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Simultaneous determination of active flavonoids and alkaloids of Tang-Min-Ling-Pill in rat plasma by liquid chromatography tandem mass spectrometry

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ABSTRACT

A rapid, sensitive and reliable liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed and validated for simultaneous determination of five active flavonoids (wogonin, chrysin, oroxylin A, naringenin, hesperetin) and four major alkaloids (berberine, coptisine, jatrorrhizine, palmatine) from Tang-Min-Ling-Pill in rat plasma. Plasma samples (100 μL) were spiked with internal standards daidzein (for flavonoids) and tetrahydropalmatine (for alkaloids), acidified with HCl and extracted by liquid–liquid extraction with acetone. Chromatographic separation was performed on a Zorbax SB-C_{18} column with the mobile phase of water (containing 0.1% of formic acid)–acetonitrile (30:70, v/v) at a flow rate of 0.3 mL/min in a run time of 7.0 min. Detection was performed by multiple reaction monitoring mode using electrospray ionization in the positive ion mode. All analytes showed good linearity over the investigated concentration range ($r > 0.9900$). The validated lower limit of quantification was 1.01 ng/mL for wogonin and oroxylin A, 0.238 ng/mL for chrysin, 1.01 ng/mL for naringenin, 0.998 ng/mL for hesperetin, 0.0505 ng/mL for berberine, 0.0996 ng/mL for coptisine, 0.0501 ng/mL for jatrorrhizine, 0.0889 ng/mL for palmatine, respectively. Intra- and inter-day precision (RSD%) was less than 15% and accuracy (RE%) ranged from −7.5% to 4.5%. The validated method was successfully applied to investigate the pharmacokinetics of the major flavonoids and alkaloids of Tang-Min-Ling-Pill after oral administration to rats.

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1. Introduction

Traditional Chinese medicine (TCM) being used clinically in Asian countries for more than 8000 years has been spread globally during the past decades [1]. Investigation of the pharmacokinetic properties of TCM is necessary to promote the efficacy and to avoid side adverse effects. Tang-Min-Ling-Pill (TMLP) is a famous traditional Chinese preparation consisting of *Radix Scutellariae*, *Rhizoma Coptidis*, *Fructus Aurantii Immaturus*, *Radix Paeoniae Alba*, etc., which is widely used for ameliorating hyperlipidemia and hyperglycemia, increasing insulin expression and antioxidant enzyme activity. Clinically, TMLP is applied in the treatment of type 2 diabetes mellitus and diabetic complications [2]. Flavonoids and alkaloids are considered to be the main pharmacological components [3–7]. Flavonoids such as wogonin (WO), oroxylin A (OR) and chrysin (CH) are the major ingredients in *Radix Scutellariae*, while naringenin (NAR) and hesperetin (HES) are present in *Fructus Aurantii Immaturus*. Protoberberine-type alkaloids of berberine (BE), coptisine (CO), jatrorrhizine (JA) and palmatine (PA) are the main bioactive components in *Rhizoma Coptidis* (Fig. 1). Therefore, simultaneous determination of these compounds in rat plasma is quite necessary to comprehensively characterize the pharmacokinetic profile of this drug.

Earlier publications described methods for analysis of some of these compounds in biological samples utilizing HPLC–UV [8], LC–MS [9] or LC–MS/MS [10–14]. However, to the best of our knowledge, there was no report on the simultaneous determination of WO, OR, CH, NAR, HES, BE, CO, JA and PA for pharmacokinetic studies.

Meanwhile, little information is available related to the pharmacokinetic profiles of WO, OR and CH. Kim et al. [11] have reported the pharmacokinetic behavior of OR and WO in rat plasma after an i.v. administration of *Radix Scutellariae* extract. The published HPLC–UV method [15] was developed for simultaneous determination of ten constituents and metabolites including WO and OR in rat plasma. However, the method incorporated a long

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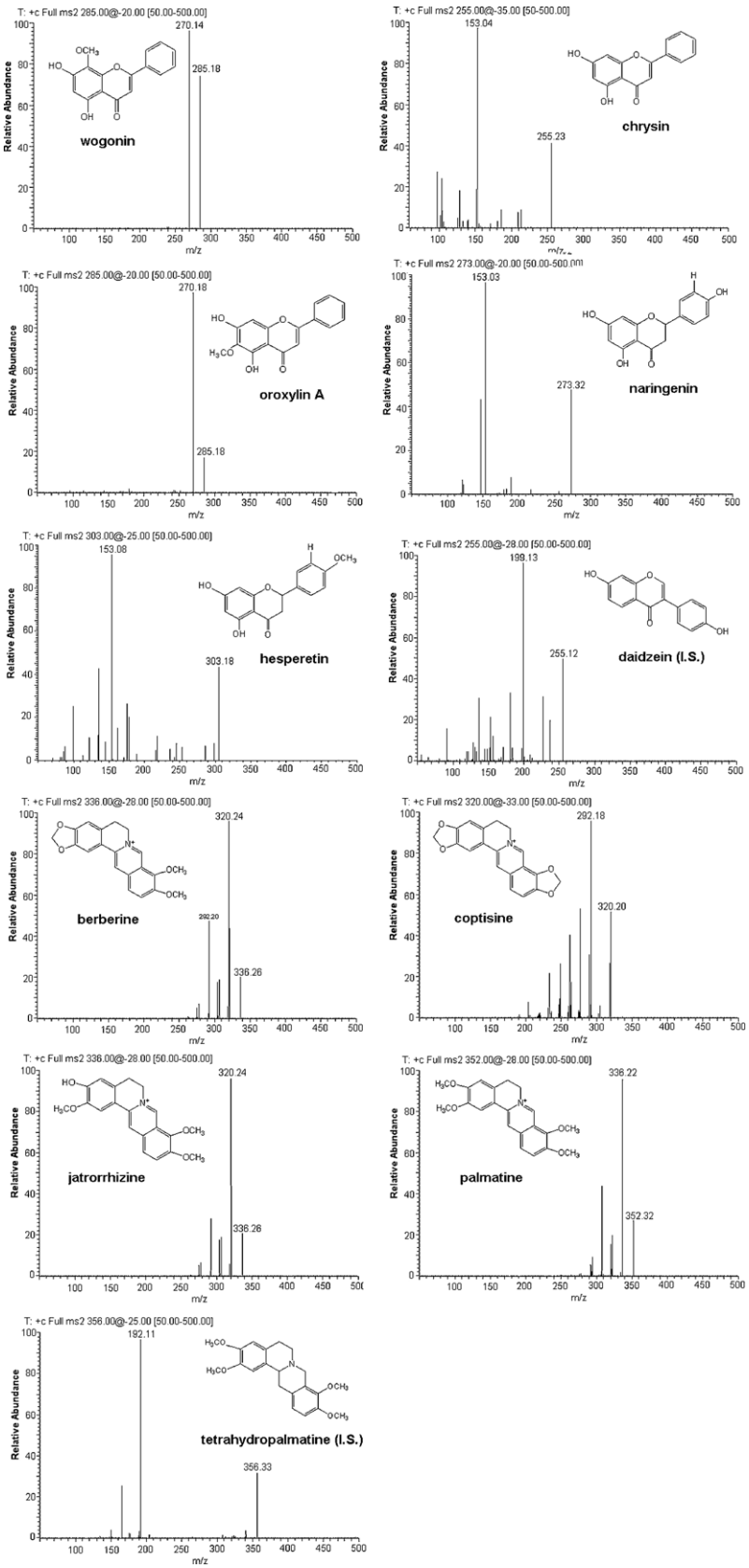

Fig. 1. Product ion mass spectra (ESI+) and ion structures of WO (a), CH (b), OR (c), NAR (d), HES (e), IS-1 (f), BE (g), CO (h), JA (i), PA (j) and IS-2 (k).

chromatographic running time of 50 min and insufficient sensitivity. T. Walle et al. [16] have investigated the disposition and metabolism of CH by HPLC–UV, but the research only aimed at administration of single substance and the representative chromatogram is not provided. Since WO, OR and CH are pharmacologically active in TMLP, it was deemed necessary to develop a method for the analysis of these flavonoids in plasma.

In this study, a rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for simultaneous quantification of WO, CH, OR, NAR, HES, BE, CO, JA and PA in rat plasma. The presented method has been used for pharmacokinetic profiling of major flavonoids and alkaloids for a study with rats receiving single dose of TMLP.

2. Experimental

2.1. Chemicals and materials

The reference standards of wogonin (WO), chrysin (CH), berberine (BE), jatrorrhizine (JA), palmatine (PA), daidzein (IS-1) and tetrahydropalmatine (IS-2) (purity >98%) were received from the National Institute for Drug Control of China (Beijing, China). oroxylin A (OR), naringenin (NAR), hesperetin (HES) and coptisine (CO) (purity >98%) were purchased from Delta Co. (Anhui, China). Acetonitrile, acetone and formic acid of HPLC grade were obtained from Tedia Co. (Fairfield, USA), other chemicals were of analytical grade. Deionized water was prepared by Milli-Q system (Millipore, MA, USA).

TMLP were supplied by Tianjin Tasly Pharmaceutical Co. Ltd. (Tianjin, China). To calculate the administration dosage, the contents of the nine major compounds in TMLP were quantitatively determined by HPLC external standard method, using a Zorbax SB-C_{18} column (250 × 4.6 mm i.d., 5 μm, Agilent, USA) with a mobile phase of water (A, consisting of 0.5% triethylamine, adjusted to pH 3.5 with acetic acid)–acetonitrile (B); The elution programme was well optimized and used as follows: 0–24 min, 19–20% B, 24–30 min, 20–25% B, 30–35 min, 25–35% B, 35–55 min, 35–55% B; UV detector at 278 nm. The content of WO, CH, OR, NAR, HES, BE, CO, JA and PA were determined to be 3.20, 0.0701, 0.733, 0.280, 0.239, 15.9, 2.20, 0.232, 1.38 mg/g, respectively.

2.2. Apparatus and LC–MS/MS conditions

The LC–MS/MS system consisted of a Surveyor™ HPLC system and a TSQ Quantum triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Finnigan, USA). Data acquisition was performed with Xcalibur 1.3 software.

Chromatographic separation was achieved on a Zorbax SB-C_{18} column (150 × 2.1 mm i.d., 5 μm, Agilent, USA) and a Luna C_{18} guard column (4 × 3.0 mm i.d., 5 μm, Phenomenex, USA) at room temperature. The isocratic mobile phase consisted of water (containing 0.1% of formic acid)–acetonitrile (30:70, v/v) at a flow rate of 0.3 mL/min in a run time of 7.0 min. The injection volume was 20 μL.

Mass spectrometer was operated in the positive mode. Quantification was obtained using multiple reaction monitoring (MRM) mode at *m*/*z* transitions of 285→270 for WO and OR, 255→153 for CH, 273→153 for NAR, 303→153 for HES, 255→199 for IS-1, 336→320 for BE, 320→292 for CO, 338→322 for JA, 352→336 for PA, and 356→192 for IS-2, respectively (Fig. 1). The standard solution (1 μg/mL) of each analyte and IS was used to optimize the MS/MS operating conditions by direct infusion. The MS parameters were as follows: spray voltage, 4.0 kV; heated capillary temperature, 350 °C; sheath gas (nitrogen), 35 psi; auxiliary gas (nitrogen): 15 psi; collision gas (argon) pressure, 1.5 mTorr; collision energy, 25 eV for WO, OA, NAR, HES and IS-1, 35 eV for CH, 30 eV for BE, CO, JA and PA and 27 eV for IS-2, respectively.

2.3. Preparation of standard and quality control samples

Stock solutions were prepared by dissolving the reference standards (1.01 mg/mL for WO, 0.595 mg/mL for CH, 0.507 mg/mL for OR, 0.503 mg/mL for NAR, 0.499 mg/mL for HES, 0.505 mg/mL for BE, 0.0498 mg/mL for CO, 0.0501 mg/mL for JA, 0.0494 mg/mL for PA) and IS (60.6 μg/mL for IS-1, 61.4 μg/mL for IS-2) in methanol. The solution was then diluted with methanol to achieve standard working solutions. The IS solution (60.6 ng/mL for IS-1 and 61.4 ng/mL for IS-2) was obtained by diluting the stock solution in methanol. All solutions were stored at 4 °C.

Calibration standards were prepared by spiking appropriate amount of the standard solutions in blank plasma (100 μL) to yield final concentrations of 10.1–5070 ng/mL for WO, 0.238–119 ng/mL for CH, 1.01–507 ng/mL for OR, 1.01–503 ng/mL for NAR, 0.998–499 ng/mL for HES, 1.01–505 ng/mL for BE, 0.0996–49.8 ng/mL for CO, 0.0501–25.0 ng/mL for JA, and 0.0889–44.5 ng/mL for PA, respectively. Three pools of quality control (QC) plasma samples were prepared containing WO (30.4, 304 and 4056 ng/mL, respectively), CH (0.714, 7.14 and 95.2 ng/mL, respectively), OR (3.04, 30.4 and 406 ng/mL, respectively), NAR (3.02, 302 and 402 ng/mL, respectively), HES (2.99, 29.9 and 399 ng/mL, respectively), BE (3.03, 30.3 and 404 ng/mL, respectively), CO (0.299, 2.99 and 39.8 ng/mL, respectively), JA (0.150, 1.50 and 20.0 ng/mL, respectively) and PA (0.267, 2.67 and 35.6 ng/mL, respectively). The spiked plasma samples (standard and quality controls) were extracted on each analytical batch along with the unknown samples.

2.4. Sample preparation

The plasma (100 μL) was spiked with 50 μL of IS solution (60.6 ng/mL for IS-1 and 61.4 ng/mL for IS-2), 50 μL of 0.5 M hydrochloric acid in methanol solution. The mixture was extracted with 800 μL of acetone by vortex-mixing for 5 min. After centrifugation at 8000 × *g* for 5 min, the organic layer was pipette-transferred and evaporated to dryness in vacuo at 40 °C. The residue was dissolved with 100 μL of 50% acetonitrile by sonicating and centrifuged. The supernatant of 20 μL was injected into the LC–MS/MS.

2.5. Method validation

Selectivity was tested by comparison of blank plasma from six individual rats with corresponding spiked plasma samples. The matrix effect was measured at three QC levels by comparing the absolute peak area of control plasma extracted and then spiked with a known amount of drug to that of neat standard samples at equivalent concentrations.

Calibration curves were confirmed by plotting the peak area ratio (y) of each analyte to IS versus plasma concentration (x) using weighted ($1/x^2$) least squares regression analysis. The lower limit of quantification (LLOQ) was defined as the lowest concentration of analytes that can be determined with acceptable accuracy within 20% deviation of the nominal concentration and precision below 20%.

Precision and accuracy were evaluated by assaying six replicates of QC samples at low, medium and high concentrations on the same day and three consecutive days. Precision was measured by intra- and inter-day relative standard deviation (RSD) and accuracy was described as relative error (RE). Extraction recoveries were determined at three QC levels with six replicates by comparing the peak area obtained from plasma sample spiked before extraction

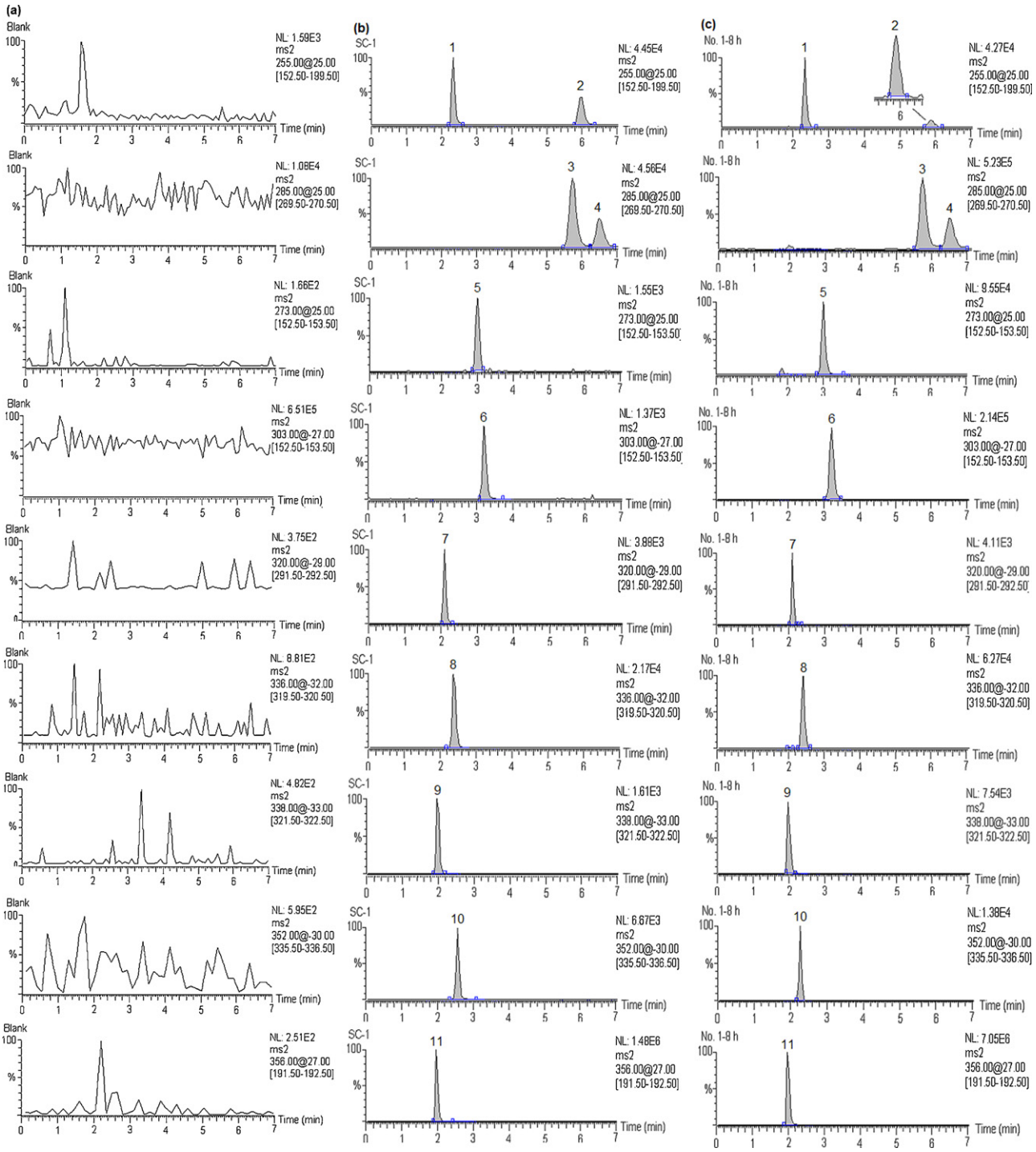

Fig. 2. Representative MRM chromatograms from (A) blank plasma sample; (B) blank plasma spiked with nine analytes at LLOQ and IS and (C) a rat plasma sample containing IS-1 (1), CH (2) 5.61 ng/mL, WO (3) 184 ng/mL, OR (4) 21.6 ng/mL, NAR (5) 134 ng/mL, HES (6) 175 ng/mL, CO (7) 0.746 ng/mL, BE (8) 8.26 ng/mL, JA (9) 0.478 ng/mL, PA (10) 0.776 ng/mL and IS-2 (11) collected at 8 h after oral administration of TMLP.

with those from plasma samples spiked after extraction. The same procedure was performed for IS.

Stability of QC samples was assessed by analyzing samples stored at −20 °C for 1 month, subjected to three successive freeze (−20 °C) to thaw (room temperature) cycles, stored at room temperature for 24 h and processed samples under autosampler condition for 12 h. Samples were considered stable with the deviation from the nominal concentration within ±15.0%.

2.6. Application of the method and pharmacokinetic study

Six male Wistar rats, weighing 200 ± 20 g, were supplied by Vital River Lab Animal Technology Co., Ltd. (Beijing, China). The rats were kept under controlled environmental conditions (temperature 22 ± 2 °C; humidity 50 ± 10%) with free access to the standard laboratory food and water. The dosing solutions were prepared by suspending the required amounts of TMLP in 0.5%

Table 1
Regression data and LLOQs of the analytes determined.

Components	Linear range (ng/mL)	Linear regression equation	Correlation coefficient (r)	LLOQ (ng/mL)
Wogonin	10.1–5070	$y = 6.68 \times 10^{-2} \times x + 3.35 \times 10^{-1}$	0.9953	1.01
Chrysin	0.238–119	$y = 2.04 \times 10^{-2} \times x + 3.94 \times 10^{-4}$	0.9956	0.238
Oroxylin A	1.01–507	$y = 2.72 \times 10^{-1} \times x + 1.15 \times 10^{-1}$	0.9961	1.01
Naringenin	1.01–503	$y = 9.78 \times 10^{-3} \times x - 2.07 \times 10^{-3}$	0.9965	1.01
Hesperetin	0.998–499	$y = 1.99 \times 10^{-2} \times x + 1.87 \times 10^{-3}$	0.9972	0.998
Berberine	1.01–505	$y = 1.23 \times 10^{-2} \times x + 8.88 \times 10^{-3}$	0.9959	0.0505
Coptisine	0.100–49.8	$y = 6.48 \times 10^{-3} \times x + 8.63 \times 10^{-4}$	0.9968	0.0996
Jatrorrhizine	0.0501–25.0	$y = 1.14 \times 10^{-2} \times x + 3.33 \times 10^{-4}$	0.9966	0.0501
Palmatine	0.0889–44.5	$y = 1.04 \times 10^{-2} \times x + 7.51 \times 10^{-3}$	0.9962	0.0889

carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution. After an oral administration of 16.0 g/kg TMLP (at a dose containing WO 51.2 mg/kg, CH 1.12 mg/kg, OR 11.7 mg/kg, NAR 4.48 mg/kg, HES 3.82 mg/kg, BE 254 mg/kg, CO 35.2 mg/kg, JA 3.71 mg/kg, and PA 22.1 mg/kg), blood samples (300 μL) were collected into heparinized tubes from each rat by the puncture of the retro-orbital sinus prior to dosage and at 0.08, 0.17, 0.33, 0.67, 1, 2, 3, 4, 6, 8, 10, 12, 24 and 30 h thereafter. Following centrifugation (4000 *g* for 10 min), plasma samples were stored at −20 °C until analysis. All pharmacokinetic parameters were processed by noncompartmental analysis using DAS 2.0 software (Chinese Pharmacological Society).

3. Results and discussion

3.1. Method development

3.1.1. LC–MS/MS optimization

According to the direct full-scan ESI mass spectra, the ion intensities of flavonoids and alkaloids obtained in positive mode were much stronger than those in negative mode. In full-scan spectra, the flavonoids produced abundant protonated molecular ions $[M+H]^+$, while the alkaloids formed predominantly quaternary ammonium ion $[M]^+$. Product ion mass spectra of the nine major compounds and IS are shown in Fig. 1. WO and OR are isomers with identical product ion at *m*/*z* 270, which corresponded to the loss of methyl group from the precursor ion *m*/*z* 285. For CH, NAR and HES, the most abundant fragment were the results of the Retro-Diels–Alder (RDA) fragmentation, all at *m*/*z* 153. The major ion of *m*/*z* 199 for IS-1 may be due to loss of two neutral CO and molecular transposition from the deprotonated molecular ion. For BE, JA and PA, the loss of a methyl group and a hydrogen was the most abundant fragment pathway. For IS-2, the most abundant fragment obtained from the precursor ion *m*/*z* 356 was detected at *m*/*z* 192. The most sensitive MRM transitions at 285→270, 255→153, 285→270, 273→153, 303→153, 255→199, 336→320, 320→292, 338→322, 352→336 and 356→192 were selected to analyze WO, CH, OR, NAR, HES, IS-1, BE, CO, JA, PA and IS-2, respectively.

Different mobile phases were tested to optimize analytical performance. Acetonitrile, instead of methanol, was found to improve

Table 2
Precision, accuracy and extraction recoveries for the analytes in rat plasma.

Components	Spiked (ng/mL)	Intra-day concentration measured (ng/mL)	Precision (%, RSD)	Accuracy (%, RE)	Inter-day concentration measured (ng/mL)	Precision (%, RSD)	Accuracy (%, RE)	Extraction recovery (%)
Wogonin	30.4	30.7 ± 3.2	10	1.0	31.4 ± 3.1	4.4	3.1	59.3 ± 5.6
	304	303 ± 19	6.8	−0.40	311 ± 21	6.5	2.3	65.3 ± 5.4
	4056	3936 ± 229	6.3	−2.9	4127 ± 282	10	1.8	63.8 ± 4.5
Chrysin	0.714	0.687 ± 0.078	9.1	−3.8	0.705 ± 0.064	8.4	−1.3	63.5 ± 5.3
	7.14	7.03 ± 0.59	7.5	−1.6	7.28 ± 0.55	7.8	2.0	69.8 ± 4.5
	95.2	91.4 ± 4.4	5.1	−4.0	94.8 ± 5.2	7.7	−0.40	68.2 ± 6.6
Oroxylin A	3.04	2.92 ± 0.16	6.6	−3.9	3.14 ± 0.25	15	3.3	61.2 ± 5.3
	30.4	29.0 ± 1.9	6.6	−4.8	30.8 ± 2.3	13	1.3	65.7 ± 3.8
	406	387 ± 28	5.6	−4.6	398 ± 24	7.7	−1.8	63.8 ± 4.5
Naringenin	3.02	2.83 ± 0.16	7.6	−6.1	2.93 ± 0.24	11	−2.9	64.5 ± 5.2
	30.2	30.6 ± 1.6	8.9	1.4	31.5 ± 2.8	8.1	4.3	66.5 ± 5.4
	402	400 ± 25	7.2	−0.70	403 ± 27	2.0	0.20	65.7 ± 3.9
Hesperetin	2.99	3.13 ± 0.21	7.8	4.5	3.13 ± 0.23	1.4	4.5	61.2 ± 6.6
	29.9	28.1 ± 1.8	7.0	−6.2	29.8 ± 2.3	12	−0.50	66.2 ± 2.5
	399	369 ± 30	7.7	−7.5	398 ± 36	15	−0.29	64.8 ± 4.1
Berberine	3.03	2.99 ± 0.27	8.3	−1.3	2.98 ± 0.26	10	−1.7	65.8 ± 5.4
	30.3	30.8 ± 2.4	9.1	1.5	30.2 ± 2.5	4.1	−0.36	68.1 ± 4.6
	404	392 ± 29	7.5	−3.1	389 ± 28	4.0	−3.8	66.7 ± 3.5
Coptisine	0.299	0.280 ± 0.024	8.8	−6.2	0.292 ± 0.026	10	−2.3	62.2 ± 6.8
	2.99	2.86 ± 0.22	6.6	−4.1	2.86 ± 0.18	2.0	−4.2	65.2 ± 5.8
	39.8	38.6 ± 2.3	6.9	−3.0	39.8 ± 2.7	6.5	0.031	63.3 ± 5.0
Jatrorrhizine	0.150	0.145 ± 0.014	10	−3.4	0.151 ± 0.014	8.9	0.54	62.5 ± 6.2
	1.50	1.50 ± 0.10	7.7	−0.94	1.47 ± 0.11	4.8	−2.1	64.5 ± 4.4
	20.0	20.7 ± 1.7	7.5	3.4	20.1 ± 1.5	8.3	0.28	65.3 ± 3.7
Palmatine	0.267	0.270 ± 0.025	8.3	1.0	0.275 ± 0.022	5.6	3.0	63.6 ± 5.6
	2.67	2.62 ± 0.18	7.6	−2.0	2.55 ± 0.19	5.8	−4.6	66.1 ± 4.6
	35.6	37.1 ± 2.7	7.2	4.3	36.5 ± 2.6	5.8	2.7	61.8 ± 3.9

the resolution of WO and OR. The addition of formic acid was proved to enhance the sensitivity and to get better peak shape. Finally, acetonitrile–water with 0.1% formic acid (70:30, v/v) was employed, and low background noise and suitable retention time were provided. As shown in Fig. 2, each chromatographic run was completed within 7.0 min. Slight cross-talk was observed between BE and JA because of similar structures and fragmentation mechanism. However, this unremarkable interference did not affect the precision, accuracy and reliability of the method due to full chromatographic separation. It is necessary to choose an appropriate IS to get high accuracy in MS quantitation. Based on the structural similarity with the corresponding analytes, daidzein and tetrahydropalmatine were chosen as IS for the flavonoids and alkaloids, respectively, giving better results for linearity and quantitation. Therefore, the developed analytical method was reliable and suitable for this study.

3.1.2. Sample preparation

Liquid–liquid extraction was investigated in our study. Different kinds of organic reagents (acetone, diethyl ether, methyl tert-butyl ether and ethyl acetate) were tested, and satisfactory recoveries were achieved by virtue of liquid–liquid extraction with acetone. Plasma acidification with HCl before extraction could help enhance the extraction efficiency of the five flavonoids, and gave cleaner samples.

3.2. Method validation

3.2.1. Specificity

Typical chromatograms obtained from blank plasma, blank plasma spiked with the analytes (at LLOQs) and IS, and plasma sample after an oral dose are presented in Fig. 2. The retention times of IS-1, CH, WO, OR, NAR, HES, CO, BE, JA, PA and IS-2 were 2.3, 5.9, 5.7, 6.5, 3.0, 3.2, 2.1, 2.3, 2.0, 2.2 and 2.0 min, respectively. No interference of endogenous peaks exhibited in drug-free specimens indicated the high selectivity of LC–MS/MS.

3.2.2. Linearity and LLOQs

The linear ranges, regression equations, LLOQs, and correlation coefficients obtained from typical calibration curves are shown in Table 1. All standard curves exhibited good linearity and the correlation coefficients (r) were higher than 0.9953. The LLOQ for WO, CH, OR, NAR, HES, BE, CO, JA, PA were 1.01, 0.238, 1.01, 1.01, 0.998, 0.0505, 0.0996, 0.0501 and 0.0889 ng/mL, with coefficient of variation 8.5%, 12.7%, 9.2%, 10.5%, 7.7%, 9.8%, 11.3%, 8.2% and 12.7%, respectively.

3.2.3. Precision and accuracy

The intra-day and inter-day precision and accuracy data are shown in Table 2. The intra-day and inter-day precisions were ranged from 5.1% to 10% and 1.4% to 15%, respectively, while the accuracy ranged from −7.5% to 4.5%. The results revealed good precision and accuracy of this present method.

3.2.4. Extraction recovery and matrix effect

As presented in Table 2, the extraction recoveries of nine analytes were in the range 53.7–74.8% at three concentration levels. The RSD values were all less than 11%. The mean extraction recovery of the IS-1 and IS-2 was 63.2 ± 5.0% and 67.1 ± 3.8%, respectively.

The observed matrix effects ranged from 101.1 to 127.9% for WO, 96.2 to 119.2% for CH, 94.9 to 119.5% for OR, 99.7 to 118.3% for NAR, 99.8 to 128.2% for hesperidin, 96.6 to 109.0% for BE, 95.8 to 111.0% for CO, 101.0 to 117.6% for JA and 104.9 to 123.7% for PA. The mean matrix effect for the IS-1 and IS-2 was 110.2% and 111.2%, respectively.

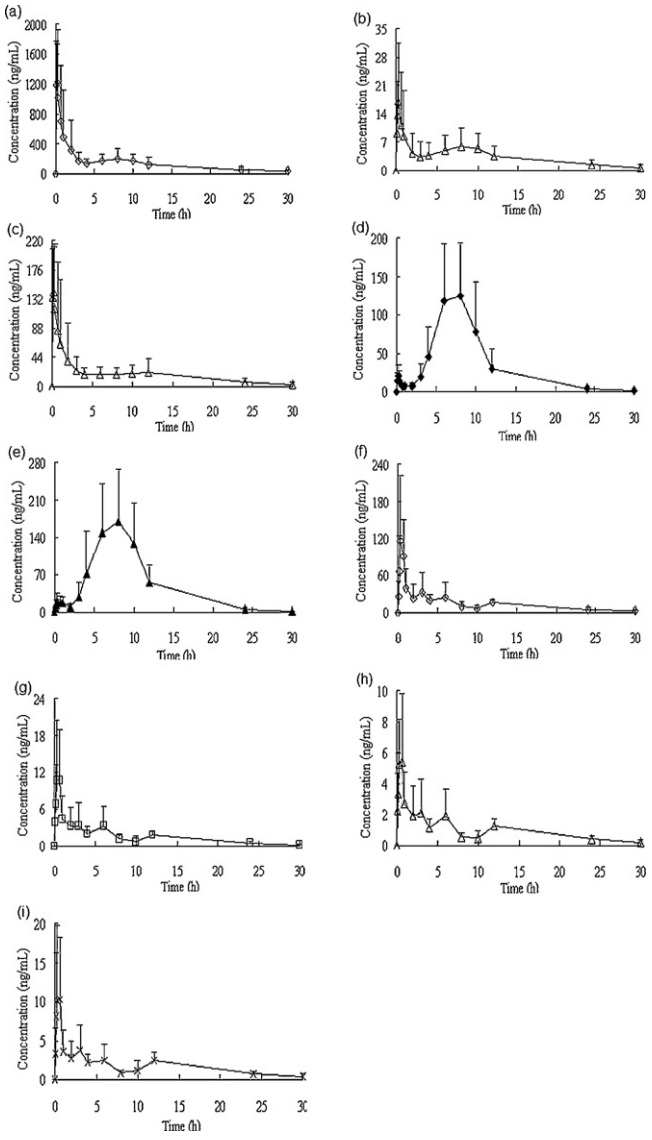

Fig. 3. Mean ± SD plasma concentration–time profiles of WO (a), CH (b), OR (c), NAR (d), HES (e), BE (f), CO (g), JA (h) and PA (i) in rat plasma after oral administration TMLP at a dose of 16.0 g/kg.

3.2.5. Stability

Table 3 summarizes the stability data of QC samples. The results showed that all the samples were stable during these tests and there were no stability related problems during the routine analysis of samples for the pharmacokinetic study.

3.3. Results of pharmacokinetic study

The validated method was sensitive enough to measure all the nine compounds in rat plasma after oral administration of TMLP simultaneously. The mean plasma concentration–time profiles of the analytes are represented in Fig. 3. Pharmacokinetic parameters are listed in Table 4. The moderately high C_{max} values of the analytes may be related to the poor absolute bioavailability of flavonoids and alkaloids after relatively high administered doses [17–19]. From the figure, all the flavonoids exhibited biphasic pharmacokinetics in pharmacokinetic behavior. It is the first time that two extrema in plasma concentration–time of WO, OR and CH have been reported. WO, OR and CH were rapidly absorbed with a maximum concentrations occurring within 0.5 h, and the other extremum appeared at 8–12 h. However, the plasma levels of NAR and HES slowly reached

Table 3
Stability of the analytes in rat plasma ($n = 5$).

Analytes	Spiked (ng/mL)	Stability (RE %)			
		Long-term (1 month at −20 °C)	Three freeze–thaw	Short-term (24 h at room temperature)	Post-treatment (12 h at room temperature)
Wogonin	30.4	3.3	−4.7	6.5	2.8
	304	−7.2	7.5	−4.9	−8.0
	4056	−4.3	10	9.1	−8.2
Chrysin	0.714	2.3	−5.3	−11	−3.5
	7.14	−3.9	−1.8	−5.1	−4.3
	95.2	3.7	2.4	6.5	−1.2
Oroxylin A	3.04	−3.6	−2.4	7.7	−10
	30.4	0.5	−0.4	−1.1	1.0
	406	6.1	8.0	−6.9	−0.57
Naringenin	3.02	−5.2	0.78	6.7	5.0
	30.2	1.8	4.8	−9.3	−7.6
	402	−4.9	3.3	−3.3	−1.6
Hesperetin	2.99	−4.8	1.7	9.4	−1.5
	29.9	−5.0	2.6	−5.0	−1.2
	399	4.7	−1.8	7.7	−5.7
Berberine	3.03	2.2	7.3	4.1	3.1
	30.3	−1.4	4.8	5.5	0.085
	404	−2.6	−3.0	−4.3	3.6
Coptisine	0.299	2.8	−9.0	−7.0	−2.6
	2.99	−7.2	−3.8	−1.4	2.3
	39.8	−2.3	−2.2	4.0	3.7
Jatrorrhizine	0.150	−3.1	1.9	−3.5	−3.1
	1.50	−1.6	−3.1	−9.0	−7.3
	20.0	11	−9.9	6.1	2.3
Palmatine	0.267	−4.6	−4.9	−4.4	−5.5
	2.67	6.1	0.64	2.0	−2.9
	35.6	3.7	−5.3	6.9	4.3

Table 4
Estimated pharmacokinetic parameters for the nine compounds in rat plasma ($n = 6$) after oral administration of TMLW.

Parameters	T_{max} (h)	C_{max} (ng/mL)	$T_{1/2}$ (h)	$AUC_{(0-t)}$ (ng h/mL)	$AUC_{(0-\infty)}$ (ng h/mL)	$MRT_{(0-t)}$ (h)	CL/F (L/h kg)
Wogonin	0.2 ± 0.1	1378 ± 693	6.5 ± 1.1	4202 ± 2909	4520 ± 3139	8.12 ± 1.32	17.5 ± 11.6
Chrysin	0.3 ± 0.1	19.47 ± 13.14	6.6 ± 0.8	100.7 ± 74.2	107.5 ± 77.9	9.80 ± 1.09	17.0 ± 11.0
Oroxylin A	0.3 ± 0.2	155.9 ± 84.8	7.6 ± 2.3	528.7 ± 459.3	566.3 ± 469.0	8.08 ± 0.97	35.9 ± 25.2
Naringenin	7.3 ± 1.4	133.8 ± 72.7	4.1 ± 1.0	999.3 ± 583.1	1007 ± 582	9.01 ± 1.02	5.84 ± 2.97
Hesperetin	7.6 ± 1.7	190.3 ± 102.4	3.4 ± 0.9	1497 ± 672	1507 ± 668	9.16 ± 1.37	3.12 ± 1.52
Berberine	0.6 ± 0.2	156.2 ± 89.6	7.3 ± 0.6	440.1 ± 113.0	473.4 ± 120.9	8.65 ± 1.62	574 ± 150
Coptisine	0.5 ± 0.3	16.94 ± 8.60	6.5 ± 1.4	47.05 ± 13.64	49.29 ± 13.64	8.17 ± 1.88	769 ± 234
Jatrorrhizine	0.6 ± 0.2	7.884 ± 3.260	7.2 ± 0.8	30.58 ± 10.14	33.10 ± 10.80	9.41 ± 1.67	121 ± 36
Palmatine	0.5 ± 0.3	16.52 ± 9.07	6.8 ± 1.4	51.95 ± 12.22	55.69 ± 14.06	9.02 ± 1.77	429 ± 125

C_{max} at 8–12 h and declined quickly, as was reported by Tong et al. [20]. Evidence was provided that glucuronidation, enteric circulation and enterohepatic circulation may contribute to the biphasic pharmacokinetics [12,13,21].

These four protoberberine-type alkaloids exhibited consistent tendency in plasma concentration–time profiles could be tentatively explained based on their similar structures. In parallel with previous literature [14], multiple plasma concentration peaks of the four alkaloids were also observed in this study, probably due to distribution re-absorption, enterohepatic circulation and interaction among multitudinous compounds in formulas. The poor absorption and extensive metabolism may contribute to the extremely low plasma concentration of BE, and possibly the other three alkaloids.

4. Conclusion

For the first time, an LC–MS/MS assay for simultaneous determination of WO, CH, OR, NAR, HES, BE, CO, JA and PA in rat plasma was developed and validated. The method was rapid, specific, sensitive, accurate and reproducible, and was successfully applied to the pharmacokinetic study of these flavonoids and alkaloids after oral administration of TMLP. The relative short chromatographic run time allows fast analysis of plasma samples.

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