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Identification of guanfu base A hydrochloride phase I and phase II metabolites in rat bile by liquid chromatography mass spectrometry¹

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KEY WORDS guanfu base A hydrochloride; glucuronides; sulfates

ABSTRACT

AIM: To study metabolites of guanfu base A hydrochloride (GFA) in rat bile. **METHODS:** An analytical method was developed to identify guanfu base A and its metabolites by liquid chromatography mass spectrometry (LC-MS) and electrospray ionization tandem mass spectrum (MS-MS). Rat bile was collected after iv injection of GFA. Phase I metabolite was identified by comparison with authentic standard for their retention time, molecular ion peaks, fragment ions, and UV spectrums. In order to identified Phase II conjugates, the aglycones were identified after rat bile was treated with either glucuronidase or sulfatase firstly. Phase II conjugates were also separated and determined for their molecular ions by LC-MS, at last they were verified by identifying characteristic product ions or precursor ions by MS-MS. **RESULTS:** Phase I metabolite, guanfu base I (GFI), was identified in rat bile. After phase II conjugates were treated with glucuronidase or sulfatase, GFA and GFI occurred in chromatograms. Quasi molecular ions m/z 606 and 510 were separated and detected in rat bile. They were indicated to be GFA glucuronide and GFA sulfate, respectively. Furthermore, GFA glucuronide was confirmed to exist in rat bile by identifying two characteristic ions, m/z 177, $[M+H]^+$ of glucuronic acid, and m/z 430, $[M+H]^+$ of GFA, as product ions of m/z 606. **CONCLUSION:** Phase I metabolite GFI and phase II conjugates, GFA glucuronide and sulfate, GFI glucuronide and sulfate, were identified in rat bile.

INTRODUCTION

Guanfu base A (GFA) is a novel anti-arrhythmic drug and is in the fourth period of clinical study now. Preclinical pharmacological study showed that various experimental ventricular arrhythmia could be protected

or arrested by GFA^[1-3]. Further electrophysiological experiment discovered GFA blocked the fast Na^+ channel and exhibited anti-arrhythmic action by direct effect on sinoatrial node^[4-6]. Besides GFA, guanfu base G (GFG) and guanfu base I (GFI) were also isolated from the tuber of *Aconitum coreanum* (Levl) Raipaics^[7]. They were all esters of the same structure of C_{20} -diterpenoid, and only differed in number of acetyls. Compared with GFA, GFG showed much more potent anti-arrhythmic effect and more toxicity, while GFI was of less potent anti-arrhythmic effect with less toxicity^[8]. It is well know that drug metabolism plays

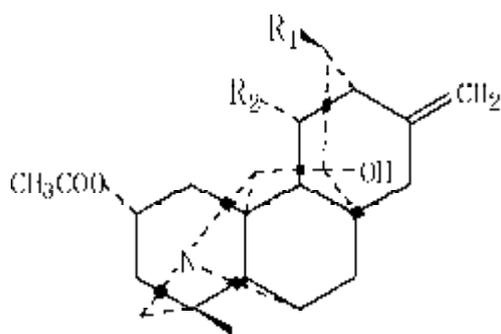
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an important role in pharmacodynamics and toxicity. However, the fate of GFA remains unknown in rats. In order to learn metabolites of GFA *in vivo*, rat biles were collected and the metabolites were investigated using liquid chromatography mass spectrometry (LC-MS) and tandem mass spectrometry (MS-MS).



	R ₁	R ₂	M _r
GFG	CH ₃ COO	CH ₃ COO	471
GFA	CH ₃ COO	OH	429
GFI	OH	OH	387

Structure of guanfu base A, guanfu base G, and guanfu base I.

MATERIALS AND METHODS

Chemicals and reagents Reference standards of GFA and GFI were provided by study group of GFA, China Pharmaceutical University; β -glucuronidase (type B-1), sulfatase (type H-1), and *D*-saccharic acid β -1,4-lactone were purchased from Sigma Chemical Co; acetonitrile: HPLC grade; Na₂CO₃, NaHCO₃, acetic acid, diethylamine, and ethyl acetate were analytical grade.

Equipments Hewlett Packard 1100 liquid chromatography mass spectrometry system, with binary pump, on-line vacuum degasser, autosampler, diode array detector (DAD), and mass spectrum detector (MSD) and PE API2000 tandem mass spectrometry (MS-MS), with syringe pump were used.

Rat bile sampling Male Sprague-Dawley rats (180-220 g, Grade II, Certificate No 006) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Science. They were housed for 1 week prior to the study. After starving overnight, rats were rendered unconscious with diethylether and anesthetized with urethane (20 %, w/v) in saline. The bile duct was cannulated as described by Mulder^[8] *et al*. Bile samples were collected from 1 to 8 h in test tubes

after rats were injected with guanfu base A hydrochloride (30 mg·kg⁻¹ body weight). All samples were frozen immediately and kept at -20 °C until use.

Extraction procedure Saturated Na₂CO₃/NaHCO₃ solution 0.2 mL was added in pooled bile 0.5 mL. The sample was mixed and extracted twice with ethyl acetate 3 mL using vortex for 2 min, and centrifuged at 1000×g for 5 min. The organic layer was combined and dried under a gentle flow of nitrogen at 50 °C. The organic extract was dissolved in mobile phase 0.2 mL, centrifuged at 10 000×g for 5 min. The upper solution 20 μ L was injected and analyzed by LC-MS.

Incubation with glucuronidase or sulfatase

After bile was extracted as in "extraction procedure." Residual bile were re-extracted with ethyl acetate 3 mL for 2 min to ensure GFA and its phase I metabolites were removed completely. To verify this, the organic layer was dried, and then the extract was analyzed by MS-MS. After extraction, the residual bile was adjusted to pH 5.0 with 20 % (v/v) acetic acid. Then aliquots of extracted residual bile was treated with 1) β -glucuronidase (1000 U); 2) β -glucuronidase in presence of *D*-saccharic acid β -1, 4-lactone 10 mmol/L (the specific inhibitor of β -glucuronidase); 3) Inactivated (boiled) glucuronidase used as control; 4) Sulfatase (50 U) in presence of *D*-saccharic acid β -1,4-lactone 10 mmol/L (there was some glucuronidase contained in sulfatase); 5) Inactivated (boiled) sulfatase used as control, in water bath at 37 °C for 12 h, respectively.

After hydrolysis (either with glucuronidase, sulfatase, or control), the reaction solution were extracted and analyzed by LC-MS.

Direct determination Extracted residual bile 1 mL was filtrated after centrifugation for 10 min (10 000×g), then was freeze dried. The residue was dissolved in mobile phase 0.1 mL, and 30 μ L was injected and analyzed by LC-MS in Scan mode. Extracted residual bile 1 mL was filled in syringe pump, analyzed by MS-MS. The product ions of *m/z* 606 were investigated.

HPLC condition Column: C8-3, 5 μ m, 250 mm×2.1 mm; Flow rate: 0.25 mL/min; Column temperature: 25 °C; Mobile phase 1: CH₃CN-H₂O (pH 3.7-4.0) (12:88); Mobile phase 2: CH₃CN-H₂O (5:95).

MSD parameters Ionization Mode: electrospray ionization (ESI); Detect ion: positive; Drying gas flow: 10 L/min; Nebulizer pressure: 40 psig; Drying gas temperature: 350 °C; Capillary voltage: 4000 V. Selected-ion monitoring (SIM) parameters: *m/z* 430 (GFA);

m/z 388 (GFI).

PE API2000 MS-MS Detect mode: scan; Detect ions: positive; Syringe injection rate: 40 $\mu\text{L}/\text{min}$; Curtain gas: 20 psig; Ion spray voltage: 4500 V; Temperature: 450 $^{\circ}\text{C}$; Ion source gas 1: 15 psig; Ion source gas 2: 40 psig.

RESULTS

Phase I metabolites Two peaks (M1, M2) were observed both on LC-MS total ion chromatogram (TIC, Fig 1A), M1 was attribute to m/z 388 (Fig 1B), and M2 was attribute to m/z 430 (Fig 1C), while the two peaks were not found for blank bile.

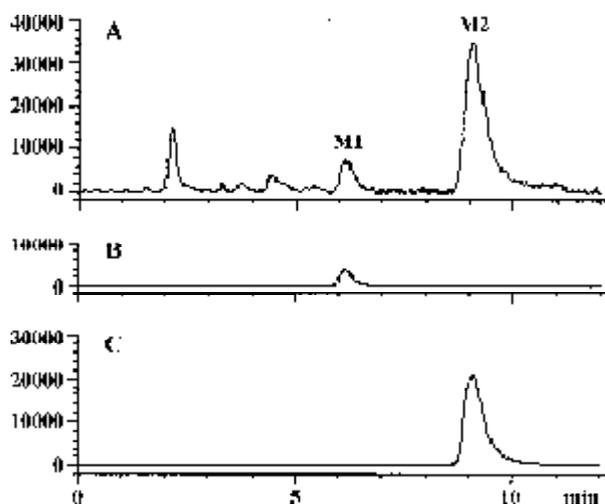


Fig 1. TIC of rat bile extract. A: SCAN; B: SIM (m/z 388); C: SIM (m/z 430).

Compound M1 showed the same retention time as GFI standard. Positive mass spectra of M1 showed its molecular ion peaks, $[\text{M}+\text{H}]^+$: m/z 388, $[\text{M}+\text{Na}]^+$: m/z 410, and $[\text{M}+\text{K}]^+$: m/z 426 (Fig 2A), in agreement with the molecular weight of GFI being 387. Besides, In SCAN mode, M1 characteristic fragment ions (CID 200V) (Fig 2B) were the same as that of the GFI standard. Moreover, M1 UV spectrum (Fig 2C) was the same as that of GFI standard. So M1 was identified as GFI. Compound M2 was identified as GFA by the same method as above. These results demonstrated the presence of GFI and GFA in the bile. GFA was the intact drug, while GFI was a metabolite of GFA, ie, a phase I metabolite.

Phase II conjugates After the bile was extracted, there was little GFA in the last organic extract (Fig 3A). It was to say organic solvent extraction had removed

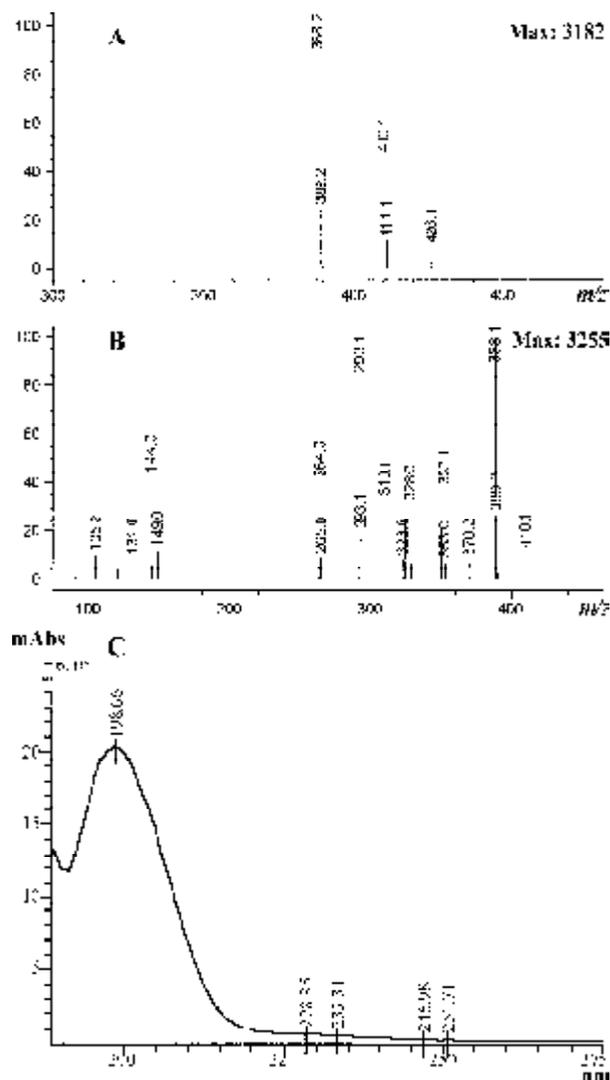


Fig 2. Spectrum of M1. A: ESI (+) mass spectrum (fragmentor 70 V); B: ESI (+) mass spectrum (CID 200 V); C: UV spectrum, consistent with authentic standard, GFI.

unconjugated phase I metabolites almost completely.

Glucuronides After one aliquot of bile was treated with glucuronidase, GFA and GFI were determined in the chromatogram (Fig 3B). Only a little of GFA and trace of GFI existing in the extracts of the other two aliquots treated with either glucuronidase in presence of its specific inhibitor *D*-saccharic acid β -1, 4-lactone or inactivated glucuronidase. It was indicated that there were glucuronide conjugates of both GFA and GFI in rat bile.

Sulfates GFA and GFI were found after one aliquot was treated with sulfatase (Fig 3C). A little of GFA and trace of GFI appeared after the other aliquot incubated with inactivated sulfatase. It was indicated that there were sulfate conjugates of both GFA and GFI

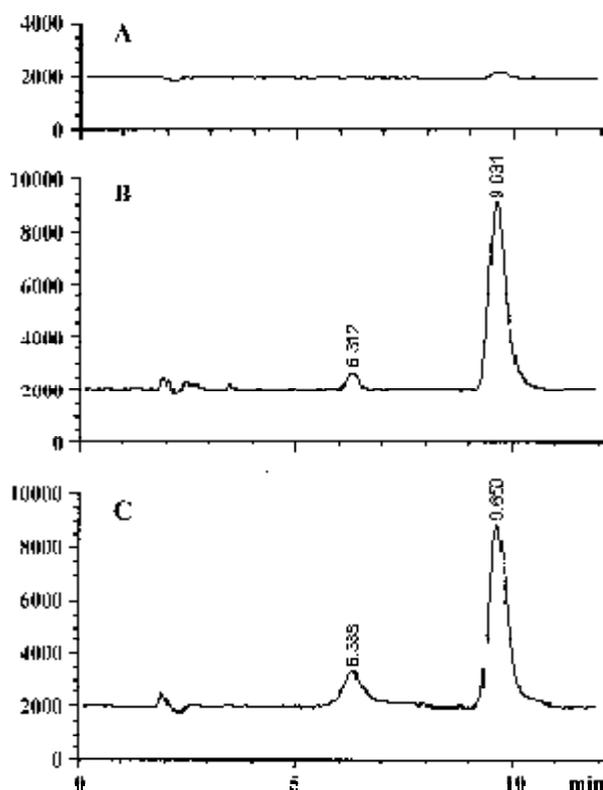


Fig 3. TIC of A: the last extract of rat bile before incubating with glucuronidase; B: rat bile after incubating with glucuronidase; C: rat bile after incubating with sulfatase in presence of sacchrolactone.

in rat bile.

Direct identification of phase II conjugates

LC-MS TIC showed a peak M3 (Fig 4A). Its positive molecular ion was m/z 606 with two-isotope ions m/z 607 and 608 (Fig 4B), which was consistent with the molecular weight of GFA-glucuronide. The compound was indicated to be GFA-glucuronide. The other TIC showed a peak M4 (Fig 5A). Its positive molecular ion was m/z 510 with an isotope ion m/z 511 (Fig 5B), which was consistent with the molecular weight of GFA-sulfate. The compound was indicated to be GFA-sulfate.

MS-MS Two characteristic ions, m/z 430 and m/z 177 were identified as the product ions of m/z 606. Ion m/z 430 was the protonated ion of GFA, while m/z 177 was the protonate ion of glucuronic acid (Fig 6). It was verified that there was GFA-glucuronide in rat bile.

DISCUSSION

It is well known that hydrolysis of esters can take

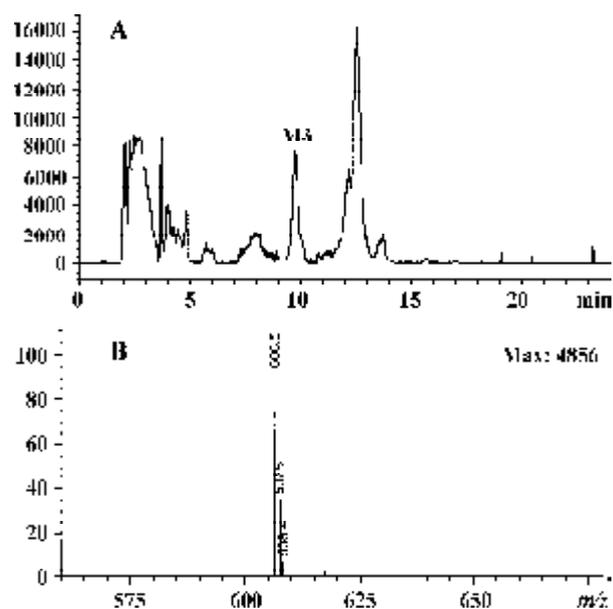


Fig 4. Metabolite M3 in rat bile. A: TIC; B: ESI (+) mass spectrum, consistent with the molecular weight (m/z 605) of GFA glucuronide.

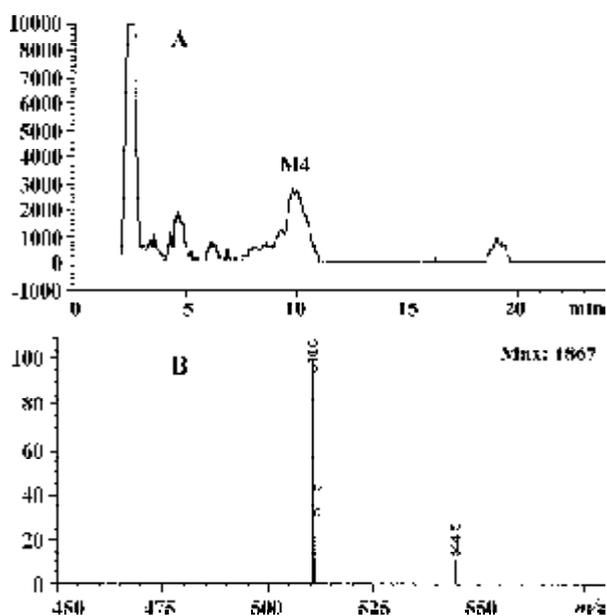


Fig 5. Metabolite M4 in rat bile. A: TIC; B: ESI (+) mass spectrum, consistent with the molecular weight (m/z 509) of GFA sulfate.

place in plasma by non-specific esterases or in liver by specific esterases. However, it was not clear whether GFI, which was found in rat bile, was a hydrolyte or metabolite of GFA. Now we are making further experiments to study the mechanism of GFA in metabolic model *in vitro* using rat liver microsomes.

Four phase II conjugates, GFA and GFI of both glucuronides and sulfates were found in rat bile.

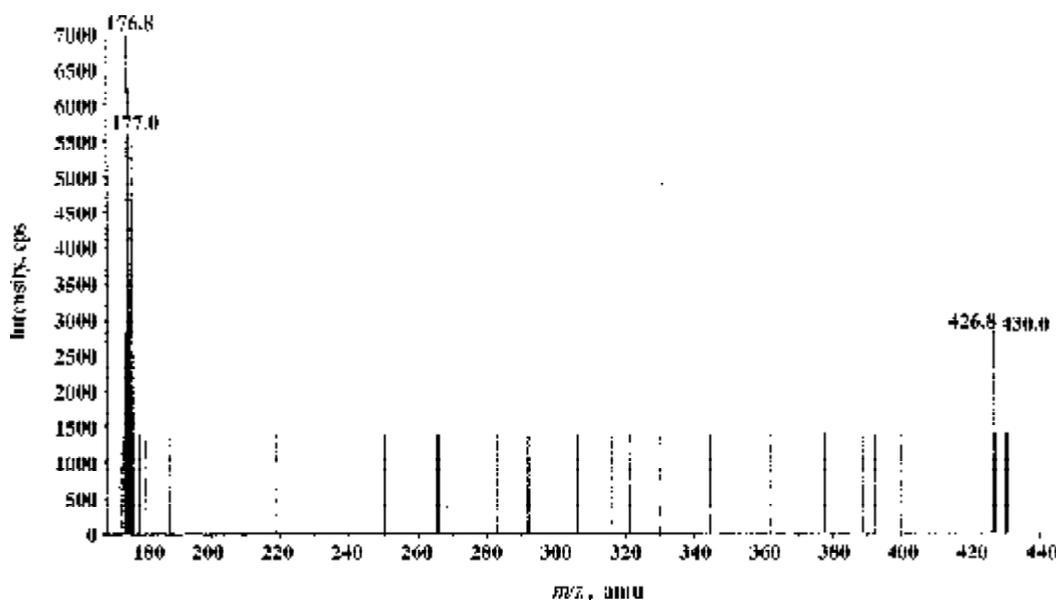


Fig 6. Product ions of m/z 606, both m/z 430, the protonated ion of GFA, and m/z 177, the protonated ion of glucuronic acid, were identified in MS/MS.

Generally, phase II conjugation always leads to a water-soluble product which can be excreted in bile or urine. There are several forms of glucuronidation, such as *O*-glucuronides, *N*-glucuronides, *S*-glucuronides, and even *C*-glucuronides. *O*-glucuronides form from phenol, alcohols, and carboxylic acids. *N*-glucuronides form from amines, amides, and sulfonamides. Sulfation is also a major conjugation path for alcohols and amines. GFA and GFI are not only alcohols, but also amines. There are proper functional groups in GFA or GFI to form the two kind of conjugates. Therefore, which position GFA or GFI link with glucuronic acid or sulfuric acid, and the mechanisms of transformation still re-

mains to be elucidated.

According to the metabolites indicated above, the pathway of GFA was proposed in rat (Fig 7).

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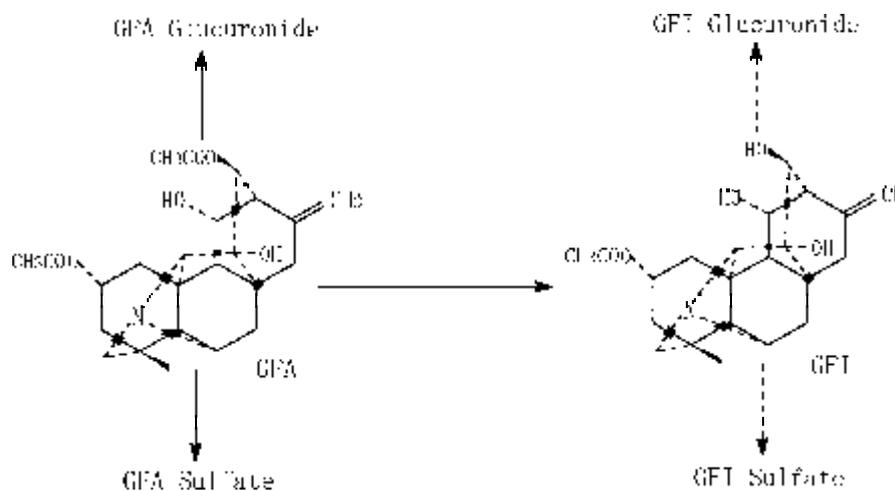


Fig 7. Proposed pathway of GFA in rat.

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