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Identification of Gambogic Acid Metabolites in Rat Bile by Liquid Chromatography-tandem Mass Spectrometry-ion Trap-time-of-flight

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[ABSTRACT] AIM: To investigate the metabolism of gambogic acid *in vivo* in rat bile. **METHODS:** Liquid chromatography-tandem mass spectrometry-ion trap-time-of-flight system and the authentic compounds were used to perform metabolism studies. **RESULTS:** Gambogic acid was rapidly metabolized to three metabolites, M1, M2 and M3, in rat bile. M1 was tentatively presumed to be 3, 4-dihydro-gambogic acid. M2 and M3 were proved to be 10-hydroxy-gambogic acid and 9, 10-epoxy-gambogic acid. **CONCLUSIONS:** Gambogic acid was metabolized to three metabolites in rat bile. M1 is firstly reported, while the structures of M2 and M3 were confirmed by comparing the tandem mass spectra and the chromatographic retention time with those of the respective authentic compounds.

[KEY WORDS] Gambogic acid; Metabolite; Identification; LCMS-IT-TOF; Synthesize

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1 Introduction

Gambogic acid (GA, CAS No. 2752-65-0) is the principal active component of gamboge, the resin from various *Garcinia* species, including *Garcinia morella* and *Garcinia hanburyi*. Our previous studies demonstrated that GA can inhibit the growth of a wide variety of tumor cells, including human gastric carcinoma SGC-7901 cells, MGC-803 cells, human lung carcinoma SPC-A1 cells, and human hepatoma SMMC-7721 cells [1-6].

Pharmacokinetic studies of gambogic acid showed that, following i.v. administration, gambogic acid was mainly distributed in organs such as the liver, lung, spleen, kidney, stomach, intestine and heart. Gambogic acid was mainly excreted through bile with approximately 36.5% being excreted within 16 h [7]. In one study, the metabolism of gambogic acid in rat liver microsomes *in vitro* was investigated and 27-hydroxy-gambogic acid and 27, 28-epoxy- gambogic acid were tentatively postulated to be the major metabolites of gambogic acid [8]. In another study, the metabolism of gambogic acid in rat bile *in vitro* was investigated and 10-hydroxy-gambogic acid and 9, 10-epoxy-gambogic acid were postulated to be the major metabolites of gambogic acid [7].

In the present study, the metabolites of gambogic acid in rat bile were identified using an LCMS-IT-TOF system. Gambogic acid was metabolized to three metabolites in rat bile. The metabolite M1 is firstly reported, while the structure of M2 and M3 have been confirmed by comparing the tandem mass spectra and the chromatographic retention time with those of the respective authentic compounds.

2 Experimental

2.1 *Chemicals, Reagents and Animals*

Gambogic acid was supplied by the Department of Medicinal Chemistry of China Pharmaceutical University. Methanol and acetonitrile were of HPLC grade and purcha-

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sed from Merck (Darmstadt, Germany). Purified water from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Other reagents were of the highest purity commercially available.

Sprague–Dawley rats (180-220 g) were obtained from the animal center of China Pharmaceutical University (Nanjing, China) and the studies were approved by the Animal Ethics Committee of China Pharmaceutical University. The animals were cared for according to the regulations of the Animal Committee under controlled conditions (20 ± 2 ℃, RH 50% ± 20%, a natural light-dark cycle and 10-15 air changes per hour).

Our lab synthesized 10-hydroxy-gambogic acid and 9,10-epoxy-gambogic acid. All the structures were confirmed by 1D and 2D NMR spectroscopy, and ESI-MS. Their structures were shown in Fig. 1. All other chemicals and reagents were purchased from commercial sources and were used as received.

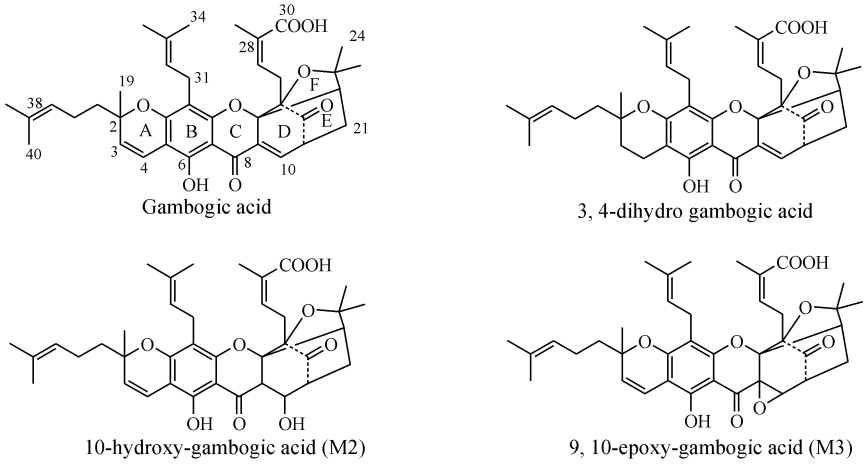

Fig. 1 Chemical structure of gambogic acid, 3, 4-dihydro-gambogic acid (M1), 10-hydroxy-gambogic acid (M2) and 9, 10-epoxy-gambogic acid (M3)

2.1.1 *Synthesis of 9, 10-epoxy-gambogic acid*

To a solution of gambogic acid (52 mg, 0.08 mmol) in methanol (2 mL), 2 mol·L^{-1} NaOH (0.5 mL, 1.0 mmol) was added, followed by 35% H_2O_2 (0.2 mL, 2.1 mmol) at room temperature. The mixture was stirred at room temperature for 10 min, diluted with 1:1 hexane/EtOAc (50 mL), washed with water, 2 mol·L^{-1} HCl and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether: EtOAc = 4:1) to yield the title compound as an oil (2.2 mg, 0.003 mmol, 4%) [9].

2.1.2 *Synthesis of 10-hydroxy-gambogic acid (M2)*

To a solution of 9,10-epoxy- gambogic acid (20 mg, 0.03 mmol) in methanol (2 mL), $HCOONH_4$ (6 mg, 0.1 mmol) was added, followed by 5% Pd-C (5 mg) at room temperature. The mixture was stirred at room temperature for 5 hours, filtered, diluted with EtOAc (10 mL) ,washed with water, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether: EtOAc = 2:1) to yield the title compound as an oil (12 mg, 0.02 mmol, 60%) [10].

2.2 *Bile Sample Collection and Preparation*

Eight rats, with the average body weight of 200 g were fasted for 12 h before dosing. All rats were implanted with a PE-10 cannula into the bile duct under anesthesia by ethyl ether, and then allowed to recover for 1 h before dosing. The blank bile was collected before administration. After recovery from the surgery, the pre-prepared drug solution was injected into vena caudalis at a dose of 6.0 mg·kg^{-1}. The bile was collected on ice for 12 h. The rats were given water occasionally during the course of bile collection [7].

Add 50.0 μL of rat bile into an Eppendorf tube, then add 1.0 mL of ethyl acetate, vortexed for 3 min and centrifuged at 3 500 r·min^{-1} for 10 min. Transfer 0.7 mL of the supernatant to another tube, evaporated to dryness. The residue was redissolved in 250.0 μL of methanol. An aliquot of 5.0 μL was injected into the LCMS-IT-TOF system.

2.3 *Instruments and Analytical Condition*

LCMS-IT-TOF system: The liquid chromatograph mass spectrometer equipped with an auto-injector (SIL-20AC), a Shimadzu solvent delivery pump (LC-20AB), a Shimadzu CTO-20AC column oven, a Shimadzu DGU-20A online degasser and a semi-micro mixer. The separation of gambogic acid and its metabolites was carried out on a Shim-pack FC-ODS (2.0 mm×150 mm) at 40 ^{o}C. The flow rate was 0.2 mL·min^{-1}. The mobile phase consisted of acetonitrile and purified water containing 0.05% acetic acid. The time program was described as follows: 20%B (0 min)-60%B (15 min)-20%B (15.10 min)-STOP (35 min). The MS acquisition was performed in scan mode of negative ions. The optimized MS operating conditions were selected as follows: CDL (curved desolvation line) temperature: 200 ^{o}C; applied volt-

age: +4.5 kV / –3.5 kV; CDL voltage: constant mode; analysis mode: MS1 measurement; Measurement range: auto MSMS mode; MS: *m/z* 100-1 000; MSMS: *m/z* 50-1 000; ion accumulation time: 30 msec; drying gas pressure：0.1 MPa; each setting of voltage for ionization mode is optimized by auto-tuning.

3 Results and discussion

3.1 *Metabolic Profiles in Rat Bile*

The characterization of the metabolites of gambogic acid was performed by LCMS-IT-TOF using the rat bile samples. Three metabolites, M1, M2 and M3, were identified from the bile samples. The resulting total ion chromatograms and the MS spectra of gambogic acid and the three metabolites were shown in Fig. 2 and Fig. 3.

3.2 *Fragmentation Mechanism of Gambogic acid*

Gambogic acid was observed as its deprotonated molecule $[M - H]^-$ at *m/z* 627.2982 (calculated for $C_{38}H_{43}O_8$ 627.295 8) with a retention time of 13.5 min. The product ions spectra of the deprotonated molecules of gambogic acid were shown in Fig. 4. MS^2 demonstrated the major product ion of gambogic acid at *m/z* 459, which corresponded to the side chains of ring A and ring D loss from the parent ion *m/z* 627. MS^3 demonstrated the major product ion of gambogic acid at *m/z* 349, which corresponded to the split of ring A and

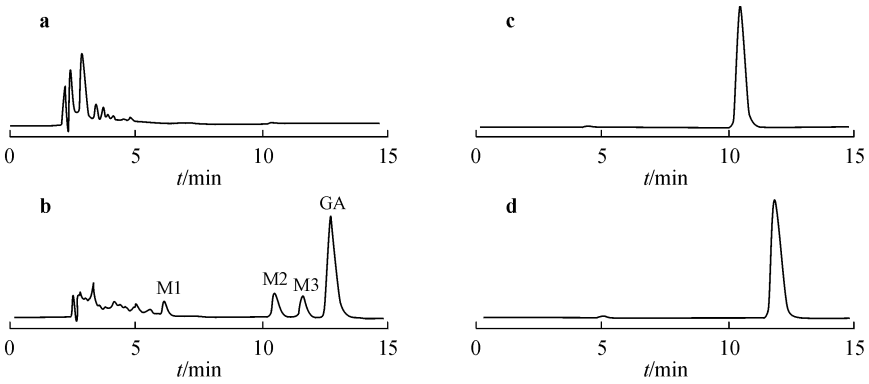

Fig. 2 HPLC chromatograms of the blank bile of rat (a), the bile sample of rat after GA iv dose (b), 10-hydroxy-gambogic acid (c), 9, 10-epoxy-gambogic acid (d)

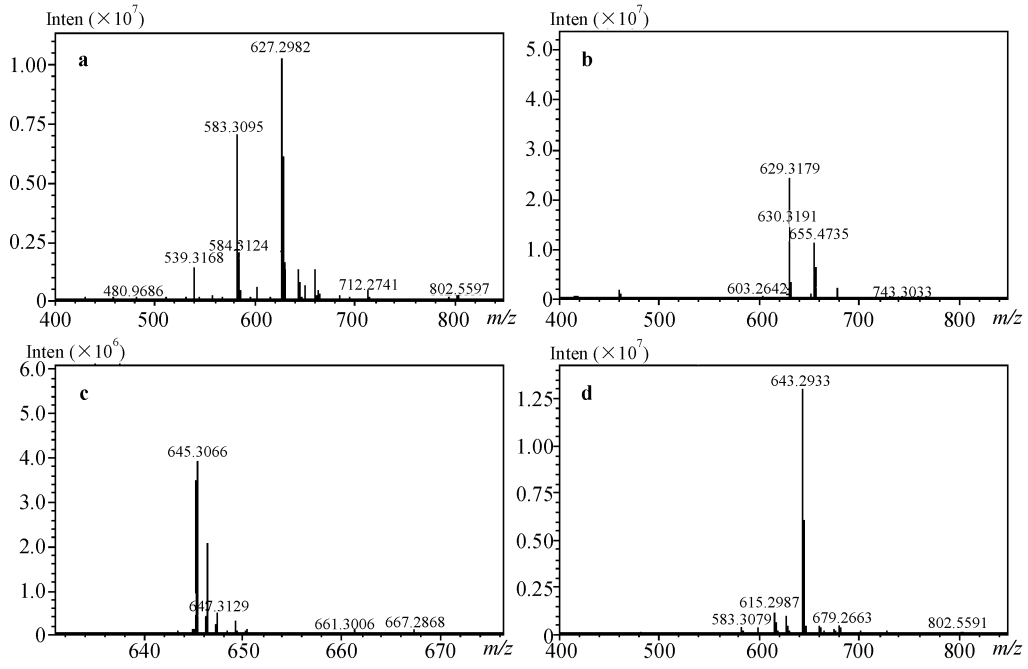

Fig. 3 Mass spectra of the depronated molecules of gambogic acid (a), M1 (b), M2 (c) and M3 (d)

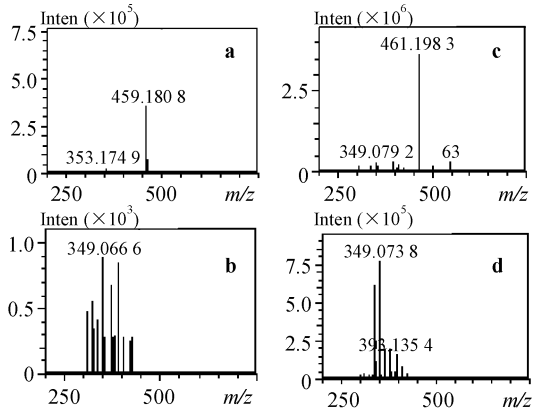

Fig. 4 MS^2 (a) and MS^3 (b) product ions spectra of the deprotonated molecules of gambogic acid, MS^2 (c) and MS^3 (d) product ions spectra of the deprotonated molecules of M1.

ring F. The proposed fragmentation mechanism of gambogic acid was illustrated in Fig. 5.

3.3 *Structural elucidation of M1*

The mass spectra of M1 exhibited deprotonated molecule [M – H]$^-$ at *m/z* 629.317 8 (calculated for $C_{38}H_{45}O_8$ 629.311 4), indicating that M1 may be the hydrogenised metabolite of gambogic acid. The MS^2 and MS^3 of M1 exhibited the fragment ions at *m/z* 461 and 349, respectively. Compared with the major product ions of gambogic acid, the hydrogenised moiety was not on the side chains of ring A and ring D, but on ring A. Therefore, M1 was tentatively postulated to be 3, 4-dihydro-gambogic acid.

3.4 *Structural determination of M2*

The mass spectra of M2 exhibited deprotonated molecules [M – H]$^-$ at *m/z* 645.306 6 (calculated for $C_{38}H_{45}O_9$ 645.306 4), indicating that M2 may be the hydrated metabolite of gambogic acid. The MS^2 and MS^3 of M2 exhibited the fragment ions at *m/z* 477 and 367, respectively. Compared with the major product ions of gambogic acid, the hydrated moiety was not on the lost side chains, indicating that M2 was probably 10-hydroxy-gambogic acid. By comparing the tandem mass spectra and the chromatographic retention time with those of the respective authentic compound, M2 was unambiguously confirmed to be 10-hydroxy-gambogic acid.

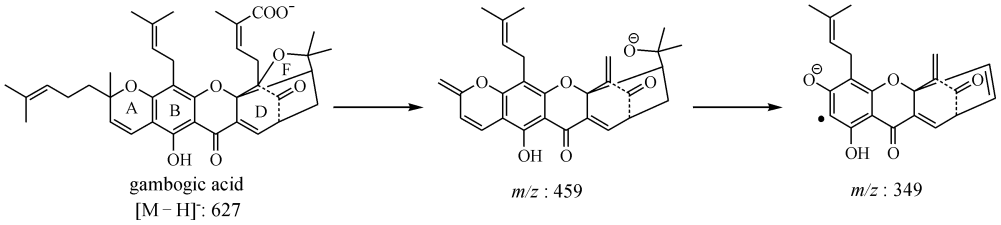

Fig. 5 Fragmentation mechanism of gambogic acid.

3.5 *Structural Determination of M3*

The mass spectra of M3 exhibited deprotonated molecules [M – H]$^-$ at *m/z* 643.293 3 (calculated for $C_{38}H_{43}O_9$ 643.290 7), indicating that M3 may be the hydroxylated or epoxidated metabolite of gambogic acid. The MS^2 and MS^3 of M3 exhibited the fragment ions at *m/z* 475 and 365, respectively. By comparing with the major product ions of gambogic acid, the hydrated moiety was not on the lost side chains, indicating that M3 was probably 9,10-epoxy-gambogic acid. By comparing the tandem mass spectra and the chromatographic retention time with that of the respective authentic compound, M3 was unambiguously confirmed to be 9,10-epoxy- gambogic acid.

4 Conclusions

In the present study, three metabolites of gambogic acid were characterized in rat bile. The results showed the formation of one hydrogenized metabolite (M1), one hydrated metabolite (M2) and one oxidative metabolite (M3). M1 was firstly reported, while the structures of M2 and M3 were confirmed by comparing the tandem mass spectra and the chromatographic retention time with those of the respective authentic compounds.

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