Original Research Article

Identification of metabolites of gardenin A in rat liver microsomes using ultra-high performance liquid chromatography coupled with linear ion-trap Orbitrap mass spectrometry

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Abstract

Purpose: To identify the metabolites of gardenin A (GA) in rat liver microsomes (RLMs) using ultra-high performance liquid chromatography coupled with linear ion-trap Orbitrap mass spectrometry (UHPLC-LTQ-Orbitrap).
Methods: The sample was prepared by incubating GA (100 µg/mL) with RLMs (0.5 mg/mL) for 8 h. Then 5 µL of the sample was injected into UHPLC-LTQ- orbitrap mass spectrometer. The metabolites of GA were tentatively identified based on accurate mass measurements, fragmentation patterns, chromatographic retention times, and bibliography data.
Results: A total of 12 metabolites were detected and identified. Based on their structures, the main reactions in the metabolism of GA are de-methoxylation and de-methylation.
Conclusion: This is the first report on in vitro metabolites of GA. These results are considered very helpful for better comprehension of the metabolism of GA and its pharmacological effects.

Keywords: Gardenin A, Metabolites, UHPLC-LTQ-Orbitrap, Rat Liver microsomes

INTRODUCTION

Murraya paniculata (L.) Jack, also called "qianlixiang in chinese" in China, belongs to the family Rutaceae. It is a very variable evergreen shrubby plant cultivated widely in gardens as an ornamental in many areas of China. The plant is officially listed in the Chinese Pharmacopoeia as a drug in Chinese Traditional medicine (TCM) [1]. Dried leaves or tender branches have been used all over China as a folk medicine due to the stimulant, astringent, antidysenteric, toothache remedy, and antidiarrheal [2-4]. Gardenin A (5-hydroxy-6, 7, 8, 3', 4', 5'-hexamethoxyflavone, GA), a hydroxylated polymethoxyflavonoid (OH-PMF), has been isolated from *Murraya*

paniculata (L.) Jack [5-6]. Pharmacological studies indicate that it possesses variety of biological activities, such as anticancer and anti-inflammatory effects [7-9].

Research on drug metabolism research is very important in the early phases of drug discovery and development. It is also important for minimizing the adverse effect of pharmaceuticals, and for maximizing their therapeutic value [10,11]. A previous report [12] demonstrated that 26 metabolites of GA were observed in *vivo*; these might be responsible for the pharmacological actions of GA. However, it is hard to establish the reliability of this observation, due to difficulties involved in obtaining enough

metabolites in vivo. Thus, it is important to obtain GA metabolites *in vitro*. To the best of our knowledge, metabolites of GA have not been investigated *in vitro*.

High performance liquid chromatography coupled with mass spectrometry has become a popular, and indeed the main method for the structural characterization of drug metabolites *in vivo* and *in vitro* due to its high efficiency, sensitivity, and selectivity. Among several different LC/MS platforms, ultra–high performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) [13-15] such as UHPLC-LTQ-Orbitrap is especially useful for the characterization of drug metabolites due to its higher and faster separation and resolution capacities.

The present study was carried out identify the metabolites of GA in rat liver microsomes (RLMs) with a view to providing better understanding of the metabolism of the drug and its pharmacological effects.

EXPERIMENTAL

Chemicals and reagents

Gardenin A, 5-hydroxy-7, 3', 4', 5'-tetramethoxyflavone and 5-hydroxy-7, 3', 4'-trimethoxyflavone was purchased from Chengdu Biopurify Phytochemicals Co, Ltd (Sichuan, China). Acetonitrile, and methanol (HPLC grade) used were products of Fisher Scientific (Fisher, Fair Lawn, NJ, USA), and ultra-pure water used throughout the experiment was produced by a Milli-Q system (Millipore, Bedford, MA, USA). The 0.22 mm membranes used in the study were purchased from Waters Corporation (USA). Reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) was supplied by Zhong Sheng Rui Tai Biotech (Beijing, China). RLMs were purchased from BD Biosciences (Bedford, MA, USA). All other reagents were of analytical grade.

Microsome incubation

Incubation conditions of microsome incubation experiment were established and controlled to provide a reproducible and linear rate of the metabolism *in vitro*. The incubation mixture in final volume of 1 mL contained GA (100 μg/mL), phosphate buffer (0.1 mol/L, pH 7.4), magnesium chloride (5 mM) and RLMs (0.5 g/mL). The incubation mixture was pre-incubated for 5 min in a water bath at 37 °C and reactions were initiated by addition of NADPH (1 mM). The reaction was terminated by adding 1 mL of ice-cold acetonitrile, vortexing and centrifuging at 15,000 rpm for 10 min at 4 °C. Aliquots of the supernatant were subjected to UHPLC-LTQ-Orbitrap MS to identify the metabolites. Blank samples were of the same composition as the test samples, but without GA. All samples were prepared in triplicate.

Instrumentation and chromatographic conditions

All UHPLC-MS analyses were carried out on LTQ/Orbitrap XL hybrid mass spectrometer (Thermo Electron, Bremen, Germany) equipped with an ESI source (Thermo Electron, Bremen, Germany). An Accela UHPLC system (ThermoFisher Scientific) was equipped with an autosampler, a vacuum de-gasser unit and a quaternary pump. Chromatographic separations were carried out on a BEH C18 column (2.1 × 50 mm, 1.7 μm) at room temperature and a flow rate of 0.2 mL/min. The mobile phase consisted of solvent A (water) and solvent C (acetonitrile). The percentage of solvent A was changed as follows: 0-2 min, 95 % A; 2-3 min, 95-80 % A; 3-20 min, 80 - 40 % A; 20-21 min, 40-20% A; 21-25 min, 20 % A; 25-26 min, 20-95 % A; 26-30 min, 95 % A; an aliquot of 5 μL of the supernatant was injected into UHPLC-LTQ-Orbitrap MS.

The optimized operating parameters in the positive ion mode were as follows: capillary voltage of 25 V, source voltage of 4.0 kV, capillary temperature of 350 °C, sheath gas flow rate of 40 (arbitrary units), auxiliary gas flow rate of 20 (arbitrary units), and tube lens of 110 V. Metabolites were detected by full-scan mass analysis from 100 to 800 m/z at a resolving power of 30,000 with data-dependent MS^2 analysis triggered by the three most-abundant ions from the precursor list of predicted metabolites followed by MS^2 analysis of the most−abundant product ions. Collision-induced dissociation (CID) was performed with an isolation width of 2 Da. The collision energy was set to 35 %.

Data processing

Thermo Xcaliber 2.1 workstation (Thermo Fisher Scientific) was used for data acquiring and processing. In order to obtain as many fragment ions as possible, the peaks detected with intensity over 50,000 were selected for identification. The chemical formulas of all parent and fragment ions of the selected peaks were calculated from the accurate mass using a formula predictor by setting the parameters as follows: C [0 - 35], H [0 - 50], O [0 - 15], S [0 - 1], N [0 - 3], and Ring Double Bond (RDB)

equivalent value [0 - 15]. Other elements such as P and Br were not considered as they were rarely present in the complex matrix. The maximum mass errors between the measured and the calculated values were < 5 ppm. Blank RLMs samples were used as control to compare with the analyzed samples, and they were all processed under the same conditions.

RESULTS

Fragmentation pathway of GA

The parent ion showed a protonated ion $[M+H]^+$ at m/z 419.13321 (-1.07 ppm, $C_{21}H_{23}O_9$) in positive ion mode. Fragmentation of this parent ion at m/z 389, m/z 404, m/z 358, m/z 371, and m/z 386 by the loss of the moiety 30 ($2CH_3^\circ$), 15 (CH_3°), 61 ($2CH_3^\circ +OCH_3^\circ$), 48 ($2CH_3^\circ + H_2O$), and 33($CH_3^\circ + H_2O$) were detected as diagnostic product ions in the MS^2 spectra. The MS^2 spectrum of GA is shown in Figure 1.

Identified metabolites

The high–resolution extracted Ion Chromato-graphy (HREIC) of blank, GA incubated in RLMS samples in 30 min are shown in the Figure 2. For the first time, a total of 12 metabolites of GA were detected and identified based on accurate mass measurements, fragmentation patterns, and chromatographic retention times in positive ion mode. The detailed information is illustrated in Table 1.

Metabolite M0, M8 and M12 were identified as GA, 5-hydroxy-7, 3', 4', 5'-tetramethoxyflavone and 5-hydroxy-7, 3', 4'-trimethoxyflavone, respectively by comparing the retention time, $[M+H]^+$ ion and MS^2 spectra with authentic references.

Metabolites M1, M2, M4, M6 and M7 were detected at 6.74, 7.54, 9.36, 14.40 and 16.44 min with protonated molecular ions $[M+H]^+$ at m/z 361.09153 (-0.72 ppm, $C_{18}H_{17}O_8$), m/z 361.09164 (-0.43 ppm, $C_{18}H_{17}O_8$), m/z 361.09157 (-0.61 ppm, $C_{18}H_{17}O_8$), m/z 361.09171 (-0.22 ppm, $C_{18}H_{17}O_8$), and m/z 361.09168 (-0.30 ppm, $C_{18}H_{17}O_8$), 58 Da ($2CH_2+OCH_2$) less than that of GA. The diagnostic product ions at m/z 346 $[M+H- CH_3^\circ]^+$, m/z 314 $[M+H- 3CH_3^\circ]^+$, and m/z 329$[M+H-2CH_3^\circ]^+$ were detected. They were tentatively identified as 8, 3', 5'-trihydroxy-5, 7, 4'-trimethoxyflavone, 5, 7, 3'-trihydroxy-8, 4', 5'-trimethoxyflavone, 5, 8, 3'-trihydroxy-7, 4', 5'-trimethoxyflavone, 5, 7, 4'-trihydroxy-8, 3', 5'-

trimethoxyflavone, and 5, 8, 4'-trihydroxy-7, 3', 5'-trimethoxyflavone, respectively [16].

Metabolites M3 and M5 were detected at 7.54 and 12.97 min respectively, with the same quasi-molecular ion $[M+H]^+$ at m/z 375.11 ($C_{19}H_{19}O_8$) that is 44 Da (CH_2+OCH_2) less than the parent compound GA. The CID product-ion spectrum of m/z 375 displayed three major fragment ions at m/z 360 ($[M+H-CH_3^\bullet]^+$), m/z 314 ($[M+H-2CH_3^\bullet-OCH_3^\bullet]^+$) and m/z 329 ($[M+H-CH_3^\bullet-OCH_3^\bullet]^+$). It was identified as the de-methoxylation and de-methylation product of GA and were tentatively confirmed as 5, 4'-dihydroxy-7, 8, 3', 5'-tetra-methoxyflavone and 5, 3'-dihydroxy-7, 8, 4', 5'-tetramethoxyflavone, respectively [12].

Metabolite M9-M11 was eluted at 21.03, 21.60, and 22.00min, respectively. Each of them showed a protonated molecule ion at m/z 389.12 ($C_{21}H_{27}O_{11}$), 30 Da (OCH_2) more than that of parent drug. The fragment ions at m/z 374 (4.2 ppm, $C_6H_9O_7$), m/z 359 (4.8 ppm, $C_6H_9O_7$) and m/z 356 (4.8 ppm, $C_6H_9O_7$) in the MS^2 spectra of metabolite M9-M11, indicated that they were a pair of hydroxy-pentamethoxyflavone isomers. Based on examination of known polymethoxy-flavonoids isolated from genus *Murraya*, *and* from bibliography data [5,12], M9 and M10 were tentatively identified as 5-hydroxy-6, 7, 3', 4', 5'-pentamethoxyflavone, 5-hydroxy-6, 7, 8, 3', 4'-pentamethoxyflavone, respectively. M11 was identified as hydroxypentamethoxy-flavone.

DISCUSSION

In the study, we identified metabolites of GA *in vitro* for the first time. These metabolites are polymethoxylated flavonoids with many methoxy groups (OH-PMFs), which facilitate their detection by ESI in positive mode. MS conditions were optimized on an UHPLC-ESI-LTQ-Orbitrap instrument using standard solution of GA (10 μg/mL). In order to obtain an appropriate elution system, two different mobile systems, acetonitrile – water and methanol – water were tested. It was found that for GA, $[M+H]^+$ ions of basically the same intensity were commonly detected with high sensitivity with these two solvent conditions. However, the mobile systems of acetonitrile – water with a gradient elution mode afforded much lower column pressure and better resolution of chromatographic peaks among the metabolites and endogenous components than that of water–methanol.

To our best knowledge, not much has been done on the elucidation of the metabolites of GA, although some studies on the metabolism of GA
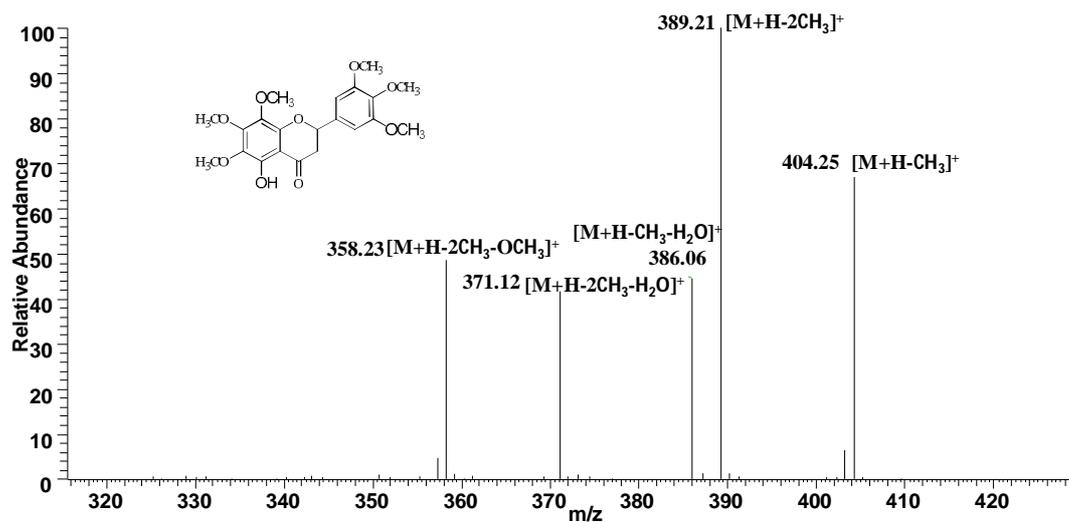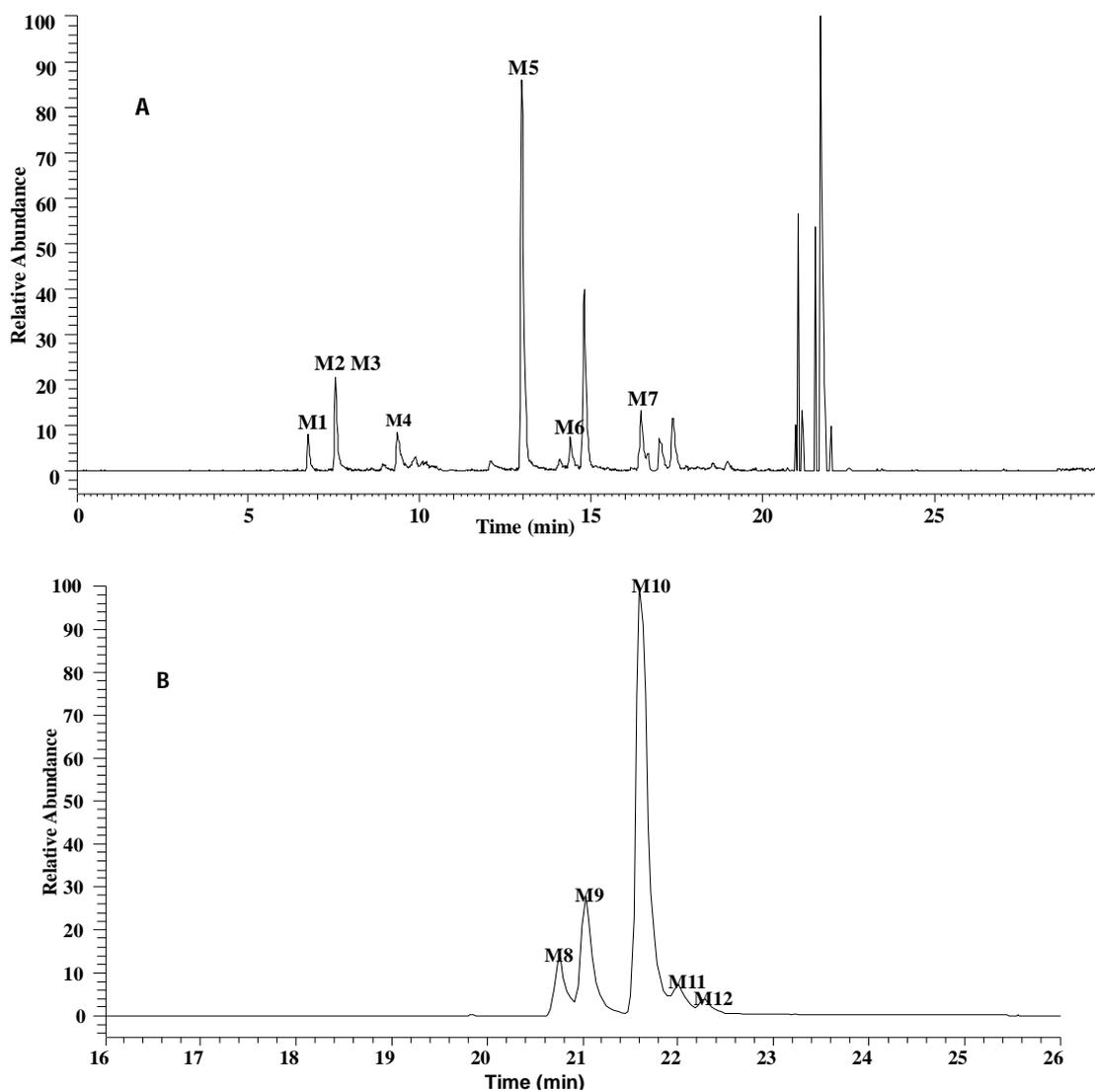
Figure 1: MS² spectrum of GA

Figure 2: HREIC in 5 ppm for the metabolites in RLMs (A) *m/z* 361.09179, 375.10744; (B) *m/z* 329.10196, 359.11253, 389.12259

Table 1: Characteristic fragment ions of GA metabolites in RLMs by UHPLC-LTQ-Orbitrap MS

Peak	t_R	Theoretical Mass *m/z*	Experimental Mass *m/z*	Error (ppm)	Formula [M-H]⁻	MS/MS fragment	Identification
0	22.76	419.13269	419.13321	-1.071	$C_{21}H_{23}O_9$	MS^2 [419]: 389(100), 404(65), 358(40), 371(38), 386(37)	gardenin A
1	6.74	361.09179	361.09153	-0.717	$C_{18}H_{17}O_8$	MS^2 [361]: 346(100), 314(26), 329(15)	8, 3', 5'-trihydroxy-5, 7, 4'-trimethoxyflavone
2	7.54	361.09179	361.09164	-0.426	$C_{18}H_{17}O_8$	MS^2 [361]: 346(100)	5, 7, 3'-trihydroxy-8, 4', 5'-trimethoxyflavone
3	7.54	375.10744	375.10721	-0.624	$C_{19}H_{19}O_8$	MS^2 [375]: 360(100), 314(82), 329(49), 347(19)	5, 4'-dihydroxy-7, 8, 3', 5'-tetramethoxyflavone
4	9.36	361.09179	361.09157	-0.606	$C_{18}H_{17}O_8$	MS^2 [361]: 346(100)	5, 8, 3'-trihydroxy-7, 4', 5'-trimethoxyflavone
5	12.97	375.10744	375.10708	-0.957	$C_{19}H_{19}O_8$	MS^2 [375]: 360(100)	5, 3'-dihydroxy-7, 8, 4', 5'-tetramethoxyflavone
6	14.40	361.09179	361.09171	-0.218	$C_{18}H_{17}O_8$	MS^2 [361]: 329(100), 346(8)	5, 7, 4'-trihydroxy-8, 3', 5'-trimethoxyflavone
7	16.44	361.09179	361.09168	-0.301	$C_{18}H_{17}O_8$	MS^2 [361]: 346(100)	5, 8, 4'-trihydroxy-7, 3', 5'-trimethoxyflavone
8	20.77	359.11253	359.11206	-1.307	$C_{19}H_{19}O_7$	MS^2 [359]:344(100), 343(26), 315(15)	5-hydroxy-7, 3', 4', 5'-tetramethoxyflavone
9	21.03	389.12259	389.12309	-1.295	$C_{20}H_{21}O_8$	MS^2 [389]: 356(100), 328(72), 374(70), 359(15)	5-hydroxy-6, 7, 3', 4', 5'-pentamethoxyflavone
10	21.60	389.12259	389.12234	-1.938	$C_{20}H_{21}O_8$	MS^2 [389]: 359(100), 374(75), 356(45), 341(41)	5-hydroxy-6, 7, 8, 3', 4'-pentamethoxyflavone
11	22.00	389.12259	389.12283	-0.679	$C_{20}H_{21}O_8$	MS^2 [389]: 374(100), 328(74), 359(48), 356(21)	hydroxy-pentamethoxyflavone
12	22.29	329.10196	329.10159	-1.139	$C_{18}H_{17}O_6$	MS^2 [329]:313(100), 314(77), 285(34)	5-hydroxy-7, 3', 4'-trimethoxyflavone

have been done in rats [12]. For example, 26 metabolites of GA were unambiguously and tentatively identified in Sprague-Dawley rats by comparison of retention times and mass spectrometry. However, it is worth mentioning that *in vitro* metabolites of GA; and the differences between *in vivo and in vitro* metabolites of GA were still unclear until now. In our study, 12 metabolites were detected and identified in RLMs. There was a good agreement between these metabolites and those obtained in *in vivo* studies. This clearly shows that it is feasible to obtain metabolites of GA *in vitro.*

CONCLUSION

This study has successfully elucidated the *in vitro* metabolism of GA in RLMs using UHPLC-LTQ-Orbitrap mass spectrometry. Among the 12 metabolites detected, 3 have been unambiguously confirmed, while the others are tentatively identified. The structures clearly indicate that de-methoxylation and de-methylation are the major metabolic fates of GA. These metabolites of GA in RLMs would facilitate better understanding of the metabolism and its pharmacological effects.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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