

Short communication

One step isolation and purification of liquiritigenin and isoliquiritigenin from *Glycyrrhiza uralensis* Risch. using high-speed counter-current chromatography

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Received 10 November 2004; received in revised form 12 January 2005; accepted 21 January 2005

Abstract

High-speed counter-current chromatography (HSCCC) technique in semi-preparative scale has been successfully applied to the separation of bioactive flavonoid compounds, liquiritigenin and isoliquiritigenin in one step from the crude extract of *Glycyrrhiza uralensis* Risch. The HSCCC was performed using a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–acetonitrile–water (2:2:1:0.6:2, v/v). Yields of liquiritigenin (98.9% purity) and isoliquiritigenin (98.3% purity) obtained were 0.52% and 0.32%. Chemical structures of the purified liquiritigenin and isoliquiritigenin were identified by electrospray ionization–MS (ESI–MS) and NMR analysis.
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Keywords: Counter-current chromatography; *Glycyrrhiza uralensis*; Plant materials; Pharmaceutical analysis; Liquiritigenin; Isoliquiritigenin

1. Introduction

Root of *Glycyrrhiza uralensis* is widely used in traditional Chinese medicine. It can be used for various purposes such as bronchitis, ague, hepatitis, phthisis and gastric ulcer [1]. Flavonoides are the main bioactive component. Pharmacological investigation concluded that they had antioxidant [2–4] and antibacterial [5,6] bioactivities. Among them, the remarkable ones such as liquiritigenin and isoliquiritigenin have reported to carry strong biological activity. Liquiritigenin and isoliquiritigenin have been shown to have a inhibition of xanthine oxidase activity in vitro [7] and dose-related anti-allergic activities [8]. In addition, isoliquiritigenin caused great interest after it was found to have effects in: inhibiting proliferation of the human non-small cell lung cancer A549 cell line, inducing apoptosis and locking cell cycle progression in the G₁ phase [9], suppressing

azoxymethane (AOM)-induced colon carcinogenesis in ddY mice, and inhibiting the growth of prostate cancer [10]. It had been suggested that isoliquiritigenin, a especial constituent in *G. uralensis*, merits investigation as a potential cancer-chemopreventive agent in humans.

The separation and purification of liquiritigenin and isoliquiritigenin from licorice by conventional methods such as column chromatography and high-performance liquid chromatography (HPLC) is tedious and usually requires multiple chromatography steps. Some flavonoides extracted from licorice have been successfully isolated and purified by HSCCC [11]. As a modern liquid–liquid chromatography without solid sorbent, high-speed counter-current chromatography (HSCCC) uses no solid support, so the adsorbing effects on stationary phase material and artifact formation can be eliminated. This technique has the maximum capacity with an excellent sample recovery and wider range of selection of solvent systems as compare to HPLC. Furthermore, it permits introduction of crude sample directly into the hollow column. Therefore, HSCCC has recently been investigated to

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effectively separate and purify a number of nature products [12–14].

The present report deals with the one-step isolation and purification of liquiritigenin and isoliquiritigenin from the crude extract of roots of *G. uralensis* Risch. for the first time, and demonstrates the rapid and efficient access of the constituents using HSCCC.

2. Experimental

2.1. Reagents and materials

All reagents used for HPLC were Chromatographic grade and were filtered and degassed prior to use. HSCCC reagents were reagent grade or higher.

The roots of *G. uralensis* Risch. were purchased from Bozhou (AnHui, China). The sample was crushed into pieces prior to extract.

2.2. Apparatus

2.2.1. HSCCC

HSCCC was performed using a Model TBE-300A HSCCC system manufactured by Tauto Biotech Co., Ltd., Shanghai, China, equipped with a 280 ml coil column made of polytetrafluoroethylene tubing (1.8 mm). The β -value of the preparative column varied from 0.42 at the internal layer to 0.63 at the external layer ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and the central axis of centrifuge). For this apparatus, the revolution radius is 130 mm). The revolution speed of the apparatus could be adjusted in a range between 0 and 900 rpm. The solvent was pumped into the column by a Model S 1007 constant-flow pump (Beijing Shengyitong Technology Development Co., Ltd.), and continuously delivered by 280 nm absorption with a Model 8823 A UV detector (Beijing Institute of New Technology Application), the data was displayed and analyzed simultaneously on a Model Anastar 2.0 chromatographic data station provided by Tianjing Autoscience Instrument Co., Ltd. A manual injection valve with a 20 ml loop was used to introduce the sample into the column.

2.2.2. HPLC

The HPLC system used employed a reversed-phase Discovery C₁₈ column (25 cm × 4.6 mm, 5 μ m) connected to a Model TSP 1000 pump, with a Model 1000 TSP UV detector. Detection was at 367 nm and data were processed using a Model Anastar 2.0 chromatographic data station provided by Tianjing Autoscience Instrument Co., Ltd.

2.3. Preparation of crude total flavonoides

The roots of *G. uralensis* Risch. were dried at 60 °C and then pulverized. Five hundred grams of sample were put

into a 3000 ml flask, to which 1200 ml 90% ethanol was added.

After soaking extraction at room temperature for 24 h, the extraction procedure was repeated twice (1200 ml of 95% ethanol each time). All the filtrates were combined, filtered and evaporated to brown syrup ointment under reduced pressure. The ointment was then dissolved in 500 ml hot water, and extracted with ether (3 × 300 ml). To desert the etheric extract, the remaining extract was continuously extracted with ethyl acetate (3 × 300 ml). The ethyl acetate extracts were combined and evaporated to dryness under reduced pressure, which yielded 15 g of crude flavonoid extracts.

2.4. Selection of two-phase solvent systems

A number of two-phase solvent systems were tested by changing the volume of the solvent to obtain the optimum composition that gave suitable partition coefficient (K) values. The partition coefficient values were determined according to the literature [13]. In brief, approximately 5 mg sample of crude extracts was weighed in a 10 ml test tube to which 3 ml of each phase of the pre-equilibrated two-phase solvent system was added. After the tube was shaken vigorously for 10 min, the solution was quietly separated for a moment. Then, the upper and lower phase were analyzed by HPLC to obtain the partition coefficient of liquiritigenin and isoliquiritigenin, respectively. K was expressed as the peak area of liquiritigenin and isoliquiritigenin in the upper phase divided by that in the lower phase.

2.5. Preparation of two-phase solvent system and sample solution

The selected solvent system (*n*-hexane–ethyl acetate–methanol–acetonitrile–water) was thoroughly equilibrated by repeatedly vigorously shaking in a separation funnel at room temperature. Two phases were separated shortly and degassed before use. The volume ratio of the five solvents is 2:2:1:0.6:2. The upper phase was used as the stationary phase, while the lower phase was used as the mobile phase.

The sample solutions were prepared by dissolving the crude flavonoid extract of *G. uralensis* Risch. in the mixture solution of upper phase and lower phase (1:1, v/v) of the solvent system used for HSCCC separation.

2.6. HSCCC separation

The multilayer coil column was first entirely filled with the upper phase (stationary phase). The lower phase (mobile phase) was then pumped into the head end of the inlet column at a flow rate of 2.0 ml min⁻¹, while the apparatus was rotated at 850 rpm. After reaching hydrodynamic equilibrium, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (80 mg of the crude extract in 12 ml of the total volume of both phase that is 1:1) was injected into the column through the sample port. The effluent from the tail end of the column was continuously monitored with a UV

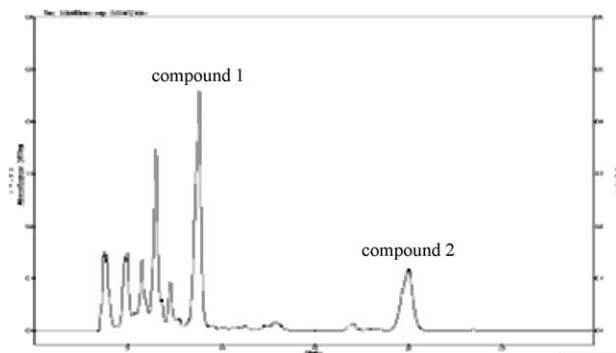


Fig. 1. HPLC analyses of the crude flavonoid extracts from *G. uralensis* Risch. HPLC conditions: a Discovery C₁₈ column (25 cm × 4.6 mm, 5 μm), column temperature: 35 °C. Mobile phase: acetonitrile–5% acetic acid (32:68, v/v), flow rate: 0.7 ml min⁻¹, monitored at 367 nm by UV. Peaks: (1) liquiritigenin, (2) isoliquiritigenin.

detector at 280 nm as stated earlier and the chromatogram was recorded. Each peak fraction was collected manually according to the elution profile and determined by HPLC.

2.7. HPLC analysis and identification of fractionated compounds

The crude flavone extract and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a Discovery C₁₈ column (25 cm × 4.6 mm, 5 μm) at column temperature of 35 °C. The mobile phase composed of acetonitrile:5% acetic acid (32:68) was isocratically eluted at a flow-rate of 0.7 ml min⁻¹, with the detector set at 367 nm. Identification of the target compounds (liquiritigenin and isoliquiritigenin) was based on MS, ¹H NMR, and ¹³C NMR spectra.

3. Results and discussion

As shown in Fig. 1, the HPLC analyses of the crude flavonoid extracts from *G. uralensis* Risch. shows several compounds where the purity of liquiritigenin and isoliquiritigenin were 31% and 22% based on HPLC peak area percentage.

Table 1

The *K* (partition coefficient) values of liquiritigenin and isoliquiritigenin in different solvents systems

Solvent system	Ratio (v/v)	<i>K</i> value ^a	
		Liquiritigenin	Isoliquiritigenin
<i>n</i> -Hexane–ethyl acetate–methanol–water	1:5:3:3	2.43	0.11
	4:2:2:4	0.83	0.21
	2:4:2:4	1.72	0.37
<i>n</i> -Hexane–ethyl acetate–methanol–acetonitrile–water	1:2:1:0.8:2	1.92	0.61
	2:2:1:2:1.2:2	1.21	0.73
	2:2:1:0.6:2	1.04	0.66

^a Expressed as: peak area_{stationary}/peak area_{mobile}.

3.1. Selection of suitable two-phase solvent system

A successful separation by HSCCC depends upon the selection of a suitable two-phase solvent system, which provides an optimum range of partition coefficient ($0.5 < K < 2$) for the targeted compounds. Several two-phase solvent systems were tested and their *K* values were measured and are summarized in Table 1. When *n*-hexane–ethyl acetate–methanol–water was used as the two-phase solvent system, the *K* value of isoliquiritigenin was small, it was eluted with other compounds together and resulted in a poor resolution. When *n*-hexane–ethyl acetate–methanol–acetonitrile–water was used as the two-phase solvent system, as shown in Table 1, all *K* values of liquiritigenin and isoliquiritigenin were suitable and both of them could be well separated from the other compounds. From the above, the two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–acetonitrile–water at a ratio of 2:2:1:0.6:2 (v/v) was found to be best.

3.2. Separation of liquiritigenin and isoliquiritigenin by HSCCC

The crude sample (80 mg) was dissolved in 12 ml of both phases. The sample solution was separated and purified by HSCCC according to the procedure described above. The retention of the stationary phase was 54.5%, and the total separation time was 600 min. Fig. 2 shows HSCCC separation of the crude extract sample, along with the HPLC chromatogram of purified compounds from HSCCC. Based on the HPLC analysis and the elution curve of the HSCCC, compound 1 corresponded to liquiritigenin and compound 2 accorded with isoliquiritigenin. A total amount of 13.8 mg of liquiritigenin (98.9% purity) and 8.5 mg of isoliquiritigenin (98.3% purity) were yielded.

3.3. The structural identification of liquiritigenin and isoliquiritigenin

The structural identification of liquiritigenin and isoliquiritigenin were carried out by ESI–MS, ¹H NMR and ¹³C NMR.

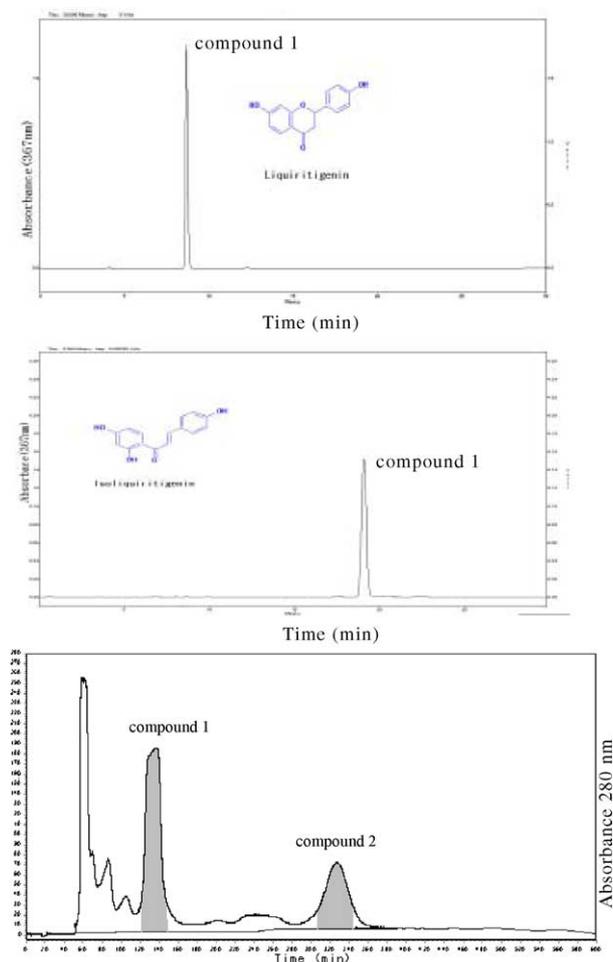


Fig. 2. Chromatogram of the crude flavonoid extracted from *G. uralensis* Risch., by HSCCC separation, along with the HPLC chromatogram of purified liquiritigenin and isoliquiritigenin from HSCCC, compound 1: liquiritigenin, compound 2: isoliquiritigenin. Conditions: column: multilayer coil of 1.8 mm i.d.; polytetrafluoroethylene tube with a total capacity of 280 ml; rotary speed: 850 rpm; solvent system: *n*-hexane–ethyl acetate–methanol–acetonitrile–water (2:2:1:0.6:2, v/v); mobile phase: the lower phase; flow-rate: 2.0 ml min⁻¹; detection: 280 nm; sample size: 80 mg; injection volume: 12 ml; retention of the stationary phase: 54.3%.

3.3.1. Compound 1

Colorless needle crystal. ESI–MS (m/z) (negative): 255 [M – 1]⁻, 511 [2M – 1]⁻; ¹H NMR ([²H₆] dimethyl sulfoxide (DMSO-*d*₆)), δ : 10.56 (br s, 7-OH), 9.57 (br s, 4'-OH), 7.64 (1H, d, J = 8.6 Hz, H-5), 7.32 (2H, d, J = 8.5 Hz, H-2', H-6'), 6.79 (2H, d, J = 8.5 Hz, H-3', H-5'), 6.50 (1H, dd, J = 8.6, 2.1 Hz, H-6), 6.33 (1H, d, J = 2.1 Hz, H-8), 5.44 (1H, dd, J = 12.8, 2.8 Hz, H-2), 3.11 (1H, dd, J = 16.8, 12.8 Hz, H-3), 2.62 (1H, dd, J = 16.8, 2.8 Hz, H-3); ¹³C NMR (100 MHz, DMSO-*d*₆), δ : 190.1 (C=O), 164.6 (C-7), 163.2 (C-8a), 157.6 (C-4'), 129.3 (C-1'), 128.4 (C-5), 128.2 (C-2', C-6'), 115.1 (C-3', C-5'), 113.5 (C-4a), 110.5 (C-6), 102.5 (C-8), 79.0 (C-2), 43.1 (C-3). The data of ¹H and ¹³C NMR was consistent with literature [15,16] about liquir-

itigenin (4H-1-benzopyran-4-one, 2,3-dihydro-7-hydroxy-2-(4-hydroxyphenyl)-, (2*S*)-).

3.3.2. Compound 2

Yellow needle crystal. ESI–MS (m/z): 257 [M + 1]⁺ (negative), 255 [M – 1]⁻; ¹H NMR (DMSO-*d*₆), δ : 13.67 (OH), 8.12 (1H, d, J = 8.9 Hz, H-6'), 7.74 (2H, d, J = 8.5 Hz, H-2, H-6), 7.73 (2H, s, H- α , H- β), 6.84 (2H, d, J = 8.5 Hz, H-3, H-5), 6.36 (1H, dd, J = 8.9, 2.1 Hz, H-5'), 6.22 (1H, d, J = 2.1 Hz, H-3'). ¹H NMR (400 MHz, [²H₅] Pyridine), δ : 14.4 (2'-OH), 8.22 (1H, d, J = 15.3 Hz, H- β), 8.19 (1H, d, J = 8.8 Hz, H-6'), 7.90 (1H, d, J = 15.3 Hz, H- α), 7.78 (2H, d, J = 8.5 Hz, H-2, H-6), 7.21 (2H, d, J = 8.5 Hz, H-3, H-5), 6.89 (1H, d, J = 2.1 Hz, H-3'), 6.78 (1H, dd, J = 8.8, 2.1 Hz, H-5'). ¹³C NMR (100 MHz, [²H₅] Pyridine), δ : 192.5 (C=O), 167.5 (C-4'), 166.7 (C-2'), 161.9 (C-4), 144.9 (C- β), 132.9 (C-6'), 131.5 (C-2, C-6), 126.7 (C-1), 117.9 (C- α), 117.0 (C-3, C-5), 114.2 (C-1'), 109.1 (C-5'), 104.0 (C-3'). The data was consistent with literature [17] about isoliquiritigenin (2-propen-1-one, 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-, (2*E*)-).

4. Conclusion

HSCCC was successfully applied to separate and purify liquiritigenin and isoliquiritigenin, two main bioactive components of the Chinese medicinal herb *G. uralensis* Risch., a particular plant species of licorice, with *n*-hexane–ethyl acetate–methanol–acetonitrile–water (2:2:1:0.6:2, v/v) as solvent system. A total amount of 13.8 mg of liquiritigenin and 8.5 mg of isoliquiritigenin was obtained from 80 mg crude sample. The purity of liquiritigenin and isoliquiritigenin were increased from 31% and 22% to 98.9% and 98.3% after only one-step separation. The overall results of the present study indicate that HSCCC is a powerful technique in separating and purifying bioactive from natural sources.

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