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Preparative isolation and purification of chuanxiongzine from the medicinal plant *Ligusticum chuanxiong* by high-speed counter-current chromatography

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Abstract

Ligusticum chuanxiong Hort has been used widely in traditional medicines for the treatment of various kinds of disorders such as cardiovascular and cerebrovascular diseases. High-speed counter-current chromatography was applied to the isolation and purification of the bioactive component chuanxiongzine from *L. chuanxiong* Hort. The crude chuanxiongzine was obtained by extraction with ethanol from the dried roots of *L. chuanxiong* Hort under sonication. Preparative high-speed counter-current chromatography with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:3:7, v/v) was successfully performed yielding 11.5 mg chuanxiongzine at 96.8% purity from 300 mg of the crude extract (4.2% chuanxiongzine) with the recovery of 91% in a one-step separation.

Keywords: *Ligusticum chuanxiong*; Counter-current chromatography; Plant materials; Preparative chromatography; Pharmaceutical analysis; Chuanxiongzine

1. Introduction

Ligusticum chuanxiong Hort (Chuanxiong in Chinese) is one of the most widely used traditional herbal medicines. Its roots have been used for the treatment of various kinds of disorders such as cardiovascular and cerebrovascular diseases [1,2]. Chuanxiongzine (i.e., tetramethylpyrazine) is the bioactive component of *L. chuanxiong* Hort, which improves the cerebral blood flow through inhibiting thrombus formation and platelet aggregation during ischemic attack [3,4]. Chuanxiongzine can significantly improve central cholinergic system function and enhance the learning and memory ability [5–7]. In addition, chuanxiongzine has anti-bacterial properties [2,8] and prophylactic effect on mice with endotoxemia [9]. It scavenges superoxide anion and decreases nitric oxide production in human polymorphonuclear leukocytes [10]. The chemical structure of chuanxiongzine is shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography, which has been applied to the separation of many natural products [11–18]. However, no report has been published on the use of HSCCC for the isolation and purification of chuanxiongzine from plants. The purpose of this study, therefore, was to develop an efficient method for the isolation and purification of chuanxiongzine from the medicinal plant *L. chuanxiong* Hort by HSCCC.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (inner diameter of tube, 2.6 mm; total volume, 325 ml). The revolution radius or the distance between the holder axis and

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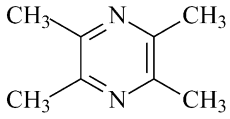

Fig. 1. Chemical structure of chuanxiongzine.

central axis of the centrifuge (R) was 7.5 cm, and the β-value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a Model Series II HPLC Pump (Pharma-Tech Research), a Model SPD-10Avp UV–vis Detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 10-ml sample loop.

2.2. Reagents

All solutions were prepared with analytical grade compounds. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. Ethyl acetate, *n*-hexane, ethanol and methanol were obtained from BDH (Poole, UK). The standard chuanxiongzine was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. The chuanxiongzine stock solution was 1.00 mg ml^{-1} which was prepared by dissolving 10.0 mg of chuanxiongzine in 10.00 ml methanol and then stored in a refrigerator. The working solutions were prepared by suitable dilution of the stock solutions with methanol.

The dried root of *L. chuanxiong* Hort was obtained from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

2.3. Preparation of crude chuanxiongzine from *Ligusticum chuanxiong Hort*

Preparation of crude chuanxiongzine was carried out according to the literature [19]. In brief, the dried roots of *L. chuanxiong* Hort were ground to powder. The powder (700 g) was extracted with 1000 ml ethanol under sonication for 1 h. The mixture was filtered with 0.22 μm film of Type GV (Millipore, USA), and then the residue was repeatedly extracted twice (1000 ml each time). The filtrate was combined, and the extract was evaporated to dryness by rotary vaporization at 42 °C. The residue (12.38 g) was stored in a refrigerator for the subsequent HSCCC separation.

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

In the present study, we selected a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in the solvent mixture of lower phase and upper phase (1:1, v/v) of the solvent system used for separation because the sample was not easily dissolved in either phase. In addition, if the sample was dissolved in the other reagent, retention of the stationary phase would be decreased.

2.5. HSCCC separation procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1000 rpm, while the lower phase (mobile phase) was pumped into the column at a flow-rate of 1.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 10 ml of the sample solution containing 300 mg of the crude chuanxiongzine was injected through the injection valve. The effluent of the column was continuously monitored with a UV–vis detector at 280 nm. Peak fractions were collected according to the elution profile.

2.6. HPLC analysis

The crude sample and each peak fraction obtained by HSCCC were analyzed by HPLC according to the literature [20]. The HPLC system used throughout this study consisted of two Waters 510 pumps (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millenium chromatography data system (Waters). The column used was a reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm, Waters). The mobile phase was methanol–water (60:40, v/v). The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 280 nm. Routine sample calculations were made by comparison of the peak area with that of the standard.

3. Results and discussion

The crude chuanxiongzine obtained from *L. chuanxiong* Hort was analyzed by HPLC, and the chromatogram is shown in Fig. 2. Peak A corresponds to chuanxiongzine. The content of chuanxiongzine was 4.2%.

The selection of the two-phase solvent system is the most important, and is also most difficult step; it is estimated that about 90% of the entire work in HSCCC is spent on that. In order to select a suitable two-phase solvent system for the successful separation of a particular compound from a complex mixture, the previous articles on the HSCCC should be consulted, and some rules need be considered. For example, the target compound should be soluble and stable in the solvent system; the settling time of the solvent system should be short (<30 s); the partition coefficient (K) of the target compound should fall with a suitable range (usually between 0.5 and 2); and the retention of the stationary phase should

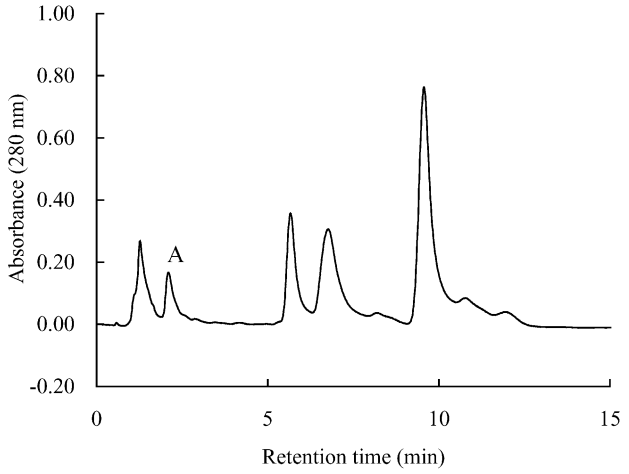

Fig. 2. Chromatogram of crude chuanxiongzine from *Ligusticum chuanxiong* Hort by HPLC analysis, A = chuanxiongzine. Conditions: column, reversed-phase symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); mobile phase, methanol–water (60:40, v/v); flow-rate, 1.0 ml min^{-1}; detection, 280 nm.

be satisfactory [21–25]. The higher the retention of the stationary phase, usually the better the peak resolution. Small *K*-value usually results in a poor resolution, while large *K*-value tends to give better resolution but broader peak and more dilute peak fraction due to a longer elution time. In this paper, *K* was expressed as C_U/C_L, where C_U was the solute concentration in the upper phase and C_L was that of the lower phase. In addition, it would save time and money if a successful solvent system was selected at an analytical level, and then scaled up to the preparative level. However, preliminary HSCCC studies would have to be carried out on preparative HSCCC instrument if there was no analytical HSCCC instrument in the laboratory.

Preliminary HSCCC studies were carried out with the two-phase solvent system composed of *n*-hexane–ethanol–water, which has been successfully used for the separation and purification of tanshinones from the herbal medicine *Salvia miltiorrhiza* Bunge [26]. Firstly, *n*-hexane–ethanol–water (10:5:5, v/v) was tested with a volume ratio of upper phase to lower phase of 8/10. The time to elute chuanxiongzine was about 2 h, but chuanxiongzine could not be separated from other compounds. As shown in Table 1, the *K*-value of chuanxiongzine in the two-phase solvent system was small (0.394). In order to increase the polarity of the mobile phase, *n*-hexane–ethanol–water (10:4:6, v/v) was attempted with a volume ratio of upper phase to lower phase of 9/9.5. Although the *K*-value was increased from 0.394 to 0.509, chuanxiongzine could not be separated from other substances. Then, *n*-hexane–ethanol–water (10:3:7, v/v) was tested with a volume ratio of upper phase to lower phase of 9.6/9.3 because the mobile phase had stronger polarity. The overlap of chromatographic peaks of chuanxiongzine with other compounds was still a question, although the *K*-value was increased to 0.638. Thus, the two-phase solvent system composed of *n*-hexane–ethanol–water at the different volume ratios (10:5:5, 10:4:6 and 10:3:7) was not suitable for the separation of chuanxiongzine from the plant. In the subsequent studies, another two-phase solvent system was thus tested.

Table 1
The *K* (partition coefficient) values of chuanxiongzine in several solvent systems

Solvent system	*K*-value
n-Hexane–ethanol–water (10:5:5)	0.394
n-Hexane–ethanol–water (10:4:6)	0.509
n-Hexane–ethanol–water (10:3:7)	0.638
n-Hexane–ethyl acetate–ethanol–water (6:4:4:6)	1.15
n-Hexane–ethyl acetate–ethanol–water (5:5:3:7)	1.45
n-Hexane–ethyl acetate–ethanol–water (4:6:2:8)	1.78

Notes: The solvent system 5 (from top to bottom) was selected. The peak resolution was poor with the first four solvent systems and the elution time was too long with the last solvent system.

Performance of the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water was evaluated in terms of peak resolution, which was more advantageous than the two-phase solvent system containing organic chloride (such as, chloroform and tetrachloromethane) when environmental and health questions were considered. It was very difficult to separate chuanxiongzine from other components with the two-phase solvent system at a ratio of 6:4:4:6 with a volume ratio of upper phase to lower phase of 9/9.9 (data not shown), although the *K*-value of chuanxiongzine was very suitable (1.15). It should be pointed that the *K*-value of chuanxiongzine in the two-phase solvent system *n*-hexane–ethyl acetate–ethanol–water (6:4:4:6) was larger than that in the two-phase solvent system *n*-hexane–ethanol–water (10:4:6, v/v). That is, the *K*-value would be increased using ethyl acetate instead of part of *n*-hexane when the contents of ethanol and water were kept constant. In addition, the *K*-value would be increased when the content of ethanol was decreased, but water was increased in two-phase solvent system composed of *n*-hexane–ethanol–water (Table 1). Thus, in order to increase *K*-value further, the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:3:7) was tested with a volume ratio of upper phase to lower phase of 8.9/10. The *K*-value was increased from 1.15 to 1.45 (Table 1), the peak resolution was satisfactory, and the retention of the stationary phase was also very good (about 61%). When the two-phase solvent system at a ratio of 4:6:2:8 was also tested with a volume ratio of upper phase to lower phase of 8.7/10.2, it required a much longer elution time (more than 7 h) to elute chuanxiongzine because the *K*-value was increased to 1.78. Fig. 3 shows the preparative HSCCC separation of 300 mg of crude sample using the solvent system *n*-hexane–ethyl acetate–ethanol–water (5:5:3:7, v/v). After chuanxiongzine was eluted, in order to save solvents and time, the remaining compounds in the column were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was not to be reused. HPLC analysis of each peak fraction of this preparative HSCCC revealed that chuanxiongzine corresponding to peak A was over

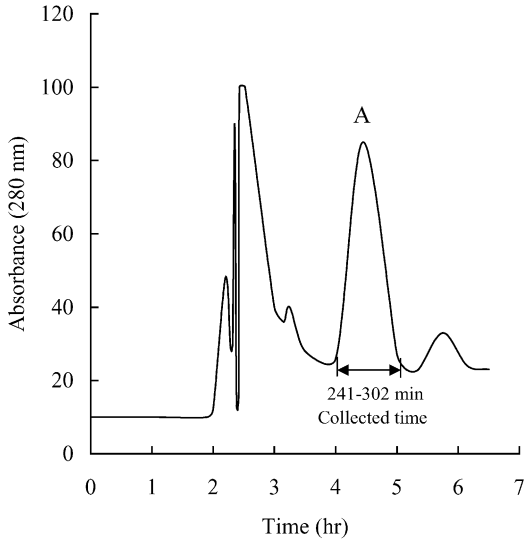

Fig. 3. Chromatogram of crude chuanxiongzine from *Ligusticum chuanxiong* Hort by HSCCC separation, A = chuanxiongzine. Conditions: column, multilayer coil of 2.6 mm i.d. PTFE tube with a total capacity of 325 ml; rotary speed, 1000 rpm; solvent system, *n*-hexane–ethyl acetate–ethanol–water (5:5:3:7, v/v); mobile phase, lower phase (water phase); flow-rate, 1 ml min^{-1}; detection, 280 nm; sample size, 300 mg; retention of the stationary phase, 61%.

96.8% pure. The yield of chuanxiongzine was 11.5 mg. The HPLC chromatogram of chuanxiongzine as purified from the preparative HSCCC is shown in Fig. 4. The elevated baseline components in Fig. 3 under peak A were not shown in Fig. 4 because peak A was cut at the sites far away from the baseline. This was why the recovery was 91% rather than 100%. Furthermore, the parts of the baseline components collected in the product were too small to be seen in Fig. 4, but the impurity could be shown from the purity (96.8%) of the product determined.

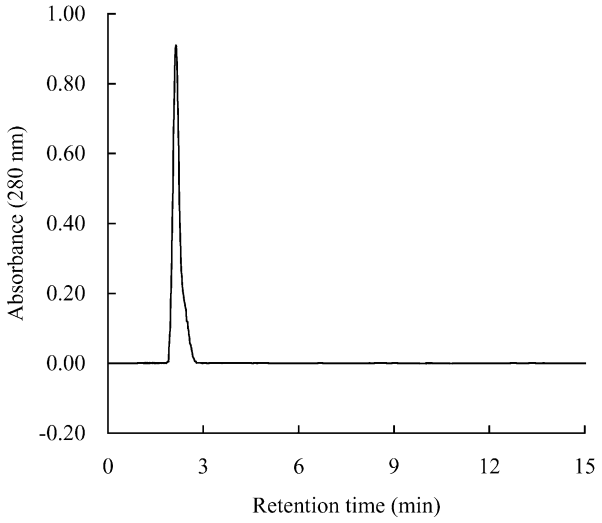

Fig. 4. HPLC chromatogram of chuanxiongzine purified from *Ligusticum chuanxiong* Hort by HSCCC. Conditions: column, reversed-phase symmetry C_{18} column (150 mm × 3.9 mm I.D., 5 μm); mobile phase, methanol–water (60:40, v/v); flow-rate, 1.0 ml min^{-1}; detection, 280 nm.

The *K*-values of chuanxiongzine in several solvent systems were measured according to the literature [22], and are given in Table 1.

In the previous method [27], sixteen steps were needed for the separation and purification of chuanxiongzine from dried roots of the medicinal plant *L. chuanxiong* Hort, such as, extraction with ethanol, evaporation, extraction with ether, back-extraction with diluted H_2SO_4, extraction with $CHCl_3$, column chromatography filled in Al_2O_3, evaporation, and sublimation. Thus, the present method was simpler and cheaper than the previous method [27].

In conclusion, HPCCC was successfully used for the isolation and purification of chuanxiongzine from the medicinal plant *L. chuanxiong* Hort, and yielded 11.5 mg chuanxiongzine at 96.8% purity from 300 mg of the crude extract with the recovery of 91% in a one-step separation.

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