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Identification of metabolites of Buyang Huanwu Decoction in rat urine using liquid chromatography-quadrupole time-of-flight mass spectrometry

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Abstract

In the present study, rapid resolution liquid chromatography was coupled with quadrupole time-of-flight tandem mass spectrometry (RRLC-Q-TOF-MS) to identify the absorbed components and metabolites in rat urine after oral administration of Buyang Huanwu decoction (BYHWD). After oral administration of BYHWD, urine samples were collected and pretreated by solid phase extraction. The mass measurements were accurate within 5 ppm of error for all the protonated molecules, and subsequent fragment ions offered higher quality structural information for interpretation of the fragmentation pathways of various compounds. A total of 50 compounds were detected in rat urine samples within 20 min, including 12 parent compounds and 38 metabolites. Except for three prototype components (Hydroxysafflor yellow A, Paeoniflorin, and Amygdalin), the metabolites identified mainly came from Radix Astragali, Radix Angelicae Sinensis, and Rhizoma Chuanxiong. The results indicated that glucuronidation and sulfation were the major metabolic pathways of isoflavonoids, while glutathione conjugation, glucuronidation and sulfation were the main metabolic pathways of phthalides. No saponin-related metabolites were detected. The present study provided important structural information on the metabolism of BYHWD. Furthermore, the results of this work have demonstrated the feasibility of the RRLC/ESI-QTOF-MS approach for rapid and reliable characterization of metabolites from herbal medicines.

Keywords

Buyang Huanwu decoction; quadrupole time-of-flight tandem mass spectrometry; metabolite profiling; urine

1. Introduction

Recently, Traditional Chinese Medicine (TCMs) has attracted considerable attention worldwide due to its high effectiveness against many diseases coupled with its low toxicity [1–3]. In Chinese herbal therapy, Chinese herbal prescriptions, comprising several crude drugs at an intrinsic mass ratio according to TCM formulation concepts, exert an important

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role in clinical applications. Buyang Huanwu Decoction (BYHWD) is one famous traditional Chinese prescription consisting of seven medicinal crude drugs, including Radix Astragali, Radix Angelicae Sinensis, Radix paeoniae Rubra, Rhizoma Ligustici Chuanxiong, Flos Carthami, Semen Persicae and Lumbricus in a ratio of 120:6:4.5:3:3:3:3. This prescription has been extensively used to improve the syndromes of Qi deficiency and blood stasis for many years in China, owing to its significant therapeutic performance [4]. In clinical applications, BYHWD has proved effective in treating cerebrovascular and cardiovascular diseases [5–7]. Previous pharmacological studies have demonstrated that BYHWD and its major components may prevent VSMC proliferation by interfering with the ERK transduction pathway [8], the formation of arterial thrombosis, and aortic vascular intimal hyperplasia after balloon catheter injury [9], as well as regulate endothelium-derived vasoactive factors in rats [4]. It is also reported that BYHWD can promote the growth and differentiation of neural cells and inhibit apoptosis of nerve cells [10, 11].

Although the pharmacokinetic parameters of BYHWD have been investigated [12], up to now the literature data on the metabolism of BYHWD is still limited. In order to get much metabolism information of BYHWD from the complex biological matrices, it is necessary to develop effective and reliable analytical methods for detection. Recently, liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS), a high-speed and sensitive technique with shorter analysis times and greater m/z value accuracy, has attracted increasing attention [13]. The LC-Q-TOF/MS has been successfully applied to identify active ingredients and metabolites in TCM [14].

In our previous work [15,16], the LC/MS analytical method was developed for quantitative and qualitative analysis of the major constituents in BYHWD, in which a rapid resolution liquid chromatography was coupled with quadrupole time-of-flight tandem mass spectrometry (RRLC-Q-TOF-MS) for elucidation of the chemical profile of BYHWD. Fifty-four major constituents were identified or tentatively characterized, enabling further *in vivo* elucidation of the metabolites in the prescription. In this investigation, we expanded the scope of our earlier study, by using an RRLC-Q-TOF/MS system to identify known and tentative metabolites in rat urine after oral administration of BYHWD. To our knowledge, it is the first time that the urinary metabolic profile of BYHWD has been established, and it will provide helpful information for further pharmacological and pharmacokinetics research on BYHWD.

2. Experimental

2.1 Herbal materials and chemicals

Radix Astragali (root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao), and Radix Angelicae Sinensis (root of *Angelica sinensis* (Oliv.) Diels) were collected from authenticated locations in Min County, Shanxi Province, and Hunyuan County, Gansu Province, in China, respectively. Rhizoma Chuanxiong (root of *Ligusticum chuanxiong* Hort.), Radix paeoniae Rubra (root of *Paeonia lactiflora* Pall.) and Flos Carthami (flower of *Carthamus tinctorius* L.) samples were collected from Sichuan Province in China. Semen Persicae (seed of *Prunus persica* (L.) Batsch) and Pheretima (*Pheretima aspergillum* (E. Perrier)) samples were bought from a local pharmacy.

The standards, hydroxysafflor yellow A, amygdalin, and paeoniflorin and ferulic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Senkyunolide H and Z-ligustilide were isolated in our laboratory from *Angelica sinensis* (Oliv.) Diels. Isoflavonoid and saponin standards were isolated from the dried roots of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao in our laboratory [17, 18]. Their structures (as shown in Figure 1) were elucidated and

unequivocally identified by spectroscopic methods (UV, IR, MS, ^1H -NMR and ^{13}C NMR). The purity of each standard compound was determined to be higher than 95% by normalization of the peak area detected by RRLC-DAD-TOFMS.

HPLC-grade acetonitrile (ACN) was supplied by Merck (Darmstadt, Germany). Formic acid (FA) (purity: 96%) was purchased from Tedia (Fairfield, OH, USA). Ethanol was purchased from the First Chemical Company of Nanjing (Jiangsu, China). Distilled water (18 M Ω) was further purified by a Milli-Q system (Millipore, Bedford, MA, USA). Other reagents and chemicals were of analytical grade.

2.2 Preparation of BYHWD

The powdered sample of BYHWD (142.5 g), consisting of Radix Astragali, Radix Angelicae Sinensis, Radix paeoniae Rubra, Rhizoma Ligustici Chuanxiong, Flos Carthami, Semen Persicae and Pheretima in a ratio of 120:6:4.5:3:3:3:3, was immersed in 2000 mL 70% ethanol for 1 h and extracted in a reflux water bath for 1 h. The mixture was refluxed for 2 h at 75°C and filtered, and the residue was refluxed again with another 2000mL 70% ethanol under the same conditions. The extracts were merged and evaporated by rotary evaporation under vacuum at 45°C. The residue was then freeze-dried. Finally, the dried residue was dissolved in water to obtain BYHWD oral solution with a concentration of 3g/mL of crude drug.

2.3 Animal experiments

Animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use. Six Sprague-Dawley rats (260 $\pm$ 15 g) were purchased from Slac Laboratory Animal Co., Ltd. (Shanghai, China). The animals were acclimatized to the facilities for 5 days and then fasted (on plastic bottom cages) with free access to water for a 12-h period prior to the experiment. Then, the blank urine sample from each rat was collected using a metabolic cage. Prior to drug administration, the experimental animals were deprived of food for 12 h once more, and then were exposed orally to the BYHWD preparation at a dose of 60 g/kg (i.e., 2 mL per 100 g body weight). The drug-containing urine samples were collected during the 0–24 h period after administration. All urine samples were centrifuged and then stored at –70°C until additional extraction and analysis.

2.4 Urine sample preparation

A solid-phase extraction (SPE) method was used to extract metabolites from the urine samples. 1.0 mL urine sample was added and flowed through LC-18 SPE columns (1 mL/100mg volume, Supelco, USA) using gravity. The SPE columns were washed with 3 mL deionized water, and then the compounds were eluted with 2 mL methanol. The eluant was then centrifuged at 12000 *g* at 4°C for 10 min, and 2 mL aliquots of the supernatants were injected into the LC/ESI-TOFMS system for analysis.

2.5 Analytical System

Chromatographic analysis was performed on an Agilent 1200 Series LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, an online degasser, an autoplate-sampler and a thermostatically controlled column compartment. Chromatographic separation was carried out at 25°C on an Agilent ZorBax SB-C18 column (4.6 $\times$ 250 mm, 1.8mm).

The chromatographic conditions were as follows: flow rate of 0.6 mL/min, sample injection volume of 2 μL , column temperature of 25 °C, mobile phase A (0.1% formic acid) and mobile phase B (100% acetonitrile). The gradient profile was optimized as the following: 0–

2.0 min, 5–10% B; 2.0–4.0 min, 10–15% B; 4.0–6.0 min, 15% B; 6.0–10.0 min, 15–28% B; 10.0–16.0 min, 28–40% B; 16.0–18.0 min, 40–80% B; 18.0–20.0 min, 80–100% B.

Detections were performed using a 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray (ESI) interface. The ESI source of the MS was operated in both positive and negative modes, and the operating parameters were: drying gas (N₂) flow rate, 10.0 L/min; drying gas temperature, 325 °C; nebulizer, 35 psig; capillary, 3000 V; Oct RFV, 750 V; and fragmentor voltage, 100 V, and skimmer voltage 65 V. All operations, acquisition, and analysis of data were monitored by Agilent LC-Q-TOF-MS MassHunter Acquisition Software Version A.01.00 (Agilent Technologies) and operated under MassHunter Acquisition Software Version B.02.00 (Agilent Technologies). Mass spectra were recorded across the range m/z 120–2000 with accurate mass measurement of all mass peaks. To optimize signals and obtain maximal structural information, the collision energy was adjusted from 5 V to 40 V for MS/MS experiments.

3. Results and Discussion

3.1 Detection and identification of components in rat urine

The screening, identification and further characterization of the metabolites of BYHWD were firstly performed by UHPLC/TOFMS in both the positive and negative ion mode. The typical total ion chromatograms (TICs) of blank urine and drug-containing urine samples in positive ion mode are shown in Figure 2(a) and (b), respectively.

Endogenous interferences from complex biological matrices usually affect the metabolite identification. In order to increase sensitivity and to facilitate data processing, the extracted ion chromatograms (EICs) of TOF/MS were used by comparison of the EICs of the drug-containing urine samples ($n=6$) with the EICs of blank urine samples, a total of 50 compounds, including 12 prototype components and 38 potential metabolites, were detected in the drug-containing urine (Table 1). The retention time of each compound was ascertained by employing its mass to generate the extracted ion chromatogram (EIC) of the drug-containing urine sample. The fragment ions of the metabolites were then correlated based on their co-elution with their corresponding protonated ions, as well as the possible metabolic or fragmentation pathways of constituents in the BYHWD preparation. Then, the most probable molecular formulas of the metabolites were determined using different criteria, including mass accuracy <5 ppm, the nitrogen rule, the double-bond equivalent index, the isotopic pattern, and modifications based on the common metabolic pathways. Furthermore, structures of the metabolites were elucidated based on product ion spectra generated via adjustment of the fragmentor voltage (5 V, 30 V, and 40 V). Ultimately, 22 of the 38 potential metabolites were tentatively identified.

3.2 Identification of parent components in drug-containing urine

By comparing the accurate masses of peaks appearing in the chromatograms of drug-containing urine with those previously identified in BYHWD [15], 12 peaks were detected as prototype components of BYHWD. As shown in Table 1, most of the prototype compounds were identified by comparison of their retention times and TOFMS data with those of the standards, and their structures are shown in Figure 1. Among these 12 compounds, there are five isoflavonoids ((6 α R, 11 α R)-9, 10-dimethoxypterocarpan-3-*O*- β -D-glucoside, Formononetin-7-*O*- β -D-glucoside, calycosin, formononetin, and (6 α R, 11 α R)-3-hydroxy-9,10-dimethoxypterocarpan), and three saponins (Astragaloside V/VI/VII, Astragaloside IV and Astragaloside II) from *Radix Astragali*, and four other types of compounds (Senkyunolide H, Hydroxysafflor yellow A, Paeoniflorin and Amygdalin) from *Radix Angelicae Sinensis*, *Flos Carthami*, *Radix paeoniae Rubra* and *Semen Persicae*.

3.3 Identification of metabolites in drug-containing urine

Beyond the 12 prototype compounds, more than 38 peaks were tentatively predicted to be metabolites of BYHWD, and could be generally divided into two groups: isoflavonoid-related and phthalide-related metabolites.

3.3.1 Identification of isoflavonoid-related metabolites—A total of 14 compounds detected in rat urine were tentatively assigned as metabolites originating from isoflavonoids. According to the aglycone type, they are generally divided into four groups named **G1**, **G2**, **G3** and **G4**, possessing calycosin, formononetin, (6*a*,11*a*)-3-hydroxy-9,10-dimethoxypterocarpan, and (3*R*)-7, 2'-hydroxy-3',4'-dimethoxy-isoflavan as the aglycone, respectively (Table 1). Ions of $[M+H]^+$, $[M+Na]^+$, or $[M+NH_4]^+$ in the positive mode and $[M-H]^-$ in the negative mode can be easily observed for most components. Moreover, diagnostic losses corresponding to conjugates were observed in the mass spectra of these metabolites, including $C_6H_8O_6$ (176Da) for glucuronide conjugates and SO_3 (80Da) for sulfate conjugates.

Metabolites of G1: Five glucuronide conjugates (M4, M8, M12, M16 and M20), one sulfate conjugate (M18), and one demethylated product (M37) were assigned as metabolites of G1 (Figure 3). Among them, M4, M16 and M18 comprised the same aglycone, calycosin, and yielded a series of characteristic ions of calycosin at m/z 285, 270, 253, 225, 213, 197, and 137. The aglycone of M12 and M37 was assigned as 4'-demethylcalycosin, due to the presence of the aglycone ion at m/z 271.0615, 14Da (CH_2) less than the protonated ion of calycosin, together with the observation of the other fragment ions shown in Table 1. Meanwhile, the aglycone ion of M8 and M20 ($[M+H-C_6H_8O_6]^+$), which appeared at m/z 301.07 ($C_{16}H_{13}O_6^+$), could have been derived from calycosin ($C_{16}H_{13}O_5^+$) via hydroxylation, suggesting that their aglycone was hydroxylated calycosin. Since the glucuronide conjugates of isoflavonoids mainly exist as 7-*O*- β -D-glucuronides *in vivo* [19,20], M12 and M16 were tentatively identified as 4'-demethyl-calycosin-7-*O*- β -D-glucuronide and calycosin-7-*O*- β -D-glucuronide. In the MS spectrum of M4, the presence of the characteristic ions of calycosin indicated that the fragment ion at m/z 447 corresponded to calycosin-7-*O*- β -D-glucoside, and, accordingly, M4 was identified as calycosin-7-*O*- β -D-glucoside-3'-*O*- β -D-glucuronide.

Metabolites of G2: Two glucuronide conjugates (M6 and M21) and one sulfate conjugate (M14) were assigned as metabolites of G2 (Figure 4). M21 yielded a peak at m/z 445.1138 in positive mode and showed product ions at m/z 269, 254, 226, 197, 181, 169, 141 and 137 in the MS/MS detection, suggesting that it possesses a formononetin aglycone. Both M6 and M14 exhibited the same product ion at m/z 255 ($C_{15}H_{11}O_4$), which was tentatively identified as daidzein, since it was 14 Da (CH_2) less than the protonated ion of formononetin; the other fragment ions shown in Table 1 were also observed. In addition, it has been well documented that formononetin can be metabolized to daidzein [21], and then to equol ($C_{15}H_{14}O_3$) [22–25]. Eventually, M6 and M21 were identified as daidzein-7-*O*- β -D-glucuronide and formononetin-7-*O*- β -D-glucuronide, respectively, due to only one hydroxyl group at the C-7 position of their skeleton.

Metabolites of G3: M25 was considered as a metabolite of G3, owing to the production of the characteristic ions of (6*a*,11*a*)-3-hydroxy-9,10-dimethoxypterocarpan at m/z 167, 301 and 152 in positive mode. Since there is only one hydroxyl group available at the C-3 position of the skeleton of G3, M25 was consequently identified as (6*a*,11*a*)-3-hydroxy-9,10-dimethoxypterocarpan 3-*O*- β -D-glucuronide.

Metabolites of G4: Three glucuronide conjugates (M3, M23, and M24) were assigned as metabolites of G4 (Figure 5). M3 and M24 produced a $[M+H-C_6H_8O_6]^+$ ion at m/z 303.1222, and further exhibited $[^{2,3}B+H]^+$ and $[^{2,3}A+H]^+$ ions at m/z 167 and 123, respectively, indicating that they possessed the same aglycone G4. Furthermore, M3 displayed the $[M+H]^+$ ion at m/z 641.1563 ($C_{29}H_{37}O_{16}$), and also yielded product ions at m/z 479 and 465, corresponding to the losses of a glucose (162 Da) and a glucuronide (176 Da). Thus, M3 was tentatively assigned as (3R)-7,2'-hydroxy-3',4'-dimethoxy- isoflavan-7-*O*- β -D-glucoside-2'-glucuronide. The aglycone ion of M23 at m/z 333.13 ($C_{18}H_{21}O_6$) was 32Da (CH_3O) higher than that of G3, implying that there is one more methoxyl in this aglycone compared with G3. According to a previous study [26], M23 was tentatively identified as 7,2'-dihydroxy-3',4',5'-trimethoxy- isoflavan- 7-*O*- β -D-glucuronide.

3.3.2 Identification of phthalide-related metabolites—Phthalides are important bioactive compounds in *Radix Angelicae Sinensis* and *Rhizoma Chuanxiong*, two components of BYHWD. A total of 7 metabolites (M29, M30, M11, M15, M19, M31, M34) were tentatively identified as phthalide-related metabolites according to their MS/MS data listed in Table 1. Figure 6 and Figure 7 displays the possible structures of these components, including two senkyunolide-related metabolites and five butylidenephthalide-related metabolites. In addition to the glucuronide conjugates and sulfate conjugate, some of these metabolites were glutathione-derived compounds, whose fragmentation patterns show a neutral loss of $C_5H_7NO_2S$ (145 Da) originating from the acetylcysteine residue.

Senkyunolide-related metabolites: M29 displayed the $[M+H]^+$ and $[M+Na]^+$ ions at m/z 370.1323 and 392.1134, respectively, indicating that the molecular formula of M29 is $C_{17}H_{23}NO_6S$. Further MS analysis of M29 yielded a product ion at m/z 207, which was formed by the loss of $C_5H_9NO_3S$ (163 Da) from the $[M+H]^+$ ion, suggesting the presence of one acetylcysteine residue ($C_5H_7NO_2S$) in M29. In addition, M29 exhibited product ions at m/z 161.0950, 179.1054, 189.0905 and 207.1014, the characteristic ions of senkyunolide in positive mode, indicating that it is a senkyunolide-related metabolite. According to previous studies [27], M29 was identified as the acetylcysteine conjugate of senkyunolide.

As for M30, the protonated ion at m/z 354.1373 ($C_{17}H_{24}NO_5S$) was 16Da (O) less than the protonated ion of M29, suggesting that it was a dehydroxylation product of M29.

Butylidenephthalide-related metabolites: M11 and M31 were considered as butylidenephthalide related metabolites owing to the production of the diagnostic ions of butylidenephthalide at m/z 189, 171, 143, 128 and 115. Furthermore, M11 yielded the aglycone ion by the loss of $C_6H_8O_6$ (176 Da), while M31 yielded the aglycone ion by the loss of $C_5H_7NO_2S$ (145 Da). According to a previous study [27], M11 and M31 were tentatively identified as 3-hydroxybutylphthalide-3-*O*- β -D-glucuronide and 3-hydroxybutylphthalide-3-*S*-acetylcysteine, respectively. Meanwhile, M15 and M19 have the same chemical formula as M11. Since no other fragment ions of M15 and M19 were found, they were tentatively assigned as the isomers of M11. In the same way, M34 was tentatively identified as the isomer of M31 due to having the same chemical formula.

3.4 Analysis of the urinary metabolite profile of BYHWD

BYHWD is a well-known traditional Chinese prescription with many good therapeutic effects. However, the lack of information on the metabolites of BYHWD has made it difficult to obtain a consistent picture of its efficacy and pharmacology. In the present study, 50 metabolites were detected in rat urine samples within 20 min. 22 of them, including isoflavonoids, phthalides and saponins, were tentatively identified based on MS data. Except for the three prototype components (Hydroxysafflor yellow A, Paeoniflorin, and

Amygdalin), the metabolites identified mainly came from the crude drugs Radix Astragali, Radix Angelicae Sinensis and Rhizoma Chuanxiong.

Isoflavonoids were the most abundant metabolites in the drug-containing urine. A total of 19 isoflavonoids, including 5 parent compounds and 14 metabolites, were identified in the drug-containing urine. The detection of two isoflavonoid glycosides (P5 and P6) demonstrated that isoflavonoid glycosides could be absorbed directly, which was supported by a previous study [26]. Based on these metabolites, the possible metabolic pathways of isoflavonoids in BYHWD were proposed and summarized in Figure 3–5. The results revealed that glucuronidation and sulfation were the main metabolic pathways of isoflavonoids in BYHWD. In addition, it showed that some Phase I reactions, such as hydrolysis, demethylation, methylation, hydroxylation and reduction also occurred in the metabolism of isoflavonoids in BYHWD.

Saponins also play an important role in BYHWD. However, only very low amounts of astragaloside II, astragaloside IV (P9) and astragaloside V/VI/VII (P8) were detected in the drug-containing urine. This can be possibly attributed to the fact that saponins were poorly absorbed directly, and were mainly excreted through feces or bile.

As for the phthalides from Radix Angelicae Sinensis and Rhizoma Ligustici Chuanxiong, both prototype components and metabolites were detected in the drug-containing urine. According to metabolism information, in addition to glucuronidation and sulfation, glutathione conjugation also occurs in the metabolism of phthalides.

Conclusion

In this study, a rapid and reliable UPLC-Q-TOF/MS method was successfully established to identify the metabolites of BYHWD in rat urine samples collected 24 h after oral administration of BYHWD. In total, 50 metabolites were detected, and 22 of them were tentatively identified. To our knowledge, this is the first study to establish the urinary metabolic profile of BYHWD by UPLC-Q-TOF/MS. The urinary metabolites and their metabolic pathways proposed in this study are very helpful in revealing the bioactive chemical basis of BYHWD. Furthermore, it was demonstrated that the high-speed and sensitive UPLC-Q-TOF/MS analytical system was a useful tool to investigate the active ingredients and metabolites of TCM.

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Figure 1.

Chemical structures of 12 parent compounds of DBT identified in the drug-containing urine.

Figure 2.
TICs of urine samples of rat by RRLC-QTOF/MS (a): The blank urine in positive mode; (b): The drug-containing urine in positive mode (*The parent constituents in Buyang Huanwu Decoction).

Figure 3.
Proposed major metabolic pathways of G1(calycosin)-related metabolites.

Figure 4.
Proposed major metabolic pathways of G2 (formononetin) -related metabolites.

Figure 5.
Proposed major metabolic pathways of G4 ((3R)-7,2'-dihydroxy-3',4'-dimethoxyiso-
flavan)-related metabolites.

Figure 6.
Proposed major metabolic pathways of senkyunolide-related metabolites.

Figure 7.
Proposed major metabolic pathways of butylidenephthalide-related metabolites.

Table 1
Metabolites identified from BYHWD in rat urine samples by RRLC-ESI-QTOF/MS

No.	<i>t_R</i> (min)	<i>m/z</i> [M+H] ⁺	<i>m/z</i> [M+Na] ⁺	Formula [M+H] ⁺	Fragment ions	ppm error	Identification
1 (P1)	3.07	225.1108		C ₁₂ H ₁₆ O ₄		-1.37	Senkyunolide H
2 (M1)	3.53	383.1319		C ₁₇ H ₁₉ O ₈ S		1.68	
3 (M2)	5.65	146.0607		C ₉ H ₈ NO		-4.32	4 (H)-quinoline or 4-hydroxyquinoline
4 (P2)	5.74	613.1764		C ₂₇ H ₃₂ O ₁₆		2.13	Hydroxysafflor yellow A
5 (P3)	6.2	458.1605		C ₂₇ H ₂₂ NO ₆		1.37	Amygdalin
6 (M3)	6.80	641.1653		C ₂₉ H ₃₉ O ₁₆	479, 465, 303, 167, 123	2.48	7,2'-dihydroxy-3',4'-trimethoxyisoflavan-7-β-O-D-glucuronide-2'-O-β-glucuronide
7 (M4)	7.15	623.1616	645.1432	C ₂₁ H ₁₉ O ₁₀	461, 447, 285, 270, 253, 225, 197, 137.	3.79	Calycosin-7-O-β-D-glucoside-3'-O-β-glucuronide
8 (M5)	8.25	247.1545	269.1364	C ₁₂ H ₂₃ O ₅		-1.93	
9 (P4)	8.32	525.1613 ([M+HCOO] ⁻)		C ₂₄ H ₂₈ O ₁₃		0.94	Paeoniflorin
10 (M6)	8.42	431.0970	453.0788	C ₂₂ H ₂₂ O ₉	253 [*] , 255, 224, 223, 195, 133	0.62	Daidzein-7-O-β-D-glucuronide
11 (M7)	9.09	453.2078	467.1884	C ₁₈ H ₃₃ N ₂ O ₉		0.08	
12 (M8)	9.64	477.1028	499.0847	C ₂₂ H ₂₁ O ₁₂	301, 285, 213, 187, 137,	-0.01	glucuronide conjugate of hydroxylated calycosin
13 (M9)	9.74	311.0699	333.0517	C ₁₃ H ₁₄ N ₂ O ₅ S		-0.86	
14 (M10)	10.18	386.2743		C ₁₇ H ₂₄ NO ₇ S		2.81	butylidenephthalide-related metabolites (glutathione-derived metabolites)
15 (M11)	10.46	383.1336 ([M-H] ⁻)	405.1148	C ₁₈ H ₂₃ O ₉	189, 171, 143, 128, 115	0.25	3-hydroxybutylphthalide-3-O-β-D-glucuronide
16 (M12)	10.61	447.0925	469.0738	C ₂₁ H ₁₉ O ₁₁	271, 253, 225, 213, 197, 137	-0.29	4'-demethylcalycosin-7-O-β-D-glucuronide
17 (M13)	11.07	479.1909	501.1727	C ₂₄ H ₃₁ O ₁₀		0.42	
18 (M14)	11.16	335.0217		C ₁₅ H ₁₁ O ₇ S	253 [*] , 255, 224, 223, 195, 167, 133,	1.02	Daidzein-7-O-sulfate or Daidzein-4'-O-sulfate
19 (M15)	11.19	383.1354 ([M-H] ⁻)		C ₁₈ H ₂₃ O ₉		0.25	isomer of 3-hydroxybutylphthalide-3-O-β-D-glucuronide
20 (M16)	11.32	461.1079	483.0903	C ₂₂ H ₂₁ O ₁₁	285, 270, 253, 225, 197, 137,	1.27	Calycosin-7-O-β-D-glucuronide
21 (M17)	11.44	597.1756	619.1574	C ₃₄ H ₂₉ O ₁₀		-0.08	
22 (M18)	11.53	365.0321		C ₁₆ H ₁₃ O ₈ S		1.15	calycosin-7-O-sulfate or calycosin-3'-O-sulfate

No.	t_R (min)	m/z [M+H] ⁺	m/z [M+Na] ⁺	Formula [M+H] ⁺	Fragment ions	ppm error	Identification
23 (P5)	12.23	463.1587		C ₂₃ H ₂₆ O ₁₀		2.47	(6a <i>R</i> , 11 <i>a,R</i>)-9, 10-dimethoxypterocarpan-3- <i>O</i> -β-D-glucoside
24 (M19)	12.33	383.1348 ([M-H] ⁻)		C ₁₈ H ₂₃ O ₉		0.26	isomer of 3-hydroxybutylphthalide-3- <i>O</i> -β-D-glucuronide
25 (P6)	12.50	431.1327		C ₂₂ H ₂₃ O ₉		2.22	Formononetin-7- <i>O</i> -β-D-glucoside
26 (M20)	12.54	477.1018	499.0844	C ₂₂ H ₂₁ O ₁₂	301, 285, 213, 187, 137,	2.00	glucuronide conjugates of hydroxylated calycosin
27 (M21)	12.66	445.1138		C ₂₂ H ₂₁ O ₁₀	269, 254, 226, 197, 181, 169, 141, 137	-1.08	Formononetin-7- <i>O</i> -β-D-glucuronide
28 (M22)	12.88	465.1379	487.1202	C ₂₂ H ₂₃ O ₁₁		2.72	
29 (P7)	13.09	285.0760		C ₁₆ H ₁₃ O ₅		-0.22	Calycosin
30 (M23)	13.09	509.1633	531.1642	C ₂₄ H ₂₉ O ₁₂	333	-2.08	7,2'-dihydroxy-3',4',5'-trimethoxyisoflavan-7-β- <i>O</i> -D-glucuronide
31 (M24)	13.34	479.1541	501.1361	C ₂₃ H ₂₇ O ₁₁	303, 167, 123.	1.57	(3 <i>R</i>)-7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7- <i>O</i> -β-D-glucuronide
32 (M25)	13.48	477.1370	499.1202	C ₂₃ H ₂₅ O ₁₁	301, 167, 152.	2.68	(6a <i>R</i> , 11 <i>a,R</i>)-3-hydroxy-9,10-dimethoxypterocarpan-3- <i>O</i> -β-D-glucuronide
33 (M26)	13.94	287.0904		C ₂₁ H ₃₀ NO ₄ S		-4.17	
34 (P 8)	14.15	947.5184		C ₄₇ H ₇₈ O ₁₉		-0.2	Astragaloside V/VI/VII
35 (M27)	14.45	381.1175	403.0988	C ₁₈ H ₂₁ O ₉		1.45	
36 (M28)	14.55	188.0709	210.0526	C ₁₁ H ₉ NO ₂		0.95	
37 (M29)	14.64	370.1321	392.1134	C ₁₇ H ₂₄ NO ₆ S	354, 207, 161, 189, 179	-0.13	acetylcyteine conjugate of senkyunolide
38 (M30)	14.76	354.1376	376.1178	C ₁₇ H ₂₄ NO ₅ S		0.19	acetylcyteine conjugate of dehydroxylated senkyunolide
39 (M31)	14.89	352.1204	374.1022	C ₁₇ H ₂₂ NO ₅ S	189, 171, 143, 129	2.56	3-hydroxybutylphthalide-3- <i>S</i> -acetylcysteine
40 (M32)	14.99	259.1643	281.1471	C ₁₂ H ₂₃ N ₂ O ₄		-2.54	
41 (M33)	15.14	216.1959	238.1777	C ₁₂ H ₂₃ NO ₂		-0.20	
42 (M34)	15.75	257.2223	279.2043	C ₁₄ H ₂₈ N ₂ O ₂		-0.18	
43 (P9)	16.30	807.4509		C ₄₁ H ₆₈ O ₁₄		1.3	Astragaloside IV
44 (M35)	16.94	373.2737	391.2839	C ₂₄ H ₃₇ O ₃		-0.03	
45 (M36)	17.21	352.1213	374.1024	C ₁₇ H ₂₂ NO ₅ S		0.1900	isomer of 3-hydroxybutylphthalide-3- <i>S</i> -acetylcysteine
46 (P10)	17.87	849.4613		C ₄₃ H ₇₀ O ₁₅		-4.4	Astragaloside II
47 (P11)	18.11	269.0823		C ₁₆ H ₁₂ O ₄		2.6	Formononetin
48 (M37)	18.41	271.0971		C ₁₆ H ₁₅ O ₄	253, 225, 213, 197, 137	-2.29	4'-demethylcalycosin

No.	<i>t_R</i> (min)	<i>m/z</i> [M+H] ⁺	<i>m/z</i> [M+Na] ⁺	Formula [M+H] ⁺	Fragment ions	ppm error	Identification
49 (P12)	18.61	301.1079		C ₁₇ H ₁₆ O ₅		-4.4	(6a <i>R</i> , 11a <i>R</i>)-3-hydroxy-9,10-dimethoxypterocarpan
50 (M38)	19.18	287.1497	309.1321	C ₁₄ H ₂₃ O ₆		-3.67	

P: The parent constituents in Buyang Huanwu Decoction; M: metabolites;

* Fragmentations were obtained in the negative ion mode.