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Simultaneous determination of GFA and its active metabolites in human plasma by liquid chromatography electrospray ionization mass spectrometry and its application to pharmacokinetic studies

Qing Huang, Guang-Ji Wang *, Jian-Guo Sun, Ji-Ye A, Wei-Bin Zha, Ying Zhang, Jing-Wei Zhang, Bei Yan, Sheng-Hua Gu, Hong-Can Ren, Lin-Sheng Liu

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210038, Jiangsu Province, China

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Abstract

A sensitive and rapid liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS) method has been developed and validated for simultaneous quantification of guanfu base A (GFA) and its metabolites guanfu base I (GFI) and guanfu alcohol-amine (AA) in human plasma with phenoprolamine hydrochloride (DDPH) as the internal standard. The analytes were extracted from human plasma by using liquid–liquid extraction with ethyl acetate and the LC separation was performed on a Diamonsil C_{18} analytical column (150 mm × 2.1 mm i.d., 5 μm). The MS acquisition was performed in selected ion monitoring (SIM) mode of positive ions. Analysis was carried out in SIM mode at *m/z* 430.25 for GFA $[M+H]^+$, *m/z* 388.25 for GFI $[M+H]^+$, *m/z* 346.25 for AA $[M+H]^+$ and *m/z* 344.20 for the IS DDPH $[M+H]^+$. The calibration curves were linear over the range of 50–5000 ng/mL for GFA and 5–1000 ng/mL for GFI and AA, with coefficients of correlation above 0.999. The lower limit of quantification for GFA was 1 ng/mL, while for GFI and AA were both 5 ng/mL. The intra- and inter-day precisions (CV) of analysis were within 9%, and the accuracy ranged from 91% to 108%. The overall recoveries for GFA, GFI and AA were about 94.2%, 87.8% and 80.6%, respectively. The total LC–MS run-time was only 5.5 min. This quantitation method was successfully applied to the simultaneous determination of GFA and its metabolites in human plasma for the metabolic study and pharmacokinetic evaluation.

Keywords: Guanfu base A; Guanfu base I; Guanfu alcohol-amine; LC–MS; Pharmacokinetics; Metabolites

1. Introduction

Aconitum coreanum (Levl.) Raipaics (Guanbaifu in Chinese) is one of the most centuries-old Chinese herbs. It has been used to treat various kinds of disorders such as cardialgia, facial distortion, epilepsia, migraine, vertigo, tetanus, infantile convulsion and rheumatic arthralgia [1]. Pharmacological studies and clinical practice demonstrated that its extract has anti-arrhythmia [2,3], analgesic and anti-inflammatory effects [4]. The bioactive constituents of the herb are diterpenoid alkaloids.

Guanfu base A (GFA, Fig. 1a) is a single chemical entity with potential anti-arrhythmic efficacy isolated from the root of *Aconitum coreanum* (Levl.) Raipaics [5]. Preclinical pharmacological study showed that various experimental ventricular arrhythmia could be protected or arrested by GFA [2,6–8]. Further electrophysiological experiment have revealed that GFA blocked the fast Na^+ channel and exhibited anti-arrhythmic action via direct effect on sinoatrial node [9–12]. Besides GFA, guanfu base G (GFG), guanfu base I (GFI) and guanfu alcohol-amine (AA) were also isolated from the tuber of *Aconitum coreanum* (Levl.) Raipaics. They were all esters of the same structure of C_{20}-diterpenoid, and only differed in number of acetyls (Fig. 1a). Compared with GFA, GFG showed much more powerful anti-arrhythmic effect but more toxicity, while GFI and AA were of less powerful anti-arrhythmic effect with less toxicity [13,14].

As compared to the extensive research on the pharmacological activities of GFA, few studies have been done on its metabolism and pharmacokinetics. Preliminary in vitro metabolism studies performed earlier in our laboratory indicated

* Corresponding author. Tel.: +86 25 85391035; fax: +86 25 85306750.
E-mail address: qqing_huang@yahoo.com.cn (G.-J. Wang).

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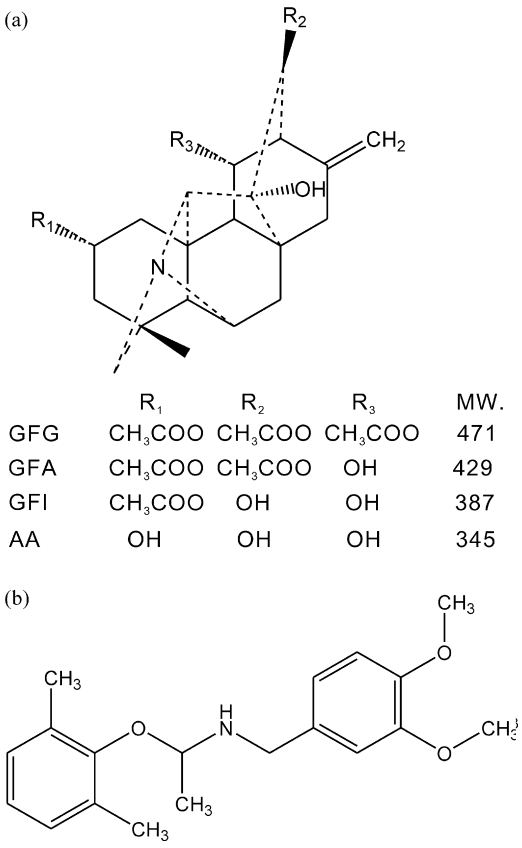

	R_1	R_2	R_3	MW.
GFG	CH_3COO	CH_3COO	CH_3COO	471
GFA	CH_3COO	CH_3COO	OH	429
GFI	CH_3COO	OH	OH	387
AA	OH	OH	OH	345

Fig. 1. (a) Chemical structures of guanfu base G (GFG), guanfu base A (GFA), guanfu base I (GFI), guanfu alcohol-amine (AA). (b) Chemical structure of the internal standard DDPH.

that GFA can be metabolized to GFI and AA in rats and humans [15–17]. A Jiye et al. reported that the phase I metabolites guanfu base I (GFI) and guanfu alcohol-amine (AA) were separated and identified in rat urine [15,16] while guanfu base I (GFI), was identified in rat bile [16] after intravenous administration of GFA.

There are some determination methods reported in the literature for the GFA in biosamples till now, such as, gas chromatographic–mass spectrometric method [18–20] and liquid chromatographic separations followed by UV [21] or MS detection [22]. While, these established methods have some disadvantages or inconvenient procedure. The GC–MS method requires extensive sample clean up as well as multi-step derivatization procedures. The sensitivity of the HPLC–UV was found to be inadequate for PK profiling of GFA by administration via conventional routes. While the HPLC–MS method for determination of GFA was reported in dog biological matrix with a relatively high limit of quantitation (420 ng/mL). Those drawbacks limit the ease of use. Moreover, there is no method available for the simultaneous determination of GFA and its metabolites in biological fluids till now. As drug metabolism plays an important role in pharmacodynamics and toxicity. Investigation of the metabolic profile of GFA and the pharmacokinetic research of the parent compound and its active metabolites in vivo is essential to clarify its mechanisms of action and to insure the safety and efficacy in clinic treatment. Therefore, it is necessary to develop a more sensitive, precise and specific method for simultaneous quantitative estimation of GFA and its metabolites in biological fluids for the metabolic study and pharmacokinetic evaluation to support the development of GFA, as well as the metabolites of GFA.

In the present study, an LC–ESI–MS method for the simultaneous quantification of GFA and its metabolites, GFI and AA, was developed and validated for its specificity, accuracy, precision and sensitivity. The method was successfully applied for the determination of GFA and its metabolites in human plasma after intravenous administration for metabolic research and pharmacokinetic investigation.

2. Experimental

2.1. Chemicals and reagents

Reference standards of GFA, GFI and AA were kindly provided by study group of GFA, China Pharmaceutical University. Phenoprolamine hydrochloride (DDPH, internal standard) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of all chemicals was proved above 95% and their chemical structures are shown in Fig. 1a and b. Acetonitrile and methanol were HPLC grade and purchased from Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Ethyl acetate and other chemicals and solvents were all of analytical grade. Blank human plasma was obtained from the Blood Supply Center (Nanjing, China) and was stored in a freezer at −20 °C until use.

2.2. Instruments and analytical conditions

The HPLC system consisted of a Shimadzu DGU-14 AM online degasser, two Shimadzu LC-10ADvp pumps with a high-pressure mixer, a Shimadzu CTO-10Avp column oven and a Shimadzu SIL-HTC autosampler (Shimadzu, Kyoto, Japan). A Shimadzu 2010A mass spectrometer (Q-array-Octapole-Quadrupole mass analyzer) equipped with an ESI interface was used for MS detection.

Chromatographic separation of analytes was achieved using a Diamonsil C_{18} analytical column (150 mm × 2.1 mm i.d., 5 μm, Metachem, Torrance, CA, USA), equipped with an ODS guard column (Security Guard, Phenomenex Inc., Torrance, CA, USA). The column and autosampler tray temperatures were set at 40 and 4 °C, respectively. A mobile phase composed of acetonitrile-0.1% glacial acetic acid (42:58, v/v) was used throughout the analysis at a flow rate of 0.2 mL/min and the sample injection volume was 5 μL. Under these conditions, GFA, GFI, AA and DDPH were eluted at approximately 3.98, 3.91, 3.80 and 4.75 min, respectively. Hence, the total run-time including column wash and equilibration was within 5.5 min for each injection.

Mass spectrometric conditions were optimized to obtain maximum sensitivity of the target. The final MS parameters were as follows: CDL (curved desolvation line) temperature, 250 °C; block temperature, 200 °C; probe temperature, 250 °C; detector

gain, 1.55 kV. Vacuum in the mass detector was obtained using a Turbo molecular pump (Edward 28, UK). Nitrogen (99.995%, Gas Supplier Center of Nanjing University, China) was used as the nebulizing gas at 1.5 L/min and curtain gas at 0.01 mPa. Mass spectra were obtained at dwell times of 0.2 s and 1 s in SIM (selective ion monitoring) and scan mode, respectively. The MS acquisition was performed in SIM mode of positive ions. Analysis was carried out using selected ion monitoring (SIM) at *m/z* 430 for GFA $[M+H]^+$, *m/z* 388 for GFI $[M+H]^+$, *m/z* 346 for AA $[M+H]^+$ and *m/z* 344 for DDPH $[M+H]^+$. Peak areas for all components were automatically integrated using LC/MS solution Version 2.04 (Copyright (C) 1997–2002 Shimadzu Corp.).

2.3. Preparation of stock solutions

Primary standard stock solutions of GFA, GFI, AA and internal standard were prepared separately at the concentration of 1.0 mg/mL, and were stored at 4 °C. A series of standard working solutions of each analyte at appropriate concentrations were obtained by mixing and further diluting of the standard stock solution with deionized water. A working solution of the internal standard (5 μg/mL) was prepared by diluting internal standard stock solution with deionized water. These diluted working standard solutions were used to prepare the calibration curve and quality control (QC) samples in human blank plasma.

2.4. Calibration standards and quality control samples

Blank human plasma was screened prior to spiking to ensure it was free of endogenous interference at the retention times of GFA, GFI, AA and DDPH.

Standard calibration samples were prepared by spiking the blank human plasma with working solutions of each analyte, to yield different concentrations over a range of 50–5000 ng/mL for GFA (i.e. 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL) and 5–1000 ng/mL for GFI and AA (i.e. 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL).

Quality control (QC) samples were prepared by adding appropriate volumes of QC working solutions (from a separate weighing to that for the calibration standard) to blank human plasma, to yield the low, medium and high concentrations (50, 500 and 5000 ng/mL for GFA and 10, 100 and 1000 ng/mL for GFI and AA.)

The concentration of the internal standard was 500 ng/mL in all samples.

2.5. Sample preparation

QC samples, calibration standards, and human plasma samples were extracted by liquid–liquid extraction with ethyl acetate. A volume of 90 μL of drug-free human plasma was added to a disposable Eppendorf tube, followed by spiking with 10 μL of the standard working solution, 10 μL of internal standard working solution and 50 μL of saturated Na_2CO_3 solution, respectively. The mixture was vortexed for 1 min using a vortex mixer (Scientific Industries Inc., USA). Then, a single step of liquid–liquid extraction was adopted to extract all the analytes from the human plasma. For this, 500 μL of ethyl acetate was added to each tube followed by vortexing for 3 min. The well-vortexed solution was then centrifuged at 15,000 rpm for 10 min and 400 μL of the upper organic layer was transferred to a new Eppendorf tube and evaporated to dryness in a Thermo Savant SPD 2010 SpeedVac system (Thermo Electron Corporation) set at 30 °C. The residue was then reconstituted in 200 μL mobile phase solution followed by centrifugation at 22,000 rpm for 10 min before analysis. An aliquot of 5 μL was injected into the LC/MS.

2.6. Method validation

Method validation was performed to evaluate the specificity, linearity, sensitivity, accuracy and precision according to the currently approved US Food and Drug Administration (FDA) bioanalytical method validation guidelines [23]. The matrix effect as well as analytes stability in plasma were also determined.

2.6.1. Specificity

The specificity of the method was investigated by analyzing six different batches of drug-free rat plasma (without IS nor analytes) for the exclusion of any endogenous co-eluting interferences at the peak region of each analyte and IS.

2.6.2. Linearity and sensitivity

The calibration standards were prepared and assayed in triplicate on three different days to demonstrate the linearity of this method. The lower limit of quantification (LLOQ) was defined as the lowest concentration at which the signal-to-noise (S/N) ratio was larger than 10 and both the precision and accuracy were less than or equal to 20% by analyzing the six replicates of samples spiked with each analyte.

2.6.3. Precision and accuracy

The precision and accuracy of the entire method were assessed at three quality control (QC) concentration levels (i.e. 50, 500 and 5000 ng/mL for GFA and 10, 100 and 1000 ng/mL for GFI and AA, respectively), each extracted and analyzed in six replicates on the same day (intra-day precision and accuracy) and on three different days within 1 month (inter-day precision and accuracy) (each along with an independent standard curve for quantification). Precision was expressed as coefficient variation (CV) and accuracy was calculated as the percentage of the ratio of the observed concentration and the nominal concentration of the QC samples. The intra- and inter-day precision and bias were set at $\leq$15%, except that at LLOQ, where it was set at $\leq$20%.

2.6.4. Recovery

The extraction recovery (absolute recovery) was determined by measuring an extracted sample against a post-extraction spiked sample and expressed as the ratio of the peak responses. For GFA, GFI and AA, the recovery experiments were performed with three QC concentrations (low, medium and high

QC concentrations), with triplicate determinations at each concentration.

2.6.5. Matrix effect

The matrix effect on the ionization efficiency of each analyte was evaluated by comparing the peak response of analytes dissolved in blank sample extract (i.e. the final solution obtained from blank plasma after extraction and reconstitution) with those for analytes dissolved to the same concentrations in deionized water. The experiment was performed in triplicate for each QC concentration. If the peak area ratios for the plasma extracts versus deionized water were <85% or >115%, a matrix effect was implied.

2.6.6. Stability

The stability testing was determined in four ways: (1) For storage stability, the QC samples were prepared and stored at −20 °C for 2 weeks. All samples were subsequently thawed and analyzed together with calibration samples, which were freshly prepared. (2) For freeze/thaw stability testing, the QC samples were determined after three freeze (−20 °C, storage temperature)/thaw (23 °C, ambient temperature) cycles and analyzed with the freshly prepared calibration samples. (3) To investigate the stability during sample processing, the QC samples were left at room temperature for 6 h, the average time required for sample preparation and then analyzed with the freshly prepared calibration samples. (4) To assess the injector stability of the processed samples, the QC samples were extracted and placed in the autosampler at 4 °C for 24 h, and then injected into the LC/MS system for analysis. The measured concentrations were then compared with those of the same QC samples, which were analyzed immediately after processing.

For each of the above stability tests, the experiments were performed at three QC concentrations (low, medium and high), with triplicate determinations for each concentration. The obtained results were compared with the nominal concentration of the analytes. A compound was considered unstable if the calculated concentration was less than the nominal concentration by more than 15%.

2.7. Pharmacokinetic study design

The validated LC–MS assay was successfully applied to a pharmacokinetics study in 8 patients with impaired renal function (mild, CrCL 51–80 mL/min, in accordance with the classification recommended by FDA [24]). The study was approved by the Helsinki Committee of the Clinical Pharmacology Research Center of the second affiliated hospital, Jiangxi Medical College. All volunteers provided informed written consent before participating in the study. Subjects who had no clinically relevant abnormalities on their physical examination, initial medical history, laboratory tests, or electrocardiographic (ECG) evaluation were enrolled. Venous blood samples were periodically collected up to 48 h after intravenous injection of 4 mg/kg GFA (Acehytisine Hydrochloride Injection, H20040329). The blood samples (3–4 mL) were collected into heparinized tubes at the time of 0, 0.0833, 0.5, 1, 1.5, 2.5, 4.0, 6.0, 9.0, 12, 24, 36 and 48 h after intravenous injection of the medicals. Following centrifugation at 8000 rpm for 10 min, resultant plasma was separated and stored at −20 °C until analysis. Plasma (100 μL) was then extracted and analyzed by the same procedure as that of calibration samples.

3. Results and discussion

3.1. Selection of IS

It is necessary to use an IS to obtain good accuracy and precision when a mass spectrometer is used as the HPLC detector. DDPH was adopted as IS because of the similarity of its retention and ionization characteristics with those of the analytes, and because of the minimal endogenous interferences in the SIM channel for DDPH ($[M+H]^+$ at *m/z* 344.

3.2. Sample preparation

LLE was advantageous because this technique not only extracted the analyte and IS with sufficient efficiency and specificity, but also minimized the experimental cost. Ethyl acetate, trichloromethane and diethyl ether were all tested as extraction solvent, and ethyl acetate was finally adopted because of its high extraction efficiency. Saturated Na_2CO_3 solution was added to the plasma in order to accelerate the drugs' dissociation from the plasma, which produces better extraction efficiency, and reduce interference since most endogenous compounds are of acidic nature. The amount of Na_2CO_3 added has been optimized. The volume 20, 40, 50 and 100 μL of Na_2CO_3 saturation solution (20 °C) were tested, and the optimal volume was 50 μL, which gives the highest extraction recovery. A mini extraction procedure was carried out in 1.0 mL Eppendorf tubes. The method has been proved to be time-saving, simple and economical using small tubes and little solvent.

3.3. Chromatography and mass conditions

GFA, GFI and AA are all esters of the same C_{20}-diterpenoid structure. Their chemical structure is quite different from any of the anti-arrhythmic we are now using in the clinic. It is difficult to analyze them with high sensitivity using HPLC methods because of their weak UV absorption and lack of fluorescence in the molecular structure. In this paper, an LC–MS method for simultaneous determination of GFA, GFI and AA in human plasma was presented, which can overcome the above-mentioned shortcomings.

To develop a sensitive LC–MS method for simultaneously quantifying GFA, GFI and AA in human plasma, ESI and APCI sources were evaluated. The ESI source produced greater sensitivity and exhibited less interference for all the analytes than those of APCI source. The mass scan spectra of GFA, GFI, AA and the IS after direct injection in mobile phase are presented in Fig. 2. It is clear that the analytes and IS both formed predominantly protonated molecules $[M+H]^+$ in the positive ion electrospray scan spectra. Therefore, *m/z* 430 for GFA, *m/z* 388

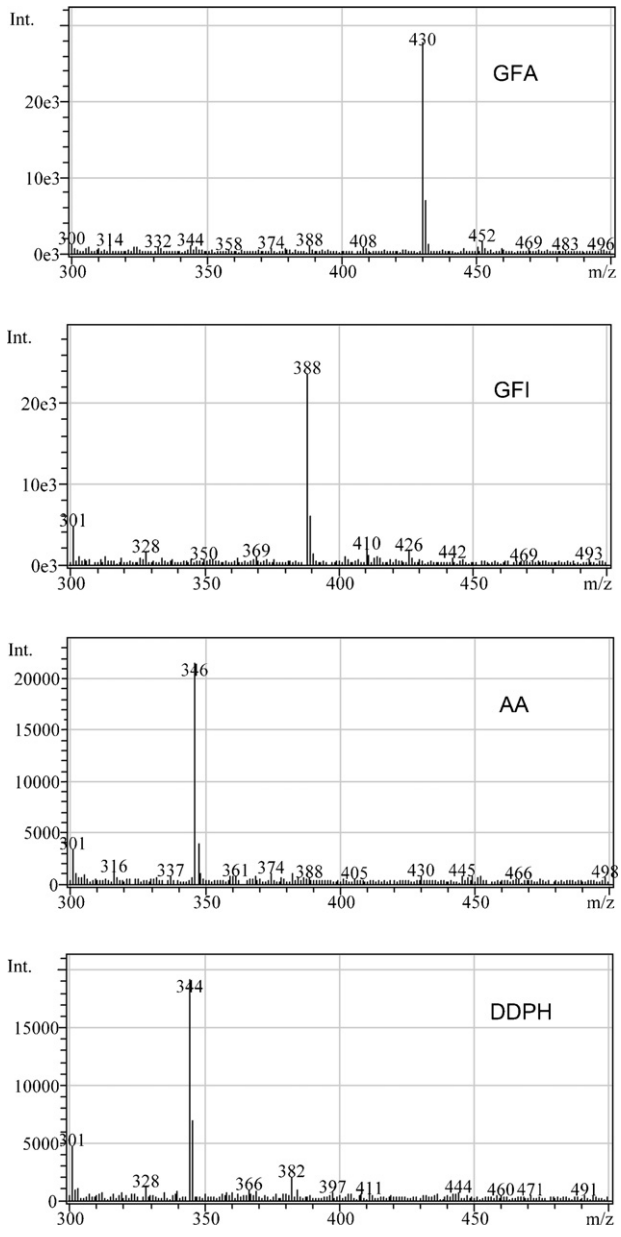

Fig. 2. Positive ion electrospray mass scan spectrum of GFA, GFI, AA and DDPH (internal standard).

for GFI, *m/z* 346 for AA and 344 for IS, were selected for the SIM acquisition, respectively.

Examples of SIM chromatograms for standard solutions and extracts of spiked blank plasma are shown in Figs. 3 and 4, respectively. Under the chromatographic conditions, GFA, GFI and AA were rapidly eluted at approximately 3.98, 3.91 and 3.80 min, respectively, while the internal standard DDPH was eluted at 4.75 min. To enhance the sensitivity of GFA, GFI and AA, solvent type (methanol versus acetonitrile) and additives (ammonium acetate, ammonium formate, acetic acid and formic acid) in various concentrations and ratios were tested. The best peak shape and ionization were achieved by the addition of 0.1% acetic acid and acetonitrile as organic solvent.

3.4. Selectivity

Under the current optimized LC–MS conditions, all analytes were eluted rapidly within 5.0 min. GFA, GFI, AA and the internal standard DDPH were eluted at retention times of 3.98, 3.91, 3.80 and 4.75 min, respectively. No interfering peaks were observed for each analyte or IS in six different lots of blank plasma samples (Fig. 5) because of the high selectivity of the SIM mode, indicating that the method possesses high specificity from endogenous substances and other concomitant agents. Representative chromatograms obtained from an extracted plasma sample of a volunteer who participated in a pharmacokinetics study conducted on 8 persons with impaired renal function are depicted in Fig. 6.

3.5. Linearity of calibration curves and LLOQ

The calibration curves were linear over the concentration range of 50–5000 ng/mL for GFA and 10–1000 ng/mL for both GFI and AA. Good linearity with a coefficient of determination r^2 exceeding 0.999 was observed for each analyte. The representative regression equations were $y = 0.0007x + 0.0004$ for GFA, $y = 0.0005 + 0.002$ for GFI, and $y = 0.0001x + 0.0017$ for AA, respectively, where y indicates the ratios of analytes to internal standard and x indicates the plasma concentrations. The slopes of the regression equations were consistent for the calibration curves prepared on three separate days. The lower limits of quantification under the optimized conditions were 1 ng/mL for GFA and 5 ng/mL for both GFI and AA, which was judged from the fact that the precision and accuracy were less than 20% and the S/N ratios were much higher than 10.

3.6. Accuracy and precision

QC samples were analyzed in six replicates at three concentrations to determine the accuracy and precision of this method. The results are shown in Table 1. The intra-day precision (CV) of the assay was less than 6% for each of the three concentrations of the QC samples; assay accuracy was in the range 91–104%. The inter-day precision (CV) of the assay was less than 9% for all the QC samples, and assay accuracy was in the range 94–108%. At these concentrations, the intra- and inter-day CVs were determined to be <10%, and the accuracy was 91–108%. These results suggest that the present method is accurate, precise and reproducible for detecting GFA, GFI and AA over the tested concentration ranges.

3.7. Recovery and matrix effect

The extraction recoveries from the rat plasma were determined at the concentrations of 50, 500, and 5000 ng/mL for GFA and 10, 100 and 1000 ng/mL for GFI and AA in triplicate. The one-step liquid–liquid extraction was proved to be simple, rapid and successful with an average recovery of over 75% for each analyte at the tested concentrations (Table 2). The %CV for recoveries was all below 15%. The possibility of matrix effect caused by ionization competition between the analytes

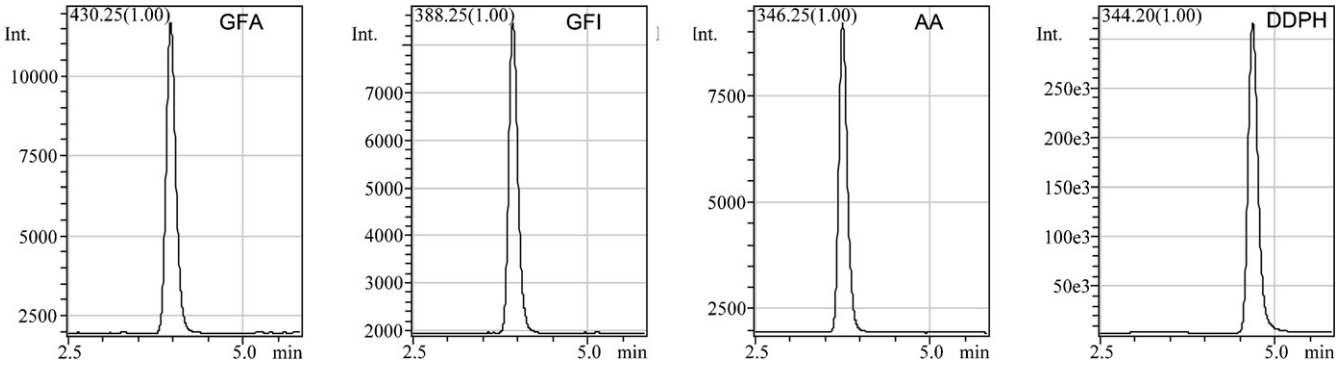

Fig. 3. Representative SIM(+) chromatograms obtained from standard solution samples of GFA, GFI, AA and DDPH (internal standard) injected directly.

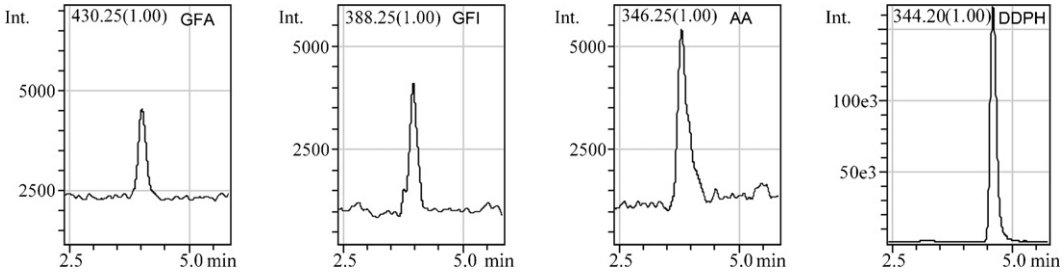

Fig. 4. Representative SIM(+) chromatograms of an extracted blank plasma sample spiked with GFA (1 ng/mL), GFI (5 ng/mL), AA (5 ng/mL) and the internal standard DDPH (500 ng/mL).

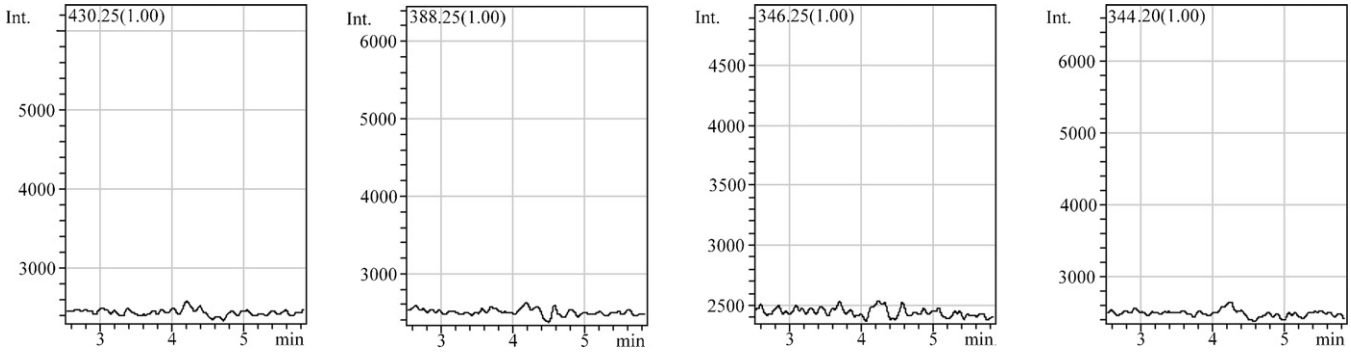

Fig. 5. Representative SIM chromatograms obtained from an extracted blank plasma sample.

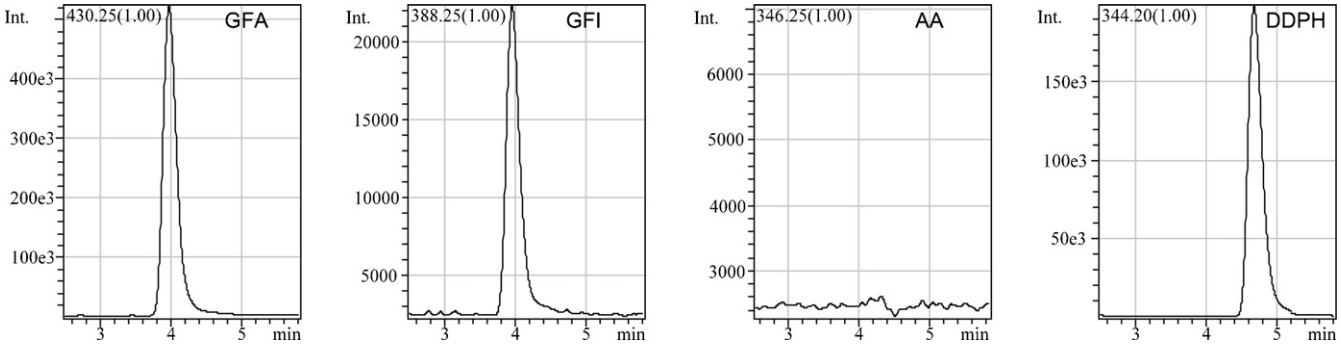

Fig. 6. Representative SIM chromatograms obtained from an extracted human plasma sample after i.v. administration of GFA.

Table 1
Accuracy and precision for the analysis of GFA, GFI and AA

Analytes	Concentration (ng/mL)	Intra-day ($n=6$)		Inter-day ($n=6$)	
		Precision (CV, %)	Accuracy (%)	Precision (CV, %)	Accuracy (%)
GFA	50	3.9	97.7	6.5	99.5
	500	3.5	92.7	7.8	101.3
	5000	2.9	91.3	4.3	96.4
GFI	10	3.9	97.7	7.6	94.6
	100	3.5	91.5	7.5	96.1
	1000	2.9	93.3	8.1	95.1
AA	10	5.7	103.7	8.9	107.9
	100	5.3	98.1	6.6	96.3
	1000	1.3	100.8	4.1	101.2

Table 2
Recoveries of GFA, GFI and AA at three different concentrations

Analytes	Concentration (ng/mL)	Recovery (%, mean ± S.D., $n=3$)
GFA	50	94.5 ± 4.1
	500	96.2 ± 3.7
	5000	91.8 ± 5.8
GFI	10	84.1 ± 7.3
	100	91.5 ± 5.4
	1000	87.8 ± 2.6
AA	10	76.8 ± 6.7
	100	82.5 ± 5.1
	1000	82.3 ± 3.0

and the endogenous co-eluents was evaluated at three concentrations in triplicate. The results of matrix effect were acquired from comparing the peak responses of the post-extraction spiked samples with those of the standard solution and suggested negligible matrix effect under the developed sample preparation and chromatographic conditions.

3.8. Stability

No significant degradation (the losses were within 10%) of GFA, GFI and AA was observed during all of the sample storage, preparation and analysis periods. Results of the stability evaluation are shown in Table 3.

3.9. Application of the method

The well-validated method described above was successfully applied to analyze plasma samples obtained from 8 patients with impaired renal function (mild, CrCL 51–80 mL/min) who received a single intravenous dose of 4 mg/kg GFA injection for the pharmacokinetics study. The chromatograms of plasma samples obtained from pre- and post-dosed patients showed that no significant interfering peak was detected at the retention times of GFA, GFI, AA and the internal standard, indicating the method was specific enough for the pharmacokinetic study.

The plasma concentration–time profile of GFA and its metabolites GFI and AA are shown in Figs. 7 and 8. The maximum plasma concentration (C_{max}) of GFA was 49.98 ± 28.29 μg/mL and the plasma concentration decreased to about 117.55 ± 20.15 ng/mL at 48 h after dosing. The main active metabolite GFI can be detected from the first sample point, 5 min post-dosing, with the average concentration of 5.02 ± 2.69 μg/mL (10% of corresponding GFA), indicating that the metabolite formation of GFI was rapid. The concentration of GFI decreased to be about

Table 3
Stability of GFA, GFI and AA during sample storage, preparation and analysis

Analytes	Concentration (ng/mL)	Remaining (%)			
		Stored at or below −20 °C for 2 weeks	Freeze/thaw (3 cycles)	Stored at room temperature for 6 h	Stored in the autosampler nack at 4 °C for 24 h
GFA	50	98.9	101.6	97.7	96.6
	500	96.6	99.8	99.2	100.9
	5000	100.4	97.9	95.3	93.8
GFI	10	96.9	94.5	99.6	99.5
	100	102.4	97.7	102.1	102.5
	1000	103.6	98.3	98.7	101.8
AA	10	90.9	90.4	92.6	90.1
	100	93.8	92.5	96.4	99.7
	1000	91.1	90.5	90.5	97.9

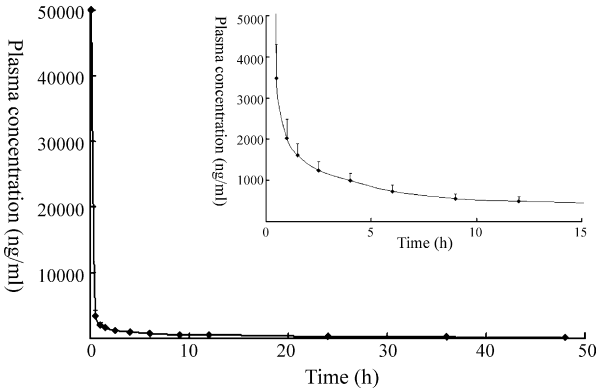

Fig. 7. Mean drug plasma concentration–time curve (mean ± S.D., $n = 8$) of GFA in human after intravenous administration of GFA (4 mg/kg, i.v.).

18.16 ± 2.42 ng/mL at 48 h after dosing. The other metabolite AA was not observed in human plasma during the experiment period.

The pharmacokinetic study show that the human plasma concentration of GFA is lower than the LOQ of some previously reported method based on HPLC–UV [21] and HPLC–MS [22]. This indicated that these methods are not satisfying the requirements of the pharmacokinetics study on GFA and its metabolites in human following intravenous administration. The present established method on the basis of LC–ESI–MS with a lower LOQ at 1, 5 and 5 ng/mL, respectively, was sensitive enough for the pharmacokinetics research of GFA and its main active metabolites GFI and AA.

GFA, GFI and AA have similar molecular structure and only differ in the number of acetyls. GFA has two acetyls and GFI has one acetyl while AA has none. It is well known that the hydrolysis of esters can take place in plasma by a non-specific esterase or in liver by specific esterase. In our experiments, after i.v. administration of GFA, GFI was found in plasma in the scan and SIM modes, suggesting that GFI may be a hydrolyte or a metabolite of GFA. We are currently conducting further experiments to study the mechanism of GFA's metabolism.

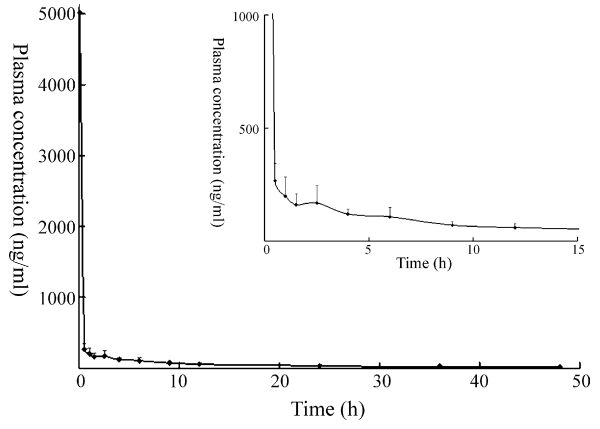

Fig. 8. Mean drug plasma concentration–time curve (mean ± S.D., $n = 8$) of GFI in human after intravenous administration of GFA (4 mg/kg, i.v.).

4. Conclusions

A simple and rapid LC–ESI–MS method for the simultaneous quantification of GFA and its metabolites GFI and AA in human plasma was developed and validated to be linear, accurate and precise. The method used a simple one-step liquid–liquid extraction and a C18 column coupled with ESI–MS for separation and detection. The simple liquid–liquid extraction procedure and short run-time can curtail test's cost and time that is very important for large sample batches. The sensitive, precise and accurate method could be modified for micro-sample analysis or trace analysis. This quantitation method was successfully applied to the simultaneous determination of GFA and its metabolites in human pharmacokinetic study. This established method was also utilized in the in vivo metabolic and pharmacokinetic study of GFA and its metabolites to clarify the detailed mechanism of metabolism and will insure the safety and efficacy in clinic therapy (forward to be published).

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