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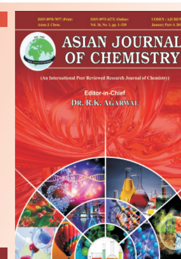

Characterization of the Constituents in Rat Biological Fluids After Oral Administration of Longshen Buyi Paste by High-Performance Liquid Chromatography/Quadrupole Time-of-Flight Mass Spectrometry

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A high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry method was established to detect as many constituents in rat biological fluids as possible after oral administration of Longshen Buyi Paste (LBP). A C18 column (3.5 μm particle size) was adopted to separate the samples and mass spectra were acquired in negative modes. First, the fingerprints of LBP were established, resulting in 20 components being detected within 70 min. Among these compounds, six compounds were tentatively identified by comparing the retention times and mass spectral data with those of reference standards and the reference literature. Other 14 components were tentatively assigned solely based on the MS data. *in vivo*, 4 components of LBP were observed in urine. This study developed a high-speed and sensitive method that was successfully utilized for screening the active ingredients of a Chinese medical formula and provided helpful chemical information for further pharmacology and active mechanism research on Chinese medicine.

Keywords: Longshen Buyi Paste, HPLC-Q-TOF/MS, Urine, Chemical constituents.

INTRODUCTION

Longshen Buyi Paste (LBP), an herbal preparation consisting of Radix Ginseng (39 g), *Radix panacis* Quinquefolii (18.6 g), *Radix rehmanniae* (37.2 g), *Radix rehmanniae* Preparata (37.2 g), *Colla carapacis* et *Plastri Testudinis* (18.6 g), *Colla corni Cervi* (18.6 g), *Radix astragali* (62 g), *Rhizoma atractylodis* Macrocephalae (55.8 g), Poria (55.8 g), *Fructus jujubae* (18.6 g), *Radix paeoniae* Alba (31 g), *Arillus longan* (31 g), *Colla corii* Asini (74.4 g), *Rhizoma chuanxiong* (18.6 g), *Fructus corni* (37.2 g), *Rhizoma cibotii* (55.8 g), *Herba cistanches* (55.8 g), *Semen cuscutae* (37.2 g), *Radix morindae* Officinalis (37.2 g), *Fructus ligustri* Lucidi (37.2 g), *Herba ecliptae* (37.2 g), *Fructus mori* (55.8 g), *Semen juglandis* (31.0 g), *Endocongha sepiae* (37.2 g), *Semen astragali* Complanati (37.2 g), *Radix ophiopogonis* (55.8 g), *Herba dendrobii* (24.8 g), *Fructus schisandrae* Chinensis (18.6 g), *Rhizoma acori* Tatarinowii (18.6 g), *Radix polygalae* (18.6 g), *Fossilia ossia* Mastodi (55.8 g), *Concha ostreae* (55.8 g), *Pericarpium citri* Reticulatae (18.6 g), *Fructus tribuli* (37.2 g), *Cortex ailanthi* (37.2 g), *Radix glycyrrhizae* (18.6 g), honey (860 g) are used in China. Longshen Buyi Paste is not a simple mixture of the 37 crude drugs, but prepared through a complicated process[1,2]. Longshen Buyi Paste carries many biological activities, including improving the symptoms of fatigue, dizziness, vertigo, tinnitus. However, there is no report on systematic analysis of its chemical constituents. Even less information is available about the metabolism of LBP after oral administration.

In order to get as many active ingredients and metabolites as possible from LBP in less time, it was necessary to set up an effective and reliable analytical method. Fortunately, a high-speed and sensitive technique with shorter analysis times and greater accuracy of the *m/z* value, high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF/MS), has attracted ever-increasing attention[3] and has been successfully applied to identify active ingredients and metabolites in traditional Chinese medicine (TCM)[4].

The aim of this study was to develop a HPLC-Q-TOF/MS method for effectively analyzing and detecting the chemical constituents of LBP and their metabolites in rats after oral administration.

EXPERIMENTAL

HPLC grade acetonitrile and formic acid were purchased from Merck (Merck, Darmstadt, Germany). Ultrapure water was purified by a Milli-Q50 SP Reagent Water System

(Millipore, Bedford, MA, USA). Longshen Buyi Paste was purchased from Yangtze River Pharmaceutical Group (Taizhou, China). The reference standards of morroniside, paeoniforin, hyperin, astragalin, ginsenoside Rg_1, physcion, ginsenoside Rb_1, ginsenoside-R_0, astragaloside A and 20(S)-ginsenoside-Rg_3 were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Instrumentation and conditions: Analysis was performed on an Agilent-1260 LC system coupled with an Agilent-6530 accurate-mass Q-TOF mass spectrometry equipped with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA). The separation of all samples was performed on an Eclipse plus C18 column (3.5 μm, 4.6 ×100 mm, Aglient) at a column temperature of 35 °C. The flow rate was 0.45 mL/min and the mobile phase consisted of 0.3 % (v/v) formic acid (A) and acetonitrile (B). The following gradient program was used: 0-45 min, 10-50 % B; 45-60 min, 50-70 % B; 60-70 min, 70-100 % B. The sample injection volume was 10 μL and the mass detection was operated in negative ion modes with parameters set as follows: drying gas (N_2) flow rate, 10 L/min; gas temperature, 330 °C; pressure of nebulizer, 10 psig; HV voltage, 3500 V and a scan range of *m/z* 50-1500. The MS/MS analysis was acquired in targeted MS/MS mode with a fixed collision energy of 20 V.

Preparation of LBP sample: 0.5 g of LBP paste was accurately weighed into a 5 mL volumetric flask and subjected to ultrasonic treatment at room temperature with 70 % methanol for 0.5 h. The methanol extraction was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected and filtered through a 0.22 μm membrane prior to HPLC-Q-TOF/MS analysis.

Animal and urine sample collection: All procedures were in accordance with the National Institute of Health's guidelines regarding the principles of animal care (2004). Twelve male Sprague-Dawley rats (200 ± 15 g) were purchased from the Slac Laboratory Animal Co., Ltd. (Shanghai, China). The animals were acclimatized to the facilities for 5 days and then fasted with free access to water for a 12 h period prior to the experiment. Longshen Buyi Paste was dissolved in water to obtain LBP oral suspension with a concentration of 0.5 g/mL of crude drug. The prepared suspension was orally administered to 6 rats at a dose of 5 g/kg and water was orally administered to 6 additional rats as a control. Urine was collected for 48 h after drug administration as well.

An aliquot of 2 mL urine sample was loaded onto an SPE cartridge (Oasis HLB 6 cc, Waters), which was preconditioned with 5 mL methanol and 5 mL water. Then the cartridge was washed with 5 mL of water and the analyte was eluted with 5 mL of methanol. The methanol effluent was collected and dried in vacuum at 40 °C. The residue was re-constituted in 100 μl of methanol and centrifuged at 12,000 rpm for 10 min. The sample was filtered through a 0.22 μm membrane and a 2 μL aliquot was injected for LC/MS analysis. Blank sample as control was prepared with the same method as the drug-containing samples.

RESULTS AND DISCUSSION

HPLC-Q-TOF/MS analysis of LBP: In our study, 20 components of LBP were separated and detected using a HPLC-Q-TOF/MS system (Fig. 2 and Table-2). Peaks 1, 4, 11, 14, 15 and 17 were, respectively attributed to morroniside, paeoniforin, physcion, ginsenoside-R_0, astragaloside A and 20(S)-ginsenoside-Rg_3, by comparison with the retention times and mass spectral data of the reference standards. Utilizing Agilent MassHunter Qualitative Analysis software and searching the Spectral Database for Organic Compounds (SDBS)[5], or comparing with the literature data[6-14], fourteen peaks were identified. They were loganin[6] (peak 2), echinacoside[7] (peak 3), cistanoside A[8] (peak 5), verbascoside[9] (peak 6), hesperidin[10] (peak 7), ginsenoside 20 (S)-Rf_2[11] (peak 8), tenuifoliside A[12] (peak 9), 2'-acetyl acteoside[13] (peak 10), pseudoginsenoside F11[11] (peak 12), ginsenoside Rg_2[11] (peak 13), astragaloside II[14] (peak 16), 20(R)-ginsenoside-Rg_3[11] (peak 18), ginsenoside-Rk_1[11] (peak 19) and ginsenoside-Rg_5[11] (peak 20). These compounds included two iridoid glycosides (**1** and **2**), four phenylethanoid glycosides (**3**, **5**, **6** and **10**), one monoterpene (**4**),

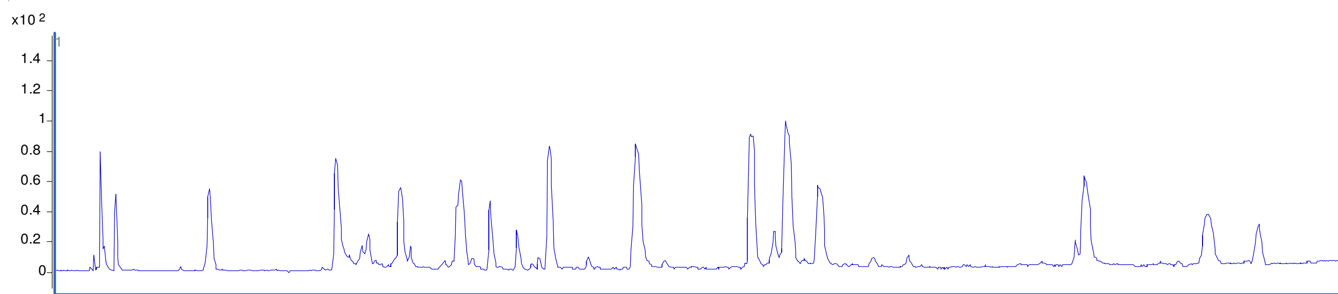


Fig.1. TIC chromatograms of reference standards in negative mode

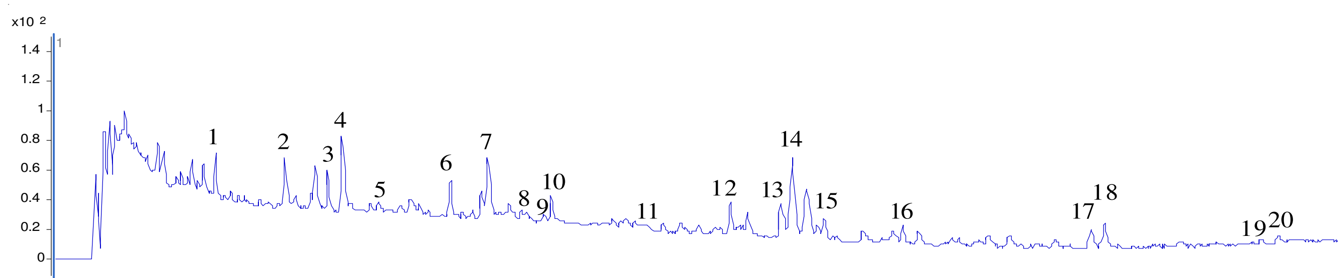


Fig. 2. TIC chromatograms of LBP extracts in negative mode

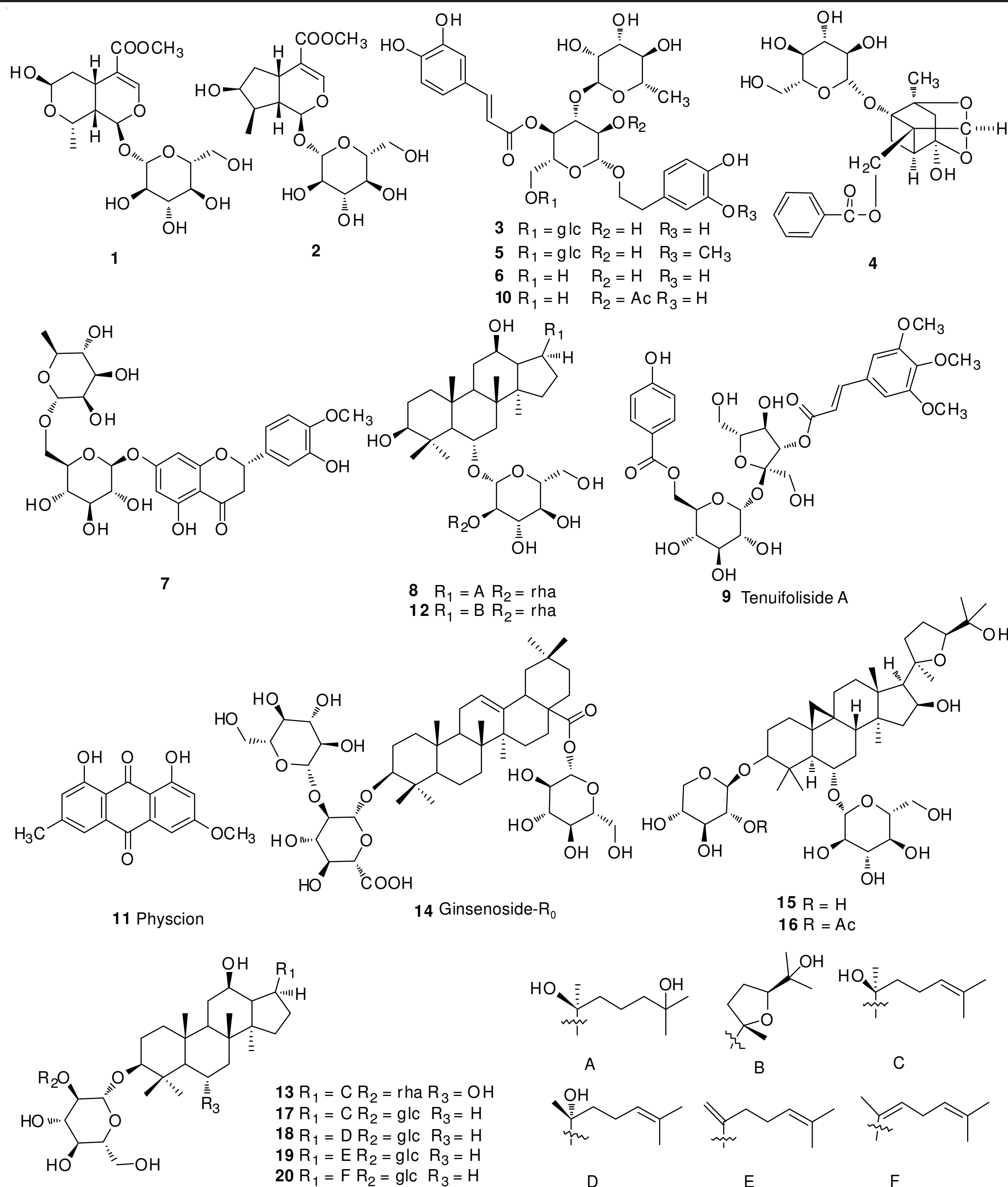


Fig. 3. Structures of compounds **1-20**

TABLE-1
HPLC-Q-TOF/MS DATA OF REFERENCE STANDARDS

RT (min)	MS (Negative)	MS/MS (Negative)	m.f.	Exact mass (calc)	Compounds
7.48	441.1170 [M + Cl]$^-$	155.0368, 141.0545, 123.0449, 101.0254	$C_{17}H_{26}O_{11}$	441.1169	Morroniside
13.33	515.1332 [M + Cl]$^-$	959.3189, 449.1440, 327.1074, 165.0551	$C_{23}H_{28}O_{11}$	515.1326	Paeoniforin
16.13	463.0881 [M-H]$^-$	300.0271, 271.0244, 255.0295, 243.0296	$C_{21}H_{20}O_{12}$	463.0882	Hyperin
18.98	447.0932 [M-H]$^-$	284.0327, 255.0299, 227.0348	$C_{21}H_{20}O_{11}$	447.0933	Astragalin
23.00	835.4617 [M + Cl]$^-$	845.4937, 799.4837, 637.4315, 475.3794, 161.0450, 101.0245	$C_{42}H_{72}O_{14}$	835.4616	Ginsenoside Rg_1
26.92	283.0611 [M-H]$^-$	268.0363, 239.0347, 211.0397, 195.0449, 183.0441, 167.0499	$C_{16}H_{12}O_5$	283.0612	Physcion
32.36	1143.5717 [M + Cl]$^-$	1107.5947, 945.5419,783.4892, 621.4336, 459.3858, 323.0977	$C_{54}H_{92}O_{23}$	1143.5723	Ginsenoside Rb_1
34.09	991.4688 [M + Cl]$^-$	955.4890, 793.4371, 731.4331, 613.3655, 569.3813, 523.3756	$C_{48}H_{76}O_{19}$	991.4675	Ginsenoside R_0
35.52	819.4302 [M + Cl]$^-$	783.4529, 651.4080, 621.3978, 489.3551	$C_{41}H_{68}O_{14}$	819.4303	Astragaloside A
47.78	819.4667 [M + Cl]$^-$	783.4900, 621.4364, 459.3835	$C_{42}H_{72}O_{13}$	819.4667	20(S)-Ginsenoside-Rg_3

one flavonoid (**7**), eight triterpenoid saponins (**8**, **12-14** and **17-20**), one sucrose ester (**9**) and one anthraquinone (**11**). The proposed fragmentation pathway of each style compounds (**3**, **7**, **8**, **9**, **13** and **16**) is postulated in Fig. 4. The LC-MS total ion chromatogram of reference standards is shown in Fig. 1 and HPLC-Q-TOF/MS data of reference standards is shown in Table-1. The LC-MS total ion chromatogram of LBP is shown in Fig. 2. The details of identified compounds are summarized in Table-2 and their chemical structures are presented in Fig. 3.

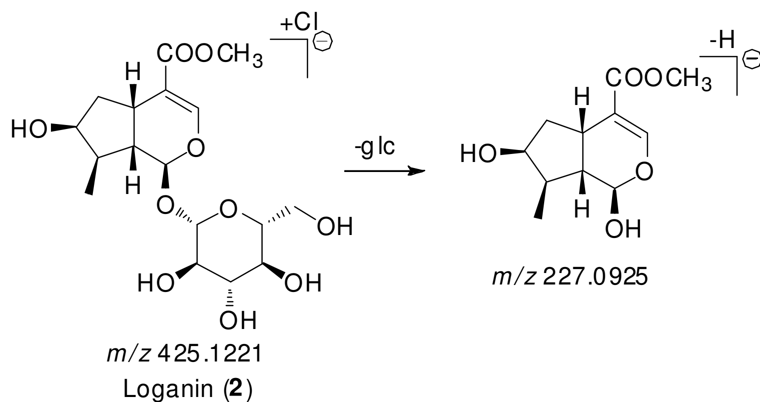
HPLC-Q-TOF/MS analysis of urine samples: By comparing the chromatograms of dosed rat urine with control urine in negative mode, 8 peaks were observed in dosed rat urine that did not appear in control urine. 4 peaks (peaks 1, 2, 11, 14) were indicated as prototype components of LBP by comparison with the chromatograms of LBP extracts (Fig. 6). The 4 target peaks at *m/z* 441.1168 $[M + Cl]^-$, 425.1217 $[M + Cl]^-$, 283.0612 $[M-H]^-$, 991.4669 $[M + Cl]^-$ were identified as morroniside, loganin, physcion and ginsenoside-R_0, respectively, by comparison with the extracted ion chromatograms of target peaks in TIC chromatograms of LBP extracts. Other target peaks at *m/z* 443.2569 $[M + Cl]^-$ (M1), *m/z* 443.2571 $[M + Cl]^-$ (M2), *m/z* 427.2621 $[M + Cl]^-$ (M3) and *m/z* 425.2460 $[M + Cl]^-$ (M4) were detected. The prototype of these 4 components could not be detected in LBP extracts. These 4 components were speculated to be endogenous sterols, which was induced to produce easily after administration.

Conclusion

In this paper, a HPLC-Q-TOF/MS method was successfully established for the separation and identification of chemical components in LBP and their metabolites in rat urine after administration. A total of 20 compounds, including morroniside (peak 1), loganin (peak 2), echinacoside (peak 3), paeoniforin (peak 4), cistanoside A (peak 5), verbascoside (peak 6), hesperidin (peak 7), ginsenoside 20 (S)-Rf_2 (peak 8), tenuifoliside A (peak 9), 2'-acetyl acteoside (peak 10), physcion (peak 11), pseudoginsenoside F11 (peak 12), ginsenoside Rg_2 (peak 13), ginsenoside-R_0 (peak 14), astragaloside A (peak 15), astragaloside II (peak 16), 20(S)-ginsenoside-Rg_3 (peak 17), 20(R)-ginsenoside-Rg_3 (peak 18), ginsenoside-Rk_1 (peak 19) and ginsenoside-Rg_5 (peak 20) were newly separated and detected (Fig. 1). *in vivo*, 4 components

TABLE-2
COMPONENTS IDENTIFIED FROM LONGSHEN BUYI PASTE

Peak	RT (min)	MS (Negative)	MS/MS (Negative)	m.f.	Exact mass (calc)	Compounds	Origin
1	7.53	441.1170 $[M + Cl]^-$	155.0351, 141.0544, 123.0451, 101.0241	$C_{17}H_{26}O_{11}$	441.1169	Morroniside	*Fructus corni*
2	10.79	425.1221 $[M + Cl]^-$	263.0955	$C_{17}H_{26}O_{10}$	425.1220	Loganin	*Fructus corni*
3	12.72	821.2279 $[M + Cl]^-$	785.2493, 623.2179,477.1618, 461.1649, 315.1032, 161.0237	$C_{35}H_{46}O_{20}$	821.2276	Echinacoside	*Herba cistanches*
4	13.38	515.1332 $[M + Cl]^-$	449.1442, 327.1075, 165.0552	$C_{23}H_{28}O_{11}$	515.1326	Paeoniforin	*Radix paeoniae* Alba
5	14.89	835.2434 $[M + Cl]^-$	799.2651, 637.2322, 491.1758, 475.1778, 179.0383, 161.0238	$C_{36}H_{48}O_{20}$	835.2433	Cistanoside A	*Herba cistanches*
6	18.32	623.1984$[M-H]^-$	461.1648, 315.1084, 271.0598, 161.0237, 135.0441, 113.0238	$C_{29}H_{36}O_{15}$	623.1981	Verbascoside	*Herba cistanches*
7	20.08	609.1825 $[M-H]^-$	301.0705	$C_{28}H_{34}O_{15}$	609.1825	Hesperidin	*Pericarpium citri* Reticulatae
8	20.91	837.4768 $[M + Cl]^-$	801.4996, 655.4395, 637.4284, 493.3874	$C_{42}H_{74}O_{14}$	837.4773	Ginsenoside 20(S)-Rf_2	Radix Ginseng
9	22.59	717.1805 $[M + Cl]^-$	681.2033, 443.1196, 281.0666, 263.0557, 239.0553, 223.0609, 179.0347, 137.0242	$C_{31}H_{38}O_{17}$	717.1803	Tenuifoliside A	*Radix polygalae*
10	22.85	665.2088 $[M-H]^-$	623.1969, 503.1748, 461.1651, 179.0336, 315.1087, 161.0239, 135.0444, 113.0242	$C_{31}H_{38}O_{16}$	665.2087	2'-Acetyl acteoside	*Herba cistanches*
11	26.97	283.0611 $[M-H]^-$	268.0361, 239.0345, 211.0394, 195.0434, 183.0441, 167.0483, 148.0157	$C_{16}H_{12}O_5$	283.0612	Physcion	*Radix morindae* Officinalis
12	31.11	835.4618 $[M + Cl]^-$	799.4827, 653.4247, 491.3738	$C_{42}H_{72}O_{14}$	835.4616	Pseudoginsenoside F_{11}	Radix Ginseng
13	33.48	819.4666 $[M + Cl]^-$	783.4881, 637.4311, 475.3772	$C_{42}H_{72}O_{13}$	819.4667	Ginsenoside Rg_2	Radix Ginseng
14	33.98	991.4672 $[M + Cl]^-$	955.4887, 793.4367, 731.4336, 613.3735, 569.3823, 523.3764	$C_{48}H_{76}O_{19}$	991.4675	Ginsenoside-R_0	*Radix panacis* Quinquefolii
15	35.54	819.4304 $[M + Cl]^-$	783.4538, 651.4066, 621.3949, 489.3505	$C_{41}H_{68}O_{14}$	819.4303	Astragaloside A	*Radix astragali*
16	39.18	861.4409 $[M + Cl]^-$	825.4692, 783.5054， 765.4348, 489.3591	$C_{43}H_{70}O_{15}$	861.4409	Astragaloside II	*Radix astragali*
17	47.78	819.4667 $[M + Cl]^-$	783.4880, 621.4359, 459.3856	$C_{42}H_{72}O_{13}$	819.4667	20(S)-Ginsenoside-Rg_3	Radix Ginseng
18	48.47	819.4665 $[M + Cl]^-$	783.4879, 621.4352, 459.3864	$C_{42}H_{72}O_{13}$	819.4667	20(R)-Ginsenoside-Rg_3	Radix Ginseng
19	55.64	801.4554 $[M + Cl]^-$	765.4786, 603.4225,	$C_{42}H_{70}O_{12}$	801.4561	Ginsenoside-Rk_1	Radix Ginseng
20	56.38	801.4559 $[M + Cl]^-$	765.4784, 603.4282	$C_{42}H_{70}O_{12}$	801.4561	Ginsenoside-Rg_5	Radix Ginseng



-H
CA moiety
PhA moiety
m/z 785.2493
echinacoside (3)
-CA
-H
m/z 623.2179
$-C_6H_{10}O_4$
-H
m/z 477.1618
-glc
-H
m/z 461.1649
-rha
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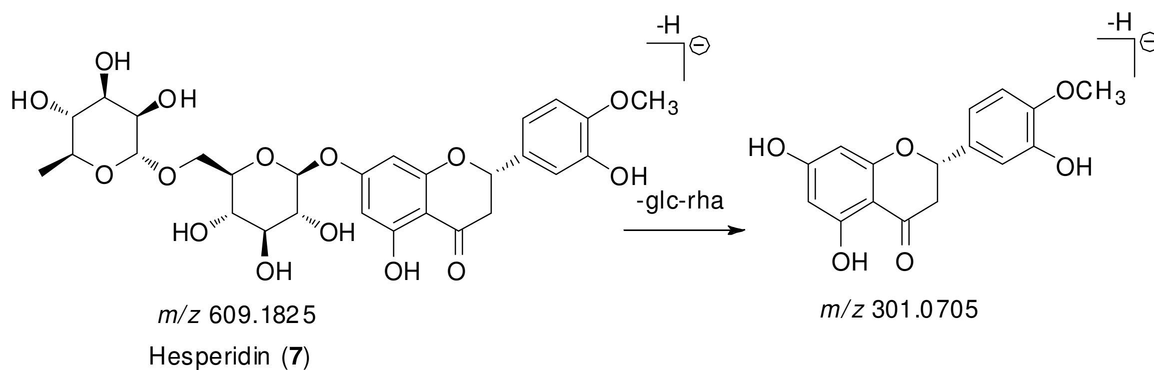

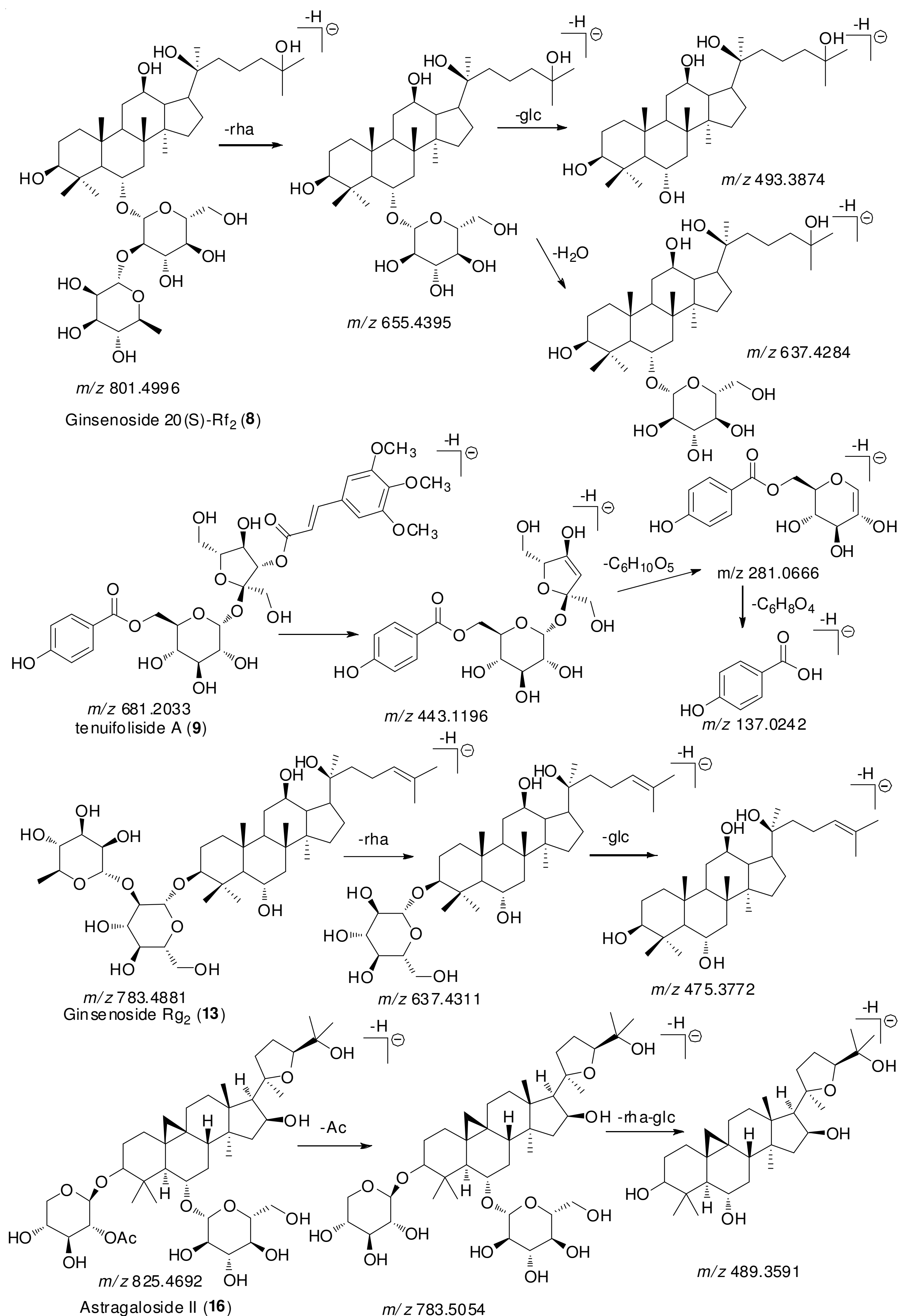

Fig. 4. Proposed fragmentation pathway of compounds **2**, **3**, **7**, **8**, **9**, **13** and **16**

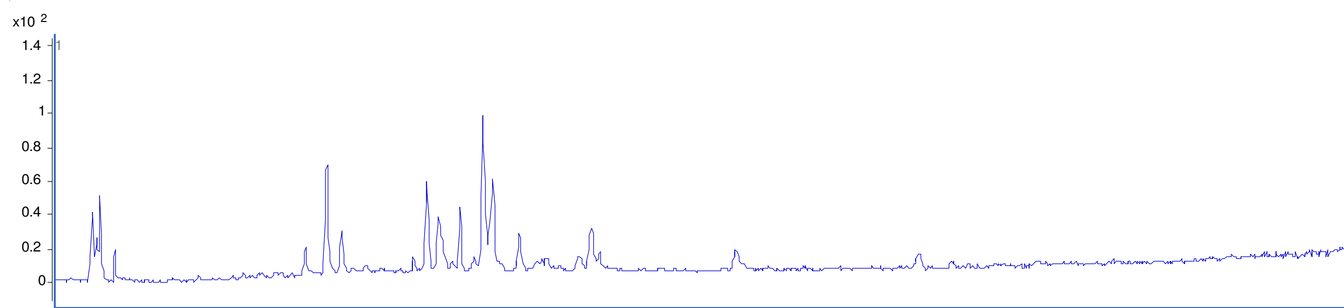

Fig. 5. TIC chromatograms of control rat urine in negative mode

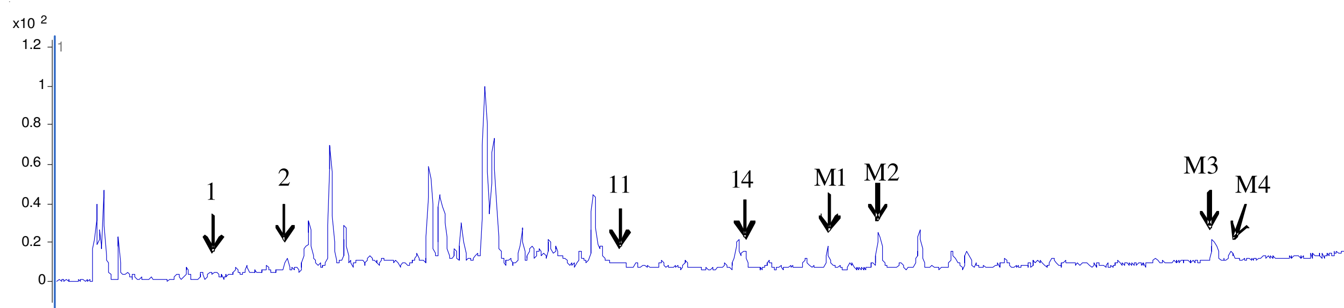

Fig. 6. TIC chromatograms of dosed rat urine in negative mode

TABLE-3
HPLC-Q-TOF/MS DATA OF M1-M4 IN URINE

Peak	RT (min)	MS (Negative)	MS/MS (Negative)	Formula	Exact mass (calc)
M1	35.72	443.2569 $[M + Cl]^-$	407.2728, 379.5349, 364.9376, 341.2478, 301.1761	$C_{24}H_{40}O_5$	443.2570
M2	38.22	443.2571 $[M + Cl]^-$	407.2802, 371.2563, 348.5195, 314.2463, 250.9675	$C_{24}H_{40}O_5$	443.2570
M3	53.58	427.2621$[M + Cl]^-$	391.2840, 345.2820, 327.2682, 311.2363	$C_{24}H_{40}O_4$	427.2621
M4	54.30	425.2460 $[M + Cl]^-$	389.2681, 371.2584, 345.2728, 327.2703, 311.2361, 295.2126	$C_{24}H_{38}O_4$	425.2464

and of LBP were observed in urine (Figs. 5 and 6). To the best of our knowledge, this is the first report on systematic analysis of the chemical constituents of LBP. The LBP paste consist of 37 crude drugs, each component is so little that we could not detecte any component in rat plasma. In order to elucidate the metabolites of Longshen Buyi Paste *in vivo*, we need to study the metabolites of each crude drug further.

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