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Short communication

Ultrasonic-assisted liquid–liquid extraction and HILIC–ELSD analysis of ginsenoside Rb_1, astragaloside IV and dulcitol in sugar-free "Fufangfufangteng Heji"

Jieping Qin[a,*], Jun Feng[a], Yaohua Li[a], Kefeng Mo[b], Shiying Lu[b]

[a] Guangxi Traditional Chinese Medical University, Nanning 530001, PR China
[b] Nanning Institute for Food and Drug Control, Nanning 530001, PR China

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ABSTRACT

A simple method for the extraction and determination of ginsenoside Rb_1, astragaloside IV and dulcitol in sugar-free "Fufangfufangteng Heji" was developed using an ultrasonic-assisted liquid–liquid extraction (UALLE) coupled with Hydrophilic Interaction Liquid Interface Chromatography and Evaporative Light Scattering Detector (HILIC–ELSD) analysis. Good chromatographic separation was achieved using a Phenomenex Luna HILIC column (250 mm × 4.6 mm i.d., 5 μm), and a mobile phase consisting of acetonitrile–water at a flow rate of 1.0 ml/min with a gradient elution within 25 min was also used. Compared to the conventional analysis method, the proposed method had the advantages of a longer column life, shorter analysis time, lower baseline noise, short sample pretreatment time and low consumption of organic solvent. The linear ranges for ginsenoside Rb_1, astragaloside IV and dulcitol were 0.0256–0.179, 0.110–0.770, 0.105–0.630 mg/ml, respectively. The recoveries of ginsenoside Rb_1, astragaloside IV and dulcitol during the pharmaceutical preparation were within the range of 97.2–100.3%, and their relative standard deviations were 1.2–3.1%.

1. Introduction

"Fufangfufangteng Heji" is a widely used oral liquid in Chinese medicine, which is made from three commonly used Chinese herbs: *Stem or Leaf of Fortune Euonymus, Astragalus Root and Panax notoginsen* [1]. In China, "Fufangfufangteng Heji" has been used as an anti-aging medicine for more than 20 years and has been officially listed in the Chinese Pharmacopoeia for a long time [1]. It has been reported to possess important and broad pharmacological activities, including anti-aging, anti-myocardial ischemia, improving microcirculation, anti-leukopenia after chemotherapy treatment and vascular modulatory activity [2–5]. Ginsenoside Rb_1, astragaloside IV and dulcitol have been reported to be the main effective constituents that contribute to the pharmacological activity [6–9]. There have been previous reports on the determination of Ginsenoside Rb_1 and astragaloside IV using HPLC–ELSD or HPLC–MS [10,11], but dulcitol is a highly polar molecule that can only be dissolved in water or a dilute methanol solution. Therefore, it is difficult to analyze dulcitol with the other effective constituents using conventional reversed-phase chromatography, because it can be poorly retained and frequently has a poor peak shape. It has been reported that astragaloside IV and dulcitol were determined using normal-phase chromatography [12], but the method employed has some disadvantages, such as lower column life and higher baseline noise. Hydrophilic interaction liquid interface chromatography (HILIC) is another alternative to these techniques. The term HILIC was first suggested by Alpert in 1990 [13] and there has been increased interest in HILIC among scientists in recent years. Unlike reversed-phase chromatography, HILIC columns retain highly polar compounds with only small amounts of water in the mobile phase. There had been a report of using a HILIC–ELSD method to separate and determine monosaccharides, and the results demonstrated that HILIC has many advantages over normal-phase chromatography [14]. "Fufangfufangteng Heji" is a liquid pharmaceutical formulation. In general, a liquid–liquid extraction is employed as the sample pretreatment method to isolate the compounds of interest from the complex sample matrix, but this method also has some drawbacks, including low repeatability, difficulty to automation and low enrichment factors that are caused by using large amounts of dispersion solvent.

In this paper, a simple ultrasonic-assisted liquid–liquid extraction (UALLE) coupled with (HILIC–ELSD) was developed to determine ginsenoside Rb_1, astragaloside IV and dulcitol in sugar-free "Fufangfufangteng Heji". Good chromatographic separation was achieved using a Phenomenex Luna HILIC column (250 mm × 4.6 mm i.d., 5 μm). This method can be used

* Corresponding author. Tel.: +86 2279189; fax: +86 3140360.
E-mail address: chinaqjp6380@yahoo.com.cn (J. Qin).

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to determine ginsenoside Rb_1, astragaloside IV and dulcitol under the same chromatographic conditions. An ultrasonic-assisted dispersion was applied to accelerate the liquid–liquid extraction process, which markedly increased the extraction efficiency and reduced the equilibrium time. It provided a good alternative method for the routine analysis of ginsenoside Rb_1, astragaloside IV and dulcitol due to its simplicity and reliability.

2. Experimental

2.1. Standards, reagents and samples

Control ginsenoside Rb_1 (Nos. 110704-200216) and astragaloside IV (Nos. 110781-200613) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); purity >99% determined by HPLC method. Dulcitol (No. 000711) was purchased from Sinopharm Chemical Reagent Co. Ltd (Beijing, China); purity 99.2% was determined by HPLC method.

HPLC-grade acetonitrile was purchased from Tedia Company, USA (No. 609499). Ultra pure water was used for all analyses. All other chemicals and reagents were of analytical grade, unless indicated otherwise.

Sugar-free "Fufangfufangteng Heji" samples were supplied by the Pharmaceutical Factory of Guangxi TCM University (Nanning, China). All samples for HPLC analysis were filtered through 0.45 μm membrane filters before injection into the HPLC. The HPLC mobile phase was prepared fresh daily and filtered through a 0.45 μm membrane filter.

An ultrasonic cleaner (KQ-250, Dianshanhu Instrument, Kunshan, China) and an analytical balance (BS110S, Sartorius, Germany) were used for sample preparation.

2.2. HILIC–ELSD analysis

Analyses were performed using a Shimadzu LC-2010 AHT liquid chromatograph (Kyoto, Japan). The Shimadzu LC-2010 AHT liquid chromatograph consists of a dual pump, an autosampler equipped with an ELSD (Model 400 ELSD, SofTA Corporation, USA) and a Phenomenex Luna HILIC column (250 mm × 4.6 mm i.d., 5 μm). The column temperature was kept constant at 30 °C, and the mobile phase flow rate was 1.0 ml/min. The parameters of the ELSD were set to the following conditions: nebulizer gas pressure = 46 psi, exhaust tube temperature = 71 °C, optical cell temperature = 70 °C, drift tube temperature = 80 °C and spray chamber temperature = 40 °C. The mobile phases consisted of water (A) and acetonitrile (B) using a gradient elution of 7% A at 0–5 min, 7–20% A at 5–15 min, and 20% A at 15–20 min, and the re-equilibration time of the gradient elution was 10 min.

2.3. Preparation of standard solutions

A stock solution of the mixture of ginsenoside Rb_1 and astragaloside IV was prepared by dissolving accurately weighted portions of the standards in methanol, transferring them to a volumetric flask and adding methanol to dilute to the specified volume. The concentrations of ginsenoside Rb_1 and astragaloside IV were 0.128 mg/ml and 0.55 mg/ml, respectively.

A stock solution of dulcitol was prepared by dissolving an accurately weighted portion of the standard in methanol–water (35:65, v/v), transferring it to a volumetric flask and adding methanol–water (35:65, v/v) to dilute to the specified volume. The concentration of dulcitol was 1.05 mg/ml.

2.4. Preparation of sample solution

2.4.1. Preparation of test solution 1

A 3.0 ml sugar-free "Fufangfufangteng Heji" sample solution was placed in a 50 ml conical flask, and 15 ml of water-saturated *n*-butanol was added to the solution. First, the solution was hand-shaken to mix, and ultrasonic vibration was subsequently applied for 20 min to ensure that the analytes were fully extracted into the *n*-butanol. The phase separation was performed easily using a separatory funnel. The sample phase was used for preparing test solution 2, which is described in greater detail in the following section. The *n*-butanol phase was extracted with a 40% ammonia–water solution to remove the pigment and other interferences. The ammonia–water layer was discarded and the *n*-butanol phase was evaporated to dryness at reduced pressure in a rotary evaporator. The residue was dissolved in 1 ml of methanol, and the supernatant was filtered through a 0.45-μm PTFE filter for LC analysis.

2.4.2. Preparation of test solution 2

A 3.0 ml sugar-free "Fufangfufangteng Heji" sample solution (after extraction using *n*-butanol) was placed in a 50 ml conical flask, and 1.5 g of diatomaceous earth was added as a dispersant. Subsequently, 20 ml of a 35% methanol–water solution was added as an extraction solvent, and the sample was sonicated for 15 min at room temperature. This extraction procedure was repeated one more time. Finally, the supernatants were filtered into a volumetric flask and diluted to 50.0 ml with the 35% methanol–water solution. Prior to injection onto the LC, the solution was filtered through a 0.45-μm PTFE filter.

3. Results and discussion

3.1. Selection of extraction conditions

Dulcitol is a polar molecule, which means that it can only be dissolved in water or a dilute methanol–water solution, while *n*-butanol is a common and good solvent for the extraction of ginsenoside Rb_1 and astragaloside IV, according to the official method [1] and the literature [15,16]. Therefore, in our experiments, the extraction and the determination procedures of the target analytes were divided into two steps.

For test solution 1, which was used to determine ginsenoside Rb_1 and astragaloside IV, *n*-butanol was selected as the extraction solvent, and the extraction method and extraction time were optimized. It was clear that one 20-min UALLE was sufficient for the complete extraction of these saponins, and it is important to note that the official method requires extracting four times [1]. The experimental results are shown in Table 1.

A series of volumes (5–20 ml) of *n*-butanol, for a 20-min UALLE procedure and different times (10, 20 and 30 min) of ultrasonic dispersion using the same extraction solvent volume of 15 ml, were investigated. The experimental results indicated that the maximum extraction efficiency was achieved using 15 ml of *n*-butanol for 20 min of ultrasonic dispersion, which was chosen for the preparation of test solution 1. The experimental results are shown in Tables 2 and 3.

Test solution 2 was used for the determination of dulcitol. In order to enable the extraction of dulcitol in higher yields and to achieve the maximum extraction efficiency, the pretreatment conditions for test solution 2 were optimized. Water, 30% ethanol, 30% methanol, 35% methanol and 35% ethanol, 40% methanol and 40% ethanol were tested in the extraction procedure. The determination results showed that water, 30% ethanol–water or 35% methanol–water enabled the extraction of dulcitol in higher yields,

Table 1
Determination results of different extraction method.

Methods	Astragaloside IV content (mg/ml)				Ginsenoside Rb_1 content (mg/ml)			
	1	2	3	Average	1	2	3	Average
Official method	0.151	0.158	0.154	0.154	0.020	0.016	0.014	0.017
Proposed method	0.154	0.156	0.157	0.156	0.018	0.017	0.016	0.017

Table 2
Determination results of different extraction solvent volume (ultrasonic dispersion for 20 min).

No.	Extraction solvent volume (ml)	Astragaloside IV content (mg/ml)			Ginsenoside Rb_1 content (mg/ml)		
		1	2	Average	1	2	Average
1	5	0.121	0.123	0.122	0.018	0.016	0.017
2	8	0.135	0.138	0.136	0.020	0.019	0.019
3	15	0.156	0.155	0.156	0.018	0.019	0.019
4	20	0.153	0.157	0.155	0.019	0.020	0.020

but the extracted solutions of water and 30% ethanol–water contained more impurities, which resulted in poor peak shape in HPLC analysis. Therefore, 35% methanol–water was chosen as the extraction solvent in the preparation of test solution 2. Furthermore, the extraction mode, extraction solvent volume and extraction time were also investigated. The test results indicated that 3.0 ml of a sample in 20 ml of a 35% methanol–water solution undergoing ultrasonic extraction twice in an ultrasonic bath for 15 min at room temperature can achieve the maximum extraction efficiency, and these conditions were chosen for the preparation of test solution 2.

3.2. Optimization of HILIC–ELSD conditions

The experimental conditions were optimized to obtain chromatograms with good resolution of adjacent peaks within a short analysis time. In this study, different mobile phase compositions (water–methanol and water–acetonitrile) and flow rates (from 0.8 to 1.0 ml/min) were examined to achieve appropriate chromatographic separation. The components could not be separated effectively using isocratic mobile phase solvents. To find an easy way to analyze the components, a gradient solvent system was employed. The best resolution was achieved with the mobile phases consisting of water (A) and HPLC-grade acetonitrile (B), and a timed gradient elution program T (min)/% A: 7% A at 0–5 min, 7–20% A at 5–15 min, and 20% A at 15–20 min, and the re-equilibration time of the gradient elution was 10 min. In addition, important parameters that affect the sensitivity of ELSD, such as the flow rate of the nebulizer gas (pressure) and drift tube temperature, were evaluated. Under the fixed chromatographic conditions, the ELSD parameters were optimized by the injection of a standard mixture solution at different drift tube temperatures (60–100 °C), nebulizer gas pressures (30–50 psi) and spray chamber temperature (30–60 °C). Finally, the selected drift tube temperature, nebulizer gas pressure and spray chamber temperature were optimized at 80 °C, 46 psi, and 40 °C, respectively, by comparing the peak area values of the analytes. These optimized parameters allowed for complete solvent evaporation and, therefore, produced negligible baseline noise. The representative HPLC chromatograms for test solution 1 (A) and test solution 2 (B) of the sample, and the chromatograms for the standard solutions (C and D) are shown in Fig. 1.

3.3. Calibration curves, limits of detection and quantification

The calibration curve was prepared with at least six appropriate concentrations. The solutions were brought to room temperature and diluted to six appropriate concentrations with methanol (test solution 1) or 35% methanol–water solution (test solution 2) and filtered through a 0.45 μm membrane. An aliquot of 10 μl was injected into the HPLC for analysis. The regression equations were calculated in the form of $Y = A \times X + B$, where Y, X are the natural logarithmic values of area and concentration injected in the ELSD chromatograms, respectively. The results are shown in Table 4. The dilute solution of the three reference compounds was further diluted to a series of concentrations to gain the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ, under the present chromatographic conditions, were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively. The LOD and LOQ for each compound are also shown in Table 4.

3.4. Precision, repeatability and accuracy

Under the optimized conditions, the intra-day and inter-day precision were determined by analyzing calibration samples during a single day and on 3 different days, respectively. The sample injection volumes were 10 μl. The concentrations of ginsenoside Rb_1 and astragaloside IV in the calibration samples 1 were 0.128 mg/ml and 0.55 mg/ml, respectively. The concentration of dulcitol in the calibration samples 2 was 0.525 mg/ml. The experimental results showed that the RSDs for the intra-day and inter-day precision of the peak area were in the range of 2.0–2.5% and 2.4–3.0%, respectively. Additionally, the results demonstrated good reproducibility for the peak area of the analytes.

To confirm the repeatability, six different test solutions 1 and test solutions 2 were prepared from the same sample (No. 20090928) and analyzed. The RSD was taken as a measure of the precision and repeatability. The results showed that the average content of ginsenoside Rb_1, astragaloside IV and dulcitol in the sam-

Table 3
Determination results of different extraction time (in 15 ml extraction solvent).

No.	Time of ultrasonic dispersion (min)	Astragaloside IV content (mg/ml)			Ginsenoside Rb_1 content (mg/ml)		
		1	2	Average	1	2	Average
1	10	0.136	0.134	0.135	0.017	0.016	0.016
2	20	0.156	0.155	0.156	0.018	0.019	0.019
3	30	0.155	0.157	0.156	0.019	0.018	0.018

Table 4
Regression data, LODs and LOQs for the three analytes in HPLC–ELSD.

Analytes	Calibration curve	r^2 (n = 6)	Test range (mg/ml)	LOD (mg/ml)	LOQ (mg/ml)
Ginsenoside Rb_1	$Y = 1.5187X + 16.149$	0.9971	0.0256–0.179	0.0035	0.0090
Astragaloside IV	$Y = 1.3786X + 16.442$	0.9958	0.110–0.770	0.0060	0.0170
Dulcitol	$Y = 1.5723X + 15.935$	0.9984	0.105–0.630	0.0050	0.0160

Y, the natural logarithmic values of area; *X*, the natural logarithmic values of concentration injected; LOD, the limits of detection; LOQ, the limits of quantification.

ple was 20 μg/ml, 158 μg/ml and 792 μg/ml with relative standard deviations (RSD) of 2.5%, 2.7% and 2.1%, respectively. These results indicate that the analytical procedure is very reproducible.

A recovery test was used to evaluate the accuracy of this method. Accurate amounts of ginsenoside Rb_1, astragaloside IV and dulcitol were added to 1.5 ml or 1.0 ml of "Fufangfufangteng Heji" samples and were then extracted and analyzed under the optimized analytical conditions, as described previously. The average recoveries were calculated using the formula: recovery (%) = (amount found − original amount)/amount added × 100%, and RSD (%) = (SD/mean) × 100%. The results showed that the average recovery (%) of ginsenoside Rb_1, astragaloside IV and dulcitol in the pharmaceutical preparation was within the range of 97.2–100.3%, and their relative standard deviations were 1.2–3.1%.

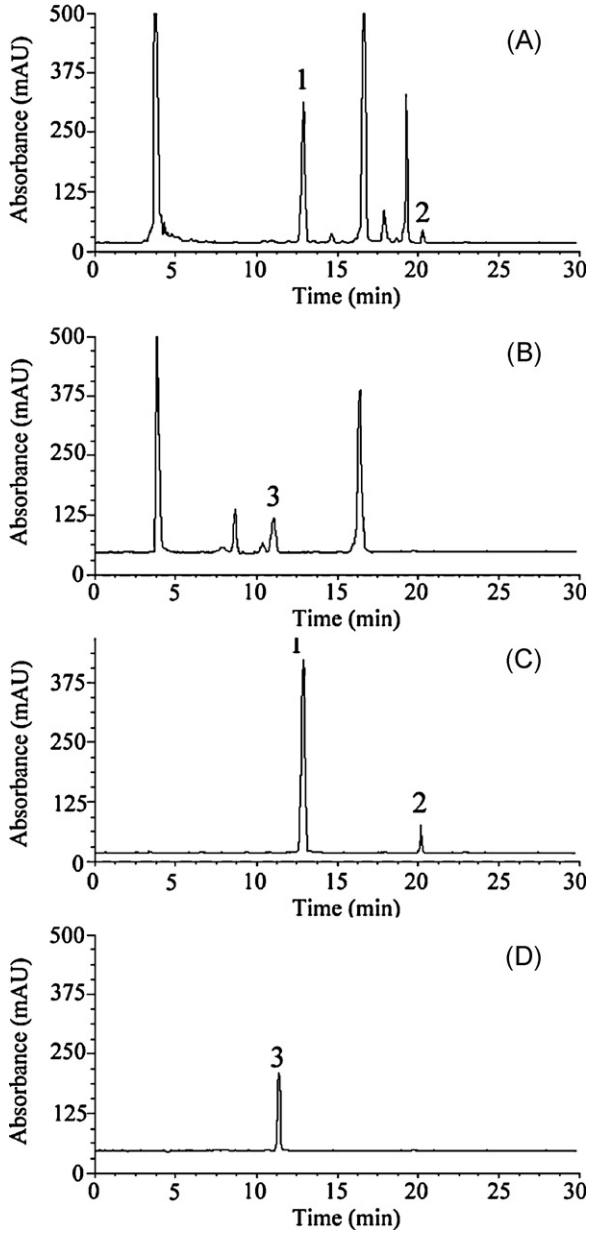

Fig. 1. Chromatograms obtained from: a test solution 1 (A) and a test solution 2 (B) of a sugar-free "Fufangfufangteng Heji" sample (No. 20090928), a standard mixture solution of ginsenoside Rb_1 and astragaloside IV (C) and a standard solution of dulcitol (D). 1 = astragaloside IV; 2 = ginsenoside Rb_1; 3 = dulcitol.

3.5. Application of the method

The developed analytical method was subsequently applied to the determination of 10 batches of samples. All samples were processed using the optimized extraction conditions proposed in the methods above. The test solution 1 and test solution 2 obtained from each sample were injected onto the instrument for analysis using the optimized chromatographic conditions. The results showed that the contents of ginsenoside Rb_1, astragaloside IV and dulcitol in 10 batches of samples ranged from 18 to 33 μg/ml, 135 to 175 μg/ml and 405 to 778 μg/ml, respectively, and the corresponding RSD% values ranged from 1.1 to 3.0%.

4. Conclusion

Dulcitol is a highly polar molecule that is impossible to analyze alongside ginsenoside Rb_1 and astragaloside IV using conventional chromatography conditions. In this paper, a HILIC–ELSD method was developed that can be used to analyze ginsenoside Rb_1, astragaloside IV and dulcitol under the same chromatographic conditions. An ultrasonic dispersion was applied to the liquid–liquid extraction process which can increase the extraction efficiency and reduce the consumption of organic solvent. The experimental results showed that the proposed method had the advantages of longer column-life, shorter analysis time and ease of operation. It is suggested that hydrophilic interaction liquid interface chromatography (HILIC) combined with evaporative light scattering detection (ELSD) can be an excellent alternative for the development of fast, sensitive, and yet inexpensive methods for a wide range of solutes, especially in the analysis of polar and hydrophilic compounds.

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