Journal of Chromatography B, 878 (2010) 2741–2750

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

UPLC–QTOF/MS-based screening and identification of the constituents and their metabolites in rat plasma and urine after oral administration of *Glechoma longituba* extract

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ARTICLE INFO

Article history:
Received 7 March 2010
Accepted 14 August 2010
Available online 21 August 2010

Keywords:
Glechoma longituba
Metabolite
UPLC–QTOF/MS
MDF
MS^E

ABSTRACT

Glechoma longituba is a widely used traditional Chinese medicine (TCM) in treating various diseases; however, the *in vivo* integrated metabolism of its multiple bioactive components remains unknown. In this paper, ultra-performance liquid chromatography (UPLC) coupled to quadrupole time-of-flight (QTOF) and the MetaboLynx™ software combined with mass defect filtering (MDF) together provide unique high throughput capabilities for drug metabolism study, with excellent MS mass accuracy and enhanced MS^E data acquisition. This rapid automated analysis method was successfully applied for screening and identification of the constituents absorbed and metabolized studies of *G. longituba* extract after oral administration to rats. The results showed that 21 parent components of *G. longituba* extract were absorbed into the blood circulation of the rats and a total of 80 metabolites of 9 parent compounds were tentatively detected *in vivo* by their MS spectra obtained at low or high collision energy scan with the comparison of the authentic standards and literature data. The developed method was simple and reliable, revealing that it could be used to rapid screen and identify the structures of active components responsible for pharmacological effects of *G. longituba* and to better clarify its action mechanism. This work suggests that the integrative metabolism approach makes a useful template for drug metabolism research of TCM.

1. Introduction

Glechoma longituba (Nakai) Kupr. is the dried overground part of a perennial plant of Labiatae widely used as traditional Chinese medicine (TCM) in China. It is recorded as "Lianqiancao" in Chinese and has an effective broad spectrum of applications as antipyretic, choleretic and diuretic agent for jaundice, as well as for the treatment of diuretic, cholagogue, lithagogue, heat-clearing, detoxicating, swelling and pain caused by traumatic injury, eliminating concretion and anti-diarrhea [1,2]. Moreover, it is also used as an ingredient of many herbal composition formulas in China to obtain the pharmacological synergism of traditional drugs in action and main ingredient in many formulas such as a famous Chinese patent medicine called Lithagogue Granules (Chinese name, Paishi Keli). The extensive phytochemical and pharmacological investigation of *G. longituba* revealed the presence of several classes of bioactive compounds such as phenolic acids, flavonoids, triterpenoid and plenty of essential oils [3–6]. Therefore, it is essential to understand how a number of chemical components of *G. longituba* are absorbed and metabolized in the body. In addition, the method should be established for investigating the absorption and metabolite components *in vivo*.

Recently, ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–ESI-QTOF/MS) system with automated MS^E data analysis software (MetaboLynx™) has been proved a powerful and reliable analytical approach for *in vivo* metabolite identification studies [7–9]. UPLC–MS has been used effectively in numerous drug metabolism studies. It is the combination of these technological developments for the identification of drug metabolites in biological matrices that produce adds a new dimension to metabolism studies enabling us to obtain better throughput, faster analysis, increased sensitivity, and increased peak resolution, which in turn will improve the data quality from the mass spectrometer desirable to the working laboratory. In MS^E experiment, two alternative scan functions are utilized for data acquisition. Namely, the first scan function acquires a wide mass range from m/z 100 to m/z 1000 at low collision energy of 6 eV and collects information on the intact ions in the sample. The second scan function acquires data over the same mass range using the collision energy ramped from 20 to 40 eV. This scan function allows for the collection of fragment ion data based on all ions in the first scan acquired, which is

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doi:10.1016/j.jchromb.2010.08.014

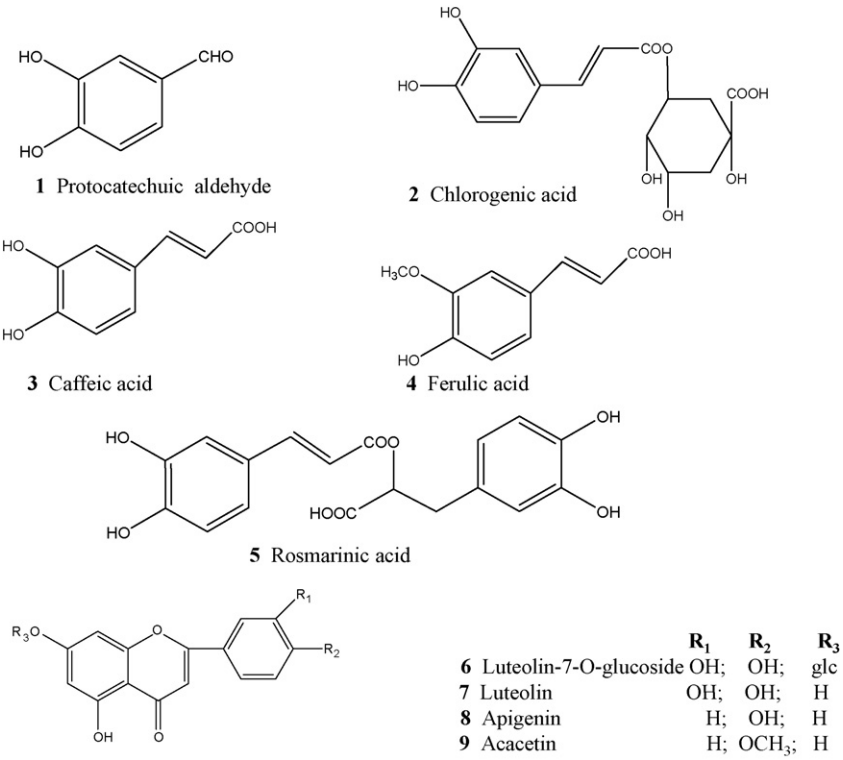

Fig. 1. Chemical structures of standard compounds.

equivalent to a non-selective tandem mass spectrometric (MS/MS) scan. MetaboLynx™ is a commercially available data processing software using mass defect filtering (MDF). The MDF approach enabled the use of MS^E data collection that has previously been shown to be more effective for the metabolite identification in a complex matrix. With the mass defect window set about ±50 mDa from that of the parent drug, the metabolite profile of biological sample was obtained with the majority of endogenous interference ions removed and the number of false-positive entries reduced. The filtered data facilitated the identification of both common and uncommon metabolites [10].

As there have been few detailed reports on the absorption, metabolism and excretion of orally administered *G. longituba* extract, therefore the purpose of the present study was to identify the multiple constituents and their potential metabolites *in vivo* after oral administration of *G. longituba* extract. We carried out a study in rats to obtain information on these processes, which should be reflected the therapeutic effects of the integration of TCM. The UPLC–ESI-QTOF/MS method including MS^E technology and automated data processing MetaboLynx™ software using the MDF approach was used to study the multiple bioactive components absorbed and metabolites *in vivo*. For the qualitative identification of bioactive compounds in TCM, the advent of new mass analyzers, hyphenated technologies and data acquisition software has created further opportunities. The absorption and metabolite components might be the bioactive substances of *G. longituba in vivo* and should be screened on pharmacological models to pave a helpful way for research on the effect related to mechanism of action.

2. Experimental

2.1. Chemical, reagents and sample

HPLC-grade acetonitrile, formic acid and methanol were purchased from Merck (Merck, Darmstadt, Germany); ultra-pure water was purified by an EPED superpurification system (Eped, Nanjing, China). The distilled water was used for the extraction and preparation of samples.

The standards of protocatechuic aldehyde (PcA), chlorogenic acid (CgA), caffeic acid (CaA), ferulic acid (FA) and rosmarinic acid (RA) were provided by Chinese National Institute for the Control of Pharmaceutical and Biological Products (CNICPBP, Beijing, China), luteolin-7-O-glucoside (Lut7G), luteolin (Lut), apigenin (Api) and acacetin (Aca) were isolated in our laboratory from *G. longituba*, and their structures were fully characterized based on chemical and spectroscopic analysis (UV, IR, NMR, MS), and the purity of each compound was more than 98% determined by HPLC analysis. The chemical structures of these standards are shown in Fig. 1.

The author in Xuyi country, Jiangsu Province, PR China, collected *G. longituba* samples, which were authenticated by Professor Jin-Ao Duan, in July 2008. The voucher specimens were deposited in the Jiangsu Key laboratory for TCM formulae Research, Nanjing University of Chinese Medicine, China.

2.2. Instrumentation and UPLC–ESI-QTOF/MS conditions

Chromatographic experiments were performed on a Waters ACQUITY™ UPLC™ system (Waters Corp., Milford, MA, USA) equipped with a 2998 photodiode array detector (PDA) together with a quaternary pump, an auto-sample injector, an on-line degasser and an automatic thermostatic column oven. UPLC separation was achieved on a Waters ACQUITY™ UPLC™ BEH C_{18} column (100 × 2.1 mm, 1.7 μm) with the column temperature set at 35 °C. The mobile phase consisted of (A) water containing 0.2% formic acid and (B) acetonitrile using a gradient elution of 1–65% B at 0–22 min, 65–99% B at 22–27 min, 99% B at 27–30 min. The flow rate was 0.4 mL/min, injection volume was 5 μL.

The MS instrument consisted of a Waters Synapt™ QTOF/MS (Waters Corp., Milford, MA, USA). Ionization was performed in the negative electrospray (ESI) mode. The mass range was set at m/z 100–1000 Da with a 0.5 s scan time. The conditions used for the ESI

source were as follows: capillary voltage, 4.0 kV; sampling cone, 30 V; extraction cone, 3.0 V; source temperature, 120 °C; and desolvation temperature, 350 °C. Nitrogen was used as desolvation and cone gas with the flow rate of 700 and 50 L/h, respectively. MS^E (where E represents collision energy) analysis was performed on ESI-QTOF/MS setup with collision energy ramp of 6–40 eV (MS^E parameters: low energy, 6 eV; and high energy, 20–40 eV). For accurate mass measurement, data were centroided during acquisition using an external reference (LockSpray™) comprising a 2 μg/mL solutions of the rutin (which were purchased by CNICPBP), which was dissolved in 50% acetonitrile and infused continuously into the ESI source at a rate of 400 μL/min via a syringe pump. The data were processed using MassLynx™ 4.1 software with MS^E program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standard solutions

Individual standard stock solutions of nine standards (containing PcA, CgA, CaA, FA, Lut7G, RA, Lut, Api and Aca) were prepared by accurately weighing the required amounts into volumetric flasks and dissolving in 60% acetonitrile. These stock solutions were filtered through a PTFE membrane filter of 0.22 μm. The mixed standard solutions were stored at 4 °C before analysis.

2.4. G. longituba extract preparation

G. longituba was chopped into pieces about 1 cm long. One-kilogramme pieces were macerated with 20-fold of 80% ethanol for 2 h, and then extracted under thermal reflux for 1 h twice. The extract was filtrated out by absorbent cotton inserted in a funnel. The two successive extracts were merged and evaporated to less than 200 mL in a rotatory evaporator R-210 (BUCHI Ltd., Labortechnik AG, Switzerland) at 45 °C under reduced pressure. Subsequently, the concentrated extracts were passed to a porcelain evaporating dish, followed by evaporating to dryness using and provide 180 g residue which were divided into two portions, A1 (20 g) and A2 (160 g). Finally, each gram of residue was equivalent to 5.6 g crude drug of *G. longituba* and stored at 4 °C for later use. A1 was dissolved with acetonitrile–water (60:40, v/v) and made up to a final concentration of 0.5 g/mL (equivalent to dry weight of raw materials). The supernatant was filtered through a 0.22 μm membrane, and an aliquot of 5 μL of the filtrate was injected for UPLC–ESI-QTOF/MS analysis. A2 was the *G. longituba* orally administrated to rats.

2.5. Animals, drug administration, biological sample collection and preparation

Ten male Sprague–Dawley (SD) rats (300 ± 10 g) were obtained from Shanghai SLAC Lab. Animal Co., Ltd. (Shanghai, China). The rats were divided into two groups (group A, drug group for blood and urine withdrawn, $n = 5$; group B, control group for blank plasma and urine, $n = 5$) and housed individually in metabolic cages for the collection of urine samples. The rats were fed with standard laboratory food as well as water ad libitum and acclimatized to the facilities for 1 week prior to the experiments. Then, the animals were fasted overnight with free access to water before the test. *G. longituba* extract was dissolved in distilled water and administered to each rat of group A by way of the traditional oral gavage route yielded satisfactorily at a dose of 3.57 g *G. longitub*a extract/kg body weight once daily for 1 week, while the equivalent volume of distilled water was orally administered to each rat of group B. On the seventh day, blood samples were collected from the common carotid artery in heparinised tubes at 1 h post-dose and then centrifuged (TGL-16G, Shanghai Precision Instruments Co., Ltd., China) at 2000 × *g* for 15 min at 4 °C. The supernatant obtained was frozen immediately and stored at −20 °C until analysis. Urine samples were collected at 12 h post-intake. After centrifuged at 600 × *g* for 15 min at 4 °C, all samples were harvested frozen immediately at −20 °C until analysis. Blood and urine were obtained from the control group animals also by using the procedure described above. All experiments were carried out in accordance with the Guidelines of the Committee on the Care and Use of Laboratory Animals of the

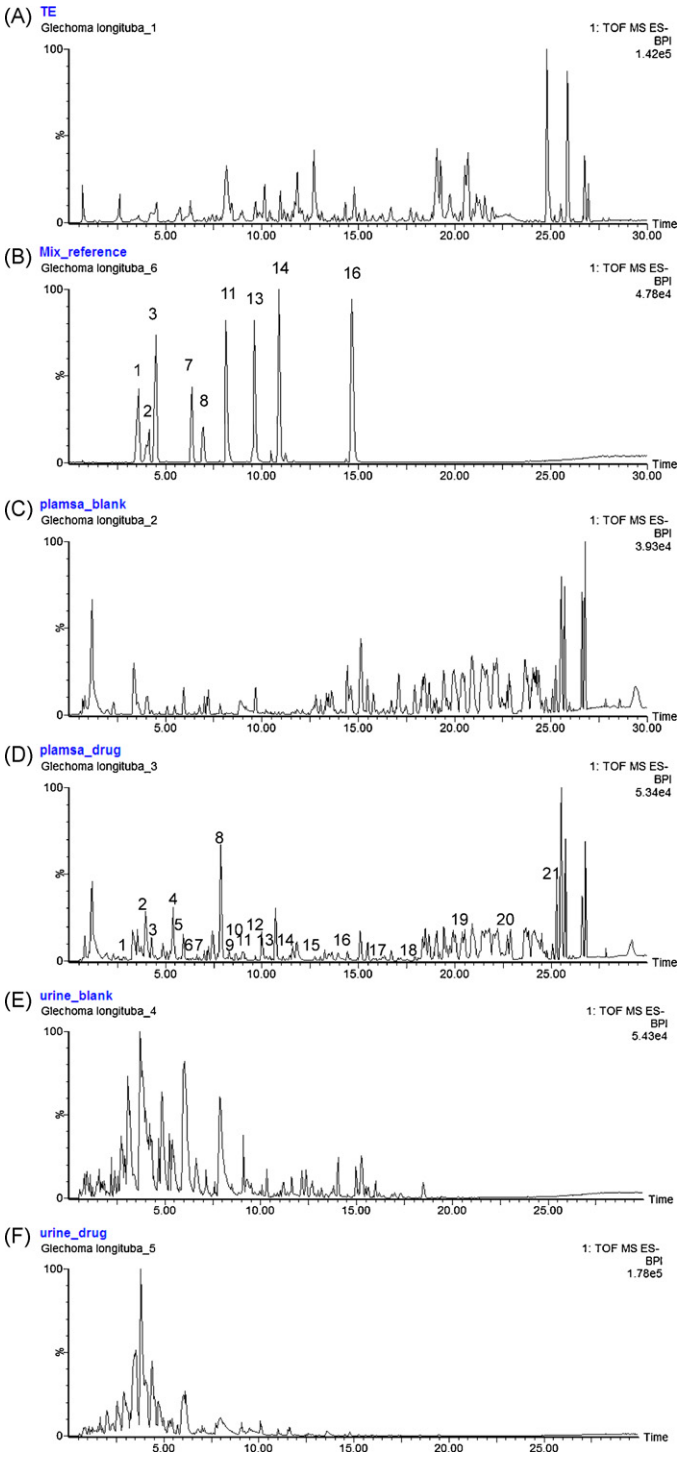

Fig. 2. The base peak intensity (BPI) chromatogram of (A) *G. longituba* extract, (B) standard mixture, (C) blank plasma and (D) plasma after oral administration of *G. longituba* extract, (E) blank urine and (F) urine after oral administration of *G. longituba* extract in negative mode. Peaks: 1, protocatechuic aldehyde; 2, chlorogenic acid; 3, caffeic acid; 7, ferulic acid; 8, rosmarinic acid; 11, luteolin-7-O-glucoside; 13, luteolin; 14, apigenin; 16, acacetin.

Table 1
Phase I and phase II biotransformations and their mass change and fractional mass difference relative to the parent compound.

Phase	Description	Mass change	Fractional mass difference/mDa	Formula change
I	Null	0	0	Parent
	Decarboxylation	−44.9977	2.3	−COOH
	Hydrolysis	18.0106	10.6	$+H_2O$
	Hydroxylation	15.9949	−5.1	+O
	Demethylation + 2 × hydroxylation	17.9742	−25.8	$-CH_2+O_2$
	Reduction	2.0157	15.7	$+H_2$
	Hydroxylation + desaturation	13.9793	−20.7	$+O-H_2$
	Alcohol to ketone	−2.0157	15.7	$-H_2$
	Demethylation	−14.0157	15.7	$-CH_2$
	Hydroxymethylene loss	−30.0106	10.6	$-CH_2O$
	Loss of O	−15.9949	5.1	−O
	2 × hydroxylation	31.9898	−10.2	$+O_2$
	Demethylation to carboxylic acid	29.9742	−25.8	$-CH_2+CO_2$
	Alcohols dehydration	−18.0106	−10.6	$-H_2O$
	Hydroxylation + methylation	30.0106	10.6	$+O+CH_2$
	Demethylation + hydroxylation	1.9793	−20.7	$-CH_2+O$
	Deethylation	−28.0313	−31.3	$-C_2H_4$
	Isopropyl dealkylation	−42.0470	−47.0	$-C_3H_6$
II	Methylation	14.0157	15.7	$+CH_2$
	Acetylation	42.0106	10.6	$+C_2H_2O$
	S-cysteine conjugation	119.0041	4.1	$+C_3H_5NO_2S$
	Glycine conjugation	57.0215	21.5	$+C_2H_3NO$
	Taurine conjugation	107.0041	4.1	$+C_2H_5NO_2S$
	Cysteine conjugation	103.0092	9.2	$+C_3H_5NO_S$
	Glucuronide conjugation	176.0321	32.1	$+C_6H_8O_6$
	Hydroxylation + glucuronide conjugation	192.0270	27.0	$+C_6H_8O_7$
	Sulfate conjugation	79.9568	−43.2	$+SO_3$
	Hydroxylation + sulfation	95.9517	−48.3	$+SO_4$
	Decarboxylation + glucuronidation	148.0372	37.2	$-CO+C_6H_8O_6$

Institute of Laboratory Animal Resources of Jiangsu (Nanjing, China) and the protocols were approved by the Animal Ethics Committee of this institution.

A 100 μL of plasma sample was thoroughly vortexed for 2 min followed by addition of acetonitrile in the ratio of 1:2 (v/v) to Eppendorf tube. After centrifuged at 12,000 × *g* for 15 min at 4 °C, the supernatant was transferred to a clean Eppendorf tube and evaporated to dryness under a stream of nitrogen at 37 °C. The residue was reconstituted in 100 μL of acetonitrile–water (60:40, v/v), the supernatant (5 μL) was directly injected into the UPLC–ESI-QTOF/MS system for analysis after centrifugation at 12,000 × *g* for 15 min. The urine sample was centrifuged at 12,000 × *g* for 15 min at 4 °C, the supernatant was transferred to a clean Eppendorf tube and evaporated to dryness under a stream of nitrogen at 37 °C. The residue was reconstituted in 100 μL of acetonitrile–water (60:40, v/v), the supernatant (5 μL) was directly injected into the UPLC–ESI-QTOF/MS system for analysis after centrifugation at 12,000 × *g* for 15 min.

2.6. Data processing

The mass defect filter (MDF) using MetaboLynx™ (Waters Corp., Milford, MA, USA) for *in vivo* metabolite identification studies processed the accurate mass full-scan raw data that were col-

Table 2
MS data of (−) ESI-MS spectra and the identification results of the constituents that appear in both *G. longituba* extract and the plasma sample.

Peak no.	RT (min) *G. longituba* extract/plasma	Ion mass peaks (*m*/*z*) *G. longituba* extract/plasma	Experimental mass (*m*/*z*)	Compounds identified
1	3.60/3.62	137/137	137.0215	Protocatechuic aldehyde[a]
2	4.15/4.19	353, 191/353, 191	353.0879	Chlorogenic acid[a]
3	4.54/4.47	179, 135/179, 135	179.0345	Caffeic acid[a]
4	5.91/5.87	563, 503, 443, 285/563, 503, 443, 285	563.1403	6-C-arabinose-8-C-glucose-apigenin
5	5.64/5.47	637, 285/637, 285	637.1047	Luteolin-7-diglucuronide
6	6.37/6.30	621, 351, 269/621, 351, 269	621.1114	Apigenin-7-diglucuronide
7	6.76/6.73	193, 149, 134/193, 149, 134	193.0519	Ferulic acid[a]
8	6.96/7.01	447, 285/447, 285	447.0713	Luteolin-7-O-glucoside[a]
9	7.24/7.26	461, 285/461, 285	461.0723	–
10	7.90/7.82	445, 269/445, 269	445.0749	–
11	8.18/8.13	359, 719, 197, 179, 161/359, 197, 179, 169, 161, 135	359.0778	Rosmarinic acid[a]
12	8.99/8.99	343/343	343.0621	–
13	9.69/9.62	285/285	285.0402	Luteolin[a]
14	10.95/10.87	269, 241, 151, 121, 117/269, 241, 151, 121, 117	269.0473	Apigenin[a]
15	12.67/12.59	329/329	329.0945	–
16	14.82/14.66	283, 268, 241, 211/283, 268, 241, 211	283.0597	Acacetin[a]
17	16.70/16.75	383/383	383.0614	–
18	19.04/18.94	293, 275/293, 275	293.0256	–
19	20.64/20.60	295/295	295.0183	–
20	22.78/22.87	555/555	555.0482	–
21	25.41/25.30	253, 237, 225, 198/253, 237, 225, 198	253.1075	Chrysophanol

RT, retention time; UPLC retention times; *m*/*z*, mass-to-charge ratio; accurate mass measurements (error < 5 ppm).
[a] Positively identified via comparison with authentic standards, "–" unknown.

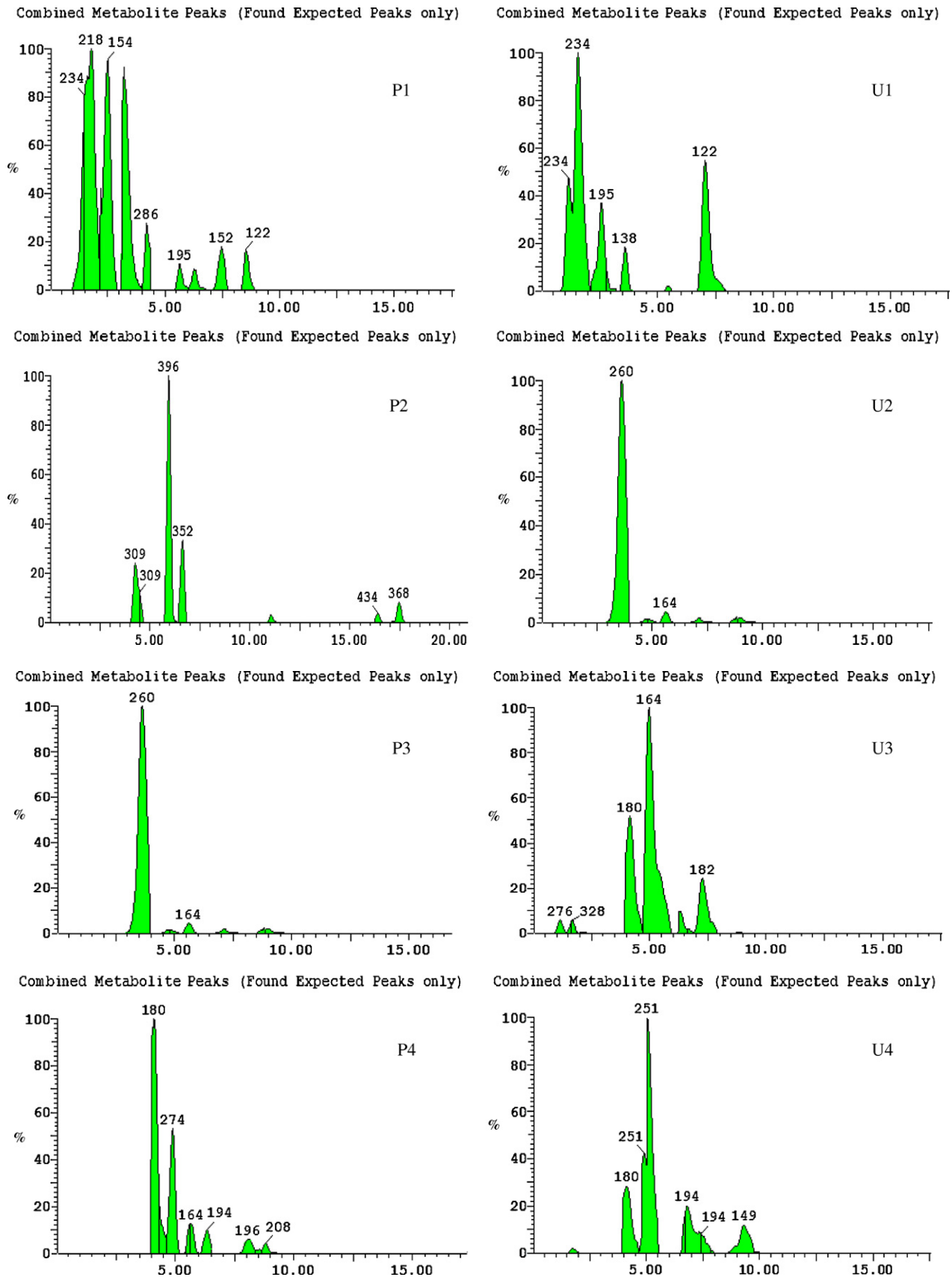

Fig. 3. Summary page from the report provided from an analysis of the metabolites of (P1, U1) protocatechuic aldehyde, (P2, U2) chlorogenic acid, (P3, U3) caffeic acid, (P4, U4) ferulic acid, (P5, U5) rosmarinic acid, (P6) luteolin-7-O-glucoside, (P7, U7) luteolin, (P8, U8) apigenin and (P9, U9) acacetin in rat plasma and urine sample after mass defect filtering. The chromatographic peaks are labeled with the mass of the molecule. U6 was not detected. P, plasma; U, urine.

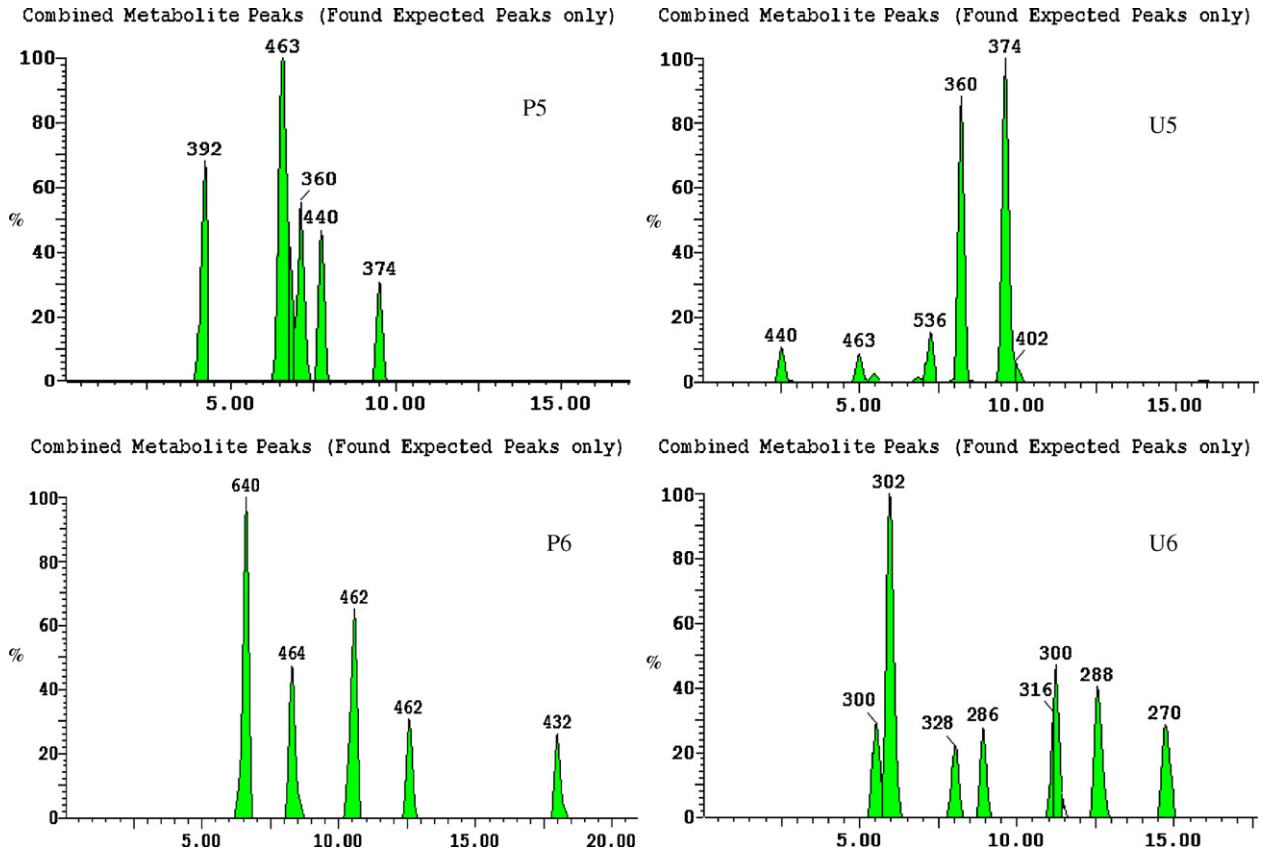

Fig. 3. (*Continued*)

lected through MS^E acquisition. Extracted ion chromatograms for each possible biotransformation (Table 1) were generated within a 5 mDa window of the mass of the test compound $[M-H]^-$ ion and were smoothed twice with a window size of ±2 scans. Peak picking of the smoothed chromatograms was accomplished with the Apex-Track Peak Integration and peaks with an absolute area of less than 1 were eliminated. The MDF window was set to ±0.10 Da around MS fractional mass filtering over a mass range or 100–1000 Da.

3. Results and discussion

3.1. Optimization of UPLC–ESI-QTOF/MS conditions

The acetonitrile–water system showed more powerful separation ability and elutive power for investigated compounds through the comparison of the methanol–water system. When formic acid was added to the mobile phase, the peak capacities and shapes of all the chromatographic peaks were enhanced remarkably. The optimal solvent systems consisting of acetonitrile and 0.1% formic acid aqueous solution, which increased the efficiency of ionization and gave satisfactory sensitivity, were ultimately selected as mobile phase. The selection of the fast UPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within a short time. The compounds of *G. longituba* belong to different chemical families and present different polarities, thus gradient elution was employed. For the ESI-QTOF/MS conditions, ESI in both negative and positive ion modes were tried and the results showed that the best analysis were obtained with the ESI negative ionization mode for getting high responses from all compounds in MS spectra.

3.2. Analysis of the absorption constituents of G. longituba extract

The rapid screening and identification of bioactive compounds in TCM is one of the most important aims of a pharmaceutical discovery process. It is well known that only the compounds successfully absorbed into the blood were thought to have a higher probability of becoming to the effective constituents for most oral administrative Chinese herbal medicines (CHMs). Thus, the purpose of using UPLC–ESI-QTOF/MS technique in this part was to study the absorption of multiple components. The typical chromatograms of the samples were shown in Fig. 2A–D. By a comprehensive analysis of the drug plasma sample, we found 21 peaks existed in both the *G. longituba* extract and the drug plasma sample (Table 2). This indicates that these components could be absorbed via the gastrointestinal tract in rat plasma and could contribute to efficacy of *G. longituba*. In these 21 absorbable compounds, 9 compounds (peaks 1–3, 7, 8, 11, 13, 14 and 16) were attributed to PcA, CgA, CaA, FA, Lut7G, RA, Lut, Api and Aca, respectively by comparing the chromatographic–mass spectrometric analysis of authentic compounds; 4 compounds (peaks 4–6, 21) were identified from several aspects, the analysis of elemental compositions of software MassLynx™ to carefully study their MS spectra of low or high collision energy scan and the comparison of literature data [11–15]. In addition, 8 compounds (peaks 9, 10, 12, 15, 17–20) were unknown and need further research. The other peaks, which were found to exist only in the drug plasma sample, would be the possible metabolites of the chemicals in the *G. longituba* extract and the endogenous metabolites owing to the pharmacological effects of the compounds present in the drug. The structure identification of those potential metabolites of unambiguous compounds was described as followed.

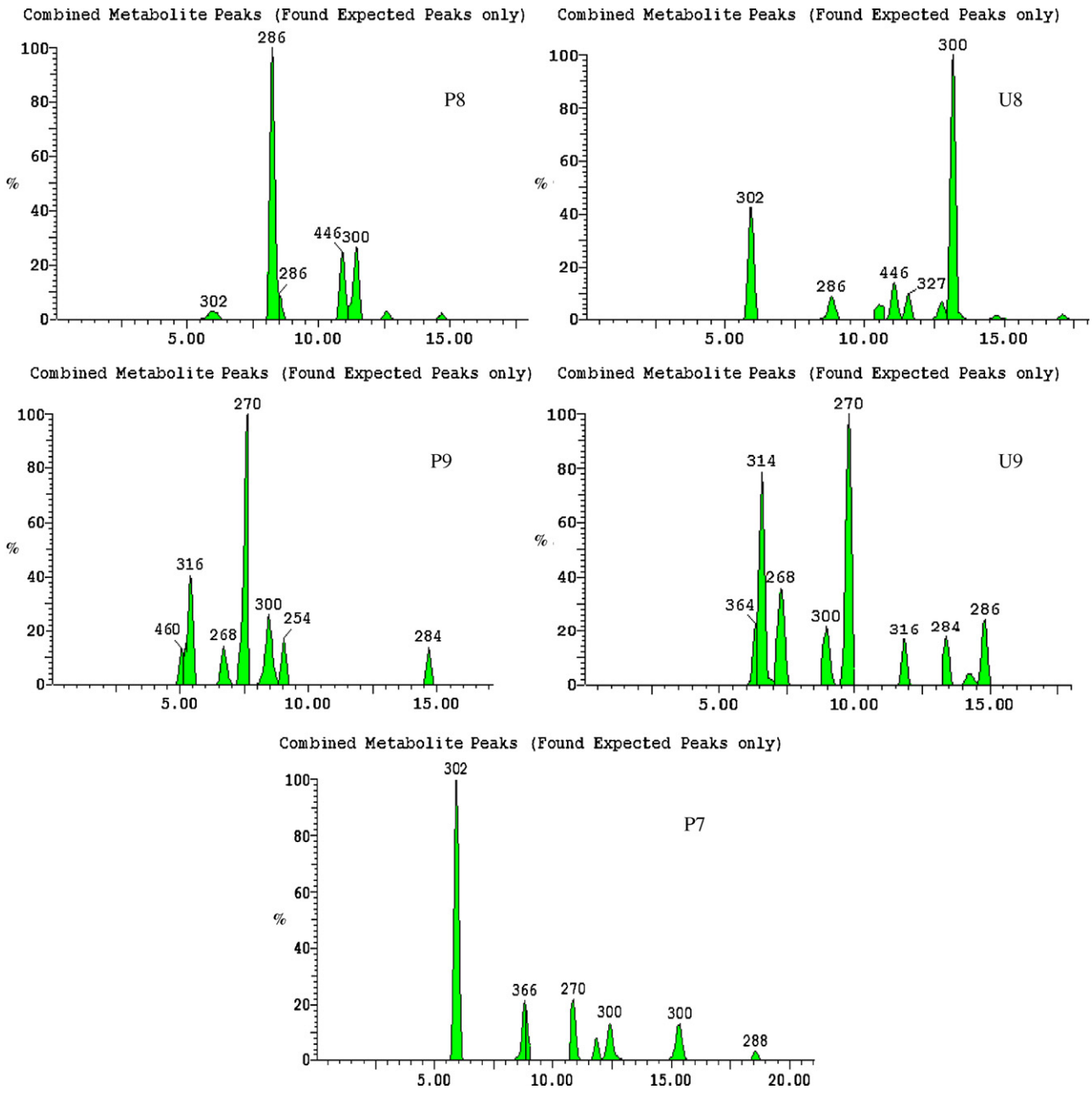

Fig. 3. (*Continued*).

3.3. *Identification of the metabolites of G. longituba extract in rat plasma and urine*

Metabolite identification plays an important and critical role in drug metabolism studies. In recent years, metabolism studies of CHMs have attracted significant attention, as they demonstrate distinctly drug efficacy, and enable the screening of bioactive components, the identification of potentially toxic metabolites, the elucidation of herb–drug interactions and the detection of drug degradation pathways [16–20].

In this study, the MS^E approach that we call 'All-in-One' analysis was applied to identify metabolite for *in vivo* samples. This type of experiment leverages the fast scanning and high mass accuracy of QTOF/MS instruments. Full-scan MS and MS/MS data is acquired from a single injection using collision energy switching with no preconceptions on the likely routes of metabolism, either manually or in a data-dependent manner, of precursor ions. The acquisition of MS/MS data is assisted by the use of UPLC. Therefore, the MS^E experiment can enhance obviously laboratorial work efficiency by associating with "chemistry aptitude" of MassLynx^{TM} informatics combination.

Furthermore, when two LC–MS post-acquisition data files were performed using MetaboLynx^{TM}, peaks present in the analyte was evaluated relative to the control samples by comparing their retention times, MS spectra and peak areas. When a peak was included as a metabolite, which could be attributed to transformation processes, the peak with an absolute area in the analyte had to be less than 1 greater than that of the control. The simplest generic approach to metabolite identification is the acquisition of full-scan LC/MS data, followed by inspection of appropriate extracted ion chromatograms (XICs) with automated software algorithms for putative drug metabolic transformations.

In order to identify the metabolites *in vivo*, probable structures were speculated in accordance with the metabolism rule of drugs.

Table 3
Summary of *G. longituba* extract metabolites identification in rat plasma and urine using MS^E with mass defect filtering.

No.	RT (min)	$[M-H]^-$	Metabolite name	Formula	MS/MS fragment ions	Source	Parent drug
M1	1.47	232.9793	Hydroxylation + sulfation	$C_7H_6O_7S$	137.0253 [M−H–SO_4]	Plasma, urine	PcA
M2	1.80	216.9835	Sulfation conjugation	$C_7H_6O_6S$	137.0569 [M−H–SO_3]	Plasma, urine	PcA
M3	2.52	153.0187	Hydroxylation	$C_7H_6O_4$	137.0244 [M−H–O]	Plasma, urine	PcA
M4	4.20	285.0591	Decarboxylation + glucuronidation	$C_{12}H_{14}O_8$	109.0300 [M−H–GlcUA]	Plasma	PcA
M5	7.25	139.0405	Reduction[a]	$C_7H_6O_3$		Urine	PcA
M6	7.49	151.0438	Methylation	$C_8H_8O_3$	137.0614 [M−H–CH_2]	Plasma, urine	PcA
M7	8.55	121.0313	Loss of oxygen[a]	$C_7H_6O_2$		Plasma, urine	PcA
M8	1.72	385.0769	2 × hydroxylation	$C_{16}H_{18}O_{11}$	223	Urine	CgA
M9	4.22	309.0974	Decarboxylation	$C_{15}H_{17}O_7$	147	Plasma, urine	CgA
M10	4.32	369.0865	Hydroxylation	$C_{16}H_{18}O_{10}$	307	Plasma	CgA
M11	4.51	449.0358	Hydroxylation + sulfation	$C_{16}H_{18}O_{13}S$	353.0143 [M−H–SO_4]	Plasma	CgA
M12	6.61	351.0707	Alcohol to ketone	$C_{16}H_{16}O_9$	189	Plasma, urine	CgA
M13	7.43	367.0641	Hydroxylation + desaturation	$C_{16}H_{16}O_{10}$	191	Urine	CgA
M14	7.64	410.1097	Glycine conjugation	$C_{18}H_{21}NO_{10}$	353.0143 [M−H–Gly]	Urine	CgA
M15	8.57	383.1021	Hydroxylation + methylation	$C_{17}H_{20}O_{10}$	369.0529 [M−H–CH_2]	Urine	CgA
M16	11.03	355.0797	Alcohols dehydration[a]	$C_{16}H_{16}O_{10}$		Plasma	CgA
M17	16.44	433.0338	Sulfate conjugation	$C_{16}H_{18}O_{12}S$	353.0319 [M−H–SO_3]	Plasma, urine	CgA
M18	17.44	367.1001	Methylation	$C_{17}H_{20}O_9$	353.0649 [M−H–CH_2]	Plasma, urine	CgA
M19	1.14	274.9884	Hydroxylation + sulfation	$C_9H_8O_8S$	179.0340 [M−H–SO_4]	Urine	CaA
M20	2.15	211.0279	2 × hydroxylation	$C_9H_8O_6$	267	Urine	CaA
M21	3.56	355.0667	Glucuronide conjugation	$C_{15}H_{16}O_{10}$	179.0348 [M−H–GlcUA]	Plasma	CaA
M22	3.71	258.9891	Sulfate conjugation	$C_9H_8O_7S$	179.0342 [M−H–SO_4]	Plasma, urine	CaA
M23	4.67	327.0707	Decarboxylation + glucuronidation	$C_{14}H_{16}O_9$	151.0448 [M−H–GlcUA]	Plasma, urine	CaA
M24	4.76	282.0479	Cysteine conjugation	$C_{12}H_{13}NO_5S$	179.0236 [M−H–Gly]	Plasma, urine	CaA
M25	5.59	163.0417	Loss of oxygen	$C_9H_8O_3$	129	Plasma, urine	CaA
M26	6.69	209.0458	Hydroxylation + methylation	$C_{10}H_{10}O_5$	195.0671 [M−H–CH_2]	Urine	CaA
M27	7.08	181.0543	Reduction	$C_9H_{10}O_4$	137	Plasma, urine	CaA
M28	8.70	134.0382	Decarboxylation[a]	$C_8H_7O_2$		Plasma, urine	CaA
M29	8.98	193.0521	Methylation	$C_{10}H_{10}O_4$	179.03139 [M−H–CH_2]	Plasma, urine	CaA
M30	1.72	385.0769	Hydroxylation + glucuronide conjugation	$C_{16}H_{18}O_{11}$	209.0482 [M−H–GlcUA]	Urine	FA
M31	4.09	179.0361	Demethylation	$C_9H_8O_4$	135	Plasma, urine	FA
M32	4.32	369.0865	Glucuronide conjugation	$C_{16}H_{18}O_{10}$	193.0514 [M−H–GlcUA]	Plasma	FA
M33	4.89	250.0741	Glycine conjugation	$C_{12}H_{13}NO_5$	193.0192 [M−H–Cly]	Urine	FA
M34	4.90	273.0100	Sulfate conjugation	$C_{10}H_{10}O_7S$	193.0522 [M−H–SO_3]	Plasma, urine	FA
M35	5.57	177.0569	Loss of oxygen	$C_{10}H_{10}O_3$	133	Plasma	FA
M36	5.65	163.0400	Hydroxymethylene loss	$C_9H_8O_3$	119	Plasma, urine	FA
M37	6.69	209.0458	Hydroxylation	$C_{10}H_{10}O_5$	165	Urine	FA
M38	7.39	296.0614	Cysteine conjugation	$C_{13}H_{15}NO_5S$	193.0009 [M−H–Cys]	Urine	FA
M39	8.02	195.0651	Reduction	$C_{10}H_{12}O_4$	151, 136	Plasma, urine	FA
M40	8.58	148.0555	Decarboxylation[a]	$C_9H_9O_2$		Plasma, urine	FA
M41	8.81	207.0685	Methylation	$C_{11}H_{12}O_4$	193.0544 [M−H–CH_2]	Plasma, urine	FA
M42	4.20	391.0680	2 × hydroxylation + methylation	$C_{19}H_{18}O_9$	377.0486 [M−H–CH_2]	Plasma	RA
M43	5.46	357.0634	Alcohol to ketone[a]	$C_{18}H_{14}O_8$		Urine	RA
M44	6.55	462.0841	Cysteine conjugation	$C_{21}H_{21}NO_9S$	359.0357 [M−H–Cys]	Plasma, urine	RA
M45	6.79	535.1089	Glucuronide conjugation	$C_{24}H_{24}O_{14}$	359.0305 [M−H–GlcUA]	Plasma, urine	RA
M46	7.77	439.0353	Sulfate conjugation	$C_{18}H_{16}O_{11}S$	359.0495 [M−H–SO_3]	Plasma, urine	RA
M47	9.53	373.0940	Methylation	$C_{19}H_{18}O_8$	359.0299 [M−H–CH_2]	Plasma, urine	RA
M48	15.88	314.0806	Decarboxylation	$C_{17}H_{15}O_6$	179, 152	Urine	RA
M49	6.61	639.1235	Hydroxylation + glucuronide conjugation	$C_{27}H_{28}O_{18}$	463.0601 [M−H–GlcUA] 301.0782 [M−H–GlcUA–Glc]	Plasma	Lut7G
M50	8.29	463.0841	Hydroxylation	$C_{21}H_{20}O_{12}$	285.0533 [M−H–Glc–O]	Plasma	Lut7G
M51	10.41	461.1089	Methylation	$C_{22}H_{22}O_{11}$	447.0486 [M−H–CH_2] 285.0854 [M–H–CH_2–Glc]	Plasma	Lut7G
M52	12.57	461.0758	Hydroxylation + desaturation	$C_{21}H_{18}O_{12}$	299.0636 [M−H–Glc]	Plasma	Lut7G
M53	17.99	431.0965	Loss of oxygen	$C_{21}H_{20}O_{10}$	269.0449 [M−H–Glc]	Plasma	Lut7G
M54	5.49	299.0213	Hydroxylation + desaturation	$C_{15}H_{12}O_6$	285.0464 [M−H–O]	Plasma, urine	Lut
M55	6.04	301.0384	Hydroxylation	$C_{15}H_{10}O_7$	285.0041 [M−H–O]	Plasma, urine	Lut
M56	8.80	365.0013	Sulfate conjugation	$C_{15}H_{10}O_9S$	285.0250 [M−H–SO_3]	Urine	Lut
M57	11.13	315.0554	Hydroxylation + methylation	$C_{16}H_{12}O_7$	301.0349 [M−H–CH_2]	Plasma, urine	Lut
M58	11.24	299.0582	Methylation	$C_{16}H_{12}O_6$	285.0540 [M−H–CH_2]	Plasma, urine	Lut
M59	12.53	287.0557	Reduction[a]	$C_{15}H_{12}O_6$		Plasma, urine	Lut
M60	12.57	461.0758	Glucuronide conjugation	$C_{21}H_{18}O_{12}$	285.0456 [M−H–GlcUA]	Plasma	Lut
M61	14.73	269.0444	Loss of oxygen[a]	$C_{15}H_{10}O_5$		Plasma, urine	Lut
M62	6.40	301.0384	2 × hydroxylation	$C_{15}H_{10}O_7$	273	Plasma, urine	Api
M63	8.23	285.0441	Hydroxylation	$C_{15}H_{10}O_6$	257	Plasma, urine	Api
M64	8.80	365.0013	Hydroxylation + sulfation	$C_{15}H_{10}O_9S$	269.0840 [M−H–SO_4]	Urine	Api
M65	10.91	445.0779	Glucuronide conjugation	$C_{21}H_{18}O_{11}$	269.0648 [M−H–GlcUA], 241	Plasma, urine	Api
M66	11.24	299.0582	Hydroxylation + methylation	$C_{16}H_{12}O_6$	285.0540 [M−H–CH_2]	Plasma, urine	Api
M67	12.57	461.0758	Hydroxylation + glucuronide conjugation	$C_{21}H_{18}O_{12}$	285.0456 [M−H–GlcUA]	Plasma	Api
M68	14.66	283.0605	Methylation	$C_{16}H_{12}O_5$	269.0462 [M−H–CH_2]	Plasma, urine	Api
M69	14.68	253.0517	Loss of oxygen	$C_{15}H_{10}O_4$	225	Urine	Api
M70	17.09	271.0618	Reduction	$C_{15}H_{12}O_5$	243	Urine	Api
M71	5.03	459.0928	Glucuronide conjugation	$C_{22}H_{20}O_{11}$	283.0876 [M−H–GlcUA]	Plasma	Aca
M72	5.38	315.0520	2 × hydroxylation	$C_{22}H_{10}O_7$	300, 273	Plasma, urine	Aca

Table 3 *(Continued)*

No.	RT (min)	$[M-H]^-$	Metabolite name	Formula	MS/MS fragment ions	Source	Parent drug
M73	6.32	363.0149	Sulfate conjugation	$C_{16}H_{12}O_8S$	283.0533 $[M-H-SO_3]$	Urine	Aca
M74	6.59	313.0738	Methylation + hydroxylation	$C_{17}H_{14}O_6$	299.0494 $[M-H-CH_2]$	Urine	Aca
M75	6.69	267.0627	Loss of oxygen	$C_{16}H_{12}O_4$	252	Plasma, urine	Aca
M76	7.64	269.0465	Demethylation	$C_{15}H_{10}O_5$	241	Plasma, urine	Aca
M77	8.47	299.0547	Hydroxylation	$C_{16}H_{12}O_6$	284, 257	Plasma, urine	Aca
M78	9.06	253.0541	Hydroxymethylene loss	$C_{15}H_{10}O_4$	226	Plasma	Aca
M79	14.17	297.0781	Methylation	$C_{17}H_{14}O_5$	283.0653 $[M-H-CH_2]$	Urine	Aca
M80	14.82	285.0742	Reduction	$C_{16}H_{14}O_5$	270, 243	Urine	Aca

PcA: protocatechuic aldehyde; CgA: chlorogenic acid; CaA: caffeic acid; FA: ferulic acid; RA: rosmarinic acid; Lut7G: luteolin-7-O-glucoside; Lut: luteolin; Api: apigenin; Aca: acacetin; RT: retention time; $[M-H]^-$: molecular ion; *m/z*: mass-to-charge ratio; GlcUA: glucuronide conjugation; Glc: glucose; Cys: cysteine conjugation; Gly: glycine conjugation. UPLC retention times, negative ion MS/MS fragmentation patterns, and identification of metabolites isolated from rat plasma and urine after oral ingestion of *G. longituba* extract. The 80 metabolites were detected in all of the plasma and urine samples from five rats. Data are representative for MS/MS fragmentation of the metabolite. Accurate mass measurements (error < 5 ppm).

[a] No MS/MS fragmentation ions for confirmation of metabolites.

Then based on comparison of their retention times, mass difference and spectral patterns of product ions for those parent drug, these probable metabolites were screened by UPLC–ESI-QTOF/MS with MS^E data acquisition and MDF processed data method (error routinely within 5 mDa). Current accurate mass UPLC–QTOF/MS technology can resolve metabolite ions and isobaric interferences when they are separated by approximately 50 mDa or more in a mass range of 100–1000 Da. Meanwhile, mass defects of phase I and phase II metabolites typically fall within 50 mDa relative to that of the parent drug. The capability of the MDF approaches to remove endogenous interferences from more complex biological matrices. In a previous study, the metabolism of some compound had been reported by this multiple combination technique method [21,22], while there has been no fully integrated study of the metabolite of multiple components in CHMs.

Metabolite identification has been greatly improved by the availability of modern mass spectrometers that are capable of providing high resolution (15,000 at full width at half maximum) and accurate mass (error < 5 ppm). Based on the drug sample in comparison with the blank sample, 9 compounds including 5 phenolic acids and 4 flavonoids were identified unambiguously, which were all parent drugs. The chromatograms of the samples in negative ESI mode were listed in Fig. 2. Furthermore, after being processed with MDF, their possible metabolites were detected in combination with related literature data or METLIN's metabolite mass spectral database [23–29] and showed in Table 3. Fig. 3 is a reproduction of the summary of the report provided for an analysis of the metabolites of 9 compounds in rat plasma and urine sample after mass defect filtering. The number of entries in the expected metabolite list is correspondingly large, since even minor metabolites have a sufficiently large peak area to be included for review. The summary table lists the potential metabolites detected, and composite extracted ion chromatograms for each type of metabolite are presented at the figure. All 80 metabolites were detected including sulfate, glucuronide, and glycine conjugates, hydroxylated, methylated and loss of oxygen. Fifty-four metabolites were from the rat plasma and 57 metabolites were from the rat urine, while 41 metabolites were detected in both rat plasma and urine. For most of the constituents $[M-H]^-$ was observed in the (−) ESI-MS spectra from MS^E data. The corresponding fragment ion were obviously identified such as $[M-H+16]^-$, $[M-H+14]^-$, $[M-H-16]^-$, $[M-H-80]^-$, $[M-H-176]^-$ and $[M-H-57]^-$ in MS spectra of high collision energy scan.

Moreover, the characteristic fragment peaks of the parent related to the metabolite were observed. For example, the high collision energy scan fragment ions of M45 (the metabolite of rosmarinic acid) at *m/z* 359 $[M-H-GlcUA]^-$ (loss of a glucuronide group), 197 $[M-H-GlcUA-179+H_2O]^-$ (loss of a caffeic acid group), 179 $[M-H-GlcUA-197+H_2O]^-$ (loss of a propanoic acid group) and 161 $[M-H-GlcUA-197-H_2O]^-$ were observed; the high collision energy scan fragment ions of M34 (the metabolite of ferulic acid) at *m/z* 193 $[M-H-80]^-$ (loss of a sulfate group), 149 $[M-H-80-CO_2]^-$ and 134 $[M-H-80-CO_2-CH_3]^-$ were observed; the high collision energy scan fragment ions of M79 (the metabolite of Aca) at *m/z* 283 $[M-H-CH_3]^-$, 268 $[M-H-2CH_3]^-$ and 241 $[M-H-2CH_3-CO]^-$ were observed; specific retro-Diels-Alder (RDA) fragments of M65 were produced such as *m/z* 269 $[M-H-GlcUA]^-$, 241 $[M-H-CO]^-$, 151 $[A_1]^-$, 117 $[B_1]^-$ and 121 $[B_2]^-$ from the metabolites of apigenin the high collision energy scan spectrum. However, the metabolite of Lut7G was not detected in the rat urine.

Meanwhile, many of the dehydroxylations/reductions occurred in plasma and urine were likely to be due to the metabolic activity of gut microflora. Some compounds such as PcA, CaA, FA, Lut, Api and Aca could be metabolized by the gut microflora in the rat intestine and their metabolites were absorbed into the blood or excreted in urine in the form of dehydroxylations/reductions. They are helpful to study on the absorption/excretion and metabolism of the compounds in the intestine explored further using experimental animal. The summary page obtained for the aforementioned phenolic acids and flavonoids potential metabolites detected were reviewed. This type of batch processing is particularly amenable to a drug metabolism and pharmacokinetic screening environment.

The majority of the metabolites of these phenolic acids and flavonoids were present in rat plasma and urine as hydroxylated, methylated, glucuronided and sulfated conjugates. Furthermore, the results described in our paper can also provide useful information concerning our own research on a new pharmacological activity of *G. longituba*.

4. Conclusions

The strategy utilizes of MDF in combination with UPLC–MS^E is a powerful approach for *in vivo* metabolite identification studies and requires minimal operator intervention, yet afford high-quality structural information in a timely fashion. The MDF technique can be widely used to analyze drug metabolites in biological material using the production mass spectrum of the parent drug as a substructure template even without standards of metabolites. With MS^E technique, the collision energy within the collision cell is switched between a low and high level in alternate scans to acquire a direct MS/MS data without the need for sample re-injection, resulting in two separate MS TIC channels. The recent developments of QTOF instruments including improvements in sensitivity, enhanced duty cycle and dynamic range (over 10^4), and the introduction of collision energy profiles for non-selective MS/MS, will likely improve the utility of this technique still further.

Through the comprehensive analysis of the absorption and metabolite components contained *in vivo* at certain time points, our present research provides the preliminary profile of the absorption

and metabolism of *G. longituba* extract *in vivo*. Twenty-one parent constituents absorbed into rat blood after oral administration of *G. longituba* extract were identified and all of 80 metabolites of the parent compounds such as PcA, CgA, CaA, FA, Lut7G, RA, Lut, Api and Aca were tentatively detected in rat plasma and urine.

In addition, the proposed method could develop an integrated template approach to analyse screening and identification of the bioactive components in plasma and urine after oral administration of CHMs. This investigation provided helpful chemical information for further pharmacology and active mechanism research on TCM.

Acknowledgements

This work was supported by the research project of the Chinese pharmacopoeia 2010 about the Quality Standard Research of *G. longituba* (NO.YS-133) from Chinese Pharmacopoeia Commission. We are grateful to Pro. Yuping Tang, Dr. Shulan Su and Dr. Sheng Guo for their kind assistance.

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