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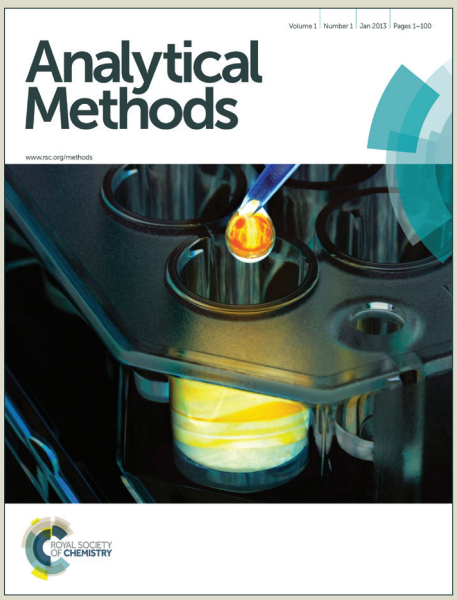

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Characterization of multiple constituents in rat plasma after oral administration of Shengmai San using ultra-performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight high-definition mass spectrometry

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Abstract

Shengmai San (SMS), a well-known traditional Chinese medical formula containing seven herbal medicines, has been used for the treatment of cardiovascular disease in Asian, however, its active chemical constituents remain perfectly unclear so far. To quickly identify the chemical constituents of SMS and to understand the chemical profiles related *in vivo* activity of SMS, a rapid and robust ultra-performance liquid chromatography coupled with electrospray ionization/ quadrupole-time-of-flight high-definition mass spectrometry (UPLC-Q-TOF-HDMS) approach has been applied for online identification of multiple components in rat plasma after oral administration of SMS. Metabolynx software that was a novel post-acquisition data processing software to detect components, was also conducted to discover bioactive components in *vivo*. A total of 30 compounds were detected in dosed rat plasma compared with blank rat plasma and tentatively characterized by comparing their retention times and MS spectra with those of authentic compounds or literature data. Furthermore, this work demonstrated the possibilities of using UPLC-Q-TOF-HDMS approach for identification of bioactive compounds from herbal medicines *in vivo*. Our present results also proved that the established method could provide helpful chemical information for further pharmacological studies of SMS.

Keywords:

UPLC-Q-TOF-MS/MS, Metabolynx, herbal medicines, multiple constituents, Shengmai San

Introduction

Traditional Chinese Medicine (TCM), an essential part of the healthcare system in most Asian countries, is increasingly being used in Western medicine [1]. Presently, there are no formal analytical methods for the identification of the chemicals in TCM. The ability to measure the multiple constituents from the herbal formulae are critical in understanding of the action of TCM [2-7]. *Shengmai San* (SMS), which was first recorded in "*Yi Xue Yuan Li*" and, is one of the most famous Chinese herbal formulae, consists of *Panax ginseng, Ophiopogon japonicas* and *Schisandra chinensis* with dosage proportion of 5:3:1.5 [8,9]. Previous studies in our laboratory have demonstrated that a total of 92 compounds were identified by comparing the accurate mass and fragments information with that of the authentic standards as well as by MS analysis and the correlative references data. These constituents included ginsenosides, lignans, steroidal saponins and homoisoflavanones [10]. Comparing with the previous studies, our research detected more compounds and presented more rapid [11]. These studies only emphasized on the chemical components *in vitro*, while which ingredient absorbed into the blood is unclear. UPLC have greatly improved the resolution, sensitivity and analytical speed, integrating with MS to form a robust platform for TCM research [12-14].

Rapid identification and structural elucidation of the chemical constituents in the TCM formulae and rat plasma may provide important experimental data for further pharmacological and clinical research [15]. Major constituents in the single herbs of SMS have been well studied, however, to the best of our knowledges, the *in vivo* chemical constituents in SMS has not been completely investigated so far. Therefore, in the present study, UPLC-Q-TOF-HDMS analysis was firstly developed to systematic investigation of the chemical components of SMS in vivo, which gave the accurate molecular weights and the fragmentation patterns acquiring from multi-stage mass fragmentation by Metabolynx^{TM} analyzer for comprehensive understanding of the multiple absorbed components in rat plasma after oral administration of SMS.

2. Experimental

2.1 Chemicals and reagents

Leucine enkephalin was purchased from Sigma–Aldrich (MO, USA). Other reagents and chemicals were of analytical grade. Formic acid and phosphoric acid (analytical grade) was purchased from the Beijing Reagent Company (Beijing, China). Acetonitrile and methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Deionized water was purified on a Milli-Q system (Millipore, Bedford, USA). The *Panax ginseng, Ophiopogon japonicas* and *Schisandra chinensis* were purchased from the Harbin Shiyitang Drugstore and were authenticated by Professor Xijun Wang of the Department of Pharmacognosy, Heilongjiang University of Chinese Medicine.

2.2. Animals

Male Wistar rats (250±20 g) were provided by the Laboratory Animal Center of Heilongjiang University of Chinese Medicine (Harbin, China). Rats were bred in a breeding room with temperature of 24±2 ◦C, humidity of 60±5%, and 12 h

dark-light cycle. They were given access to tap water and normal chow *ad libitum*. All the experiment animals were housed under the above conditions for 7 days acclimation, and were fasted overnight before the experiments. The animal facilities and protocols were approved by the Institutional Animal Care and Use Committee, Heilongjiang University of Chinese Medicine.

2.3. Preparation of SMS samples for analysis

SMS sample was prepared by combining the *Panax ginseng, Ophiopogon japonicas* and *Schisandra chinensis*. Then the mixture was extracted three times with 1000 mL, 800 mL and 600 mL water for 1 h, respectively. The extracts were combined and concentrated to approximate 0.8 g/mL, and this concentration solution was used for oral administration. All sample solutions were stored at −20 °C and used at room temperature.

2.4 Preparation of plasma sample for analysis

Freeze-dried powder of SMS was dissolved with distilled water as stock solution (0.8g/mL), and was orally administrated to male Wistar rats (1mL/100g body weight), and the control rats were orally administered with physiological saline in the same way [16]. One hour after drug administration, the animals were anaesthetized by intraperitoneal injection of 1% pentobarbital sodium (0.15mL/100g body weight). The 5 mL blood was collected from the hepatic portal vein and then centrifuged at 13 000 rpm for 10 min at 4 °C. All plasma samples from one group of rats were combined into one sample to eliminate the individual variability. The plasma samples were pretreated by solid phase extraction before LC/MS analysis. The 1mL plasma was processed on a pre-activated OASIS HLB solid phase extraction C_{18} column(30μm, 60mg, Waters Corporation, USA), washed with 4 mL of water and 2 mL of 20% methanol and abandoned the eluents, then eluted with 3 mL of 100% methanol. The 100% methanol eluents were collected and dried under nitrogen gas at 35 °C. The residues were redissolved in 100 μL of methanol and then centrifuged at 13 000 rpm for 10 min at 4 °C. The 3μL supernatant obtained was finally used as UPLC-Q-TOF-HDMS samples.

2.5 UPLC-Q-TOF-HDMS analysis

2.5.1 Liquid Chromatography Conditions—UPLC was performed with a Waters ACQUITY UPLC™ system (Waters Corporation, Milford, USA), equipped with quaternary pump, vacuum degasser, autosampler, diode-array detector. The chromatography was performed on a Waters ACQUITY HSS T_3 column (2.1mm×100mm, 1.8μm). The column temperature was maintained at 45 ◦C. The mobile phase consisted of (A) 0.01% formic acid in water and (B) ACN containing 0.01% formic acid used gradient elute procedure as follows: 0-2.5min, A 1-20%; 2.5-5.5min, A 20-32%; 5.5-8.5min, A 32-43%; 8.5-12.5min, A 43-55%; 12.5-20 min; 55-99 %. The flow rate was 0.50 mL/min and 3 μL aliquot of each sample was injected into the column.

2.5.2 Mass Spectrometry Detection—Waters Micromass Q-TOF-micro™ Synapt High Definition Mass Spectrometer (Manchester,UK) equipped with electrospray ion source operating in positive ion and negative ion mode. For the UPLC-Q-TOF-HDMS analysis, the optimal conditions were as follows: In positive ion mode, the source temperature was

set at 105 °C with a cone gas flow of 50 l/h, a desolvation gas temperature of 350 °C and a desolvation gas flow of 600 l/h, the capillary voltage was set at 2400 V, the sample cone voltage was set at 35 V and the extraction cone voltage was set at 3.0 V. In negative ion mode , the source temperature was set at 115 °C with a conegas flow of 50 l/h, a desolvation gas temperature of 300 °C and a desolvation gas flow of 500 l/h, the capillary voltage was set at 2200 V, the sample cone voltage was set 30 V and the extraction cone voltage was set 3.0 V. All MS data were acquired using the LockSpray™ to ensure mass accuracy and reproducibility. The mass spectrometer was calibrated using a lock-mass of leucine enkephalin at a concentration of 200 pg/mL in acetonitrile (0.1% formic acid): H_2O (0.1% formic acid) (50:50, v/v) for the positive ion mode ($[M+H]^+$=556.2771) and negative ion mode ($[M-H]^-$=554.2615) were employed at a flow rate of 100μL min^{-1} via a lock spray interface.

2.6 Data analysis

Post-acquisition analyses were performed using a MetaboLynxTM (v4.1) program which is able to show the presence of a wide range of metabolites, to generate a series of extracted ion chromatograms (XICs). These XICs are compared between the control and sample to eliminate those chromatographic peaks in the sample that also appear in the control. The data file from the dosed plasma and the control plasma was specified as ‘analyte’ and ‘control’. Peaks presented in the analyte were evaluated by their retention times compared to control sample, meanwhile, the peak area in the analyte had to be at least 5 times greater than that of the control. First, the acquired data were processed using a user-defined parameter file, to generate a preliminary report file. Second, this report was displayed in the browser, and the output refined by a variety of data filters. Finally, a large number of peaks were generated, and it was necessary to determine manually whether they were likely to be compound-related metabolites.

3. Results and discussion

3.1 Analytical consideration for UPLC-Q-TOF-HDMS

To acquire UPLC chromatograms with good separation, various chromatographic columns, mobile phases, column temperatures and flow rates that were optimized. For analysis of SMS, a Acquity UPLC® HSS T_3 column was found to be better than Acquity UPLC BEH c_{18} column. It was found that a mixture of 0.01 % formic acid/H_2O and 0.01 % formic acid/acetonitrile (gradient) produced better chromatographic separation for SMS, control and dosed plasma samples. The temperature and flow rate were 45 °C and 0.5 mL/min respectively for better separation. The spectrometric parameters were also optimized for max sensitivity achievement of most components. The analysis was carried out in both positive and negative mode by following the optimized MS parameters to get high responses from all compounds in MS spectra.

3.2 Optimization of sample preparation

Various approaches were adopted to prepare the plasma samples in order to select an efficient method to obtain a better recovery of the target compounds, such as using protein precipitation, SPE, and liquid-liquid extraction. The sensitivity of MS spectra was reduced by protein precipitants such as acetonitrile, methanol and acetone sample extracts etc., and the outcome of recovery and relative standard deviation of SPE was found better than the other method utilizations. Eventually, SPE was chosen to process the simultaneous extraction of the components and ensured less interference from the co-eluted endogenous matrices. The blood samples were collected from each rat from the hepatic portal vein at 0.25, 1, 2, 3, 5, 8 and 12h after oral administration. In this experiment, peaks and responses detected in the LC-MS spectrum at 1 h post-dose were more and higher than at other points of time, which indicated the extent of SMS absorption reached maximum approximately. Therefore, LC-MS chromatography post-dose at 1h was adopted as blood collecting time to study the multiple absorbed bioactive components and metabolites in rat plasma.

3.3. Identification of constituents of SMS in vivo by UPLC–Q-TOF/HDMS

A significant aim for pharmaceutical discovery is the rapid screening and identification of potential bioactive compounds in TCM [17-21]. Thus, the purpose of using UPLC–ESI-Q-TOF/MS technique in this part was to discover the components absorbed. Comparing with the chromatograms between the dosed and control plasma samples to find different peaks as the components absorbed into blood including prototype (parent) compounds. Under the optimized conditions, the typical chromatograms (both in positive and negative modes) of the SMS samples are shown in Fig. 1 in selected ion monitoring (SIM) mode, including 17 components (13 in the positive mode, 4 in the negative mode) marked with numerals 1–17 in the chromatogram with a comprehensive comparison between their peaks. The components in rat plasma after oral administration of SMS were well separated and identified by using their retention time and mass spectra. From a comprehensive analysis of the chromatograms of SMS, comparing individual peak retention times and the online MS spectra with those of authentic compounds, 13 peaks were found in positive mode and 4 peaks in negative mode common both in the SMS spectra and dosed plasma spectra respectively, which demonstrated that the 17 components were absorbed into the rat's blood from SMS in the prototype. Moreover, another 13 peaks were detected using MetaboLynx software package (Waters Corp., Milford, MA, USA). Compared with the conventional manual inspection, Metabolynx analyzer in a much shorter time frame and more chemical compounds, avoiding the omission of some compounds at the lower concentration levels, and could comprehensively understand the material basis of SMS. It is concluded that a valid and robust platform based on UPLC-Q-TOF-HDMS analytical technique was established, which gives high sensitivity and resolution that is useful for identification of multiple compounds of SMS *in vivo*.

3.4 Structural characterization of proposed bioactive components

In accordance with the optimized chromatographic and mass spectrum conditions, MassLynx V4.1 workstation is used to conduct structure analysis of chromatographic peaks in the total ion chromatogram of the transmitting components in SMS blood. Specific procedure is as the following: first of all, use the MS method to measure the precise mass of the compounds, get the element composition of corresponding compounds within the error range of measurement (less than 5ppm), and calculate the degree of un-saturation; then, search in the compound database by molecular formula or molecular mass, get possible compound structure, combine it with the MS/MS data and screen out the most possible one or several types of compounds; at last, by comparing the optimized chromatographic and mass spectrum behaviors with the reference substances, or through corresponding references, or searching in the database, confirm the compound structure. Through the structure analysis of chromatographic peaks conducted with the method mention above, we find that gensenosides component can be tested out under the negative mode, and appears as the parent ion of $[M+H]^-$ and $[M-H+HCOOH]^-$; lignins type component can only be tested out under the positive ion mode, and appears in the form of $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+Na]^+$. According to the standards and data recorded in literature, we had identified the 30 peaks of prototype components and metabolites. Fig.2A showed the MS spectra of reference substance ginsenoside Rh1. Fig.2B showed the MS/MS information and structure analyzing process of ginsenoside Rh1, which represented for ginsenosides. According to the mass spectra and fragment assignment at high collision energy (Fig.3A), the low collision (Fig.3B) and proposed fragmentation pathways (Fig.3C) in positive mode, the compound was identified as Schisantherin A. Similarly, other compounds could also be characterized according to the above-mentioned methods.

3.5 Application of MetaboLynx to analysis compounds in vivo

Based on the above parameters, the UPLC-Q-TOF-HDMS chromatograms were processed under positive and negative mode by the application of Metabolynx respectively. After the good filtering of each peak in mass spectrum, background interferences were removed, and the blood component peaks was easily to identify, meanwhile, the extracted ions were listed in the window ‘Expected Metabolites’ according to the isotope peaks and fragment ions for further manual investigation. Metabolynx can be used to extract the internal composition and metabolite of TCM. It can target screening the metabolites in accordance with the set metabolic pathway, and give potential compounds to be screened in accordance with other known metabolic pathways, and in this way realizing the purpose of holographic testing. Application of Meyabolynx, 13 compounds were screened out besides the intuitional comparison shown in table 1. Chemical compositions of the same type in TCM has similar biological resources and pathways, and also has similar metabolic processes within the body.

Several analytical methods have been established to purify and indentify the metabolites from TCM *in vivo* [22-26]. In the current study, mass spectrometry along with UPLC was used to identify SMS chemicals and metabolites in plasma. Given its high sensitivity and reliability, this approach should then be applied to investigate the chemicals composition of other

herbal remedies along with their metabolites. This work could provide a scientific basis for elevating the quality of herbs and their compound preparations and optimizing the dosage regimens, as well as contributing the knowledges of searching active metabolites for drug discovery. In addition, the method could be developed as an integrated template approach to analysis for screening and identification of the bioactive components in blood after oral administration of herb and provided helpful chemical information for further pharmacology and active mechanism research on TCM.

4. Conclusion

In this paper, we described a method using UPLC–Q-TOF/HDMS with automated data analysis (Metabolynx^{TM}) for fast analysis of metabolic profile of SMS in rat plasma after oral administration. It provides unique high throughput capabilities for drug metabolism study, with excellent MS accuracy and enhanced data acqui-sition. A total 30 compounds including 23 prototype components and 7 metabolites were successfully separated and characterized tentatively. This method could provide a rapid and valid platform for identification of multiple components for TCM. This identification and structural elucidation of the constituents in SMS and rat plasma provided essential data for further active chemical constituents identification and pharmacological research of SMS. It would be also helpful to better understand the pharmacodynamic profile of SMS, which will facilitate its clinical usage and quality control during production.

Acknowledgments

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Competing financial interests

The authors declare no competing financial interests.

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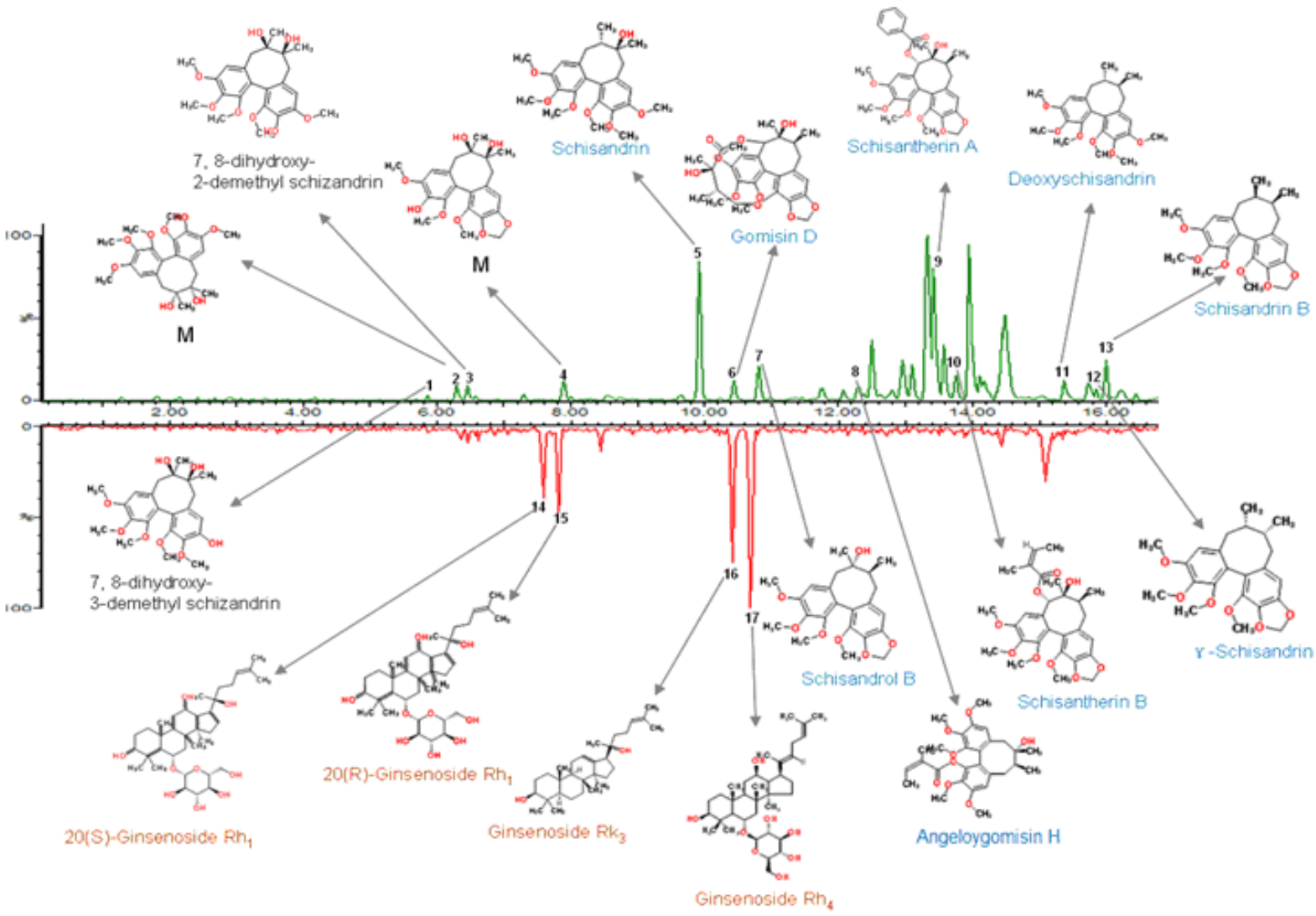


Fig.1 The UPLC-Q-TOF-HDMS chromatograms of the sample in rat serum after oral administration of SMS in the positive (up) and negative (down) ion mode.

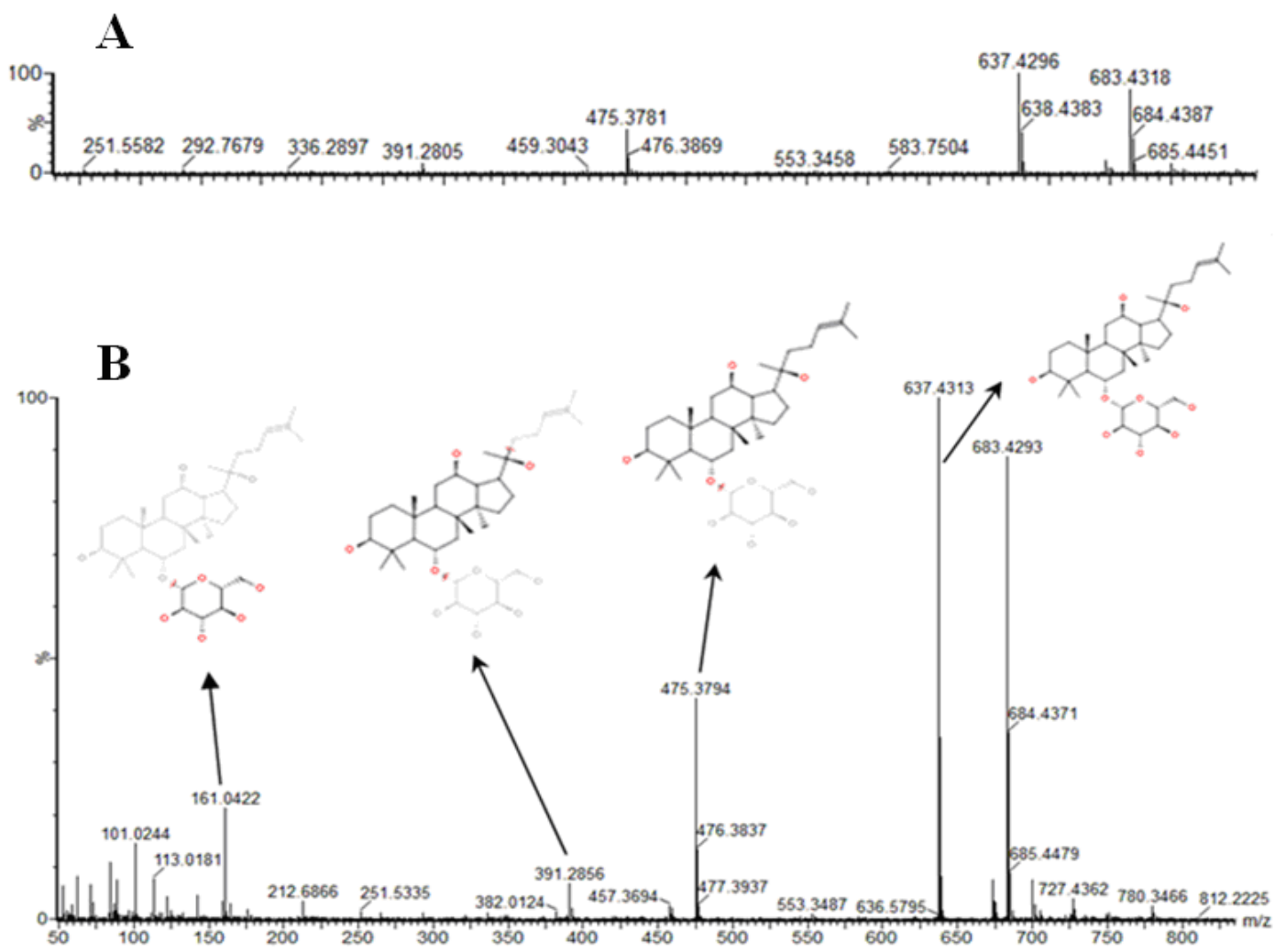
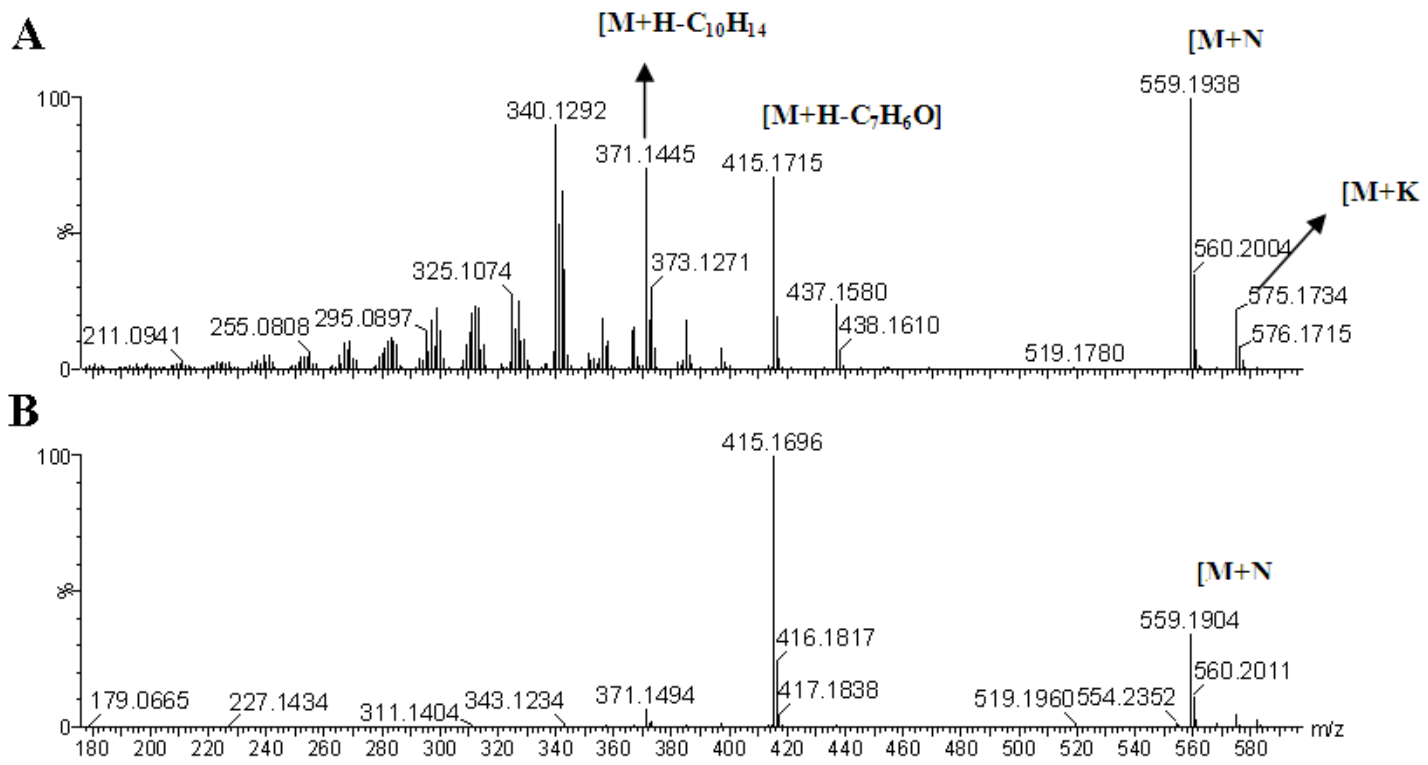


Fig.2. MS spectra of reference substance ginsenoside Rh1 (A) and MS/MS information and structure analyzing process of Ginsenoside Rh1(Peak14) (B).



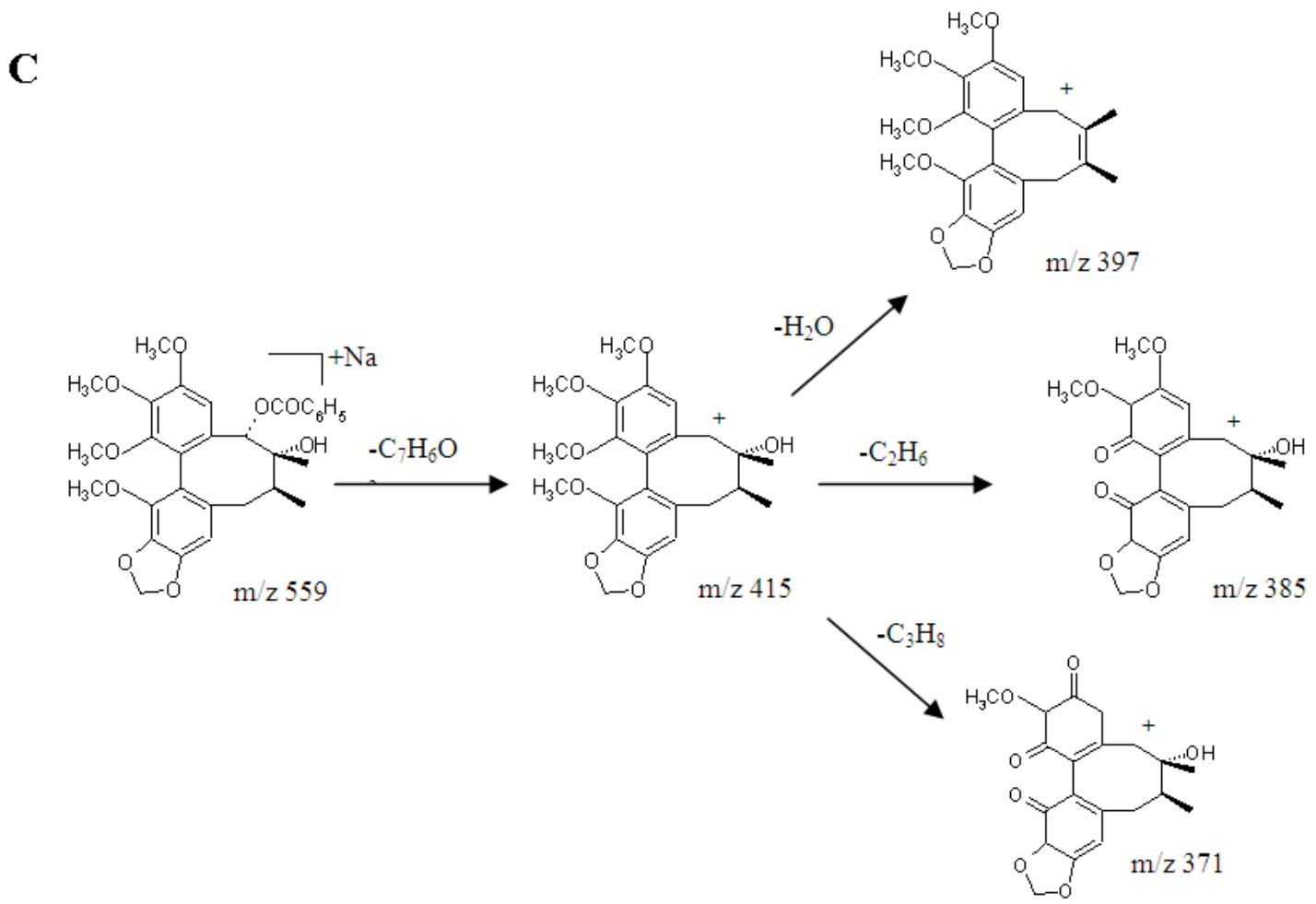


Fig.3 The mass spectra of Schisantherin A in positive mode.

The mass spectra and fragment assignment at high collision energy (A); the mass spectrum at low collision (B); proposed fragmentation pathways of Schisantherin A (C).

Table 1. The constituents in rat serum after oral administration of SMS

Peak No.	Rt (min)	Name	Molecular formula	Molecular Weight	$[M+Na]^+$/ $[M-H]^-$	Error (ppm)
1	5.86	7,8-dihydroxy-3-demethyl schizandrin ☆	$C_{23}H_{30}O_8$	434.4795	457.1823	-3.3
2	6.31	7,8-dihydroxy-2-demethyl schizandrin ☆	$C_{23}H_{30}O_8$	434.4795	457.1826	-2.6
3	6.46	7,8-dihydroxy-2-demethyl schizandrin isomer☆	$C_{23}H_{30}O_8$	434.4795	457.1830	-1.7
4	7.91	7,8-dihydroxy-schizandrin ☆	$C_{24}H_{30}O_7$	448.5061	471.1987	-1.7
5	9.94	Schisandrin	$C_{24}H_{32}O_7$	432.5067	455.2035	-2.4
6	10.45	Gominsin D	$C_{28}H_{34}O_{10}$	530.5636	553.2035	-2.7
7	10.83	Schisandrol B	$C_{23}H_{28}O_7$	416.4642	439.1729	1.3
8	12.34	Angeloygomisin H	$C_{23}H_{28}O_6$	500.5806	523.2306	-0.4
9	13.66	Schisantherin A	$C_{30}H_{32}O_9$	536.5697	559.1939	-0.9
10	13.78	Schisantherin B	$C_{24}H_{32}O_6$	514.5642	537.2092	-1.7
11	15.42	Deoxyschisandrin	$C_{23}H_{28}O_6$	416.5073	439.2085	-2.7
12	15.89	γ-Schisandrin	$C_{23}H_{28}O_6$	400.4648	423.1779	-1.2
13	16.05	Schisandrin B	$C_{36}H_{62}O_9$	400.4648	423.1789	1.2
14	7.56	20(S)-Ginsenoside Rh1	$C_{36}H_{62}O_9$	638.8721	637.4310	-0.9
15	7.81	20(R)-Ginsenoside Rh1	$C_{36}H_{60}O_8$	638.8721	637.4326	1.6
16	10.37	Ginsenoside Rk3	$C_{36}H_{60}O_8$	620.8568	619.4210	-0.5
17	10.66	Ginsenoside Rh4	$C_{24}H_{32}O_6$	620.8568	619.4233	3.7
18	6.60	Gomisin P isomer ☆	$C_{23}H_{28}O_8$	432.4636	455.1674	-1.8
19	8.02	Gomisin H isomer ☆	$C_{23}H_{30}O_7$	418.4801	441.1889	1.1
20	8.98	Isoschisandrin	$C_{24}H_{32}O_7$	432.5067	455.2075	1.1
21	9.65	Schisandrin isomer ☆	$C_{24}H_{32}O_7$	432.5067	455.2029	-3.7
22	10.99	Unknown	$C_{27}H_{32}O_9$	500.5376	523.1945	0.2
23	11.28	Unknown	$C_{28}H_{36}O_{10}$	532.5794	555.2202	0.7
24	11.48	Unknown	$C_{28}H_{36}O_{10}$	532.5794	555.2198	-1.4
25	11.75	Tigloylgomisin H	$C_{28}H_{36}O_8$	500.5806	523.2295	-2.5
26	12.39	Epi-Gomisin O	$C_{23}H_{28}O_7$	416.4642	439.1728	-1.1
27	12.63	Benzoylgomisin Q	$C_{31}H_{36}O_9$	552.6121	575.2265	1.4
28	12.84	Angeloylgomisin Q	$C_{29}H_{38}O_9$	530.6066	553.2419	0.9
29	13.49	Gomisin F	$C_{28}H_{34}O_9$	514.5642	537.2095	-1.1
30	16.32	Schisandrin C	$C_{22}H_{24}O_6$	384.4224	407.1473	0.5

Note: ☆, metabolite