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Dynamic pH junction high-speed counter-current chromatography coupled with microwave-assisted extraction for online separation and purification of alkaloids from *Stephania cepharantha*

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ABSTRACT

A simple and efficient dynamic pH junction high-speed counter-current chromatography method was developed and further applied to the online extraction, separation and purification of alkaloids from *Stephania cepharantha* by coupling with microwave-assisted extraction. Mineral acid and organic base were added into the mobile phase and the sample solution, respectively, leading to the formation of a dynamic pH junction in the column and causing focus of alkaloids. Selective focus of analytes can be achieved on the basis of velocity changes of the pH junction through appropriate selection of solvent systems and optimization of additive concentrations. The extract can be directly introduced into the HSCCC for the online extraction, separation and purification of alkaloids from *S. cepharantha*. Continuous separation can be easily achieved with the same solvent system. Under the optimum conditions, 6.0 g original sample was extracted with 60 mL of the upper phase of hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v) containing 10% triethylamine under 50 °C and 400 W irradiation power for 10 min, the extracts were directly separated and purified by high-speed counter-current chromatography. A total of 5.7 mg sinomenine, 8.3 mg 6,7-di-O-acetylsinococuline, 17.9 mg berbamine, 12.7 mg isotetrandrine and 14.6 mg cepharanthine were obtained with purities of 96.7%, 93.7%, 98.7%, 97.3% and 99.3%, respectively. The online method provides good selectivity to ionizable compounds and improves the separation and purification efficiency of the high-speed counter-current chromatography technique. It has good potential for separation and purification of effective compounds from natural products.

1. Introduction

High-speed counter-current chromatography (HSCCC) is a liquid chromatography technique that uses liquid stationary phase. Biphasic liquid systems are used to separate solutes. The big advantage of a liquid stationary phase is that the solutes have access to the whole volume of the phase. HSCCC is useful for the separation and purification of target compounds from many fields such as natural products [1–3]. The conventional HSCCC separation is solely based on the difference in the partition coefficients of solutes between the two phases of a solvent system. Generally, a systematic search for the two-phase solvent systems for HSCCC is focused on the hydrophobicity of the solvent system for providing a proper range of partition coefficients of solutes. For the separation of ionizable solutes such as alkaloids, however, it is difficult to obtain a satisfactory two-phase solvent system using conventional HSCCC method due to the solubility limitations in common solvents [4] and an additional adjustment is required with respect to the pH and ionic strength of the solvent system. Historically, halogen-containing organic solvents, such as chloroform and dichloromethane, have been widely used for the separation of alkaloids by HSCCC because of their relatively strong proton-donor character provides better solubility for alkaloids [5], and yet chloroform is considered to be an environmentally unfriendly chemical, its use in routine applications may contribute to health and environmental hazards.

Regarding the ionization state, the pH-related HSCCC techniques such as pH-modulated stepwise elution [6,7], pH-gradient elution [8–10] were established to separate ionic compounds. These techniques offer various advantages over conventional HSCCC methods, such as higher partition efficiency, larger sample capacity and less separation time. The pH-zone-refining HSCCC was firstly developed for the large-scale separation of ionizable compounds, including alkaloids and organic acids in the 1990s by Ito [11]. It elutes the solutes according to their pK_a values and hydrophobicity, and produces a succession of concentrated rectangular solute peaks while impurities are concentrated at the peak boundaries. Moreover, the sample solution was loaded immediately after filling the column with the stationary phase without pre-equilibrating the column, which can shorten the whole

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operation time and increase the retention of stationary phase [7]. This method has been successfully applied to the separation of natural and synthetic products including acidic and basic derivatives of amino acids, oligopeptides, hydroxyxanthene dye, alkaloids, indole auxin, and structural, geometrical and optical isomers, and so on [12,13]. Dynamic pH junction method is a velocity-difference induced focusing method in capillary electrophoresis (CE) and the concept relies on a substantial difference in mobility of the analytes at different pHs [14,15]. A difference in the pH between the sample and electrolyte can be applied to stacking ionizable components at the interface. For example, a weak acid is protonated at low pH and ionized at high pH and therefore it is possible to concentrate the weak acids on the dynamic pH junction that moves through the sample by using an acidic sample matrix [16,17]. When using a biphasic liquid system in HSCCC, the partition coefficient of solutes is a vital parameter that significantly affects the elution velocity and thus the purification efficiency of analytes. The partition coefficient and the elution velocity of an ionizable compound, such as alkaloid, is obviously different in acidic and in basic solvent system. And then, the moving rate of ionizable compounds and the dynamic pH junction between acid and base in the solvent system was also different and it can be used for the selective focusing of ionizable compounds. As a result, high-speed counter current chromatography based on dynamic pH junction would be benefit for the separation and purification of alkaloids in natural products.

A self-designed online coupling of microwave-assisted extraction (MAE) and HSCCC system was used to study the dynamic pH junction HSCCC method. Microcomputer and sample injector valve assembly with electric motor were self-constructed to facilitate fully automatic sample injections. Our previous research has demonstrated that the system composed of extraction, concentration and separation modules was an efficient preparative apparatus for consecutive separation and purification [18]. However, the concentration step was usually very time-consuming, which might result in a longer operation time.

The present work developed a dynamic pH junction high-speed counter current chromatography, which have good sample capacity and easy to conduct consecutive separation. The analytes can also be selective focused on the basis of velocity changes of the pH junction. Moreover, a simple and efficient online method based on MAE and dynamic pH junction HSCCC for the extraction, separation and purification of analytes from original natural plants was developed. *Stephania cepharantha* was selected as testing materials to evaluate the dynamic pH junction HSCCC and the online method. It has been widely used in traditional Chinese medicines for the treatment of parotiditis, gastric ulcer and leucopenia [19]. The alkaloids in *S. cepharantha* have antiproliferative and proapoptotic effects against a diverse range of tumors both in vitro and in vivo [20–22]. Three primary alkaloids in *S. cepharantha*, berbamine (BBM), isotetrandrine (ITD) and cepharanthine (CEP), were selected as models to study the influence of pH on the chromatographic retention behavior of basic analytes in the dynamic pH junction HSCCC.

2. Experimental

2.1. Apparatus

MAE experiments were performed on a MAS-II microwave oven (2450 MHz, Sineo, Shanghai, China) consisting of continuous microwave non-pulsed power supply, advanced IR temperature sensor and uniform temperature throughout the cavity.

A J-type HSCCC instrument (Emilion, Beijing, China) equipped with a 100 m multilayer coil (1.6 mm I.D.) with a total capacity of 240 mL was utilized for HSCCC. The β values of this preparative column varied from 0.8 at the head terminal to 0.5 at the tail terminal ($\beta = r/R$, where r is the spool radius and R is the rotor radius). The HSCCC system was equipped with a Model NS-1007 constant-flow pump (UE Biotech., Beijing, China), a UV-3000 detector (ChuangXinTongHeng, Beijing, China). HW-2000 chromatography data software (Qianpu, Shanghai, China) was employed to carry out data acquisition.

HPLC analysis was performed on a LC-2010C system (Shimadzu, Kyoto, Japan) with a SCL system controller, a low pressure gradient solvent pump, an auto-sampler, a column oven, a UV–vis detector, and a CLASS-VP software. Mass spectroscopy (MS) identification was performed on a Shimadzu LC/MS 2010A system with a single quadruple MS with ESI probe (Q-array-Octapole-Quadruple mass analyzer). The NMR data were obtained on a Mercury Plus 300 spectrometer (Varian, USA).

2.2. Reagents and materials

All chemical solvents used were of analytical grade (Guangzhou Chemical Reagent Factory, Guangdong, China) and distilled water was used. Chromatographic grade methanol and acetonitrile used for HPLC analysis were purchased from Merck (Darmstadt, Germany) and Lab-scan (Bangkok, Thailand), respectively. *S. cepharantha* was purchased from Qingping traditional Chinese medicine market in Guangzhou (Guangdong, China). Samples were all dried at 45 °C for 24 h and ground into powder before extraction.

2.3. Dynamic pH junction HSCCC procedure

The two-phase solvent system of HSCCC was selected according to the partition coefficient K of each target compounds determined by HPLC. The partition coefficient of each alkaloids were obtained in high pH matrix so that the compounds were in their molecular form and the K-value was calculated by peak area obtained from the upper phase to that obtained from the lower phase.

The selected two-phase solvent system composed of hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v) was thoroughly equilibrated in a separation funnel at room temperature, it was separated and degassed by ultrasonic bath for 30 min before use. Hydrochloric acid (HCl) of 5.0 mmol/L was solely added into the lower phase. The column was first filled with the stationary phase (upper phase of the solvent system) in the absence of triethylamine (TEA) and pre-equilibrated with the acidic mobile phase, and then the sample solution with triethylamine was introduced into the solvent system.

2.4. Microwave-assisted extraction coupling with HSCCC for online separation and purification

Microwave-assisted extraction was used to prepare crude extract from *S. cepharantha*. An online coupling system as described in our previous work [18] was employed for the online separation and purification procedure. All the MAE extracts were directly used as HSCCC sample solution and the concentration module was not used in the present study. The modules in the system were connected by some peristaltic pumps (Jieheng, Chongqing, China) and polytetrafluoro ethylene tube (3.0 mm ID × 5.0 mm OD) (Longer, Baoding, China). The microcomputer (89E516RT, SST, China) was used to automatically control all pumps and valves in the online system. 3.0 g sample was extracted with 30 mL upper phase of hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v) containing 3.0 mL TEA at 50 °C for 10 min, the power of microwave irradiation was 400 W. After the extraction was completed, the extract was directly introduced into the HSCCC system, which was operated in head-to-tail mode using the upper phase as stationary phase. The rotation speed of the multilayer-coiled column was set at 800 rpm and the flow rate of mobile phase was 1.5 mL/min. Chromatograms were recorded at 282 nm and the fractions were