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PREPARATIVE ISOLATION AND PURIFICATION OF THREE GLYCINE-CONJUGATED CHOLIC ACIDS FROM PULVIS FELLIS SUIIS BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY COUPLED WITH ELSD DETECTION

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Abstract

Coupled with evaporative light scattering detection, a high-speed counter-current chromatography (HSCCC) method was developed for preparative isolation and purification of three glycine-conjugated cholic acids, glycochenodeoxycholic acid (GCDCA), glycohyodeoxycholic acid (GHDCA) and glycohyocholic acid (GHCA) from Pulvis Fellis Suis (Pig gallbladder bile) for the first time. The separation was performed with a two-phase solvent system consisted of chloroform-methanol-water-acetic acid (65:30:10:1.5, v/v/v/v) by eluting the lower 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, 2 ml/min and 25 °C, respectively. In a single operation, 33 mg of GCDCA, 38 mg of GHDCA and 23 mg of GHCA were obtained from 200 mg of crude extract with the purity of 95.65%, 96.72% and 96.63%, respectively, in one step separation. The HSCCC fractions were analyzed by high-performance liquid chromatography (HPLC) and the structures of the three glycine-conjugated cholic acids were identified by ESI-MS, ¹H NMR and ¹³C NMR.

Keywords

High-speed countercurrent chromatography; Separation and purification; Two-phase solvent system; Pulvis Fellis Suis; Glycine-conjugated cholic acids

INTRODUCTION

Pulvis Fellis Suis (Pig gallbladder bile), a traditional Chinese medicine and officially listed in the Chinese Pharmacopoeia, 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

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pharmaceutically relevant compounds.^[2–6] According to our previous research, the contents of glycine-conjugated cholic acids were higher than taurine-conjugated cholic acid in Pulvis Fellis Suis, and therefore it is necessary to develop an efficient method to isolate and purify the single glycine-conjugated cholic acid with high purity for quality control and pharmacological research. However, it is difficult to obtain pure single glycine-conjugated cholic acid by conventional separation methods, such as column chromatography (CC) and thin-layer chromatography (TLC) because of their similarity in chemical structure.

High-speed counter-current chromatography (HSCCC) is a liquid-liquid partition chromatography technique that is free of solid support matrix. Therefore, it can eliminate the risk of irreversible adsorption of sample components that is often the case with solid supports used in conventional column chromatography. In HSCCC the separation process is entirely based on the composition of the two-phase solvent system, which should provide an ideal partition coefficient of the target compound between the mobile and stationary phases. The method has advantages of high efficiency, high recovery and ease of scaling-up.^[7–12] Therefore, HSCCC has been used widely in the preparative separation of various natural products.^[13–16] However, to the best of our knowledge, no report has been published on the isolation and purification of glycine-conjugated cholic acids in Pulvis Fellis Suis by HSCCC.

The aim of this study is to establish a method for the separation and purification of glycochenodeoxycholic acid (GCDCA), glycohyodeoxycholic acid (GHDCA) and glycohyocholic acid (GHCA) (Figure 1) from Pulvis Fellis Suis. The chemical structures of these compounds are verified by UV ESI-MS, ¹H NMR and ¹³C NMR.

EXPERIMENTAL

Apparatus

The preparative HSCCC instrument employed in the study is a TBE-300A high-speed counter-current chromatography (Tauto Biotechnology Company, 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, and R , the revolution radius). 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 ambient 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 detection. The data were collected with a Model N2000 chromatography workstation (Zhejiang University, Hangzhou, China).

The high-performance liquid chromatography (HPLC) equipment used was a Waters Alliance 2695 system (Waters, Milford, MA, USA) including a vacuum degasser, a low pressure quaternionic pump, an autosampler and a dual- λ absorbance detector, controlled by “Empower” software. A Welchrom C₁₈ column (250×4.6 mm, 5 μ m) was used to separate conjugated cholic acids.

Reagents and Materials

All organic solvents used for preparation of crude sample 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).

Preparation of Crude Sample

Pulvis Fellis Suis (175 g) was loaded into a glass column (10 cm × 70 cm) containing 3500 g aluminum oxide active neutral, and 45 L of 80% (v/v) ethanol solution was eluted through the column. The effluents were combined and evaporated to dryness by rotary evaporator under reduced pressure. Finally, 64.8 g crude sample was obtained for further HSCCC separation.

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 was weighed into a 10 ml test tube to which 2 ml of each phase of the pre-equilibrated two-phase solvent system were added. 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 to separate test tubes and evaporated, the residue of which was dissolved in the equal volume of methanol and analyzed by HPLC. The *K* values were calculated by dividing the peak areas of the upper phase with that of the lower phase for corresponding peaks.

Preparation of Two-Phase Solvent Systems and Sample Solution

The two-phase solvent system composed of chloroform-methanol-water-acetic acid (65:30:10:1.5, v/v/v/v) was selected and used for HSCCC separation. The solvent mixture was thoroughly equilibrated in a separatory funnel by vigorously shaking several times at room temperature. Then it was left overnight and the two phases were separated and degassed by sanitation for 20 min prior to use. The upper phase was used as the stationary phase and lower phase as the mobile phase. The sample solution was prepared by dissolving 200 mg of the crude extract in 10 ml solvent mixture consisting of equal volumes of each phase of the two-phase solvent system used for HSCCC separation.

HSCCC Separation Procedure

In each separation, the multilayer coil column was first completely filled with the upper phase. Then the HSCCC apparatus was run at a revolution speed of 800 rpm, while the lower phase was pumped into the column inlet at a flow rate of 2.0 ml/min in a head-tail elution mode. 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 detection. The tube temperature of ELSD was 40°C and a gas flow rate set at 2.8 ml/min. Peak fractions were collected by a fraction collector set at 3 min for each tube. The analyses of fractions were performed by HPLC and all fractions containing each target compound were combined and evaporated under pressure. The ambient temperature was controlled at 25°C during the whole experiment. Each purified fraction was stored at 4 °C before ESI-MS and NMR analysis.

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 consisted 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 μ L.

The identification of HSCCC peak fractions was carried out by ^1H NMR, ^{13}C 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.

RESULTS AND DISCUSSION

Selection of Two-Phase Solvent System

The successful separation by HSCCC largely relies on the selection of a suitable two-phase solvent system to provide the ideal partition coefficient (K) for the applied compounds. The optimum K value is usually 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, more dilute peaks with a longer elution time^[8, 10]. In our research, in order to resolve three glycine-conjugated cholic acids with similar structures, several two-phase solvent systems were tested, and their K values are summarized in Table 1. The results indicated that the two-phase solvent system of chloroform-methanol-water (4:3:2, or 4:4:2, v/v/v) gave all target compounds too large K value which would distribute the target compounds mostly into the stationary aqueous phase. Therefore, 1-butanol was added to the system. Among the solvent systems with different volume ratio of n-butanol, the K values of GHCA were suitable, but the K values of GHDCA were too small and that of GCDCA too large. When ethyl acetate-n-butanol- water (4:1:5, v/v/v) and ethyl acetate-n-butanol-methanol-water (4:1:0.5:6, v/v/v/v) were used as two-phase solvent system, the K values of GHCA were suitable, but K values of GHDCA became too large and that of GCDCA too small. The two-phase solvent system chloroform-methanol-water-acetic acid (6.5:2.5:1:0.2, v/v/v/v) gave acceptable K values for each target compound but with similar K values between GHCA and GCDCA. However, when the solvent ratio was changed to 6.5:3:1:0.15, v/v/v/v, the K values were much improved so that all target compounds could be well separated from each other. Therefore, the two-phase solvent system composed of chloroform-methanol-water-acetic acid (6.5:3:1:0.15, v/v/v/v) was selected as the two-phase solvent system for the HSCCC separation.

The effects of flow rate of the mobile phase and revolution speed on HSCCC separation were also investigated. The result showed that the optimum separation conditions were 2.0 ml/min of the flow rate, 800 rpm of revolution speed and 25 °C of column temperature.

HSCCC Separation

The crude sample (200 mg) was dissolved in a 10 ml mixture of upper and lower phases (1:1, v/v) and separated by HSCCC in the head to tail elution mode. The retention of the stationary phase was 61%, and the separation time was 130 min. Figure 2 showed the HSCCC separation of the crude sample using chloroform-methanol-water-acetic acid (6.5:3:1:0.15, v/v/v/v) solvent system. This separation yielded 33 mg compound 1 (fraction collected during 87–97 min), 38 mg compound 2 (fraction collected during 98–108 min) and 23 mg compound 3 (fraction collected during 115–128 min), with purity of 95.65%, 96.72%, and 96.63%, respectively, which were directly analyzed by NMR. Figure 3 shows the HPLC chromatogram of the crude sample and the combined fractions of glycine-conjugated cholic acids.

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): 472.33 $[\text{M}+\text{Na}]^+$. ^1H NMR spectrum (400 MHz, DMSO, ppm): 0.69 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.99 (3H, d, CH_3), 3.35 (2H, m, CH_2), 3.88 (2H, m, CH_2), 7.72 (1H, s, NH). ^{13}C NMR spectrum (400 MHz, DMSO, ppm): 36.59 (C-1), 31.39 (C-2), 72.89 (C-3), 40.80 (C-4), 43.21 (C-5), 35.93 (C-6), 69.10 (C-7), 41.10 (C-8), 34.08 (C-9), 36.26 (C-10), 21.82 (C-11), 41.84 (C-12), 43.72 (C-13), 51.57 (C-14), 24.67 (C-15), 29.28 (C-16), 57.38 (C-17), 12.21 (C-18), 23.44 (C-19), 36.89 (C-20), 18.93 (C-21), 33.13 (C-22), 33.81 (C-23), 177.19 (C-24), 48.40 (C-25), 173.22 (C-26). Compound 1 was identified as glycochenodeoxycholic acid.

Peak 2 in Fig. 3: ESI-MS (m/z): 472.42 $[\text{M}+\text{Na}]^+$. ^1H NMR spectrum (400 MHz, DMSO, ppm): 0.58 (3H, s, CH_3), 0.82 (3H, s, CH_3), 0.87 (3H, d, CH_3), 3.29 (2H, m, CH_2), 3.80 (2H, m, CH_2), 7.46 (1H, s, NH). ^{13}C NMR spectrum (400 MHz, DMSO, ppm): 35.85 (C-1), 29.71 (C-2), 70.46 (C-3), 28.14 (C-4), 43.29 (C-5), 66.33 (C-6), 35.34 (C-7), 34.79 (C-8), 40.39 (C-9), 36.00 (C-10), 20.86 (C-11), 40.60 (C-12), 42.80 (C-13), 56.35 (C-14), 24.35 (C-15), 30.81 (C-16), 56.05 (C-17), 12.33 (C-18), 24.01 (C-19), 35.43 (C-20), 18.75 (C-21), 31.96 (C-22), 32.75 (C-23), 172.41 (C-24), 48.71 (C-25), 172.1 (C-26). Compound 2 was identified as glycohyodeoxycholic acid.

Peak 3 in Fig. 3: ESI-MS (m/z): 488.42 $[\text{M}+\text{Na}]^+$. ^1H NMR spectrum (400 MHz, DMSO, ppm): 0.58 (3H, s, CH_3), 0.81 (3H, s, CH_3), 0.88 (3H, d, CH_3), 3.11 (2H, m, CH_2), 3.43 (2H, m, CH_2), 3.58 (2H, m, CH_2), 7.47 (1H, s, NH). ^{13}C NMR spectrum (400 MHz, DMSO, ppm): 36.00 (C-1), 31.10 (C-2), 71.36 (C-3), 28.19 (C-4), 43.26 (C-5), 68.97 (C-6), 70.86 (C-7), 40.57 (C-8), 40.16 (C-9), 36.05 (C-10), 20.75 (C-11), 40.36 (C-12), 42.45 (C-13), 50.37 (C-14), 23.58 (C-15), 32.01 (C-16), 56.06 (C-17), 12.10 (C-18), 23.55 (C-19), 35.55 (C-20), 18.78 (C-21), 32.67 (C-22), 32.77 (C-23), 172.5 (C-24), 48.5 (C-25), 172.22 (C-26). Compound 3 was identified as glycohyocholic acid.

CONCLUSION

The overall results of our studies indicated that HSCCC was successfully used for separation and purification of glycochenodeoxycholic acid, glycohyodeoxycholic acid and glycohyocholic acid from Pulvis Fellis Suis in one step operation in less than 2.5 h. By using the HSCCC method described above, these glycine-conjugated cholic acids could be obtained with high purities which might be useful for further chemical research and pharmacological studies as well as for reference substances. HSCCC is a fast, effective and powerful technique for the separation and purification of bioactive compounds from the natural products.

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Figure 1.
Chemical structure of three glycine-conjugated cholic acids

Figure 2.

HSCCC chromatogram of the crude extract of Pulvis Fellis Suis. Peak 1, compound 1; peak 2, compound 2; Peak 3, compound 3. Experimental conditions: two-phase solvent system: chloroform-methanol-water-acetic acid (65:30:10:1.5, v/v/v/v); stationary phase, upper phase; mobile phase, lower phase; flow rate, 2 mL/min; rotary speed, 800 rpm; ELSD condition: drift tube temperature, 40°C; gas flow rate, 2.8 L/min; sample, 200 mg of crude extract dissolved in 10 ml of a mixture of upper and lower phase(1:1, v/v) of the solvent system used for HSCCC.

Figure 3.

HPLC analyses of crude sample and HSCCC fractions. (A) : HPLC analysis of crude extract from Pulvis Fellis Suis; (B) HSCCC fraction of peak 1; (C) HSCCC fraction of peak 2; (D) HSCCC fraction of peak 3. HPLC conditions: Welchrom C₁₈ column (250×4.6 mm, 5 μm), mobile phase methanol – 0.03 mol/L monobasic sodium phosphate solution (70:30, v/v), add phosphate acid to adjust to pH 4.4, flow rate 1.0 mL/min, column temperature 25 °C, detection wavelength 200 nm.

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

Solvent system (v/v)	<i>K</i> value		
	GCDCA	GHDCa	GHCA
Chloroform-methanol-water (4:3:2)	9.07	86.57	88.49
Chloroform-methanol-water (4:4:2)	5.3	63.88	65.21
Chloroform- n-butanol-methanol-water (4:0.1:3:2)	2.99	0.46	1.53
Chloroform- n-butanol-methanol-water (4:0.2:3:2)	10.87	0.37	1.36
Chloroform- n-butanol-methanol-water (4:0.3:3:2)	5.02	0.23	0.75
Acetic ether- n-butanol- water (4:1:5)	0.06	3.67	1.84
Acetic ether- n-butanol-methanol-water (4:1:0.5:6)	0.07	3.97	1.96
Chloroform-methanol-water-acetic acid (6.5:2.5:1:0.2)	0.60	0.67	1.03
Chloroform-methanol-water-acetic acid (6.5:3:1:0.15)	0.92	1.15	1.98