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On-line coupling of dynamic microwave-assisted extraction with high-speed counter-current chromatography for continuous isolation of nevadensin from *Lyeicnotus pauciflorus* Maxim.

Xing Tong, Xiaohua Xiao, Gongke Li *

School of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou 510275, China

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ABSTRACT

An on-line method based upon dynamic microwave-assisted extraction (DMAE) coupled with high-speed counter-current chromatography (HSCCC) was developed for continuous isolation of nevadensin from *Lyeicnotus pauciflorus* Maxim. The DMAE parameters were optimized by means of the Box–Behnken design. The maximum extraction yield was achieved using 30:1 ml/g of liquid–solid ratio, 10 ml/min of solvent flow rate and 200 W of microwave power. The crude extracts were then separated by HSCCC with a two-phase solvent system composed of n-hexane–ethyl acetate–methanol–water (7:3:5:5, v/v/v/v). 13.0 mg of nevadensin was isolated from 15.0 g original sample by HSCCC with five times sample injection in 12 h, and the isolation yield of nevadensin was 0.87 mg/g. The average purity of nevadensin was higher than 98.0%. The chemical structure of collected fraction was identified by HPLC, ESI-MS and ^{1}H NMR. The results indicated that this on-line method was effective and fast for high-throughput isolation of nevadensin from *L. pauciflorus* Maxim.

1. Introduction

Lysionotus pauciflorus Maxim. (Chinese name: Shidiaolan) was species of Gesneriaceae genus of Lysionotus, and it was an important crude herb used in traditional Chinese medicines (TCMs). It has been proven to be effective in the treatment of lymph node tuberculosis, cough with tachypnoea and rheumatic pains [1,2]. Nevadensin (5,7-dihydroxy-6,8,4′-trimethoxyflavone, Fig. 1) with good antibacterial, anti-inflammatory, antihypertensive and free radical-scavenging activities effects [3–5] was the major active constituent of *L. pauciflorus* Maxim. Therefore a high-throughput method for the isolation of nevadensin was required.

Several methods have been developed to analyze nevadensin in *L. pauciflorus* Maxim. [3–5]. In these studies, maceration at room temperature [4,5], heating reflux extraction [6] and ultrasonic-assisted extraction [7] were used for extraction of nevadensin from *L. pauciflorus* Maxim. Those methods were time-consuming and low efficient. Microwave assisted extraction (MAE), which could provide good extraction efficiency and large treating amount, was used as an alternative method for the effective extraction of various natural products [8–10]. MAE could be performed by either static or dynamic mode. In the static MAE, the sample was extracted in a closed-vessel system, and the recovery of targeted component mainly relied on its rate constant of desorption in the extraction solvent [11]. In recent years, the dynamic MAE (DMAE) was gradually developed, and it could continuously supply the fresh extraction solvent to the extraction vessel [12–18]. Moreover, the DMAE suggested a possibility of the automation in the sample isolation step [19].

Although DMAE extraction method was fast and had a higher extraction yield, the next separation processes, such as silica gel, polyamide and preparative HPLC, were tedious, time-consuming, and required multiple chromatographic steps [20]. As a support-free liquid–liquid partition chromatographic technique, HSCCC has been widely used to separate natural products from medicinal plants. Compared with traditional separation methods, HSCCC eliminated the complications coming from the solid support matrix, such as irreversible adsorptive sample loss, deactivation, tailing of solute peaks and contamination [21–23]. Recently, the supercritical fluid extraction (SFE) [24–26] and MAE [8–10] have been used as independent pretreatments on the extraction of target compound before it was separated by HSCCC. However, there were no reports on the isolation of target compounds by on-line coupling DMAE with HSCCC.

The main purpose of this study was to develop an on-line method to improve and simplify the isolation of nevadensin from *L. pauciflorus* Maxim. by coupling DMAE with HSCCC. The purity of obtained nevadensin was determined with HPLC and its chemical structure was identified with ESI-MS and ^{1}H NMR.

* Corresponding author. Tel.: +86 20 84110922; fax: +86 20 84115107.
E-mail address: cesgkl@mail.sysu.edu.cn (G. Li).

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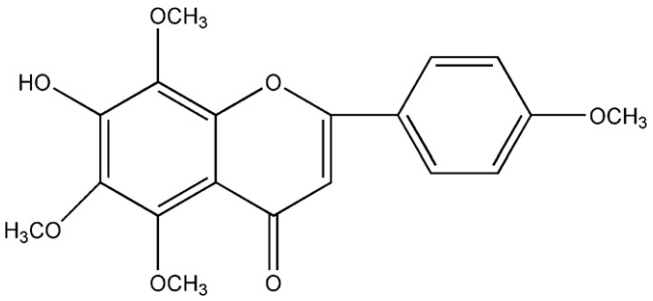

Fig. 1. The chemical structure of nevadensin.

2. Experimental

2.1. Chemicals and samples

Standard nevadensin was purchased from Guangdong Institute for Drug Control (Guangzhou, China). Dryness *L. pauciflorus* Maxim. was purchased from the Caizhiling medicinal material emporium in Guangzhou (Guangdong, China). Chromatography grade acetonitrile was purchased from Merck (Darmstadt, Germany). All the other organic solvents of analytical grade were purchased from Guangzhou Chemical Factory (Guangdong, China). Stock solution of the standard (52 μg/ml) was prepared by dissolving nevadensin in acetonitrile. They were stored in a refrigerator at 4 °C.

2.2. Apparatus

The on-line DMAE-HSCCC system composed of extraction, concentration and separation module was self-designed and constructed by our laboratory (Sun Yat-sen University, Guangzhou, China). The schematic diagram was show in Fig. 2. The three modules were connected by some peristaltic pumps (pump 1–5 in Fig. 2, 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 on-line system.

The extraction module mainly performed on a MAS-II microwave oven from Sineo Microwave Chemistry Technology Company (Shanghai, China) with a frequency magnetron of 2450 MHz and a maximum output power of 1000 W. It was an open system equipped with a temperature and power feedback control. The temperature was monitored by an infrared probe inside the microwave oven.

The concentration module with an auto concentrator and a temperature transducer was constructed by our laboratory, and included an AutoScience AP-02B vacuum pump (Tianjin, China) and an IKA basic-2 magnetic force stirrer (Deutschland, Germany).

The HSCCC used in the separation module was a GS10A (Beijing UE Biotech., Beijing, China) equipped with PTFE multilayer coil (110 m × 1.6 mm ID, 224 ml). A manual sample injection valve (valve 1 in Fig. 2) with sample loop equipped in GS10A was modified to be an automatic sample injection valve by adding electric motor (TH37JB555, Tianheng Company, China), and it used to introduce the sample into the coil automatically. Furthermore, the solvent was pumped into the column with a Model NS-1007 constant-flow pump (pump 6 in Fig. 2, Beijing UE Biotech., Beijing, China). The out flow of HSCCC was detected by an 8823A-UV Monitor (Beijing UE Biotech., Beijing, China) at 280 nm and recorded by a HW-2000 chromatography workstation (Shanghai, China), then collected by a BSZ-100 fraction collector (Shanghai, China).

2.3. On-line coupling of DMAE with HSCCC

5 g samples of *L. pauciflorus* Maxim. were accurately weighted and then put into the extraction vessel. 50 ml methanol was pumped into the vessel first for immersion of the samples. Then the microwave oven was started with the power of 200 W. At the same time, the pump 1 and the pump 2 were simultaneous activated and the extraction solvent (methanol 150 ml) was passed through extraction vessel with a flow rate of 2.0 ml/min. The extraction was completed when the 200 ml extraction solvent all pumped into the auto concentrator. Then 5 g fresh original samples were put into extraction vessel for the next time extraction.

When the 600 ml of extraction solvents was condensed to dry extracts at 50 °C in auto concentrator, the dry extracts were dissolved with the 50 ml lower phase of the HSCCC solvent system of hexane–ethyl acetate–methanol–water (7:3:5:5, v/v/v/v) which pumped from the mobile phase bottle 1 by pump 3. The most of dry extracts dissolved in the lower phase were transferred to sample bottle for storage and then introduced into sample loop of automatic sample injection valve. When the sample injection was complete, the pump 6 started to run and the samples in sample loop were introduced into the HSCCC column by switching valve 1. After 20 min, the samples were completely pushed into the HSCCC column and the valve 1 was switched back to initial state for getting ready for next sample injection. The effluent from HSCCC was monitored at 280 nm. After HSCCC separation, the collected fractions were analyzed by HPLC.

Briefly, the isolation yield of nevadensin was defined as following:

$$\text{Isolation yield } (\%) = \frac{\text{Mass of nevadensin obtained in isolation}}{\text{Mass of the original samples}} \times 100\%$$

2.4. The selection of the solvent system

The selection of the solvent system for separation of the target compounds was the most important step in HSCCC. The solvent system for HSCCC separation was selected according to the difference of partition coefficients (K) of each target compound between the two-phase systems. The K value was determined as follows: two-phase solvent systems with different ratios of organic solvent and water were prepared. Upper and lower phases (2 ml each) were placed in test tubes and 1 mg dry extracts added. Each test tube was shaken for 1 min and then left for the phases to separate. 500 μl of each phase was removed and concentrated to dryness. The residue was re-dissolved in 1 ml methanol and 10 μl analyzed by HPLC with UV detection at 280 nm. The K value was defined as the concentration of nevadensin in the upper phase (C_U) divided by the concentration of nevadensin in the lower phase (C_L) at the same retention time in the HPLC chromatogram, that was $K = C_U/C_L$. Furthermore, a successful separation in HSCCC largely depended on the separation factor α, which was defined as $\alpha = K_{\text{nevadensin}}/K_{\text{impurity}}$.

The solvent systems of HSCCC were prepared with the appropriate solvent volumes. Each solvent mixture was thoroughly equilibrated in a separation funnel for more than 12 h at room temperature.

2.5. HPLC analysis and identification of HSCCC fraction

A Shimadzu LC-2010 system (Tokyo, Japan) with UV detector was used for the analysis of the extracts of *L. pauciflorus* Maxim. The fractions and the collected nevadensin were filtrated through a 0.45 μm micro porous membrane. Chromatographic separation was performed on a Diamonsil C18 column (200 mm × 4.6 mm ID, 5 μm) equipped with an EasyGuard C18 guard column (10 mm × 4.6 mm ID) at 25 °C. The conditions of HPLC analy-