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Original Research Article

Pharmacokinetics and tissue distribution of N-3-methoxybenzyl-palmitamide in rat: A macamide derived from Lepidium meyenii

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Abstract

Purpose: *To study the pharmacokinetics and tissue distribution of N-3-methoxybenzyl-palmitamide (MPM) derived from Lepidium meyenii (Maca)*
Methods: *MPM and N-benzylpalmitamide (BPM, as the internal standard, IS) were prepared by one-pot synthesis method and characterized. For the analysis of MPM in rat plasma and tissue samples, a rapid ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method was developed and validated by optimizing sample preparation conditions and UPLC conditions. Finally, the pharmacokinetics and biodistribution of MPM after oral administration in rats were studied.*
Results: *The lower limit of quantification (LLOQ) and limit of detection (LOD) of the UPLC-MS/MS method were 1.2 and 5.0 ng/mL, respectively. Good linear relationship of calibration curve ($r > 0.9951$) was achieved over the range of 5 – 5000 ng/mL. In pharmacokinetics, plasma concentration-time curve of MPM showed double peaks. The highest distribution of MPM after absorption was in the stomach, followed by lung. The absorption and eliminate rate of MPM were slow in rats. In fact, MPM displayed a lung targeting property.*
Conclusion: *The developed UPLC-MS/MS method is suitable for plasma and tissue distribution studies of MPM in rats. The present study can provide guidance for the further development and utilization of Maca tuber.*

Keywords: *Macamide, Maca tuber, Lepidium meyenii, Pharmacokinetics, Tissue distribution, UPLC-MS/MS*

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INTRODUCTION

Maca, an Andean plant of the crucifer family, is heavily cultivated in some South American countries, and is also called "Peruvian ginseng" [1]. Indigenous Peruvians have traditionally used Maca for centuries as an adaptogenic plant to manage infertility, anemia, menstrual, anticancer, female hormone balance and sexual disorders [1-3]. In recent years, Based on the historical use of above facts, a series of commercial dietary supplements containing Maca have gained popularity in Japan, Europe, the US and China [4]. Numerous secondary metabolites of the Maca have been found over the years, and macamide, one of the secondary metabolites,

was first found in Maca as the typical marker. On the chemical structure, macamide is composed of various fatty acids and their benzylamine.

In recent years, macamide has been proposed to be the biologically active substance, because of its multiple functions, such as fatty acid amide hydrolase inhibitor [5], improving sexual performance [6], endurance capacity and anti-fatigue [7]. So far, 22 kinds of macamide is separated and identified from Maca tuber, and N-3-methoxybenzyl-palmitamide (MPM) is a representative substance [8,9].

In this study, MPM was further researched on its pharmacokinetics and tissue distribution in rats, in order to deepen the understanding of macamide, and to provide guidance for the development and utilization of Maca tuber.

EXPERIMENTAL

Chemicals and reagents

Palmitoyl chloride, benzylamine and 3-Methoxybenzylamine were purchased from Sinopharm Chemical Reagent Co. Ltd., China. MPM and BPM (purity > 98.0 %) were synthesized in our laboratory. Their chemical structures are presented in Figure 1. Acetonitrile of high performance liquid chromatography (HPLC) grade was acquired from Merck KGaA (Darmstadt, Germany). The other solvents and chemicals were all of analytical reagent grade. The water (resistance > 18 MΩ) came from a Millipore Milli-Q water purification machine (Bedford, MA, USA).

Synthesis of MPM and BPM

Synthesis of MPM: Anhydrous diethyl ether (15 mL) was placed to a round bottom flask which was attached to a Claisen adapter, and palmitoyl chloride (4.05 mL, 0.012 mol) was injected through the Claisen adapter septum. The mixture was stirred over 15 min in an ice bath. Subsequently, a mixture of 3-Methoxybenzylamine (1.99 mL, 0.018 mol) and anhydrous diethyl ether (1.50 mL) was injected drop by drop into the reaction bottom flask, and then a white precipitate was formed at the end. The precipitate was washed with 10 % sodium hydroxide and 10 % hydrochloric acid. Finally, the product was recrystallized from ethanol with ca. 81 % yield. The spectroscopic data (UV, FT-IR, ESIMS) of the product was characterized. Product purity was assayed using HPLC.

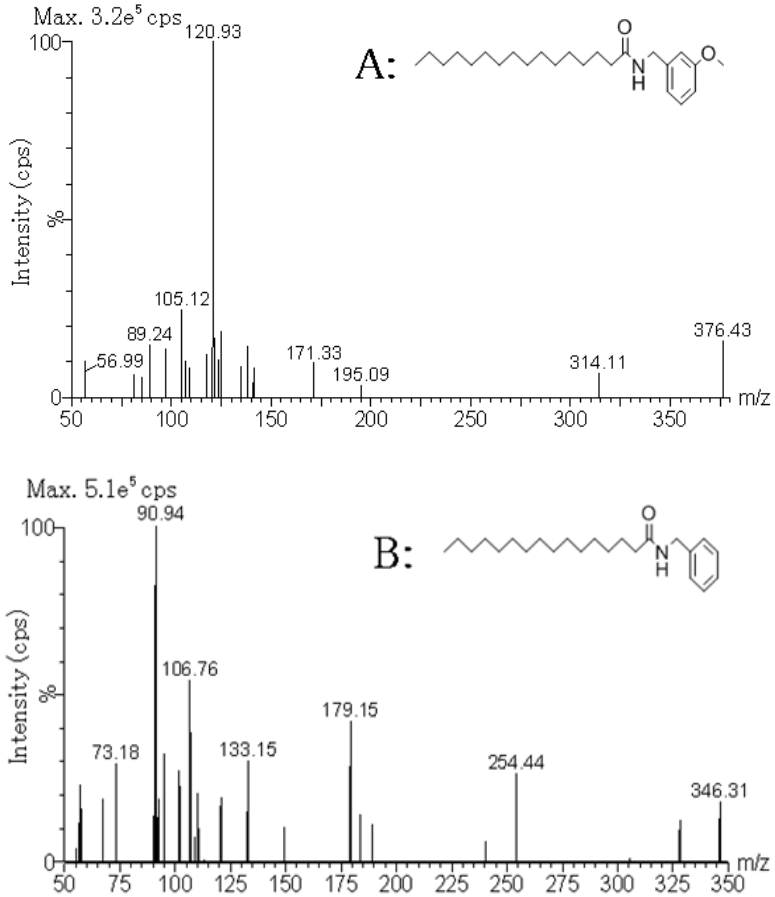

Figure 1: Production mass spectra of (A) MPM and (B) BPM, and their chemical structures

Synthesis of BPM: BPM's synthesis process was the same with MPM, with benzylamine instead of 3-methoxybenzylamine [9].

Structure confirmation of MPM and BPM

MPM: white powder; UV (MeOH solvent): λ_{max} 213, 273 nm; IR (KBr powder) cm^{-1}: υ_{max} 3294 (N-H), 2919, 2849, 1640 (C=O), 1534, 1469, 1261, 1154, 1049, 778, 693 cm^{-1}; ESIMS, *m/z*376.4 ($[M + H]^+$).

BPM: white powder; UV (MeOH solvent): λ_{max} 209 nm; IR (KBr powder) cm^{-1}: υ_{max}3306 (N-H), 2917, 2849, 1638 (C=O), 1550, 1458, 1248, 1118, 1081, 744, 698 cm^{-1}; ESIMS, *m/z* 346.3 ($[M + H]^+$). The above structure data of MPM and BPM corresponded to data previously reported [9,10].

Instrumentation and conditions (for UPLC-MS/MS method)

Liquid chromatography was performed on an Acquity ultra performance liquid chromatography (UPLC) unit (Waters Corp., Milford, MA, USA) with an Acquity BEH C18 column (2.1 × 50 mm, 1.7 µm particle size). The mobile phase consisted of acetonitrile and water containing 0.05 % trifluoroacetic acid (TFA). A gradient elution program was conducted for chromatographic separation with 0.05 % TFA aqueous solution (mobile phase A) and acetonitrile (mobile phase B) as follows: 0 - 0.5 min (30 % A), 0.5 - 2.0 min (30 - 10 % A), 2.0 - 2.5 min (10 - 30 % A). The flow rate was 0.40 mL/min and the overall run time was 2.5 min.

A XEVO TQD triple quadruple mass spectrometer was used to carry out mass spectrometric analysis (Waters Corp.). Multiple Reaction Monitoring (MRM) mode is used in mass analyzers with an electro-spray ionization. The MRM transitions was *m/z* 376.4→120.9 and *m/z* 346.3→90.9 for MPM and IS, respectively. Data acquisition and instrument control adopted Masslynx 4.1 software.

Method validation

The method was fully validated for specificity, matrix effect, sensitivity, linearity, precision, accuracy, recovery and stability in accordance with the guidelines set by Food and Drug Administration of the United States [11]. Each validation run consisted of six replicates of quality control plasma samples (QC) or one set of calibration standards.

The specificity was determined by analysis of blank plasma samples (or blank tissue samples), blank sample spiked and the plasma sample obtained after oral administration of MPM with six different rats. Moreover, the possible interference from endogenous substances was confirmed. To evaluate the matrix effect (ME) of blank plasma (or tissues), generalized standard addition method was performed. Six different blank rat plasma (or tissues) was spiked with the analytes. ME was calculated at three QC levels (10, 100 and 1000 ng/mL) by comparing the peak areas (B/C × 100 %, B = the peak area of blank plasma samples (or tissues) spiked with analytes after extraction, C = the peak area of the pure standard solutions at the same concentrations). The ME of IS was calculated at the concentration (100 ng/mL) in a similar manner.

The sensitivity of the method was evaluated by determination in terms of LLOQ, and signal-to-noise ratio (SNR) was used to calculate the LLOQ. Calibration curves were obtained using these spiked calibration samples on three separate days in succession. Peak area ratio of MPM to IS was plotted against MPM concentrations. The correlation of all calibration curves was required more than 0.99. The precision and accuracy of the method were evaluated by analyzing the QC samples with known concentrations of MPM and IS (10, 100 and 1000 ng/mL) with six replicates. The precision was defined by relative standard deviation (RSD), and the accuracy was presented as a relative error (RE).

The extraction recoveries of MPM at three QC levels (10, 1000, 1000 ng/mL) were calculated by comparing peak area ratios of two different sets of samples. One was the samples spiked with the MPM prior to extraction, and the other was the samples spiked with the MPM after extraction at same content. The extraction recovery of IS was determined in the same manner. The stabilities of MPM in rat sample were analyzed by using six replicates of plasma samples spiked with MPM at known concentrations (10, 100 and 1000 ng/mL). Those samples were exposed to following four conditions. They were respectively the short-term stability (exposing samples to 25 °C for 24 h), the freeze–thaw stability (subjecting samples to five freeze–thaw cycles), the long-term stability (samples stored at - 80 °C for 21 days prior to extraction), and the processed sample stability (samples stored at 4 °C for 72 h).

Animals

The Sprague-Dawley rats (210 ± 20 g) were provided by the Laboratory Animal Center of Wenzhou Medical University, and were

performed to study the pharmacokinetics and biodistribution of MPM. Before the experiment, the diet was banned for 12 h, but the water was free. The animal care and use committee of our university approved the experiment protocols and procedures (ethical approval code: WMU20160817). These experiments are in line with rules in the NIH Guide for the Care and Use of Laboratory Animals [12].

Pharmacokinetics study

Six rats were used for pharmacokinetics study at a dose of 80 mg/kg MPM prepared with 1.0 % sodium carboxymethylcellulose. Collect whole blood (0.5 ml) from the caudal vein into plastic centrifuge tubes (pretreated with heparin sodium) at pre-dose, 0.08, 0.16, 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h after oral administration of MPM. Then the whole blood was centrifuged timely at 3000 g for 10 min for isolation of the plasma. Those samples were stored at - 80 °C until further preparation.

Tissue distribution studies

Eighteen SD rats were randomly assigned to three groups with six rats per group. They were used for tissue distribution study at a dose of 80mg/kg MPM prepared with 1.0 % sodium carboxymethylcellulose. Rats were sacrificed under ether anesthesia at 0.5, 2, 12 h after dosing and brain, lung, heart, stomach, kidney, liver, spleen or reproductive organ were harvested. All of the samples were timely stored at - 80 °C until further preparation.

Sample preparation

The 250 µL plasma sample (or 0.25 g milled tissue sample) was added to a 5 mL centrifuge tube, and 5 µL of IS methanol solution (5 µg/mL) and 2 × 2.5 mL ethanol solution (containing 0.2 % formic acid) was added. Then the tubes were vortexed for 1.0 min and centrifuged at 5,000 g for 10 min. The twice extracted solutions were merged and concentrated. It was purified by pure silica solid-phase extraction (SPE) column washed with 2 × 1 mL of water, 1 mL of acetonitrile – water (20 : 80, v/v) and finally with 3 × 1 mL of acetonitrile, and the eluate was blow-dried with nitrogen at 50 °C and the residue was redissolved in 250 µL of ethanol. This extracted solution was injected into UPLC/MS system.

Data analysis

After UPLC/MS determination, the peak areas of analytes were integrated, and the peak area ratio of MPM to IS was calculated. Parameter values were calculated and expressed with mean ± SD (upper and lower CI 95 %). Precision RSD (%) = SD/mean × 100. Accuracy RE (%) = (conc. measured – conc. spiked)/conc. spiked × 100.

RESULTS

Optimized sample preparation and UPLC conditions

Extract reagent played a critical role when the sample was prepared to get higher recovery and lower matrix interferences for the analyte. The reagent effect on extraction yield was investigated, including methanol, alcohol, acetonitrile, acetone and n-hexane. In order to study the effect of pH, each of these solvents added 0.2 % formic acid and 0.2 % ammonia respectively. Ethanol solution (containing 0.2 % formic acid) had the highest recovery rate, but also had a high matrix interference especially in tissue sample. In order to remove the matrix interference, SPE method was adopted, and it was valid that plasma sample and tissue sample all had negligible matrix interference.

To achieve more efficient separation of isolates, chromatographic conditions of chromatographic column and mobile phase were optimized. We investigated four mobile phases, and acetonitrile - water (containing 0.05 % TFA) resulted in the production of better peaks due to higher sensitivities. Furthermore, We tested three types of chromatographic column with different polarity. Finally Acquity BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) produced better separation within a short time. In addition, we optimized mobile phase gradient elution with acetonitrile - water containing 0.05 % TFA in 400 µL/min flow rate, within 2.5 min.

Specificity and matrix effect

Three samples of the representative extract ion chromatograms (XIC) from mass spectrometry were shown in Figure 2. Three samples of blank plasma sample, plasma sample spiked at LLOQ level and plasma sample collected 15 min after oral administration represented three different concentrations, and endogenous interferences for the analyte were all not observed. The matrix effect for MPM at concentrations of 10, 100 and 1000 ng/mL, were 95.1 ± 3.7 %, 106.4 ± 4.2 % and 102.7 ± 4.3 % (n=6), respectively. The ME for IS (100 ng/mL) was 105.3 ± 3.6 % (n=6). It followed that matrix effect from plasma was negligible in this method.

The use of SPE method resulted in a significant reduction of endogenous interferences of tissue

samples. The extract ion chromatograms of tissue sample were similar to the plasma samples'.

Linearity and sensitivity

Using weighted ($1/x^2$) linear regression analysis, we established calibration curves based on the peak area ratio of MPM to IS. MPM presented a good linear relationship with the range of 5 - 5000 ng/mL. The regression equations, the linear ranges, and the correlation coefficients for MPM in plasma and tissues are listed in Table 1. The LLOQ of MPM in plasma and tissue was 5 ng/mL with the RSD and RE of 8.5 and 5.6 %, respectively. The LOD of MPM in plasma and tissues was 1.2 ng/mL.

Precision, accuracy and recovery

RSD taken as the measures of precision for QCs was calculated over three days of validation tests at three concentration levels. In the same way, relative error (RE) was taken as the measures of accuracy. The results of the precision, accuracy and recovery were showed in Table 2. All results of precision for the samples were in the range of - 9.2 ~ 11.8 % within the acceptable criteria of ± 15 %. These data demonstrated that the assay was reproducible and accurate for MPM determination in rat plasma or tissue samples.

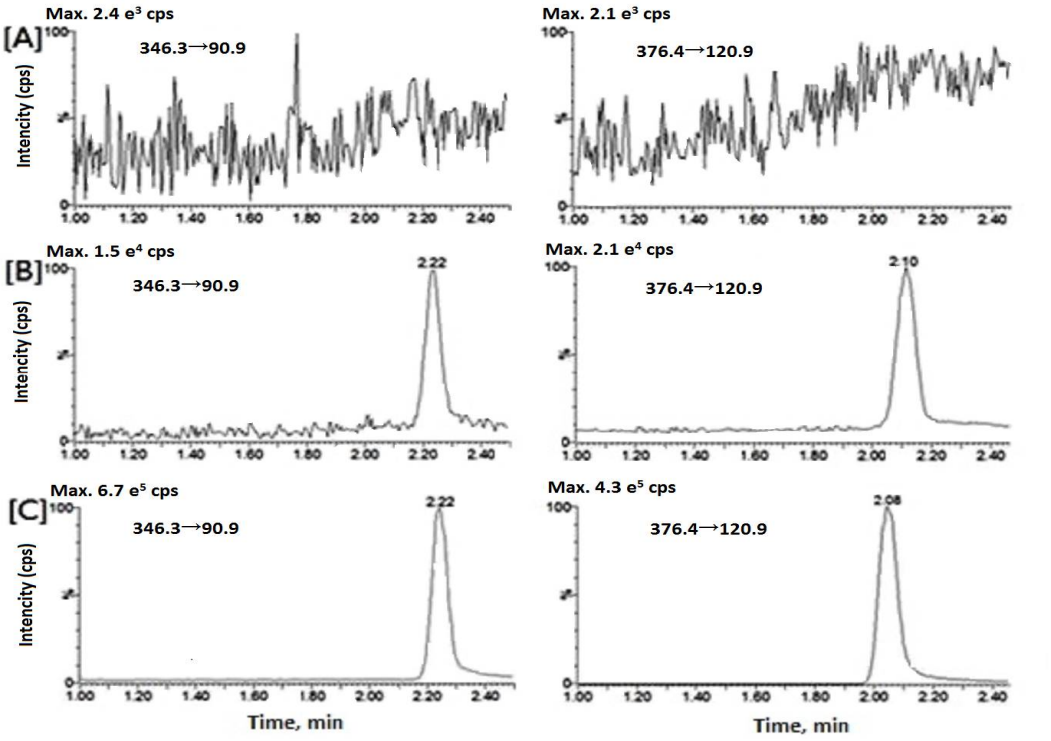

Figure 2: Representative XIC from mass spectrometry: (A) a blank plasma sample; (B) plasma sample spiked at LLOQ level; (C) plasma sample collected 15min after oral administration of MPM at the dose of 80mg/kg. The XIC showed MPM at m/z 376.4 → 120.9 and IS at m/z 346.3 → 90.9

Table 1: Calibration parameters for MPM in rat plasma and tissues

Sample	Regression equation	Correlation coefficient (r)	Linear range (ng/mL)
Plasma	y = 0.0001562321x + 0.00024597	0.9969	5 - 5000
Brain	y = 0.0001610634x + 0.00032861	0.9991	5 - 5000
Lung	y = 0.0001547624x + 0.00021632	0.9951	5 - 5000
Heart	y = 0.0001496412x + 0.00023037	0.9959	5 - 5000
Stomach	y = 0.0001595638x + 0.00019053	0.9977	5 - 5000
Kidney	y = 0.0001475628x + 0.00020531	0.9969	5 - 5000
Liver	y = 0.0001555281x + 0.00029651	0.9992	5 - 5000
Spleen	y = 0.0001630921x + 0.00017976	0.9987	5 - 5000
Reproductive organ	y = 0.0001450331x + 0.00013992	0.9971	5 - 5000

Note: y = the peak area ratio of MPM to IS; x = the concentration of MPM

Table 2: Precision, accuracy and recovery for the determination of MPM in rat sample (n = 6)

Sample	Conc. spiked (ng/mL)	RSD (%)		RE (%)		Recovery (%)
		intra-day	inter-day	intra-day	inter-day	
Plasma	10	10.1	10.6	-8.6	7.8	82.3
	100	5.5	6.1	6.7	5.5	87.8
	1000	2.6	3.2	2.4	-3.3	85.1
Brain	10	9.3	11.2	-8.2	9.2	80.3
	100	6.0	6.4	6.2	-6.0	81.5
	1000	3.2	3.6	2.7	-3.2	86.2
Lung	10	9.2	10.1	-8.7	7.9	80.9
	100	5.1	6.3	7.2	-6.8	83.8
	1000	3.9	2.8	2.1	-2.9	84.2
Heart	10	8.7	10.3	-9.2	8.3	82.1
	100	6.6	6.1	7.8	-5.7	88.8
	1000	2.1	3.7	3.0	-3.2	85.2
Stomach	10	10.5	12.3	-9.1	8.6	90.4
	100	6.5	5.1	7.3	6.7	84.8
	1000	3.9	4.1	3.6	-3.1	87.2
Kidney	10	9.7	9.6	-3.2	7.3	80.9
	100	5.1	6.5	7.8	-5.3	85.8
	1000	4.9	3.6	2.7	-3.9	84.1
Liver	10	9.5	10.2	-8.2	7.4	83.4
	100	5.9	6.4	5.9	5.8	83.8
	1000	2.3	3.7	2.8	-3.3	84.7
Spleen	10	9.9	9.8	-8.0	7.7	91.4
	100	5.4	5.7	5.5	5.4	85.8
	1000	3.0	3.2	4.6	-3.7	85.2
Reproductive	10	9.9	11.8	-8.4	9.2	87.1
organ	100	6.5	7.3	6.8	-6.9	82.4
	1000	3.9	3.9	-4.7	4.8	83.1

In rat plasma and tissue samples, the minimum recovery of MPM was 80.3 %. The recovery of the IS (100 ng/mL) was 83.7 % or higher. The above results indicated that sample preparation method could obtain satisfying extraction efficiency for MPM in rat plasma and tissue sample.

Stability

The stability of MPM in rat sample were evaluated by analyzing six replicates of rat samples which were all exposed to long-term stability, short-term stability freeze–thaw stability, and the processed sample stability. The re-inspection yield {RIY = (conc. measured later/conc. measured first) × 100 %} was used to express the stability. Calculated results showed that the RIY was in the range of 90.5 – 111.2 %. The data indicated that there was no significant degradation and the established methods were acceptable when samples of MPM were exposed to different conditions. The method was successfully applied to determine the concentration of MPM in rat's plasma and tissue samples in the following experiments.

Plasma pharmacokinetics

The mean plasma concentration-time profile is shown in Figure 3 and the primary pharmacokinetic parameters are summarized in Table 3. Plasma concentration-time curve of MPM showed double peaks. The peak plasma concentration of MPM after oral administration at 0.5 h was 600.3 ± 167.1 ng/mL. The area under the curve ($AUC_{0\rightarrow t}$) and elimination half-life of MPM in plasma was 6485.75 ± 737.72 ng/mL•h and 7.44 ± 1.12 h, respectively.

Tissue distribution

The tissue distribution of MPM in rats after oral administration of 80 mg/kg dose is presented in Figure 4. The MPM concentrations in stomach after 0.5, 2 and 12 h were 3105.0 ± 267.1, 2782.2 ± 205.6 and 404.5 ± 53.1 ng/g, respectively. The MPM concentrations in lung were 300.5 ± 17.4, 2200.7 ± 152.9 and 415.2 ± 43.3 ng/g, respectively. The MPM concentrations in spleen were 205.3 ± 15.8, 769.3 ± 94.1 and 175.2 ± 24.1 ng/g, respectively. The MPM concentration in other tissues of brain, heart, kidney, liver, reproductive organ was low, and even its highest concentration also was under 250 ± 37.4 ng/g.

DISCUSSION

MPM is a hydrophobic organic compound with a long aliphatic chain. Usual method to prepare biological sample with adding 200 µL methanol

or acetonitrile to100 µL plasma sample was tried and infeasible because of the low recovery rate. Several kinds of reagents with different volume were investigated. Finally, high proportion of extracting solution and addition of formic acid were adopted together, to raise the extraction rate and recovery rate of MPM. The optimum combination ratio was 1 : 20 (v : v) corresponding to biological sample : ethanol (containing 0.2 % formic acid).

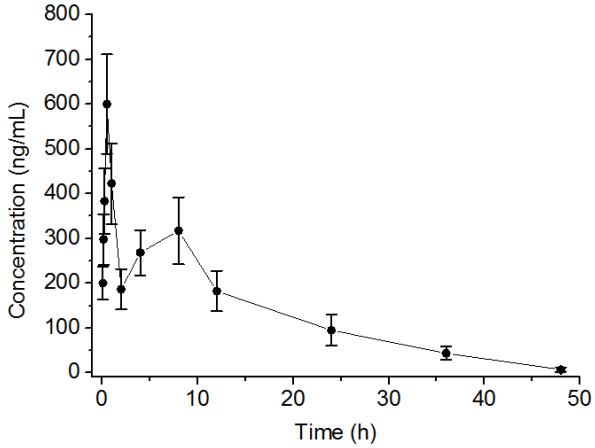

Figure 3: Mean plasma concentration-time plots at a single dose of 80 mg/kg MPM of oral administration in rats (n = 6)

Table 3: The main pharmacokinetic parameters at a single dose of 80 mg/kg MPM of oral administration in rats (n = 6)

Parameter	Mean ± SD
Absorption	
T_{max} (h)	0.50 ± 0.18
C_{max} (ng/mL)	600.3 ± 167.1
Elimination	
$T_{1/2el}$ (h)	7.44 ± 1.12
$AUC_{0\rightarrow t}$ (ng/mL•h)	6485.75 ± 737.72
$AUC_{0\rightarrow \infty}$ (ng/mL•h)	6560.91 ± 807.36
V_d (L/kg)	130.98 ± 15.29
CL (L/h/kg)	12.19 ± 1.92

In pharmacokinetic experiment, plasma concentration-time curve of MPM presented a double peak, probably because the rat's organization concentration of MPM is much higher than its plasma's, which could cause MPM from organization transporting to plasma [13]. We inferred there might be distribution, re-absorption and entero-hepatic circulation in rats [14]. Pharmacokinetic of MPM could be evaluated by two compartment model. The elimination half-life of 7.44 ± 1.12 h showed that the elimination of MPM was slow in rats.

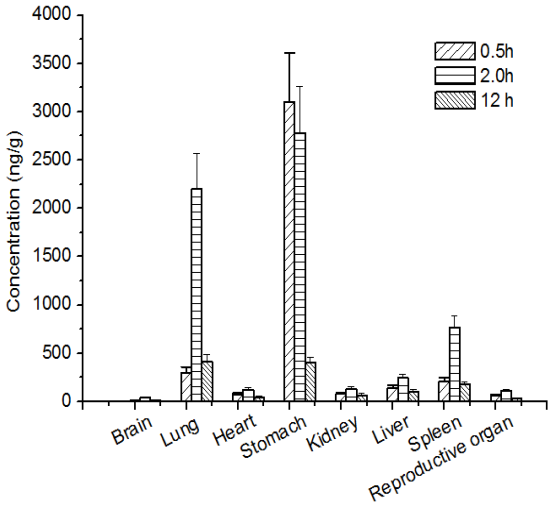

Figure 4: Rat tissue concentration of MPM after single oral administration of 80 mg/kg dose

The value of AUC (6485.75 ± 737.72 ng/mL•h) is not significant with a dose of 80 mg/kg compared to other reported drugs [15-17]. Unfortunately, there are missing data of absolute bioavailability, because direct intravenous drug delivery was unsuccessful in this experiment. By intravenous administration of MPM ethanol solution (at the dose of 1 mg/kg MPM), all the rats died within 5 min. After excluding the toxicity of the MPM [18], the poor solubility of MPM in blood that could lead MPM to precipitating and blocking the blood vessels might be the main cause of death of the rats.

The results of tissue distribution showed that stomach had the dominant distribution, and that the absorption of MPM in stomach was slow and incomplete because of a higher concentration at 12 h after dosing. The second was in lung with relatively high concentration, even higher than the concentration of stomach at the point of 12 h. The phenomenon showed that MPM in lung was eliminated at a slow rate. Lipophilic MPM could remained in lung tissue with a longer time, because lung tissue has good lipophicity [13]. Thus, MPM has a high targeting property and is widely distributed in other tissues with low concentration.

CONCLUSION

The developed UPLC-MS/MS method is suitable for plasma and tissue distribution studies of MPM in rats. The MPM pharmacokinetic and tissue distribution data obtained would provide guidance for the further development and utilization of Maca tuber.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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