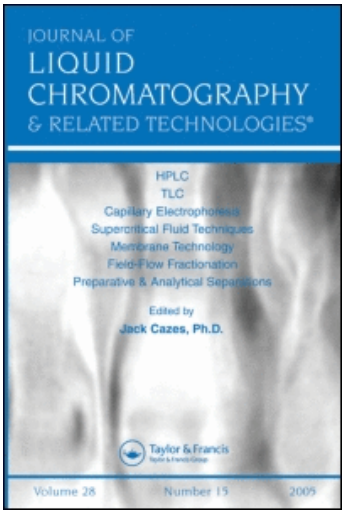

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Isolation and Characterization of Related Impurities of Monoammonium Glycyrrhizinate and Development of RP-HPLC Method for Quality Control Study

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Abstract: An HPLC method was developed for the determination of impurities of monoammonium glycyrrhizinate in bulk drugs and pharmaceutical formulations. The separation was accomplished on a Shimadzu Shim-pack C_{18} (150 mm × 4.6 mm, 5 μm) column. The mobile phase was methanol: (0.2 M) ammonium acetate (70:30; v/v, pH = 4.5) and a UV detector set at 250 nm was used for detection. The unknown impurities were isolated by preparative HPLC and characterized by FT-IR, ESI-MS, ^{1}H NMR and ^{13}C NMR spectral data. The proposed method was validated and successfully applied to the analysis of commercial bulk drugs and formulations, thus it supplied a simple and sensitive method to ensure the quality assurance for the manufactures and researches.

Keywords: Glycyrrhizin, Isolation and characterization, Monoammonium glycyrrhizinate, Related impurities

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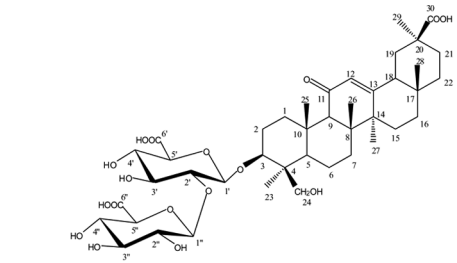
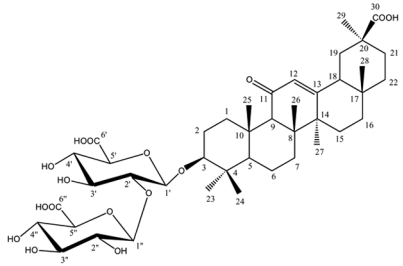
INTRODUCTION

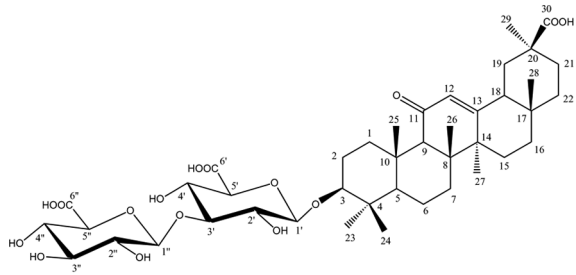
Glycyrrhizin (GL, CAS 1405-86-3) a triterpene glycoside from licorice root, is composed of a molecule of glycyrrhetic acid and two molecules of glucronic acid. It has been reported to have a variety of pharmacological activities, including anti-inflammatory, anti-allergic and anti-viral activities.[1–4] In Japan, Stronger Neo-Minophagen CTM (SNMC), a GL-containing preparation, has been used as a treatment for chronic hepatitis for more than 30 years.[5–8] The pharmaceuticals similar to SNMC are widely used in China.[9,10] The raw material of these pharmaceuticals is monoammonium glycyrrhizinate, and the official standard set for the raw material is that the monoammonium glycyrrhizinate not to be less than 73.0% by HPLC determination.[11] What are the impurities occupied 27.0%? Do they also exist in the preparations? According to the thorough literature search, there is no report on the analysis of the relative impurities of monoammonium glycyrrhizinate. But with the further research and development on the medicine, it is necessary to develop analytical methods for separation and determination of the related impurities for the evaluation of its quality. Thus a comprehensive study to separate and purify the unknown impurities was undertaken, and they were identified as saponin G2 (24-Hydroxycyrrhetic Acid-3-O-ß-Glucuronopyranosyl(1→2)-ß-Glucuronopyranoside; SG) and uralsaponin B (Cyrrhetic Acid-3-O-ß-Glucuronopyranosyl(1→2)-ß-Glucuronopyranoside; UB). They were isolated and characterized by ESI-MS, ^{1}H, ^{13}C NMR and FT-IR spectra. A simple HPLC method was developed to assay the content of the three main components in the bulk drugs and the pharmaceutical formulations. The structures of them were shown in Fig. 1. Several assays have been developed to detecting GL in licorice or its herbal preparations, such as HPLC-UV or DAD,[12–15] and even nano-LC.[16] HPLC-MS, or MS/MS.[17–19] Capillary zone electrophoresis method[20–22] and immunochromatographic separation[23–25] were also developed. However, among these methods, except HPLC-UV and DAD, the equipments used in other methods are either complicated or expensive for the manufactories to control the prompt intrinsic quality of the bulk drugs and pharmaceuticals. In some manufactories, the compulsory criterion of the quality control is to determine GL only. Obviously, it cannot well ensure the products' safety and batch-to-batch uniformity; thereby a novel approach for the multiple components determination is urgently needed. This work focuses on developing a simple, effective and low cost HPLC method of simultaneously determination of GL and the impurities for the routine analysis and quality evaluation of the bulk drugs and pharmaceutical formulations.



saponin G_2



Glycyrrhizin



uralsaponin B

Figure 1. The structures of saponin G_2, and uralsaponin B.

EXPERIMENT

Materials

HPLC grade acetonitrile and methanol were purchased from Merk (Merk, Darmstadt, Germany); ammonia, ammonium acetate, hydrochloric acid, acetic acid and phosphoric acid were of analytical grade from Nanjing Chemicals Co. Ltd., (Nanjing, China); deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

Monoammonium glycyrrhizinate S, supplied by Xinjiang Tianshan Co. Ltd., (Xinjiang, China). Compound ammonium glycyrrhetate injection, supplied by No. 7 Wuxi Pharm Co. Ltd., (Wuxi, China).

Instruments

FT-IR Spectroscopy

All experimental absorbance spectra were recorded on a Nicolet Impact 410 FTIR with an Everglo^{TM} (Nicolet Instrument Co., US) mid-IR source, a 9 mm aperture, a Ge-on-KBr beamsplitter, and a DGTS detector. An optical retardation velocity of 0.6 cm s^{-1} was used.

MS Spectrometry

The ESI-MS analyses were performed on an Agilet 1100 LC-DAD system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump and automatic sampler. The parameters were set with a drying gas (N_2) flow of 9.0 L/min, nebulizer pressure of 35 psi, drying gas temperature of 350°C, capillary voltage of 4 kV and fragmentor voltage of 70 V. Negative ionization with selected-ion monitoring (SIM) mode was used for all analyses. The scan range was within 120–100 m/z.

The separation was carried out on a Shimadzu Shim-pack C18 (250 mm × 4.6 mm, 5 μm) with the mobile phase consisted of acetonitrile: 0.2% acetic acid (40:60; v/v) at a flow-rate of 1.0 mL/min.

NMR Spectroscopy

NMR spectra were recorded on a Bruker DRX500 Fourier transform NMR spectrometer (Bruker, Fallanden, Switzerland). Tetramethylsilane (TMS) (0.2–0.3 mmol) in pyridine-d5 (C_5D_5N) was used as an internal reference standard. Chemical shifts were given in δ-values in ppm downfield from tetramethylsilane ($\delta_{TMS} = 0$). ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{13}C-^{13}C COSY correlation techniques and homonuclear decoupling technique were conducted to assign the chemical shifts of proton and carbon atoms of the analytes.

Semi-Preparative Chromatography

A Shimadzu LC-8A Preparative Liquid Chromatograph equipped with SPD-10A VP, UV-VIS detector (Shimadzu Co., Analytical Instruments Division, Kyoto, Japan) was used. Separation and isolation of the impurities was carried out on a preparative PREP-ODS C18 (250 mm long × 20 mm i.d.) column packed with 10 μm particle size (GL Sciences Inc., Tokyo, Japan), using methanol: 0.1% acetic acid (70:30; v/v) as

mobile phase at a flow rate of 10 mL/min and the detector was maintained at 250 nm.

Analytical Chromatography

The analytical chromatography was carried on a Shimadzu LC-10A Liquid Chromatograph equipped with SPD-10A VP, UV-vis detector (Shimadzu Co., Analytical Instruments Division, Kyoto, Japan). The Shimadzu Shim-pack C18, (150 mm × 4.6 mm, 5 μm) was adopted and methanol: (0.2 M) ammonium acetate (70:30; v/v, pH = 4.5) was used as mobile phase at a flow rate of 1 mL/min and the detector was maintained at 250 nm.

Solutions (180.6 μg/mL) of GL, (15.60 μg/mL) of saponin G2 and (36.75 μg/mL) of uralsaponin B were prepared in the mobile phase as the standard solutions. 1 mL of Compound ammonium glycyrrhetate injection was diluted to 10 mL as the working solution.

RESULT AND DISCUSSION

The chemical structures of glycyrrhizinic acid and the impurities saponin G2, uralsaponin B are shown in Fig. 1. The present study is aimed at developing a separate and quantitative determination method of GL, SG, and UB. Further studies will be carried out to characterize their structures.

Optimization of the Analytical Conditions

According to the published papers and the preliminary experiments in our lab, GL and the impurities were subjected to separation by RP-HPLC on commercial C18 columns. The mobile phase systems were tried as follows:

(A) acetonitrile: (0.1%) phosphoric acid (40:60; v/v);
(B) methanol: (0.2 mol/L) ammonium acetate (70:30; v/v, pH = 4.5);
(C) methanol: (1%) acetic acid (70:30; v/v); and
(D) acetonitrile: (0.01 mol/L) acetic acid (38:62; v/v)

All of these mobile phases could achieve good resolution and peak symmetry, as well as a short run time for the interest analytes. Considering the cost and environment effects, B was selected for the determination analysis and C was adopted for the preparative HPLC system.

Isolation of Impurities by Preparative HPLC

Pretreatment of Samples

Monoammonium glycyrrhizinate can be described as R–NH_4, and when methanol: (1%) acetic acid (70:30; v/v) was used as the mobile phase, the following equation is occurred:

$$R\text{–}NH_4 + HAc \rightleftharpoons RH + NH_4Ac$$

As the strong acidity of the mobile phase, the equation is incline to turn to the right side, that means monoammonium glycyrrhizinate is exist as GL in the mobile phase. Thus, while the sample is vacuum distilled, methanol, water and acetic acid would be distilled in turn, and the above equation would go to the right side, then the end product would be the mixture of GL and ammonium acetate. The ammonium acetate has to be removed in order to purify GL, but the routine method to get rid of it would make a great loss of the GL inevitably. So it is recommended to change monoammonium glycyrrhizinate to GL before it enters into the preparative chromatographic system. The detailed process is as follows:

5.00 g monoammonium glycyrrhizinate was put in 150 mL deionized water and then appropriate volume of ammonia was added till the pH was around 9.50–9.60. While the solution was totally dissolved, hydrochloric acid was used to adapt the pH to 2–3 and then there will appear white powder. Centrifugated the white powder at $3000 \times g$ for 10 min and collected the underlayer. Washed it with 50 mL distilled water and then centrifugated again. After washing for two times, the underlayer is vacuum distilled at $-40°C$. Finally, 4.74 g white loose powder was obtained.

The Sample Solution

The above loose powder was dissolved in the mobile phase, and the concentration of saturation solution is about 70 mg/mL. The concentration of the sample solution was set at 50 mg/mL according to the chromatographic peak shape and the loading capability of the column.

Preparation Process

For concentration 50 mg/mL was used, total 100 runs were performed using 0.2 mL (10 mg) in each loading on to the preparative LC column. The major peaks were isolated individually at $7.80 \sim 8.70$ min, $9.65 \sim 11.65$ min, $14.60 \sim 15.80$ min. All isolated fractions that have maximum purity were pooled together for individual impurities. All isolated and final pooled fractions were analyzed by analytical HPLC.

The solvent evaporation was performed under high vacuum using Buchi rotavapor R-124 equipped with vacuum system B-178 and water bath B-480. The concentrated fractions were dried using dryer (Christ, Type 100400, Martin Christ Gefriertrock-nungsanlagen GmbH, Osterode, Germany) and the samples were used for identification purpose. Finally, 0.52 g of GL, 0.14 g of SG and 0.22 g of UB were isolated in solid form.

Characterization of Glycyrrhizin, Saponin G_2 and Uralsaponin B

Glycyrrhetic acid is the aglycone of GL. In order to verify the structure of GL, the spectrums of ^{1}H NMR and ^{13}C NMR of glycyrrhetic acid were investigated and compared to those of GL, SG and UB, as shown in Tables 1 and 2.

Glycyrrhizin

GL gave a deprotonated molecular ion $[M–H]^-$ at m/z 821 and the peak of $[M + Na–2H]^-$ at 843 m/z in ESI-MS (negative mode). The IR spectrum showed 3437 cm^{-1} (O–H, stretch, broad), 1726 cm^{-1} (C=O, stretch, strong), 1652 cm^{-1} (C=C, stretch, strong), 1172 cm^{-1} and 1051 cm^{-1} (C–O, stretch, strong).

The data of ^{1}H NMR and ^{13}C NMR of GL were most similar to those of authentic samples of glycyrrhetic acid. With the comparison of the ^{13}C NMR spectra of GL and glycyrrhetic acid, it was found that C3 (δ 89.1 ppm) downfield shifted to (δ 78.7 ppm) but C2, C4 upfield shifted a little. It can be concluded that this carbon atom is the glycosyl site. The HMBC and H-H COSY experiments further confirmed this site. The HMBC data showed the correlations between C1″ (δ 106.8) and H3′ (δ 4.23 ~ 4.39), and the same between C2′ (δ 84.4) and H1″ (δ 5.42); while the H-H COSY spectrum indicated the correlations between H1″ (δ 5.42) and H2′ (δ 4.22 ~ 4.41). Therefore, it can be confirmed that the linkform of the glycosyl is 1→2. The ß configuration of the linkage between the glycosyl and aglycone was defined by the coupling constant J = 7.5 Hz and 7.6 Hz between H1′ and H1′. On the basis of the above analysis and comparison with the literatures or authentic samples, it was characterized as glycyrrhizin (Cyrrhetic Acid-3-O-ß-Glucuronopyranosyl (1→2)-ß-Glucuronopyranoside).

Saponin G2

SG gave a deprotonated molecular ion $[M–H]^-$ at m/z 837 and the peak of $[M + Na–2H]^-$ at 859 m/z in ESI-MS (negative mode). The IR

Table 1. ^{1}H NMR spectroscopic data of glycyrrhizin, saponin G2, uralsaponin B and glycyrrhetic acid (500 MHz, in pydine-d_5)

H	Glycyrrhizin	Saponin G2	Uralsaponin B	Glycyrrhetic acid
H-1a	3.02 d	2.98 m	3.01 d	2.99 m
H-1b	1.05 o	1.02 o	1.01 o	1.01 o
H-2a	2.06 o	2.09 o	2.07 o	2.10 o
H-2b	2.26 o	1.70 o	2.30 o	2.11 o
H-3	3.22 m	3.20 m	3.18 m	3.12 m
H-5	0.71 d	0.84 m	0.67 d	0.69 d
H-6	1.46 o	1.53 m	1.22 o	1.36 o
H-7a	1.22 m	1.23 m	1.22 o	1.26 m
H-7b	1.46 o	1.23 m	1.50 o	1.43 o
H-9	2.42 s	2.41 s	2.37 s	2.33 s
H-12	5.93 s	5.92 s	5.76 s	5.80 s
H-15a	2.06 o	3.49 m	2.07 o	2.10 o
H-15b	1.70 o	2.25 o	2.30 o	1.81 o
H-16a	1.05 o	1.70 o	1.01 o	1.01 o
H-16b	0.91 m	1.02 o	0.85 o	0.96 m
H-18	2.49 t	2.48 dd	2.19 o	2.11 o
H-19a	2.06 o	2.09 o	2.44 t	1.81 o
H-19b	1.70 o	1.70 o	1.63 m	1.81 o
H-21a	2.26 o	2.25 o	2.19 o	2.10 o
H-21b	1.46 o	2.09 o	1.35 m	1.36 o
H-22a	1.70 o	1.70 o	1.67 m	1.43 o
H-22b	1.46 o	1.42 m	1.43 m	1.33 m
H-23	1.39 s	1.40 s	1.36 s	1.30 s
H-24	1.22 s	3.71 d	1.22 s	1.24 s
H-25	1.17 s	1.18 s	1.18 s	1.12 s
H-26	1.01 s	1.01 s	1.02 s	1.03 s
H-27	1.32 s	1.46 s	1.34 s	1.36 o
H-28	0.75 s	0.74 s	0.83 s	0.76 s
H-29	1.39 s	1.32 s	1.38 s	1.30 s
Glc I-1′	5.03 d (7.6)	5.01 d (7.2)	5.02 d (7.6)	/
2′ ~ 5’	4.22 ~ 4.65	4.23–4.59	4.23 ~ 4.63	/
Glc I-1″	5.42 d (7.6)	5.68 d (5.7)	5.42 d (7.5)	/
2″ ~ 5″	4.22 ~ 4.65	4.23–4.59	4.23 ~ 4.63	/

The chemical shifts (δ values) are given in parts per million (ppm).
The coupling constant (J values) are reported in hertz (Hz).
S, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet; o, overlapped.

spectrum showed 3445 cm^{-1} (O–H, stretch, broad), 1728 cm^{-1} (C=O, stretch, strong), 1652 cm^{-1} (C=C, stretch, strong), 1164 cm^{-1} and 1049 cm^{-1} (C–O, stretch, strong).

Similar to GL, the ^{13}C NMR, HMBC and H−H COSY data analysis indicated that the glycosyl site is at C3. The coupling constant J = 7.5 Hz

Table 2. ^{13}C NMR spectroscopic data of glycyrrhizin, saponin G2, uralsaponin B and glycyrrhetic acid (500 MHz, in pydine-d5)

C	Glycyrrhizin	Saponin G_2	Uralsaponin B	Glycyrrhetic acid
C-1	39.4	39.4	39.5	39.5
C-2	26.6	26.5	26.5	25.7
C-3	89.1	89.6	89.1	78.7
C-4	39.8	43.4	39.9	38.7
C-5	55.4	55.9	55.4	55.0
C-6	17.5	18.4	17.6	17.1
C-7	32.8	33.2	32.9	32.1
C-8	43.4	44.0	45.5	44.8
C-9	62.0	61.9	62.0	61.1
C-10	37.1	36.9	37.2	36.6
C-11	199.3	199.1	199.3	198.9
C-12	128.6	128.5	128.8	127.2
C-13	169.3	169.4	169.0	169.4
C-14	45.4	45.4	43.5	43.0
C-15	26.6	26.5	26.6	26.9
C-16	26.7	26.7	26.7	26.0
C-17	32.0	32.0	32.5	31.4
C-18	48.6	48.6	46.7	48.0
C-19	41.6	41.6	39.8	40.6
C-20	44.0	44.3	42.5	42.8
C-21	31.5	31.5	29.7	30.3
C-22	38.3	38.3	35.9	37.4
C-23	28.5	23.4	28.1	27.7
C-24	16.6	63.3	16.7	16.1
C-25	16.7	16.4	16.8	15.9
C-26	18.7	18.5	18.8	18.3
C-27	28.0	22.9	23.4	22.9
C-28	28.6	28.6	28.5	28.3
C-29	23.4	28.5	19.8	28.1
C-30	179.0	178.9	180.6	177.5
Glc I-1′	105.0	104.5	105.0	/
2′	76.7	81.7	76.8	/
3′	77.6	75.7	84.4	/
4′	72.9	72.9	73.0	/
5′	77.5	77.5	77.3	/
6′	171.9	172.1	172.0	/
Glc I-1″	106.8	105.2	106.8	/
2″	77.2	81.9	77.6	/
3″	84.4	77.8	77.7	/
4″	73.2	73.0	73.2	/
5″	78.3	77.6	78.3	/
6″	172.2	172.1	172.3	/

and 7.6 Hz between H1′ and H1″ showed the ß configuration of the linkage between the glycosyl and aglycone. Compared with the data of ^{13}C NMR of GL, this compound had no CH_3 signal at δ 16 ppm, on the contrary, there is a CH_2OH signal at δ 63.3 ppm. Hence, it can be presumed that the hydroxymethyl replaced methyl at C24 or C23 site. Therefore, the compound was elucidated as saponin G2, 24-Hydroxycyrrhetic Acid-3-O-ß-Glucuronopyranosyl(1→2)-ß-Glucuronopyranoside.

Uralsaponin B

SG gave a deprotonated molecular ion $[M-H]^-$ at m/z 821 and the peak of $[M+Na-2H]^-$ at 843 m/z in ESI-MS (negative mode). The IR spectrum showed 3443 cm^{-1} (O−H, stretch, broad), 1720 cm^{-1} (C = O, stretch, strong), 1645 cm^{-1} (C = C, stretch, strong), 1168 cm^{-1}, 1051 cm^{-1}(C−O, stretch, strong).

The HMBC data showed the correlations between C1″ (δ 106.8) and H3′ (δ 4.23 ~ 4.39), and the same between C3′ (δ 84.4) and H1″ (δ 4.55–4.63); while the H-H COSY spectrum indicated the correlations between H1′ (δ 4.55–4.63) and H3′ (δ 4.23 ~ 4.39). Thus, it can be confirmed that the linkform of the glycosyl is 1→3. Same as GL and UB, the coupling constant between H1′ and H1″ characterized the ß linkage between the glycosyl and aglycone. From the data analysis and the literatures, this compound was considered to be uralsaponin B (Cyrrhetic Acid-3-O-ß-Glucuronopyranosyl(1→2)-ß-Glucuronopyranoside).

Validation

The developed RP-HPLC method was validated in terms of specificity, linearity, recovery and precision.

System Suitability and Specificity

The system suitability was examined using 2.0% w/w of all the impurities spiked to GL (100 µg/mL) and evaluated by making five replicated injections. The system was suitable for use if the tailing factor for GL was <1.20 and the resolutions for all the compounds were >2.0. The assay of GL for three determinations was found to be 99.78% with 0.23% relative standard deviation (RSD), while in the presence of impurities (2.0% w/w) it was 99.67% with a RSD of 0.37%. The resolution was 2.61 between GL and SG, and it was 2.30 between GL and UB. This suggests that the assay results should not change in the presence of

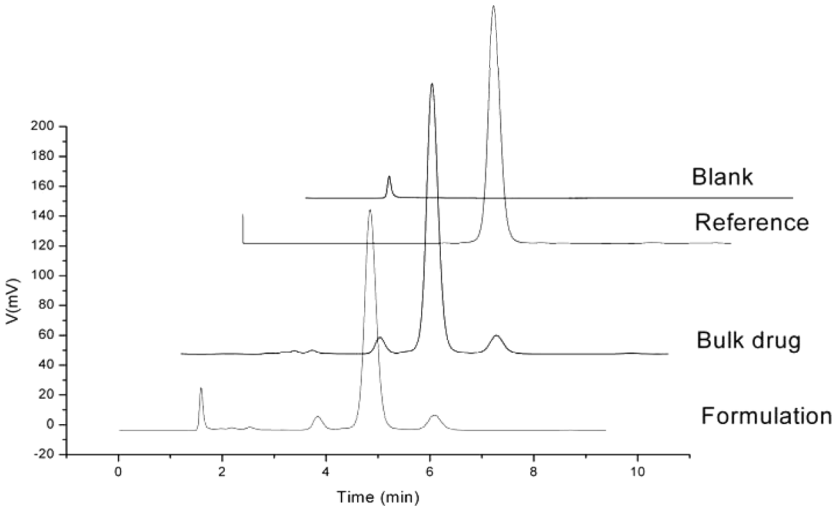


Figure 2. Typical chromatography.

impurities. The excipients in formulations did not interfere with the peaks of interest (see Fig. 2) and were well separated from GL and other impurities.

Linearity and Limits of Detection and Quantification

The lineary of detector response to different concentrations of GL and impurities was studied at six different levels. The data were subjected to statistical analysis using a lineary-regression model. The regression equations and correlation coefficients r are given in Table 3. The results indicated good linearity.

Limits of detection (LOD) represents the concentration of the analyte that would yield signal to noise ratio (S/N) of 3.

Table 3. Linearity data of the analytes

Compound	Retention time (min)	Linear equation	R	Range (μg/mL)	LOD (μg/mL)
Glycyrrhizinic acid	4.8	$A = -20429.0 + 8178.5 \times C$	0.9997	12.04–180.60	0.040
Saponin G_2	3.8	$A = -2334.3 + 6419.3 \times C$	0.9999	1.04–15.60	0.080
Uralsaponin B	6.1	$A = -14136.9 + 60231.7 \times C$	0.9992	2.45–36.75	0.100

A: peak area; C: concentration.

Recovery

The recovery of the method was studied by calculating the mean recoveries of the analytes using the standard addition method. The reference standards were added at three different concentration levels (approximately equivalent to 0.8, 1.0, 1.2 times of the concentration of the matrix) with three parallels at each level. The recoveries of the three compounds were tested with one sample of formulation as the matrix. The total amount of each analyte was calculated from the corresponding calibration curve, and the recovery of each analyte was calculated by the following equation:

$$\text{Recovery } (\%) = (\text{amount determined} - \text{amount original}) / \text{amount spiked} \times 100\%$$

where amountdetermined is the determined total amount of each analyte, amountoriginal is the original amount of each analyte in formulation measured above, and amountspiked is the spiked amount of each analyte.

In Table 4, it was found that the overall recoveries of the analytes were of 99.5–102.7%. The results of recovery test indicated that the method developed was accurate enough for the determination of GL and the impurities in bulk drugs.

Precision

The RSD was taken as a measure of precision. The precision of the method was tested by six ($n = 6$) injections of the working solution

Table 4. Recovery of the assay

Analyte	Original (μg)	Spiked (μg)	Determined mean (μg)	Recovery mean (%)	RSD (%)
Glycyrrhizinic acid	42.1	38.53	81.65		
	42.3	48.16	90.69	99.7	1.03
	41.7	57.80	99.21		
Saponin G_2	2.65	3.33	6.07		
	2.68	4.16	6.82	100.8	0.92
	2.66	4.99	7.79		
Uralsaponin B	6.20	7.84	14.16		
	6.24	9.80	15.94	101.5	1.12
	6.27	11.76	17.93		

Table 5. Results of GL and impurities in bulk drugs and formulations (n = 3)

	Content (%, mean)			Content (μg/ampoule, mean)		
Analyte	Bulk-drug-1	Bulk-drug-2	Bulk-drug-3	Injection-1	Injection-2	Lyophilized powder for injection
Glycyrrhizinic acid	82.42	82.90	84.34	846	1692	859
Saponin G2	5.28	6.30	5.04	54.8	111.3	54.7
Uralsaponin B	11.61	11.54	11.37	12.8	27.2	130

and the RSD of peak areas were determined. The RSD ranged from 0.82% to 1.06%. The precisions in determination of GL and the two impurities were studied by intra-day and inter-day variations. The intra-day variations were determined by analyzing the six replicates on the same day and inter-day variations were determined in three consecutive days. The RSD ranged from 1.02% to 1.27%. These RSD data indicated that the method was precise, accurate and sensitive enough for simultaneous quantitative evaluation of three major components in bulk drugs.

Analysis of the Bulk Drugs and Formulations

The developed HPLC analytical method was subsequently used to simultaneously determine the three components in bulk drugs of monoammonium glycyrrhizinate and GL containing formulations.

The samples of bulk drugs were prepared by dissolving 6.0 mg powder in 50 mL mobile phase and the samples of injections were prepared by dilute 1 mL to 10 mL with the mobile phase. An aliquot (20 μL) of the filtrate was directly subjected to HPLC analysis. Each sample was determined in triplicate. The content of each analyte was calculated from the corresponding calibration curves. The representative HPLC chromatograms of the samples are shown in Fig. 2, and the results as shown in Table 5 were in accordance with the manufactory's criterion.

CONCLUSION

Two unknown related impurities were isolated and purified by preparative HPLC from bulk drugs of GL, and characterized by FT-IR, ESI-MS, ^{1}H NMR, and ^{13}C NMR analysis. A simple HPLC method has been developed and validated for determination of GL, the two

related substances in bulk drugs and pharmaceuticals. The method was found to be selective, sensitive, precise, accurate and linear. It could be used as a convenient, effective technique to control the quality of the bulk drugs and pharmaceuticals. This study provided an approach to develop a chromatographic profile of the main compounds to ensure the quality of commercial GL. It strongly reminded us to systematically control the content of the main compound and the related impurities in bulk drugs and pharmaceuticals so as to insure its therapeutic effects during preparation and application since multiple constituents are responsible for the therapeutic effects or the side effect.

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