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Study on Metabolism of Natural Medical Components *In Vivo*: Metabolism Study in Rat After Oral Administration of Rhubarb Decoction and Characterization, Identification of the Rat Metabolite of *Scutellaria baicalensis*

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1. Introduction

Rheum palmatum L., one of the commonly used Traditional Chinese Medicines (TCMs), which is called Dahuang in Chinese, has been successfully used in China for thousands of years. The crude material of *R. palmatum* called rhubarb was widely used as laxative to treat constipation. Furthermore, it has various other pharmacological actions, such as antibacterial [1, 2], anti-inflammatory [3, 4], antiviral [5], anti-angiogenic [6], antioxidative [7], immunomodulatory [8], protecting effects in rat with chronic renal failure [9] and anti-diabetic effects on diabetic mice [10]. Although many investigations have been conducted in the fields of pharmacology, clinical trials, and phytochemistry about rhubarb, researchers still do not know what its effective constituents are, how many compounds are absorbed into blood after intragastric administration of the decoction, and what the fate of the decoction in the body is. The limited knowledge about the metabolism of rhubarb decoction restricts the deeper pharmacological mechanism study and wider clinical use of rhubarb.

It was well accepted that TCMs expressed its effects through multi-components and multi-targets. Rapid, sensitive and selective analytical method is needed to simultaneous determine of multiple components of TCMs in biological matrix with low concentrations. In recent years, HPLC/ESI/MSⁿ has been proved to be a modern and powerful method for the identification of compounds in biosamples or TCM extracts [11, 12]. Metabolism of rhubarb has been reported [13], but data on pharmacokinetics are scanty. So, our interest was to utilize a solid phase extraction (SPE) and HPLC/ESI/MSⁿ techniques for the detection of potentially active compounds in urine, plasma and tissue samples and for the

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pharmacokinetic study of rhubarb. Total of 39 compounds were identified as absorbed bioactive constituents. The excretion of M22 and M39 reached more than 1000 µg in urine samples after intragastric administration of the decoction.

2. Experiments

2.1 Chemicals and materials

Standards of the rhapontin, aloe-emodin, chrysophanol, emodin, rheochrysidin and rhein were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). The plasma, urine and tissue samples were extracted using Supelco™ LC-18 solid-phase extraction (SPE) tubes (1ml/100mg, USA). Acetonitrile (HPLC grade) was purchased from the Dikma Company (Dikma, USA). Water was deionized and double distilled. All other chemicals were of analytical grade. Blank rat plasma (drug free) was prepared in our laboratory.

2.2 Equipment

An HPLC-MS system (Agilent 1100, Agilent Technologies, Wilmington, DE, USA) with an electrospray ionization (ESI) ion source was used. The LC/MSD Trap software (version 5.3) was used for data acquisition and processing. An API 4000 triple quadrupole instrument (Applied Biosystems, Toronto, Canada) was used for the mass spectrometric detection using an electrospray ionization (ESI) source in the negative mode. A high-speed bench-top centrifuge (Sorvall ST16, Thermo Fisher Scientific, Germany) was used for centrifuge biological samples.

2.3 Preparation of rhubarb decoction

200 g rhubarb was immersed in 2000 mL distilled water and then refluxed for 1 h at 100°C. The aqueous extract was filtered and the residue was refluxed for 30 min with another 2000 mL of distilled water under the same conditions. The two water extracts were combined and condensed to 200 mL and stored at 4°C until use.

2.4 Standard preparation

The standard of rhapontin, aloe-emodin, chrysophanol, emodin, rheochrysidin and rhein were dissolved in methanol at a concentration of 100 µg/ml to obtain the standard stock solutions.

2.5 Animals

30 male Sprague-Dawley rats (200±20 g) were obtained from the Animal Center of Shanghai Institute of Materia Medica, Shanghai, China. They were kept under standard laboratory conditions (12/12 h light/darkness, 22±2°C room temperature, 50-60% humidity) for one week prior to the experiments. All animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of Shanghai Institute of Materia Medica.

Six male rats were orally administered a single dose of rhubarb decoction at 10 g/kg. After dosing, the rats were housed in separate glass metabolic cages with free access to water. Urine was collected at -8 to 0 h predose and during 0-2, 2-4, 4-6, 6-8, 8-24, 24-36, 36-48 h postdose.

Other 24 male rats were divided into four groups at random. They were used in determining the concentration of rhubarb in blood and tissues. One group of rats was killed predose to provide control blood and tissues for analysis. Other three groups of rats were orally administered of rhubarb decoction at a dosage of 17.6 g/kg. Six rats each were anesthetized with ether and blood samples were collected from abdominal aorta of the rats at 1.0, 2.0, and 3.0 h after dosing, respectively. The following tissues and organs were collected and weighed at the time of killing for determination of rhubarb concentration: heart (HE), liver (LV), spleen (SP), lung (LU), kidney (KI), brain (BR), small intestine (SI), large intestine (LI), stomach (ST), and testis (TE). The whole tissues were homogenized with PBS to yield final concentration of 20% w/v. All the homogenates were stored at -80°C.

2.6 Extraction of rat biological samples

All samples were centrifuged at 3000×g for 10 min. The supernatants were loaded onto a C18 SPE cartridge. Before use, an SPE column was conditioned and washed with 2 ml of methanol and then 2 ml of deionized water. Then 600 µl of the selected supernatant sample was applied to the SPE well. The SPE well was washed with 1 ml of water, and then the analytes were eluted with 1 ml of methanol. The eluent was evaporated at 37°C under a gentle stream of nitrogen. The dry residue was then reconstituted with 200 µl methanol and vortex-mixed for 20 s. The solution was then centrifuged at 14000×g for 10 min, and an aliquot of 10 µl supernatant was injected into the HPLC/ESI/MS system for analysis.

2.7 HPLC condition

The metabolites of rhubarb decoction were separated on a Grace Apollo C18 reversed-phase column (250 mm×4.6 mm, 5 µm) equipped with an EasyGuard Kit C18 (4×2 mm) guard column. The column was maintained at 25°C. The mobile phase consisted of 0.5% formic acid-water (A) and acetonitrile (B) and was delivered at a flow rate of 1.0 ml/min. The detection wavelengths were set at 190-400 nm. Gradient elution was used as follows: a linear gradient from 35% to 100% B in the first 30 min, followed by 100% B for 10 min, and finally a linear gradient to 35% B at 45 min that was held for 5 min. The total run time was 50 min.

2.8 Mass spectrometry analysis for qualification

The HPLC system used was an Agilent 1100 series LC/MSD Trap mass spectrometer (Agilent Technologies, Wilmington, DE, USA), connected to an Agilent 1100 HPLC instrument via an electrospray ionization (ESI) source. The LC/MSD Trap software (version 5.3) was used for system operation and data collection. The operating parameters in the negative ion mode were as follows: collision gas, ultra-high purity helium (He); nebulizing gas, high purity nitrogen (N₂); capillary voltage, 3.5 kV; end plate offset, 500 V; nebulizer, 30 psi; drying gas flow rate, 10 l/min; drying gas temp., 350°C. Trap: ICC, target, 30000, max accu. time, 300.00ms, averages, 5. Auto Ms. 4; MS/MS frag. ampl., 1.00V; auto MS (n>2): frag. ampl., 0.77V. Smart frag: start ampl., 30.0%; end ampl., 200%. For full-scan MS analysis, the spectra were recorded in the range of *m/z* 50-1500.

2.9 Mass spectrometry analysis for quantitation

An API 4000 Qtrap mass spectrometer (Applied Biosystems, Toronto, Canada) equipped with a pneumatically assisted ESI interface was linked with the HPLC system. Initially optimization of the parameters for the ESI/MS and ESI/MS/MS analyses of the standards

and the samples was performed by direct infusion into the ES ionization source. The operating parameters were as follows: negative ion scan mode, curtain gas (CUR) 30 psi, collision gas (CAD) medium, ion source gas 1 (GC1) 45 psi, ion source gas 2 (GC2) 45 psi, ion spray voltage (IS) 5000 V, entrance potential (EP) 10 V, declustering potential (DP) 160 V, collision energy 60 V, collision cell exit potential (CXP) 3 V and temperature (TEM) 350°C.

3. Results

3.1 Optimization of HPLC and MS conditions

To obtain HPLC chromatograms with good separation and peak shape, different mobile phase compositions were screened. Given the acidity of phenolic compounds, it was found that acetonitrile and 0.5% aqueous formic acid were the most suitable eluting solvent system. The proposed method was acceptable as well as adequate for further MS/MS analysis. To acquire maximum sensitivity for most compounds, MS parameters such as spray voltage, capillary temperature, sheath gas and auxiliary gas pressure, source CID, collision gas pressure and collision energy were optimized using methanol extraction of rhubarb decoction by flow injection analysis (FIA). It was found that the negative ion mode was more sensitive than positive ion mode for most of the compounds.

3.2 System suitability

The system suitability test is performed to assure that the analytical method can be executed with the existing HPLC system. A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of a system suitability/calibration standard (at concentration of 10 µg/ml) were made. Area and retention time relative standard deviation, asymmetry factor t_a and efficiency (as plate number N) for the five injections was determined. For all samples analyses, the asymmetry factor t_a was ≤ 1.4 , efficiency ≥ 3000 and area % R.S.D. $\leq 1.0\%$.

3.3 Optimization of sample preparation

Solid-phase extraction was used as an important step of the sample preparation. Quantitative elution of standard samples from SPE cartridge are apparent after 1.0 ml of methanol. The reproducibility and recovery of solid-phase extraction was determined from five repetitions. The reproducibility expressed as R.S.D. was 0.6-0.9% and recovery was 84.8-97.2% for concentration of 10 µg/ml of standard samples.

3.4 Limits of detection (LOD)/ linearity/accuracy/precision for related substances

The limit of detection of the six standard components (based on a detector signal-to-noise ratio 3:1) was 0.1 µg/ml. The method was found to be linear with correlation coefficients (R^2) of 0.986-0.997 for the six standard components, with slopes near unity and y -intercepts near zero for these low-level determinations. Accuracy and precision for the standard components were satisfactory at three concentrations studied. Accuracy and intra-day and inter-day precision of the six standard components were less than 10%.

3.5 Stability of standard and sample solutions

Prepared samples and standards have been shown to be stable for at least 2 weeks when stored refrigerated. Additionally, the standard solutions have been shown to be stable while

in use for assays for at least 72 h. The stability of the six standard sample solutions was evaluated after 1 and 2 weeks under refrigerated condition. The results obtained for refrigerated standard solution were 100.1 to 101.2% of the initial concentration for 1 and 2 week time points, respectively. The result for the refrigerated sample solution were 102.1 to 96.8% of its initial concentration after 1 and 2 week's storage, respectively. No degradation products were observed for any of the solutions tested. The standard solutions that had been held at room temperature for 72 h were stable with responses of 98.7-100.9%.

3.6 Fragmentation behavior of the parent compounds

To interpret the mass spectra of the metabolites using the LC/MSⁿ technique, it is necessary to fully understand the fragmentation behavior of the parent compound. Rhapontin, aloemodin, chrysophanol, emodin, rheochrysidin and rhein were selected as representative parent compounds in this study. It was found that the negative ion mode was more sensitive than positive ion mode for most of the compounds with triple quadrupole mass spectrometer, so negative ion mode was chosen. The HPLC/ESI/MSⁿ spectra of [M-H]⁻ ion of the six parent compounds were shown in Fig.1. As most of these metabolites remained the structural features of the parent compounds, the analysis of the fragmentation pathways greatly facilitated the identification of metabolites from rhubarb decoction.

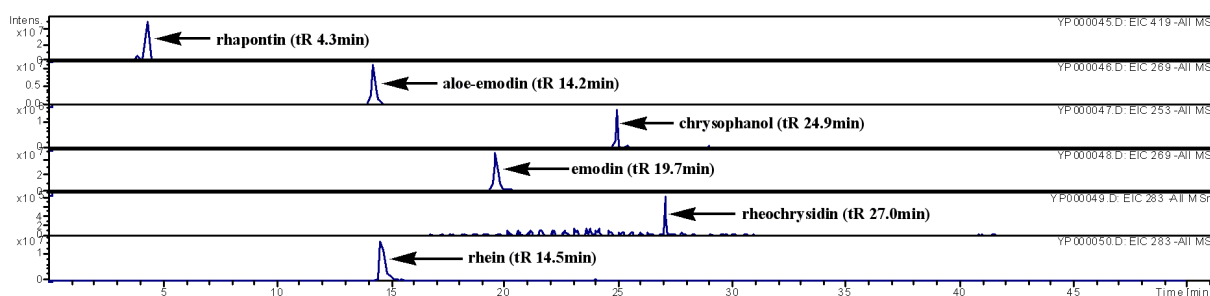


Fig. 1. The HPLC/ESI/MS spectra of the parent compounds

3.7 Identification of metabolites

Possible metabolite structures were considered based on the parent structure and the known metabolic pathways. As for MS detection, the potential metabolites were detected by comparison of the chromatograms of the drug-containing samples with those of blank samples. The retention time of each potential metabolite was ascertained by employing its mass to generate its extracted ion chromatogram (EIC) of the drug-containing sample. Compared with blank samples, a total of 39 compounds were detected in extracted ion chromatograms from the drug-containing urine, plasma and tissue samples (Table 1).

The identification of the metabolites and elucidation of their structures were performed mainly based on their MSⁿ fragmentations. Possible metabolite structures were considered based on the parent structure and the known metabolic pathways. Then, rat urine, plasma and tissue samples after i.g. administration of rhubarb decoction were analyzed and compared with blank samples by HPLC/ESI/MSⁿ in negative mode. Typical chromatograms resulting from the analysis of various biological samples are shown in Fig.2. Ultimately, the 39 metabolites could be generally divided into three groups: glucoside, glucuronidation and sulfation metabolites. The MS² spectra of the 39 compounds were shown in Fig. 3. Metabolite M4, M10 and M20 were selected as examples and the structural

elucidations of the other metabolites were carried out similarly. The postulated fragmentation pathways of M4, M10 and M20 were shown in Fig.4.

Metabolites	t_R (min)	[M-H] ⁻	UR	PL	Tissues										ESI-MS data (% base peak) (<i>m/z</i>)	
					HE	LV	SP	LU	KI	BR	SB	LB	ST	TE		
M1	3.3	591	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [591]:253(100),175(14.4) MS ³ [253]:253(100)
M2	3.3	383	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [383]:303(34.7),207(100),113(52.1) MS ³ [207]:207(52.8),163(100),122(10.8)
M3	3.3	445	-	-	-	-	-	-	-	-	-	-	+	-	-	MS ² [445]:427(8.0),283(100),175(4.5) MS ³ [283]:283(100),239(17.4)
M4	3.4	479	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [479]:303(100),174(20.9) MS ³ [303]:303(100),259(9.8) MS ² [417]:373(100),330(12.5),241(41.0), 175(3.7)
M5	3.4	417	+	-	-	+	-	-	-	-	-	-	-	-	-	MS ³ [373]:373(9.1),330(100)
M6	3.5	313	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [313]:313(12.8),233(100),175(3.6) MS ³ [233]:233(100),189(11.2)
M7	3.5	459	+	+	-	-	-	-	+	-	-	-	-	-	-	MS ² [459]:283(100),175(3.6) MS ³ [283]:283(100),239(9.1)
M8	3.7	475	-	-	-	-	-	-	-	-	-	-	+	-	-	MS ² [475]:431(7.2),311(57.0),269(100) MS ³ [269]:269(100)
M9	3.8	287	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [287]:287(8.3),207(100) MS ³ [207]:207(85.5),163(100)
M10	4.1	621	-	+	-	-	-	-	-	-	-	-	-	-	-	MS ² [621]:575(3.5),445(100),269(21.3) MS ³ [445]:311(7.8),269(100)
M11	4.1	375	-	-	+	-	-	-	-	-	-	-	-	-	-	MS ² [375]:332(18.8),243(100) MS ³ [243]:243(100)
M12	4.2	445	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [445]:269(100),175(2.1) MS ³ [269]:269(100)
M13	4.2	268	+	-	+	-	-	-	+	-	-	-	-	-	-	MS ² [269]:189(100)
M14	4.3	417	+	+	-	-	-	-	-	-	-	-	-	-	-	MS ² [417]:399(8.1),241(5.1),175(100) MS ³ [175]:113(100)
M15	4.5	435	-	-	-	-	-	-	-	-	-	-	+	-	-	MS ² [435]:273(100),167(4.3) MS ³ [273]:273(100),167(53.0)
M16	4.6	431	-	-	-	-	-	-	-	-	-	-	+	-	-	MS ² [431]:269(100) MS ³ [269]:269(100)
M17	4.6	459	+	-	-	-	-	-	-	-	-	-	+	-	-	MS ² [459]:283(100) MS ³ [283]:283(100),257(1.9)
M18	4.7	297	+	+	+	-	-	+	+	-	-	-	-	-	-	MS ² [297]:175(83.7),113(100) MS ³ [113]:95(80.0),85(100)
M19	4.9	401	-	+	-	+	-	+	+	-	+	-	-	-	-	MS ² [401]:358(100),296(4.2),225(32.4), 174(60.5)
M20	5.2	525	-	+	-	-	-	-	-	-	-	-	-	-	-	MS ³ [358]:313(13.5),174(100) MS ² [525]:445(100),349(20.3),269(73.5) MS ³ [445]:269(100)
M21	5.3	349	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [349]:269(100) MS ³ [269]:269(100),240(5.8)
M22	5.3	363	+	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [363]:283(100) MS ³ [283]:283(100),239(7.0)
M23	5.4	433	-	+	-	-	-	-	-	-	-	-	-	-	-	MS ² [433]:415(7.4),257(100),175(60.5) MS ³ [257]:257(100)
M24	5.6	321	-	+	+	+	-	-	-	-	-	+	-	-	-	MS ² [321]:241(100),121(87.7) MS ³ [241]:241(12.3),121(100)
M25	6.0	473	-	+	-	-	-	-	-	-	-	-	-	-	-	MS ² [473]:426(6.2),335(9.5),297(87.7), 253(100) MS ³ [253]:253(100)

M26	6.0	363	+	+	+	-	-	-	+	-	-	-	-	-	MS ² [363]:283(100) MS ³ [283]:283(100),239(4.7)
M27	6.3	407	-	-	-	-	-	-	-	-	-	-	-	+	MS ² [407]:369(18.7),245(100) MS ³ [245]:245(100),230(50.4)
M28	6.6	431	-	-	-	-	-	-	-	-	-	-	-	+	MS ² [431]:340(3.3),269(100) MS ³ [269]:269(100)
M29	6.6	429	+	+	-	-	-	-	+	-	-	-	-	-	MS ² [429]:253(100),175(6.8) MS ³ [253]:253(100)
M30	6.8	415	-	-	-	-	-	-	-	-	-	-	-	+	MS ² [415]:253(100) MS ³ [253]:253(100)
M31	7.0	429	+	+	+	+	-	+	+	-	-	-	-	-	MS ² [429]:253(100),175(6.8) MS ³ [253]:253(100)
M32	8.2	313	-	-	-	-	-	-	-	-	-	-	-	+	MS ² [313]:269(100),201(5.4) MS ³ [269]:269(100)
M33	8.4	445	+	+	+	+	-	-	+	-	-	-	-	-	MS ² [445]:269(100) MS ³ [269]:269(100)
M34	8.4	459	+	-	-	-	-	-	-	-	-	-	-	-	MS ² [459]:283(100),175(7.0) MS ³ [283]:283(100)
M35	10.2	443	-	-	-	-	-	-	-	-	-	-	-	-	MS ² [443]:411(100),267(87.5),253(91.5), 157(10.9) MS ³ [411]:335(30.2),267(100),253(88.9)
M36	11.5	297	+	+	+	+	-	-	+	-	+	-	+	-	MS ² [297]:253(100) MS ³ [253]:253(100)
M37	12.9	349	+	-	-	-	-	-	-	-	-	-	-	+	MS ² [349]:269(100) MS ³ [269]:269(100)
M38	14.3	561	+	-	-	-	-	-	-	-	-	-	-	-	MS ² [561]:269(100),240(9.5) MS ³ [269]:269(100)
M39	14.6	283	+	+	+	+	-	+	+	-	+	-	+	+	MS ² [283]:257(100),239(66.9) MS ³ [257]:257(100),239(41.0)

tR: retention time; +:found; -:not found; [M-H]: negatively charged molecular ion

Table 1. The metabolites detected in rat urine, plasma and tissues after intragastric administration of rhubarb decoction

M4, which was only detected in plasma samples produced a [M-H]⁻ at m/z 479 with a retention time of 3.4 min. It was found that the [M-H-176]⁻ ion plus the ion at m/z 303 were observed in its MS² spectrum. So, M4 was identified as the metabolite that conjugated with one molecule of glucuronic acid (GlcA). The ion at m/z 303 could produce fragment ion at m/z 259. The spectroscopy (MSⁿ) data of M4 was different from those reported in the published literature before [14]. According to the data above, M4 was conjectured to be glucuronide conjugate of methyl catechin.

M10 gave a [M-H]⁻ signal at m/z 621 in the mass spectrum and fragmentation of the ion with the peak at m/z 621 gave the ion peak at m/z 445 (-176 Da), involving the loss of glucuronic acid. Then, the MS³ fragmentation yielded the ion peak at m/z 269 (-176 Da), involving another loss of glucuronic acid. The quasi-molecular ion of m/z 621 lost a 46 Da and a 30 Da fragmentation sequentially forming the product ions at m/z 575 and 591, suggesting that the mother nucleus was aloe-emodin, and the hydroxymethyl was not conjugated. Therefore, the compound M10 was identified as bisdesmoside conjugate of aloe-emodin.

The mass spectrum of the compound M20 displayed a signal at m/z 525 ([M-H]⁻). A product ion spectrum of M20 displayed fragment ion at m/z 445, 400, 349 and 269. Fragmentation of the ion at m/z 525 gave the ion peak at m/z 349 (-176 Da), suggesting the loss of glucuronic acid. Meanwhile, fragmentation of the quasi-molecular ion yielded an ion peak at m/z 445 (-80 Da), involving the presence of sulfate. Glucuronic acid group and sulfate were not conjugated with hydroxymethyl of the parent compound, which was judged by sequential

loss of 44 Da and 46 Da. Therefore, the structure of M20 was conjectured to be glucuronide and sulfate conjugate of aloe-emodin, glucuronic acid group and sulfate occurred at the 1 and 8-hydroxy, respectively.

Based on the discussions above, the proposed metabolic pathways of rhubarb in rats are shown in Fig.5. The parent compound rhein was found in urine, plasma and most of the tissue samples.

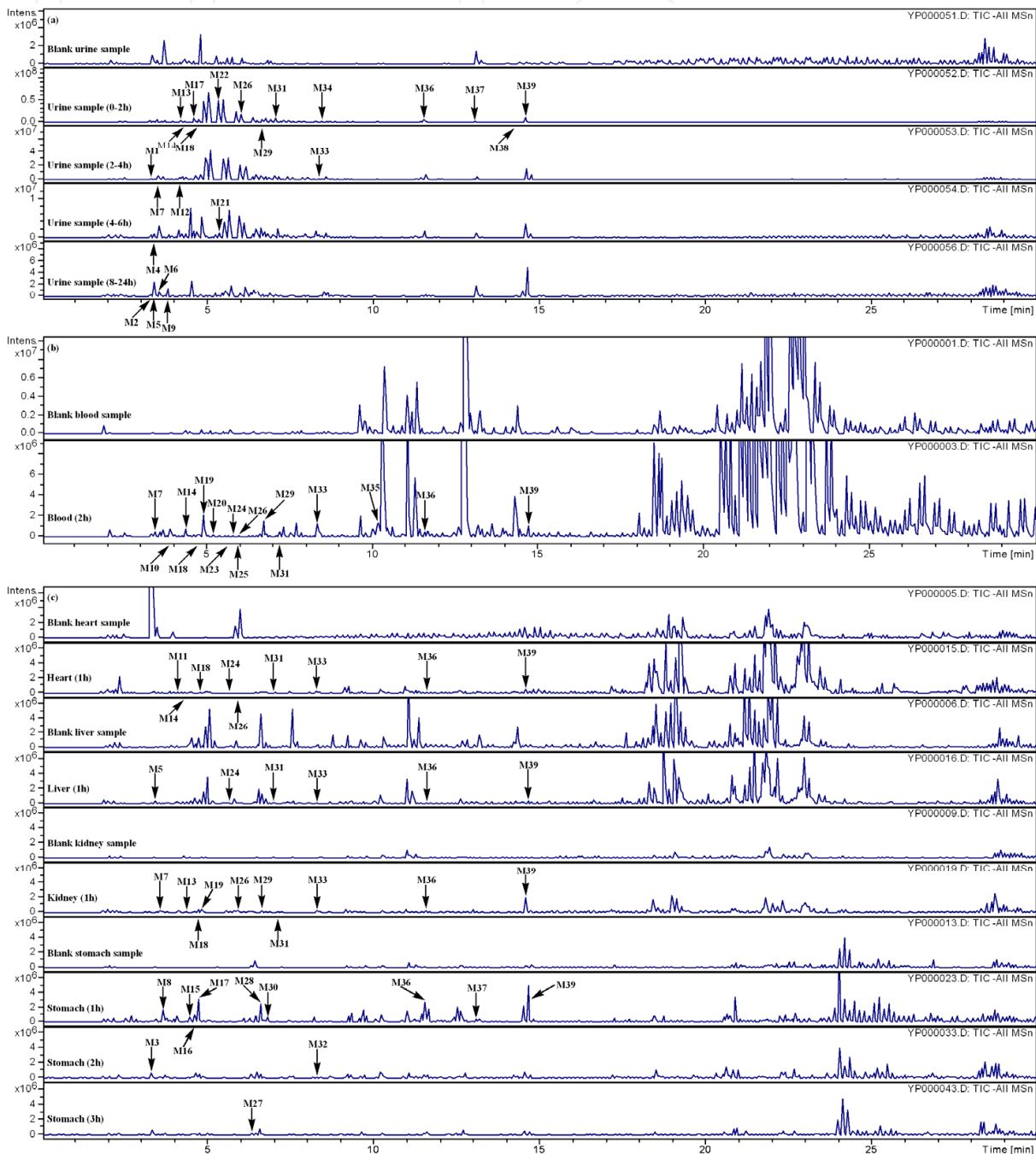


Fig. 2. Typical chromatograms of biological samples (a) chromatograms of urine samples after i.g. administration of 10 g/kg rhubarb decoction; (b) chromatograms of plasma samples after i.g. administration of 17.6 g/kg rhubarb decoction; (c) chromatograms of some tissue samples after i.g. administration of 17.6 g/kg rhubarb decoction

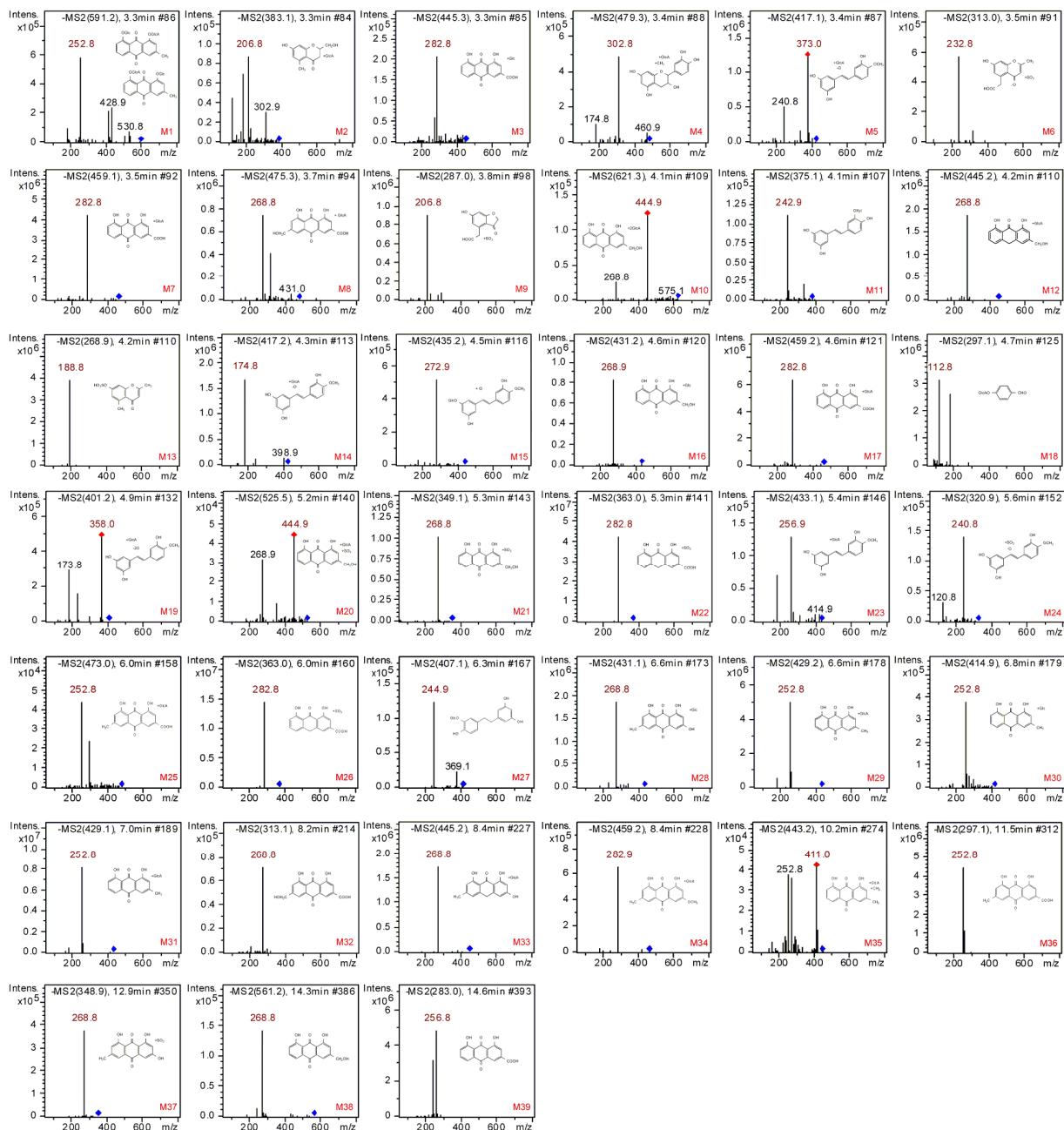


Fig. 3. The MS² spectra of the 39 compounds

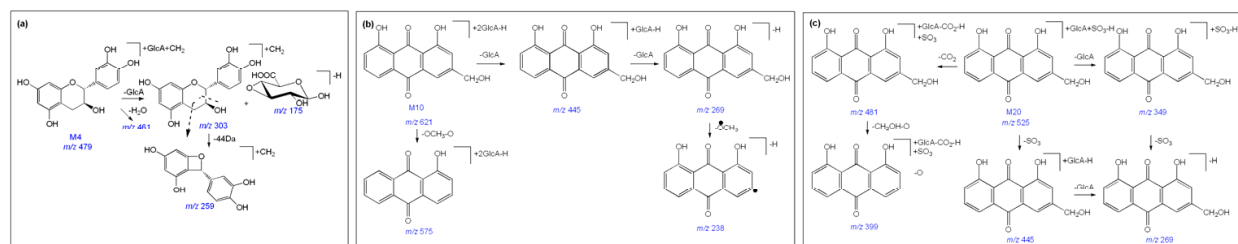


Fig. 4. Postulated fragmentation pathways of the selected metabolites (a) M4; (b) M10; (c) M20

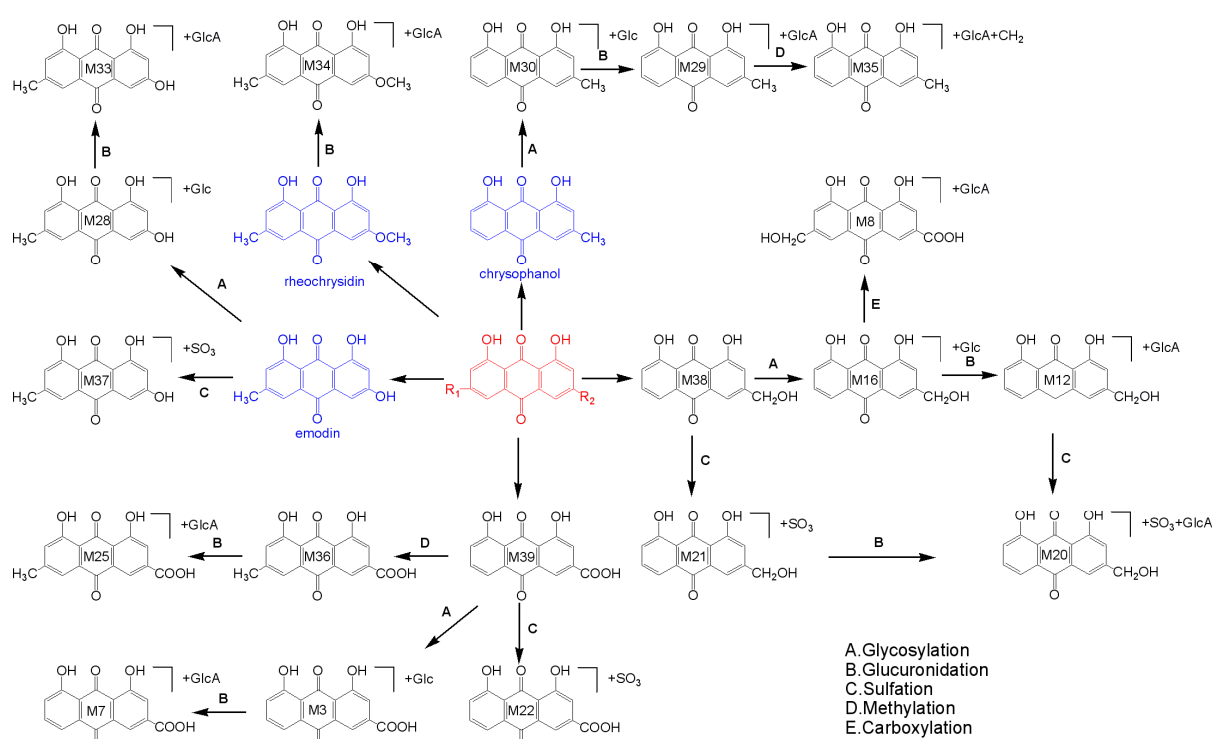


Fig. 5. The proposed transformation pathway of rhubarb in rats

3.8 Urinary excretion of rhubarb

The urinary excretion of rhubarb following a single intragastric administration of rhubarb decoction to rats is summarized in Fig.6. Among the 21 compounds detected in urine samples, the excretion of M22 and M39 were the largest, reaching more than 1000 μg in 48 h, the excretion of M5 and M26 has reached more than 500 μg .

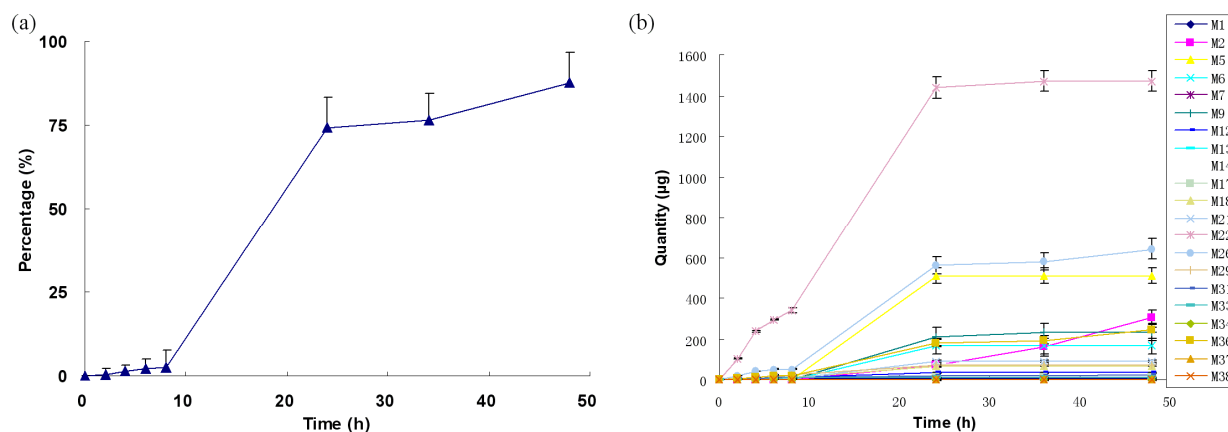


Fig. 6. Cumulative excretion curves of rhein (M39) and the metabolites of rhubarb following the intragastric administration of 10 g/kg rhubarb decoction to SD rats (a) the prototype component M39; (b) metabolites detected in urine samples

3.9 Plasma concentration of rhubarb and the metabolites

Among the 16 constituents detected in plasma, only rhein (M39) was the parent compound. The peak rhein concentration (C_{max}) in plasma was 7.23 $\mu\text{g}/\text{ml}$. The peak concentration of

M19, a metabolite of emodin, reached 8.05 µg/ml at 2 h after i.g. administration of rhubarb decoction. The concentrations of the 16 compounds were shown in Table 2.

Time (h)	M7	M10	M14	M18	M19	M20	M23	M24	M25	M26	M29	M31	M33	M35	M36	M39
1	2.55	0.72	1.76	6.53	4.14	0.33	1.02	1.11	0	4.01	3.40	1.34	3.90	0.80	3.56	7.23
2	1.46	3.57	2.93	0	8.05	0.89	0.19	0.40	0.30	0	5.33	1.48	5.44	0	2.79	3.19
3	0	4.07	1.40	1.18	5.53	0.65	0.11	0	0.35	0	3.89	1.64	0	0	0.62	1.27

Table 2. The concentration of the 16 compounds detected in plasma (µg/ml)

3.10 Tissue distribution of rhubarb

Tissue distribution of the metabolites at 1.0, 2.0 and 3.0 h after a single intragastric administration of 10 g/kg rhubarb decoction to SD rats are also studied. Among the 24 metabolites found, M36 and M39 were distributed in most of the tissues, tissue concentrations of M36 and M39 are shown in Fig.7. At 1.0 h after intragastric dosing, tissue concentrations in most tissues were higher than the corresponding concentrations at 2.0 and 3.0 h. M7, M13, M18, M26, M29 and M33 reached the highest amount of concentration in kidney, M3, M8, M15, M16, M17, M27, M28, M30 and M37 reached the highest amount in stomach at 1.0 h after intragastric dosing. A total of nine compounds were detected in heart samples, seven in liver samples, four in lung samples, ten in kidney samples, thirteen in stomach samples, one in large intestine samples, three in small intestine samples and one in testis samples. The metabolites detected were shown in Table 1. The highest level of most of the metabolites was observed in the stomach, while none of the metabolites was found in spleen and brain.

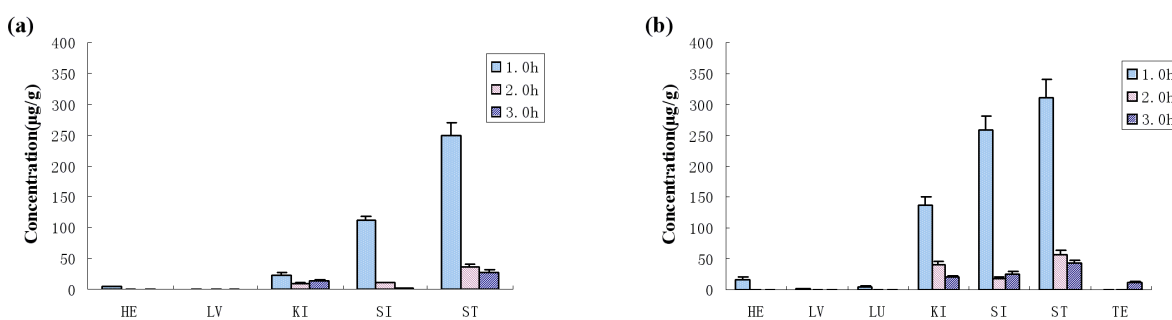


Fig. 7. Distribution of (a) M36, (b) M39 at 1.0h, 2.0h and 3.0h following intragastric administration of 10 g/kg rhubarb decoction to SD rats. Each value represents mean and SD of six animals.

4. Discussion

Many medicinal herbs have a long history of clinical use. However, the safety and efficacy of most of these herbs in relation to their pharmacological activities are poorly understood. Furthermore, knowledge of the pharmacokinetics of the main bioactive ingredients of these herbs is extremely limited. Dahuang is one of the most commonly prescribed Chinese medicinal herbs for the treatment of constipation. However, pharmacokinetics of the main bioactive ingredients in this herb is largely unknown.

In this paper, we developed a triple quadruple MS method for analysis and identification the main bioactive components and their possible metabolites in urine, plasma and tissues. Following a single intragastric administration of rhubarb decoction to rats, 21 constituents in urine samples and 16 compounds in plasma samples were identified. Some constituents of rhubarb were rapidly absorbed into blood and tissues, indicating that the compounds might be responsible for curative effects of rhubarb decoction. Ten compounds were detected in kidney samples, it may elucidate that rhubarb decoction has the effects of anti-diabetic and protecting chronic renal failure in rats. [9, 10]. Their conjugations of glucosides, glucuronide and sulfates were also detected and two of the parent compound aloe-emodin and rhein. The developed method was simple, reliable and sensitive, which revealed that it will be appropriate for rapid analysis and identification the characterization of main bioactive components and their metabolites in biosamples. However, the present study still demonstrated the analytical potential of this approach for identification of metabolites. This identification and structure elucidation of these metabolites provided essential data for further pharmacological and clinical studies of rhubarb and related preparation.

5. Introduction

The root of *Scutellaria baicalensis*, called Huangqin in Chinese, is one of the most commonly used traditional Chinese medicines for the treatment of hepatitis, tumors, diarrhea, and inflammatory diseases [15]. It originated from Shennong Materia Medica, the earliest pharmacopoeia of China in Eastern Han (24-220 A.D.), and has been officially listed in the Chinese Pharmacopoeia for a long time. The major chemical constituents of Huangqin are flavonoids. Modern pharmacological studies have demonstrated that flavones have wide biological activities, such as anti-oxidants, anti-cancer, anti-inflammatory, etc [16,17,18]. Few data are available on the metabolism and metabolites of *S. baicalensis* extract *in vivo*. Therefore it is important to explicate the biotransformation of flavonoids *in vivo* so as to clarify the mechanism of pharmacological action and to promote its availability as well.

A simple and rapid high-performance liquid chromatographic-electrospray ionization (ESI) tandem mass spectrometric method has been developed for elucidation of the structures of the metabolites in rat plasma, urine samples. LC-MS/MS is a more powerful analytical tool for the identification of drug metabolites in biological matrices by comparing changes in molecular masses (ΔM), retention-times, and spectral patterns of product ions with those of the parent drug [19,20].

6. Experimental

6.1 Chemicals and reagents

The roots of *Scutellaria baicalensis* Georgi were collected from Xi'an, ShanXi province, China, and authenticated by Professor Shen Jingui of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. HPLC grade acetonitrile was purchased from Dikma Company (Dikma, USA). Water was deionized and double distilled. Other reagents used are of analytical grade.

6.2 Instrumentation

HPLC-MS experiments were performed with a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) was connected to a Agilent 1100 HPLC

instrument via an ESI source. The software Xcalibur version 1.2 (Finnigan) was applied for system operation and data collection. A high-speed desktop centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge urine, plasma sample. The urine, plasma sample were extracted on a C18 solid-phase extraction (SPE) cartridge (1ml/100mg, Supelco).

6.3 Chromatographic and mass spectrometric conditions

An Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a UV detector, and a column compartment was used for analyses. The samples were separated on a Apollo C₁₈ column (5µm, 4.6 × 250mm, Grace), including an EasyGuad Kit C₁₈ (4 × 2mm) guard column. The column was maintained at 25°C. Detection wavelengths were set at 280nm. The flow rate was 0.8ml/min. A gradient elution of 0.5% aqueous formic acid (A) and acetonitrile (B) was used as follows: 20% B in the first 10min, 20%~25% B at 10~11 min, then B held at 25% for 14min, linearly gradient to 35% B at 26 min and hold for 15min, 35%~55% B at 41~45 min, linearly gradient to 100%B at 50min and hold for 5min. The mass spectra were recorded in negative modes, drying gas flow rate 10L/min, drying gas temperature 35°C, nebulizer 35 psig., capillary voltage 4000V, fragmentor 100V, mass range 50-1500 m/z.

6.4 Administration

Male Sprague-Dawley (SD) rats (220 ± 10g body weight, laboratory Animal Center of Shanghai University of Tradition Chinese Medicine) were divided into a blank group and drug group. Prior to oral administration, each rat was fasted for 24h in a metabolic cage with free access to water, and were then administered 1.2g/Kg the extract of *S. baicalensis* by i.g. Urine were collected separately at 2h, 4h, 6h, 8h, 10h and 24h. Samples were stored at -20°C until analysis. Blood samples were collected at 0.25, 0.5, 1, 3, 5, 7, and 24h after dosing from the caudal vein of the rats, then shaken and centrifuged at 4000rpm for 10min. The supernatant was decanted, and immediately frozen at -20°C until analysis.

6.5 Sample preparation

Solid phase extraction (SPE) with C18 cartridge (1ml/100mg, Supelclean™, Dikma) was used to purify the above supernatants of urine, plasma samples, for LC-MS/MS analysis. Before use, SPE columns were conditioned by 4ml methanol, 2ml deionized water. Then the selected supernatant sample was loaded, and the column was washed with 3ml deionized water to elute the impurity and 1ml methanol to elute the analytes in turn. The eluent was evaporated to dryness at 37°C in vacuum, and the residue dissolved in 100µl of 100% methanol. After centrifugation at 8000rpm. for 10min, 10µl of the supernatant was introduced into the HPLC system for HPLC-MS.

7. Results and discussion

7.1 Identification of metabolites

To elucidate the active constituents responsible for the pharmacological action, it is necessary to perceive the metabolic changes *in vivo* and chemical constituent profile in biological system. Therefore, the full-scan mass spectrum total-ion current chromatogram obtained from rat urine and plasma (Figs. 8(c), 9(b)) after *i.g.* of extract of *S. baicalensis* was

compared with that from blank urine to find probable metabolites. The results show that the total peaks and corresponding peak areas in metabolic chromatograms were different when collected at different periods, and the most abundant metabolites were found at 4-6h in urine samples. These compounds were then analyzed by LC-MS/MS. Using negative ion electrospray tandem mass spectrometry, a total of 12 and 6 metabolites were detected in drug-containing urine and plasma samples in comparison with the extract of *S. baicalensis* and blank sample.

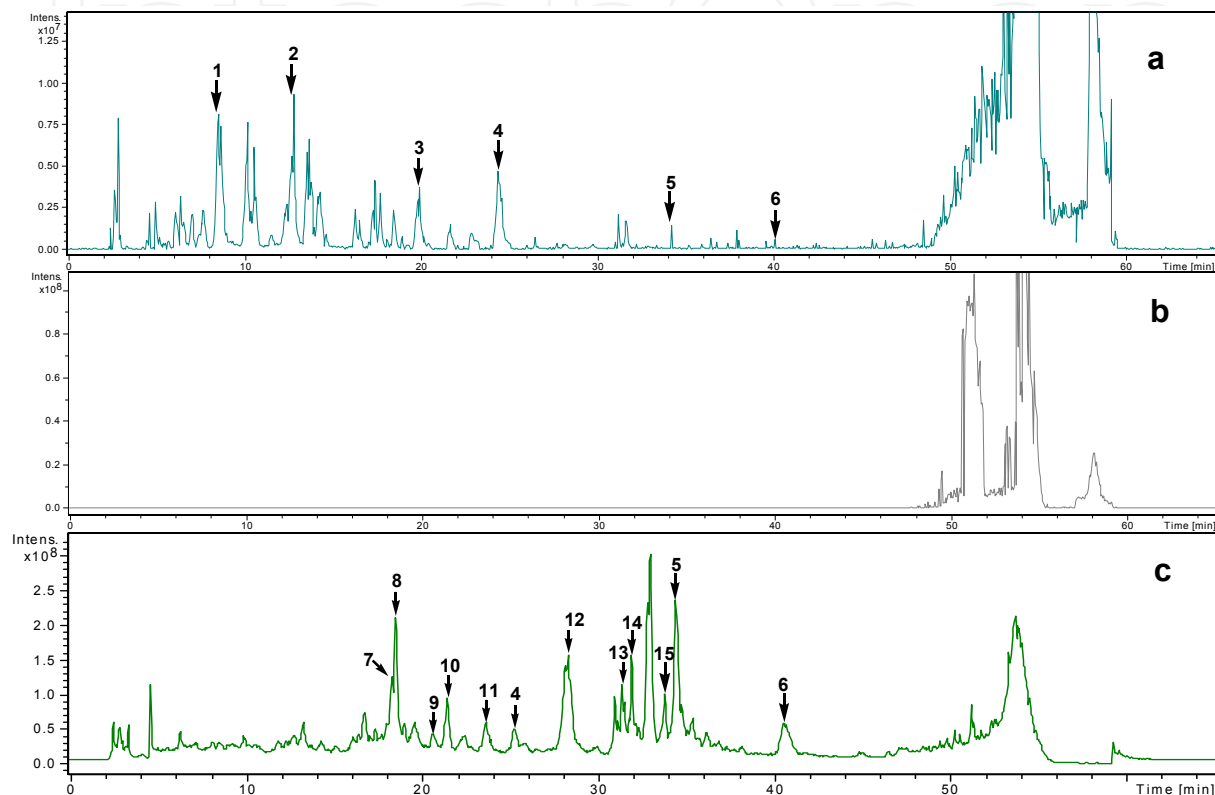


Fig. 8. HPLC-MS total ion current of the *S. baicalensis* decoction and its metabolites in negative mode. (a) The decoction of *S. baicalensis*; (b) Blank rat urine; (c) Urine sample after oral administration.

Peak 4 and 5, 6 were confirmed as original components, baicalin (4), wogonoside (5) and baicalein (6). The $[M-H]^-$ ion of baicalein at m/z 269 produced an ion at m/z 251, which should result from the loss of H_2O . The ion at m/z 241 was due to the loss of CO, and the m/z 223 ion was due to the successive loss of H_2O and CO.

Peak 6 showed $[M-H]^-$ m/z 445.3, with retention time of Baicalin is an O-glucuronide. Upon CID, the glycosidic bond was easily cleaved to generate an ion at m/z 269, which resulted from the neutral loss of a glucuronic acid ($\Delta m = 176u$). In the MS^3 experiment, ion at m/z 269 produced the same ions as baicalein described above.

Wogonin is a methoxylated flavone. It exhibited a significant radical anion $[M-H-CH_3]^-$ as the base peak. In MS^3 spectra, we observed the significant ion at m/z 239 $[M-H-CH_3 - COH]^-$, and the low signal intensity ions at m/z 163 ($^{0,2}A^-$), 212 $[M-H-CH_3 - 2CO]^-$, 223 $[M-H-CH_3 - CO_2H]^-$, 240 $[M-H-CH_3 - CO]^-$, which is identical with the previous report.

Peak 7 showed a $[M-H]^-$ m/z 607.4, with retention time at 18.3min on HPLC. Its MS^2 produced an ion at m/z 431 $[M-H-176]^-$, indicating the existence of a glucuronic acid.

Additionally, the successive yielded the ion at m/z 269 [M-H-176-162]⁻. Based on these data and by the knowledge on the flavones in *S. baicalensis*, compounds 1 were identified as Baicalein glucoside glucuronide conjugate.

Peak 8 gave a [M-H]⁻ ion at m/z 621.4, with retention time at 18.5min on HPLC. CID of this compound produced an ion of [M-H-176]⁻ at m/z 445, which resulted from the neutral loss of a glucuronic acid residue. In the MS³ of m/z 445, fragments of m/z 430 and 269 indicated the presence of a H₂O and a glucuronic acid, respectively. Therefore, this compound was thus proposed as Baicalin glucuronide conjugate.

With a retention time at 19.6min on HPLC, peak 9 generated a [M-H]⁻ at m/z 635.3 in MS spectrum and a [M-H]⁻ at m/z 459 ([M-H]⁻ -176, loss of a glucuronic acid) in MS². In the MS³ experiment, ion at m/z 283 produced the same ions as wogonoside described above. Therefore, compound 9 was thus proposed as Wogonoside glucuronide conjugate.

Peak 10 displayed a [M-H]⁻ ion at m/z 577.4, with retention time at 20.6min on HPLC. The MS² and MS³ spectra gave ions at m/z 401 and 269, suggesting sequential losses of glucuronic acid (176 Da) and arabinose (132 Da) residues, respectively. This compound was thus identified as Baicalein xyloside glucuronide conjugate.

Peak 11 showed a [M-H]⁻ ion at m/z 417.3, with retention time at 21.4min on HPLC. In the MS² spectrum of m/z 417.3, the fragment ion of 241 and 399 was generated by natural loss of a glucuronic acid and a H₂O, then the successive yielded the ion at m/z 199 [M-H-GlcA-C₂H₂O]⁻ in MS³. Therefore, it was tentatively identified as pinosylvin 2-hydroxymethyl glucuronide conjugate.

Peaks 12 and 15 are sulfation metabolites. Peak 12 with a retention at 23.6 min on HPLC, showed a [M-H]⁻ m/z 539.4. In the MS² spectrum, the fragment ion of 363 and 459, indicating loss of a glucuronic acid and SO₃, respectively. Based on these data and by the knowledge on the flavones in *S. baicalensis*, this compound were identified as wogonoside sulfate conjugate. Peak 16 with a retention time at 33.7min on HPLC, yield MS fragments at m/z 363.2 and [M-H]⁻ m/z 282.9 ([M-H]⁻ - 80 Da, loss of a SO₃) in MS². Based on these data and by the knowledge on the flavones in *S. baicalensis*, this compound could be confirmed as wogonin sulfate conjugate.

Peak 13 gave a [M-H]⁻ ion at m/z 447.3, with retention time at 31.3min on HPLC. CID of this compound produced an ion of [M-H-176]⁻ at m/z 270.9, which resulted from the neutral loss of a glucuronic acid residue. Based on these data and by the knowledge on the flavones in *S. baicalensis*, this compound was thus proposed as baicalein glucuronide conjugate.

Peak 14, appearing at 31.8min on HPLC, had a [M-H]⁻ at m/z 446 and yield a major ion at m/z 268.9 ([M-H]⁻ - 176 Da, loss of a glucuronic acid unit) in MS² and a MS³ fragment [M-H]⁻ at m/z 251.0 ([M-H]⁻ -176 Da-18 Da, loss of a glucuronic acid unit and one molecule of H₂O). Therefore it was presumed as apigenin glucuronide conjugate, which acts as one of the major apigenin metabolites in urine.

7.2 Plasma metabolites

The direct comparison of the TIC chromatograms of the blank rat plasma and the rat plasma samples collected at 0.5, 1, 2, 3, 4h after the oral administration of the extract of *S. baicalensis*. It showed that the information in the chromatogram at 1h post administration was more sufficient (Fig.9(b)). Including two original components, two metabolites, were tentatively elucidated as baicalin glucuronide conjugate (16), baicalin (4), norwogonoside glucuronide conjugate (17), wogonoside sulfate conjugate (18), wogonoside (5).

Peak 17 with retention time at 33.7min on HPLC, showed a $[M-H]^-$ ion of m/z 621.1. The MS^2 and MS^3 spectra gave ions at m/z 445 and 269, suggesting sequential losses of two glucuronic acid (2×176 Da) residues. In the MS^3 experiment, ion at m/z 225 produced $[M-2Glc A-CO-H]^-$. Base on these data, the structure of 17 could be identified as norwogonoside glucuronide conjugate.

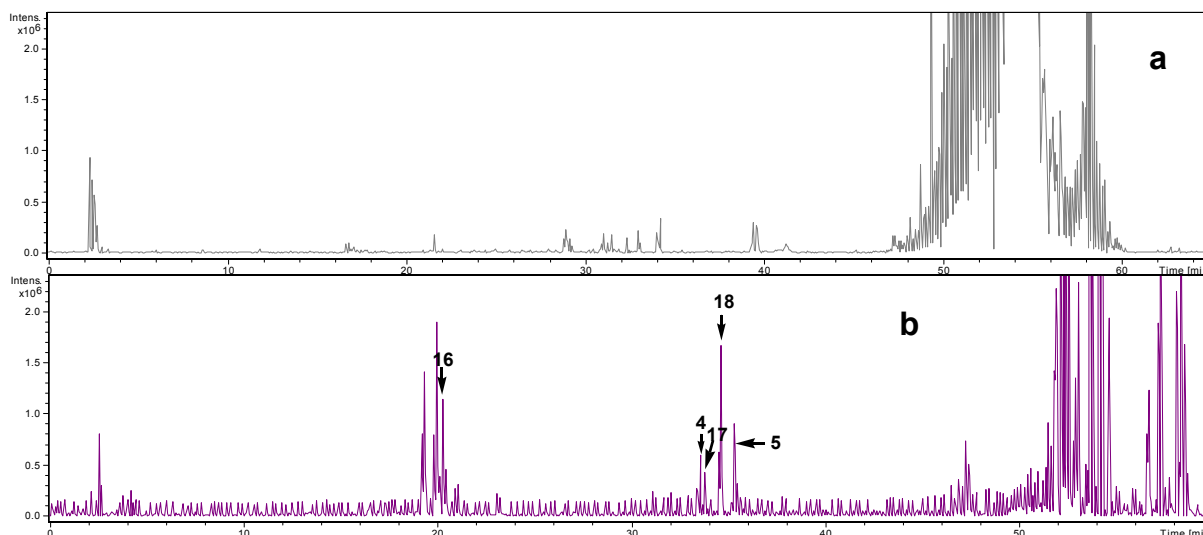


Fig. 9. HPLC-MS total ion current of the *S. baicalensis* decoction and its metabolites in negative mode. (a) Blank rat plasma; (b) Plasma sample after oral administration.

7.3 Identification of Baicalin metabolites in rat blood

Some reported that baicalin are the main active components of *S. baicalensis* [21]. In order to further study of baicalin metabolism *in vivo*. The metabolites of baicalin were studied further in rat plasma.

We identified three metabolites and the parent drug in rat plasma after administration of baicalin by healthy rats. Their protonated molecular ions ($[M-H]^+$) were at m/z 621, 643, and 564, respectively. MS^n spectra of metabolites, obtained by CID of their molecular ions, were used for more precise structural identification of metabolites. Among them, the retention time, the MS^2 and MS^3 spectra of the molecular ion at m/z 445 (M0) were the same as those of baicalin. Therefore, M0 is the unchanged parent drug.

M1 was observed at the retention time of 19.9 min and gave an deprotonated molecule $[M-H]^-$ at m/z 621. The ion at m/z 621 was increased by 176Da compared to that of unchanged baicalin and glucuronidation was a very common metabolic pathway of drug *in vivo*, indicating that M1 might be a conjugate of baicalin with glucuronic acid, which was confirmed by the characteristic fragment ions presented in the following MS^n spectrum. The $[M-H]^-$ ion at m/z 621 of M1 generated the base peak at m/z 445 in the MS/MS spectrum, attributed to the loss of 176 Da, suggesting the loss of a glucuronic acid. Then the product ion at m/z 445 was subjected to MS^3 analysis and produced an intense ion at m/z 268 by loss of a glucuronic acid group (176Da) again. Therefore, M1 was elucidated as baicalin glucuronide conjugate.

M2, eluted at 20.1 min, exhibited $[M-H]^-$ signal at m/z 643 which was 198 Da greater than that of parent compound, which indicated that M2 was a Na glucuronide metabolite of baicalin. The MS^2 spectrum of m/z 643 produced a significant radical ion at m/z 467 (Fig.10)

via the loss of glucuronic acid (176Da). Then the ion at m/z 467 was successively subjected to MS³ analysis and produced ion at m/z 269 by the loss of Na and glucuronic acid (198 Da). At the same time, the base peak at m/z 241 was observed in the MS⁴ spectrum by the loss of CO (28Da) from the ion at m/z 269. Therefore, M2 was tentatively proposed to be the baicalin Na glucuronide conjugate.

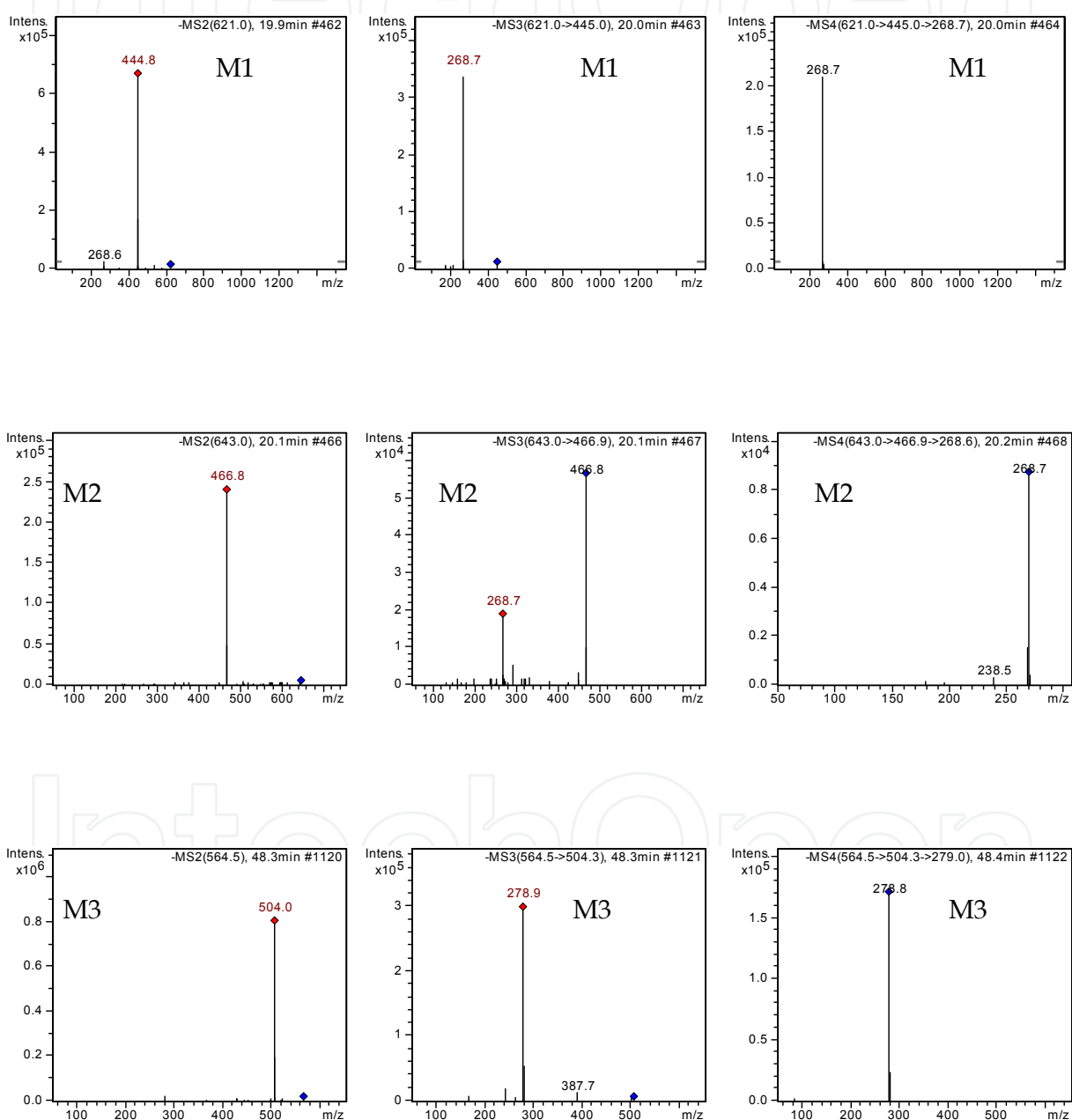


Fig. 10. Negative ion ESI-MSⁿ spectra of baicalin metabolites in rat plasma

M3 was detected as a deprotonated molecule $[M-H]^-$ at m/z 564, with the retention time of 48.3 min. The molecular ion at m/z 564 was increased by 119 Da compared to that of the parent compound and there may be cysteine (119Da) reactions occurring at the baicalin skeleton. The molecular ion at m/z 564 by loss of the neutral fragment $C_2O_2H_3N$ (60Da) produced the product ion at m/z 504 (Fig.10C) in the MS² spectrum. The obtained ion was subjected to MS³ fragmentation, in which the base peak at m/z 389 was observed by the losing a glucuronic acid(176 Da). Based on the above results, M3 could be characterized as the baicalin cysteine conjugate.

8. Conclusion

In this paper, we describe a strategy using liquid chromatographic-electrospray ionization (ESI) tandem ion trap mass spectrometric for fast analysis of the metabolic profile of *S. baicalensis* and baicalin. Using negative ion mode and applying the MS fragmentation rules of flavonoids reported before, 3 organic components, 9 metabolites in urine, 2 organic and 3 metabolites in plasma, were identified or tentatively identified in the extracts of *S. baicalensis*. 3 metabolites were identified in baicalin. However, several new analogues were identified in the present study, which proved that HPLC-MS is a powerful and rapid method to discover new constituents in Chinese medicinal herbs and its metabolites, help to lay the foundation of further study *in vivo*.

9. Acknowledgements

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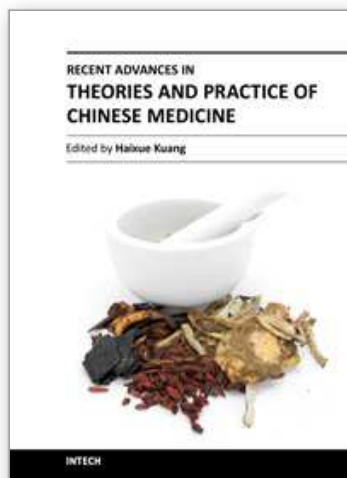
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During the recent years, traditional Chinese medicine (TCM) has attracted the attention of researchers all over the world. It is looked upon not only as a bright pearl, but also a treasure house of ancient Chinese culture. Nowadays, TCM has become a subject area with high potential and the possibility for original innovation. This book titled *Recent Advances in Theories and Practice of Chinese Medicine* provides an authoritative and cutting-edge insight into TCM research, including its basic theories, diagnostic approach, current clinical applications, latest advances, and more. It discusses many often neglected important issues, such as the theory of TCM property, and how to carry out TCM research in the direction of TCM property theory using modern scientific technology. The authors of this book comprise an international group of recognized researchers who possess abundant clinical knowledge and research background due to their years of practicing TCM. Hopefully, this book will help our readers gain a deeper understanding of the unique characteristics of Chinese medicine.

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