Preparative Separation of Aurantio-obtusin and Rhein from *Semen cassiae* by High Speed Counter-current Chromatography Using pH-gradient Elution

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ABSTRACT:OBJECTIVE To establish an efficient method for preparative separation of the characteristic constituent aurantio-obtusin from the crude extract of *Semen cassiae*.**METHODS** High speed counter-current chromatography was employed and the optimum two-phase solvent system methyl *tert*.-butyl ether-water was selected for the target compounds with a pH-gradient elution.**RESULTS** 28 mg aurantio-obtusin at over 98.7% purity and 30 mg of rhein at over 95.5% purity by high-performance liquid chromatography (HPLC) analysis were isolated by preparative high speed counter-current chromatograpy from 150 mg of the crude extract and their structures were identified by ^{1}H-NMR.**CONCLUSION** A simple and quick preparative separation method was presented for purification of hydroxyanthraquinones from the traditional Chinese herb.

KEY WORDS:counter-current chromatography; preparative chromatography; *semen cassiae*; aurantio-obtusin

pH 梯度洗脱高速逆流色谱制备性分离决明子中的橙黄决明素和大黄酸

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摘要：目的 建立一种从决明子提取物中分离特征性成分橙黄决明素的高效制备性分离方法。**方法** 采用高速逆流色谱技术，针对目的化合物选择合适的两相溶剂体系，水相为流动相并采用 pH 梯度洗脱。**结果** 采用制备性高速逆流色谱技术一次性从 150 mg 决明子提取物中分离得到了 28 mg 橙黄决明素以及 30 mg 大黄酸，其纯度分别为 98.7%和 95.5%。**结论** 采用 pH 梯度洗脱的方法能快速的分离得到中草药中的蒽醌类化合物。

关键词：逆流色谱；制备色谱；决明子；橙黄决明素

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1 INTRODUCTION

Semen cassiae is a traditional Chinese medicinal herb, and is officially listed in the Chinese Pharmacopoeia. Pharmacological test revealed that *Semen cassiae* had pharmacological activities such as cathartic, anti-psychotic, anti-inflammatory, antimicrobial, epatoprotective and so on[1]. The major active components of the herb are hydroxyanthraquinones (HAQs), including aurantio-obtusin, rhein, emodin and chrysophanol, which are often used as criteria in the quality control of *Semen cassiae*. However, rhein, emodin and chrysophanol are not the distinctive components of *Semen cassiae*. They are also active components in a large number of plant-derived drugs such as *Rheum*, *Aloe* and *Polygonum* species[2-3]. Only aurantio-obtusin is the characteristic constituent of *Semen cassiae*. In light of this background, an efficient preparative separation method for aurantio-obtusin from *Semen cassiae* is warranted. The conventional methods such as column chromatography followed by crystallization are tedious and often require several steps. High-speed counter-current chromatography (HSCCC) uses no solid support, so the adsorbing effects on stationary phase material and artifact formation can be eliminated. This technique has the maximum capacity with an excellent sample recovery and can be employed for preparative-scale separation in a completely straightforward manner. Furthermore, it permits introduction of crude samples directly into the hollow column[4]. Five HAQs weighed 2-9 mg had been isolated from 100 mg of ethanol extract of Cassia seeds[5]. The present paper reports on the one-step separation of the two other bioactive HAQs (see Fig 1), namely aurantio-obtusin and rhein, from the extract of *Semen cassiae* by preparative HSCCC using pH-gradient elution. This is the first report that the two HAQs aurantio-obtusin and rhein was isolated from *Semen cassiae* by the preparative HSCCC.

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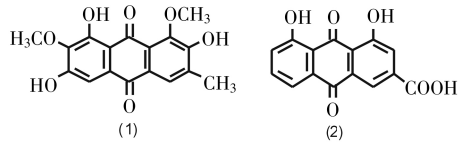

Fig 1 Chemical structure of aurantio-obtusin (1) and rhein (2)
图 1 橙黄决明素(1)和大黄酸(2)结构式

2 EXPERIMENTAL

2.1 Apparatus

A Model TBE-300A high-speed counter-current chromatography (Shanghai Tauto Biotechnique, Shanghai, China) equipped with three preparative multilayer coils (270 mL,wound with 1.6 mm I.D. PTFE tubing) was used. The β values of this column range from 0.46 to 0.73 ($\beta=r/R$,R=6.5 cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder shaft and central axis of the centrifuge). The columns of HSCCC were installed in a vessel that was retained at 25℃ by a Model HX-1050 constant-temperature controller. The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Shengyitong Technique Co. Ltd., Beijing, China). Continuous on line monitoring of the effluent was achieved with a Model UV-II detector Monitor (Shanghai Institute of Biochemistry of Academy of Science, Shanghai, China) at 254 nm. A manual sample injection valve with a 20 mL loop was used to introduce the sample into the column. N2000 workstation (Zhejiang University Zhida Information & Technology Co. Ltd., Hangzhou, China) was employed to record the chromatogram. Eluate was collected with a Model BSZ-100 fraction collector (Shanghai Huxi Tech, Shanghai, China), 6 mL for each fraction.

The high performance liquid chromatography (HPLC) used was a CLASS-VP Ver.6.1 system (Shimadzu, Japan) comprised a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver.6.1 workstation.

2.2 Reagents and Materials

All organic solvents used for HSCCC were of analytical grade and purchased from Hangzhou HuiPu Chemical Factory, Hangzhou, China. Methanol used for HPLC analysis was of chromatographic grade. *Semen cassiae* were purchased from local drugstore.

2.3 Extraction of Crude Sample

Preparation of the crude sample was carried out according to the literature[6]. The dried *Semen cassiae* (0.5 kg) was ground to powder (about 50 mesh) and it was extracted under reflux with the mixture of 20% HCl and chloroform (1∶5) for three times (1 h period of each time). The chloroform extracts were combined and evaporated under reduced pressure to about 200 mL. Then the chloroform extract was extracted with 5% NaOH five times. The aqueous solution was acidified with 20% HCl to pH 3 and then the acidified aqueous solution was extracted with ethyl ether for five times. The ethye ether extract was evaporated to dryness to give 1.65 g of the original sample. The crude sample was directly subjected to HSCCC.

2.4 Preparation of Two-phase Solvent System and Sample Solution

For the present study, we selected a two-phase solvent system composed of methyl *tert.*-butyl ether-water (1∶1). The solvent mixture was thoroughly equilibrated in a separation funnel at the same temperature as in the vessel of HSCCC and the two phases separated shortly before using. After the two phases were separated, the organic phase was used as stationary phase, and the aqueous phase as the mobile phase. For performing stepwise elution, a portion of the aqueous phase was basified by adding 5.0% $NaHCO_3$ and 0.7% Na_2CO_3, and these two mobile phases at different pH values were successively eluted through the column in an increasing pH value for the separation. The experiment of selecting the solvent to obtain the optimum composition that gave suitable partition coefficient (K) values of the target compounds was performed as in the literature[7].

The sample solution was prepared by dissolving the crude extract in the mixture solution of lower phase and upper phase(1∶1) of the solvent system used for HSCCC separation.

2.5 Separation Procedure

The multilayer-coiled column was first entirely filled with the upper phase as a stationary phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow-rate of 2.0 $mL\cdot min^{-1}$, while the apparatus was run at a revolution speed of 800 $r\cdot min^{-1}$. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution [150 mg dissolved in 12 mL mixture solution of lower phase and upper phase (1∶1) of the solvent system] was

injected through the sample port. Then, the stepwise elution was started by successively eluting the column with two different mobile phases containing 140 mL 5.0% $NaHCO_3$ and 300 mL 0.7% Na_2CO_3, respectively, in an increasing order of pH as mentioned above. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was manually collected according to the elution profile and determined by HPLC. After the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized air.

2.6 HPLC Analysis and Identification of HSCCC Peak Fractions

The crude extract from *Semen cassiae* and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm, 5 μm). The mobile phase was of methanol–0.1% H_3PO_4 (80:20) eluted with isocratic elution. The flow-rate was 1.0 mL·min^{-1} and the effluent was monitored by a Shimadzu SPD10Avp UV detector at 254 nm.

Identification of HSCCC peak fractions was carried out by ^{1}H-NMR spectra as well as comparing the HPLC retention time with those of standards. NMR spectra were recorded on a Bruker Advance 500 MHz spectrometer with TMS (tetramethylsilane) as internal standard.

3 RESULTS AND DISCUSSION

The method uses HSCCC with pH-gradient elution based on HAQs' characteristic acidity which is determined by the position of the phenolic hydroxyl group in the molecule as well as the number of carboxylic and phenolic hydroxyl groups[8]. The characteristic acidity of the HAQs molecules is different from each other. With phenolic hydroxyl groups in molecule, HAQ is a kind of acid compound[9]. The solubility of them in solutions with different pH is different from one another. So they can be separated by changing pH value of the mobile phase of HSCCC. Under optimum HSCCC separation condition, a 150 mg quantity of the crude extract was separated by HSCCC. The retention of the stationary phase was 51.1%, and the separation time was 200 min for a separation run. Fig 2 shows the result obtained from 150 mg of the crude extract of *Semen cassiae* by preparative HSCCC. After this separation, the fractions containing aurantio-obtusin and rhein were collected, respectively.

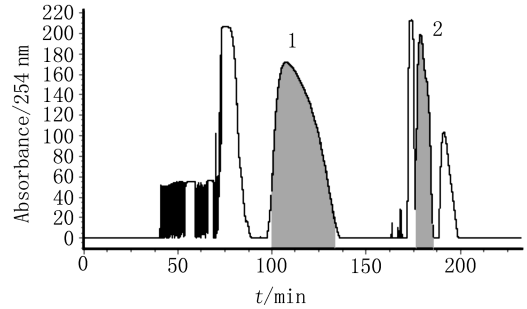

Fig 2 Chromatogram of the crude sample from *Semen cassiae* by HSCCC

1-aurantio-obtusin; 2-rhein.

图 2 高速逆流色谱分离色谱图

1-橙黄决明素；2-大黄酸

The crude extract of *Semen cassiae* was analysed by HPLC. The result indicated that under UV 254 nm the crude contained several compounds among which aurantio-obtusin represented 21.2% and rhein represented 23.5% of the total. After HSCCC separation, the fractions containing aurantio-obtusin and rhein were collected. After separation, each purified compound from the peak fraction was extracted with chloroform after acidification by concentrated hydrochloric acid. The analysis indicated that the peak 1 fraction contained aurantio-obtusin, which weighed 28 mg, at over 98.7% purity, and peak 2 fraction contained rhein, which weighed 30 mg, at over 95.5% purity, as determined by HPLC (Fig 3).

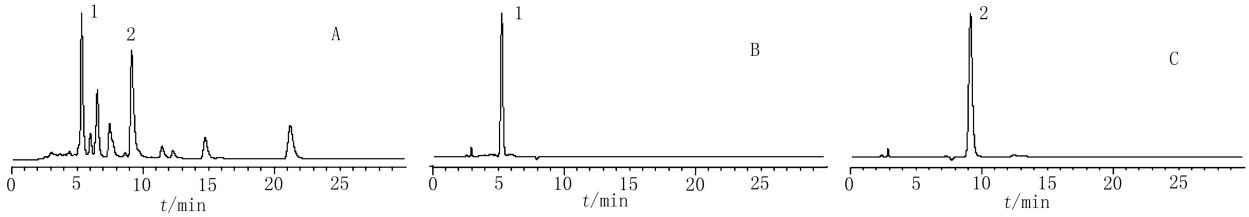

Fig 3 Results of HPLC analyses of the crude sample of *Semen cassiae* and its HSCCC fraction

A-The crude sample;B-HSCCC fraction from Peak 1(Fig 2);C-HSCCC fraction from Peak 2 (Fig 2)

图 3 液相色谱分析结果

A-粗提物；B-逆流色谱峰 1；C-逆流色谱峰 2

The structural identification of the two compounds was carried out by ^{1}H-NMR spectra as well as comparing the HPLC retention time with those of standards. Peak 1: ^{1}H-NMR (500 MHz, DMSO-d_6) δ ppm: 13.26 (1H, *s*, 8-OH), 10.95 (1H, *brs*, 6-OH), 10.28 (1H, *brs,* 2-OH), 7.78 (1H, *s*, H-4), 7.18 (1H, *s*, H-5), 3.84, 3.80 (3H, *s*, 1, 7-OCH_3), 2.29 (3H, *s*, 3-CH_3). After comparing the data with spectral information from literature[10], the first component was confirmed as aurantio-obtusin. Peak 2: ^{1}H-NMR (500 MHz, DMSO-d_6) δ ppm:13.84(1H,brs, -COOH),11.87 (2H, s, 1, 8-OH), 8.09 (1H, s, H-4),7.73(1H, s, H-2), 7.82(1H, t, *J* =8.0 Hz,H-6),7.71(1H, d, *J*=8.0 Hz, H-5), 7.38(1H, d, *J*=8.0 Hz,H-7). Comparing with the reported data, the spectra data of the second component was in agreement with those of rhein[9].

4 CONCLUSIONS

An efficient method for preparative isolation of aurantio-obtusin and rhein from *Semen cassiae* using HSCCC with pH-gradient elution was established. The two-phase solvent system was methyl tert.-butyl ether-water (1∶1) and the mobile phase was the lower phase modulated with 5% $NaHCO_3$ and 0.7% Na_2CO_3. 28 mg aurantio-obtusin at over 98.7% purity and 30 mg of rhein at over 95.5% purity was separated from 150 mg of the crude extract of *Semen cassiae*. The result of our studies described above clearly demonstrated that HSCCC is very successful in the preparative separation of bio-active constituents from natural medicinal herbs.

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