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Identification and quantitative determination of a major circulating metabolite of gambogic acid in human

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ABSTRACT

Gambogic acid (GA), a promising anticancer candidate, is a polyprenylated xanthone abundant in the resin of *Garcinia morella* and *Garcinia hanburyi*. The major circulating metabolite of GA in human, 10-hydroxygambogic acid (10-OHGA), was identified by comparison of the retention time and mass spectra with those of reference standard using liquid chromatography–tandem mass spectrometry. The reference standard of 10-OHGA was isolated from bile samples of rats after intravenous injection of GA injection, and its structure was confirmed by NMR. Then, a selective and sensitive method was developed for the quantitative determination of this metabolite in human plasma. After liquid–liquid extraction by ethyl acetate, the analyte and the internal standard were separated on a Sepax HPC18 column (100 mm × 2.1 mm i.d., 3.0 μm) with a mobile phase of 10 mM ammonium acetate water solution containing 0.1% formic acid–acetonitrile (20:80, v/v). The detection was performed on a single quadrupole mass spectrometer equipped with electrospray ionization (ESI) source. The calibration curve was linear over the range of 3–2000 ng/mL for 10-OHGA. The developed quantification method can now be used for the pharmacokinetic and pharmacological studies of 10-OHGA after intravenous infusion of GA injection in human.

1. Introduction

Gambogic acid (GA, see Fig. 1) is a polyprenylated xanthone abundant in the resin of *Garcinia morella* and *Garcinia hanburyi* with a long history of use as a complementary and alternative medicine [1,2]. The antitumor activity of GA has been well demonstrated [3–5]. Many reports suggest that GA inhibits angiogenesis and may be a viable drug candidate in antiangiogenesis and anticancer therapies [6–8], while others demonstrate that GA inhibits NF-κB, a protein complex that controls the transcription of oncogene, and potentiates apoptosis through its interaction with the transferrin receptor [9–11].

In vitro study [12] indicates that GA is rapidly metabolized in rat liver microsome and a hydration metabolite is crucial for the elimination of GA and CYP1A2 is responsible for this pathway. In vivo studies [13,14] suggest that GA is metabolized to two main phase I metabolites in rat bile and their structures are elucidated as 10-hydroxygambogic acid (10-OHGA, see Fig. 1) and 9,10-epoxygambogic acid. However, human metabolic study of GA has never been reported so far. In this study, we identified 10-OHGA as the major circulating metabolite of GA in human.

To further characterize the clinical pharmacokinetic and pharmacodynamic profile of GA in post-approval phase II studies, the quantification methods with selectivity and sensitivity sufficient for determination of GA and its main circulating metabolite in human plasma are indispensable in order to consider the efficacy and safety of these compounds after administration of GA injection in human [15]. Several HPLC–UV methods have been described for quantification of GA in rat plasma and tissues [16] and in dog plasma [17]. Previously, we have reported an LC–APCI–MS method for quantification of GA in human plasma and it has been successfully applied for the clinical pharmacokinetic study [18]. However, the quantitative method for the determination of GA metabolite in bio-fluid has not been reported up to now. Thus, the main purpose of this study is to develop an LC–MS method for the determination of the identified circulating metabolite of GA in human plasma for pharmacokinetic study.

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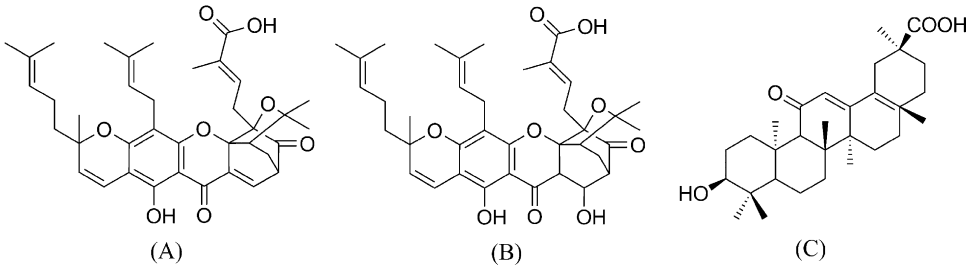

Fig. 1. Chemical structures of GA (A), 10-OHGA (B), and glycyrrhetic acid (C).

2. Experimental

2.1. Chemicals and reagents

GA was kindly supplied by Kanion Pharmaceutical Co., Ltd. (Lianyungang, China). Glycyrrhetic acid, the internal standard (IS, see Fig. 1), was purchased from the National Institution for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile was obtained from Merck KGaA (Darmstadt, German). Ammonium acetate was analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (SCRC, Shanghai, China). Formic acid and ethyl acetate were analytical grade purity and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

2.2. Instrument and conditions

2.2.1. LC–DAD–MS/MS for the qualitative analysis

The HPLC was performed using a Finnigan Surveyor LC Pump with an autosampler (Thermo Finnigan, San Jose, CA, USA). Isocratic chromatographic separation was performed on a Luna C18 column (150 mm × 2.0 mm i.d., 3.0 μm, Phenomenex, Torrance, CA, USA) with a security C18 guard column (4 mm × 2.0 mm i.d., Phenomenex, Torrance, CA, USA); the mobile phase was composed of acetonitrile–10 mM ammonium acetate (68:32, v/v) with a flow rate of 0.25 mL/min. The column temperature was maintained at 25 °C. Sample injection volume was 10 μL. The DAD detector was used in the qualitative analysis and absorption was measured at 320 nm.

A TSQ Quantum Discovery MAX triple-quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an ESI source, was used in negative ion mode with full scan and product scan for the further identification. The spray voltage was set to −3.0 kV, and the capillary temperature was maintained at 320 °C. Nitrogen was used as the sheath gas (30 Arb) and auxiliary gas (10 Arb) for nebulization. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of 1.5 mTorr. The collision energy in the in-source CID mode was set at 7 eV. Analytical data were acquired using Xcalibur 2.0.7 software.

2.2.2. LC–ESI–MS for the quantitative analysis

The LC–ESI–MS method was performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA, USA), including an Agilent 1100 G1312A binary pump, vacuum degasser (model G1322A), G1316A injection temperature controlled column compartment, Agilent 1100 autosampler (model G1313A), and an Agilent 1100 MSD single quadrupole mass spectrometer equipped with an electrospray source (model G1956B). A Sepax HPC18 column, 100 mm × 2.1 mm i.d., 3 μm (Sepax Technologies, Inc, Newark, USA) protected by a SecurityGuard C18 column, 4 mm × 2.0 mm i.d., 5 μm (Phenomenex, Torrance, CA, USA) was used for all measurements. The Signal acquisition, peak integration and concentration determination were performed using the ChemStation software (10.02 A) supplied by Agilent Technologies.

The mobile phase was 10 mM ammonium acetate water solution containing 0.1% formic acid–acetonitrile (20:80, v/v) at a flow rate of 0.3 mL/min. The column temperature was maintained at 35 °C. The LC–ESI–MS was carried out using nitrogen to assist nebulization. The quadrupole mass spectrometer equipped with an ESI source was set with the drying gas (N_2) flow of 10 L/min, nebulizer pressure of 40 psig, drying gas temperature of 350 °C, capillary voltage of 3.5 kV and the negative ion mode. The fragmentor voltage was set at 140 V. The ESI–MS was performed in the selected-ion monitoring (SIM) mode using the target ions of m/z 627.3 for 10-OHGA and m/z 469.3 for glycyrrhetic acid (IS), respectively.

2.3. Preparation of 10-OHGA reference standard

The reference standard 10-OHGA was isolated from bile samples of rats after intravenous injection of GA injection and purified by preparation HPLC and its chemical structure was confirmed by UV, ESI–MS/MS and ^{1}H NMR.

2.3.1. Bile collection

Under light anesthesia with chloral hydrate (350 mg/kg), bile fistulas of male Sprague–Dawley rats (Qinglongshan Experimental Animal Center, Nanjing, China) were cannulated with PE-5 polyethylene tubing for collection of bile. The rats were allowed about 1 h to recover from anesthesia before receiving a 2 mg/kg intravenous dose of GA injection in saline. The bile samples were collected in 0–12 h post-dose into polypropylene vials on ice. All the biliary samples were pooled and stored at −20 °C until analysis. The animal study was approved by the Animal Ethics Committee of China Pharmaceutical University.

2.3.2. Isolation of 10-OHGA

The combined bile samples were extracted with equal volume ethyl acetate. The organic phase was collected and evaporated to dryness and then the residue was dissolved in 30 mL acetonitrile. The reconstituted solution was subjected to P6000™ preparation HPLC (C18, 150 mm × 40.5 mm i.d.,10 μm, ChuangXinTongHeng Science And Technology Co., Ltd., Beijing, China) equipped with A1359 manual sampler and a UV6000 detector (ChuangXinTongHeng Science And Technology Co., Ltd., Beijing, China) with a detection wavelength set at 320 nm. The separation was performed with a mobile phase of acetonitrile–water (80:20, v/v) at flow rate 10 mL/min, and the metabolite 10-OHGA was collected at the time region of 32–36 min. Six injections were made. The eluted samples were collected into the glass vials and evaporated to dryness, and 11 mg residue of 10-OHGA was obtained. The purity of 10-OHGA was 97% based on HPLC analysis by area normalization method. The NMR spectra were recorded at 303 K on Bruker ACF-500 NMR spectrometer (500 MHz) equipped with a 5 mm probe, using DMSO-d_6 as solvent and TMS as internal standard.

The spectra data of the isolated metabolite ($C_{38}H_{46}O_9$, light yellow powder) were as follows:

UV λ_{max} (nm): 275, 320 (acetonitrile). ESI–MS (neg.): MS *m/z* 645, MS/MS *m/z* 627, 583, 539. ^{1}H NMR δ:12.02 (1H, br, H-30), 6.56(1H, d, *J* = 10H, H-3), 6.49(1H, t, *J* = 10 Hz, H-27), 5.79(1H, br, H-6), 5.61(1H, d, *J* = 10H, H-4), 5.09(1H, t, *J* = 13 Hz, H-32), 4.99(1H, t, *J* = 13 Hz, H-37), 4.56(1H, d, *J* = 5 Hz, H-10), 3.26(2H, m, H-31), 3.05(2H, m, H-26), 2.52(1H, d, *J* = 5.5 Hz, H-9), 2.44(1H, d, *J* = 8.5 Hz, H-11), 2.03(2H, t, *J* = 13 Hz, H-36), 1.85(1H, m, H-22), 1.83(3H, s, H-29), 1.70(3H, s, H-39), 1.66(2H, m, H-21), 1.60(3H, s, H-34), 1.59(3H, s, H-40), 1.50(3H, s, H-35), 1.33(3H, s, H-24), 1.25(3H, s, H-25), 1.03(3H, s, H-19). The spectra data of the isolated metabolite were consistent with that of 10-OHGA reported in the literatures [13,14].

2.4. Preparation of standard and quality control sample

The stock solution of 10-OHGA was prepared in acetonitrile to give a concentration of 1.0 mg/mL. The solution was then successively diluted with acetonitrile to achieve standard working solutions at concentrations of 0.1, 1.0, 10, 100 μg/mL for 10-OHGA. The IS working solution (5.0 μg/mL) was prepared by diluting the 1 mg/mL stock solution of the IS also with acetonitrile. All the solutions were stored at −20 °C and were brought to room temperature before use. Matrix-matched calibration standards of 10-OHGA at concentration levels of 3, 10, 30, 100, 300, 1000, and 2000 ng/mL were constructed by spiking appropriate amount of 5–15 μL working solutions in seven aliquots of 0.5 mL blank human plasma, respectively. All quality control samples (QCs) used in the validation and during the pharmacokinetics study were prepared in the same way as the calibration standards before analysis. Plasma concentrations of the QCs were 5, 500, and 1600 ng/mL for 10-OHGA. The spiked plasma samples (the standards and QCs) were prepared freshly and extracted on each analytical batch along with the unknown samples.

2.5. Sample preparation

2.5.1. Qualitative analysis

For metabolites identification, plasma samples collected 0–4 h after GA intake were used (see Section 2.7). A 1 mL aliquot plasma sample was extracted with 4 mL ethyl acetate. Following centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen in a water bath of 30 °C. The residue was reconstituted in 100 μL of mobile phase, and a 10 μL aliquot was injected onto the LC–DAD–MS/MS system.

2.5.2. Quantitative analysis

To a 0.5 mL aliquot plasma sample, 30 μL of internal standard (5 μg/mL glycyrrhetic acid in acetonitrile) was added. The mixture was vortex mixed for 30 s, and vortex extracted with 3 mL of ethyl acetate for 5 min. After centrifugation at 4000 rpm for 10 min, the organic phase was transferred into another tube and evaporated to dryness under a stream of nitrogen in a water bath of 30 °C. The residue was reconstituted in 120 μL of the mobile phase. A 4 μL aliquot of the solution was injected onto the LC–ESI–MS system.

2.6. Method validation

The selectivity of the method was evaluated by comparing the chromatograms of the blank plasma samples from six different batches with the corresponding spiked plasma samples. Each blank plasma sample was tested using the proposed preparation procedure and the LC–ESI–MS conditions to ensure no interference of the analyte from the plasma.

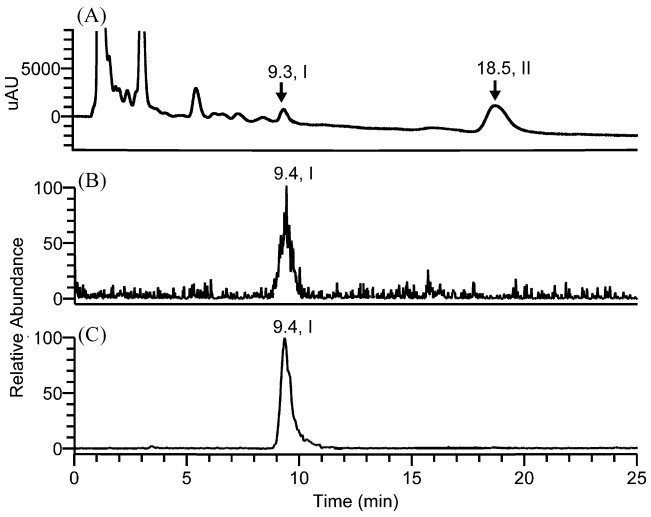

Fig. 2. HPLC chromatograms of human plasma sample after intravenous infusion of GA injection (45 mg/m^2), (A) UV chromatogram at 320 nm, chromatographic peaks of 10-OHGA (I) and GA (II) were directed by the black arrows respectively; (B) extraction ion (*m/z* 645) chromatogram of 10-OHGA (I), and (C) extraction ion (*m/z* 645) chromatogram of the authentic standard of 10-OHGA.

The matrix effect of the method was evaluated by comparing the peak areas of the analytes resolved in blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution) (*A*) with that resolved in mobile phase (*B*). The ME was calculated by using the formula: ME (%) = $A/B \times 100\%$. Three different concentration levels of 10-OHGA at 5, 500 and 1600 ng/mL and the IS at 300 ng/mL were evaluated by analyzing five replicates at each level. The blank plasmas used in this study were from five different batches of healthy human blank plasma.

The extraction recoveries (*R*) of 10-OHGA at 5, 500 and 1600 ng/mL and the IS at 300 ng/mL were determined by comparing two different sets of samples. In set 1, the analytes were spiked to the blank plasma and prepared as the procedures described in Section 2.5, and the obtained peak areas of the analytes were defined as A. In set 2, the analytes were resolved in the mobile phase, and the obtained peak areas of the analytes were defined as *B*. The extraction recoveries were calculated using the formula: *R* (%) = $A/B \times 100\%$.

Calibration curves were constructed by analyzing spiked calibration samples. Samples were quantified using the ratio of the peak area of 10-OHGA to that of the IS. The peak area ratios were plotted against nominal concentration of the analyte, and standard curves were calculated using linear regression analysis with $1/x^2$ weighting.

Accuracy and precision were assessed by the determination of QCs at three concentration levels in five replicates in three validation batches. The precision was expressed by relative standard deviation (*RSD*) and the accuracy by relative error (*RE*). The intra- and inter-batch precisions were required to be below 15%, and the accuracy to be within ±15%, except at the lower limit of quantification (LLOQ), where precision should be below 20% and accuracy within ± 20%.

The stabilities of 10-OHGA in human plasma were evaluated by analyzing replicates ($n = 3$) of plasma samples at the concentrations of 5.0, 500, 1600 ng/mL for 10-OHGA which were exposed to different conditions. These results were compared with those obtained from freshly prepared plasma samples. The analytes were considered stable in the biological matrix when 85–115% of the initial concentrations were found. The short-term stability was determined after the exposure of the spiked samples at room temperature for 8 h and the ready-to-inject samples (post preparation)

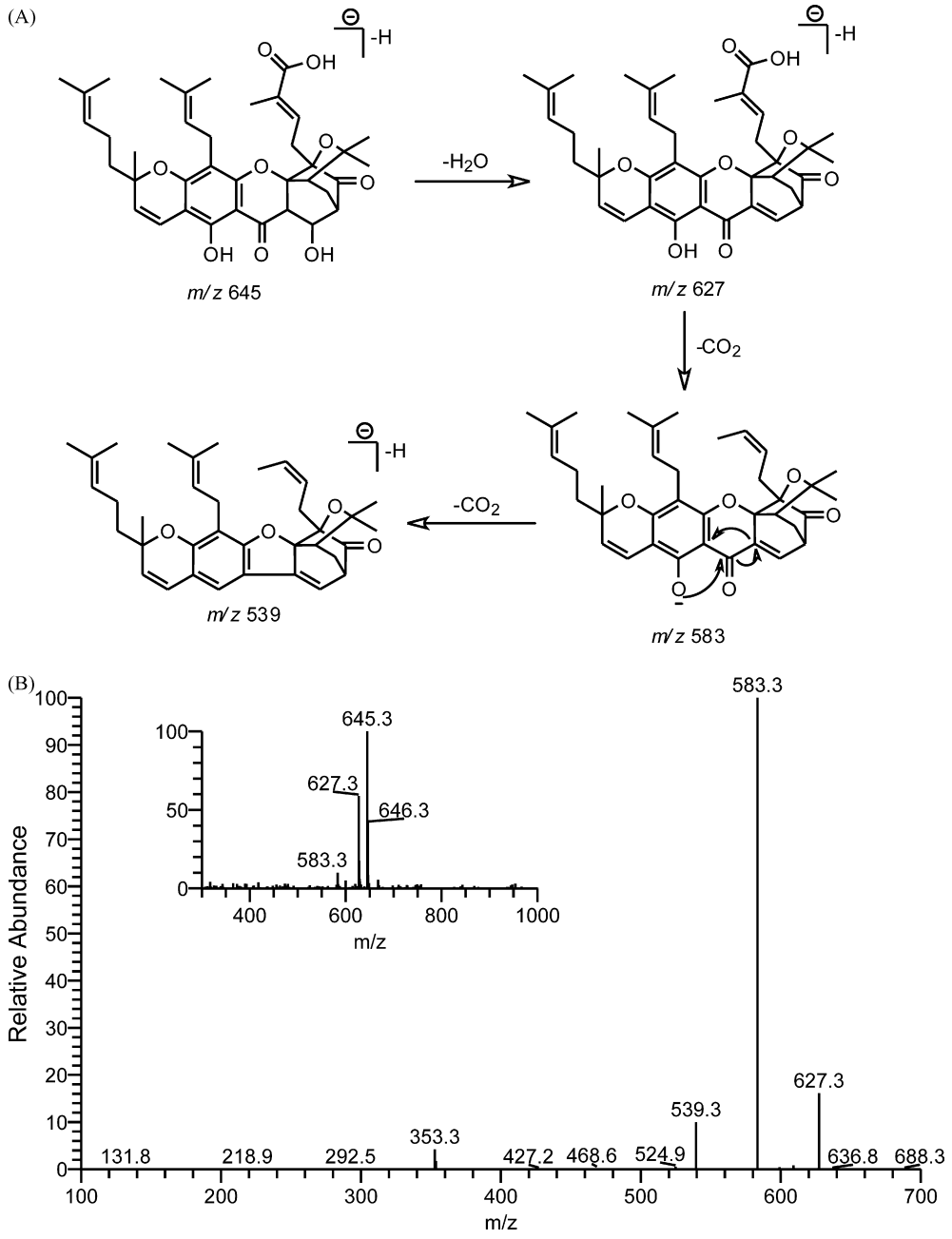

Fig. 3. Proposed fragmentation pathway of 10-OHGA (A) and the full scan product ion spectrum of 10-OHGA $[M-H]^-$ (B) in negative ion ESI mode. (Inset) Q1 full scan spectrum of 10-OHGA in the same ionization mode.

in the HPLC autosampler at 8 °C for 10 h. The long-term stability was assessed after storage of the standard spiked plasma samples at −20 °C for 2 months. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days. The stability of stock solution was also investigated at −20 °C.

2.7. Application

The clinical study protocol was reviewed and approved by the Ethics Committee of Cancer Hospital Affiliated to Chinese Academy of Medical Sciences. All volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Several Chinese patients with malignant tumours participated in the study. Following an overnight fast, each volunteer received a single intravenous dose of 45 mg/m^2 of the GA injection. The intravenous infusion of the GA injection was designated to finish within 2 h. Blood was sampled pre-dose and at 1, 2, 2.5, 3, 4, 6, 9, 12, 24, 36, and 48 h following dosing for determination of plasma concentrations of GA [18] and 10-OHGA. Model-independent pharmacokinetic parameters were calculated for 10-OHGA. The maximum plasma concentration (C_{max}) and the time to it (t_{max}) were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. The elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentrations (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as follow: $AUC_{0-\infty} = AUC_{0-t} + C_t/k$.

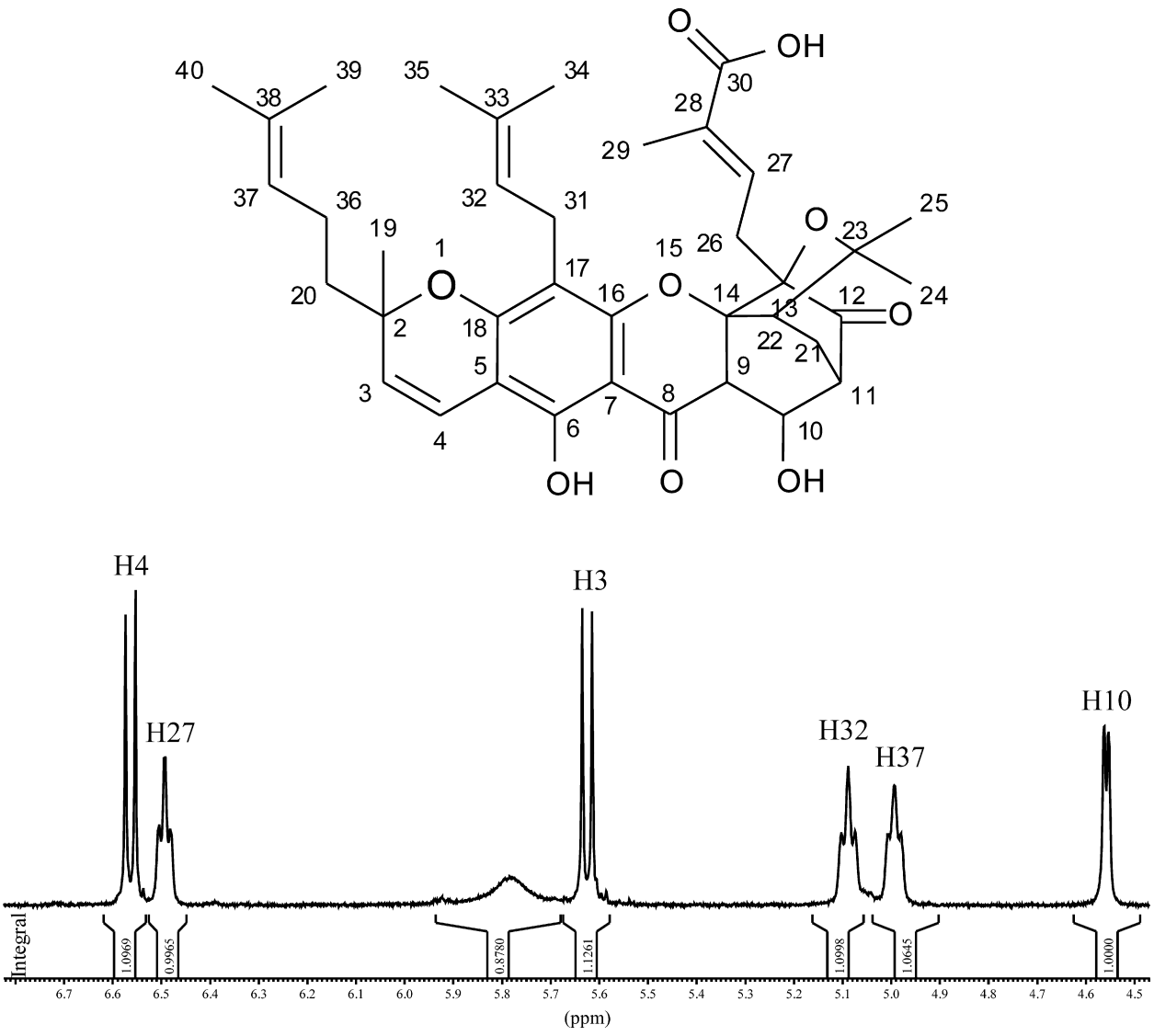

Fig. 4. Downfield region in ^{1}H NMR spectrum of the isolated metabolite 10-OHGA in DMSO-d_6.

3. Results and discussion

3.1. Identification of a major circulating metabolite of GA in human

To effectively identify the metabolites of GA in human plasma, the possible metabolic pathways were proposed according to its metabolism in animals [13,14]. Then, LC–DAD–MS/MS system was used for analyzing human plasma samples. By carefully comparing the difference in the chromatograms and corresponding extract spectra between human plasma samples and the blank controls, a major metabolite of GA was discovered in human plasma. The extracted ion chromatogram of detected GA metabolite was shown in Fig. 2 using negative ion ESI mode. The full scan spectrum showed that its deprotonated molecule ion $[M-H]^-$ at m/z 645 and the product ion scan spectrum of m/z 645 showed that the major fragment ions at m/z 627, 583 (the base peak), 539. Moreover, DAD scan (200–400 nm) result showed that the maximum absorptions of this metabolite were 275, 320 nm. All of these results suggested that this circulating metabolite could be tentatively identified as 10-OHGA [13,14].

To further elucidate the chemical structure of the detected circulating metabolite in human, a comparison of the HPLC retention time, as well as MS and MS/MS spectra of putative metabolite with those of the reference standard is necessary. Thus, the reference standard of 10-OHGA was prepared.

The MS analyses of the isolated metabolite also exhibited a $[M-H]^-$ ion at m/z 645 and the same fragmentation pattern as 10-OHGA (Fig. 3). The diagnostic fragment ion at m/z 583 was the base peak (Fig. 3), corresponding to the loss of H_2O and successive elimination of CO_2 (44 Da) from the carboxylic acid functional group in the structure [19–21]. Further increase of the collision energy led to marked decrease of the intensity of this ion and increase of the intensity of the fragment ion at m/z 539 by successive charge transfer process (Fig. 3). The fragmentation pattern of this analyte in negative ion ESI mode has been confirmed by the data in a recently published paper using LC–ESI–IT–TOF [14]. The ^{1}H NMR spectrum of the isolated metabolite (Fig. 4) was also consistent with that of 10-OHGA in the literature [13]. Conclusively, this isolated metabolite was identified as 10-OHGA and used as the reference standard for qualitative and quantitative analyses.

Due to the retention time and mass spectra of the main circulating metabolite of GA in human were consistent with those of reference standard of 10-OHGA (Fig. 2), it was finally identified as 10-OHGA.

3.2. Optimization of LC–ESI–MS for quantitative determination of 10-OHGA in human plasma

Comparing with capillary voltage, desolvation gas (drying gas) flow and temperature, fragmentor voltage is considered to be the most important instrumental parameter in optimization of ionization settings [22,23]. As it is increased, ions are accelerated quickly through the vacuum region, undergoing collisions with solvent vapor and desolvation gas and resulting in some fragmentation [24]. At first, mass spectrometric condition selection was conducted by changing the fragmentor voltage. Over the range of 50–140 V, the base peak in the mass spectra of 10-OHGA obtained was the deprotonated molecule ions $[M-H]^-$ at m/z 645.3. At the same time, the product ion $[M-H-H_2O]^-$ of the analyte at m/z 627.3 could be seen in the mass spectra as well and its relative abundance was lower than the former (see Fig. 5). When the fragmentor voltage was further increased, the product ion $[M-H-H_2O]^-$ at m/z 627.3 could be fragmented by successive loss of CO_2 to give the product ion at m/z 583.3. Although the highest absolute intensity could be achieved by monitoring the deprotonated molecule ion $[M-H]^-$ at m/z 645.3, there were significant interferences from endogenous substances observed at the retention time of 10-OHGA in this SIM channel. By monitoring the product ion $[M-H-H_2O]^-$ at m/z 627.3, interferences from endogenous substances could be avoided. Finally, the product ion m/z 627.3 at fragmentor voltage of 140 V was selected as the target ion for the determination of 10-OHGA.

Due to the selected target ion of 10-OHGA at m/z 627.3 was also the deprotonated molecule ion of GA [18], GA and 10-OHGA needed to be well separated [25]. In the method development, GA was, therefore, spiked into the human blank plasma as well and chromatographic conditions were carefully optimized. A number of reverse phase columns (Lichrospher C18, Zorbax extend C18, Zobax SB-C18, Sepax GPC18 and Sepax HPC18) were evaluated, and the Sepax HPC18 column, 3 μm, 100 mm × 2.1 mm i.d. was finally selected. Since preliminary test showed that both 10-OHGA and its parent drug possessed strong retention on these columns even using the 100% methanol, methanol was not used in the later method development. So, the optimization was carried out using the water–acetonitrile system. Finally, high selectivity and good separation were obtained using 10 mM ammonium acetate water solution containing 0.1% formic acid–acetonitrile (20:80, v/v) as mobile phase. Data acquisition was stopped when 10-OHGA was eluted completely, and the total chromatographic run time was set at 9 min to prevent the next injection from the inference by GA, which was eluted following the former.

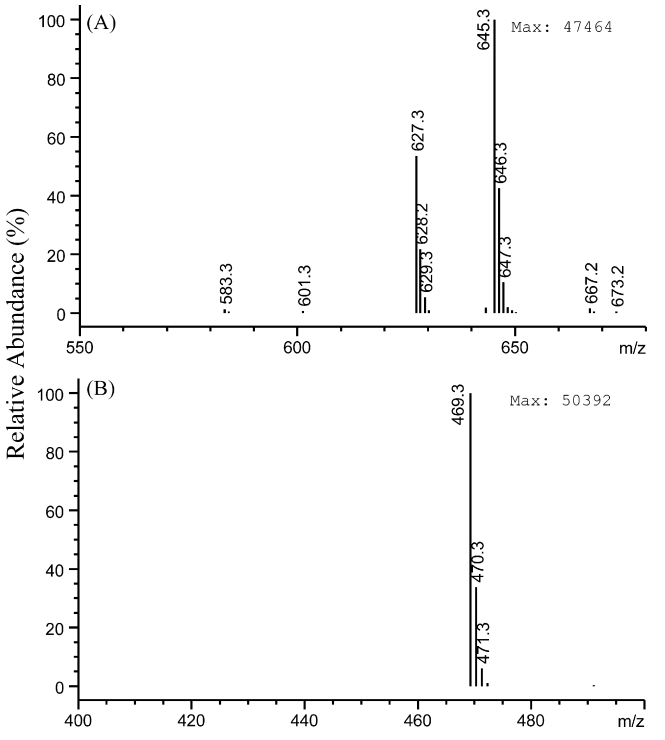

Fig. 5. Mass spectra of the negative ions of 10-OHGA (A) and glycyrrhetic acid (IS, B) at 140 V fragmentor voltage.

3.3. Method validation

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma samples. Fig. 6 shows the typical chromatograms of a blank plasma sample, blank plasma spiked with 10-OHGA at the LLOQ and the IS, and a plasma sample obtained at 2 h after intravenous infusion of GA injection (45 mg/m^2) to a Chinese volunteer. No interfering endogenous substances were observed at the reten-

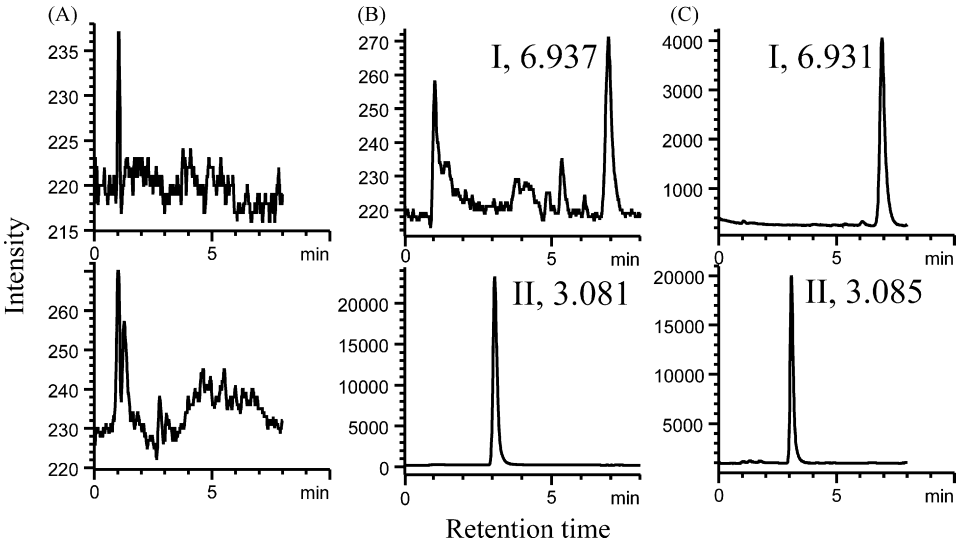

Fig. 6. Typical SIM chromatograms of 10-OHGA (I) and glycyrrhetic acid (IS, II) in human plasma samples, (A) blank plasma sample; (B) plasma sample spiked with 10-OHGA at LLOQ (3 ng/mL, respectively) and the IS at 300 ng/mL; (c) plasma sample 2 h after intravenous infusion of GA injection (45 mg/m^2) to a Chinese volunteer.

Table 1
Precision and accuracy data for the analysis of 10-OHGA in human plasma (five replicates per run).

Analyte	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Intra-batch *RSD* (%)	Inter-batch *RSD* (%)	*RE* (%)
10-OHGA	5.199	5.043	3.9	14.1	−3.0
	519.9	530.1	4.2	5.8	2.0
	1664	1713	5.8	1.6	3.0

Note: RSD: relative standard deviation; *RE*: relative error.

Table 2
Summary of stability of 10-OHGA in human plasma under various storage conditions (n = 3).

Analyte	Conditions	Concentration (ng/mL)		*RSD* (%)	*RE* (%)
		Added	Measured		
10-OHGA	Room temperature, 8 h	5.199	5.058	6.2	−2.7
		519.9	491.4	4.7	−5.5
		1664	1649	11.9	−0.9
	−20 °C, 2 months	5.199	5.379	5.3	3.5
		519.9	557.2	2.6	7.2
		1664	1611	8.0	−3.2
	Three freeze–thaw	5.199	5.355	3.5	3.0
		519.9	488.6	1.8	−6.0
		1664	1610	1.5	−3.2
	Auto sampler Ambient, 10 h	5.199	5.142	8.7	−1.1
		519.9	498.4	8.5	−4.1
		1664	1673	5.0	0.6

Note: *RSD*, relative standard deviation; *RE*, relative error; *n*, number of replicates.

tion times of the analyte and the IS. The chromatograms presented in Fig. 6 indicated that the method was selective.

3.3.2. Matrix effect

The matrix effect data of five different batches of human plasma were 94.6 ± 5.1%, 93.4 ± 2.4%, and 103.1 ± 7.7% for 10-OHGA at concentrations of 5, 500, and 1600 ng/mL, respectively. The matrix effect for the IS was 98.8 ± 6.2%. The results indicated that no coeluting substances significantly influenced the ionization of 10-OHGA and the IS.

3.3.3. Extraction recovery

Mean extraction recoveries of GA were 73.5 ± 7.4%, 75.9 ± 5.7%, and 71.7 ± 3.7% (n = 5) at concentrations of 5, 500, and 1600 ng/mL, respectively. Mean extraction recovery of the IS was 64.8 ± 5.7%.

3.3.4. Calibration curves and LLOQ

The selected assay ranges for the analyte fulfilled the criteria for the LLOQ concentration and the calibration curve [26]. The typical linear regression equation of the calibration curves was as follows: 10-OHGA: $y = -0.002061 + 0.001795x$, $r = 0.9987$. Where y represents the ratio of 10-OHGA to that of the IS and x represents the nominal concentration of 10-OHGA in human plasma.

The LLOQ was 3 ng/mL for 10-OHGA. The precision and accuracy at LLOQ were 11.5% and −5.0% for 10-OHGA, respectively (n = 5).

3.3.5. Precision and accuracy

The precision of the method was determined by calculating *RSD* for the QCs at three concentration levels over three validation batches using a one-way analysis of variance (ANOVA). Intra-day precision was or less 5.8% for 10-OHGA, and the inter-day precision was or less 14.1% for 10-OHGA at each QC level.

The accuracy of the method, expressed in terms of *RE*, ranged from −3.0% to 3.0% for 10-OHGA at each QC level. Assay performance data were presented in Table 1. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise [26].

3.3.6. Stability

The stability tests of the analyte were designed to cover anticipated conditions for the preservation of the clinical samples. The stability results showed that 10-OHGA spiked into human plasma was stable for 8 h at room temperature, for up to 2 months at −20 °C, and during three freeze–thaw cycles. Stability of the analyte extracts in the sample solvent on autosampler was also observed over a 10 h period. The stock solutions of 10-OHGA and the IS were stable for at least 2 months at −20 °C. The results of stability experiments were shown in Table 2.

3.4. Application

The present method was successfully applied to the pharmacokinetics study in which plasma concentrations of 10-OHGA in human were determined up to 36 h after intravenous infusion of GA injection (45 mg/m^2). The plasma concentration–time curve of 10-OHGA of a Chinese volunteer are shown in Fig. 7. After administration of a single dose of GA injection, the C_{max}, $AUC_{0-36\,h}$, $AUC_{0-\infty}$, $t_{1/2}$ and t_{max} of 10-OHGA in this volunteer are 474.3 ng/mL, 1119 μg h/L, 1134 μg h/L, 7.7 h and 2 h, respectively.

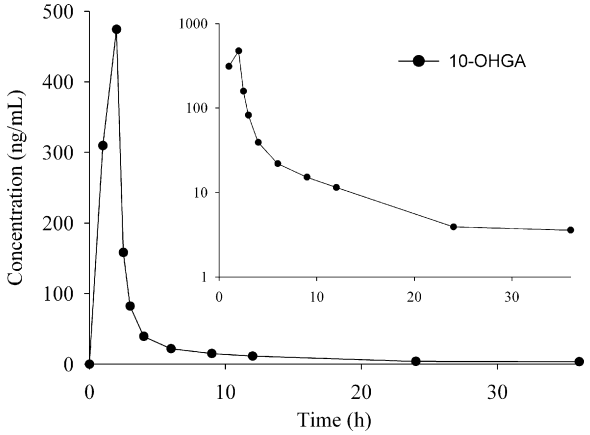

Fig. 7. The plasma concentration–time curve of 10-OHGA of a Chinese volunteer after intravenous infusion of GA injection (45 mg/m^2). (Inset) Semilogarithmic scale.

4. Conclusion

Using a systematic approach, a major circulating metabolite in human was found and identified as 10-OHGA, and an LC–ESI–MS method for the quantitative determination of this metabolite in human plasma was then developed and could now be used for the pharmacokinetic and pharmacological studies of 10-OHGA after intravenous infusion of GA injection in human.

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