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Simultaneous determination of berberine and palmatine in rat plasma by HPLC–ESI-MS after oral administration of traditional Chinese medicinal preparation Huang-Lian-Jie-Du decoction and the pharmacokinetic application of the method

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Abstract

A sensitive and specific liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method has been developed and validated for the identification and quantification of berberine and palmatine in rat plasma. After the addition of the internal standard (IS) and alkalization with 0.5 M sodium hydroxide solution, plasma samples were extracted by ethyl ether and separated by HPLC on a Shim-pack ODS (4.6 μm, 150 mm × 2.0 mm i.d.) column using a mobile phase composed of A (0.08% formic acid and 2 mmol/l ammonium acetate) and B (acetonitrile) with linear gradient elution. Analysis was performed on a Shimadzu LC/MS-2010A in selected ion monitoring (SIM) mode with a positive electrospray ionization (ESI) interface. $[M]^+ = 336$ for berberine; 352 for palmatine and $[M + H]^+ = 340$ for IS were selected as detecting ions, respectively. The method was validated over the concentration range of 0.31–20 ng/ml for berberine and palmatine. Inter- and intra-CV precision (R.S.D.%) were all within 15% and accuracy (%bias) ranged from −5 to 5%. The lower limits of quantification were 0.31 ng/ml for both analytes. The extraction recovery was on average 68.6% for berberine, 64.2% for palmatine. The validated method was used to study the pharmacokinetic profile of berberine and palmatine in rat plasma after oral administration of Huang-Lian-Jie-Du decoction.
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Keywords: Berberine; Palmatine; LC–ESI-MS; Huang-Lian-Jie-Du decoction; Pharmacokinetics; Traditional Chinese medicine

1. Introduction

Traditional Chinese medicine (TCM) is the natural therapeutic agent used under the guidance of the theory of traditional Chinese medical sciences, which has played an indispensable role in the prevention and treatment of diseases in China. In clinical application, most herbal medicines are prescribed in combination to obtain the synergistic effects or to diminish the possible adverse reactions.

Huang-Lian-Jie-Du decoction is the extract of *Coptidis rhizoma*, *Scutellariae radix*, *Phellodendri cortex* and *Gardeniae fructus*. In the clinical practice of TCM, the decoction has been used to treat gastrointestinal disorders, inflammation, liver disease, hypertension and cerebrovascular disease over a very long period of time [1].

The quaternary protoberberine-type alkaloids berberine and palmatine (structures shown in Fig. 1) are the main active components of *C. rhizome*, *P. cortex*. Despite the extensive literature on the pharmacology of berberine and palmatine, little information is available relating to their pharmacokinetics.

Development of a sensitive method to determine berberine and palmatine in biological fluids was a prerequisite to the pharmacokinetic evaluation. Earlier publications have described methods for analysis of berberine in biological samples using HPLC-UV [2–9], HPLC-fluorimetry [10], field desorption-mass spectrometry [11] and GC–chemical ionisation-mass spectrometry [12]. The poor absorption and extensive metabolism [8,10,13] caused extremely low plasma concentration of berberine (ng/ml level) after oral administration to rat, Beagle dog and human [2,3,5,7]. To our knowledge, the above methods were

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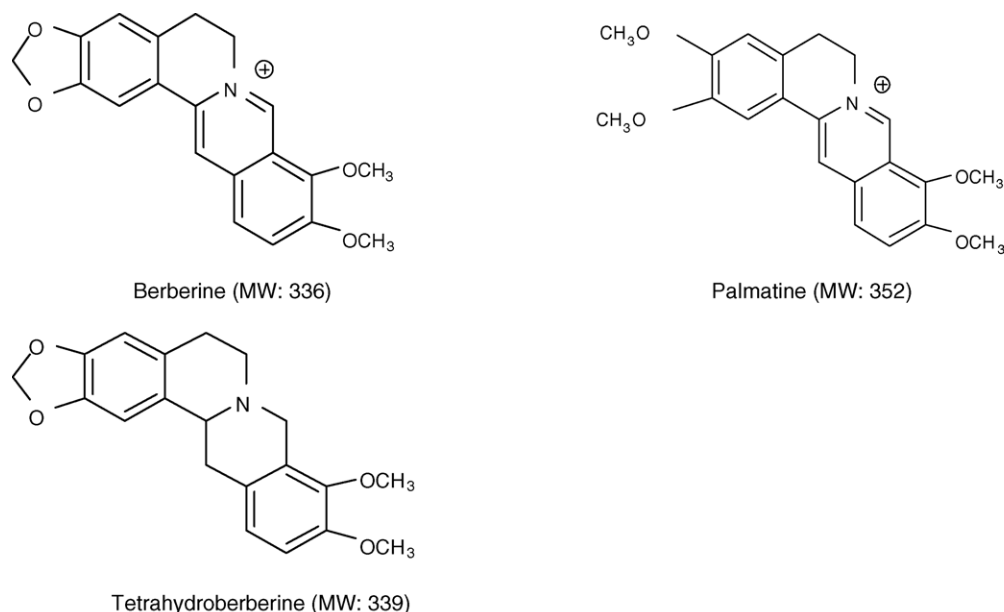

Fig. 1. Chemical structures of berberine, palmatine and internal standard tetrahydroberberine.

not sensitive enough for oral pharmacokinetic study of berberine and there was no report about the pharmacokinetic behavior of palmatine so far. In this paper, a selective and sensitive liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method is presented for the simultaneous determination of berberine and palmatine in rat plasma after oral administration of Huang-Lian-Jie-Du decoction.

2. Experimental

2.1. Herbal materials

Huanglian (*Coptis chinensis* French.), Huangqin (*Scutellaria baicalensis* Georgi.), Huangbai (*Phellodendron amurense* Rupr.), Zhizi (*Gardenia jasminoides* Ellis.) were purchased from Kai-xin Pharmacy (Nanjing, China) and identified by Dr. Li-Na Chen (Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China).

2.2. Chemicals and reagents

The reference standards of berberine and palmatine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The internal standard (IS), tetrahydroberberine (structure in Fig. 1) was kindly provided by Dr. Can Zhang (Department of Medicinal Chemistry, China Pharmaceutical University, Nanjing, China). Acetonitrile was of chromatographic grade (Fisher Company Inc., USA). All other reagents were of analytical grade. Milli-Q water (Millipore, Bedford, MA) was used throughout the study.

2.3. Preparation of Huang-Lian-Jie-Du decoction extract

Huanglian 30 g, Huangqin 20 g, Huangbai 20 g, Zhizi 30 g were extracted twice by refluxing with boiling water (1:10 and then 1:5, w/v) for 1 h, and the solution obtained was concentrated to give an extract (21.9 g). The dried powder was stored at 4 °C before use.

2.4. Content of berberine and palmatine in Huang-Lian-Jie-Du decoction extract

To calculate the administered dose, the contents of berberine and palmatine in the extract were quantitatively determined. One hundred milligrams of the dry powder was extracted by 100 ml of 1:100 mixture of hydrochloric acid and methanol at 60 °C for 15 min, ultrasounded for 30 min and then diluted 100 times. The mixture was centrifuged for 10 min at 15,000 rpm. Twenty microliters of the supernatant was injected into the HPLC system. The HPLC analysis of the berberine and palmatine was a modified version of a previously published method [14]. The contents of berberine and palmatine were 5.6 and 1.36 g/100 g extract, respectively.

2.5. Instrumentation

The LC/MS system consisted of a Shimadzu LC-10AD HPLC series liquid chromatograph and a Shimadzu LC/MS-2010A single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface and a Q-array-Octapole-Quadrupole mass analyzer. Shimadzu LCMS solution Version 2.02 was used for data acquisition and processing.

2.6. Chromatographic conditions

LC separation was achieved using a Shim-pack ODS (4.6 μm, 150 mm × 2.0 mm i.d. Shimadzu) column maintained at 40 °C. The mobile phase consisted of A (0.08% formic acid and 2 mmol/l ammonium acetate) and B (acetonitrile) with linear gradient elution. The gradient cycle consisted of an initial 3 min

isocratic segment (70% A and 30% B). Then, the linear gradient was started, increasing solvent B to 80% within 0.5 min and maintained from 3.5 to 5.5 min. After changing back to 30% solvent B at 6 min, the mobile phase gradient was maintained at this composition from 6 to 8 min for column equilibration. The initial 1.5 min was switched to the waste. The flow rate was 0.2 ml/min during the whole gradient cycle.

2.7. Mass spectrometer conditions

The effluent from the HPLC column was directed into the ESI probe. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The curve dissolution line (CDL) temperature and the block temperature were maintained at 250 and 200 °C, respectively. The probe voltage (capillary voltage), CDL voltage and detector voltage were fixed at 4.5 kV, −10 V and 1.65 kV, respectively. Vacuum was obtained by a Turbo molecular pump (Edwards 28, UK). Liquid nitrogen (99.995%, Nanjing University, China) was used as the source of nebulizer gas (1.5 l/min) and drying gas (curtain gas) (4.0 l/min). Analytes were quantitated in selected ion monitoring (SIM) mode. $[M]^+ = 336$ for berberine; 352 for palmatine and $[M+H]^+ = 340$ for IS were selected as detecting ions. Mass spectra were obtained at a dwell time of 0.2 s in SIM and 1 s in scan mode.

2.8. Preparation of standard solutions

Stock solutions of the analytes and IS were prepared by dissolving 10.0 mg of each authentic samples in 10 ml methanol producing a concentration of 1.0 mg/ml and were stored at 4 °C.

Working solution of the analytes was prepared at concentrations of 3.12, 6.25, 12.5, 25, 50, 100 and 200 ng/ml by diluting the stock solutions with methanol. A solution containing 200 ng/ml IS was also prepared in methanol.

2.9. Calibration curves and quality control samples

The samples for standard calibration curves were prepared by spiking the blank rat plasma (100 μl) with 10 μl of the appropriate working solutions to yield the following concentrations: 0.31, 0.625, 1.25, 2.5, 5, 10 and 20 ng/ml. Quality control (QC) samples were prepared from blank plasma at concentrations of 0.625, 2.5 and 10 ng/ml.

2.10. Sample preparations

Plasma samples, calibration standards and QC samples were extracted employing a liquid–liquid extraction technique. Plasma samples were removed from −20 °C storage and immersed in a 37 °C water bath for 5 min to thaw. After vortexing, 100 μl plasma samples were extracted with 1.25 ml ethyl ether after addition of 10 μl IS and 50 μl 0.5 M sodium hydroxide solution. Following centrifugation and separation, the organic phase (1 ml) was evaporated to dryness in a 50 °C water bath. The residue was reconstituted with 100 μl mobile phase. An aliquot of 10 μl was injected into the LC–MS system.

3. Application to pharmacokinetic study

The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Five male Sprague–Dawley rats, weighing 250–280 g, were supplied by Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China). The rats were maintained in an air-conditioned animal quarter at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%, free access to water, and feeding with a laboratory rodent chow (Nanjing, China). The animals were acclimatized to the facilities for 5 days, and then fasted, free access to water for 12 h prior to experiment. Huang-Lian-Jie-Du decoction extract was dissolved in 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution and was administered to the rats (4.38 g extract/kg body weight, containing 245 mg berberine and 60 mg palmatine/kg body weight) by oral gavage. Blood samples (300 μl) were obtained from the oculi chorioideae vein before dosing and subsequently at 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 12 and 24 h following administration, transferred to a heparinized eppendorf tube and centrifuged at 5000 rpm for 10 min. The plasma obtained was frozen at −20 °C until analysis.

4. Data analysis

To determine the pharmacokinetic parameters of berberine and palmatine, the concentration–time data were analyzed by non-compartmental methods using the Bioavailability Program Package (BAPP, Version 2.0, Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University). C_{max} and t_{max} values were obtained directly from the observed concentration versus time data. All results were expressed as arithmetic mean ± standard deviation (S.D.).

5. Results and discussion

5.1. Optimization of LC–MS for quantitative analysis

The selection of mobile phase was a critical factor in achieving good chromatographic behavior (peak shape and resolution) and appropriate ionization. Modifiers such as formic acid and ammonium acetate alone or in combination in different concentrations were compared. The best peak shape and ionization were achieved using 2 mmol/1 ammonium acetate buffer, with pH adjusted to 3.5 with formic acid. Linear gradient elution was used to elute endogenous substances residue from the column.

In the positive ion scan mode, the molecular ions $[M]^+$ for berberine and palmatine and the protonated molecular ion [M + H] for IS were the most abundant ions. Therefore, these were used in the SIM acquisition.

Tetrahydroberberine were chosen as IS for its similarity with the analytes in structure (Fig. 1), chromatographic behavior and mass spectrographic behavior (Figs. 2–4).

The analytes and IS were easily extracted from plasma sample under alkaline conditions. Ethyl ether and ethyl acetate were compared and ethyl ether was adapted because of lower level of chemical noise of the sample and easier sample preparation. Fifty microliters of 0.5 M sodium hydroxide was added to basify

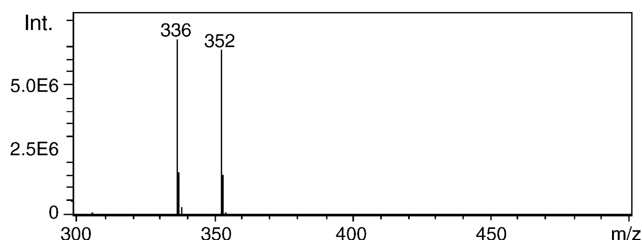

Fig. 2. Positive ion electrospray mass scan spectrum of berberine and palmatine.

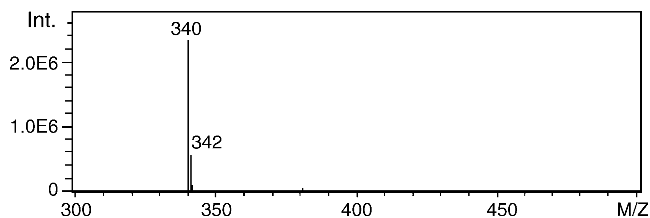

Fig. 3. Positive ion electrospray mass scan spectrum of tetrahydroberberine.

the sample to achieve reduced interference of acidic endogenous compounds.

5.2. Method validation

The method validation assays were carried out according to the currently accepted Chinese State Food and Drug Administration (SFDA) bioanalytical method validation guidance (2005.3). The validation experiments and results obtained are described below.

5.2.1. Selectivity

Assay selectivity was evaluated by analyzing blank plasma samples obtained from six rats. All samples were found to be free of interferences with the compounds of interest.

Positive ion electrospray mass scan spectra of the analytes and IS are shown in Figs. 2 and 3. $[M]^+ = 336$ for berberine; 352 for palmatine and $[M+H]^+ = 340$ for IS were chosen for quantification due to their high stability and intensity. Representative SIM chromatograms are shown in Fig. 4. The retention times of berberine, palmatine and IS were 4.4, 4.1, 4.0 min, respectively.

5.2.2. Linearity of calibration curves and lower limit of quantitation

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within ±15% bias of nominal concentration and precision not exceeding 15% CV, was 0.31 ng/ml for the analytes. The calibration curves were constructed using unweighted linear regression of the peak area ratio of the analytes to IS (*Y*) against the corresponding spiked plasma concentrations of the analytes (*X*,

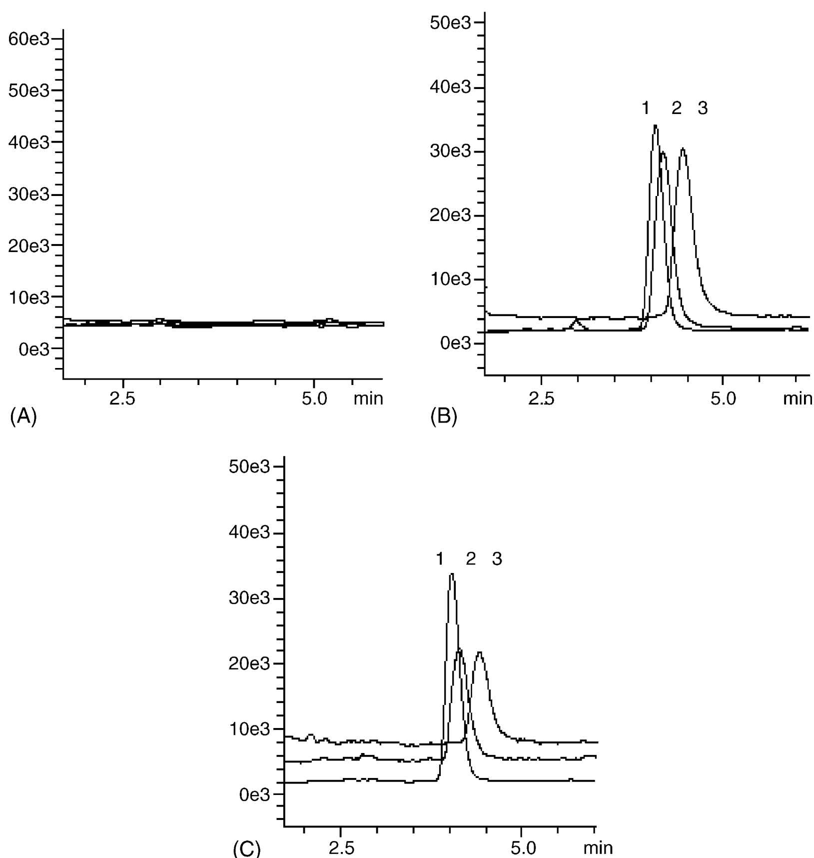

Fig. 4. SIM chromatograms of (A) blank plasma; (B) blank plasma spiked with berberine, palmatine (5 ng/ml) and tetrahydroberberine (20 ng/ml); (C) samples 3 h after oral administration of Huang-Lian-Jie-Du decoction. (1) Tetrahydroberberine; (2) palmatine; (3) berberine.

Table 1
Precision and accuracy of the determination of berberine and palmatine in rat plasma (inter-day $n = 5$; intra-day $n = 5 \times 3$)

Sample	Spiked concentration (ng/ml)	Measured concentration (mean ± S.D.)	R.S.D. (%)	Bias (%)
Berberine				
Inter-day	0.625	0.638 ± 0.061	9.56	−2.22
	2.5	2.52 ± 0.109	4.34	−0.89
	10	9.64 ± 0.58	5.88	−0.35
Intra day	0.625	0.614 ± 0.064	10.48	−1.71
	2.5	2.48 ± 0.078	3.14	−0.61
	10	10.28 ± 0.31	3.03	−2.85
Palmatine				
Inter-day	0.625	0.641 ± 0.025	3.98	−2.60
	2.5	2.424 ± 0.047	1.97	3.01
	10	9.87 ± 0.51	5.16	−1.29
Intra-day	0.625	0.641 ± 0.028	4.33	−2.61
	2.5	2.39 ± 0.051	2.11	4.16
	10	9.78 ± 0.33	3.36	2.15

ng/ml) over the range 0.31–20 ng/ml. The regression equations were $R = 0.0626C + 0.0132$ ($r = 0.9992$, $n = 5$) for berberine and $R = 0.0976C - 0.0016$ ($r = 0.9999$, $n = 5$) for palmatine. The concentrations in unknown samples were calculated using these calibration lines.

5.2.3. *Assay precision and accuracy*

Precision and accuracy of the assay were determined by replicate analyses ($n = 5$) of QC samples on the same day (intra-day) and also on 3 consecutive days (inter-day). The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentration (C_{obs}) as follows: bias (%) = $[(C_{obs} - C_{nom})/(C_{nom})] \times 100$. The intra- and inter-day precision and accuracy are summarized in Table 1. The results demonstrate that the precision and accuracy of this assay were acceptable.

5.2.4. *Extraction recovery*

The extraction recovery (absolute recovery) of analytes from rat plasma after the extraction procedure was assessed in quintuplicate by comparing the analyte/IS peak area ratio of extracted analytes (R_1) with those of blank plasma extracts spiked with standard solution (R_2). IS was spiked before extraction in both cases. QC samples at three concentrations were evaluated. The extraction recovery was expressed as $(1.25R_1/R_2) \times 100\%$. The data are shown in Table 2. The extraction recovery of the analytes was shown to be consistent and reproducible.

5.2.5. *Matrix effect*

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence ionization of the analyte) was examined by comparing the peak areas of the analytes and IS between two different sets of samples. In set 1, analyte standards were dissolved in the reconstitution solvent and analyzed at concentrations of 0.625, 2.5 and 10 ng/ml for the analyses and 20 ng/ml for the IS. These analyses were repeated five times at each concentration. In set 2, blank plasma samples obtained from five rats were extracted and then spiked with the same concentrations of analytes and IS in the reconstitution solvent. Deviation of the mean peak areas of set 2 versus set 1 would indicate the possibility of ionization suppression or enhancement for analyses and IS; this is called an ‘absolute’ matrix effect [15].

As shown in Table 3, no matrix effect was observed for the six blank plasma lots, indicating that the extracts were “clean” with no co-eluting “unseen” compounds that could influence the ionization of the analytes.

Table 2
Extraction recovery of berberine and palmatine in rat plasma ($n = 5$)

Sample	Spiked concentration (ng/ml)	R_1 (mean ± S.D.)	R_2 (mean ± S.D.)	Recovery (%) $1.25R_1/R_2$
Berberine	0.625	0.055 ± 0.004	0.101 ± 0.008	68.2
	2.5	0.173 ± 0.007	0.322 ± 0.035	67.1
	10	0.657 ± 0.02	1.168 ± 0.067	70.3
	0.625	0.069 ± 0.008	0.135 ± 0.007	64.2
Palmatine	2.5	0.268 ± 0.022	0.538 ± 0.044	62.4
	10	1.086 ± 0.093	2.035 ± 0.082	66.7

Table 3
Matrix effect evaluation of berberine, palmatine and IS in rat plasma ($n = 5$)

Sample	Spiked concentration (ng/ml)	Set 1 (mean ± S.D.)	Set 2 (mean ± S.D.)	Absolute matrix (%)
Berberine	0.625	21507 ± 1486	20554 ± 2436	95.6
	2.5	75079 ± 2117	72879 ± 2963	97.1
	10	290147 ± 15991	285192 ± 13921	98.3
Palmatine	0.625	26823 ± 2675	27093 ± 2678	101
	2.5	105193 ± 2725	104644 ± 3239	99.5
	10	434095 ± 24912	423684 ± 22421	97.6
IS	20	443484 ± 31330	427225 ± 6991	96.3

5.2.6. Stability

The stability of berberine and palmatine was evaluated under conditions mimicking situations likely to be encountered during sample storage and the analytical process by analyzing five replicates of QC samples for both analytes. QC samples were frozen and stored at −20 °C for a week. The concentration variations found after one cycle of freezing and thawing were within ±15% of nominal concentrations, indicating no significant substance loss during thawing and freezing. When processed samples were stored in the autosampler at 4 °C, berberine and palmatine showed good stability evidenced from that the responses varied no more than ±10% at QC concentrations during 24 h. After storage at ambient temperature for 12 h, the concentrations of berberine and palmatine in plasma deviated less than ±10% from those in freshly spiked plasma.

6. Results of pharmacokinetic study

After oral administration of Huang-Lian-Jie-Du decoction (4.38 g extract/kg) to five rats, plasma concentrations of berberine and palmatine were simultaneously determined by the described LC–ESI-MS. The mean plasma concentration–time profiles ($n = 5$) are represented in Fig. 5. Pharmacokinetic parameters are listed in Table 4.

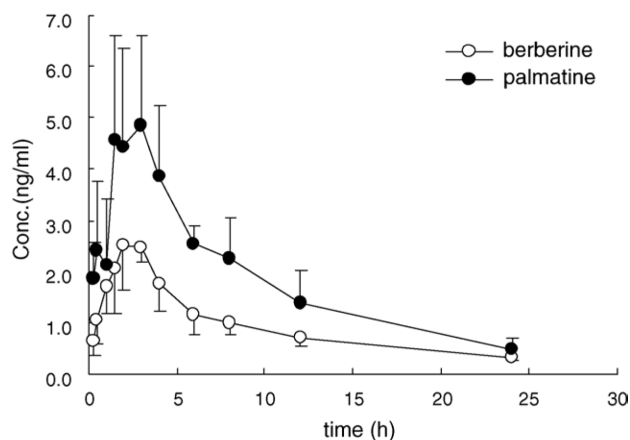

Fig. 5. Mean plasma concentration–time profiles of berberine and palmatine in five male rats after oral administration of 4.38 g extract/kg, each point and bar represents the mean ± S.D. ($n = 5$).

Table 4
Pharmacokinetic parameters of berberine and palmatine after oral administration of 4.38 g extract/kg, each value represents the mean ± S.D. ($n = 5$)

Sample	t_{max} (h)	C_{max} (ng/ml)	AUC (0-T) (ng h/ml)	$t_{1/2}$ (h)
Berberine	2.5 ± 1.0	6.2 ± 1.2	44.6 ± 10.1	8.0 ± 2.9
Palmatine	2.4 ± 0.6	3.1 ± 0.6	22.2 ± 2.2	10.5 ± 2.8

7. Conclusion

Quantification of components at low level was the barrier in the study of active components of TCMs in biological fluids. The only use of chromatography was sometimes time consuming and not sensitive and selective enough. In the present study, a highly selective and sensitive LC–ESI-MS method was developed and validated for the simultaneous quantification of berberine and palmatine in rat plasma after oral administration of Huang-Lian-Jie-Du decoction. Application with some modifications of the described methodology will be probably suitable for the determination of also other alkaloids of TCM in plasma.

Acknowledgements

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