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Semi-Preparative Isolation and Purification of Three Tauro-Conjugated Cholic Acids from *Pulvis Fellis Suis* by HSCCC Coupled with ELSD Detection

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Abstract

Coupled with evaporative light scattering detection, a high-speed counter-current chromatography (HSCCC) method was applied to the separation and purification of three tauro-conjugated cholic acids of taurochenodeoxycholic acid (TCDCA), taurohyodeoxycholic acid (THDCA) and taurohyocholic acid (THCA) from *Pulvis Fellis Suis* (Pig gallbladder bile) for the first time. The two-phase solvent system composed of chloroform-methanol-water-acetic acid (4:4:2:0.3, v/v/v/v) was selected for the one-step separation where the lower phase was used as the mobile phase in the head to tail elution mode. The revolution speed of the separation column, flow rate of the mobile phase and separation temperature were 800 rpm, 1.5 ml/min and 25°C respectively. From 100 mg of the crude extract, 10.2 mg of TCDCA, 11.8 mg of THDCA and 5.3 mg of THCA were obtained with the purity of 94.6%, 96.5% and 95.4%, respectively. In one step separation the HSCCC fractions were analyzed by high-performance liquid chromatography (HPLC) and the structures of the three tauro-conjugated cholic acids were identified by ESI-MS, ¹H NMR and ¹³C NMR.

Keywords

High-speed counter-current chromatography; *Pulvis Fellis Suis*; Tauro-conjugated cholic acids

1. Introduction

Pulvis Fellis Suis (Pig gallbladder bile) is a traditional Chinese medicine used widely in China. According to the Chinese Pharmacopoeia, *Pulvis Fellis Suis* is the dried bile of *Sus scrofa domestica* Brisson and has been used extensively for the treatment of acute pharyngitis, jaundice, whooping cough, asthma, diarrhea, dysentery and constipation [1,2]. Bile acids, including free cholic acid and conjugated cholic acid, are the major components in *Pulvis Fellis Suis* and these are also the pharmaceutically relevant compounds [2-6]. Modern pharmacological research indicates that these bile acids exhibited a wide range of biological activities, such as antibacterial [7], anti-inflammatory [8], anti-infectious [9], and

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inhibitory proliferation of human promyelocytic leukemia cell line HL-60 [10] as well as accelerating bile secretion, treating gallstones, and lowering blood lipids [7,11-13]. Considering such various biological activities of bile acids, it is important to develop an efficient method to isolate and purify each bile acid with high purity for quality control and pharmacological research. Generally, repeated column chromatography over silica gel has been frequently used for the purification of compounds from traditional Chinese medicines (TCM). However, this separation method always consumes large volumes of organic solvents and may cause irreversible adsorption of samples onto solid phase. Therefore, a green and preparative separation method is of great interest in recent years.

High-speed counter-current chromatography (HSCCC), a kind of liquid-liquid partition chromatography, has been widely used in preparative isolation of pure compounds from natural materials. It is free of solid support matrix so as to eliminate the risk of irreversible adsorption of sample components that is often caused by solid supports used in conventional column chromatography. The separation process is entirely based on the composition of the two-phase solvent system, which provides an ideal partition coefficient of the target compound between the mobile and stationary phases. It has advantages of high efficiency, high recovery and easy to scale-up [14-18]. Therefore, this technique has been widely used for the preparative separation of various natural products [19-22].

To the best of our knowledge, no report has been published on the isolation and purification of tauro-conjugated cholic acids in *Pulvis Fellis Suis* by HSCCC. The aim of this study was to establish a method for the separation and purification of taurochenodeoxycholic acid (TCDCA), taurohyodeoxycholic acid (THDCA) and taurohyocholic acid (THCA) (Figure 1) from *Pulvis Fellis Suis*. The chemical structures of these compounds were verified by UV ESI-MS, ^1H NMR and ^{13}C NMR.

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument employed in the study is a TBE-300A high-speed counter-current chromatography (Tauto Biotech Co. Ltd., Shanghai, China) with three multilayer coil separation columns connected in series (I.D. of the tubing = 1.5 mm, total volume = 260 ml) and a 20 ml sample loop. The β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX-1050 constant-temperature circulating implement was used to control the separation temperature. The solvent was pumped into the column with a model TBP5002 constant flow pump and continuous monitoring of the effluent was achieved with an Alltech 800 evaporative light scattering detector. The data were collected with the Model N2000 chromatography workstation (Zhejiang University, Hangzhou, China).

The high-performance liquid chromatography (HPLC) analysis was performed in a Waters Alliance 2695 system (Waters, Milford, MA, USA) equipped with a vacuum degasser, a low pressure quaternary pump, an autosampler and a dual- λ absorbance detector, controlled by "Empower" software. A Welchrom C₁₈ column (250×4.6 mm, 5 μm) was used to separate conjugated cholic acids.

The nuclear magnetic resonance (NMR) spectrometer was a Bruker DRX-500 spectrometer (Bruker BioSpin, Rheinstetten, Germany).

2.2. Reagents and materials

All organic solvents used for sample preparation and HSCCC separation were of analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC analysis was of chromatography grade and purchased from Supervision of Tianjin Kermel Chemical Reagents Development Center (Tianjin, China). Monobasic sodium phosphate used for HPLC analysis was of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Water was purified in a Milli-Q plus system (Millipore, Madrid, Spain). Pulvis Fellis Suis was purchased from Xi'an Wanshou Road Chinese crude drug market (Shaanxi, China).

2.3. Preparation of crude sample

Pulvis Fellis Suis (120 g) was added into a glass column (10 cm × 70 cm, contained 3500 g aluminum oxide active neutral), 80% (v/v) ethanol as elution solution was passed through glass column, and then the effluents were combined and evaporated to dryness by a rotary evaporator under reduced pressure. Finally, 32.5 g of crude sample was obtained for the subsequent HSCCC isolation and separation.

2.4. Determination of partition coefficients (K)

The two-phase solvent system was selected according to the partition coefficient (*K*) of each target component. The *K* values were determined by HPLC analysis as follows: a suitable amount of crude samples were weighed into a 10 ml test tube, and then 2 ml of both phases of the pre-equilibrated two-phase solvent system was added to the tube. Then the test tube was capped and shaken vigorously for 1 min to thoroughly equilibrate the compounds between the two phases. An equal volume of each phase was transferred and evaporated separately. The residue of each phase was dissolved in an equal volume of ethanol and analyzed by HPLC. The *K* values were calculated by the ratio between the peak areas, and expressed as the area of the upper phase divided by that of the lower phase.

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

The two-phase solvent system composed of chloroform-methanol-water-acetic acid (4:4:2:0.3, v/v/v/v) was used for HSCCC separation. The solvent mixture was thoroughly equilibrated in a separatory funnel by vigorously shaking at room temperature. Then the solvent mixture was left overnight and the two phases were separated and degassed by sonication for 20 min prior to use. The upper aqueous phase was used as the stationary phase and the lower organic phase as the mobile phase. The sample solution was prepared by dissolving 100 mg of crude extract into 10 ml of solvent consisting of the equal volume of each phase used for HSCCC separation.

2.6. HSCCC separation procedure

In order to isolate and purify the target compounds from the crude sample, the multilayer coil column was first completely filled with the upper phase of the solvent system as stationary phase. Then the HSCCC apparatus was rotated at a revolution speed of 800 rpm. Meanwhile, the lower phase was pumped into the head inlet of the coiled column at a flow rate of 1.5 ml/min as mobile phase. After the mobile phase front emerged at the tail outlet and hydrodynamic equilibrium was established throughout the column, the sample solution was injected into the separation column through the injection valve. The effluent from the tail end of the separation column was continuously monitored with an Alltech 800 ELSD detector. The tube temperature of ELSD was 40°C and the gas flow rate was set at 2.8 ml/min. Peak fractions were collected by an automatic fraction collector set at 3 min for each tube. The analyses of fractions were performed by HPLC and all fractions containing the same pure compound were combined and evaporated under pressure. The separation

temperature was controlled at 25°C during the whole experiment. Each purified compounds were stored at 4°C before ESI-MS and NMR analysis.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude sample and each purified fraction from the preparative HSCCC separation were analyzed by HPLC with a Welchrom C₁₈ column (250×4.6 mm, 5 μm) at 25°C. The mobile phase was composed of methanol-0.03 mol/L monobasic sodium phosphate (70:30, v/v) to which phosphoric acid was added to adjust pH at 4.4. The flow rate was kept at 1.0 mL/min and UV detection was set at 200 nm. The injection volume was 20 μL.

The identification of HSCCC peak fractions was carried out by ¹H NMR, ¹³C NMR and ESI-MS. NMR spectra were obtained with a Bruker Avance 400 NMR. ESI-MS experiments were carried out using a Thermo Scientific LTQ XL ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source.

3. Results and discussion

3.1. Selection of two-phase solvent system

In a HSCCC separation, the most important first step is to select a suitable two-phase solvent system which can provide the ideal partition coefficient (*K*) for the target compounds. since it is crucial for efficient separation. The appropriate *K* value is usually considered in the range of 0.5–2. If the *K* value is too small, the analytes will be eluted close to the solvent front with poor resolution, while a larger *K* value tends to give better resolution but broader and more dilute peaks due to a longer elution time [14,16]. In our research, considering the similar structures of the three tauro-conjugated cholic acids, a series of two-phase solvent systems were carefully screened to optimize the solvent system for HSCCC separation and their *K* values of the target compounds were listed in Table 1. The results indicated that the two-phase solvent system of ethyl acetate-*n*-butanol-water (4:1:4, 4:1:5, 4:1:6, v/v/v) gave too small *K* values to all target compounds which were mostly distributed in the lower phase. When chloroform-methanol-water (4:3:2, 4:4:2, v/v) were used as the two-phase solvent systems, the *K* values of the target compounds were too large, and therefore a modifier was added to each system. Among the solvent systems with different modifiers, chloroform-methanol-water-acetic acid (4:4:2:0.5, v/v/v/v) were found to have a suitable *K* value, but the retention of the stationary phase was too low to give a peak resolution. Among all two-phase solvent systems examined, chloroform-methanol-water-acetic acid at a volume ratio of 4:4:2:0.3 was found to be satisfactory for separation of target compounds from the crude sample.

Other factors, such as the flow rate of the mobile phase, the temperature and the revolution speed of the separation column, were also investigated. The result showed that the optimum separation conditions were 1.5 ml/min of the flow rate, 800 rpm of revolution speed and 25°C of column temperature.

3.2. Separation of the three tauro-conjugated cholic acids by HSCCC

The crude sample (100 mg) was dissolved in 10 ml of both upper and lower phases (1:1, v/v) and separated by HSCCC. The HSCCC separation was achieved in the head to tail elution mode. The retention of the stationary phase was 42%, and the separation time was 410 min. Figure 2 showed the HSCCC separation of the crude sample using chloroform-methanol-water-acetic acid (4:4:2:0.3, v/v/v/v) solvent system. This separation yielded 10.2 mg compound 1 (fraction collected during 275-330 min), 11.8 mg compound 2 (fraction collected during 335-378 min) and 5.3 mg compound 3 (fraction collected during 380-400 min), with purity of 94.6%, 96.5%, 95.4%, respectively, which were directly used for NMR

analyses. Figure 3 shows the HPLC chromatogram of the crude sample and the combined fractions of tauro-conjugated cholic acids.

3.3. The structure identification

The chemical structures of the fractions were identified by ESI-MS, ^1H NMR and ^{13}C NMR. Data of each compounds were given as follows.

Peak 1 in Fig. 3: ESI-MS (m/z): 522.42 $[\text{M}+\text{Na}]^+$. ^1H NMR spectrum (400 MHz, DMSO, ppm): 0.66 (3H, s, CH_3), 0.91 (3H, s, CH_3), 0.96 (3H, d, CH_3), 3.06 (2H, m, CH_2), 3.55 (2H, m, CH_2), 3.49 (H, m, CH), 3.86 (H, m, CH). ^{13}C NMR spectrum (400 MHz, DMSO, ppm): 35.24 (C-1), 28.42 (C-2), 68.39 (C-3), 38.08 (C-4), 46.65 (C-5), 31.62 (C-6), 64.97 (C-7), 39.14 (C-8), 30.96 (C-9), 31.90 (C-10), 19.58 (C-11), 39.72 (C-12), 46.75 (C-13), 51.63 (C-14), 24.73 (C-15), 26.46 (C-16), 55.28 (C-17), 14.91 (C-18), 20.28 (C-19), 36.10 (C-20), 17.46 (C-21), 29.22 (C-22), 29.30 (C-23), 173.73 (C-24), 32.14 (C-25), 51.25 (C-26). Compound 1 was identified as taurochenodeoxycholic acid.

Peak 2 in Fig. 3: ESI-MS (m/z): 522.42 $[\text{M}+\text{Na}]^+$. ^1H NMR spectrum (400 MHz, DMSO, ppm): 0.67 (3H, s, CH_3), 0.91 (3H, s, CH_3), 0.95 (3H, d, CH_3), 3.29 (2H, m, CH_2), 3.60 (2H, m, CH_2), 3.48 (H, m, CH), 3.99 (H, m, CH). ^{13}C NMR spectrum (400 MHz, DMSO, ppm): 36.86 (C-1), 33.04 (C-2), 72.46 (C-3), 29.16 (C-4), 48.36 (C-5), 68.69 (C-6), 35.62 (C-7), 35.49 (C-8), 41.31 (C-9), 36.91 (C-10), 21.88 (C-11), 44.04 (C-12), 48.58 (C-13), 57.65 (C-14), 25.20 (C-15), 31.03 (C-16), 57.13 (C-17), 12.49 (C-18), 24.14 (C-19), 36.14 (C-20), 18.82 (C-21), 35.47 (C-22), 33.09 (C-23), 178.13 (C-24), 37.63 (C-25), 50.67 (C-26). Compound 2 was identified as taurohyodeoxycholic acid.

Peak 3 in Fig. 3: ESI-MS (m/z): 538.42 $[\text{M}+\text{Na}]^+$. ^1H NMR spectrum (400 MHz, DMSO, ppm): 0.58 (3H, s, CH_3), 0.81 (3H, s, CH_3), 0.86 (3H, d, CH_3), 3.11 (2H, m, CH_2), 3.56 (2H, m, CH_2), 3.26 (H, m, CH), 3.35 (H, m, CH), 3.89 (H, m, CH). ^{13}C NMR spectrum (400 MHz, DMSO, ppm): 36.05 (C-1), 32.65 (C-2), 71.38 (C-3), 40.16 (C-4), 42.45 (C-5), 68.95 (C-6), 70.85 (C-7), 40.37 (C-8), 39.95 (C-9), 35.90 (C-10), 23.54 (C-11), 40.57 (C-12), 48.49 (C-13), 51.06 (C-14), 28.18 (C-15), 31.10 (C-16), 55.97 (C-17), 12.09 (C-18), 23.58 (C-19), 38.68 (C-20), 18.76 (C-21), 31.93 (C-22), 35.46 (C-23), 172.61 (C-24), 36.00 (C-25), 50.36 (C-26). Compound 3 was identified as taurohyocholic acid.

4. Conclusion

The overall results of our study clearly demonstrate that HSCCC can provide highly efficient preparative separation of taurochenodeoxycholic acid, taurohyodeoxycholic acid and taurohyocholic acid from Pulvis Fellis Suis in one step operation. By using the HSCCC method described above, these tauro-conjugated cholic acids could be obtained with high purities which might be used for further chemical research and pharmacological studies or as reference substances. HSCCC has been demonstrated to be a fast, effective and powerful technique for the separation and purification of active compounds from the natural medicines.

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Fig. 1.
Chemical structures of three target cholic acids.

Fig. 2.
HSCCC separation of crude samples of Pulvis Fellis Suis.

Fig. 3.
HPLC analyses of crude sample and HSCCC peak fractions.

Table 1
The *K* values of target components measured in several solvent systems

Solvent system (v/v)	<i>K</i> value		
	TCDCA	THDCA	THCA
Ethyl acetate- n-butanol- water (4:1:4)	0.11	0.10	0.32
Ethyl acetate- n-butanol- water (4:1:5)	0.09	0.06	0.13
Ethyl acetate- n-butanol- water (4:1:6)	0.07	0.05	0.10
Chloroform-methanol-water (4:3:2)	25.75	19.98	19.03
Chloroform-methanol-water (4:4:2)	3.82	5.25	3.86
Chloroform- n-butanol-methanol-water (4:0.1:3:2)	8.74	12.94	4.59
Chloroform- n-butanol-methanol-water (4:0.2:3:2)	6.36	8.77	5.52
Chloroform- n-butanol-methanol-water (4:0.3:3:2)	6.25	9.30	6.30
Chloroform-methanol-water-acetic acid (4:4:2:0.1)	2.65	3.44	2.28
Chloroform-methanol-water-acetic acid (4:4:2:0.3)	1.76	1.95	1.48
Chloroform-methanol-water-acetic acid (4:4:2:0.5)	1.40	1.66	1.24