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Simultaneous determination of glycyrrhizin, a marker component in radix Glycyrrhizae, and its major metabolite glycyrrhetic acid in human plasma by LC–MS/MS

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Abstract

Glycyrrhizin (GLY) which has been widely used in traditional Chinese medicinal preparation possesses various pharmacological effects. In order to investigate the pharmacokinetic behavior of GLY in human after oral administration of GLY or licorice root, a liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous determination of GLY and its major metabolite glycyrrhetic acid (GA) in human plasma. The method involved a solid phase extraction of GLY, GA, and α-hederin, the internal standard (IS), from plasma with Waters Oasis MCX solid phase extraction (SPE) cartridges (30 mg) and a detection using a Micromass Quattro LC liquid chromatography/tandem mass spectrometry system with electrospray ionization source in positive ion mode. Separation of the analytes was achieved within 5 min on a SepaxHP CN analytical column with a mobile phase of acetonitrile:water (50:50, v:v) containing 0.1% formic acid and 5 mM ammonium acetate. Multiple reaction monitoring (MRM) was utilized for the detection monitoring 823 → 453 for GLY, 471 → 177 for GA and 752 → 456 for IS. The LC–MS/MS method was validated for specificity, sensitivity, accuracy, precision, and calibration function. The assay had a calibration range from 10 to 10,000 ng/mL and a lower limit of quantification of 10 ng/mL for both GLY and GA when 0.2 mL plasma was used for extraction. The percent coefficient of variation for accuracy and precision (inter-run and intra-run) for this method was less than 11.0% with a %Nominal ranging from 87.6 to 106.4% for GLY and 93.7 to 107.8% for GA. Stability of the analytes over sample processing (freeze/thaw, bench-top and long-term storage) and in the extracted samples was also tested and established.

Keywords: Glycyrrhizin; Glycyrrhetic acid; Metabolite; LC–MS/MS

1. Introduction

The production of traditional, complementary, and alternative medicines, in particular those based on plant materials, is a global business. According to the report by the World Health Organization, the use of these types of medicine has been increasing [1]. Traditional Chinese medicines (TCM) have been used in clinical practice for several thousand years and the healing benefits of more than 7000 kinds of herbs have been documented. Recently, the use of traditional Chinese herbs as therapeutic agents has gained strong scientific supports in many placebo-controlled and double-blind clinical studies [2–8].

Glycyrrhizin (GLY), a triterpene saponin and major (marker) component of radix Glycyrrhizae (licorice root) which has been widely used in traditional Chinese medicinal preparation, possesses various pharmacological effects such as anti-inflammatory, anti-ulcerous and anti-allergic effects [9–15]. It has recently received attention as a potential therapeutic agent for several virus diseases including chronic hepatitis and acquired immunodeficiency syndrome (AIDS) [11,16–18]. GLY is known to be metabolized to glycyrrhetic acid (GA), the aglycone of GLY, by human intestinal bacte-

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ria prior to absorption [19]. In order to investigate the pharmacokinetic behavior of GLY after oral administration of GLY or licorice root in humans, simultaneous determination of GLY and GA in human plasma is inevitable. Detection of GLY and GA in biological fluids by high-performance liquid chromatography (HPLC) with ultraviolet detection [20–25] has been reported, but it was time consuming and lacked sensitivity. In addition, detection using capillary electrophoresia [25,26] and micellar electrokinetic chromatography [27] has been demonstrated in other studies. Although quantitative liquid chromatography/tandem mass spectrometry (LC–MS/MS) has become a common analytical tool for various compounds, however, an LC–MS/MS method suitable for the routine analysis of GLY and GA has not been reported. The present study describes a novel, simple, sensitive and reliable LC–MS/MS method for the simultaneous determination of GLY and GA in human plasma.

2. Experimental

2.1. Chemicals and reagents

GLY ammonium salt (~75% purity), GA (>98% purity), and α-hederin hydrate, the internal standard (IS), were purchased from Sigma (St. Louis, MO, USA). The chemical structures of GLY, GA, and IS are shown in Fig. 1. Type I water was prepared by an ultra high quality polishing system unit (UHQ-PS) (High Wycombe, Bucks, England). Acetonitrile, methanol, ammonium hydroxide, and hydrochloric acid were purchased from Fisher Scientific (St. Louis, MO, USA). Ammonium acetate was bought from Aldrich (Milwaukee, WI, USA) and formic acid (FA), from Burdick & Jackson (Muskegon, MI, USA). Blank human EDTA plasma was from Bioreclamation Inc. (Hicksville, NY, USA) and was stored in a freezer at −20 °C. All mobile phase solvents were HPLC grade and all reagents were analytical reagent grade.

2.2. Calibration standard solutions and quality control samples

Standard solutions and quality control samples (QCs) were made from two separate stock solutions (1 mg/mL in water). Working calibration standard solutions at concentrations of 10, 20, 50, 100, 500, 1000, 5000 and 10,000 ng/mL were fresh prepared in plasma daily. For the determination of inter- and intra-run accuracy and precision, six concentrations of QC samples, 10, 20, 50, 500, 5000 and 8000 ng/mL, were prepared in plasma, aliquoted, and stored at −20 °C. For the determination of dilution linearity, QC at 15,000 ng/mL that exceeded the upper limit of quantitation (ULOQ) was prepared.

2.3. LC–MS/MS methods

LC–MS/MS analyses were performed using a Waters 2690 HPLC system (Milford, MA, USA) coupled to a Micromass Quattro LC triple-quadrapole mass spectrometer

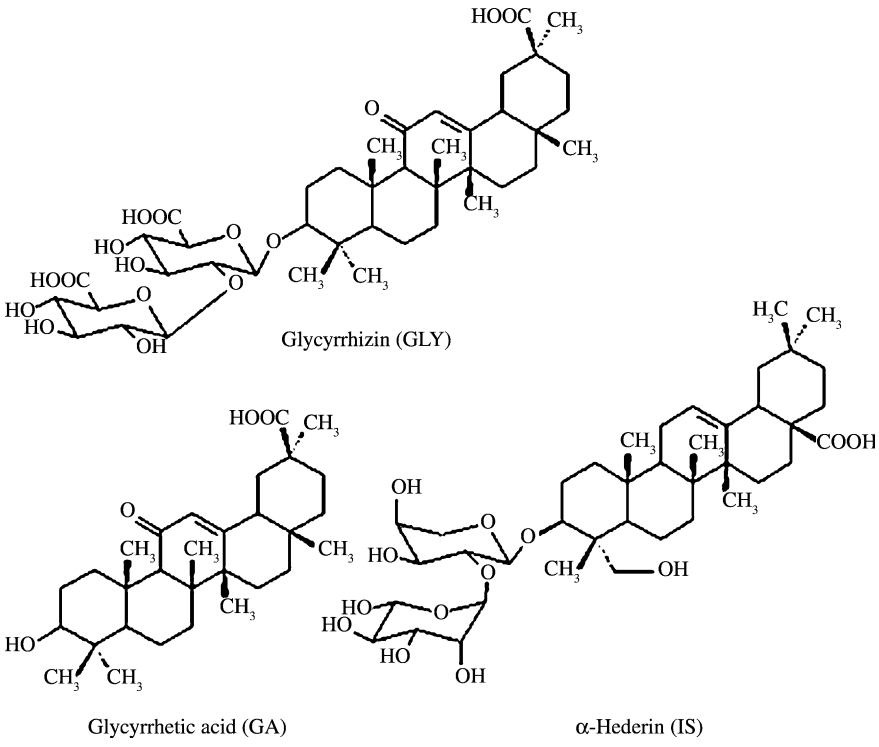

Fig. 1. Chemical structures of glycyrrhizin (GLY), glycyrrhetic acid (GA) and the internal standard (IS).

(Manchester, UK). The mass spectrometer was operated using an electrospray atmospheric pressure ionization source in positive ion mode (ESI^+) with multiple reaction monitoring (MRM). The analytical column was a SepaxHP CN, 100 mm × 2 mm, 3 μm (Newark, DE, USA) and column temperature was kept at room temperature. The mobile phase consisted of acetonitrile:water (50:50, v:v) with 5 mM ammonium acetate and 0.1% FA and the flow rate was 0.3 mL/min. The sample injection volume was 10 μL and run time was 5 min.

Sensitivity of MRM was optimized by infusing a mixture of GLY and GA, 1 μg/mL each, in the mobile phase. The capillary voltage was maintained at 3.8 kV. The cone and the extractor voltages were set to 35 and 3 V, respectively. The desolvation and ion source temperatures were 250 and 120 °C, respectively. Ions were activated at 15 eV of collision energy and at 2.3×10^{-3} Torr of indicated argon pressure. To assay all analytes, both quadrupoles were maintained at unit resolution and the transitions (precursor to daughter) monitored were *m/z* 823 → 453 for GLY, *m/z* 471 → 177 for GA, and *m/z* 752 → 456 for IS. The dwell time for each transition was 200 ms and the interchannel delay was 20 ms. MRM data were acquired and the chromatograms were integrated using MassLynx™ NT, version 3.2 software. A weighted 1/concentration^2 quadratic regression was used to generate a standard calibration curve and calculate sample concentrations.

2.4. Sample preparation

All samples, quality control samples, and standards with volume of 0.2 mL were spiked with IS (5000 ng/mL in methanol), acidified by addition of 0.5 mL of 0.1 N HCl water solution, and vortex-mixed in glass tubes. The samples were then loaded onto the Oasis MCX SPE cartridges (30 mg) (Milford, MA, USA) which were pre-conditioned with methanol and followed by water. The cartridges were washed with 0.1 N HCl water solution and dried by centrifuging at 3000 rpm for 15 min. Analytes were eluted with 2% NH_4OH in methanol to a set of clean glass tubes. The eluent was evaporated to dryness under nitrogen gas and redissolved in 0.1 mL mobile phase. After transfer into glass inserts of autosampler vials and centrifugation for 5 min at 4000 rpm, an aliquot of 10 μL of each sample was injected onto the LC–MS/MS system.

2.5. Validation of the LC–MS/MS method

The method fulfilled the analytical validation criteria for accuracy, precision, sensitivity, specificity, calibration function, and reproducibility according to the FDA guideline for bioanalytical methods validation for human studies [28]. The method validation was performed in three separate batch runs over a concentration range of 10–10,000 ng/mL using eight calibration standards, each containing the two analytes of interest, and six replicates of QC samples at each concentration level. Each batch run also contained additional samples such as stability samples for processing and storage.

Analyte stability test was conducted using QCs for multiple freeze/thaw cycles (F/T cycles), short-term stability (kept at room temperature for 4 h), and long-term storage (stored at −20 °C in the freezer for 1 month). Post-preparative stability and stock solution stability were also determined. The extraction recovery of GLY and GA was calculated by comparing the peak areas of extracted plasma standards to those of post-extraction plasma blanks spiked with corresponding concentrations. The overall absolute recovery in human plasma was determined by comparing the peak areas of extracted plasma standards to those of extracted standards prepared in mobile phase. The method specificity and matrix lot-to-lot reproducibility were evaluated by analyzing six lots of blank plasma and plasmas spiked with LLOQ concentration of GLY and GA, respectively.

3. Results and discussion

3.1. LC–MS/MS method

A SepaxHP CN analytical column with the mobile phase of acetonitrile and water (50:50, v:v) with 5 mM ammonium acetate and 0.1% FA provided good separation of GLY, GA, and IS, and maintained good peak shapes.

The electrospray ionization gave the optimum sensitivity for GLY and GA in positive ion mode. Electrospray in negative ion mode in appropriate solvent was also assessed but provided no advantages over positive mode. The desolvation temperature was kept low at 250 °C to prevent GLY from thermal decomposition at a higher temperature that would have sacrificed sensitivity of GA and IS. The Q1 mass spectrum of GLY, GA, and IS showed protonated molecular ions $[M + H]^+$ at *m/z* 823, 471, and 752, respectively (Fig. 2). The

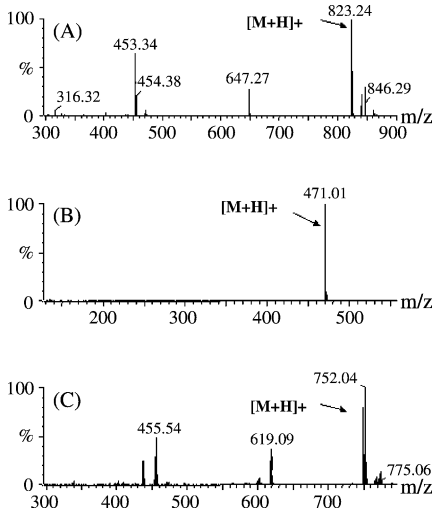

Fig. 2. Q1 scan spectra of glycyrrihizin (A), glycyrrihetic acid (B) and IS (C).

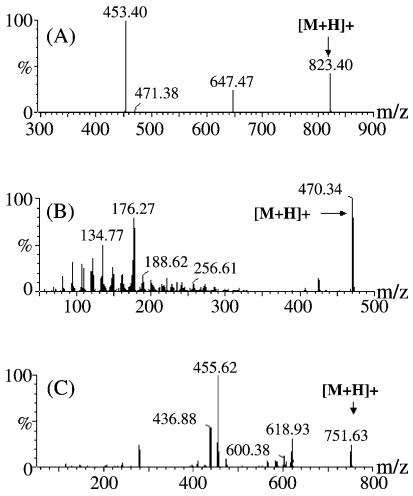

Fig. 3. Product Ion scan spectra of *m/z* 823 for glycyrrihizin (A), *m/z* 471 for glycyrrihetic acid (B) and *m/z* 752 for IS (C).

product ion scan spectrum of *m/z* 823 for GLY, *m/z* 471 for GA and *m/z* 752 for IS showed high abundant fragment ions at *m/z* 453, 177 and 456, respectively (Fig. 3). The ion transitions of *m/z* 823 → 453 for GLY, *m/z* 471 → 177 for GA and *m/z* 752 → 456 for IS were chosen for multiple reaction monitoring.

3.2. Specificity, sensitivity and calibration function

Six human blank plasmas or those spiked with IS, or with GLY and GA were extracted and used as a true blank (double blank without spiking analytes and IS) or as a single blank spiking only IS or analytes, respectively, for the analysis. There were no endogenous peaks that interfered with the quantitation of GLY and GA. There was no interference from IS contributing to the GLY and GA *m/z* channels or vice versa. In order to demonstrate matrix lot-to-lot reproducibility, six different blank plasmas were spiked with the two analytes at LLOQ, 10 ng/mL. The percent coefficient of variation (%CV) varied from 3.3 to 14.3% for GLY and 0.5 to 7.5% for GA and %Nominal ranged from 89.4 to 98.8% for GLY and 91.6 to 113.0% for GA (Table 1). This result indicated that there was no significant lot-to-lot variation in matrix effect. The ratio of signal to noise from extracted LLOQ samples was at least 20 for GLY and

Table 1
Matrix lot-to-lot reproducibility ($n = 3$) at LLOQ (10 ng/mL)

Matrix lot	GLY			GA		
	Mean	%CV	%Nominal	Mean	%CV	%Nominal
1	9.62	6.9	96.2	9.77	4.6	97.7
2	9.21	3.3	92.1	9.16	3.3	91.6
3	8.98	10.6	89.8	10.1	0.5	101.0
4	9.88	6.7	98.8	10.5	5.3	105.0
5	8.94	14.3	89.4	11.3	6.3	113.0
6	9.38	4.7	93.8	9.74	7.5	97.4

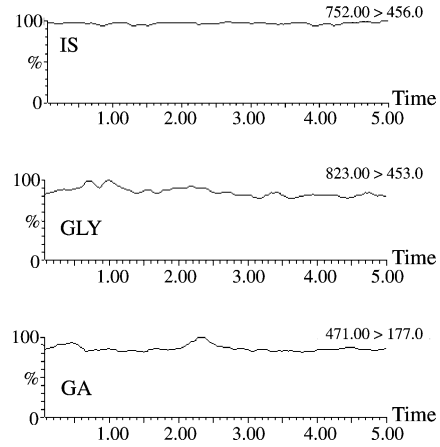

Fig. 4. Chromatograms of an extracted blank human plasma sample. (A) IS channel: *m/z* 752 → 456; (B) GLY channel: *m/z* 823 → 453; (C) GA channel: *m/z* 471 → 177.

GA. The calibration curve was constructed over concentration range of 10–10,000 ng/mL using a quadratic regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$) for GLY and GA. The typical calibration equations were $-2.34917e{-}8x^2 + 0.00145808x + 0.000726713$ for GLY with the coefficients of determination of 0.9964 or better and $-5.22350e{-}8\,x^2 + 0.00247339x + 0.0113784$ for GA with the coefficients of determination of 0.9956 or better, respectively. Representative chromatograms of extracted blank plasma, plasma spiked with IS only, or with LLOQ and ULOQ of GLY and GA standards are shown in Figs. 4–7.

3.3. Precision, accuracy and dilution linearity

Table 2 shows the validation data on accuracy and precision of each standard concentration. The inter-run %CV for the back-calculated calibration standards ranged from 3.2 to 8.1% for GLY and 3.6 to 11.0% for GA and the %Nominal ranged from 91.1 to 116.1% for GLY and 93.8 to 106.8% for

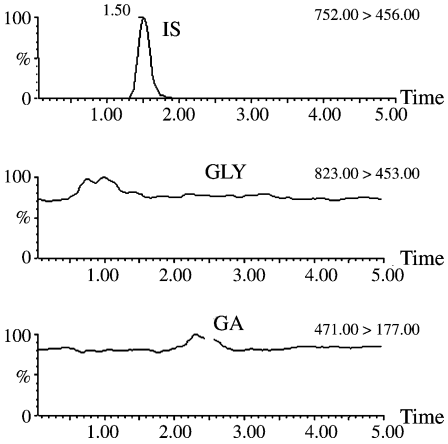

Fig. 5. Chromatograms of an extracted human plasma spiked with internal standard only. (A) IS channel: *m/z* 752 → 456; (B) GLY channel: *m/z* 823 → 453; (C) GA channel: *m/z* 471 → 177.

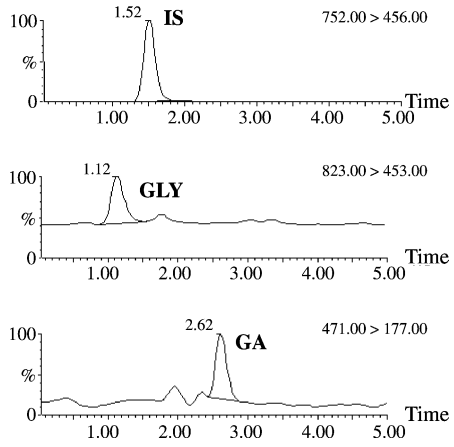

Fig. 6. Chromatograms of an extracted human plasma spiked with GLY, and GA at LLOQ (10 ng/mL). (A) IS channel: *m/z* 752 → 456; (B) GLY channel: *m/z* 823 → 453; (C) GA channel: *m/z* 471 → 177.

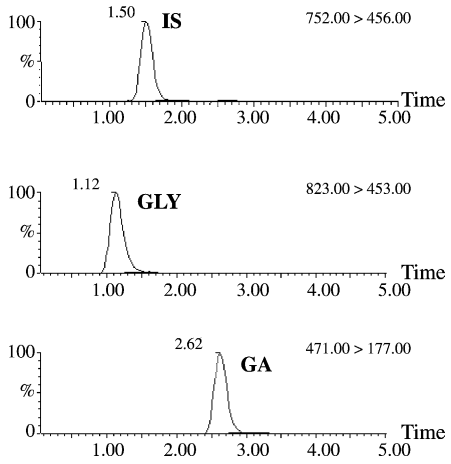

Fig. 7. Chromatograms of an extracted human plasma spiked with GLY, and GA at ULOQ (10,000 ng/mL). (A) IS channel: *m/z* 752 → 456; (B) GLY channel: *m/z* 823 → 453; (C) GA channel: *m/z* 471 → 177.

Table 2
Precision and accuracy of calibration standards ($N = 6$)

Nominal (ng/mL)	GLY			GA		
	Mean	%CV	%Nominal	Mean	%CV	%Nominal
10	9.11	7.9	91.1	9.52	11.0	95.2
20	23.2	3.2	116.1	21.4	9.5	106.8
50	52.2	4.9	104.5	52.4	3.6	104.9
100	101	3.7	100.9	106	3.6	106.3
500	469	6.4	93.8	469	4.2	93.8
1000	949	8.1	94.9	938	6.6	93.8
5000	4738	5.2	94.8	4772	6.2	95.4
10000	10448	3.8	104.5	10469	5.8	104.7

GA. The precision and accuracy data for QCs are summarized in Table 3. Intra-run %CV values were less than 9.2% for GLY and 6.1% for GA and %Nominal ranged from 87.6 to 98.2% for GLY and 93.7 to 105.1% for GA. Inter-run %CV values were less than 11.0% for GLY and 8.5% for GA and %Nominal ranged from 91.7 to 106.4% for GLY and 96.9 to 107.8% for GA. These %CV and %Nominal values indicated that LC–MS/MS conditions were reproducible and that the assay was consistent and reliable. For partial volume analysis, QC samples (15,000 ng/mL) were diluted 10-fold with blank plasma instead of water prior to extraction in order to keep the same matrix as the calibration standards. The dilution integrity data showed that the %CV for was 1.5 and 3.1% for GLY and GA, respectively, with a %Nominal of 98.2 and 98.7% for GLY and GA, respectively. These results support that sample dilution up to 10-fold for analysis was acceptable.

3.4. Recovery and matrix effect

Waters Oasis mixed-mode MCX SPE cartridge with cation exchange and reversed-phase sorbent was used to clean up

Table 3
Precision and accuracy of quality control samples

Nominal (ng/mL)	GLY			GA		
	Mean	%CV	%Nominal	Mean	%CV	%Nominal
Intra-run ($N = 5$)						
10	9.30	9.2	93.0	9.41	4.5	94.1
20	17.5	4.4	87.6	18.7	6.1	93.7
50	45.2	3.2	90.4	49.7	4.0	99.3
500	441	3.9	88.1	499	2.2	99.8
5000	4670	8.6	93.4	5256	5.1	105.1
8000	7034	7.5	87.9	8027	4.1	100.3
15000[a]	14729	1.5	98.2	148113	3.1	98.7
Inter-run ($N = 3$)						
10	10.6	7.6	106.4	10.1	8.5	101.4
20	19.3	7.1	96.7	20.6	1.1	102.9
50	47.5	11.0	95.0	48.9	6.9	97.8
500	459	4.7	91.7	485	5.2	96.9
5000	5306	1.8	106.1	5388	5.9	107.8
8000	8085	10.8	101.1	8181	2.6	102.3

[a] Samples were diluted 10-fold with blank plasma prior to analysis.

Table 4
Stability of GLY and GA

	GLY			GA		
	20	500	8000	20	500	8000
4 h short-term at 25 °C						
Mean (n = 3)	22.3	466	7563	18.4	505	7963
%CV	4.8	2.5	1.8	7.8	4.9	2.8
%Nominal	111.4	93.2	94.5	92.0	101	99.5
1 month at −20 °C						
Mean (n = 3)	20.7	438	7589	19.4	461	8736
%CV	9.6	3.1	6.0	3.6	2.9	1.8
%Nominal	103.4	87.6	94.9	97.0	92.4	109.2
Three freeze/thaw cycles						
Mean (n = 3)	21.7	483	7667	18.6	509	7901
%CV	7.6	4.0	3.0	7.5	2.9	3.7
%Nominal	108.5	96.6	95.8	93.0	101.8	98.8
24 h post-preparative						
Mean (n = 3)	20.3	482	8042	19.8	467	7887
%CV	4.6	5.9	7.5	7.6	7.9	5.2
%Nominal	101.5	96.3	100.5	98.8	93.3	98.6

plasma sample. The extraction recovery was determined by comparing the peak areas of extracted plasma standards at 20, 500 and 10,000 ng/mL to those of post-extraction blank plasmas spiked with corresponding concentrations. The mean extraction recovery was 77.7, 74.6, and 83.6% for GLY, GA and IS, respectively. The mean overall absolute recovery was 32.3, 54.1 and 96.0% for GLY, GA and IS, respectively. The mean overall absolute recovery for GLY and GA was low, which was probably due to matrix effects. However, matrix effects did not cause quantitation bias as evidenced by the %CV and %Nominal values. Therefore, attempts to further improve the matrix effect were not pursued.

3.5. Stability of the analytes

The stability tests of the analytes were designed to cover anticipated conditions that clinical samples might experience. Stability data are summarized in Table 4 and indicated that the analytes were stable for at least the length of time under different conditions listed. Briefly, three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 4 h prior to sample preparation appeared to have no effect on the quantitation of GLY and GA. QCs stored in a freezer at −20 °C remained stable for at least 1 month. Extracted analytes were allowed to stand at ambient temperature for 24 h prior to LC–MS/MS analysis and no effect on quantitation was observed. Stability of stock solutions was also investigated. When stock solutions of GLY and GA in water were stored at a nominal temperature of 4 °C for 1 month or at room temperature for 6 h, the analytes were stable.

4. Conclusions

An LC–MS/MS method for quantitation of GLY and its metabolite GA in human EDTA plasma has been successfully developed and validated. Plasma samples were cleaned up using Waters Oasis MCX SPE cartridges. The lower limit of quantitation for GLY and GA in 0.2 mL plasma sample was 10 ng/mL. The method described here clearly demonstrates greatly improved sensitivity and analysis speed compared to the conventional HPLC methods. It, therefore, is suitable for routine quantitation of GLY and GA in human plasma over a concentration range of 10–10,000 ng/mL to support clinical trials of GLY or radix Glycyrrhizae.

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