome. They had neither suffered from any illness nor taken any other medicines in the preceding 2 weeks. The study was approved by the Ethical Committee of the People's Hospital of Liaoning Province (Shenyang, China).

Study Design. Each subject swallowed a 60 mg dose of scutellarin dripping pills (Qiu, et al., 2005) with 200 ml of water. Serial blood samples were collected into heparinized vacuum tubes before dosing and at 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11, 13, 15 and 24 h after dosing, and were immediately centrifuged for 10 min at 2500 *g* at 4 °C to separate the plasma fractions. The obtained plasma samples (1.5 ml) were immediately transferred into a plastic tube containing 100 μl of 8 *M* phosphoric acid, then vortex mixed. The acidified plasma samples were stored frozen at –20°C until analysis. The volunteers voided immediately before the dose was administered, and urine was collected from 8 to 12 h over wet ice into tubes. The collected urine samples were acidified by 8 *M* phosphoric acid, then were stored frozen at –20° until analysis.

LC/MS^n Procedures for Metabolites Analysis. A Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source system and an Agilent 1100 HPLC system (Wilmington, DE, USA) consisting of a vacuum degasser, a binary pump and an autosampler was employed to identify the metabolites of scutellarin in human urine or plasma samples. The LCQ interface was adjusted to the following conditions: ion mode, positive; spray voltage, 4.5 kV; capillary temperature, 220°C; sheath gas (nitrogen), 50 Arb; auxiliary gas (nitrogen), 5 Arb; The full-scan mass spectrum to obtain the protonated molecules $[M + H]^+$ of each potential metabolite was collected in the mass range from *m/z* 150 to 900. Furthermore, MS/MS and MS^3 spectra were obtained for selected precursor ions through incidental collision

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with neutral gas (helium) molecules in the ion trap, which could provide characteristic fragment ions of each metabolite. The relative collision energy was set at 30-40%.

To simultaneously detect phase I and phase II metabolites, a gradient of two solvent systems, A and B, was used. Solvent A was water containing 0.1% formic acid; solvent B was methanol containing 0.1% formic acid. The gradient started at 50% A, 50% B for 13 min, followed by a linear increase of solvent B to 80% in 1 min. This rate composition was maintained from 14 min to 30 min, and the initial conditions were returned to in 1 min and followed by a stable phase of 5 min. The flow-rate was delivered at a constant flow rate of 0.5 ml/min. Chromatographic separation was achieved using on a 200×4.6 mm, 5 μm Diamonsil C_{18} column (Dikma, Beijing, China) with a 4×3.0 mm SecurityGuard C_{18} (5 μm) guard column (Phenomenex, Torrance, CA, USA). The column temperature was maintained at 25°C.

A simple solid-phase extraction (SPE) procedure was used to extract scutellarin and its metabolite from urine or plasma samples. Aliquots (1.0 ml) of urine or plasma samples collected from volunteers were applied to Supelclean LC-18 solid-phase extraction (SPE) column (2 ml, Supelco, Bellefonte, PA, USA) preconditioned with 2-ml aliquots of methanol and water. After loading the sample, the column was washed with 1 ml of water. Metabolites were eluted with 1 ml of methanol containing 0.5% formic acid. The eluate was evaporated to dryness at 40°C under a gentle stream of nitrogen, and the residue was reconstituted by addition of 200 μl of the initial mobile phase. A 20-μl aliquot of the solution was injected onto the LC/MS^n system.

Preparation and Identification of Metabolites. A semi-preparative HPLC system was used to purify metabolites, consisting of two LC-6AD solvent delivery units, a DGU-14A degasser unit, an SPD-10A VP UV-Vis detector and an FRC-10A fraction collector. A Bruker AMX 300 NMR spectrometer (Faellanden, Switzerland) was used to record ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra. Resonances are reported relative to DMSO at 2.50 ppm.

Quantification of Scutellarin and Its Major Metabolite in Human Plasma. Scutellarin and its major metabolite (iso-scutellarin) were simultaneously determined in human plasma using a sensitive and selective LC/MS/MS method. Briefly, to a 500 μl aliquot of acidified plasma, 100 μl of the internal standard (400 ng/ml baicalin) and 200 μl of water were added. This mixture was extracted with 3 ml of ethyl acetate. The organic phase was separated, evaporated to dryness, reconstituted, and injected into the

LC/MS/MS system, which consisted of a Shimadzu (Kyoto, Japan) LC-10AD solvent delivery pump, an SIL-HT_A autosampler and a Thermo Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer fitted with a Ion Max^{TM} ESI interface. The mass spectrometer was operated in the positive ion detection mode. Selected reaction monitoring (SRM) included *m/z* 463 → *m/z* 287 for scutellarin and the metabolite, *m/z* 447→ *m/z* 271 for baicalin, respectively, with a dwell time of 0.20 s per transition. The same column described above was used for isocratic chromatographic separation. The mobile phase was composed of methanol and water (65: 35, v/v) with 0.1% formic acid at a flow rate of 0.6 ml/min.

For preparation of standard curves, the stock standard solutions of scutellarin and the metabolite, iso-scutellarin, were prepared by dissolving the accurately weighed standard compounds in methanol to give final concentrations of 400 μg/ml for each analyte. The solutions were then successively diluted with a mixture of methanol–water–formic acid (50: 50: 0.5, v/v/v) to achieve standard working solutions at concentrations of 500, 250, 100, 40.0, 15.0, 5.0, 2.0 ng/ml for scutellarin, and 4000, 2000, 1000, 300, 100, 40, 15.0, 5.0 ng/ml for iso-scutellarin. The obtained standard working solutions of scutellarin and iso-scutellarin (50 μl each) were used to spike acidified blank plasma samples (0.5 ml) and vortex-mixed. The mixed plasma samples were treated as described above, both in prestudy validation and during the pharmacokinetic study for the preparations of standard curves. The final standard concentrations in human plasma were 0.20 – 50.0 ng/ml for scutellarin and 0.50 – 400 ng/ml for iso-scutellarin. The quality control (QC) samples were prepared using a different stock solutions to obtain the plasma concentrations of 0.50, 5.0 and 45.0 ng/ml for scutellarin and 1.0, 20.0 and 360 ng/ml for iso-scutellarin, representing low, medium and high concentration of QC samples, respectively.

During prestudy validation, the calibration curves were defined in three days based on triplicate assays of the spiked plasma samples, and QC samples were determined in replicates (n = 6) on the same day. Peak area ratios of each analyte to the internal standard were utilized for the construction of calibration curves, using $1/x^2$ weighted linear least-squares regression of plasma concentrations. Excellent linearity was obtained over the concentration range of 0.20 – 50.0 ng/ml for scutellarin and 0.50 – 400 ng/ml for iso-scutellarin in human plasma. Typical equation of calibration curves

were as follows: scutellarin: $y = (3.236 \pm 0.316) \times 10^{-3}x + (4.093 \pm 1.185) \times 10^{-4}$, $r = 0.9970 \pm 0.0012$; iso-scutellarin: $y = (2.569 \pm 0.258) \times 10^{-3}x + (3.545 \pm 0.225) \times 10^{-4}$, $r = 0.9987 \pm 0.0001$.

The lower limit of quantification (LLOQ) for determination of scutellarin and iso-scutellarin in plasma, defined as the lowest concentrations analyzed with accuracy within ±15% and a precision ≤ 15%, were 0.20 and 0.50 ng/ml, respectively.

The intra- and inter-day relative standard deviation, calculated from QC samples, were below 10.2% for scutellarin and below 5.3% for iso-scutellarin. The inter-day relative error as determined from QC samples was within ±2.3% for each analyte. Mean extraction recoveries for scutellarin at concentrations of 0.50, 5.0 and 45 ng/ml were 52.4 ± 4.6, 48.2 ± 3.7, 48.7 ± 2.1% (n = 6), respectively; the recoveries for the metabolite at 1.0, 20.0 and 360 ng/ml levels were 45.6 ± 2.8, 45.3 ± 4.6, 47.2 ± 3.4% (n = 6), respectively. The results of stability experiments showed that no significant degradation occurred during chromatography, extraction, and three freeze–thaw cycles and sample storage processes (–20°C for 60 days) for scutellarin and the metabolite in acidified plasma samples (deviating no greater than 10% at low, high nominal concentrations for each analyte). The analytical data demonstrated excellent reproducibility, specificity, sensitivity, and precision for measurement of scutellarin and its metabolite.

The above-validated method was applied to the pharmacokinetic study of scutellarin and its metabolite after an oral dose of 60 mg scutellarin to 20 healthy volunteers. During the routine analysis, one set of calibration standards and two sets of QC samples were extracted and assayed along with the unknown samples. The plasma concentrations of unknown samples and QC samples were calculated according to the standard curve within each run.

Pharmacokinetic Analysis. The main pharmacokinetic parameters were calculated by model-independent methods. The peak plasma concentration (C_{max}) and the time to reach it (T_{max}) were taken directly from the data. The elimination half-life ($T_{1/2}$) was calculated from the equation $T_{1/2} = \ln 2/k$, using the terminal monoexponential log-linear slope of the time *vs* concentration curve of each subject for the estimation of k by least-squares regression. The area under the plasma concentration–time curve (AUC_{0-24}) was calculated by the linear trapezoidal method. The total body clearance (Cl_T/F) and steady-state volume of distribution (V_{ss}/F) were calculated as follows: $Cl_T/F =$

Dose/AUC and V_{ss}/F = mean residence time $\times$ Cl_T/F. All data are expressed as mean $\pm$ SD.

Results

Identification of Scutellarin Metabolites. The metabolism of scutellarin has been investigated in rats. Four metabolites were identified, including two sulfate conjugates of scutellarin, 6-methyl-scutellarin and 6-methyl-scutellarein (6-methyl of aglycone) (Zhang, et al., 2003). However, studies on the metabolism of scutellarin in humans have not been reported until now. In this experiment, an ion trap mass spectrometer was utilized to identify the major metabolites of scutellarin in humans, as it does not suffer the sensitivity losses during the full-scan mode that are unavoidable to a triple-quadrupole MS. Scutellarin is a flavone 7-*O*-glucuronide, and its MS signal intensity obtained in the positive mode was higher than that in the negative mode. In addition, ESI source revealed much higher signals for the protonated molecule of scutellarin at *m/z* 463 compared to APCI source.

In order to profile for potential metabolites of scutellarin, the urine or plasma samples were firstly analyzed by LC/MS^n method *via* full scan mode in the mass range from *m/z* 150 to 900. No appreciable metabolite peaks were observed in the total ion current (TIC) chromatogram. Therefore, possible metabolites of scutellarin were screened from TIC chromatogram by selected ion monitoring (SIM) mode, including conjugates of scutellarin with one or two sulfate groups (*m/z* 543 or 623), with glucuronic acid (*m/z* 639 or 815), with glucose (*m/z* 625 or 787) or methylated metabolites (*m/z* 477 or 491), and aglycone (scutellarein, *m/z* 287), as well as methylated aglycone (*m/z* 301) or aglycone conjugates with sulfuric acid or glucuronic acid (*m/z* 367 or 463). The SIM chromatogram by monitoring the $[M + H]^+$ ion at *m/z* 463 showed six peaks (Fig. 2B). But by comparison of blank urine, only three peaks with the retention times of 7.31, 9.03 and 9.83 min, respectively, were identified as the metabolites of scutellarin in humans and designated as M-1, M-2 (major peak) and M-3 (minor peak), respectively. Compared with blank urine, another metabolite showing $[M + H]^+$ at *m/z* 477 was observed, and named as M-4 (t_R = 27.05 min) (Fig. 2B). To further investigate the structures of the four metabolites full scan MS/MS and MS^3 modes (Fig. 3) were applied to obtain the characteristic fragments ions. The MS/MS spectra of M-1, M-2 and M-3 all yielded relatively simple fragment ion at *m/z* 287 derived from the neutral loss of glucuronic acid moiety (–176 u), which was identical to the MS/MS spectrum of scutellarin. And M-1 also had the same HPLC retention time (7.31 min) with the parent drug. Based on these data, M-1 was identified as scutellarin, and M-2 and M-3 as its

isomeric forms that arose from variation in the positioning of the glucuronyl moiety on the flavonol ring. Scutellarin has three potential sites for glucuronidation at 5, 6 and 4'-OH groups, respectively. The exact position of the glucuronosyl moiety could not be identified at this stage.

The MS/MS spectrum of M-4 gave an abundant peak at *m/z* 301 due to loss of glucuronic acid. Its $[M + H]^+$ ion, 14 Da higher than that of the parent drug, suggested that M-4 could be a methylated scutellarin. Furthermore, full scan MS^3 spectrum of *m/z* 477→301 yielded a major fragment ion at *m/z* 286 (Fig. 3), which is the characteristic fragment ion of methylated flavonoids. The potential sites of methylation for M-4 could not be elucidated only based on MS^n data.

Four metabolites could be detected in urine samples of all subjects. But only M-2 and trace amount of parent drug were found in plasma samples. Time-course analysis of scutellarin and its metabolites showed that the predominant metabolite in plasma and urine was M-2. To investigate scutellarin pharmacokinetics in humans, it is necessary to further identify the structure of M-2 and obtain its plasma concentration-time curves.

The collected urine samples were initially acidified to pH 2-3 by 8 *M* phosphoric acid and further extracted using ethyl acetate. The extract was removed under reduced pressure. The obtained residue was dissolved in methanol and subjected to XAD-2 chromatography using a gradient of H_2O and methanol system to obtain M-1-containing fractions. These fractions were subjected repeatedly to silica gel chromatography using methanol-H_2O (1: 1) and finally were purified by semi-preparative HPLC on a Shim-pack PRC-ODS column (250 × 20 mm i.d., Shimadzu, Kyoto, Japan) using a gradient of methanol and H_2O (1: 4→7: 3) to give the compound M2 (c.a. 70 mg). Its structure was characterized by MS and NMR spectra. The positive ESI/MS spectrum showed that the purified compound corresponds to the mono-glucuronide derivative and has the same molecular weight as that of the parent drug. The ^{13}C NMR spectrum of M-2 showed six carbon signals assigned to be a glucuronic acid moiety, including one anomeric carbon (104.3 ppm) and one carboxyl carbon (170.9 ppm), besides those of an aglycone moiety (Table 1). In the ^1H NMR spectrum, a sequential *trans*-1,2-diaxial relationship of H-1''–H-5'' (4.87–3.60 ppm, J = 6.3–9.2 Hz) indicated the presence of a *β*-D-glucuropyranosyl moiety in M-2. The complete identification of the structure of M-2, especially located position for glucuronyl group was established by ^1H- and ^{13}C-NMR and UV absorption analysis. Table 1 presents the ^{13}C and ^1H NMR chemical

shift values and ^{1}H NMR coupling constants from the parent drug and the major metabolite M-2. Comparison of the chemical shift values of the aromatic protons of M-2 with the ^{1}H NMR spectrum of scutellarin reveals that only the C8-H, represented by a singlet at 7.04 ppm shifts by -0.5 ppm towards 6.54 ppm. The ^{1}H NMR signals of C2'-H, C3'-H, C5'-H, C6'-H and C3-H are almost similar to those of the parent drug, which indicated that the glucuronic acid moiety of M-2 was conjugated with hydroxyl group in A ring. Therefore, this metabolite can be either the 5-*O*- or 6-*O*-glucuronosyl of scutellarein. In addition, similar as that of the parent drug, ^{1}H NMR spectrum of this metabolite shows a broad singlet signal at 12.77 ppm, which derived from a resonance of the 5-OH group of the flavonides (Abe, et al., 1990), because of lack of rapid exchange with solvent due to strong hydrogen bonding of the OH-hydrogen to the 4-keto moiety. As a result, the metabolite M-2 cannot be the 5-*O*-glucuronosyl of scutellarein and was confirmed as scutellarein 6-glucuronide, named as iso-scutellarin. The shift difference in the PPM value of C5-OH (-0.46 ppm) was observed between ^{1}H NMR spectra of scutellarin and M2 due to the glucuronosyl moiety attached at C6-OH. Additionally, in ^{13}C NMR spectrum of M-2, the different shifts of C-5, C-6 and C-7 can be explained by the glycosidation shift rule of phenolic hydroxyl group, which leads to the upfield shift (–2.1 ppm) of the *ipso*-carbon signal (C-6) and the downfield shift (+6.2, +7.2) of the *ortho*-carbon signals (C-5, C-7). The structural elucidation of the metabolite was further supported by UV absorption spectroscopy. When 5 μ*M* $AlCl_3$ was added to methanol solution of M-2, the absorbance peaks of M2 at 323 and 283 nm increased and shifted to 338 and 293 nm, respectively. Furthermore, followed by 1 *M* HCl, the UV peak did not shift. These results indicated that M-2 did not contain *ortho*-dihydroxyl substitutions, but has a free 5-OH group.

The reference substance of the metabolite M-3 was not obtained in this experiment as its amount in urine samples from the volunteers was very low. Since the LC/MS^n data indicated it to be an positional isomer of scutellarin, and the 6-*O*- and 7-*O*-monoglucuronosyls were already identified, the one residual monoglucuronosyl for M-3 only can be the 5-*O*- or 4'-*O*-glucuronosyl of scutellarein, which both contained an *ortho*-dihydroxyl substitution. Therefore, the UV absorption spectrum was not suitable to elucidate the glucuronosyl position of M-3. Due to the strong hydrogen bonding of the 5-OH-hydrogen with the 4-keto, conjugation to 5-OH does not readily occur. This leaves the 4'-position as the only remaining possibility for monoglucuronosyl. Taking

these considerations into account, the metabolite M-3 was proposed as scutellarin 4'-*O*-glucuronide.

Stability of Scutellarin and Its Major Metabolite in Plasma Samples. Although some methods have been reported to determine scutellarin in animal or human plasma, there is little data available on stability of scutellarin in plasma or urine samples and solutions. In this experiment, it was observed that scutellarin in human plasma degraded more than 30% at room temperature within only 5 min and about 50% at –20°C for 20 days. The low stability of scutellarin in plasma might be attributed to its two hydroxyl groups, situated *ortho* to each other in the structure, which are highly susceptible to oxidation to produce an *ortho*-quinone-like compound. The major metabolite, iso-scutellarin, was relatively stable in plasma under the storage conditions and in $AlCl_3$ solution after exposure of the solution to room temperature for 1.0 h, which is in accordance with its structure of *meta*-diphenol group. Hence, plasma or urine samples of scutellarin from the volunteers must be pre-treated by addition of an antioxidant to improve its stability in the process of storage or sample extraction. Xing *et al* (2005) reported that acidification could stabilize flavonoids containing *ortho*-hydroxyl groups in biological samples and solution. In this experiment, it was found that scutellarin was stable in pH 2-3 plasma or urine samples or methanol solution adjusted by addition of phosphoric acid or formic acid. In addition, the controlled lower pH values of biological sample could increase the extraction recovery of the analytes.

Pharmacokinetic Results. After a single oral administration of 60 mg scutellarin to 20 healthy subjects, plasma concentrations of scutellarin and its major metabolite (iso-scutellarin*)* were determined by the above described LC-MS/MS method. The mean C_{max} value of the parent drug was less than 5.0 ng/ml, and its plasma concentration–time curve was anomalous. Fig. 4A shows a typical individual plasma concentration–time curve. The metabolite iso-scutellarin could be detected in plasma samples from all subjects. Its mean plasma concentrations exceeded those of scutellarin by approximately 30-fold and there were markable interindividual differences in the $AUC_{0\text{-}24\ h}$ and C_{max} values. The C_{max} values were 38.1-128.1 ng/ml for iso-scutellarin. Its elimination from plasma was monophasic, with half-lives of 3.08 ± 0.55 h. The mean plasma concentration–time curve is displayed in Fig. 4B. The corresponding main pharmacokinetic parameters are presented in Table 2. As described above, there was no evidence of oxidative metabolism of scutellarin.

Discussion

Flavonoids are polyphenol compounds. Their pharmacological effects have been reported to be highly dependent on the number and position of the hydroxyl moieties in the molecule (Bors, et al., 1990; Rice-Evans, et al., 1996). The hydroxyl groups in the structure are mainly metabolized as conjugates with a glucuronic acid or sulfuric acid during the phase II biotransformation pathway in vertebrates (Havsteen, 2002; Morand, et al., 1998). Therefore, the difference in conjugated positions can be expected to influence their biological activities. It has been reported that glucuronidation of the 3'- or 4'-hydroxyl group in the B-ring of flavonoids decreased the antioxidant activity, whereas glucuronidation in the A-ring affected antioxidant activity to a less extent (Brown, et al., 1998).

It has been demonstrated that the preferential conjugation sites exist in the conjugates formation for flavonoid compounds, which may be related to the steric configuration of the flavonoids or the UDP-glucuronosyl-transferase involved (species difference) (Boersma, et al., 2002; Havsteen, 2002). Although the *in vitro* and *in vivo* experiments revealed that the glucurono- or sulfo-conjugated metabolites could occur at any hydroxyl group of flavonoids, the 7-OH position on the A-ring is predominantly conjugated to form a glucuronide or sulfate compared to 3- and 5-OH positions (Boersma, et al., 2002; O'Leary, et al., 2003; Yodogawa, et al., 2003; Gradolatto, et al., 2004; Chen, et al., 2005). For some flavonoids, such as naringin or naringenin (Zhang, et al., 2004), the major metabolites in rat urine were 4'-*O*-glucuronide, followed by 7-*O*-glucuronide. Due to the low acidity, 5-OH position was well-known to be the least reactive for glucuronidation. Only one literature reported that a minor 5-*O*-glucuronide was observed *in vivo* when rats were fed an isoflavone rich diet (Fang, et al., 2002). Until now, few data about the 6-OH metabolic reactivity was available. A 6-*O*-glucuronosyl type metabolite was identified when investigating the metabolism of baicalein and baicalin (baicalein 7-*O*-glucuronide) in human or rat urine (Abe, et al., 1990; Che, et al., 2001). But no quantitative comparison of the glucuronidation capacity for 6-OH and 7-OH groups has been made in the literature. In our study, three mono-glucuronide isomers of the aglycone substituted on 6-, 4'- or 7-OH were onserved in human urine after an oral administration of scutellarin. Among them, the major metabolite was scutellarein-6-*O*-glucuronide, followed by scutellarein-4'-*O*-glucuronide, and 7-*O*-glucuronide (the parent drug) as a minor metabolite, implying that 6-OH group

of scutellarein (or scutellarin) was the preferential site for glucuronosyl conjugation in humans. Similar sites for mono-glucuronidation of scutellarin were observed in rat urine, but the ratio of the formed products was different from that of the present study. The parent drug (scutellarein-7-*O*-glucuronide) was a major mono-glucuronoconjugate, and only a small amount of 6-*O*-glucuronide was detected in rat urine. Until now, no scutellarein-6-*O*-glucuronide was found in plant kingdom. These results illustrate that the regioselectivity of glucuronosyl conjugation to scutellarin is species different (variable UDP-glucuronosyl-transferase selectivity). To the flavonoids containing 6-OH group, the 6-*O*-glucuronide conjugation might be an important metabolic pathway in humans. The effect of conjugation on biological activities remains to be investigated.

No sulfo-conjugates were detected in this experiment by LC/MS^n and specific enzymatic hydrolysis (β-glucuronidase-sulfatase/D-saccharic acid 1,4-lactone) methods (Yodogawa, et al., 2003), probably due to the inhibitory effect of scutellarein on phenol sulfotransferase (Walle, et al., 1995).

There has been some controversy regarding how flavonoids are absorbed, and it has been suggested that some flavonoids, mainly those with glucose molecules, might be absorbed intact (Hollman, et al., 1999). However, flavonoids such as rutin, hesperidin, naringin, narirutin, and baicalin, which contain sugar (rutinoses, neohsperidoses or glucuronic acid) chains are most likely hydrolyzed to their aglycones by intestinal enzymes of bacterial origin, such as α-rhamnosidases and β-glucuronidase (Choudhury, et al., 1999; Hollman, et al., 1999; Akao, et al., 2000; Erlund, et al., 2000; Spencer, 2003; Walle, 2004) and are then absorbed into the intestinal cells by passive mechanisms. The absorbed aglycone might be efficiently conjugated as glucuronides, sulfates or methylated derivatives, however, it is not clear where the conjugation of the aglycone occurred, especially glucuronidation, as both human intestinal and liver tissue have been shown to posses UDP-glucuronosyltransferase activity. It has been reported that glucuronidation process for catechin, resvertrol, quercetin and luteolin occurred mainly in the small intestine (Donovan, et al., 2001; Kuhnle, et al., 2000; Spencer, 2003). In our study, after an oral administration of scutellarin, the major component in plasma is its isomer, and the parent drug was hardly detectable. The peak plasma concentrations of the major metabolite were reached between 5.0 to 11 h (mean value 7.85 h) and part of individual profiles (~30%) showed double peaks. These results indicated that scutellarin was hydrolyzed to the corresponding aglycone, which was

absorbed from the small intestine or the colon, followed by a regioselective glucuronidation to form scutellarein-6-*O*-glucuronide to enter blood stream. From our data, we cannot determine whether the iso-scutellarin is formed in the intestine and/or liver.

It was reported that human liver cells possessed β-glucuronidase and UDP-glucuronosyltransferase activity, which could further deglucuronidated some glucuronides and then reglucuronidated in liver (O'Leary, et al., 2003). Therefore, it was inferred that the major metabolite iso-scutellarin could be hydrolyzed to the aglycone by *β*-glucuronidase in liver, then reconjugated with glucuronic acid to give scutellarein-4'-*O*-glucuronide and scutellarein-7-*O*-glucuronide (original form), which both were found in human urine. The re-formation of the parent drug in liver might result in its anomalous plasma concentration-time curve (Fig. 4A).

The C_{max} and AUC values of iso-scutellarin exhibited wide inter-individual variation. We hypothesize that these variations were caused by gastrointestinal microflora or their glucuronidation capacity. Large interindividual variation in bioavailability has also been reported to occur for quercetin-rutinoside (Erlund, et al., 2000).

In summary, the use of LC/ESI–MS in multi-stage full scan mode allowed to identify the metabolites of scutellarin in human urine and plasma samples. Two positional isomers were observed as its mono-glucuronide metabolites in human urine, in which the isomer conjugated with 6-OH group was the major metabolite *in vivo*, and could be used as biomarker of scutellarin intake. Pharmacokinetic behavior of iso-scutellarin indicated that scutellarin is most likely hydrolyzed by intestinal β-glucuronidase of bacterial origin, followed by a reconjugation step in the intestinal cell and/or in the liver with glucuronic acid after absorption of the aglycone, which showed the positional selectivity and species difference. In humans, 6-OH group of the aglycone of scutellarin was the preferential site for glucuronosyl conjugation, compared with 4'-, 5-, and 7-OH groups.

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Footnotes

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Legends for figures

FIG. 1. Chemical structures of scutellarin (I), its major metabolite iso-scutellarin (II) and baicalin, internal standard (III)

FIG. 2. LC–MS^n analysis of scutellarin and its major metabolites in human urine. (A) Blank urine; (B) a urine sample collected within 8-12 h after an administration of 60 mg scutellarin to a healthy subject. (C) a blank urine sample spiked with 1000 ng/ml scutellarin.

Channel I: Total ion current chromatogram; Channel II: Full scan MS/MS chromatogram of *m/z* 463; Channel III: Full scan MS/MS chromatogram of *m/z* 477.

FIG. 3. Full scan MS^n spectra corresponding to the metabolite peaks. (A) MS/MS spectrum of the peak eluting at 7.31 min; (B) MS/MS spectrum of the peak eluting at 27.05 min; (C) MS^3 spectrum of the peak eluting at 27.05 min.

FIG. 4. Plasma concentration–time profiles of scutellarin and its major metabolite after an oral dose of 60 mg scutellarin. (A) typical individual plasma concentration–time profile of the parent drug; (B) mean plasma concentration–time profile of iso-scutellarin (n = 20). Each point represents the mean ± SD.

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TABLE 1

The ^{1}H and ^{13}C-NMR data of scutellarin and its major metabolite iso-scutellarin (δ in DMSO-d_6)

No.	**Carbon signals**		Proton signals	
	Scutellarin	Metabolite	Scutellarin	Metabolite
2	164.3	163.7		
3	102.7	102.4	6.74 (H, s)	6.76 (H, s)
4	182.4	182.0		
5	147.0	153.2		
6	130.6	128.5		
7	151.1	158.3		
8	93.8	94.7	7.04 (H, s)	6.54 (H, s)
9	149.1	152.6		
10	106.0	103.4		
1’	121.4	121.3		
2’	128.5	128.5	7.94 (H, d, *J* = 8.3Hz)	7.91 (H, d, *J* = 8.5Hz)
3’	116.1	116.0	6.96 (H, d, *J* = 8.3Hz)	6.92 (H, d, *J* = 8.5Hz)
4’	161.3	161.2		
5’	116.1	116.0	6.96 (H, d, *J* = 8.3Hz)	6.92 (H, d, *J* = 8.5Hz)
6’	128.5	128.5	7.94 (H, d, *J* = 8.3Hz)	7.91 (H, d, *J* = 8.5Hz)
1’’	100.2	104.3	5.26 (H, d, *J* = 6.8Hz)	4.87 (H, d, *J* = 6.3Hz)
2’’	72.9	73.5	3.45 (H, o)	3.38 (H, o)
3’’	75.6	76.1	3.38 (H, o)	3.28 (H, o)
4’’	71.4	71.7	3.50 (H, o)	3.43 (H, o)
5’’	75.4	75.7	4.11 (H, d, *J* = 9.2Hz)	3.60 (H, d, *J* = 9.2Hz)
6’’	170.1	170.9		
5-OH			13.23 (H, s)	12.77 (H, s)

TABLE 2

The main pharmacokinetic parameters of iso-scutellarin after an oral administrsation of 60 mg scutellarin to 20 healthy Chinese subjects.

Parameters	Mean	SD	Max. values	Min. values
AUC_{0-t} (ng/ml×h)	459.3	151.4	707.4	160.7
$AUC_{0-\infty}$ (ng/ml×h)	464.0	154.0	713.0	162.1
$T_{1/2}$ (h)	3.08	0.55	4.05	2.22
$AUMC_{0-t}$ (ng/ml×h^2)	4017	2029	7717	1070
$AUMC_{0-\infty}$ (ng/ml×h^2)	4102	2090	7824	1093
MRT (h)	8.87	2.10	11.94	6.35
CL/F (l/h)	147.9	75.8	370.1	84.2
VD/F (L)	690.1	490.7	2165	270.1
VDss/F (L)	1206	481.9	2496	717.5
Tmax (h)	7.85	1.62	11.00	5.00
Cmax (ng/ml)	87.01	29.14	128.1	38.11

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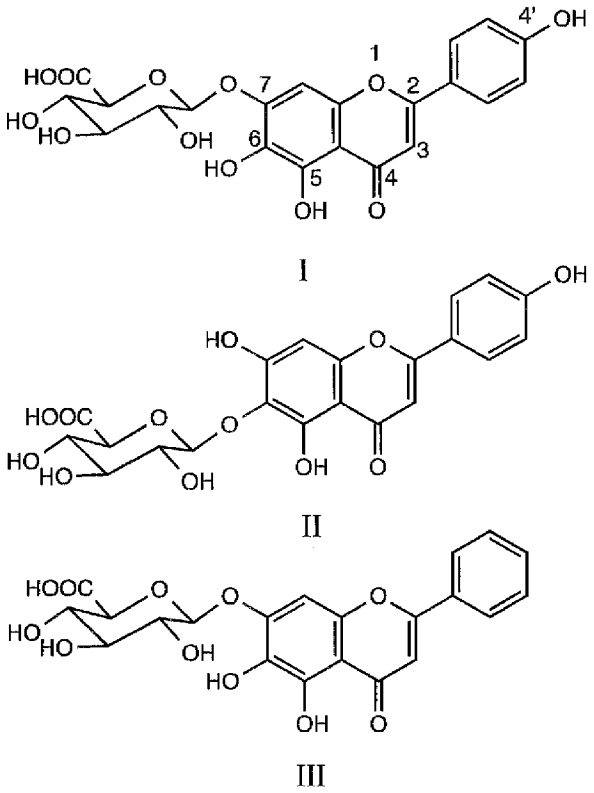

FIG. 1.

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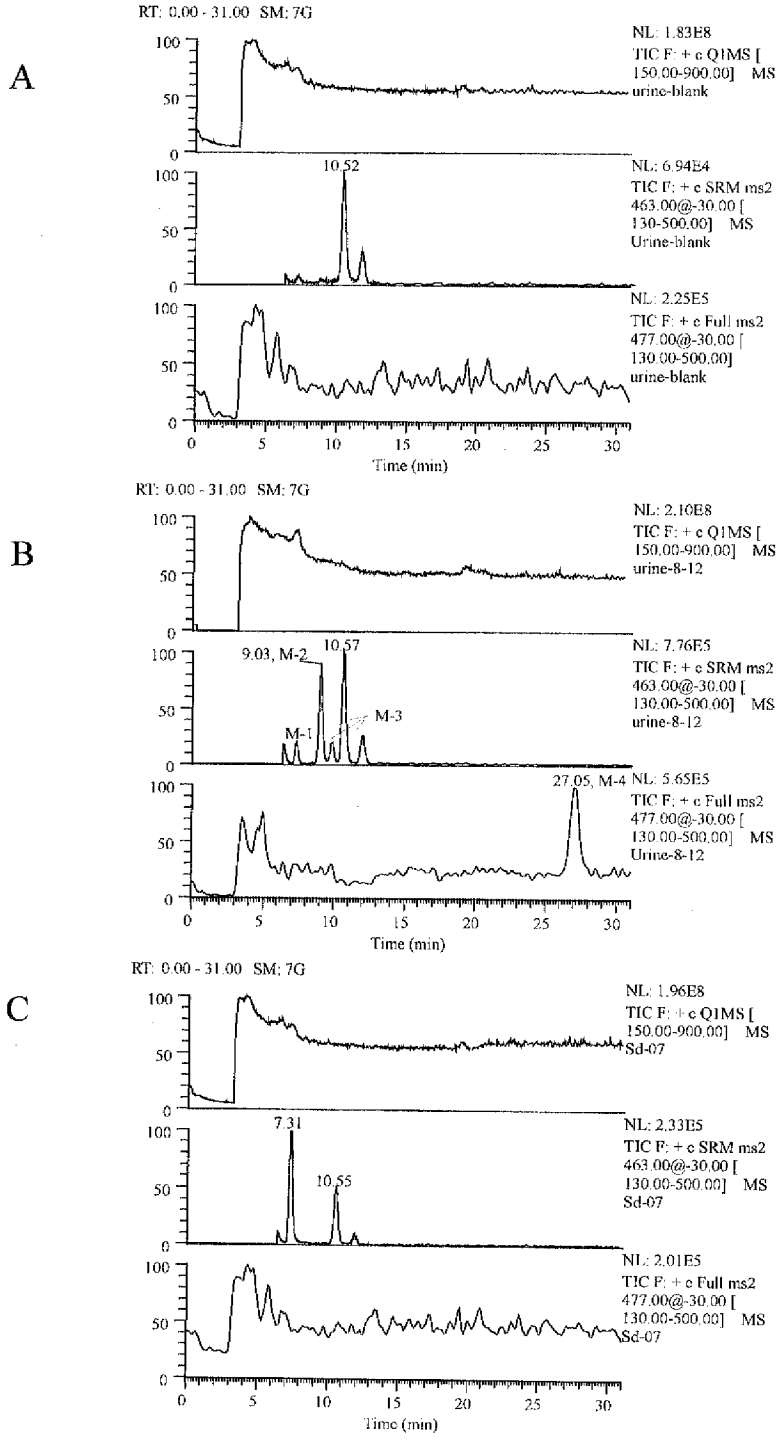

FIG. 2.

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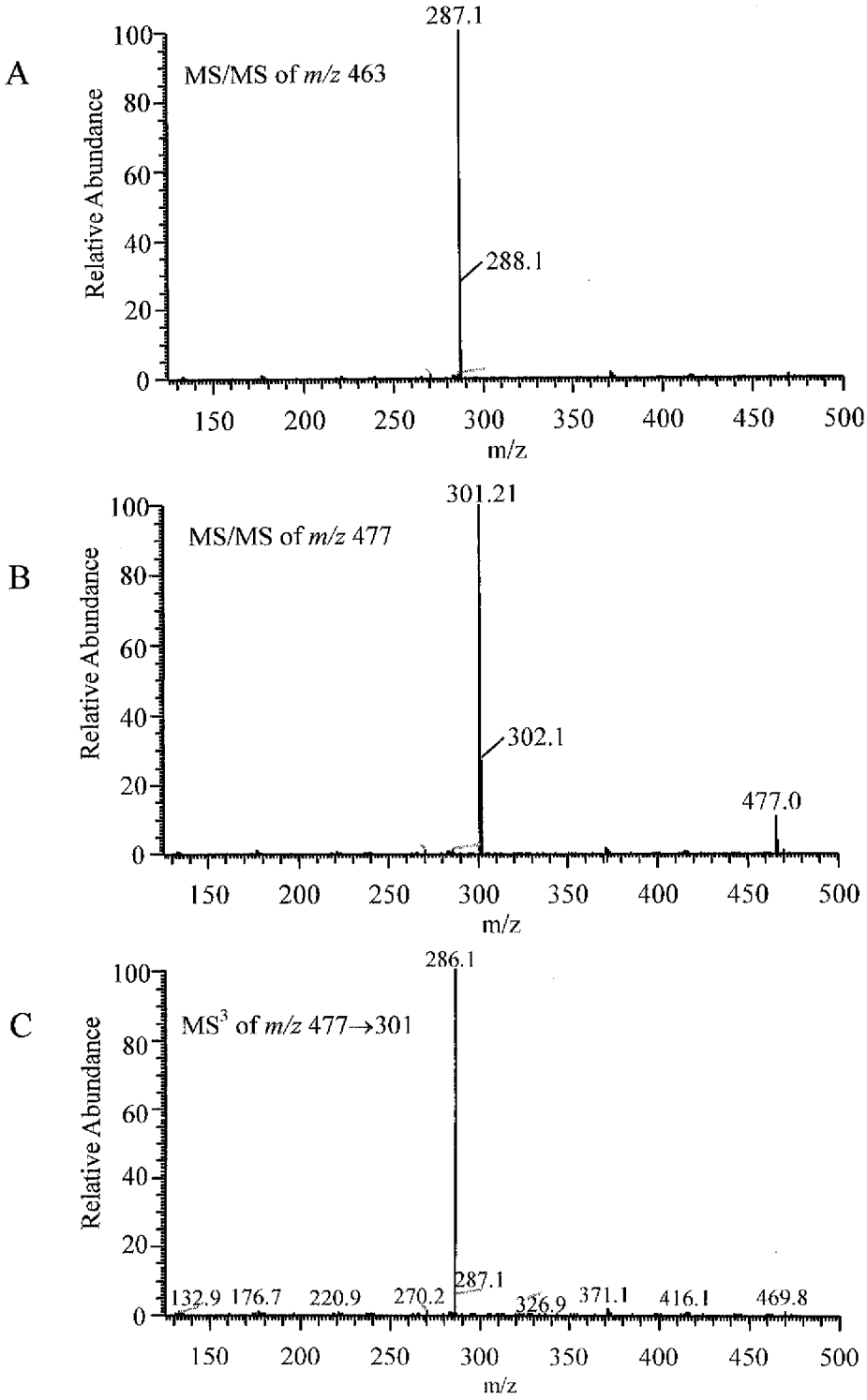

FIG. 3.

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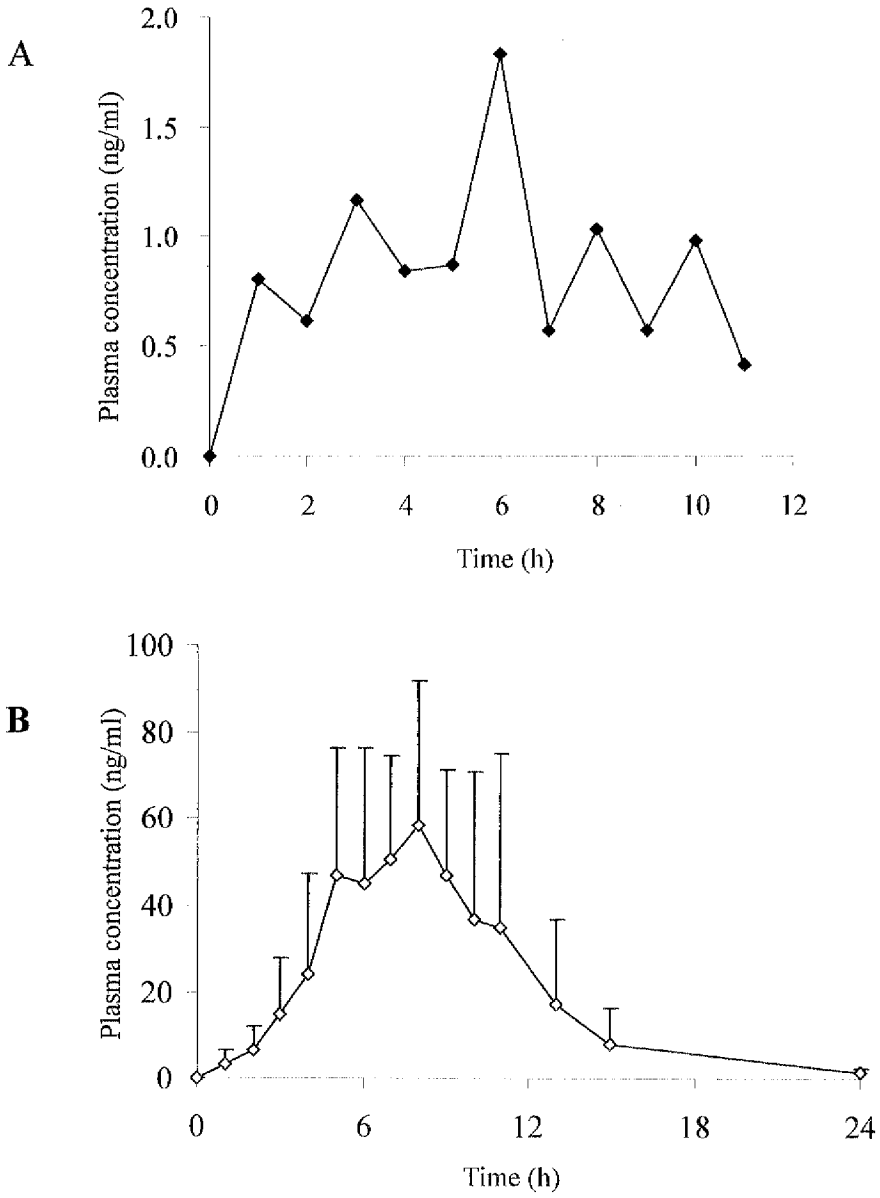

FIG. 4.