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Determination of sinomenine sustained-release capsules in healthy Chinese volunteers by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive and selective liquid chromatographic tandem mass spectrometric method was developed and validated for the determination of sinomenine in human plasma. Plasma samples were precipitated using methanol with metronidazole as internal standard. Separation was carried out on an Inertsil ODS-3 column using a mixture of 0.2% ammonium acetate solution (A) and methanol (B) as the mobile phase with linear gradient elution as follows: 0 min (50%B) → 1.5 min (80%B) → 4.5 min (80%B) → 4.6 min (50%B) → 6.0 min (50%B). All mass data were obtained in the positive ion mode, and the fragmentation transitions for the selective multiple reaction monitoring were m/z 330 → 181 and 172 → 128 for sinomenine and metronidazole, respectively. The method was fully validated to be accurate and precise with a linear range of 0.5–500 ng/mL and applied to a single- and multiple-dose pharmacokinetics study of sustained-release capsules of sinomenine hydrochloride in 20 healthy Chinese volunteers. After oral administration of a single 60-mg dose, the T_{max}, C_{max}, AUC_{0-96} and $t_{1/2}$ were 7.9 ± 2.0 h, 123 ± 22 ng/mL, 3032 ± 682 ng h/mL and 13.4 ± 1.6 h, respectively. After oral administration of the 60 mg capsules twice-daily for 7 consecutive days, these parameters were 4.4 ± 3.6 h, 279 ± 69 ng/mL, 7333 ± 2096 ng h/mL and 15.1 ± 1.3 h, respectively. The AUC and C_{max} values after multiple-dose treatment were significantly higher than those after a single-dose treatment ($P < 0.01$), with an accumulation factor of 2.49 ± 0.77.

1. Introduction

Sinomenine [(9alpha, 13alpha, 14alpha)-7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinan-6-one; Fig. 1] is an alkaloid extracted from the stem of the Chinese medicinal plant, *Sinomenium acutum*, Rehder & E.H. Wilson (Family Menispermaceae). In vitro and in vivo studies have indicated that sinomenine has an array of biological activities, including immunosuppression [1], anti-inflammatory [2,3], anti-arthritic effect [4,5], inhibition of lymphocyte proliferation [6] and prevention of antagonize cartilage degradation and chondrocyte apoptosis [7]. It has shown significant beneficial effects as monotherapy in the treatment of rheumatoid arthritis [8–11]. Although the enteric-coated tablet of 20-mg sinomenine hydrochloride has been marketed in China for many years, the very detailed information about human pharmacokinetics was limited.

Similar to the non-steroidal anti-inflammatory drugs (NSAIDs), conventional formulations of sinomenine hydrochloride occasionally caused adverse effects in the digestive system [11]. Accordingly, it is necessary to develop sustained-release formulation to overcome some of the pharmacokinetic limitations and safety concerns associated with the conventional formulation [12,13]. The formulation in this study is a hard gelatin capsule containing pellets coated with ethylcellulose for film-controlled release of sinomenine hydrochloride. Multiparticulate formulations have the significant advantage of less intra- and inter-subject variability in terms of pharmacokinetics than their monolithic counterparts. The twice-daily formulation assessed in this study has been characterized in vitro as a stable and sustained-release drug, which is active for at least 12 h.

To characterize the pharmacokinetics of sinomenine, a highly sensitive and selective method is required. Currently, the primary focus of quantification of sinomenine hydrochloride in biological samples is based on the high-performance liquid chromatography with ultraviolet detector (LC–UV) [14–20], which required either a complicated extraction or long analysis time (>10 min). Furthermore, the lower limits of quantitation (LLOQ) of these methods in a range of 6–320 ng/mL were not suitable for sinomenine

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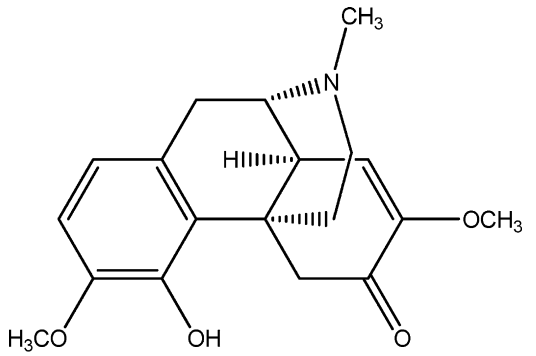
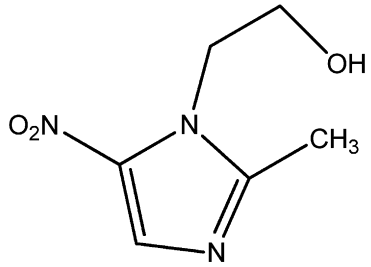


sinomenine



metronidazole

Fig. 1. Chemical structures of sinomenine and internal standard (metronidazole).

pharmacokinetic study. Some more sensitive methods using LC/MS have been reported to detect this drug in rat's brain [20] or skin [21]. Only one study using ion trap mass spectrometry has been published for the determination of sinomenine in human plasma with an LLOQ of 0.5 ng/mL [22], but the analyses were performed only with spiked plasma samples so pharmacokinetic parameters were not available.

The present study developed and fully validated a selective and sensitive high performance liquid chromatography–electrospray ionization-tandem mass spectrometric (LC/MS/MS) method for the determination of sinomenine in human plasma. The established LC/MS/MS method was applied to the pharmacokinetic study of the 60-mg sinomenine hydrochloride sustained-release capsules after a single and multiple oral doses in 20 healthy Chinese volunteers.

2. Materials and methods

2.1. Reagents and materials

Sinomenine hydrochloride standard substance (purity > 99% HPLC) and sinomenine hydrochloride sustained-release capsules were supplied by Jiangsu Chiatai Qingjiang Pharmaceutical Co., Ltd. (Jiangsu, Huai'an, PR China). Internal standard, metronidazole was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (lot 100191-200305, Beijing, PR China). Methanol of HPLC/Spectro grade was obtained from Tedia Company Inc. (Fairfield, OH, USA). Other chemicals were all of analytical grades, and purchased from Nanjing Chemical Reagent Factory (Nanjing, PR China). Deionized water was purified through a PL5242 Purelab Classic UV (PALL Co., Ltd, USA) before use. Blank plasma was supplied by the Red Cross Society of China, Nanjing Branch.

2.2. LC/MS/MS conditions

A Shimadzu LC-2010CHT system (Shimadzu, Kyoto, Japan) was hyphenated with a Waters Quattro-micro tandem mass spectrometer equipped with electrospray ionization (ESI) source (Micromass, Manchester, UK). Data acquisition was performed with Masslynx software (Version 4.0). An Inertsil ODS-3 column (5 μm, 250 mm × 4.6 mm i.d.) was used for the chromatographic separations. The mobile phase A was 0.2% ammonium acetate solution (pH 6.6), and the mobile phase B was methanol. The linear gradient elution program was performed as follows: 0 min (50%B) → 1.5 min (80%B) → 4.5 min (80%B) → 4.6 min (50%B) → 6.0 min (50%B) with a flow rate of 1.0 mL/min and 30% of the effluent was split into the MS inlet for the determination. To assure the reproducibility of the retention time, the column temperature was maintained at 30 °C. For optimal stability, the auto-sampler temperature was set at 4 °C.

All analyses were carried out in positive-ion ESI and monitored in multiple selective reactions monitoring (SRM) mode. The MS conditions were optimized as follows: the spray voltage was set at 2.5 kV with the source temperature at 120 °C, and the desolvation nitrogen gas temperature was set at 400 °C with a desolvation gas flow of 450 L/h and a cone gas flow of 30 L/h. The $[M+H]^+$ ion of sinomenine was monitored with a transition of m/z 330 → 181, cone voltage of 38 volts (V) and collision energy of 35 electrovolts (eV). The $[M+H]^+$ ion of metronidazole (internal standard) was monitored with a transition of m/z 172 → 128, cone energy of 38 V and collision energy of 15 eV. The extractor voltage was 2 V and RF lens voltage 0.1 V. Argon gas of 0.2 Pa was used for collision-induced dissociation.

2.3. Sample preparation

To an aliquot 200 μL plasma samples in 1.5 mL Eppendorf tube, 20 μL of methanol (when preparing calibration and quality control (QC) samples, standard solution was added instead of methanol), 20 μL of IS solution (400 ng/mL) and 600 μL methanol were added and vortex-mixed for 1 min for protein precipitation and centrifuged at 10,000 × g for 10 min. An aliquot of 20 μL of the supernatant was injected into the LC/MS/MS system.

2.4. Preparation of standard solutions, calibration and QC samples

Standard stock solution of sinomenine was prepared by dissolving an accurately weighed appropriate amount in a 25 mL volumetric flask with methanol to achieve a concentration of 500 μg/mL. Stock solution of the IS was also prepared in methanol and diluted to 400 ng/mL. The sinomenine standard solutions of 5–5000 ng/mL were prepared by serial dilution of the stock solution. All of the solutions were stored at 4 °C, and brought to room temperature before use. Plasma calibration standards of 0.5–500 ng/mL were prepared by spiking 20 μL each of the standard solutions with aliquots of 0.200 mL blank human plasma. QC samples were prepared in the same way with four levels of 2.5, 20, 150, and 400 ng/mL.

2.5. Method validation

2.5.1. Selectivity

Selectivity was demonstrated by the chromatograms of six different blank plasma samples obtained from six subjects with reference to those spiked with sinomenine and metronidazole.

2.5.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves for sinomenine in plasma were prepared in the range from 0.5 to 500 ng/mL. The linearity of each

calibration curve was determined by plotting the peak area ratios (y) of sinomenine versus metronidazole (IS) with the nominal concentrations (x) of sinomenine in plasma. The calibration curves were constructed by weighted least square linear regression with a factor of $1/x^2$.

The LLOQ was defined as the lowest concentration on the calibration curve with an acceptable accuracy (%) ranging from 80% to 120%, and precision (RSD) below 20% were obtained.

2.5.3. Precision and accuracy

The intra-batch precision and accuracy were determined by analyzing five sets of spiked plasma samples of sinomenine at each QC level (2.5, 20, 150 and 400 ng/mL) in a batch. The inter-batch precision and accuracy were determined by analyzing five sets of spiked plasma samples of sinomenine at each QC level (2.5, 20, 150 and 400 ng/mL) in three consecutive batches. The concentration of each sample was calculated by using the calibration curve prepared, and analyzed in the same batch. The precision was expressed as the relative standard deviation (RSD). The accuracy of the method was evaluated by analyzing the QC samples spiked with standard solutions, and expressed as a percentage error of measured concentrations versus nominal concentrations.

2.5.4. Recovery and matrix effect

The absolute recovery of sinomenine from plasma through the protein precipitation procedures was determined at four concentration levels (2.5, 20, 150 and 400 ng/mL) each with five replicates by spiking a known amount of sinomenine into blank plasma prior to the protein precipitation as described in Section 2.3. The absolute recovery of sinomenine and IS were respectively calculated by comparing the peak areas of sinomenine or IS of deproteinized samples to the peak areas of the corresponding standards in the same amount of the mobile phase.

In order to evaluate the matrix effects (ME) on the ionization of the analytes, i.e. the potential ion suppression or enhancement due to the matrix, sinomenine at four concentration levels was added to 0.2 mL of the blank plasma sample's reconstituted solution, the corresponding peak areas (A) were compared with those of the sinomenine standard solutions dried directly, and reconstituted with the same amount of the mobile phase (B). The ratio (A/B × 100)% was used to evaluate the matrix effects. The matrix effect of IS was also evaluated using the same method.

2.5.5. Stability

Short-term stability: It was assessed by determining QC samples of two concentration levels (2.5 and 150 ng/mL) kept at room temperature (25 °C) for 2, 4 and 8 h, which covered the maximum span for plasma sample preparation.

Long-term stability: It was evaluated by determining QC samples of two concentration levels (2.5 and 150 ng/mL) kept frozen (−20 °C) for 30, 60 and 90 days.

Post-preparative stability: It was measured by determining QC samples of two concentration levels (2.5 and 150 ng/mL) post preparation and kept in the auto-sampler (4 °C) for 2, 4, 8, 12, 24 h, and kept at the room temperature (25 °C) for 2, 4, 8 h, respectively.

Freeze–thaw stability: It was tested by analyzing QC samples of two concentration levels (2.5 and 150 ng/mL) undergoing three freeze (−20 °C)–thaw (room temperature) cycles on consecutive days. For the first freeze–thaw cycle, the samples were refrozen at −20 °C for 24 h and then thawed unassisted at room temperature. For the second and the third freeze–thaw cycles, the samples were refrozen at −20 °C for 12 h and then thawed unassisted at room temperature.

Stock solution stability: Sinomenine and the IS were evaluated by analyzing their working solutions kept at 4 °C for 30 and 90 days, respectively. All the samples were analyzed, and the experimental concentrations were compared with the nominal values.

2.6. Subjects

Healthy, nonsmoking male Chinese volunteers aged to 40 years with body weight > 50 kg and body mass index from 19 to 24 kg/m^2 were eligible for inclusion. Health screening included a medical history, complete physical examination, electrocardiogram (ECG), and standard laboratory tests (hematology, blood chemistry, and urinalysis). Subjects were required to abstain from alcohol and caffeine-containing products before and during the study periods, and not taking any prescription or non-prescription drugs or being treated for medical conditions. Then written informed consent was obtained from all the subjects prior to the study. Twenty healthy men were enrolled with following body characteristics (mean ± SD with range in parentheses) age 34 ± 2 years (31–38); height 169 ± 2 cm (165–173); body weight 62 ± 2 kg (59–65).

2.7. Clinical study design

The clinical study was conducted at Xijing Hospital, Xi'an, PR China. In the single-dose study, each subject was orally given a 60 mg sinomenine hydrochloride sustained-release capsule. Blood samples were collected from the forearm vein for the determination of plasma concentrations of sinomenine from 0 to 96 h at predetermined time intervals. In the multiple-dose study, 60 mg sinomenine hydrochloride sustained-release capsules were given to each subject twice-daily for 7 consecutive days with only one morning dose on the last day, and blood samples were drawn before the morning dose on days 4, 5, 6, and 7 to determine the steady state and until 96 h at the predetermined time intervals as in the single dose study after the last morning dose. The pharmacokinetic parameters, including C_{max}, T_{max}, AUC, MRT, $t_{1/2}$, CL and V_d, were calculated using noncompartmental analysis. The study protocol was approved by the hospital Ethical Review Committee in accordance with the principles of the Declaration of Helsinki.

3. Results and discussion

3.1. LC/MS/MS optimization and sample preparation

Reversed phase LC with acetonitrile or methanol as the elution modifier in combination with different volatile buffer solutions, such as ammonium acetate, ammonium formate or methanoic acid with pH ranged from 2.5 to 7.0, along with altered gradient pattern were tested for optimum MS responses and chromatographic retention of the analytes. At the chosen mass spectrometry conditions, the predominately protonated molecule ions were obtained by the positive ESI scan. The most sensitive ion transition m/z 330–181 was selected for the monitoring of sinomenine, which was different from the transition (m/z 330 → 255 → 223) chosen by Yao et al. [22]. This was perhaps due to the different fragmentation mechanisms between the two mass analyzers.

Several liquid–liquid extractions or protein precipitation procedures [14–22] were exploited for plasma sample preparation. Acetonitrile, methanol and 7% perchloric acid were chosen and tested for protein precipitation because of high throughput characteristics and the suitable sensitivities obtained. However, the chromatographic peak shapes and retentions of sinomenine and the IS were easily affected by the strong acidity of perchloric acid or elution ability of acetonitrile. Finally, methanol was selected as the protein precipitation reagent for plasma sample pretreatment for it was compatible with and also one of the ingredients of the mobile phase.

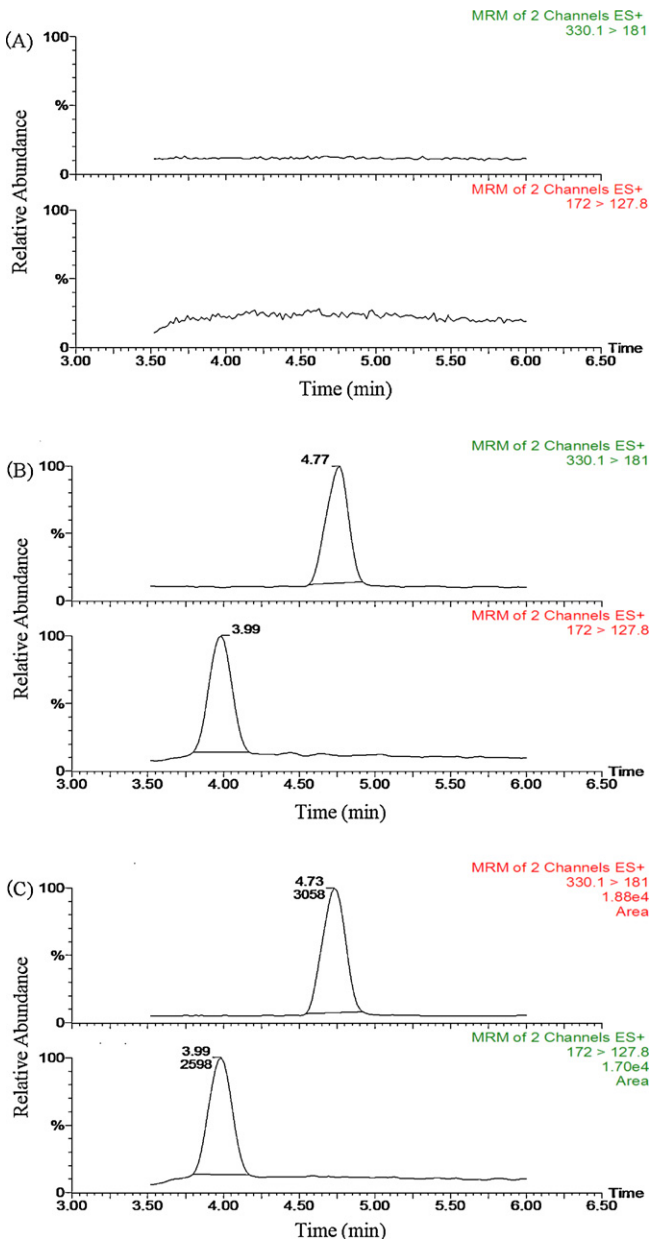


Fig. 2. Chromatograms by selective reaction monitoring (SRM): (A) blank plasma (sinomenine and IS free); (B) blank plasma spiked with sinomenine (20 ng/mL, t_R = 4.77 min) and IS (40 ng/mL, t_R = 3.99 min); (C) plasma sample of a subject 2 h after oral administration of 60-mg sinomenine hydrochloride sustained-released capsules (sinomenine 39.17 ng/mL, t_R = 4.73 min).

3.2. Method validation

3.2.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma samples with the corresponding spiked plasma samples. As shown in Fig. 2, no interference from endogenous substances was observed for both sinomenine and IS.

3.2.2. Linearity and LLOQ

The best linear fit and least-squares residuals for the calibration curve over the concentration range 0.5–500 ng/mL were achieved with a weighing factor of $1/x^2$, giving a mean linear regression equation for the calibration curve as $y = 0.02160x + 0.01905$ ($r = 0.996$), where x was the concentration of the analyte and y was the peak area ratio of the analyte to IS. The precision and accuracy of LLOQ (0.5 ng/mL) was 6.4% (RSD) and 94.0%, respectively. The limit of detection (LOD) for sinomenine was about 0.05 ng/mL.

Table 1
Stability data of short-term, long-term, post-preparative, freeze–thaw and stock solutions (n = 3).

Sample type	Concentration spiked (ng/mL)	C found (mean, ng/mL)		
		2 h	4 h	8 h
Short-term	2.5	2.48	2.27	2.46
	150	151.6	153.1	156.2
Sample type	Concentration spiked (ng/mL)	C found (mean, ng/mL)		
		30 days	60 days	90 days
Long-term	2.5	2.55	2.56	2.74
	150	150.2	153.4	157.6
Sample type	Concentration spiked (ng/mL)	C found (mean, ng/mL)		
		4 h	12 h	24 h
Post-preparative (4 °C)	2.5	2.82	2.62	2.80
	150	154.7	152.2	155.2
Sample type	Concentration spiked (ng/mL)	C found (mean, ng/mL)		
		2 h	4 h	8 h
Post-preparative (25 °C)	2.5	2.60	2.48	2.45
	150	146.2	149.5	152.2
Sample type	Concentration spiked (ng/mL)	C found (mean, ng/mL)		
		1 cycle	2 cycles	3 cycles
Freeze–thaw	2.5	2.64	2.82	2.58
	150	148.2	145.1	149.7
Sample type	Concentration spiked (ng/mL)	C found (mean, ng/mL)		
			30 day	90 day
Stock solutions (4 °C)				
Sinomenine	500.8 μg/mL		498.3	498.4
Metronidazole (IS)	501.0 μg/mL		495.8	505.8

3.2.3. Precision and accuracy

The intra-batch precision for sinomenine at QC levels of 2.5, 20, 150 and 400 ng/mL were 3.5%, 3.7%, 2.7%, 6.2%, respectively, and those of inter-batch analysis were 8.9%, 5.4%, 3.1%, 6.9%, respectively, with an accuracy ranging from 93.0% to 115.1%. The results of the precision and accuracy of the proposed method were acceptable for clinical pharmacokinetics.

3.2.4. Recovery and matrix effect

The extraction recovery and matrix effect of sinomenine and IS were within 80–109%. In terms of matrix effect, all the ratios of A/B × 100% defined as in Section 2 were between 92% and 107%, therefore, there was no obvious matrix effect for both sinomenine and IS for this LC/MS/MS determination.

3.2.5. Stability

The stock solutions of sinomenine and IS were found to be stable at 4 °C for 90 days. The results from all stability tests presented in Table 1 demonstrated a good stability of sinomenine over all steps of the determination. The method was therefore proved to be feasible for routine analyses.

Table 2
Pharmacokinetic parameters in healthy male Chinese volunteers after a single- and BID multiple-doses oral administration at steady-state of the 60 mg sinomenine hydrochloride sustained-released capsules (mean ± SD, $n = 20$).

Parameters	Single-dose (day 1)	Multiple-dose (day 7)
C_{max} (ng/mL)	123 ± 22	279 ± 69*
C_{min} (at 12 h, ng/mL)	108 ± 21	250 ± 61
T_{max} (h)	7.9 ± 2.0	4.4 ± 3.6*
$t_{1/2}$ (h)	13.4 ± 1.6	15.1 ± 1.3*
MRT_{0-96} (h)	21.1 ± 2.6	21.2 ± 2.3
AUC_{0-96} (ng h/mL)	3032 ± 682	7333 ± 2096*
$AUC_{0-\infty}$ (ng h/mL)	3064 ± 620	7436 ± 2140*
CL (L/h)	20.6 ± 4.8	8.7 ± 2.2*
V_d (L)	370.3 ± 57.6	178.6 ± 59.3*
C_{av} (ng/mL)	–	240 ± 59
AUC_{ss} (ng h/mL)	–	2886 ± 707
DF		0.10 ± 0.12

* $P < 0.05$ versus single-dose.

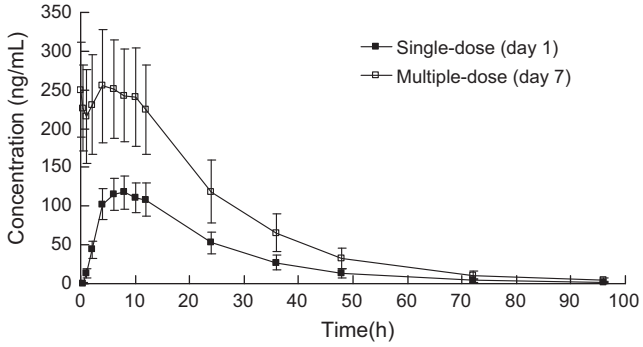


Fig. 3. Mean (SD) plasma concentration–time profiles after a single and twice-daily multiple doses oral administration at steady-state of the 60-mg sinomenine hydrochloride sustained-release capsules in healthy male Chinese volunteers ($n = 20$).

3.3. Application in pharmacokinetics study

The main pharmacokinetic parameters are presented in Table 2 and the mean plasma concentration–time curves are presented in Fig. 3.

The single-dose pharmacokinetics of orally administered conventional sinomenine hydrochloride has been characterized previously [14,15]. Sinomenine hydrochloride sustained-release capsules were developed to provide sustained plasma concentrations of sinomenine over a longer period of time than with conventional formulations. As expected, after a single oral dose administration, this sustained-release formulation demonstrated a significantly longer T_{max} (7.9 h) than those of the conventional forms (1.0 h [14], 2.3 h [15]). Meanwhile, the $t_{1/2}$ of 13.4 h was also numerically longer than values reported (3.6 h [15]). The values in the present study appeared to have been influenced by delayed absorption of the sustained-release formulation. Furthermore, the C_{max} values obtained from this study (123 ± 22 ng/mL) was approximately half compared with those reported for conventional formulations (246 ± 71 ng/mL [14]), suggesting a lower likelihood for the occurrence of adverse reactions that have been associated with higher plasma concentrations.

In the multiple-dose cases, the steady state valley concentrations on days 4, 5, 6, and 7 were 277.1 ± 63.6, 207.1 ± 45.8, 229.8 ± 54.5, and 249.8 ± 61.3, respectively, which demonstrated steady state was reached after four consecutive days twice-daily administration. Significant differences were found for all pharmacokinetic parameters between the single dose and the steady state. The $t_{1/2}$ was prolonged from 13.4 to 15.5 h; CL was decreased from 20.61 to 8.68 L/h. The AUC and C_{max} at steady state appeared to be about 2.5 times higher than those after a single dose, respectively. Hence, there was obvious accumulation with repeated dosing (accumulation factor about 2.49 ± 0.77). Therefore, clinical dosage regimen should be closely monitored and dose adjustment may be necessary when the drug is administered for long term. In addition, the T_{max} value on day 7 (4.4 h) was significantly shorter than that in the single-dose study (7.9 h); this was also due to the high steady-state average concentration (C_{av}, 240 ± 59 ng/mL).

4. Conclusions

The LC/MS/MS methods described in this paper achieved a LLOQ for sinomenine in human plasma as low as 0.5 ng/mL. Circumventing the complicated solvent extraction for plasma sample preparation, this method using just one simple step of protein precipitation procedures also offers advantages with much improved accuracy and precision, which are important for high throughout clinical investigations. The method has been fully validated and the assay performance results indicate that it is precise and accurate for routine determination of sinomenine in human plasma. The clinical pharmacokinetics results showed that the sinomenine hydrochloride sustained-release capsules could maintain adequate sinomenine levels in the plasma for at least 12 h. However, accumulation was observed after repeated dosing, therefore attentions should be given to the clinical dosage regimen.

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