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Determination of berberine in human plasma by liquid chromatography–electrospray ionization–mass spectrometry

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Abstract

A liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) method for the determination of berberine in human plasma using chlorobenzylidine as the internal standard (IS) has been developed and validated. The plasma samples were prepared by LLE and the analytes were chromatographically separated on a Hanbon Lichrospher 5-C18 HPLC column under gradient elution with a mobile phase consisted of acetonitrile and 10 mm ammonium acetate buffer containing 0.1% formic acid. Berberine was determined with electrospray ionisation–mass spectrometry (ESI–MS). LC–ESI–MS was performed in the selected-ion monitoring (SIM) mode using target ions at M^+ *m/z* 336.1 for berberine and M^+ *m/z* 464.1 for the IS. Calibration curve was linear over the range of 0.020–3.0 ng/ml. The lower limit of quantification (LLOQ) was 0.020 ng/ml. The intra- and inter-run variability values were less than 6.7 and 7.7%, respectively. The method has been successfully applied to determine the plasma concentration of berberine in healthy Chinese volunteers.
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Keywords: Berberine; LC–ESI–MS; Pharmacokinetics

1. Introduction

Berberine (Fig. 1A), a well-known alkaloid, was found in medicinal herbs such as *Coptis chinensis* and *Hydrastis Canadensis*. Currently, the predominant clinical uses of berberine preparations include the treatment of bacterial diarrhea, intestinal parasite infections and ocular trachoma infections [1]. Other pharmacological effects of berberine, such as antiarrhythmic [2], anti-inflammatory [3], anticancer [4], immunosuppressive [5], vasorelaxant and antiproliferative [6] have also been reported. Several methods have been reported for the qualitative and quantitative analysis of berberine in herbs and berberine preparations, which include thin-layer chromatography (TLC) [7], capillary electrophoresis coupled with laser-induced native fluorescence [8], capillary electrophoresis with electrospray mass spectrometry (CE-MS) [9] and ion-pair supercritical fluid chromatography (IP-SFC) on-line coupled with ion-pair supercritical fluid extraction (IP-SFE) [10]. The methods reported for the determination of berberine in animal biological specimen include TLC [11], HPLC [12–14] and LC–ESI–MS [15] methods, in which the most sensitive method is LC–ESI–MS with an LLOQ of 0.31 ng/ml. Recently, Zuo et al. [16] reported an more sensitive LC/MS–MS method for the determination of berberine in rat plasma with an LLOQ of 0.05 ng/ml. Though there are almost one hundred of papers published about the determination of berberine, very few methods are reported for the determination of berberine in human plasma. Miyazaki et al. [17] reported a GC–MS method with an LLOQ of 1 ng/ml for the determination of berberine in human urine. Yu et al. [18] developed an HPLC method for the determination of berberine in human urine and plasma, in which the limit of detection (LOD) was 0.1 ng/ml. In this paper, they reported a valuable data obtained in their pilot study that the steady-state concentration of berberine in human plasma was about 0.3 ng/ml after an oral multi-dose of 300 mg berberine per day for 7 days. The pilot pharmacokinetic study results in our laboratory showed that the C_{max} of berberine in human plasma was about 0.4 ng/ml and the concentration levels of berberine on the terminal elimination phase were below 0.1 ng/ml after the single oral dose of 400 mg berberine. So, to evaluate the pharmacokinetics of

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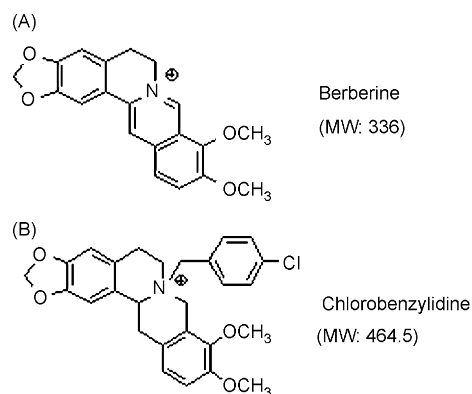

Fig. 1. Chemical structures of berberine (A) and chlorobenzylidine (B).

berberine in human, a more sensitive method for the determination of berberine in human plasma is required. The objective of this study is to develop and validate an LC–ESI–MS method for the measurement of berberine in human plasma in support of the clinical investigations. In this study, we established a more sensitive method with the linear range of 0.020–3.0 ng/ml. The method was fully validated for its accuracy, precision, recovery, stability studies and matrix effect, and successfully applied to study the pharmacokinetics of berberine in healthy volunteers.

2. Experimental

2.1. Materials and reagents

Berberine hydrochloride was purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Chlorobenzylidine hydrochloride was obtained from Medicinal Chemistry Research Center of China Pharmaceutical University. The test berberine hydrochloride tablet containing 0.1 g berberine per tablet was provided by Yunnan Gulin Pharmaceutical Co., Ltd. (Yunnan, China). And the reference berberine hydrochloride tablet containing 0.1 g berberine per tablet was provided by Guangdong Huanan Pharmaceutical Co., Ltd. (Guangdong, China). Acetonitrile of HPLC grade was purchased from Merck KGaA (Darmstadt, Germany). Ethyl acetate, ammonium acetate and formic acid were analytical grade purity and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrument and conditions

LC–ESI–MS analyses were performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA) with a Hanbon Lichrospher 5-C18 HPLC column, 5 μm, 250 mm × 4.6 mm i.d. (Jiangsu Hanbon Science & Technology Co., Ltd., China). The LC–ESI–MS was controlled by a computer employing the HP Chemstation software (10.02 A) supplied by Agilent. The mobile phase consisted of A (acetonitrile) and B (10 mm ammonium acetate solution containing 0.1% formic acid) was used under gradient elution. The gradient cycle consisted of an initial 5.5 min isocratic segment (39% A and 61% B). Then, the gradient was started, solvent A was increased to 80% within 0.2 min and maintained from 5.7 to 10 min. After changing solvent A back to 39% at 10.2 min, the mobile phase gradient was maintained at this composition from 10.2 to 12 min for column equilibration. The flow rate was 1 ml/min during the whole gradient cycle. The column temperature was kept at 25 °C. A quadrupole mass spectrometer equipped with an electrospray ionization source was set with a drying gas (N_2) flow of 12 l/min, nebulizer pressure of 40 psi, drying gas temperature of 350 °C, capillary voltage of 3 kV and the positive ion mode. The fragmentor voltage was 160 V. LC–ESI–MS was performed in selected-ion monitoring (SIM) mode using target ions at M^+ m/z 336.1 for berberine and M^+ m/z 464.1 for the IS. The typical full-scan ESI mass spectrum of berberine and IS was showed in Fig. 2(A and B).

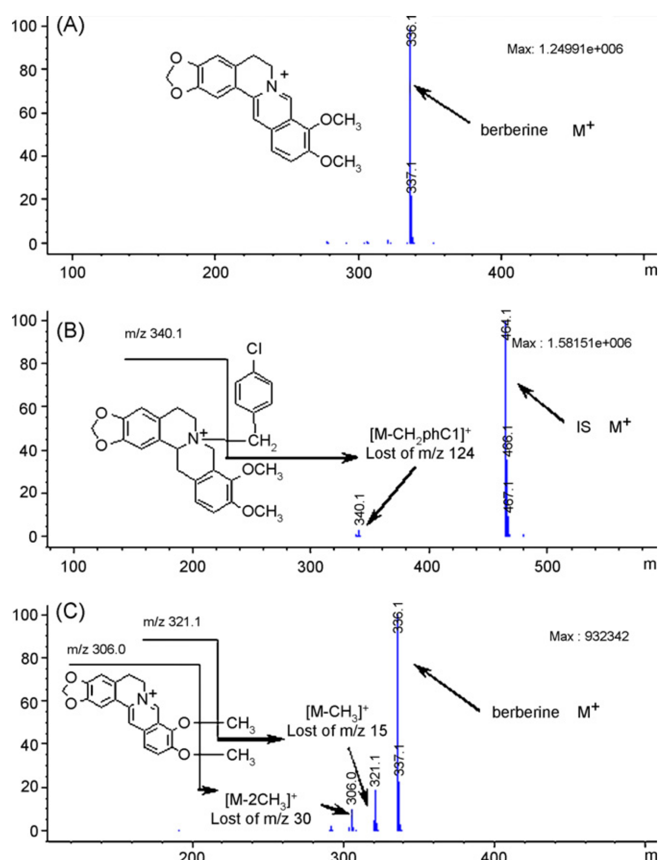

Fig. 2. Mass spectra of the positive ions of berberine (A) and IS (B) at 160 V, berberine (C) at 250 V fragmentor voltage.

2.3. Preparation of stock and working solutions

The stock solution of berberine (1.0 mg/ml) and the IS (0.5 mg/ml) were prepared in acetonitrile and stored at −20 °C. Standard solution of berberine with concentrations of 100.0 μg/ml, 10.0 μg/ml, 1.0 μg/ml, 100.0 ng/ml, 10.0 ng/ml and 1.0 ng/ml, were made by serial dilution of the berberine stock solution with acetonitrile in separate 10 ml volumetric flasks. A solution containing 100.0 ng/ml IS was also obtained by further

dilution of IS stock solution with acetonitrile. All the solutions were stored at −20 °C.

2.4. Sample preparation

A 1-ml aliquot of human plasma sample was mixed with 80 μl of the IS working solution (100.0 ng/ml). Then, 5 ml ethyl acetate was added and the mixture was vortex-mixed for 3 min. The sample was centrifuged at 16,000 rpm for 8 min. The organic phase was separated and evaporated to dryness under a nitrogen stream in a water bath of 40 °C. The residue was reconstituted in 120 μl of mobile phase (39% A and 61% B) and a 30-μl aliquot was injected into the LC–ESI–MS system for analysis.

2.5. Preparation of calibration curves and quality control samples

Calibration standards of berberine were prepared by spiking appropriate amounts of the working solutions in 1 ml blank plasma obtained from healthy volunteers. Standard curves were prepared in the range of 0.020–3.0 ng/ml for berberine at concentrations of 0.020, 0.050, 0.10, 0.30, 1.0, 2.0, 3.0 ng/ml. The calibration curves were prepared and assayed along with quality control (QC) samples and each batch of clinical plasma samples. The QC samples were prepared in 1 ml blank plasma at concentrations of 0.030, 0.30 and 2.5 ng/ml for berberine.

3. Results and discussion

3.1. Method development and optimization

The goal of this study was to develop and validate a sensitive method for the extraction and quantification of berberine in human plasma, which is suitable for determination of pharmacokinetics of berberine in clinical study. To achieve the goal, during method development, different options were evaluated to optimize MS parameters, chromatography condition and sample extraction method.

3.1.1. Mass spectrometry

A positive ion-monitoring mode was adopted in the LC–ESI–MS assay, for berberine is a basic compound. In order to select the target ion for monitoring berberine, the ESI mass spectra obtained by the scan monitoring at different fragmentor voltage were investigated. The test results showed that, though the intensity of fragment ions at *m*/*z* 321.1 and *m*/*z* 306.0 (Fig. 2C) became higher and higher as the increasing of the fagmentor voltage, the base peak (the highest ion peak in the mass spectrum, which can be selected as the target ions of the analytes) in the mass spectra of berberine obtained at different fragmentor voltages was of the same ion at *m*/*z* 336.1, which was the molecular ion M^+ of berberine. Therefore, the molecular ion M^+ at *m*/*z* 336.1 was selected as the target ion for berberine in the SIM. Fig. 2(A) shows a typical mass spectrum of the positive ions of berberine at 160 V fragmentor voltage obtained by the scan monitoring. In order to determine the optimal fragmentor voltage, the intensities of the molecular ion of berberine M^+ at *m*/*z* 336.1 were compared at the fragmentor voltages of 50, 80, 100, 110, 120, 130, 150, 160, 180, 200 and 250 V. The result showed that the highest sensitivity was obtained using a fragmentor voltage of 160 V. So, the fragmentor voltage was set at 160 V in the ESI–MS assay for berberine. At this fragmentor voltage, the base peak in the mass spectrum of IS was at M^+ *m*/*z* 464.1 that was the molecular ion of the IS (Fig. 2B). Therefore, the positive ion M^+ *m*/*z* 464.1 of the IS was selected as the target ion in the SIM for the IS. At this fragmentor voltage, when nebulizer pressure was set at 40 psi, drying gas (N_2) flow was set at 12 l/min with drying gas temperature set at 350 °C, the highest sensitivity of the assay could be achieved.

3.1.2. Chromatography and extraction method

Chlorobenzylidine was chosen as the internal standard because it is structurally similar to berberine, is well resolved from the berberine and other peaks, and mimics the berberine in any sample preparation steps. Because of their alkalinity, berberine and IS would easily appear as tailing peaks in their chromatograms. Several experiments show that the employment of an appropriate ratio of ammonium acetate buffer solution in the mobile phase may improve the chromatographic peak shapes [19–22]. So, the different concentrations of ammonium acetate buffer solution at levels of 10, 20 and 40 mm were tested in the mobile phase. The test results showed that the 10 mm ammonium acetate buffer was sufficient enough to improve the chromatographic peak shapes of berberine and the IS, and result in the symmetric chromatography peaks of the analytes. The test result showed that the weak acidic condition of the mobile phase is beneficial to the separation of berberine with the endogenous interference compounds from the plasma. Therefore, different concentrations of formic acid at levels of 0.05, 0.1 and 0.2% were tested in the mobile phase. Finally, good separation of target compounds was obtained with a mobile phase of acetonitrile (A) and 10 mm ammonium acetate solution containing 0.1% formic acid (B) (39:61, v/v). However, the retention time for the IS was as long as 15.5 min at this chromatography condition. So, the gradient elution was essential to reduce the run time. Finally, a gradient elution was developed for the assay. The gradient cycle consisted of an initial 5.5 min isocratic segment (39% A and 61% B). Then, the gradient was started, solvent A was increased to 80% within 0.2 min and maintained from 5.7 to 10 min. After changing solvent A back to 39% at 10.2 min, the mobile phase gradient was maintained at this composition from 10.2 to 12 min for column equilibration. The flow rate was 1 ml/min during the whole gradient cycle. After maintaining the mobile phase of acetonitrile (A) and 10 mm ammonium acetate solution containing 0.1% formic acid (B) (39:61, v/v) for the initial 5.5 min, acetonitrile was increased to 80% within 0.2 min and maintained for 4.3 min, the retention time for the IS could be reduced to 9.2 min. At the same time, since the ratio of acetonitrile was increased to 80%, the background noise could be reduced and the endogenous peak interference could also be avoid in the next injection. The last 1.8 min of the run was long enough to restore the chromatography system to the initial eluting condition prior to next injection.

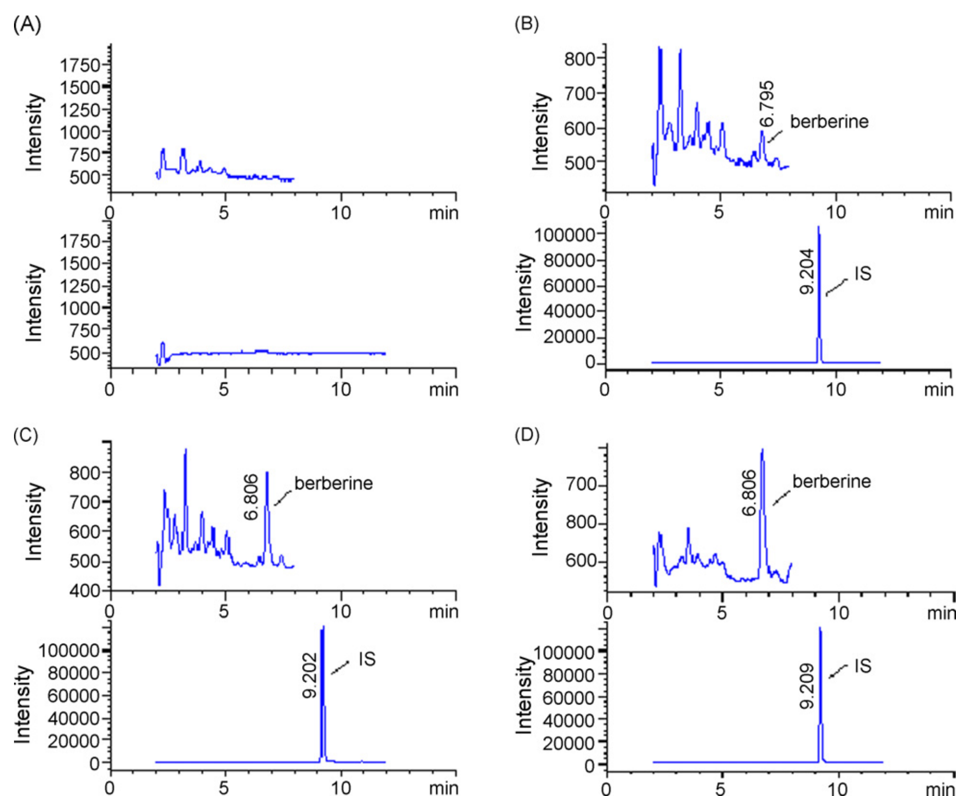

Fig. 3. Typical chromatogram of blank plasma (A), LLOQ for berberine in plasma (0.02147 ng/ml) and IS (B), blank plasma spiked with berberine (0.096 24 ng/ml) and IS (C), plasma obtained from a healthy volunteer at 1 h after an oral dose of 400 mg berberine test tablets, the plasma concentration of berberine was estimated to be 0.08593 ng/ml (D).

The most widely employed biological sample preparation techniques currently are liquid–liquid extraction (LLE), LLE cannot only purify but also concentrate the sample. The plasma samples containing berberine were prepared by LLE procedure. Several solvents were tested for the extraction. Finally, ethyl acetate was chosen as the extraction solvent that can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for berberine from the plasma.

3.2. Method validation

3.2.1. Selectivity

The typical chromatograms of blank plasma, LLOQ for berberine in plasma sample (0.02147 ng/ml), blank plasma spiked with berberine (2.116 ng/ml) and IS, and a plasma sample from a healthy volunteer were presented in Fig. 3. The selectivity of the assay was evaluated by analyzing six different batches of blank human plasma. All samples were found to have no interference at the retention times of the berberine or the IS.

3.2.2. Linearity of calibration curve and lower limit of quantification

Calibration standards of seven berberine concentration levels at 0.020, 0.050, 0.10, 0.30, 1.0, 2.0, 3.0 ng/ml were extracted and assayed. The berberine calibration curve was constructed by plotting the peak-area ratios of berberine to the IS versus the concentrations of berberine, using weighed least squares linear regression (the weighing factor was 1/C). Eight calibration curves, which related the concentrations of berberine to the area ratio of berberine to IS, showed good linearity over the range of 0.020–3.0 ng/ml.

The LLOQ was defined as the lowest concentration on the calibration curve at which the standard deviation was within 20% and accuracy was within ±20% [23], and it was established using five samples independent of standards. The LLOQ for berberine was 0.020 ng/ml. Assay precision was calculated using the relative standard deviation (R.S.D. %). The accuracy is the degree of closeness of the determined value to the nominal true value under prescribed conditions. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (R.E. %). It was calculated by using the formula: R.E. % = $(E - T)/T \times 100$. The R.S.D. % and R.E. % of the LLOQ of the assay are showed in Table 1. Those data show that the

Table 1
Accuracy and precision for the analysis of LLOQ ($n = 5$)

Concentration added (ng/ml)	Concentration found (ng/ml)	Mean (ng/ml)	R.S.D. (%)	R.E. (%)
0.01940	0.02164	0.02092	9.1	11.6
0.01940	0.01860			−4.1
0.01940	0.01917			−1.2
0.01940	0.02277			17.4
0.01940	0.02242			15.6

Note: R.S.D., relative standard deviation; R.E., relative error; number of replicates.

Table 2
Intra- and inter-run precision and accuracy for the analysis of berberine ($n = 15$)

Concentration added (ng/ml)	Mean concentration found (ng/ml)	R.E. (%)	Intra-assay R.S.D. (%)	Inter-assay R.S.D. (%)
0.02910	0.02942	1.1	6.7	7.7
0.2910	0.2765	−5.0	4.0	5.9
2.425	2.426	0.1	4.5	5.6

method is sensitive enough for pharmacokinetic study of berberine.

3.2.3. Precision and accuracy

Validation samples were prepared and analyzed on 3 consecutive days (one run per day) to evaluate the accuracy and the intra- and inter-run precision of the analytical method. The accuracy as well as the intra- and inter-run precision of the method was determined by analyzing five replicates at 0.030, 0.30 and 2.5 ng/ml of berberine along with one standard curve on each of 3 days. These QC samples were also assayed along with clinical samples in each run to monitor the performance of the assay and to assess the integrity and validity of the results of the unknown clinical samples analyzed. The intra- and inter-run precision and accuracy are summarized in Table 2. The standard deviation was calculated by using one-way-ANOVA. The results in Table 2 demonstrate that the precision and accuracy of the assay are within the acceptable range [23] and the method is accurate and precise.

3.2.4. Recovery and stability

The extraction recovery of berberine was evaluated by analyzing five replicates at 0.030, 0.30 and 2.5 ng/ml of berberine. Recovery was calculated by comparison of the peak areas of berberine extracted from plasma samples with those of injected standards. Ethyl acetate was chosen as the extraction solvent for its higher extraction efficiency to the two target compounds. The recovery values of berberine from human plasma with ethyl acetate, determined at three concentrations of 0.030, 0.30 and 2.5 ng/ml were 84.6 ± 11.6, 77.6 ± 6.6 and 68.9 ± 4.9% ($n = 5$), respectively.

The stability of berberine in plasma was studied under a variety of storage and handling conditions at low (0.030 ng/ml) and high (2.5 ng/ml) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low- and high-concentration samples that were thawed at room temperature and kept at this temperature for 6 h. Berberine in the reconstituted solution was found to be stable in the autosampler at 10 °C up to 10 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. Three aliquots at each of the low- and high-concentrations were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low- and high-concentrations stored at −20 °C for a month. The results in Table 3 showed that no significant degradation occurred after being kept at room temperature for 6 h and during the three freeze–thaw cycles for the berberine plasma samples. Berberine in plasma at −20 °C was stable for at least 1 month.

3.2.5. Matrix effect

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [23]. In set 1, analytes were resolved in the blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution). In set 2, analytes were resolved in mobile phase. The matrix effect of the method was determined by comparing the peak areas of set 1 (A) and set 2 (B). ME was calculated by using the formula: ME (%) = A/B × 100. The matrix effect of the method was evaluated at three concentration levels of 0.030, 0.30 and 2.5 ng/ml, and five samples at each level were analyzed. The blank plasma samples used in this study were from five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an exogenous matrix effect is implied. The data in Table 4 showed there was no matrix effect of the analytes observed in this study.

3.3. Applications

The method described above was successfully applied to the bioequivalence study of berberine in 20 healthy Chinese male volunteers. The Ethics Committee of Hospital of the Kunming Military Medical University approved the clinical pharmacokinetic study. All volunteers gave written informed consent to participate in the study according to the principles of the Decla-

Table 3
Stability data of berberine in human plasma under various storage conditions ($n = 3$)

Storage conditions	Concentration added (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	R.E. (%)
Room temperature (6 h)	0.02910	0.03011	7.8	3.5
	2.425	2.347	4.6	−3.2
Three freeze–thaw cycles	0.02910	0.03087	5.3	6.1
	2.425	2.485	6.8	2.5
Autosampler (10 °C for 10 h)	0.02910	0.03165	2.5	8.8
	2.425	2.237	3.1	−7.8
One month at −20 °C	0.02910	0.03133	5.1	7.7
	2.425	2.359	0.7	−2.7

Table 4
The matrix effect data of berberine and IS in human plasma ($n = 5$)

Sample	Concentration added (ng/ml)	Peak area				Matrix effect (%)
		Set 1		Set 2		
		Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)	
Berberine	0.02910	4920 ± 269	5.5	5077 ± 307	6.1	96.9
	0.2910	33090 ± 3360	10.2	32948 ± 2506	7.6	100.4
	2.425	255006 ± 11718	4.6	258158 ± 6092	2.4	98.8
IS	5.250	1521484 ± 81093	5.3	1452696 ± 70879	4.9	104.7

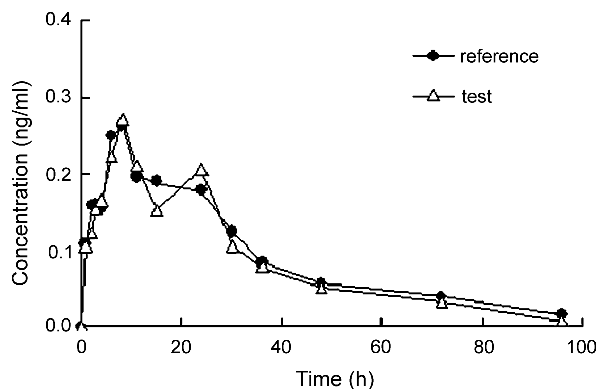

Fig. 4. Mean berberine plasma concentration–time profiles in 20 healthy volunteers after a single oral dose of 400 mg test and reference tablets ($n = 20$).

ration of Helsinki. Plasma samples were periodically collected up to 96 h after single oral administrations of 400 mg test and reference tablets to 20 healthy volunteers in each phase. Blood was sampled pre-dose and at 1, 2, 3, 4, 6, 8, 11, 15, 24, 30, 36, 48, 72 and 96 h post-dose for determination of plasma concentration of berberine. The mean plasma concentration–time curves of test and reference formulations in 20 healthy volunteers are shown in Fig. 4. The method was sensitive enough to monitor the berberine plasma concentration up to 96 h. Calibration curves were prepared and analyzed with each run of clinical samples and QC samples, the precision and accuracy for the QC samples were well within the acceptable limits. The main pharmacokinetic parameter values of the test and reference formulations are presented in Table 5. The relative bioavailability of test preparation was 94.4 ± 12.6%, based on the test-reference ratios of $AUC_{0–96}$. These pharmacokinetic parameters confirm the bioequivalence of the 400 mg test and reference formulations in terms of the rate and extent of absorption. After 400 mg oral dose of berberine, the mean maximum plasma concentration (C_{max}) in twenty volunteers was about 0.4 ng/ml. This result is comparable with that reported in the reference 18, in which Yu et al. [18] described that the steady-state plasma concentration of berberine in human plasma was about 0.3 ng/ml after an oral multi-dose of 300 mg berberine per day for 7 days. These results indicate that the absolute bioavailability of oral administration of berberine is extremely low. This characteristic of berberine is also demonstrated by Zuo et al. [16] in rats. In fact, the low bioavailability is a common characteristic of the drugs containing the quaternary ammonium groups in their structures.

Table 5
The main pharmacokinetic parameters of berberine in 20 healthy volunteers after a single oral dose of 400 mg test and reference tablets ($n = 20$)

PK parameters	Mean ± S.D.	
	Test	Reference
C_{max} (ng/ml)	0.4356 ± 0.4194	0.4171 ± 0.2792
$t_{1/2}$ (h)	28.6 ± 9.5	31.8 ± 12.2
t_{max} (h)	9.8 ± 6.6	9.6 ± 5.2
$AUC_{0–96}$ (h ng/ml)	7.835 ± 3.743	8.461 ± 4.180
$AUC_{0–\infty}$ (h ng/ml)	9.179 ± 3.767	9.803 ± 4.322

4. Conclusions

In the study presented here, we report the development and validation of an LC–ESI–MS method for measurement of berberine in human plasma. Validation results demonstrated that this method is specific, reproducible and reliable. The basic underlying advantage of this method is that the LLOQ for berberine is as low as 0.020 ng/ml. No significant interferences and matrix effect caused by endogenous compounds were observed. The method is suitable for pharmacokinetic study of berberine in human subjects.

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