Journal of Chromatography B, 907 (2012) 146–153

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Metabolite discovery of helicidum in rat urine with XCMS based on the data of ultra performance liquid chromatography coupled to time-of-flight mass spectrometry

Qingfei Liu[a,*], Yun Shi[b], Tuo Guo[c], Yong Wang[a], Wenjuan Cong[d], Jingjie Zhu[a]

[a] School of Medicine, Tsinghua University, Beijing 100084, China
[b] The First Affiliated Hospital, Guangzhou University of TCM, Guangzhou 510405, China
[c] Computer Department, Guilin University of Electronic Technology, Guilin 541004, China
[d] Engineering Research Center of Modern Preparation Technology of TCM, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 13 June 2012
Accepted 16 September 2012
Available online 20 September 2012

Keywords:
Helicidum
Metabolites
Urine
UPLC/TOF MS
XCMS

ABSTRACT

The present study demonstrates the use of XCMS (various forms (X) of chromatography coupled to mass spectrometry), an open-source software tool primarily used in bioinformatics, on the data of ultra-performance liquid chromatography connected online with a mass spectrometer (UPLC/MS) for the discovery of the metabolites of helicidum in urine after oral single dosage to rats. Helicidum (formaldehydephenyl-O-β-D-pyranosyl alloside) is the major active component of the fruits of *Helicid hilagirica Beed*. In China, it is often used in the clinic to treat neurasthenic syndromes, vascular headache, and trigeminal neuralgia with high efficacy and low side effect and toxicity. The urine samples of five rats were collected during 0–4, 4–8, 8–12, 12–16, 16–20, 20–24, 24–32, 32–40, and 40–48 h, respectively, after oral administration of helicidum at a dosage of 25.0 mg/kg. A UPLC coupled to time-of-flight MS (UPLC/TOF MS) was used to analyze the samples. Concerning XCMS, the ".raw" format files were preliminarily converted to the open mzXML format using massWolf-4.3.1 (http://sourceforge.net/projects/sashimi/files/massWolf%20(MassLynx%20converter)/). For converting lots of files a time, we wrote a tool rawTomzXML which also uses massWolf-4.3.1. The data were processed using XCMS version l.26.0 (http://www.bioconductor.org/packages/2.8/bioc/html/xcms.html) running under R version 2.13 (http://http://www.r-project.org/) which provided the running platform for XCMS. The "centWave" method from XCMS was used for chromatographic peak detection. Based on the *m*/*z* data of the metabolites obtained by XCMS, MS was used to identify the molecular formula. Nine metabolites were finally found and identified. For six of them, the bio-transformation mechanisms of the parent compound was elucidated: glucuronide conjugation ($C_{19}H_{24}O_{14}$), reduction ($C_{13}H_{18}O_7$), oxidation ($C_{13}H_{16}O_8$), methylation ($C_{14}H_{18}O_7$), and the mixed transformation of reduction, methylation, and acetylation ($C_{16}H_{22}O_8$). For the other three metabolites, $C_{11}H_{19}N_3O_9$, $C_{11}H_{21}N_3O_9$, and $C_{14}H_{15}NO_7$, the bio-transformation mechanisms remain unknown and need further investigation. Calculated as mass of helicidum, the cumulative urine excretion rate of the metabolites was 8.39%. The amount of oxidized helicidum was more than 50% among the metabolites while the parent compound helicidum was 13.28% and the reduced helicidum 11.72%, indicating that oxidation was the major bio-transformation that occurred *in vivo*.

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1. Introduction

Helicidum (or helicid), formaldehydephenyl-O-β-D-pyranosyl alloside, is the major active component of the fruits of *Helicid hilagirica Beed* [1]. The chemical structure of helicidum is shown in Fig. 1. In China, it is often used in the clinic to treat neurasthenic syndromes, vascular headache, and trigeminal neuralgia [2–5]. We have reported the major metabolites of helicidum in plasma after oral administration to Beagle dogs with high performance liquid chromatography/ultra-violet/electrospray ionization-ion trap mass spectrometry (HPLC/UV/ESI-ITMS). Two metabolites of helicidum were identified and determined, formylphenyl-O-β-D-pyranosyl alloside and hydroxylmethylphenyl-O-β-D-pyranosyl alloside, with the bio-transformations of oxidation and reduction, respectively [6]. Jia et al. determined helicidum in rat biosamples

* Corresponding author. Tel.: +86 10 62797450; fax: +86 10 62797450.
E-mail addresses: liuqf@biomed.tsinghua.edu.cn, qingfeiliu@sina.com.cn (Q. Liu).

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http://dx.doi.org/10.1016/j.jchromb.2012.09.029

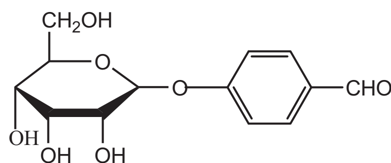

Fig. 1. The chemical structure of helicidum.

such as plasma, lung, kidney, and brain by liquid chromatography electro-spray ionization mass spectrometry [7,8], however no metabolites were reported. It has been well known that the *in vivo* biochemical transformation of the drug is complicated and various transformations occur such as oxidation, reduction, methylation, glucuronide conjugation, and sulfation, leading to multiple metabolites [9,10]. Some metabolites even have bioactivities or toxicities. For example, calcium oxalate monohydrate, a metabolite of ethylene glycol, is toxic for renal mitochondrial function [11]. It is therefore highly significant for drug development, dosage form design, and clinical application, to investigate the metabolites so as to know the *in vivo* fate of the drug.

Ultra performance liquid chromatography coupled with mass spectrometry (UPLC/MS) is a recently developed analysis technology, which can provide a higher peak capacity, increased sensitivity, and higher speed of analysis compared with conventional HPLC. Additionally, UPLC/MS can provide more information on molecular formulas, chemical structures, and contents of compounds. It has been widely used in environmental monitoring, food detection, toxic drug assays, botanic and biological sample analysis, *etc.* [12–20].

For LC/MS data analysis, much of the software currently available is either proprietary or restricted to a particular vendor's instruments. By contrast, XCMS (various forms (X) of chromatography coupled to mass spectrometry), a software tool used in bioinformatics and especially the metabolomics, is an open-source tool developed by the Scripps Research Institute (USA) for locating biomarkers in LC/MS data. It has been designed to be independent of instrument vendor. That enables identical data analysis to be undertaken and repeated using instruments from a variety of manufacturers. While other programs include tools for peak identification and visualization, they may be designed in a very rigid manner that does not allow automated analysis of many samples [21].

The novel advantage of XCMS is that it incorporates nonlinear retention time alignment, peak detection, and peak matching which permits the comprehensive discovery of chromatographic peaks that may be markers for a particular condition [22–24]. Based on the LC/MS data sets from an enzyme knockout study and a large-scale analysis of plasma samples, XCMS, without using internal standards, could dynamically identify hundreds of endogenous metabolites for use as standards, calculating a nonlinear retention time correction profile for each sample. Following retention time correction, the relative metabolite ion intensities were directly compared to identify changes in specific endogenous metabolites, such as potential biomarkers [21]. In addition, XCMS can be used for non-metabolomic work, for example, to look for LC/MS peaks that may be markers for associating or differentiating various dichlor samples [25].

Based on the advantages of XCMS and UPLC/MS mentioned above, the present paper therefore used them to find, identify, and quantify the metabolites of helicidum in rat urine after oral administration of helicidum to get more information about the bio-transformations of helicidum that occurred *in vivo*. The general strategy of the preprocessing and analysis of LC/MS data is shown in Fig. 2. The samples were separated and detected by UPLC/MS. The data were then processed with XCMS, including de-noising, baseline correction, peak detection, deconvolution, retention time correction, and alignment. Based on the result of statistical analysis, m/z data of peaks were found corresponding to the metabolites. With MS, the molecular formulae of these peaks were identified and the bio-transformations could be deduced.

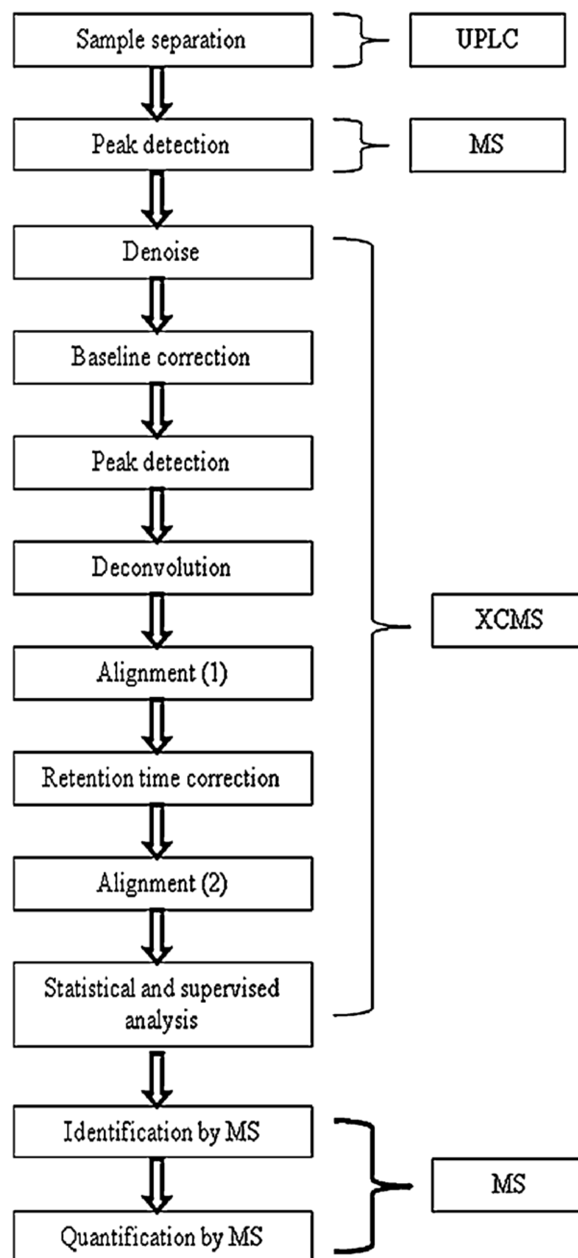

Fig. 2. General strategy of the preprocessing and analysis of LC/MS data.

2. Experimental

2.1. Chemicals and reagents

Helicidum was purchased from Kunming Institute of Botany, the Chinese Academy Sciences (Yunnan, China). Helicidum standard (99% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile was of HPLC grade (Fisher, USA). HPLC grade water was obtained using a water purification system (Milli-Q Reagent Water System, MA, USA). All the other chemicals were of analytical

reagent grade. All mobile phases were filtered through 0.22 μm micropore filters (Jinteng Ltd., Tianjin, China) prior to use.

2.2. Equipment and chromatographic conditions

The UPLC/MS system "LCT Premier™ XE" comprised a Waters UPLC system connected inline with a time of flight mass spectrometer (TOF MS) (Waters Ltd., USA). MassLynx™ software (version 4.1, Waters, Milford, MA, USA) was used to control the instruments and for data acquisition and processing. The column utilized was an Acquity BEH C_{18} (100 × 2.1 mm, 1.7 μm), maintained at 50 °C. Mobile phases were A (0.2% formic acid) and B (acetonitrile). LC gradient conditions were as follows: 0–5 min: 5 → 50% (B); 5–7 min: 50 → 95% (B); 7–8 min: 95 → 95% (B); 8–10 min: 95 → 5% (B); 10–15 min: 5 → 5% (B). The flow rate was set at 0.5 mL/min. Samples were maintained at 4 °C throughout.

Analytes were quantified using TOF MS under negative-ion mode. Source parameters included capillary voltage of 2.2 kV, cone voltage of 35 V, source temperature of 120 °C, desolvation temperature of 350 °C, cone gas flow of 10 L/h, and desolvation gas flow of 700 L/h. Product ions were monitored in W^- extended mode. In the full scan mode, the mass spectrometer was operated over a range of *m*/*z* 50–1000. Injection volumes for samples and standards were 4 μL with needle overfill.

2.3. Animals

Five male Sprague-Dawley (SD) rats, body weight of 250–270 g, were purchased from Beijing Mars Biotechnology Co., Ltd. (Beijing, China), housed and cared for under a constant temperature (22 ± 1 °C) and humidity (50 ± 10%). Food was withheld for 1 day before the experiment, while water was freely available. The entire experiment was performed in accordance with the Regulations of the Animal Ethical Committee of Tsinghua University.

2.4. Administration and preparation of urine samples

Helicidum was dispersed in deionized water at 5.0 mg/mL by sonication (KQB-100, Kunshan Supersonic Instrument Co., Kunshan, China). Each rat was orally administrated the solution as 25.0 mg helicidum per kg body weight. After administration, food was withheld for 48 h for all rats while water was freely available. The whole urine of each rat was collected during 0–4, 4–8, 8–12, 12–16, 16–20, 20–24, 24–32, 32–40, 40–48 h, respectively, and the volume was assayed. Blank urine was collected before the administration of helicidum.

Urine samples were centrifuged at 4500 rpm for 10 min at 4 °C (TGL 16C, Medical centrifuge Co., Beijing, China). 100 μL of the upper layer was mixed with 400 μL of methanol by vortexing for 1 min, and then centrifuged at 10,000 rpm for 15 min at 4 °C. The upper layer was transferred to another tube and mixed with 500 μL of water. The mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. 4 μL of the supernatant was injected into the UPLC system for analysis.

2.5. Standard solution, calibration curves, and quality control samples

A standard stock solution (1.0 mg/mL) was prepared by dissolving 10.0 mg of helicidum in 10.0 mL of methanol. A series of standard working solutions with low concentration range of 0.125, 0.25, 0.500, 1.00, 2.50, and 5.00 μg/mL, and high concentration range of 5.00, 10.0, 25.0, 50.0, 75.0, and 100 μg/mL were obtained by further dilution of the standard stock solution with methanol. All solutions were stored at 4 °C. A series of 200 μL standard working solutions were evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residues were reconstituted in 20 μL of blank urine, 80 μL methanol, and 100 μL water in the sequence. The mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. 4 μL of the supernatant was injected into the UPLC system for analysis. Linearity was demonstrated by statistical analysis of a linear regression model: $y = ax + b$, where x represents the calculated concentration of helicidum and y represents the peak area of helicidum. In order to semi-qualify the metabolite of helicidum, the concentration of helicidum in the regression equation is expressed with unit of μmol/mL (for helicidum, 1 μmol/mL is equal to 284.09 μg/mL because of its molecular weight of 284.0896).

Quality control (QC) samples were prepared in the same way with concentrations of 0.125, 5.00, and 100 μg/mL.

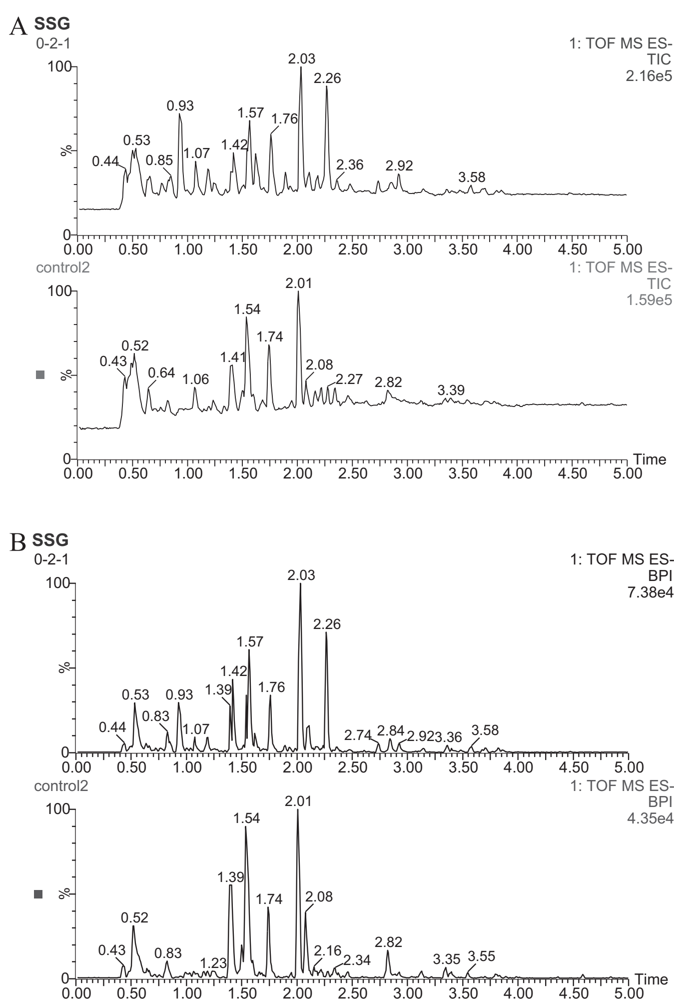

Fig. 3. Total ion current (TIC) (A) and base peak intensity (BPI) (B) chromatograms of urine sample of 0–4 h after a single oral dose of helidicum (25.0 mg/kg) to rats. The column utilized was an Acquity BEH C_{18} (100 × 2.1 mm, 1.7 μm), maintained at 50 °C. Analytes were quantified using TOF MS under negative-ion mode. Source parameters included capillary voltage of 2.2 kV, cone voltage of 35 V, source temperature of 120 °C, desolvation temperature of 350 °C, cone gas flow of 10 L/h, and desolvation gas flow of 700 L/h. Product ions were monitored in W^- extended mode.

2.6. Method validation

The limits of quantification (LOQ) and detection (LOD) were defined as helicidum responses which yielded a signal to noise ratio, without smoothing, of greater than 10 and greater than 3, respectively.

The QC samples with low, middle, and high concentrations of 0.125, 5.00, and 100 μg/mL were determined. The standard

Table 1
Identification of helicidum and its metabolites in urine after a single oral dose of helicidum to rats.

Peak no.	R_t (min)	Basic peak $[M-H]^-$	m/z	Molecular formula	Exact mass	Identification
Standard	1.20	329.0850	283.1138 (M−H) 329.0850 (M+FA−H) 613.2092 (2M+FA−H)	$C_{13}H_{16}O_7$	284.0896	Helicidum
1	1.19	329.1064	329.1064 (M+FA−H) 613.1780 (2M+FA−H)	$C_{13}H_{16}O_7$	284.0896	Helicidum
2	0.75	475.1105	475.1105 (M−H) 521.1194 (M+FA−H) 951.2303 (2M−H)	$C_{19}H_{24}O_{14}$	476.1166	Glucuronide conjugation
3	0.83	331.1029	331.1029 (M+FA−H) 617.2109 (2M+FA−H)	$C_{13}H_{18}O_7$	286.1052	Reduction
4	0.91	345.1017	345.1017 (M+FA−H) 599.1954 (2M−H) 899.2980 (3M−H)	$C_{13}H_{16}O_8$	300.0845	Oxidation
5	2.89	297.1089	297.1089 (M−H) 343.1200 (M+FA−H) 595.2229 (2M−H)	$C_{14}H_{18}O_7$	298.1052	Methylation
6	1.78	387.1298	387.1298 (M+FA−H) 683.1893 (2M−H)	$C_{16}H_{22}O_8$	342.1315	Reduction + methylation + acetylation
7	1.64	336.1087	336.1087 (M−H) 382.1086 (M+FA−H) 673.1537 (2M−H)	$C_{11}H_{19}N_3O_9$	337.1121	Unknown
8	1.89	338.1240	338.1240 (M−H) 384.1241 (M+FA−H) 677.2045 (2M−H)	$C_{11}H_{21}N_3O_9$	339.1278	Unknown (reduced C11H19N3O9)
9	1.67	354.0849	308.0777 (M−H) 354.0849 (M+FA−H) 617.1097 (2M−H)	$C_{14}H_{15}NO_7$	309.0849	Unknown

working solutions at the same concentrations were also assayed. The recovery of the method was evaluated with the ratio of determined peak area of the QC samples to that of the standard working solutions at the same concentrations.

Intra-day precision was tested by analysis of the QC samples at different time during the same day and inter-day precision was determined by repeated analysis of the same sample over two consecutive days. The concentration of each sample was determined using calibration standards prepared on the same day. The precision was assessed by relative standard deviation (RSD).

The stability of the urine samples was examined at room temperature for 0, 4, 8, and 12 h, respectively. The amount of helicidum in the urine samples was determined using a newly prepared calibration curve.

2.7. Data processing

The UPLC/TOF data of the samples collected during 0–4 h as well as the blank control urine sample were processed with XCMS by the following method. The data were first preprocessed prior to XCMS analysis. The proprietary data format (*.raw) generated by the MS instrument control software was preliminarily converted to the open mzXML format using massWolf-4.3.1 (http://sourceforge.net/projects/sashimi/files/massWolf%20(MassLynx%20converter)/). For converting lots of files at a time, we wrote a tool rawTomzXML which also used massWolf-4.3.1 and was much more convenient.

The data were processed using XCMS version l.26.0 (http://www.bioconductor.org/packages/2.8/bioc/html/xcms.html) running under R version 2.13 (http://http://www.r-project.org/) which provided the running platform for XCMS. The "centWave" method from XCMS was used for peak detection. The "centWave" method needs the chromatographic peak width range and the XCMS group() command needs the bandwidth (half width at half

Table 2
Recoveries and precision of QC levels of helicidum ($n = 3$).

Concentration (μg/mL)	Recovery (%)	Intra-day precision (RSD%)	Inter-day precision (RSD%)
0.125	97.23 ± 1.76	6.42	6.61
5.00	97.78 ± 1.52	5.27	5.08
100	98.16 ± 1.04	5.19	4.92

Table 3
Pharmacokinetic parameters of helicidum and its metabolites in urine after a single oral dose of helicidum (25.0 mg/kg) to rats ($n = 5$, calculated as body weight of 250 g).

Metabolite	Exact mass	$T_{1/2}$ (h)	K_e (h^{-1})	Cumulative urine excreted amount (μg)	Cumulative urine excreted amount calculated as helicidum (μg)	Cumulative urine excreted rate calculated as helicidum (%)	Percentage in the total metabolites (%)
Helicidum	284.0896	13.904 ± 24.632	0.206 ± 0.124	154.63 ± 86.08	154.63 ± 86.08	2.47 ± 1.38	13.28
$C_{19}H_{24}O_{14}$	476.1166	11.250 ± 0.352	0.107 ± 0.071	28.53 ± 12.78	17.02 ± 7.63	0.27 ± 0.12	1.46
$C_{13}H_{18}O_7$	286.1052	4.270 ± 2.561	0.204 ± 0.094	137.49 ± 70.33	136.53 ± 69.83	2.18 ± 1.12	11.72
$C_{13}H_{16}O_8$	300.0845	3.314 ± 1.507	0.241 ± 0.092	676.40 ± 268.54	640.35 ± 254.22	10.25 ± 4.07	54.98
$C_{14}H_{18}O_7$	298.1052	7.042 ± 0.823	0.100 ± 0.012	46.97 ± 15.72	44.77 ± 14.98	0.72 ± 0.24	3.84
$C_{16}H_{22}O_8$	342.1315	13.429 ± 13.328	0.079 ± 0.036	29.11 ± 26.87	24.17 ± 22.31	0.39 ± 0.36	2.08
$C_{11}H_{19}N_3O_9$	337.1121	10.216 ± 5.347	0.088 ± 0.050	57.08 ± 22.75	48.10 ± 19.17	0.77 ± 0.31	4.13
$C_{11}H_{21}N_3O_9$	339.1278	7.971 ± 2.333	0.094 ± 0.033	89.82 ± 63.59	75.24 ± 53.27	1.20 ± 0.85	6.46
$C_{14}H_{15}NO_7$	309.0849	30.292 ± 31.609	0.037 ± 0.017	25.91 ± 4.68	23.82 ± 4.30	0.38 ± 0.07	2.05
Sum					1164.63	18.63	100.00

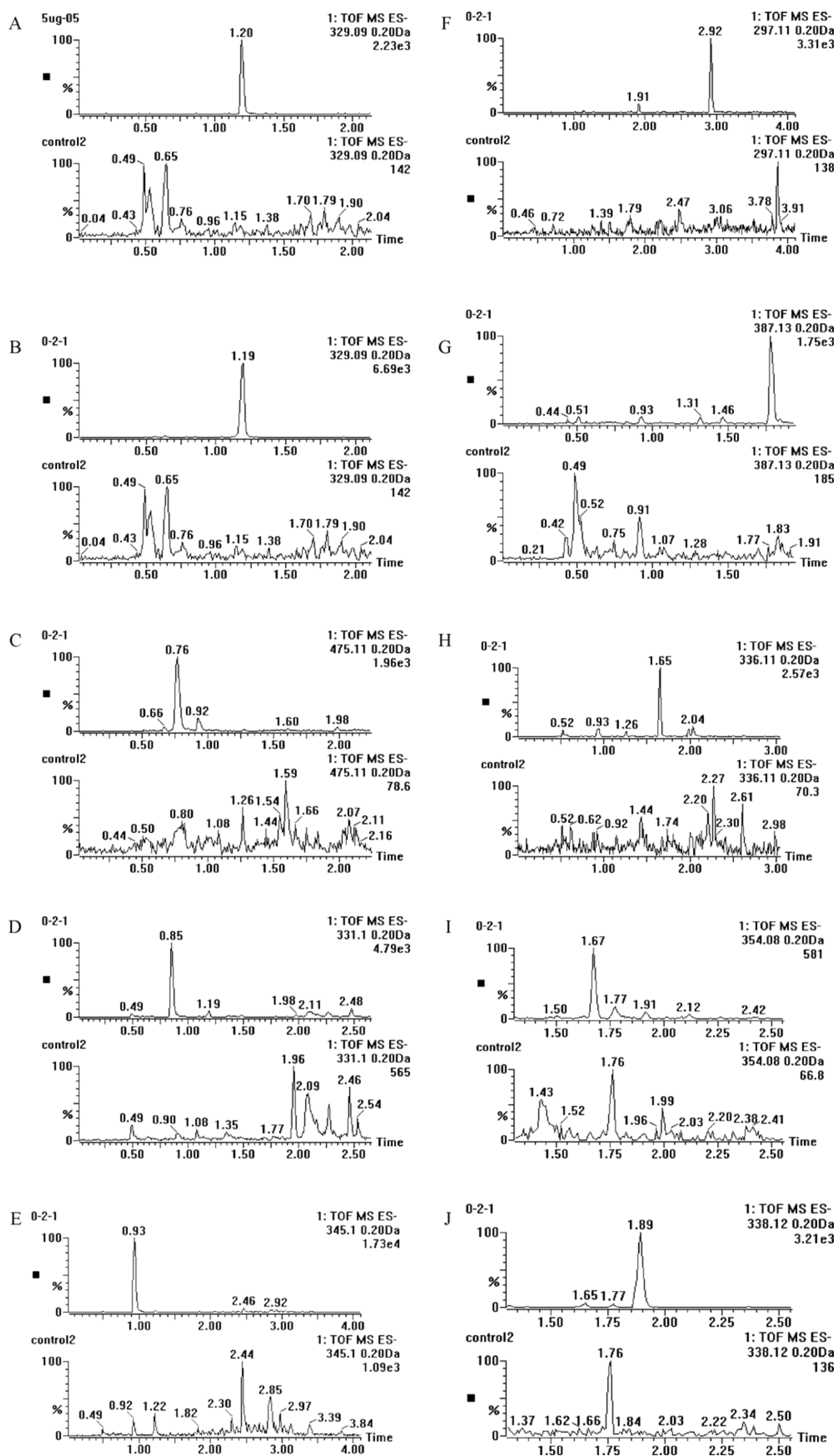

Fig. 4. EIC and MS of helicidum and its metabolites in rat urine after a single oral dose of helidicum to rats (A) standard of helicidum, (B) helicidum, (C) *m/z* 475.11, (D) *m/z* 331.10, (E) *m/z* 345.10, (F) *m/z* 297.11, (G) *m/z* 387.13, (H) *m/z* 336.11, (I) *m/z* 354.08, and (J) *m/z* 338.12.

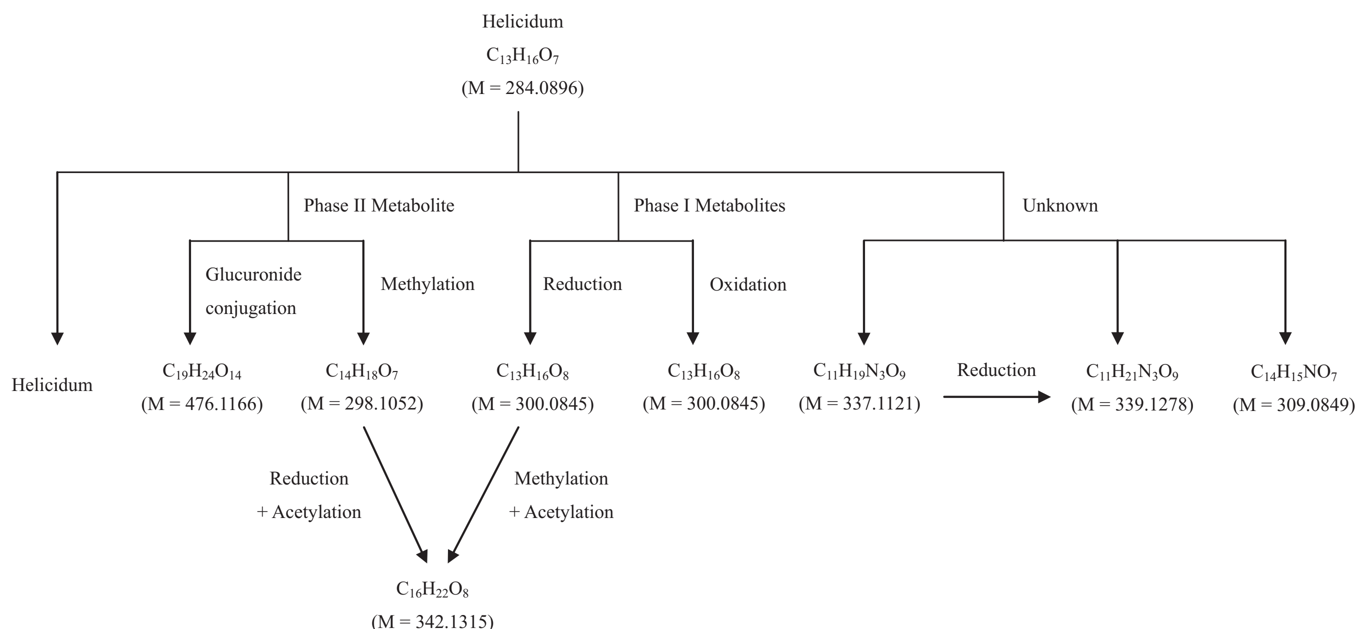

Fig. 5. Metabolism profile of helicidum in urine after a single oral dose of helidicum to rats.

maximum) of a Gaussian smoothing kernel. Therefore, plotChrom( ) of XCMS was at first employed to observe the chromatogram. The optimized XCMS parameters were as follows: maximal tolerated *m/z* deviation in consecutive scans in parts per million (ppm) 15.0, peak width range (peakwidth) c (6, 16), signal-to-noise cutoff (snthresh) 6.0, Gaussian smoothing-function width (bw) 2.0, minimum number of samples (minsamp) 1, width of overlapping *m/z* slice (mzwid) 0.01, and minimum fraction of samples in at least one of the sample groups (minfrac) 0.2. The parameters for ppm and snthresh were set according to the TOF MS instrument.

All the samples collected during 0–4, 4–8, 8–12, 12–16, 16–20, 20–24, 24–32, 32–40, 40–48 h were also processed with the method above. After the *m/z* data of the metabolites were obtained by XCMS, further identification of the metabolites was carried out by TOF MS.

2.8. Identification of the metabolites

Using the MS data and Masslynx software installed on the instrument, the molecular formulae of helicidum and its metabolites were identified. The potential biochemical transformations between the metabolites and the parent compound could be deduced based on the identified molecular formulae.

2.9. Metabolism profile of helicidum

The concentrations of the metabolites of helicidum were calculated by the method of semi-quantification with the equations of linear regression for helicidum. The urine volumes of the rats were obviously different, which could lead to a significant difference of the drug concentration even the same time interval. Therefore, the amounts of the metabolites (M, μg) were calculated ($M = C \times MW \times V$) based on the molecular weight (MW), the concentration (C, μmol/mL), and excreted urine volume (V, mL). Furthermore, all data on the amount of helicidum and the metabolites were normalized to the same body weight of 250 g ($M_{250g} = M/W \times 250$, where W was the actual body weight). Pharmacokinetic parameters were calculated by the method of urine excretion using DAS (ver. 2.0) pharmacokinetic software provided by the Chinese Pharmacology Association.

3. Results and discussion

3.1. Identification of helicidum and the metabolites

With the tool of raw TomzXML we wrote based on massWolf-4.3.1, batches of files could be conveniently converted at the same time. The UPLC/TOF MS data were processed using XCMS version l.26.0 running under R version 2.13. From the comparison of urine samples collected during 0–4 h to the blank control, 9 major peaks could be found. Besides these 9 peaks, there were no more new peaks found by the comparison of urine samples collected during other time intervals to the blank control. Helicidum and its metabolites were identified by the mass spectrometry data. The total ion current (TIC) and base peak intensity (BPI) chromatograms for urine sample are presented in Fig. 3 and the extracted ionic current (EIC) chromatograms are shown in Fig. 4.

The molecular formulae and the mass information of the metabolites including *m/z* of the base peaks, adducts, or dimmers are shown in Table 1. For the MS information of helicidum standard, it could be found that helicidum was conjugated with formic acid from the mobile phase with 329 *m/z* of $[M+HCOOH-H]^-$ and 613 *m/z* of $[2M+FA-H]^-$. The peak with *m/z* of 283 for the parent compound $[M-H]^-$ was very weak and for the urine sample it could not even be found, given the low concentration or different micro-environment of the test sample. The information on the peaks of the helicidum standard could help us find and identify the metabolites more exactly. For the peak with *m/z* of 475, based on 951 *m/z* of $[2M-H]^-$, 521 *m/z* of $[M+FA-H]^-$, and 475 *m/z* of $[M-H]^-$, it could be identified as the parent drug conjugated with glucuronide with the formula of $C_{19}H_{24}O_{14}$. The methyl helicidum $C_{14}H_{18}O_7$ could be identified from 595 *m/z* of $[2M-H]^-$, 343 *m/z* of $[M+FA-H]^-$, and 297 *m/z* of $[M-H]^-$. For the peak with *m/z* of 331, 617 *m/z* of $[2M+FA-H]^-$ and 331 *m/z* of $[M+FA-H]^-$ showed the reduced helicidum with the formula of $C_{13}H_{18}O_7$. The oxidized helicidum $C_{13}H_{16}O_8$ could easily be found from 899 *m/z* of $[3M-H]^-$, 599 *m/z* of $[2M-H]^-$, and 345 *m/z* of $[M+FA-H]^-$. Based on 387 *m/z* of $[M+FA-H]^-$ and 683 *m/z* of $[2M-H]^-$, we could find $C_{16}H_{22}O_8$ with MW of 342 with possible mechanism of mixed bio-transformations of reduction, methylation, and acetylation. It could be produced from either the reduced helicidum or methyl helicidum.

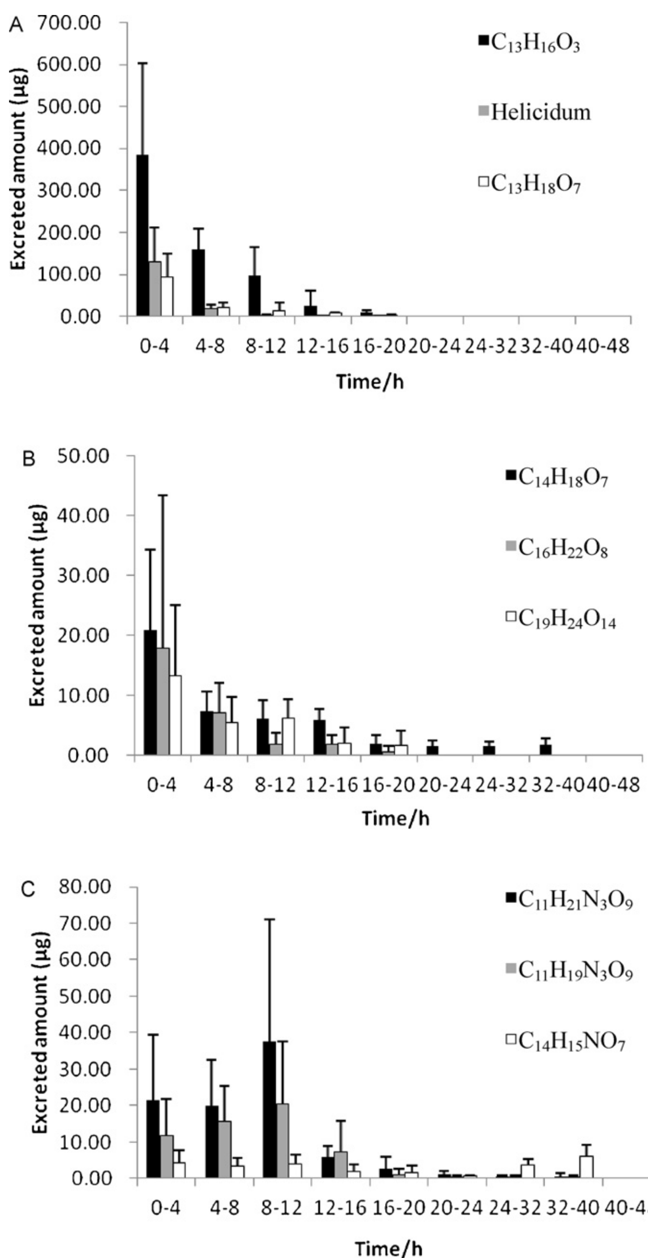

Fig. 6. Excreted amount of helicidum and its metabolites in rat urine after a single oral dose of helidicum (25.0 mg/kg) to rats ($n = 5$). (A) Helicidum, $C_{13}H_{16}O_8$, and $C_{13}H_{18}O_7$, (B) $C_{14}H_{18}O_7$, $C_{16}H_{22}O_8$, and $C_{19}H_{24}O_{14}$, (C) $C_{11}H_{21}N_3O_9$, $C_{11}H_{19}N_3O_9$, and $C_{14}H_{15}NO_7$.

Three metabolites containing the element of nitrogen could be identified as well, with the formulae of $C_{11}H_{19}N_3O_9$, $C_{11}H_{21}N_3O_9$, and $C_{14}H_{15}NO_7$, respectively. $C_{11}H_{21}N_3O_9$ might be the reduced product of $C_{11}H_{19}N_3O_9$. The bio-transformation mechanisms of these metabolites were not clear and needed further investigation. The metabolism profile of helicidum is described in Fig. 5.

Based on the previous analysis, it was obvious that for helicidum with the molecular weight of 284, the highest m/z was 329 of $[M+FA-H]^-$ rather than 283 of $[M-H]^-$. For most metabolites of helicidum, the fragments with the tallest peaks were also $[M+FA-H]^-$ rather than $[M-H]^-$. Therefore, manual identification and comparison must be applied to find and identify the real molecular formulae to avoid false results.

3.2. Quantification and validation of the analysis method

Using reference standards, the metabolites were quantitatively determined by the method of semi-quantification with the equations of linear regression for helicidum. Two regression equations of the standard curves were obtained: $y = 3082x + 1.087$ (correlation coefficient $r = 0.9989$, $n = 3$) for the concentration range of 0.000440–0.00176 μmol/mL (0.125–5.00 μg/mL) for helicidum, and $y = 1448.4x + 33.266$ ($r = 0.9939$, $n = 3$) for the concentration range of 0.00176–0.0352 μmol/mL (5.00–100 μg/mL), where x was the concentration of helicidum (μmol/mL) and y was the peak area. They showed good liner relationships between y and x. The assay was considered adequately specific, for no endogenous urine components eluted at the same retention time of the analytes of interest. For helicidum, LOQ and LOD were 0.440 nmol/mL (0.125 μg/mL) and 0.176 nmol/mL (0.050 μg/mL), respectively. The recoveries, intra-day precision, and inter-day precision of low, middle, and high QC samples (0.125, 5.00, and 100 μg/mL) are shown in Table 2. The urine samples were stable at room temperature for at least 8 h with RSD of 3.12%.

3.3. Urine pharmacokinetics of the metabolites

The excreted amounts and cumulative amounts of helicidum and the metabolites are shown in Fig. 6. It was obvious that helicidum, $C_{13}H_{16}O_8$, $C_{19}H_{24}O_{14}$, $C_{16}H_{22}O_8$, and $C_{13}H_{18}O_7$ were rapidly excreted within 4 h and could not be found in urine sample after 24 h. The other metabolites could still be found during the 32–40 h interval.

The pharmacokinetic parameters of helicidum and the other metabolites were calculated and the main parameters are shown in Table 3. The urine excretion rate of helicidum was (2.47 ± 1.38)%. Calculated as helicidum, the cumulative urine excretion rate was 18.63%. In the total metabolites, the amount of helicidum was about 13.28% (w/w) and the major metabolite was $C_{13}H_{16}O_8$, oxidized helicidum, at 54.98%. The metabolite of glucuronide-conjugated helicidum, $C_{19}H_{24}O_{14}$, was only 1.46%.

4. Conclusion

The present study used XCMS, an open-source software tool, to analyze the data of UPLC/MS for the discovery of the metabolites of helicidum in urine after a single oral dose to rats. UPLC/TOF MS was used to analyze the samples in negative ion mode. Nine metabolites were finally found and identified. For six of these, the bio-transformation mechanisms of the parent compound were identified as glucuronide conjugation, reduction, oxidation, methylation, and the mixed transformation of reduction, methylation, and acetylation, respectively. For the other three metabolites, the bio-transformation mechanisms remain unknown and need further investigation. The cumulative urine excretion rates of the metabolites compared to the oral dose were determined, calculated as mass of helicidum. The amount of oxidized helicidum was more than 50% among all the metabolites, indicating that oxidation was the major bio-transformation occurring *in vivo*.

XCMS is written in the R statistical programming language and can be freely used under an open-source license. It is distributed through both the Metlin Metabolite Database and the Bioconductor bioinformatics project. The openness of the software allows it to be easily customized for different data analysis or optimized for a particular application. Alterations can be made without requiring significant investment to develop the infrastructure and algorithms that XCMS already provides [21]. Therefore, this type of open software architecture will be increasingly developed and applied to more data-intensive research.

Acknowledgment

The project was supported by National Natural Science Found (No. 30500666).

References

[1] D.Q. Zhou, Chin. Tradit. Pat. Med. 18 (1996) 21.
[2] F.Q. Tang, X.Z. Wu, Y.A. Xiang, Q.M. Chen, H.C. Luo, X.Y. Zhao, Chin. J. Com. Wes.-Chin. Med. 18 (1998) 170.
[3] Q. Wang, X.S. Wu, Liaoning J. Med. 15 (2001) 220.
[4] P. Liu, J.M. Geng, J. Li, D.Q. Na, L. Shang, Chin. Tradit. Pat. Med. 24 (2002) 278.
[5] P. Liu, J. Li, D.Q. Na, J.M. Geng, L. Shang, Chin. Tradit. Herb. Drugs 33 (2002) 238.
[6] Q.F. Liu, X.D. Liu, G.A. Luo, W.W. Tian, Y.M. Wang, J. Chromatogr. B 832 (2006) 185.
[7] Y.W. Jia, H.T. Xie, G.J. Wanga, J.G. Sun, W. Wang, H. Qing, X. Wang, H. Yang, M.J. Xu, Y. Gu, C. Yao, J. Shen, J. Chromatogr. B 878 (2010) 791.
[8] Y.W. Jia, G.J. Wang, H.T. Xie, X.C. Dai, Y.H. Wang, W. Wang, M.J. Xu, R. Wang, C. Yao, J. Chromatogr. B 847 (2007) 72.
[9] S.J. Bruce, P. Jonsson, H. Antti, O. Cloarec, J. Trygg, S.L. Marklund, T. Moritz, Anal. Biochem. 372 (2008) 237.
[10] X.J. Wang, H.T. Lv, H. Sun, B. Yang, W. Sun, P. Wang, D. Zhou, L. Zhao, S. Dou, G. Zhang, H. Cao, J. Pharm. Biomed. Anal. 48 (2008) 1161.
[11] C.G. Guo, K.E. McMartin, Toxicology 208 (2005) 347.
[12] H. Chang, S.M. Wu, J.Y. Hua, M. Asami, S. Kunikane, J. Chromatogr. A 1195 (2008) 44.
[13] Z.X. Cai, Y. Zhang, H.F. Pan, X.W. Tie, Y.P. Ren, J. Chromatogr. A 1200 (2008) 144.
[14] R.R. González, A.G. Frenich, J.L.M. Vidal, Talanta 76 (2008) 211.
[15] B. Avula, Y.H. Wang, R.S. Pawar, Y.T.J. Shukla, T.J. Smillie, I.A. Khan, J. Pharm. Biomed. Anal. 48 (2008) 722.
[16] G.X. Xie, Y. Ni, M.M. Su, Y.Y. Zhang, A.H. Zhao, X.F. Gao, Z. Liu, P.G. Xiao, W. Jia, Metabolomics 4 (2008) 248.
[17] M. Dan, M.M. Su, X.F. Gao, T. Zhao, A.H. Zhao, G.X. Xie, Y.P. Qiu, M.M. Zhou, Z. Liu, W. Jia, Phytochemistry 69 (2008) 2237.
[18] J. Tang, R.H. Zhu, R.K. Zhao, G. Cheng, W.X. Peng, J. Pharm. Biomed. Anal. 47 (2008) 923.
[19] P.M.W. Lam, T.H. Marczylo, M.E. Talatini, M. Finney, V. Nallendran, A.H. Taylor, J.C. Konje, Anal. Biochem. 380 (2008) 195.
[20] Q.F. Liu, Y. Shi, Y. Wang, J. Lu, W.J. Cong, G.A. Luo, Y.M. Wang, Talanta 80 (2009) 84.
[21] C.A. Smith, E.J. Want, G. O'Maille, R. Abagyan, G. Siuzdak, Anal. Chem. 78 (2006) 779.
[22] R. Tautenhahn, G.J. Patti, E. Kalisiak, T. Miyamoto, M. Schmidt, F.Y. Lo, J. McBee, N.S. Baliga, G. Siuzdak, Anal. Chem. 83 (2011) 696.
[23] H. Yao, P.Y. Shi, L. Zhang, X.H. Fan, Q. Shao, Y.Y. Cheng, Mol. Biosyst. 6 (2010) 1061.
[24] F. Kieken, G. Pinel, J.P. Antignac, A.C. Paris, P. Garcia, M.A. Popot, M. Grall, V. Mercadier, P.L. Toutain, Y. Bonnaire, B.L. Bizec, Metabolomics 7 (2011) 84.
[25] C.G. Fraga, B.H. Clowers, R.J. Moore, E.M. Zink, Anal. Chem. 82 (2010) 4165.