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Full Length Research Paper

Pharmacokinetics, tissue distribution and oral bioavailability evaluation of isoimperatorin by high-performance liquid chromatography coupled with mass spectrometry

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A simple and specific high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) method was developed to investigate the plasma pharmacokinetics, tissue distribution and oral bioavailability of isoimperatorin (ISOIM), and osthole was employed as internal standard (IS). The separations were performed on a Shim-pack (VP-ODS) C_{18} analytical column (150 × 2.0 mm, 5 µm) with the column temperature kept at 25°C. The determinations were performed by a quadrupole mass spectrometer operated under selected ion monitoring (SIM) mode. The mobile phase was a mixture of acetonitrile and 0.5% formic acid solution (75:25, v/v) at a flow rate of 0.2 ml/min. The method was linear over the concentration range 2.0 to 5000 ng/ml (correlation coefficients >0.99). The RSDs of the overall intra- and inter-day precisions were less than 3.75%. The recoveries for ISOIM were within the range 82.70 to 97.74%. The pharmacokinetic data were successfully fitted to two-compartment models with first-order absorption. The absolute bioavailability of ISOIM is 68.17%. At 60 min after oral administration, ISOIM was found in all the seven investigated tissues.

Key words: Isoimperatorin, pharmacokinetics, tissue distribution, bioavailability, high performance liquid chromatography coupled with mass spectrometry (HPLC-MS).

INTRODUCTION

Isoimperatorin (ISOIM) is one of the main bioactive constituents in *Radix Angelicae dahuricae* (BaiZhi), the dried root of *A. dahurica* (Fisch. ex hoffm.) Benth. Et Hook. f. and *A.. dahurica* (Fisch. Ex hoffm.) Benth. Et Hook. f. var. formosana (Boiss.) Shan et Yuan. *Radix Angelicae dahuricae* is a well known traditional Chinese medicine (TCM) with the function of treatment of headache, toothache, nose congestion resulting from cold and the reduction of swelling and pain from sores and wounds and originated from Shennong Materia Medica, the earliest Pharmacopoeia of China in Eastern Han (24 to 220 AD) (Zhao et al., 2000). The major constituents in *Radix A. dahuricae* are furanocoumarins and isoimperatorin (ISOIM) is one of the bioactive components (Ketai et al., 2001; Piao et al., 2004; Figueroa et al., 2007; Feng et al., 2008). It has been reported that ISOIM possesses a variety of pharmacological and biochemical properties, such as inhibits the cyclooxygenase-2 (COX-2) and COX-1-dependent phases of prostaglandin D2 (PGD2) generation in bone marrow-derived mast cells (BMMC), inhibits the production of leukotriene C4 (LTC4) (Moon et al., 2008), competitively inhibits cytochrome P450 1B1 (CYP1B1) (Mammen et al., 2005), inhibits tumor cell proliferation (Kim et al., 2007), possesses a potent hepatoprotective effect against AFB1 (Pokharel et al., 2006), selectively inhibits TNF-alpha-induced expression of VCAM-1 (Moon et al., 2011). For these reasons, ISOIM is always treated as marker compound for the

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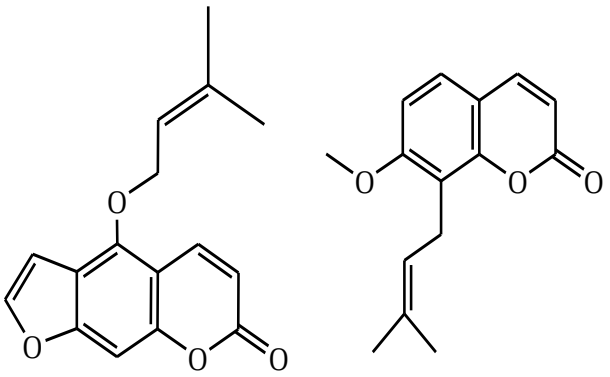

Figure 1. Structures of isoimperatorin and IS osthole.

quality control of *Radix A. dahuricae* and its preparations. A number of studies have been performed on the isolation, identification and determination of ISOIM in *Radix A. dahuricae*. Particularly analytical studies mostly deal with chromatography (GC) coupled with mass spectrometry (MS) methods (Wang et al., 2007), high-speed counter-current chromatography (HSCCC) (Yang et al., 2000; Liu et al., 2004; Wei et al., 2006), high performance liquid chromatography coupled with ultraviolet detector (HPLC-UV) (Shi et al., 2004; Tammela et al., 2004; Qian et al., 2007). Compared with LC-MS methods, HPLC-UV methods have several inherent limitations—lower sensitivity and lack of specificity. MS based techniques are now the mainstay for such biological fluids studies, especially as the cost of the instruments has declined significantly over the past few years. This article proposed a LC-ESI-MS method which employed a simple sample preparation (without any plasma extraction) with high specificity. This method was successfully applied to determine the concentration of ISOIM in rat plasma and tissues and further to evaluate the pharmacokinetics and bioavailability of ISOIM after oral administration and I.V. injection.

EXPERIMENTAL

Chemicals and reagents

ISOIM was isolated from CO_2 Super-critical fluid extract (SFE) of *Radix A. Dahuricae*, its chemical structure was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and purity was over 99% by HPLC analysis, ISOIM reference standard and internal standard (IS) osthole were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), their chemical structures are shown in Figure 1.

Acetonitrile was of HPLC-grade and purchased from Merck (Darmstadt, Germany), formic acid was purchased from Tedia (Ohio, USA), ultrapure water was prepared by a Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA) for the preparation of samples and buffer solutions. Other reagents were of analytical grade.

LC-MS conditions and instrumentation

A Shimadzu HPLC system with photodiode array detector was coupled with an electrospray ion (ESI) mass spectrometer (LCMS-2010EV Shimadzu, Kyoto, Japan), equipped with LC-20AB pumps, DGU-20A3 vacuum degasser, SIL-20A auto sampler, CTO-20A column homeothermia box, SPD-M20A photodiod arry detector (Shimadzu, Kyoto, Japan). The separations were performed on a Shim-pack (VP-ODS) C_{18} analytical column (150 × 2.0 mm, 5 µm, Shimadzu Corporation, Kyoto, Japan) with the column temperature kept at 25°C. The mobile phase was a mixture of acetonitrile and 0.5% (volume percentage) formic acid solution (75:25, v/v) at a flow rate of 0.2 ml/min and the whole portion was delivered into the ion source of mass spectrometry.

The operating parameters of the ESI interface in positive mode were optimized, temperatures were set as followed: 400°C for interface, 25°C for CDL, 200°C for heatblock , while the voltages were set as followed: +4.5 kV for interface, 20 V for CDL, 1.5 kV for detector, nebulizing gas was 1.5 L/min. Detection was operated under SIM mode. Two qualifying ions were selected for analytes under investigation: m/z 271 ($[M+H]^+$) for ISOIM and m/z 245 ($[M+H]^+$) for osthole were abundant and used for quantification. System management and hardware interface for data acquisition were carried out by the LCMS-solution software package from Shimadzu.

Animals and bio-sample collection

26 male Sprague-Dawley rats (250 ± 10 g) were obtained from Laboratory Animal Center of Xi'an Jiaotong University (Shaanxi, 710061, China). They were kept in an environmentally controlled breeding room for 5 days before starting the experiments and fed with standard laboratory food and water. All rats were dosed following an overnight fast (except for water). All the animal studies were approved by the Animal Ethics Committee of Xi'an Jiaotong University.

Before the surgeries, the animals were anesthetized with Pentobarbital Sodium (PS) by intraperitoneal injection at the dose of 40 mg/kg and fixed on wooden boards. Wounds were sutured after catheterization to the femoral artery. Small cotton blankets were used to keep the animals warm. ISOIM was dissolved in 0.9% saline immediately before intravenous injection or oral administration and the injected volume was adjusted at 0.5 ml/100 g for rats. The doses are 5 mg/kg for intravenous administration and 25 mg/kg for oral administration, the administrations were carried out after the animals came to their senses. After the experiment, all animals were sentenced to death, the bodies were collected by the Laboratory Animal Center. Blood samples (about 0.1 ml) were withdrawn in heparinized eppendorf tubes from the femoral arteries at 5, 10, 15, 30, 45, 60, 90, 180, 360, 720 and 1440 min after oral administration and 1, 2, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 720 min after I.V. injection. Plasma was separated by centrifugation at 3000 × g for 5 min and the plasma was stored at −20°C until LC/MS assay.

To determine tissue distribution, 25 mg/kg of ISOIM was administered orally to 6 rats. At 60 min after oral administration, tissues samples (brain, spleen, liver, heart, lung, intestine, and kidney) were harvested after anesthetized with PS and stored in refrigerator at -20°C until analysis.

Standard stock, work solutions and QC samples

A stock solution of ISOIM was prepared with methanol, obtained a

Table 1. Precisions and recoveries of isoimperatorin in rat plasma (n = 5).

Sample	Added amount (ng/ml)	Intra-day			Inter-day		
		Found (ng/ml)	RSD (%)	Recovery (%)	Found (ng/ml)	RSD (%)	Recovery (%)
Plasma	20	17.69	1.87	88.45	16.95	2.88	84.75
	2000	1897.2	1.26	94.86	1846.4	1.72	92.32
	5000	4885.7	0.92	97.71	4839.1	1.54	96.78
Brain	20	17.98	3.25	89.90	16.88	3.33	84.40
	500	473.32	2.12	94.66	469.87	2.28	93.97
	1000	952.25	1.95	95.23	958.55	2.11	95.86
Lung	20	17.35	3.33	86.75	16.87	3.44	84.35
	500	480.22	2.21	96.04	477.54	2.42	95.51
	1000	965.25	2.03	96.53	958.27	2.18	95.83
Liver	20	16.77	3.55	83.85	16.54	3.68	82.70
	1000	974.17	2.57	97.42	970.55	2.66	97.06
	2000	1945.23	1.33	97.26	1922.26	1.46	96.11
Spleen	20	17.44	3.05	87.20	17.18	3.27	85.90
	500	466.77	2.68	93.35	462.47	2.74	92.49
	1000	975.34	1.75	97.53	977.35	1.87	97.74
Kidney	20	17.44	2.79	87.20	17.28	2.81	86.40
	1500	1425.39	2.28	95.03	1414.52	2.34	94.30
	3000	2879.51	1.35	95.98	2869.48	1.33	95.65
Cerebellum	20	16.72	3.66	83.60	16.77	3.75	83.85
	500	462.35	2.74	92.47	462.11	2.72	92.42
	1000	963.57	1.58	96.36	962.81	1.48	96.28
Heart	20	16.89	3.64	84.45	16.78	3.68	83.90
	500	465.59	2.82	93.12	459.51	2.84	91.90
	1000	968.42	1.36	96.84	966.54	1.25	96.65

concentration of 100 µg/ml. The appropriate volumes of this solution were placed in glass tubes, and the solvent was evaporated under a compressed air stream. The dried analytes were reconstituted using blank plasma or blank tissue homogenates to yield 8 sets (each set contains 7 concentrations, the linear ranges were listed in Table 4) of final concentrations. 8 sets (each set has three concentration levels: low, middle and high, listed in Table 4) of QC samples were also prepared as above, these calibration standards and QC samples were then treated as described above.

The stock solution of IS osthole was prepared at a concentration of 100 µg/ml in acetonitrile and was further diluted to give a working concentration of 250 ng/ml and stored at 4°C prior to use. All the solutions were preserved in brown glass containers and stored in dark places.

Bio-sample procedure

Protein precipitation of plasma samples (40 µl) were performed by adding 160 µl IS solution each. After stirring on a vortex mixer (XW-80A Vortex mixer, Shanghai) for 30 s, all samples were centrifuged at 16,000 × g for 10 min, Then the supernatants were transferred to glass tubes and evaporated and reconstituted in 100 µl mobile phase. After centrifuged again, 5 µl of the supernatants were injected into the LC-MS system for analysis.

Tissue samples (about 0.5 g of each tissue was taken) were homogenized under iced bath with normal saline (1:9, w:v) in an Ultra-Turrax apparatus. Aliquots (0.2 ml) of each tissue homogenate were transferred to glass tubes,100 µl IS solution and 700 µl acetonitrile were added, after stirring on a vortex mixer (XW-80A Vortex mixer, Shanghai) for 30 s, the mixtures were centrifuged at

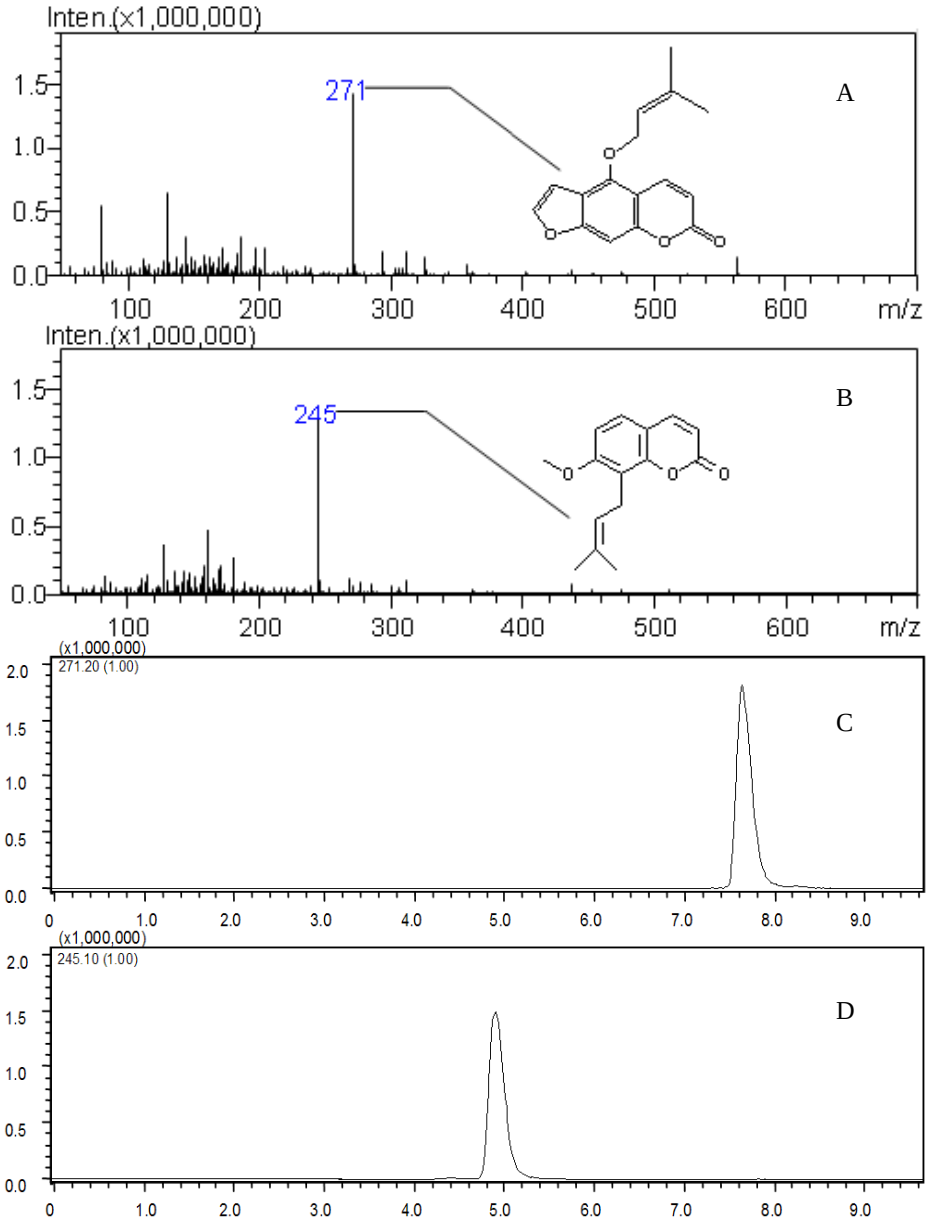

Figure 2. Mass spectrogram of A osthole, B imperatorin, and selected ion monitoring chromatograms of C isoimperatorin at m/z: 271, D osthole at m/z: 245.

16,000 × g for 10 min and the supernatants were transferred to a glass tubes and evaporated using a Speed Vac (Savant, USA). The dried samples were reconstituted in 100 µl mobile phase, 5 µl of the supernatants were injected into the LC-MS system for analysis.

RESULTS AND DISCUSSION

Chromatography

Typical selected ion monitoring (SIM) mass spectrogram chromatograms and structures for ISOIM and internal standard (IS) were shown in Figure 2. The retention time were 7.62 min for ISOIM and 4.84 min for IS osthole, the selected ions were m/z 271 for ISOIM and m/z 245 for IS osthole. Blank samples were also analyzed to confirm that there is no ignorable interference. It is obvious that the LC–SIM method simplifies the chromatograms very efficiently and provides single peaks for identification.

Linearity and lower limit of quantitation

The calibration curves were constructed by calculating the peak area ratios (y) of ISOIM to IS against ISOIM concentrations (x). Satisfactory linearities were observed

Table 2. Freeze-thaw stability and long term stability of isoimperatorin in rat plasma at -20°C.

Sample	Nominal concentration (ng/ml)	After three freeze -thaw cycle (n=5)		After storing at -20°C for 20 days (n=5)	
		Calculated concentration (ng/ml)	RSD (%)	Calculated concentration (ng/ml)	RSD (%)
Plasma	20	17.2	2.13	16.4	2.56
	2000	188.7	1.97	185.4	2.24
	5000	4869.2.	1.65	4854.1	1.86
Brain	20	17.33	2.79	17.02	2.94
	500	469.13	2.22	466.71	2.45
	1000	978.56	1.27	975.28	1.42
Lung	20	17.02	3.11	16.99	3.64
	500	476.28	2.22	463.27	2.48
	1000	957.55	1.83	958.77	1.43
Liver	20	17.25	3.22	16.88	3.15
	1000	958.71	2.63	947.13	2.40
	2000	1942.56	1.42	1896.28	1.53
Spleen	20	16.43	3.51	16.92	3.52
	500	466.25	2.54	470.23	2.15
	1000	962.44	1.63	966.41	1.36
Kidney	20	17.24	3.28	17.17	3.48
	1500	1415.17	2.01	1403.20	2.33
	3000	2887.45	1.44	2839.76	1.52
Cerebellum	20	17.16	3.24	16.94	3.35
	500	477.51	2.35	463.18	2.61
	1000	979.62	1.58	958.46	1.69
Heart	20	17.55	3.24	17.19	3.66
	500	469.18	2.36	455.37	2.54
	1000	972.54	1.69	964.15	1.72

for each kind of bio-sample, linear ranges and regression equations were listed in Table 4, with correlation coefficient over 0.99, which showed good linear relationships between the peak area ratios and the concentrations. The lower limits of quantitation (LLOQs) were defined as the lowest concentration points in the calibration curves, and were 2.0 ng/ml for each bio-sample.

Precision and recovery

In order to get accuracy results, it is necessary to estimate the recovery under different situations. The precision and recovery of the method were studied by analyzing the QC samples with low, medium and high ISOIM concentrations in rat plasma and tissue homogenates. The measured concentrations of QC samples were calculated from the calibration curves. The precisions were evaluated by the intra-day and inter-day variability with relative standard deviation (RSD). The results were shown in Table 1. The RSDs of the overall intra- and inter-day precisions were less than 3.75%. The recoveries for ISOIM were within the range 82.70 to 97.74%.

Stability

The stabilities of ISOIM and IS stock solutions were evaluated by calculating the concentration changes at room temperature for 24 h and at 4°C for 20 days. The calculated concentrations were within the range from 99.52 to 99.73% and 98.65 to 99.28% for ISOIM at room temperature and 4°C, respectively.

The calculated concentrations were within the range from 99.22 to 99.58% and 98.49 to 99.37% for IS at room temperature and 4°C, respectively. The tests were parallelly processed five copies of the two stock solutions (n = 5).

The stabilities of ISOIM in plasma and tissue homogenates were evaluated following freeze–thaw cycles, the stability evaluations were conducted by comparing the concentrations of ISOIM determined in plasma and tissue homogenates immediately after spiking with the samples, which had beenfrozen at -20°C for 24 h, then thawed naturally at room temperature. The freeze–thaw cycles were repeated three times, and for

Table 3. Pharmacokinetic parameters of isoimperatorin after oral administration (25 mg/kg, n=6) and I.V. injection (5 mg/kg, n = 6).

Parameter	Oral administration		Intravenous administration	
	Mean	S.D.	Mean	S.D.
Ka (1/min)	0.028	0.003	/	/
t1/2α (min)	38.35	7.95	3.64	0.92
t1/2β (min)	883.15	198.27	44.46	12.25
t1/2ka (1/min)	24.65	4.26	/	/
AUC 0-∞(μg/ml)•min	364.14	232.18	106.84	23.44
CL(s) (mg/kg/min/<μg/ml>)	0.069	0.014	0.047	0.012
Tpeak (min)	49.68	13.36	/	/
Cmax (μg/ml)	1.18	0.24	/	/
V/f(c) (<mg/kg>/ <μg/ml>)	12.10	2.33	/	/
V(c) (<mg/kg>/ <μg/ml>)	/	/	1.26	0.31

Table 4. Calibration curves of isoimperatorin in Bio-samples (n = 3).

Sample	Regression equation	Correlation coefficient	Linear range (ng/g *ng/ml)
Plasma	y = 0.246x - 0.011	r = 0.997	2–5000*
Brain	y = 0.722x-0.045	r = 0.990	2–1000
Lung	y = 0.685x-0.143	r = 0.995	2–1000
Liver	y = 0.893x-0.052	r = 0.992	2–2000
Spleen	y = 0.657x-0.013	r = 0.993	2–1000
Kidney	y = 0.751x+0.012	r = 0.998	2–3000
Cerebellum	y = 0.893x- 0.025	r = 0.991	2–1000
Heart	y = 0.785x-0.032	r = 0.999	2–1000

Regression equations were expressed as y = Ax + B, where A and B are the constant values, x is the isoimperatorin concentration, y is the peak aera ratio of isoimperatorin against IS osthole.

long term stability, the spiked samples were frozen at -20ºC for 20 days. The stability results were summarized in Table 2, which showed that ISOIM is stable either in plasma or in tissue homogenates under the storage condition.

Matrix effect

Matrix effect (ME) was investigated by comparing the peak areas of ISOIM and IS between two different sets of samples. In set 1, ISOIM and IS were dissolved in the mobile phase and analyzed at the concentration of 20 ng/ml for imperatorin and 50 ng/ml for IS. In set 2, blank plasma samples and tissue homogenates obtained from five rats were extracted and then spiked with the same concentrations of ISOIM and IS. The assessment of the relative MEs were made by comparing the ISOIM peak area values between different sources of samples. Ratios of the mean peak areas of set 2 to that of set 1 would indicate the possibilities of ionization suppression or enhancement for ISOIM and IS. The ratios are from 89.65 to 98.37% for ISOIM and from 91.25 to 97.42% for IS, which indicate that no significant exogenous matrix effect was implied (Matuszewski et al., 2003).

Pharmacokinetics and tissue distribution

The concentration–time curves of ISOIM in rat plasma after oral administration and I.V. injection were shown in Figure 3. The pharmacokinetic data were successfully fitted to two-compartment models with first-order absorption and Pharmacokinetic parameters are summarized in Table 3. The absorption rate constant (K_a) value is 0.028 1/min, which indicates that absorption is very fast, ISOIM reached its maximum plasma concentration (C_{max}=1.18 μg/ml) at 49.68 min (T_{peak}) after oral administration. The absolute bioavailability (AB) of ISOIM was calculated with the following equation:

$$AB = \frac{AUC_{oral}}{AUC_{iv}} \times \frac{iv\ dose}{oral\ dose} \times 100$$

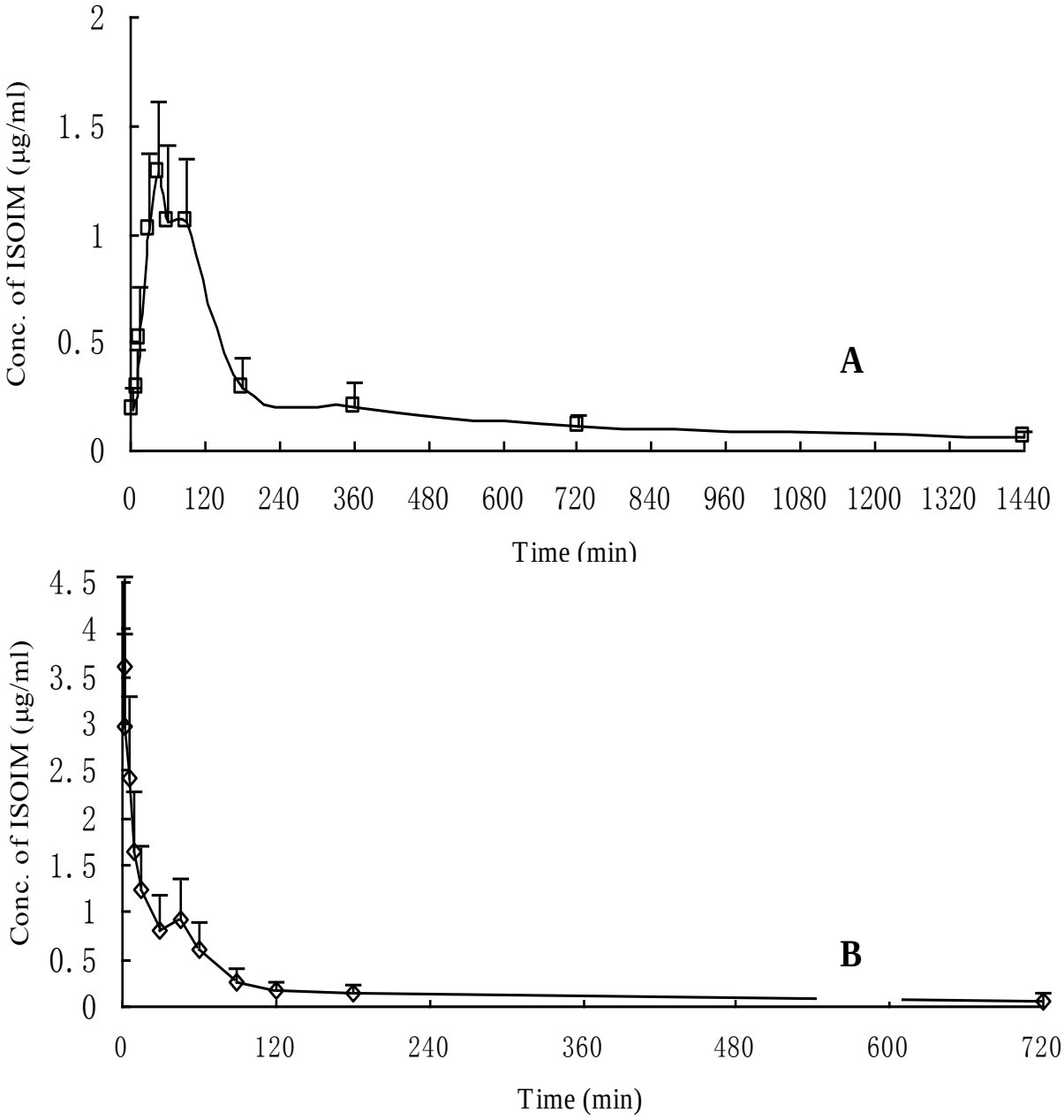

Figure 3. Time-Concentration curves of ISOIM after oral administration (A, 25 mg/kg) and I.V. injection (B, 5 mg/kg).

Where oral is the oral administration and iv is the intravenous injection, the absolute bioavailability of ISOIM is 68.17%, it is easily for ISOIM to enter haemal circulation.

Tissue distribution result of ISOIM at 60 minutes after oral administration was shown in Figure 4, ISOIM distributed in the 7 investigated tissues, since ISOIM was found in brain and cerebella, blood-brain barrier could not impede the transfer of ISOIM from blood to central nervous system. Concentrations of ISOIM in kidney and liver are relatively higher than the other tissues and plasma, such a result occurred might because of the rich blood flow and higher tissue affinity.

Conclusions

Herein we presented a sensitive and reliable HPLC-MS method to quantitate ISOIM in rat plasma and tissues; it was successfully applied to study the pharmacokinetics and tissue distribution of ISOIM in rats. A suitable internal standard (osthole) was choosed for the quantitation of

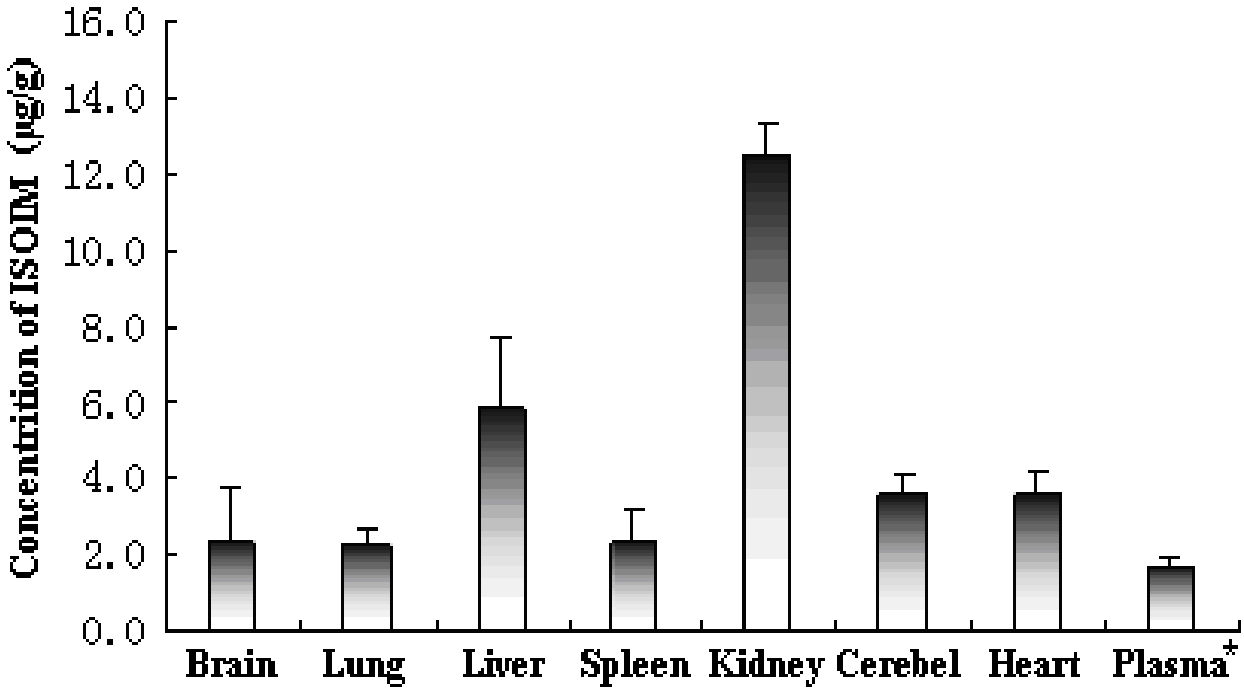

Figure 4. Mean tissue distributions of isoimperatorin in rat brain, lung, liver, spleen, kidney, cerebellum, heart and plasma (*μg/ml) at 60 minutes after oral administration isoimperatorin (25 mg/kg, n = 6).

ISOIM in different bio-samples. Because of the high specificity and sensitivity, this method could be further used for the pharmacokinetics research of the Chinese materia medica preparations as ISOIM the marker compound.

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