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Separation and Purification of α-Cyperone from *Cyperus rotundus* with Supercritical Fluid Extraction and High-Speed Counter-Current Chromatography

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Abstract: α-Cyperone was separated and purified for the first time from essential oil of the traditional Chinese medicinal plant *Cyperus rotundus* L. by high-speed counter-current chromatography (HSCCC). Essential oil was obtained by extraction with supercritical carbon dioxide under the pressure of 20 MPa and temperature of 40°C. The separation was performed in one step with a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:0.2:1.1:0.2, *v*/*v*), in which the lower phase was used as the mobile phase at a flow-rate of 2.0 ml/min in the head-to-tail elution mode. A total of 60 mg α-cyperone at 98.8% purity was yielded from 0.9 g essential oil as determined by HPLC analysis. The structure of the target compound was performed by electron impact ionization mass spectrometry (EI-MS) and further conformed by comparison with an authentic sample (National Institute of the Control of Pharmaceutical and Biological Products, Beijing, China).

Keywords: Counter-current chromatography, α-Cyperone, *Cyperus rotundus*, preparative chromatography

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INTRODUCTION

Rhizoma Cyperi (Xiangfu in Chinese), the dried rhizome of *Cyperus rotundus* L. (family *Cyperaceae*), is one of the popular Chinese medicines and is officially listed in the Chinese Pharmacopoeia (1). It has been widely used for smoothing liver, removing stagnation of qi, regulating menstruation, and relieving pain (1). The essential oil is considered as an effective part of *Cyperus rotundus* L. and it is suggested that α-cyperone (Fig. 1) is selected as one of the major bioactive constituents for control of quality of *Cyperus rotundus* L. and its products (2). Reports show that α-cyperone is an important bioactive compound, having many pharmaceutical actions such as antimalarial activity, antimicrobial activity, emmenagogue, and so on (2–5). Further studies on pharmacological and clinical effects of α-cyperone necessitate the development of an efficient preparative separation method of this drug. However, the preparative separation and purification of α-cyperone from other constituents of the plant by classical methods are tedious, requiring multiple chromatographic steps resulting lower recovery. High-speed countercurrent chromatography (HSCCC) is a unique liquid–liquid partition chromatography technique that uses no solid support matrix. HSCCC eliminates the irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column (6). This method has been successfully used for the preparative separation of natural products such as traditional Chinese medicinal herbs (7–16). No report has been seen on the use of HSCCC for the isolation and purification of α-cyperone from plants. We herein report an efficient method for the preparative isolation and purification of α-cyperone from *Cyperus rotundus* L. by HSCCC.

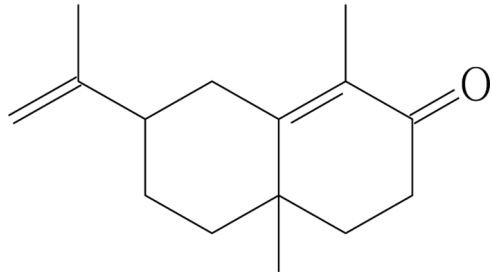

Figure 1. Chemical structure of α-cyperone.

EXPERIMENTAL

Reagents and Materials

Organic solvents including *n*-hexane, ethanol, petroleum ether (60–90°C), ethyl acetate, and methanol were all of analytical grade and were purchased from Guangcheng Chemical Factory, Tianjin, China. Methanol used for HPLC analysis was of chromatographic grade and purchased from Siyou Tianjin Chemical Factory, Tianjin, China. Reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions. The standard α-cyperone was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China.

The rhizome of *Cyperus rotundus* L. was purchased from a local drug store. The species was identified by Prof. Fengqin Zhou, Shandong University of Traditional Chinese Medicine, China.

Apparatus

HSCCC was carried out using a Model GS10A2, with a multilayer coil of 1.6 mm I.D. and 110 m in length with a total capacity of 230 ml. The β values of this preparative column range from 0.5 at internal to 0.8 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R ($R = 8$ cm) is the revolution radius or the distances between the holder axis and central axis of the centrifuge) (Beijing UE Biotech Co., Beijing, China). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm, and 800 rpm was used in the present studies. A ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with Prime view 5.0 Chromatography Workstation (Amersham Pharmacia Biotechnique Group, Sweden).

The high-performance liquid chromatography (HPLC) equipment used was a Waters Millennium^{32} system including a Waters 996 Photodiode Array Detection (DAD) system, a Waters 600 Multisolvent Delivery System, a Waters 600 System controller, a Waters 600 pump, and a Millennium^{32} work-station (Waters, Milford, USA).

A *Spe-ed*^{TM} supercritical fluid extraction (SFE) system (Applied Separations, Inc., USA) was used for extracting essential oil from the rhizome of *Cyperus rotundus* L.

Preparation the Sample

Preparation of the crude extract was carried out according to the literature (17). Air-dried and ground rhizome (500 g) of *Cyperus rotundus* L. was extracted with supercritical carbon dioxide under the pressure of 20 MPa and temperature of 40°C which yielded 12.8 g of essential oil for further isolation and separation.

Selection of Two-Phase Solvent System

A number of two-phase solvent systems were tested by changing the volume ratio of the solvent to obtain the optimum composition that gave suitable partition coefficient (K) values. The partition coefficient values were determined according to the literature (6,9). In brief, two ml of each phase of the equilibrated two-phase solvent system was added to approximately 2 mg of crude sample placed in a 10-ml test tube. The test tube was caped, and was shaken vigorously for 1 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficients. The partition coefficient value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

Preparation of a Two-Phase Solvent System and Sample Solution

The selected solvent system was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at room temperature. The two phases were separated shortly prior to use. The lower phase was used as the mobile phase, while the upper phase was used as the stationary phase. The sample solution was prepared by dissolving the essential oil in the mixture solution of upper phase and lower phase (1:1, v/v) of the solvent system used for HSCCC separation.

Separation Procedure

The separation was initiated by filling the entire column with the stationary phase using the pump, and then loading the sample dissolved in a mixture of stationary and mobile phases. The mobile phase was then pumped into the column at 2 ml/min while the column was rotated at 800 rpm in the combined head to tail elution mode. The absorbance of the eluate was continuously monitored at 254 nm. The fractions were collected according to the chromatogram and were analyzed by

HPLC. After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas.

Separation and Purification of α-Cyperone by Silica Gel Column

The SFE extracts (11 g) were subjected to silica gel column (200 g), elution with petroleum ether (60–90°C)-ethyl acetate (100:6, v/v), and afforded five fractions according to HPLC results. Fraction 5 containing α-cyperone was repeatedly chromatographed on silica gel column (200 g) eluted with same solvent to afford α-cyperone (210 mg).

HPLC Analysis

The crude sample and each purified fraction from the HSCCC separation were analyzed by HPLC with a Shim-pack VP-ODS column (250 × 4.6 mm, I.D.) at 254 nm and column temperature of 25°C. The mobile phase, a solution of methanol and water (80:20, v/v), was set at a flow-rate of 1 ml/min. The effluent was monitored by a photodiode array detector.

MS Analysis

Identification of HSCCC peak fractions was carried out by EI-MS on an Agilent 5973N mass spectrograph. Typical MS operating conditions were: ionization energy 70 eV, MS source temperature 230°C, MS quadrupole temperature 150°C, and electron multiplier voltage 3000 V.

RESULTS AND DISCUSSION

The crude extract obtained from *Cyperus rotundus* L. by SFE was analyzed by HPLC, and the chromatogram is shown Fig. 2a. The result indicated that the crude sample contained several compounds among which α-cyperone represented 6.9% of the total extract. The composition of the crude extract was similar to that described by Liang et al. (18).

Selection of the Two-Phase Solvent System

The selection of the two-phase solvent system is the most important, and is also the most difficult of steps. It is estimated that about 90% of the entire

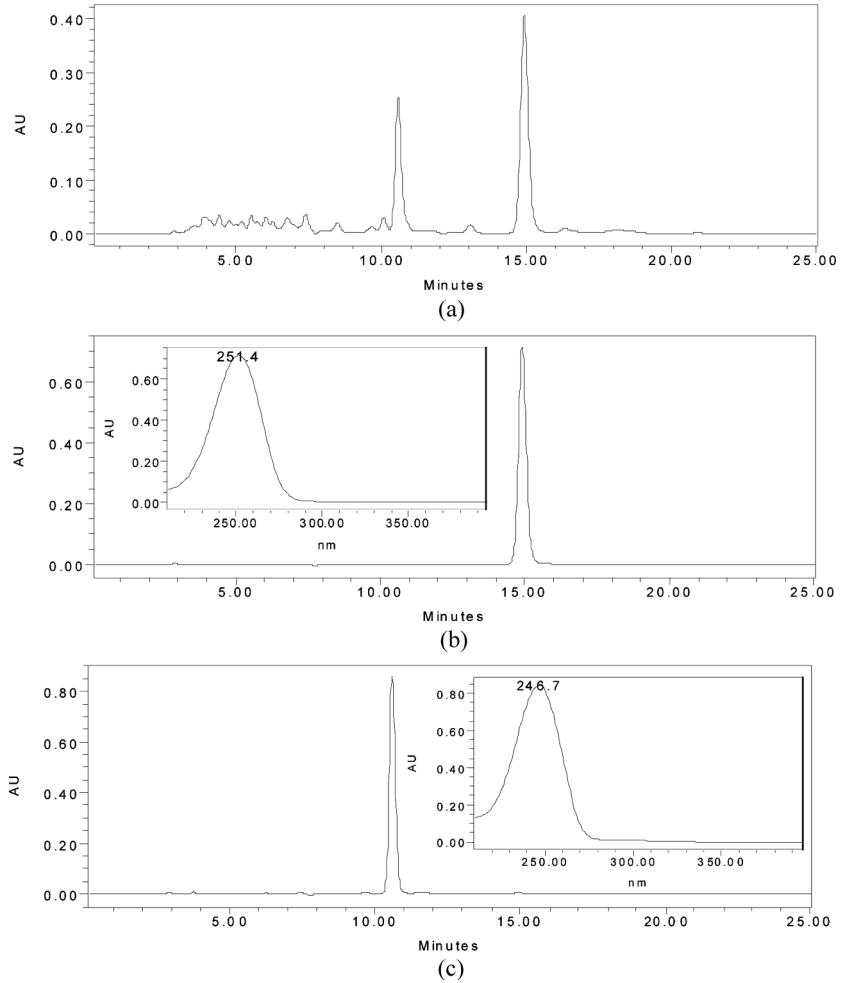

Figure 2. The results of HPLC analyses and UV spectrum of the crude α-cyperone extracted from *Cyperus rotundus* and purified by HSCCC fraction shown in Figure 3. Peak 1–3 and 5: unknown compounds; Peak 4: α-cyperone.

work in HSCCC is invested in solvent system selection (6,7). A suitable two-phase solvent system requires the following considerations (6,19):

1. retention of the stationary phase should be satisfactory;
2. the settling time of the solvent system should be short (i.e. <30 s); and
3. the partition coefficient (K) of the target compound should be close to 1.

Small K values usually result in a poor peak resolution, while large K values tend to produce excessive sample band broadening. The K value for a pure compound can be determined simply by measuring the UV absorbance of each phase after partitioning between the two phases. When the compounds to be separated are not available in a pure form, as in the present case, their K values can be determined using HPLC by computing the ratio of the peak heights (or areas) between the corresponding peaks.

Table 1 shows the partition coefficients of α-cyperone in different solvent systems. Among them, the two-phase solvent systems composed of *n*-hexane, ethyl acetate, methanol, and water at ratios of 1:1:1:1, 3:2:3:2, 1:2:2:1, had high K values which required a long time to elute α-cyperone. When petroleum ether (60–90°C)-ethanol-water at ratio of 10:17:3 (v/v) and *n*-hexane-ethyl acetate- methanol-water at ratio of 1:0.2:1.1:0.2 (v/v) were used as solvent system, the K values were suitable and α-cyperone could be well separated from other compounds. After trying the two solvent systems, the solvent system at ratio of 1:0.2: 1.1:0.2 (v/v) was found to be the best to effect the separation. Figure 3 showed the separation of HSCCC using this solvent system.

Separation of α-Cyperone with HSCCC

The crude sample was dissolved in 8 ml of a mixture of both lower phase and upper phase (1:1, v/v) of the solution system used for the HSCCC separation. The upper phase was used as the stationary phase while the lower phase was used as the mobile phase in the head to tail elution mode. The retention of the stationary phase was 66%, and the separation time was 240 min in each separation run. Based on the HPLC analysis and elution curve of the preparative HSCCC (Fig. 3), peak 4 was identified as α-cyperone by congruence of its retention time and UV spectra with that of authentic α-cyperone. It was further conformed by electron

Table 1. The partition coefficient (K) values of α-cyperone in different solvent systems

No.	Solvent system	Ratio	K Values
1	*n*-Hexane-ethyl acetate-methanol-water	1:1:1:1	26.11
2	*n*-Hexane-ethyl acetate-methanol-water	3:2:3:2	23.03
3	*n*-Hexane-ethyl acetate-methanol-water	1:2:2:1	4.91
4	Petroleum ether(60–90°C)-ethanol-water	10:17:3	1.12
5	*n*-Hexane-ethyl acetate-methanol-water	1:0.2:1.1:0.2	1.20

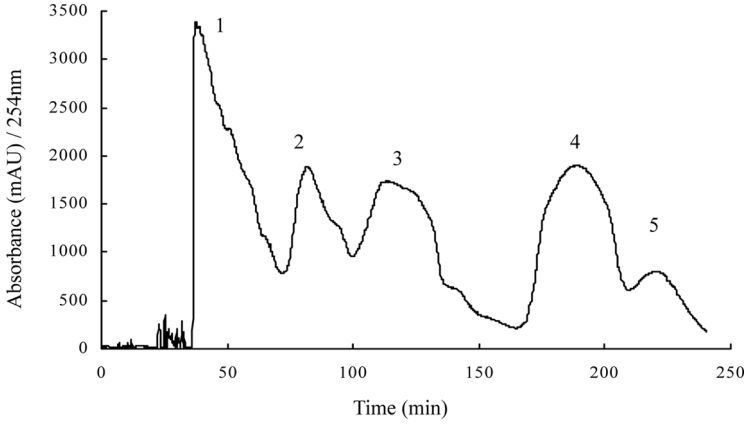

Figure 3. HSCCC separation of the extract from *Cyperus rotundus*.

impact ionization mass spectrometry as follows: EI-MS (m/z) 218, 203, 175, 161, 147, 133, 121, 105, 91, 79, 55, which matched with the reported EI-MS data for α-cyperone (5). A 900 mg quantity of SFE extract of *Cyperus rotundus* was separated by HSCCC, which yielded 60 mg α-cyperone with 98.8% purity and 95% recovery (Fig. 2b). Peak 3 fraction contained an unknown compound which weighed 30 mg, with over 92% purity, as determined by HPLC (Fig. 2c).

Separation of α-Cyperone with Conventional Silica Gel Column

The SFE extracts (11 g) were separated and purified by conventional silica gel column and 210 mg of α-cyperone was obtained after several separation steps. The separation time was about 70 h and the recovery was low (about 28%). However, HSCCC could separate and purify α-cyperone from the crude extract in one-step with 95% recovery. The separation time was only 4 h in each separation run. In comparison with open-column silica gel, HSCCC represents very low solvent consumption for a sample of the size and short separation time used in this experiment.

CONCLUSIONS

α-Cyperone was extracted from the traditional Chinese medicine *Cyperus rotundus* by the supercritical fluid extraction technique. From 900 mg crude SFE extract, 60 mg of α-cyperone was obtained with 98.8% purity

by HSCCC with a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:0.2:1.1:0.2, v/v) in one step. The results of the present study demonstrate that SFE and HSCCC are very useful techniques for the extraction, separation, and purification of α-cyperone from *Cyperus rotundus*.

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