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Purification of salvianolic acid B from the crude extract of *Salvia miltiorrhiza* with hydrophilic organic/salt-containing aqueous two-phase system by counter-current chromatography

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Abstract

Establishment of hydrophilic organic/salt-containing aqueous two-phase system and purification of salvianolic acid B from crude extract of *S. miltiorrhiza* by counter-current chromatography with said system were studied. Ethanol and *n*-propanol were selected to constitute biphasic systems with ammonia sulphate, sodium chloride and phosphate separately, and related system characteristics including phase diagrams, phase ratio, separation time were tested. The partition coefficient of crude salvianolic acid B was also tested in above systems and further finely adjusted by altering the constitution of phosphate in *n*-propanol/phosphate system. Salvianolic acid B was purified to 95.5% purity by counter-current chromatography in 36% (w/w) *n*-propanol/8% (w/w) phosphate system with the ratio between dipotassium hydrogen phosphate and sodium dihydrogen phosphate of 94:6. One hundred and eight milligrams of salvianolic acid B was purified from 285 mg crude extract with the recovery of 89%.

Keywords: Counter-current chromatography; Hydrophilic organic/salt-containing aqueous two-phase system; Salvianolic acid B; Purification

1. Introduction

Counter-current chromatography (CCC) is a continuous liquid–liquid partition chromatography with no solid support matrix, the stationary phase of which is retained in the separation columns by gravity and centrifugal force field [1,2], and therefore avoids the disadvantages arising from the interaction of samples with the solid support, such as absorption and denaturation of target products. CCC has the unique features of high recovery, high efficiency and the ease to scale-up, and has been widely used in the separation and purification of natural products, antibiotics and rare elements with organic/aqueous systems [3,4].

The selection of separation system is crucial for successful purification of target product in CCC, which is usually based on the characteristics of the target product, especially the polarity [5]. A large variety of organic/ (organic/) aqueous two-phase systems with rather wide range of polarity from most hydrophobic system of hexane/ethanol/water (5:4:1) to most polar system of 1-butanol/water (1:1) have been established and successfully used for the separation of natural products [5–7]. However, when separating natural products with even higher polarity, including those that could form salt at high pH, such as glycyrrhizic acid [7] and salvianolic acid with high pharmacological value, the polarity of common used systems such as 1-butanol/water system was not strong enough, which may lead to unsatisfied separation or prolonged separation time.

Recently, a new biphasic system composed of water-soluble hydrophilic organic solvent and inorganic salt solution has been proposed and used for the partition study of protein [9], amino acid [10] and hydrophilic natural products [8,11]. These biphasic systems have the advantages of higher polarity compared with conventional organic/aqueous systems and lower cost compared with aqueous polymer two-phase systems, as well as the relatively low environmental toxicity.

In this paper, we tested the establishment and characteristics of several hydrophilic organic/salt-containing aqueous two-phase systems with low environmental toxicity and tested the

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partition of a polar natural product, salvianolic acid B (Sal B) in said systems and further purified it by CCC with *n*-propanol/phosphate system. Sal B is the water-soluble bioactive components of the traditional Chinese medicine, *Salvia miltiorrhiza*, which has recently been addressed and shown to exhibits endothelium-dependent vasodilation in the aorta and may be useful in the treatment of hypertension while its salt magnesium salvianolic acid B has a potent hepato-protective activity and shows an improved effect on uremic symptoms [6,12,13]. Sal B is a phenolic acid compound and its structure is shown in Fig. 2C.

2. Experimental

2.1. Chemicals

Ethanol (absolute), *n*-propanol, ammonia sulphate, sodium chloride, dipotassium hydrogen phosphate, sodium dihydrogen phosphate were of reagent grades from Shanghai Chemical Reagent (Shanghai, China). De-ioned water was used for all solutions, except those for HPLC analysis, which used double distilled water.

The crude extract of *S. miltiorrhiza* was purchased from Guanghan Bencao Plant Chemical Co. Ltd. (Guanghan, China). In brief, the extract was prepared by refluxing *S. miltiorrhiza* powder with water followed by filtration. The filtrate was concentrated in rotatory evaporator under reduced pressure and was spray dried.

Sal B standard (>98%) was purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Equipments

Synchronous CCC apparatus TBE-300V was from Tauto Biotech (Shanghai, China), three coiled separation columns were connected in series (I.D. of PTFE tube = 2.6 mm, revolution radius = 50 mm, β value = 0.51–0.74, total volume = 120 mL) with a 20 mL sample loop. The rotation speed of separation columns was adjusted between 700 and 1000 r/min and the revolution speed of the columns was the same as the rotation speed. The temperature of separation columns was controlled by water circulator (HX-1050, Boyikang Lab Instrument, Beijing, China).

TBE-300V was coupled to Äkta Prime system (GE Healthcare) for liquid pumping, sample detection and fraction collection.

2.3. Preparation of hydrophilic organic/salt-containing aqueous two-phase systems

Hydrophilic organic/salt-containing aqueous two-phase systems were prepared by thoroughly mixing desired amount of alcohol with inorganic salt solutions in separatory funnel at room temperature, allowing for two clear phases to form. Each phase was degassed with ultrasonic water bath for 15 min before use.

2.4. Preparation of sample solutions for CCC

The crude extract powder (500 mg) was dissolved in 50 mL de-ioned water and ultrasonicated for 10 min. The resultant supernatant was extracted with 15 mL ethyl acetate for three times. The upper ethyl acetate phase was collected and combined, and then evaporated to dryness by vacuum rotary evaporator at 50 °C. Before separation, 4 mL mobile phase of the intended separation systems was added to the resultant dried extract and shaked until all solid dissolved.

2.5. CCC operation

The separation columns of counter-current chromatograph was filled with the stationary lower phase, followed by pumping mobile upper phase in tail to head mode at preset flow-rate; meanwhile the apparatus was rotated at the desired speed. The effluent from the outlet of the column was monitored at 280 nm. After the mobile phase flowed out of the outlet and the absorbance became stable, indicating that the equilibration between mobile and stationary phase had been established in the column, the volume of flowing-out stationary phase was noted. The retention of stationary phase before loading sample is defined as the ratio between the volume of the station phase retained in the separation columns and the total volume of the separation columns. The sample solution was then injected through the sample loop. The elution peak was collected by the fraction collector. After purification the biphasic solvent system in the separation column was blown out with compressed air into a measuring cylinder and the retention of stationary phase after purification was recorded again.

2.6. Determination of partition coefficient

One milligram of ethyl acetate extract (crude Sal B) prepared in Section 2.4 was added to a test tube with 1 mL of each phase for every individual hydrophilic organic/salt-containing aqueous two-phase system. The contents were thoroughly mixed and allowed to settle at room temperature until two clear layers formed. 0.5 mL of each phase was pipetted and diluted when necessary with corresponding phase (also served as blank) to determine the absorbance at 280 nm using a Shimadzu spectrophotometer UV-2401 (Shimadzu, Japan). The partition coefficient was defined as the ratio between the absorbance value of upper and lower phase ($K = A_{Upper}/A_{Lower}$).

2.7. HPLC analysis

The crude Sal B and elution peak obtained by CCC was analyzed by HPLC at room temperature according to the literature [14]. The column used was Ultrasphere C18 column (150 mm × 4.6 mm I.D., 5 μm, Shimadzu, Japan). The mobile phase was methanol–5% acetic acid (35:65) and the flow-rate was 1.0 mL/min. The effluent was monitored at 280 nm.

Table 1
Composition and characteristics of alcohol/inorganic salts systems

System	Concentration (%, w/w)	pH	Phase ratio	Phase separation time	K
Ethanol/$(NH_4)_2SO_4$	26, 17	6	1.17	85	5.7
Ethanol/NaCl	26, 17	7	n/a	n/a	n/a
Ethanol/phosphate	16, 26	6	1.47	98	36.7
		7	1.28	56	12.5
		9	0.91	44	7.8
n-Propanol/$(NH_4)_2SO_4$	34, 8	6	1.00	51	4.9
n-Propanol/NaCl	34, 8	7	0.79	65	2.1
n-Propanol/phosphate	34, 8	6	0.81	83	2.8
		7	0.91	70	2.4
		9	1.15	56	0.8

3. Results and discussion

3.1. Characteristics of hydrophilic organic/salt-containing aqueous two-phase system and establishment of corresponding CCC system

Two hydrophilic alcohols, ethanol and *n*-propanol, with low environmental toxicity were used to form hydrophilic organic/salt-containing aqueous two-phase systems with three inorganic salts, i.e. ammonia sulphate, sodium chloride and phosphate, separately.

Firstly, the binodal curves of the intended systems were constructed, two typical curves were formed that are shown in Fig. 1, which could be used for the selection of the alcohol and inorganic salt concentration to constitute systems with appropriate phase ratio for further studies.

The binodal curves of *n*-propanol/inorganic salt systems are typically more closed to the origin than the ones for ethanol/inorganic salt systems, indicating that lower concentration of alcohol and salt will be needed to form two separated phases, which is due to the relatively high hydrophobicity of *n*-propanol.

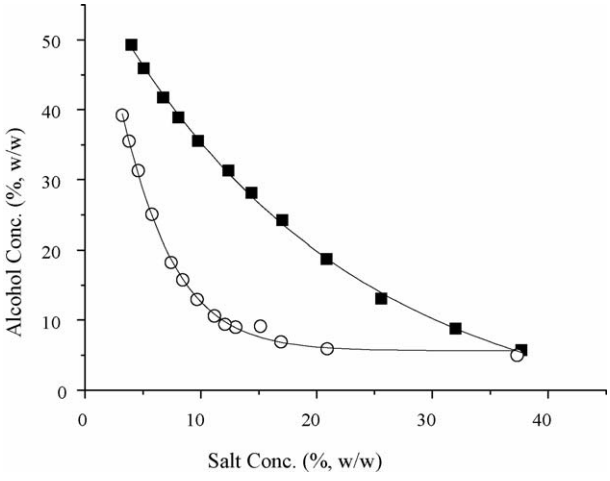

Fig. 1. Binodal curves of ethanol/ammonia sulphate (■) and *n*-propanol/phosphate (◯) systems.

System concentrations were determined for each intended system based on the binodal curves and the characteristics of those systems and the partition coefficient of crude Sal B were tested and shown in Table 1.

Ethanol can form stable and adjustable organic/aqueous two-phase system with inorganic salt solutions except with sodium chloride, which formed salt precipitation and may be due to the extreme low solubility of sodium chloride in ethanol. *n*-Propanol formed stable biphasic systems with all the three inorganic salt solutions. The phase separation time of the biphasic systems in Table 1, a major determinant for the retention of stationary phase in CCC, is much shorter than that of aqueous two-phase system [15] and fall into the time order of organic/organic system [5], which indicated that relatively higher retention of stationary phase may be attained. The characteristics of above organic/aqueous two-phase systems, such as pH, phase ratio and ionic strength can be easily adjusted by altering the composition of inorganic salt and/or concentration of both to have optimal partition of target product.

From Table 1, the partition coefficients of crude Sal B in *n*-propanol/phosphate system would be suitable for CCC purification, and then were further tuned based on the composition of phosphate (Table 2). In order to facilitate the preparation of organic/aqueous two-phase system, the composition of phosphate was expressed as weight percentage.

With the decrease of NaH_2PO_4/K_2HPO_4 ratio, the partition coefficient of crude Sal B was thereby finely down-adjusted. Considering the alcohol-rich upper phase could facilitate following process, it was chosen as mobile phase for CCC purification, and the composition of 6% (w/w) NaH_2PO_4 with the partition coefficient of 1.5 was chosen for further CCC purification study.

Table 2
Partition coefficient of Sal B in 34% (w/w) *n*-propanol/8% (w/w) phosphate systems with different phosphate composition

No.	NaH_2PO_4 (%)	K_2HPO_4 (%)	pH (approximately)	K
1	25	75	7.1	2.4
2	18	82	7.3	2.0
3	12	88	7.5	1.8
4	6	94	7.8	1.5
5	0	100	9.2	0.8

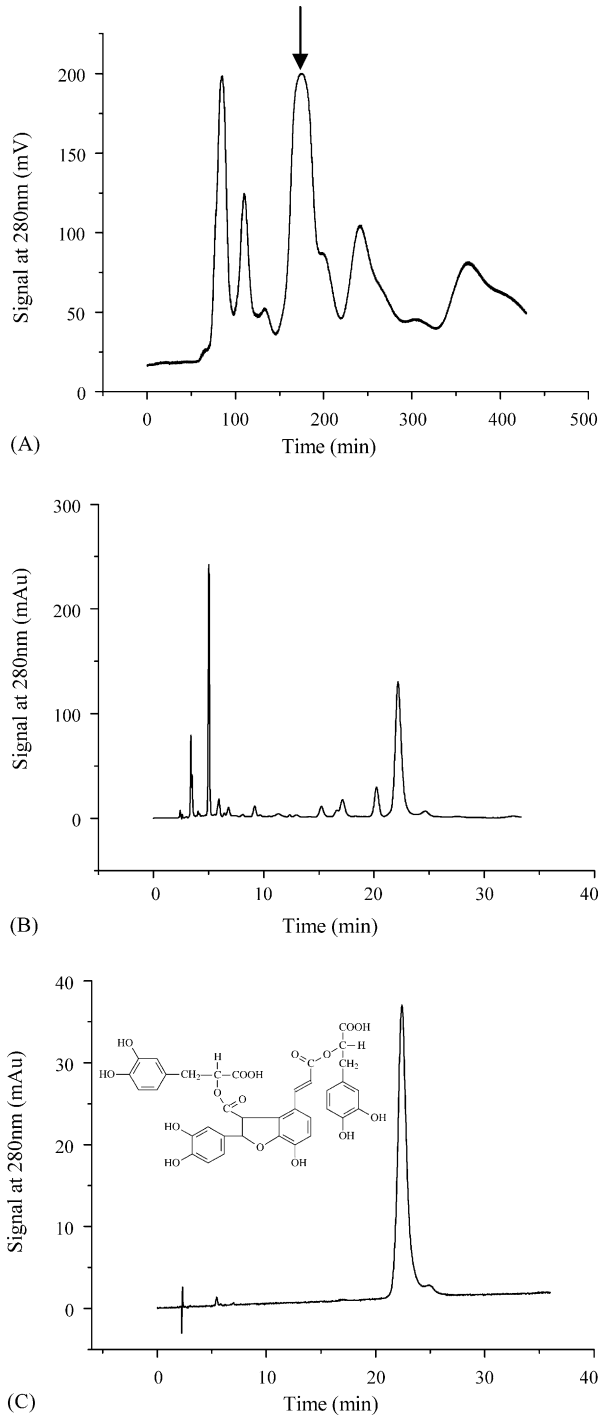

Fig. 2. CCC purification of Sal B in *n*-propanol/phosphate systems (A) and HPLC determination of purity in crude Sal B (B) and CCC purified Sal B (C): arrow in (A) indicates the elution peak of Sal B. Experimental conditions for CCC include the following: 4 mL sample solution, prepared as described in Section 2; solvent system, 36.0% (w/w) *n*-propanol/8% (w/w) phosphate (NaH_2PO_4:K_2HPO_4 = 6:94, w/w); mobile phase, upper phase; flow-rate, 1.2 mL/min; revolution, 880 r/min; retention of stationary phase before loading sample and after purification, 52% and 48%, respectively.

3.2. CCC purification of Sal B in n-propanol/phosphate organic/aqueous two-phase system

n-Propanol/phosphate organic/aqueous two-phase system composed of 34% (w/w) *n*-propanol, 0.48% (w/w) NaH_2PO_4 and 7.52% (w/w) K_2HPO_4 as suggested above was used to perform CCC purification of crude Sal B and the result was shown in Fig. 2A.

The elution peaks were collected and sampled onto the TLC plate together with the Sal B standard and developed with chloroform:acetic ether:formic acid (4:3:1). The major peak between 150 and 200 min was found to be the one for Sal B, which was further analyzed on HPLC to determine its purity. The result was shown in Fig. 2C. The purity of Sal B elution peak was determined to be 95.5% by HPLC with the recovery of 89%, which is calculated based on the sample-loading amount (285 mg), the content of Sal B in crude sample determined by HPLC (42.6%) and the amount of purified Sal B (108 mg).

4. Conclusion

Hydrophilic organic/salt-containing aqueous two-phase systems were established and successfully used to purify Sal B by counter-current chromatography with high recovery and high purity, which indicates the promising potential of said systems in the purification of compounds with high polarity and widens the selection range of biphasic systems for CCC purification of natural products.

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