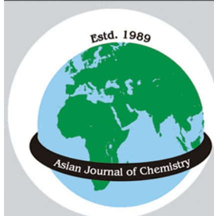

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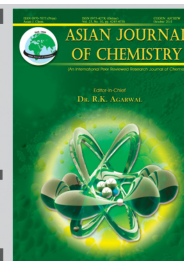

A Time-Saving and Plasma-Saving Method for Determination of Glycyrrhetic Acid in Human Plasma by HPLC-MS/MS

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A sensitive, time-saving and plasma-saving HPLC-MS/MS method was established and validated for the quantification of glycyrrhetic acid in human plasma using oleanolic acid as the internal standard. Dichloromethane as the organic solvent was used to extract the glycyrrhetic acid and internal standard from 100 μL plasma. After liquid-liquid extraction, the post-treatment samples were analyzed on a Luna CN column interfaced with HPLC-MS/MS in negative selected reaction monitoring mode. The mobile phase was composed of acetonitrile and water (containing 0.05 % acetic acid and 0.02 % ammonium acetate) (95:5 v/v) at a flow-rate of 0.2 mL/min throughout the experiment. The linearity ranged from 2.5 to 160 ng/mL and the correlation coefficients for the calibration curves ranged from 0.998 to 1.000. More than 500 plasma samples were prepared and analyzed within 2 days. This method was successfully employed in pharmacokinetic study after oral administration of 150 mg glycyrrhetic acid to 19 healthy volunteers.

Key Words: Glycyrrhetic acid, HPLC-MS/MS, Pharmacokinetic.

INTRODUCTION

Licorice is used widely in the world, which is one of the oldest and most popular herbal medicines among many Asian and European countries including China[1,2], Japan, United Kingdom and others[3]. As flavouring additives, licorice is also widely used in food, pharmaceutical and particularly tobacco industries due to its pleasant aromatic sweet taste[4,5]. Licorice is extensively used in the traditional Chinese medicines and appears as a component herb in *ca.* 60 % of all traditional Chinese medicines prescriptions. Its marked preparation, diammonium glycyrrhizinate capsule, is used clinically for the treatment of chronic hepatitis, allergic disorder and inflammation[3,5-7]. Extensive pharmacological studies provide scientific evidence of glycyrrhetic acid, which as the main active component of licorice extraction[8] and hydrolysis product of diammonium glycyrrhizinate, possesses various pharmacological effects such as antiulcer, antiinflammatory, antiallergenic, antioxidative, neuroprotective, antiviral and antimicrobial properties[9-12]. It was demonstrated that licorice is completely transformed to its active metabolite glycyrrhetic acid by human intestinal bacteria prior to absorption when oral administration[11,13] and the latter exhibits a higher antiherpes activity than licorice[8,14].

Several detection methods such as HPLC for the pharmacokinetic[11,12,15-18] and bioavailability[19,20] studies in experimental animals and human plasma have been reported many times[21-25]. Raggi *et al.*[10] reported a rapid, facile and economic HPLC method for the simultaneous analysis of glycyrrhizin and glycyrrhetic acid in liquorice roots and confectionery products. Yoshikazu *et al.*[26] published an HPLC method to determinate the concentration of glycyrrhetic acid in human plasma. The LC/MS and LC/MS/MS methods were much more sensitive and practical for the pharmacokinetic study of glycyrrhetic acid. Ding *et al.*[27] reported a LC-ESI-MS method to evaluate the pharmacokinetic of glycyrrhetic acid in human plasma. Wei *et al.*[28] reported a LC/MS method for simultaneous determination of 18α- and 18β-glycyrrhetic acid in human plasma and applied it to the pharmacokinetic study. But in their experiments 1 mL of human plasma was extracted with 5 mL extractant each time in sample preparation and analysis of each sample needed 5 min or even more.

In this paper, we reported a more sensitive, convenient, time-saving and plasma-saving method to determinate the concentration of glycyrrhetic acid in human plasma, in which 100 μL plasma was extracted with 1 mL dichloromethane each time and analysis of each sample required within 3.5 min. The method may save much more time and plasma in batch processing and successfully applied to evaluate the pharmacokinetic of glycyrrhetic acid in healthy Chinese volunteers.

EXPERIMENTAL

Glycyrrhetic acid (Fig. 1A) and oleanolic acid (Fig. 1B) were supplied by the National Institute for the Control of

Pharmaceutical and Biological Products (Beijing, China). HPLC-grade dichloromethane and ammonium acetate were purchased from TEDIA (Fairfield, USA); HPLC-grade methanol and acetonitril were purchased from Merck Company (Dermstadt, Germany). Distilled water, prepared from demonetarized water, was employed throughout the whole experiment. Blank plasma was provided by The First Affiliated Hospital of Anhui Medical University (Hefei, China).

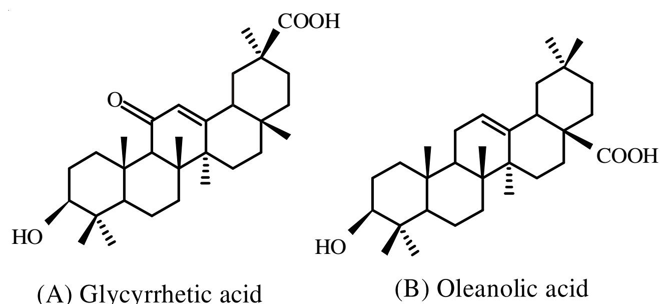

Fig 1. Stuctures of glycyrrhetic acid and oleanolic acid

A TSQ Quantum Ultra AM triple quadrupole tandem mass spectrometer (Thermo Finnigan, USA), coupled with electrospray ionization source, a Finnigan Surveyor LC pump and Finnigan Surveyor autosampler, was used for LC-MS/MS analysis. Data acquisitions were performed with Xcalibur 1.4 software (Thermo Finnigan, USA). The system was operated at ambient temperature 20 ºC.

Chromatographic separation and mass spectrometric conditions: Reversed-phase LC separation was performed using a Finnigan Surveyor LC pump, with a Luna CN column (5 µm 150 mm × 2.0 mm i.d.; Phenomenex, USA) and the column temperature was maintained at 25 ºC. The mobile phase was composed of acetonitrile and water (containing 0.05 % acetic acid and 0.02 % ammonium acetate) (95:5 v/v) at a flow-rate of 0.2 mL/min throughout the experiment.

Mass spectrometric detection was carried out using nitrogen to assist nebulization. A triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was performed in negative ion mode with selected reaction monitoring (SRM) using target ions at m/z 469.40→469.40 for glycyrrhetic acid and m/z 455.47→455.47 for oleanolic acid (the internal standard, IS). The product ion spectra of $[M-H]^-$ ions of glycyrrhetic acid and internal standard were analyzed. The optimal parameters were summarized in the following: capillary voltage of 3800 V, capillary temperature of 270 ºC, source CID of 10 eV. Nitrogen was used as both sheath and auxiliary gas at the pressures (arbitrary units) of 10 and 3, respectively. Argon was used as collision gas at a pressure of 1.0 mTorr. The collision energy of 10 eV was set for glycyrrhetic acid and internal standard. The scan width for SRM was 0.2 and scan time was 0.35 s.

General procedure

Preparation of standard solutions, calibration standard solutions and quality control samples: Stock solution of glycyrrhetic acid (1.6 µg/mL) was prepared by dissolving the accurately weighted glycyrrhetic acid in methanol. Solution of internal standard was prepared in methanol at concentration of 20 µg/mL and diluted to 1 µg/mL with methanol for sample preparation. All the stock solutions were stored at -20 ºC and stable for 2 months at least until analysis.

Standard solution of 800, 400, 200, 100, 50, 25 ng/mL glycyrrhetic acid were prepared by diluting the 1.6 µg/mL glycyrrhetic acid with methanol step by step. Calibration curves were prepared by spiking appropriate amount of the standard solutions in blank plasma to obtain concentrations of 2.5, 5, 10, 20, 40, 80, 160 ng/mL.

Quality control (QC) samples were prepared by spiking blank plasma at concentrations of 5, 20, 80 ng/mL, representing low-, medium-, high-concentration quality control samples, respectively. All the calibration standard solutions and quality control samples were stored at -20 ºC.

Sample preparation: Plasma sample (100 µL) was collected in 2 mL clean centrifugal tube and 10 µL of internal standard solution (1 µg/mL) was added. The samples were briefly mixed for 30 s and 1 mL dichloromethane was added. The mixture was vortex-mixed for 3 min. After centrifugation at 13,400 rpm for 10 min, the lower organic phase was removed to another 1.5 mL centrifugal tube and evaporated to dryness under a stream of nitrogen in a 40 ºC water bath. The residue was reconstituted in 80 µL of the mobile phase. After vortex for 1 min and centrifugation for 3 min, 10 µL of the aliquot was injected into the LC-ESI-MS/MS system for analysis.

Detection method: The validation was carried out according to the recommendations and definitions provided by the 'Guidance for Industry-Bioanalytical Method Validation' (FDA, USA)[29].

For the calibration standards, peak area ratios (the analyte: internal standard) were plotted against nominal plasma concentrations and fitted by weighted ($1/x^2$) least-squares linear regression. Plasma calibration curves were prepared and assayed in triplicate on three separate days. In addition, six blank plasma samples were analyzed to confirm absence of interferences.

The lower limit concentration of quantification (LLOQ) was evaluated by analyzing samples preparing in quintuplicate (n = 5) at which both precision and accuracy were not more than 20 %.

Accuracy was assessed by determining the quality control (QC) samples at low-, medium-, high-concentration prepared in quintuplicate at three separate days. Relative standard deviation (RSD) and relative error (RE) were used to characterize the accuracy which were calculated using the formula: RSD % = (standard deviation)/(mean concentration) × 100 %; RE % = (mean observed concentration - spiked concentration)/(spiked concentration) × 100 %. The acceptable criterion was 15 % at maximum.

For the evaluation of recovery, peak areas of glycyrrhetic acid obtained were compared from the spiked-after-precipitation samples with those from the pure standard solutions at the same concentration level. Low-, medium-, high-quality control samples were evaluated in triplicate for every concentration.

Owing to the components of the sample matrix, signal suppression or enhancement may occur. Matrix effect was assessed by comparing peak areas of glycyrrhetic acid obtained from the spike-after-precipitation samples with those from the pure standard solutions at the same concentration level.

Stability experiments were performed by analyzing quality control samples in quintuplicate after exposure to different temperature and timing conditions which including the following conditions: three freeze-thaw cycles, short-term storage stability (20 ºC for 4 h), 24 h storage stability (prepared samples at ambient temperature for 24h), long-term storage stability (-20 ºC for 30 days).

Pharmacokinetic study: A pharmacokinetic study was conducted with 19 healthy male volunteers who had received a single oral dose of 150 mg diammonium glycyrrhizinate tablets. The drug was administrated under fasting condition. Blood samples were taken at 0, 4, 6, 8, 10, 11.5, 13, 14.5, 16, 18, 24, 36, 48 and 60 h after ingestion. Plasma was obtained through centrifugation at 3000 rpm for 10 min and then stored at -20 ºC prior to analysis.

RESULTS AND DISCUSSION

Mass spectrometric conditions: Owing to the carboxyl group existing in the glycyrrhetic acid, it was much prone to losing a proton. Therefore, the signal intensities obtained in negative ion mode were much higher than those in positive ion mode. In our experiment, compared with selected ion monitoring (SIM) mode, selected reaction monitoring (SRM) mode was chosen because in this mode, analyte can be filtered twice to eliminate impure ions and make signal *versus* noise (S/N) stronger. Because of difficult fragmentation of glycyrrhetic acid and oleanolic acid, m/z 469.40→469.40 (glycyrrhetic acid) and 455.47→455.47 (internal standard) were set as pair ions, respectively. In order to gain strong response in spectrum, collision voltage was optimized to values ranging from 5 to 45 eV with a step of 5 eV according to response in signals. At last, 10 eV was adopted as the collision voltage for both glycyrrhetic acid and internal standard.

Chromatographic separation conditions: Several columns were investigated at the beginning of the experiment to screen a suitable column which was best fit for the chromatographic separation. In this study, three columns were evaluated, including Luna C18 (5 µm) column, Luna CN (5 µm) column and Synergi 4 µm Fusion-RP column. The contrast results of columns appeared at Fig. 2A-C. From Fig. 2, the Luna CN column was found to give shaper peaks, stronger signal/noise and suitable retention time, which became the best choice in chromatographic separation.

Multiple components of mobile phase were assessed to increase the sensitivity and obtain better separation. For mobile phase, water-methanol system was compared with water-methanol-acetonitrile system and water-acetonitrile system. It was found that water-acetonitrile system gave more symmetric peak shapes and suitable retention time. In addition, by comparing additives of formic acid, acetic acid, ammonium acetate and triethylamine at different percentages, we found 0.05 % acetic acid and 0.02 % ammonium acetate in water had a great effect on retention time and signal intensity. In order to obtain high accuracy, the use of the internal standard was necessary when a mass spectrometer was equipped with HPLC as detector. Based on the previous work, ursolic acid, oleanolic acid, acetaminophen, domperidone and enalapril maleate were tested. Under these experimental conditions,

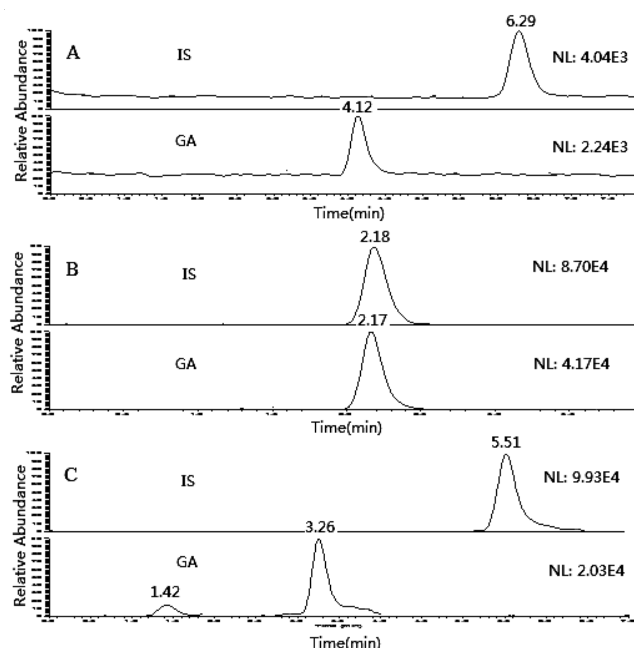

Fig. 2. SRM chromatograms for glycyrrhetic acid and internal standard on three different columns at the same concentration; (A) Luna C18 (5 µm) column; (B) Luna CN (5 µm) column; (C) Synergi (4 µm) column

oleanolic acid was adopted in the end for its similar structure, chromatographic character and extraction efficiency to the glycyrrhetic acid. Finally, the optimal parameters for HPLC determination described above were obtained.

Selectivity: Under the optimal mass spectrometric and chromatographic condition, the results for selectivity were shown in Fig. 3. Fig. 3A showed a typical chromatogram for blank plasma. Fig. 3B showed the chromatogram of blank plasma spiked with glycyrrhetic acid (2.5 ng/mL) and internal standard (100 ng/mL). Fig. 3C showed the chromatogram for a volunteer plasma sample 4 h after an oral dose of 150 mg glycyrrhetic acid. From Fig. 3, it appeared that there were no endogenous substances in blank plasma at the same retention time as glycyrrhetic acid and internal standard.

Linearity and the LLOQ: Linearity was evaluated by calibration curves ranging from 2.5-160 ng/mL for the analyte. Peak area ratios (glycyrrhetic acid:internal standard) were plotted against glycyrrhetic acid concentrations and fitted by weighed ($1/x^2$) least-squares linear regression. To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on three consecutive days. Calibration curves appeared good linearity, such as $y = 0.021x + (0.892 \pm 0.044)$. The calibration coefficient fell between 0.9980 and 1.0000.

The lower limit concentration of quantification (LLOQ) was measured with acceptable accuracy and precision at 2.5 ng/mL (S/N > 10) in human plasma, that appeared the inter-day precision (RSD) was 13.34 % and the accuracy (RE) was -2.2 %.

Precision and accuracy: The inter-day precision and accuracy of this method was evaluated by analyzing 45 quality control samples at three concentration levels, while the intra-day precision and accuracy was evaluated by analyzing quality control samples in quintuplicate at three concentration levels. Table-1 summarized the mean values of precision and accuracy for both inter- and intra-day assays.

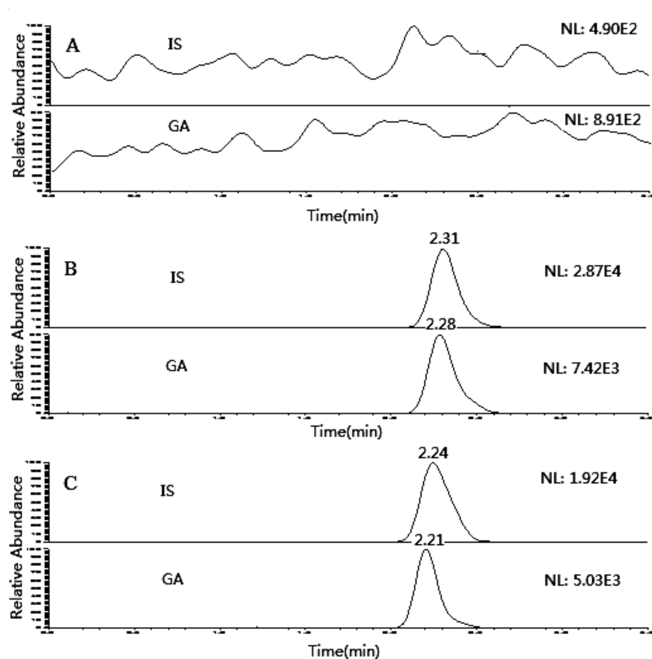

Fig. 3. SRM chromatograms for glycyrrhetic acid and internal standard in human plasma; (A) blank plasma; (B) blank plasma spiked with standard glycyrrhetic acid (2.5 ng/mL) and internal standard (100 ng/mL); (C) human plasma sample after administration of glycyrrhetic acid (4 h) and spiked with internal standard

TABLE-1
PRECISION AND ACCURACY FOR ASSAY OF GLYCYRRHETIC ACID IN HUMAN PLASMA

Date		Concentration of glycyrrhetic acid (ng/mL)		
		Low quality control	Medium quality control	High quality control
		5.000	20.000	80.000
Day 1	Mean (ng/mL)	5.056	19.473	74.191
	RSD (%)	7.497	5.191	3.810
	RE (%)	1.131	-2.636	-7.261
Day 2	Mean (ng/mL)	4.803	19.212	75.257
	RSD (%)	9.031	8.718	4.125
	RE (%)	-3.949	-3.942	-5.929
Day 3	Mean (ng/mL)	5.245	18.731	79.790
	RSD (%)	11.036	7.153	3.183
	RE (%)	4.904	-6.344	-0.262
Inter-day RSD (%)		9.437	6.828	4.753
Inter-day RE (%)		0.695	-4.307	-4.484

RSD = Relative standard deviation; RE = Relative error.

Recovery and matrix effect: Unlike others' sample processing, we chose dichloromethane as liquid-liquid extracting agent and obtained preferable recovery. Dichloromethane as extracting agent can make the processed samples more clean, which was good for chromatography and mass spectrometer. The recovery of glycyrrhetic acid ranged from 72.4 to 77.1 % at three quality control concentration levels, respectively. In terms of matrix effect, 90.45-106.2 % was obtained for glycyrrhetic acid, which demonstrated ion suppression or enhancement from plasma matrix was negligible for this method.

Storage stability: Three freeze-thaw cycles, short-term, 24 h and long-term storage stability were evaluated by analyzing quality control samples at different condition. The results for the stability of glycyrrhetic acid were given in Table-2, which demonstrated reliable stability through all the experimental procedure.

TABLE-2
STORAGE STABILITY FOR ASSAY OF GLYCYRRHETIC ACID IN HUMAN PLASMA (n = 5)

Storage condition	Concentration (ng/mL)		RSD (%)	RE (%)
	Spiked	Found		
4h stability	5	4.974 ± 0.098	1.844	-0.529
	20	19.718 ± 2.296	10.157	-1.410
	80	70.846 ± 1.353	1.871	-11.443
24h stability	5	5.308 ± 0.361	6.568	6.161
	20	20.965 ± 0.878	3.813	4.827
	80	80.518 ± 6.653	7.207	0.647
Three freeze/thaw cycle	5	5.548 ± 0.310	4.906	10.970
	20	19.396 ± 1.516	6.778	-3.019
	80	80.484 ± 7.680	12.024	0.605
Long term stability	5	5.046 ± 0.127	2.296	0.920
	20	20.031 ± 1.497	6.533	0.155
	80	88.583 ± 1.352	1.345	10.728

RSD = Relative standard deviation; RE = Relative error.

Application: This method was successfully applied to determine the plasma concentration of glycyrrhetic acid after oral administration of 150 mg glycyrrhetic acid. The mean plasma concentration-time curve of glycyrrhetic acid was shown in Fig. 4. The pharmacokinetic parameters received as follows: the maximum plasma concentration (C_{max}) 43.664 ± 21.209 ng/mL; area under the curve from 0 h to the last measurable concentration (AUC_{0-t}) 1110.679 ± 621.584 ng h/mL; area under the curve from 0 h to maximum plasma concentration ($AUC_{0-\infty}$) 1247.362 ± 779.432 ng h/mL; half-time ($T_{1/2}$) 15.675 ± 7.412 h.

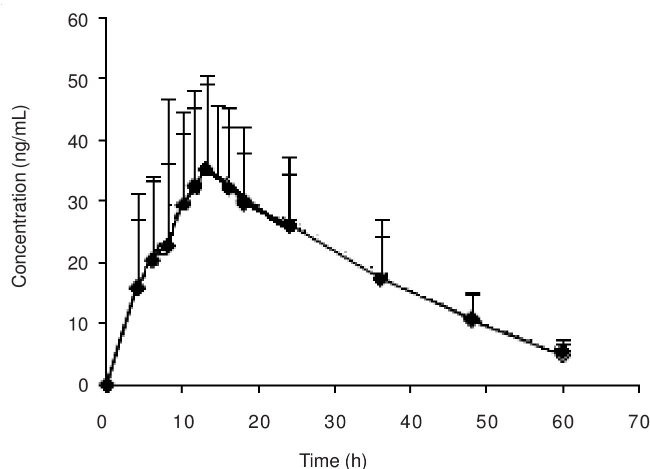

Fig. 4. Mean plasma concentration-time profiles of glycyrrhetic acid from 19 healthy volunteers after oral administration of 150 mg glycyrrhetic acid

Conclusion

We established a simple LC-MS/MS method for the quantification of glycyrrhetic acid in human plasma and applied it in pharmacokinetic study. This method offered interesting features of high sensitivity and selectivity, saved time and plasma.

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