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Short communication

Preparative isolation and purification of triterpene saponins from *Clematis mandshurica* by high-speed counter-current chromatography coupled with evaporative light scattering detection

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Abstract

Preparative high-speed counter-current chromatography (HSCCC) coupled with evaporative light scattering detection (ELSD) was successfully applied to the isolation and purification of four triterpene saponins from *Clematis mandshurica* by one-step separation. The solvent system was composed of ethyl acetate–*n*-butanol–ethanol–0.05% TFA (5:10:2:20, v/v). The purities of all the saponins obtained were better than 97%. The structures of the compounds were elucidated by the means of spectroscopic methods.

Keywords: *Clematis mandshurica* Rupr.; High-speed counter-current chromatography; Triterpene saponins

1. Introduction

Clematis mandshurica Rupr., the roots and rhizomes of the plant named "Weilingxian" in traditional Chinese medicine (TCM), is widely distributed in the northeast of China. It is commonly used as an anti-inflammatory, antitumor, and analgesic agent in TCM. Our preliminary pharmacological studies suggested that the total saponins prepared from the 50% ethanol extract of the roots and rhizomes of *C. mandshurica* showed significant inhibitory activity against cyclooxygenase–2 (COX–2). Chromatographic separation of the saponin fraction has resulted in the isolation of seven triterpene saponins [1]. But all the saponins isolated from *C. mandshurica* contain 7–9 mononsaccharides, which make them difficult to be purified by conventional column chromatography. It usually needs repeated chromatographic steps on a silica gel or other columns, which is tedious and time consuming. Most of all, the overall yield of this method is always poor because of the serious irreversible adsorption.

Compared to the conventional column chromatography, HSCCC is a support-free liquid–liquid partition chromatographic technology, which eliminates the irreversible adsorption of the sample onto solid support and has an excellent sample recovery. So it is gaining increasing interest recently, and is used more and more frequently in the isolation of bioactive components from crude materials. A literature investigation revealed that HSCCC equipments are always coupled with an UV detection, which is of benefit to the isolation of the components with UV absorptions including flavonoids, coumarins, lignans etc., while the isolation of the components without UV absorption using HSCCC are very sparse. In the previous investigation, we have successfully isolated many phenolic components from crude materials by HSCCC coupled an UV detection [2–7]. Herein, we report a successful method to isolate triterpene saponins from the roots and rhizomes of *C. mandshurica* using HSCCC coupled with ELSD.

2. Experimental

2.1. Apparatus

Preparative HSCCC was performed using a model GS10A2 multilayer coil of 110 m long, 1.6 mm I.D. polytetrafluoroethylene (PTFE) column, and a model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing,

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Table 1
The *K*-values of compounds **1**–**4** in different two-phase solvent systems used in HSCCC.

Solvent system	K_1	K_2	K_3	K_4
Ethyl acetate–*n*-butanol–methanol–water (10:10:2:20, v/v)	0.25	0.27	0.39	0.45
Ethyl acetate–*n*-butanol–ethanol–water (10:10:2:20, v/v)	0.22	0.23	0.38	0.42
Ethyl acetate–*n*-butanol–ethanol–water (5:10:2:20, v/v)	0.46	0.51	0.68	0.72
Ethyl acetate–*n*-butanol–ethanol-0.05% TFA (5:10:2:20, v/v)	0.52	0.58	0.70	0.73

K_1, K_2, K_3, and K_4 represent the *K*-values of compounds **1**, **2**, **3** and **4**, respectively. All the *K*-values were measured by HPLC.

China) with a 20 ml injection valve (Tianjin High New Science technology Company, Tianjin, China). Continuous monitoring of the effluent was achieved with an Alltech 2000 evaporative light scattering detection (nebulizer gas flow: 3.0 ml/min, probe temperature: 105 °C, split ratio of the splitter: 25:1). The high-performance liquid chromatography (HPLC) equipment used was a Waters Millennium^{32} system consisting of an Alltech 2000 evaporative light scattering detection and a Waters Delta 600 pump (Waters, Milford, MA, USA).

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Acetonitrile used for HPLC analysis was of chromatographic grade. The roots and rhizomes of *C. mandshurica* Rupr. were collected in Heilongjiang Province, China. The plant was identified by Professor Pengfei Tu (School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing, China). A voucher specimen was kept in the herbarium of Peking University Modern Research Center for Traditional Chinese Medicine (CM200208).

2.3. Preparation of sample solution

The dried roots and rhizomes of *C. mandshurica* (200.0 g) were pulverized into the powder and extracted with 50% ethanol three times (2 h, 1 h and 1 h, respectively) at 80 °C. The extract was combined and evaporated at 60 °C in vacuum to give a dry residue (15.6 g). The residue was defatted with acetone, and yielded the total saponins (12.8 g), 1.5 g of which was subjected to HSCCC. Sample solution for HSCCC was prepared by dissolving the residue in the lower phase solvent at a concentration of 50 mg/ml.

2.4. Preparation of the two-phase solvent system

The solvent systems utilized in the present study were prepared by mixing ethyl acetate–*n*-butanol–ethanol–0.05% TFA (5:10:2:20, v/v), and thoroughly equilibrating in a separatory funnel at room temperature. The two phases were separated shortly before use.

2.5. Separation procedure

HSCCC was performed as follows. The multilayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column at a flow-rate of 2.0 ml/min, while the apparatus was run at 900 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, 30 ml of sample solution (50 mg/ml) was injected using an injection valve in 10 times run. The effluent from the tail end of the column was continuously monitored with an ELSD. Peak fractions were manually collected according to the chromatogram.

2.6. HPLC analysis

The crude 50% ethanol extract from the roots and rhizomes of *C. mandshurica* and peak fractions from HSCCC were ana-

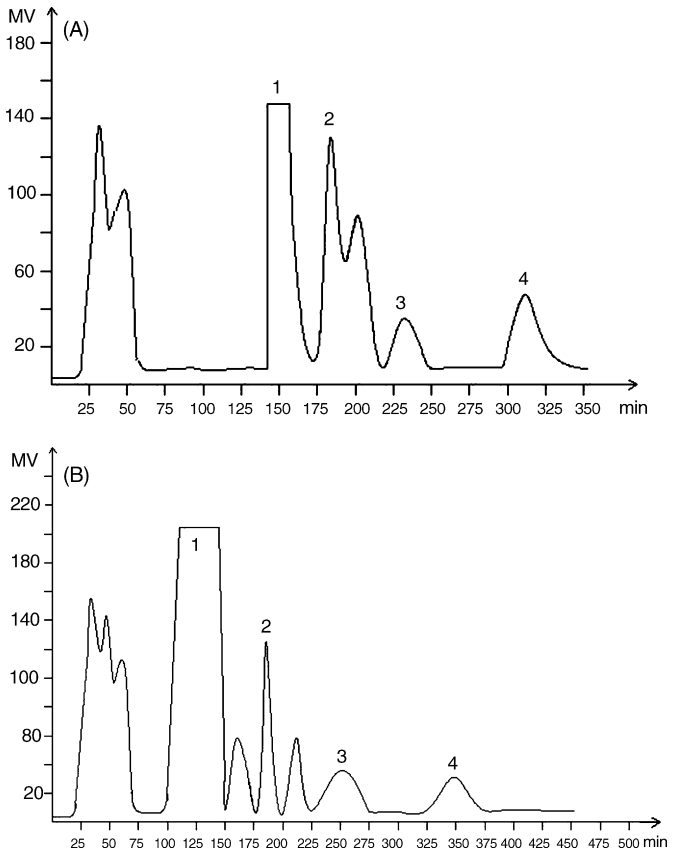

Fig. 1. HSCCC chromatogram of total saponins prepared from *C. mandshurica*. Solvent system: (A) ethyl acetate–*n*-butanol–ethanol–water (5:10:2:20, v/v); (B) ethyl acetate–*n*-butanol–ethanol–0.05% TFA (5:10:2:20, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml min^{-1}; revolution speed: 900 rpm; sample: 150 mg dissolved in 3 ml lower phase; retention of the stationary phase: 45%. Peaks 1–4 represent compounds **1**–**4**, respectively.

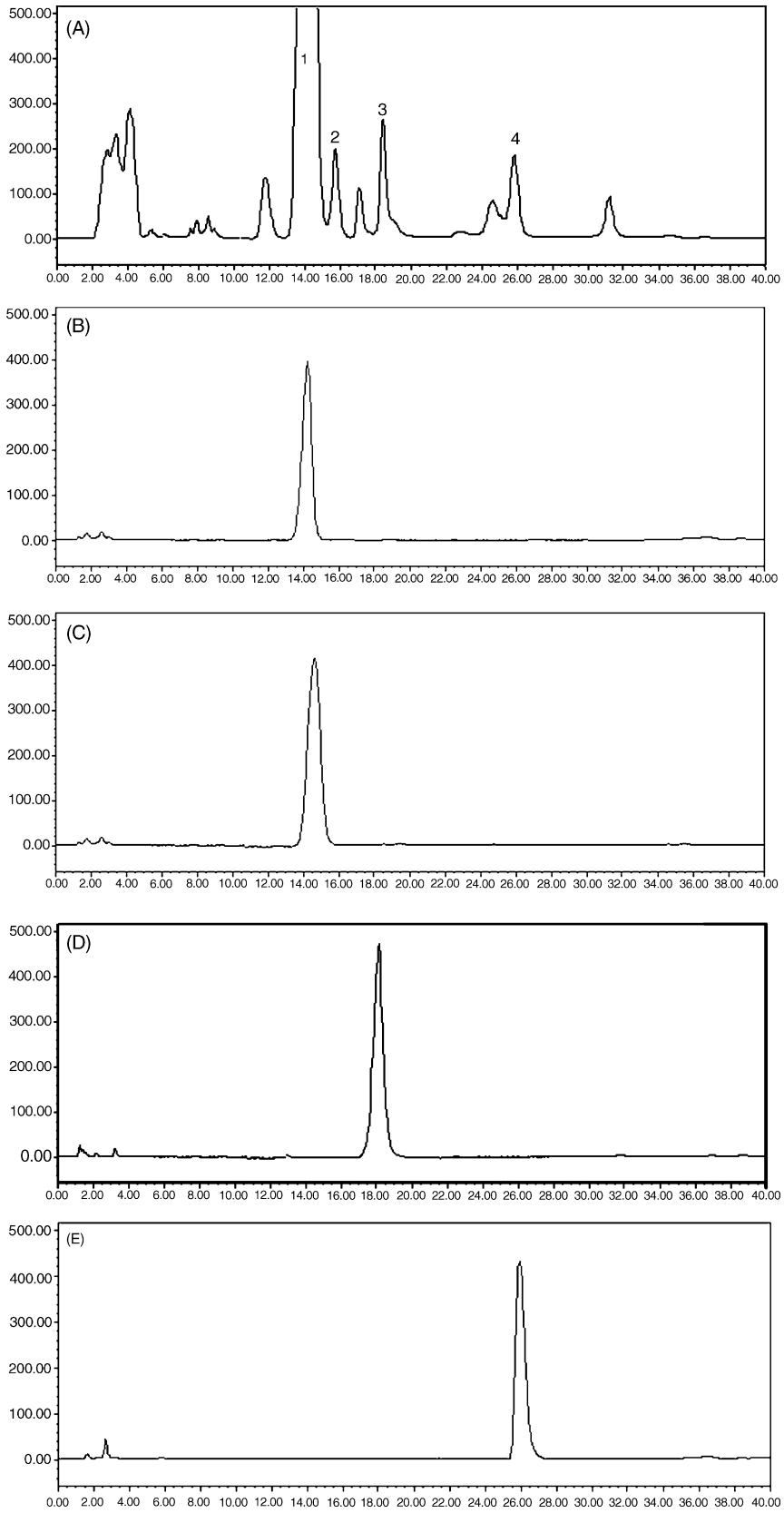

Fig. 2. (A): HPLC analysis of total extract from the roots and rhizomes of *C. mandshurica*; (B), (C), (D) and (E): HPLC analysis of the target compounds; column: Alltech C18 (5 μm, 250 mm × 4.6 mm I.D.); mobile phase: acetonitrile–0.05% TFA solution (31:69, v/v); flow-rate: 1.0 ml min^{-1}.

lyzed by HPLC. The analyses were performed with an Alltech C18 column (250 mm × 4.6 mm I.D., 5 μm) at room temperature. The mobile phase composed of acetonitrile–0.05% TFA solution (31:69, v/v) was eluted at a flow rate of 1.0 ml/min, and the effluent was continuously monitored by an ELSD. The concentration of the target compounds **1**–**4** was estimated by the peak area percent.

3. Results and discussion

3.1. Selection of the extraction process

The roots and rhizomes of *C. mandshurica* possess rich sources of triterpene saponins, and these saponins can be perfectly extracted by 50% ethanol. But the 50% ethanol extracts contained lots of fat soluble components except these triterpene saponins. In order to remove the fat soluble components and get a comparatively pure total saponins, the 50% ethanol extracts was defatted with acetone (see Section 2.3). As a result, four saponins, with a considerable amount, were isolated from 1.5 g of the total saponins by HSCCC.

3.2. Selection of the two-phase solvent system

To achieve a successful separation using HSCCC, the two-phase solvent must completely laminate in a short time, and the target compounds must have an appropriate partition coefficient (*K*) between the two phases. Saponins in the roots and rhizomes of *C. mandshurica* containing more, comparatively, monosacchrides, which make these saponins to have a comparatively larger polarity and a satisfactory solubility in hydrophilic solvents. In order to make the target compounds have a perfect partition between the two phases, both of the phases must have a certain hydrophilicity. So the following solvent systems have been scanned: (1) *n*-butanol–water, (2) *n*-butanol–ethanol–water, (3) *n*-butanol–methanol–water, each at various volume ratios. Unfortunately, all the systems have a poor reservation in the column of HSCCC. For the purpose of shorting the lamination time and enhancing the reservation of the stationary phase in the column, ethyl acetate was added into the systems mentioned above. Finally, the system of ethyl acetate–*n*-butanol–ethanol–water (5:10:2:20, v/v) was picked out and practiced on HSCCC apparatus, but the purities of compounds **1** and **2** afforded under the condition were 80 and 75%, respectively. So the solvent system was optimized which resulted in a perfect separation for compounds **1** and **2** using the system of ethyl acetate–*n*-butanol–ethanol–0.05% TFA (5:10:2:20, v/v). The *K*-values of compounds **1**–**4** in different two-phase solvent systems are presented in Table 1. Fig. 1A and B show the preparative HSCCC separation of the total extraction using the two different solvent systems mentioned above. This separation resulted in the isolation of four saponins: **1** (22 mg), **2** (8 mg), **3** (12 mg) and **4** (16 mg), which were having purities over 97% according to HPLC analysis. The chromatograms of HPLC analysis are shown in Fig. 2B–E, respectively.

3.3. Selection of the detection

In previous investigations, the compounds without UV absorption were always analyzed by TLC [8]. Only few literatures reported the method of HSCCC coupling with ELSD [9,10]. Saponins in the *C. mandshurica* are oleanic-type triterpene saponins and only have absorption at 210 nm, so it cannot be satisfactorily detected by an UV detection. We have attempted to use a differential refractive index detection (RID), but it was with poor sensitivity. So, ELSD detection was selected and a satisfactory result was achieved.

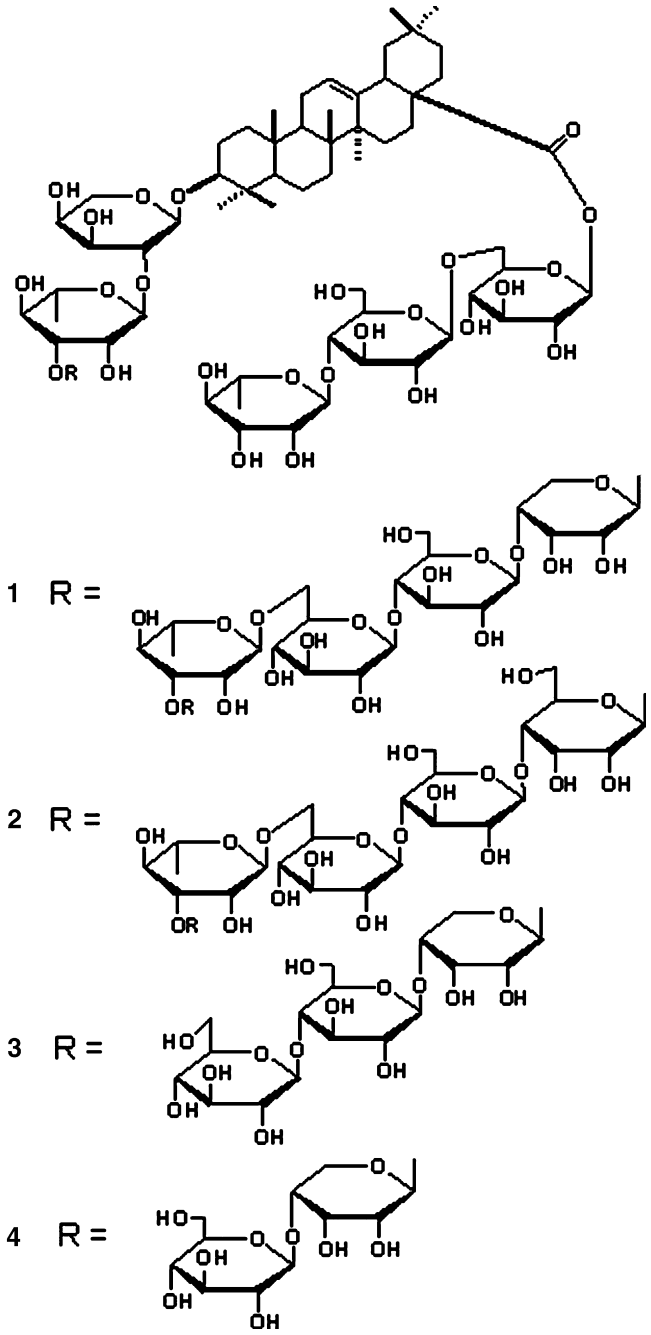

Fig. 3. Structures of compounds **1**–**4** from *C. mandshurica*.

3.4. Identification of compounds *1–4*

Compounds **1–4** (Fig. 3) were all obtained as a white amorphous powder, with a positive reaction when were dealt with the Liebermann–Burchard regents. Their structures were identified by comparison of their spectroscopic values with those of reported in literatures [1,11,12], confirmed by chemical hydrolysis and the comparison with authentic samples on HPLC.

4. Conclusions

The study indicated that HSCCC coupled with ELSD was successfully used in the isolation and purification of triterpene saponins from *C. mandshurica*. It also demonstrated that HSCCC coupled with ELSD is an effective method to isolate components without UV absorptions from natural products.

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