Preparative isolation and purification of 12,13-dihydroxyeuparin from *Radix Eupatorii Chinensis* by high-speed counter-current chromatography

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**KEYWORDS**

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**Abstract**

An efficient method for the isolation and purification of 12,13-dihydroxyeuparin from *Radix Eupatorii Chinensis* by high speed counter-current chromatography (HSCCC) was established in this paper. The ether extracts of *Radix Eupatorii Chinensis* were purified by HSCCC with a solvent system of hexyl hydride–ethyl acetate–methanol–water (1:2:1:2, v/v/v/v). The upper phase was used as the stationary phase and the lower phase as the mobile phase. About 8.4 mg of 12,13-dihydroxyeuparin was obtained from 200 mg of ether extracts from *Radix Eupatorii Chinensis* in one-step HSCCC separation, with the purity of 96.71%, as determined by HPLC. After methanol–water recrystallization, the purity of 12,13-dihydroxyeuparin reached 99.83%. Such a simple and effective method was fairly useful to prepare pure compound as reference substances for related study on *Radix Eupatorii Chinensis*.

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**1. Introduction**

*Radix Eupatorii Chinensis*, the dried roots of *Eupatorium chinense* L., has been recorded in Standard of Traditional Chinese Medicinal Materials for Guangdong Province [1]. It has effects of antipyretic and anti-dotal and has been widely used for the treatment of acute and chronic faucitis and amygdalitis in clinic of Guangdong area [2,3]. Recent studies demonstrated that it also has anti-inflammatory activity [4], as well as antibacterial and antivirus effect [5]. However, the active components in *Radix Eupatorii Chinensis* were still unknown and there was no official assay until now. Initially, the reported determination of *Radix Eupatorii Chinensis* was adenosine, which is of low specificity [6]. Lately, Xie [7] has
established an accurate and convenient method for the simultaneous determination of 12,13-dihydroxyeuparin and glycyrrhizic acid for the quality control of Yanyanfang mixture (A preparation containing *Radix Eupatorii Chinensis*). 12,13-dihydroxyeuparin was selected as a marker of *Radix Eupatorii Chinensis* because of good specificity in not only the herb but also some preparations containing *Radix Eupatorii Chinensis*. Therefore, high purity preparation of 12,13-dihydroxyeuparin is of great interest for pharmacological study and quality control.

Xie [8] had prepared 12,13-dihydroxyeuparin as reference substance of *Radix Eupatorii Chinensis* and Yanyanfang mixture (A preparation containing *Radix Eupatorii Chinensis*) by high pressure preparative chromatography, but this method cost high, and its pre-treatment used the traditional silica gel column chromatography, which was time consuming and low efficient. High-speed counter-current chromatography (HSCCC) is a continuous efficient allocation of liquid chromatographic separation technology. Due to using no support matrix, it eliminates the irreversible adsorptive loss of the samples onto solid support and has an excellent sample recovery [9]. Moreover, it has a large scale of injection. Multiform relatively pure substances can be obtained at one time in large amounts. Many successful applications have been reported on the purification of different kinds of active compounds from natural products [10–13]. However, no report has been published on the use of HSCCC for isolation and purification of 12,13-dihydroxyeuparin from plants. The aim of this study, therefore, was to develop an efficient method for the isolation and purification of 12,13-dihydroxyeuparin from *Radix Eupatorii Chinensis* by HSCCC.

2. Materials and methods

2.1. Materials and reagents

Reference substance of 12,13-dihydroxyeuparin was provided by Guangdong Natural Product Reference Material Research and Development Central Lab with purity of 98.0% (Batch number: 100518). Acetonitrile (HPLC grade) was purchased from SK Chemicals (Korea). Ultrapure water was obtained from a Milli-Q purification unit (Millipore, Bedford, MA, USA). Other reagents were of analytical grade and purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China).

*Radix Eupatorii Chinensis* was purchased from Guangzhou medicinal materials company. The botanical identification was made by Dr. Xin-Jun Xu, Sun Yat-Sen University, China.

2.2. Apparatus

The HSCCC instrument used was QuikPrepTM Chassis Mk5 high-speed counter-current chromatography (The unit utilized had four identical coils of 3.2 mm o.d. and 2.16 mm i.d. Each coil had a volume of approximately 115 mL. Each coil in this configuration had a beta range of 0.85–0.62. All coils could be used individually, or as any combination of numbers in series or in parallel. Quattro CCC are manufactured by AECS-QuikPrep Ltd., Bristol, UK) with a series II HPLC pump (SSI, USA), a sample injection valve with a 10 mL sample loop and an SPD-10AVP detector (SHIMADZU, Japan), and a N2000 work station (Zhejiang University, China).

HPLC analysis was carried out on a Lab alliance HPLC system (1500 pump, AS1000 autosampler, UV6000 detector, SSI, USA). SB25-12DTD ultrasound machine (Xinzi Biotechnical. Ltd., Ningbo, China).

Electronic balance (KERN ABT 220-5DM, 0.1 mg, Germany). RE-300 rotational vacuum concentrator (Shanghai Yarong, China).

2.3. Preparation of the crude extracts from *Radix Eupatorii Chinensis*

The process of extraction of *Radix Eupatorii Chinensis* is shown in Fig. 1. The ether extracts were used for HSCCC separation.

2.4. Selection of two-phase solvent system

The two-phase solvent system was selected according to the partition coefficient (K) of the target components. The K values were determined by HPLC analyses as following [14,15]: about 1 mg of crude sample was weighted into a 5 mL test tube to which 2 mL of each phase of the equilibrated two-phase solvent system was added. The test tube was capped and shaken vigorously for several minutes to thoroughly equilibrate the sample between two phases. Then, equal volume (20 μL) of the upper and lower phase was separately evaporated to dryness. The residues were dissolved with 50% methanol-water to 1 mL and analyzed by HPLC to determine K value of 12,13-dihydroxyeuparin. The peak area of the upper phase was recorded as $A_U$ (area of upper phase) and that of the lower phase was recorded as $A_L$ (area of lower phase). The K value was calculated according to the following equation: $K=A_U/A_L$.

2.5. Preparation of two-phase solvent system and sample solution

The selected two-phase solvent system of hexyl hydride-ethyl acetate-methanol–water was prepared by adding all the solvents to a separation funnel according to the volume ratios of 1:2:1:2 and thoroughly equilibrated by shaking repeatedly. Then, the two phases of the selected solvent system were separated shortly and degassed by sonication prior to use. The sample solution for HSCCC separation was prepared by dissolving about 200 mg of crude extract in 10 mL the lower phase of the solvent system used.

2.6. HSCCC separation

The multilayer-coiled column was first entirely filled with the upper phase as stationary phase. The lower aqueous phase was then pumped into the head end of the column at a suitable flow rate of 2.0 mL/min while the apparatus was rotated at...
860 rpm. After reaching hydrodynamic equilibrium, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (about 200 mg of the crude extract in 10 mL of lower phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 240 nm and the chromatogram was recorded. Each peak fraction was collected according to the elution profile and analyzed by HPLC.

2.7. HPLC conditions

A Dikma-Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) with a C_{18} guard column (10 mm × 4.6 mm, 5 μm) was used. The binary mobile phase consisted of acetonitrile (B) and ultrapure water (A). The system was run with a gradient program at 1 mL/min: 0–20 min: 20%B→30%B, 20–30 min: 30%B, 30–40 min: 30%B→60%B, 40–60 min: 60%B. The effluent was monitored by a DAD detector. The sample injection volume was 20 μL and the column temperature was set at 35 °C.

3. Results and discussion

3.1. Optimization of HPLC

When we used the gradient program at 1 mL/min: 0–20 min: 20%B→30%B, 20–30 min: 30%B, 30–40 min: 30%B→60%B, 40–60 min: 60%B; 12,13-dihydroxyeuparin got good separation. Fig. 2 shows the HPLC chromatogram of ether extracts from Radix Eupatorii Chinensis.

3.2. Optimization of two-phase solvent system and other conditions of HSCCC

According to the physicochemical properties of 12,13-dihydroxyeuparin and the rule of selecting a two-phase solvent system in reference [9], hexyl hydride–methanol–water and hexyl hydride–ethyl acetate–methanol–water were tested for HSCCC separation in this paper. HPLC was employed to measure the concentration in each phase, from which the K values of the target compounds were calculated. Results are summarized in Table 1. When hexyl hydride–methanol–water was used as the two-phase solvent system, the K value of target compound was too small, indicating that it was not a suitable system for the separation. Then K values tested in hexyl hydride–ethyl acetate–methanol–water with different ratios were proved to be better. Solvent systems no. 2 and 3 were not suitable because the K values of target compound were still small. When solvent system no. 4 was used as two-phase solvent system, 12,13-dihydroxyeuparin was not separated well from the former compound. Finally, when solvent system no. 5 was used, in which the K value was 2.22, good resolution and acceptable separation time could be obtained. As shown in Fig. 3, 200 mg of the crude sample was successfully separated using the optimized solvent system of hexyl hydride–ethyl acetate–methanol–water.

![Figure 2](image_url)

Figure 2 The HPLC chromatogram of ether extracts from Radix Eupatorii Chinensis as well as the chemical structure of 12,13-dihydroxyeuparin. Chromatographic conditions: column, Dikma-Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) with a C_{18} guard column (10 mm × 4.6 mm, 5 μm); the binary mobile phase consisted of acetonitrile (B) and ultrapure water (A) 0–20 min: 20%B→30%B, 20–30 min: 30%B, 30–40 min: 30%B→60%B, 40–60 min: 60%B; flow rate: 1 mL/min; DAD detector; injection volume: 20 μL; column temperature: 35 °C.

| No. | Solvent system                        | Ratio (v/v/v/v) | K*  |
|-----|--------------------------------------|-----------------|-----|
| 1   | Hexyl hydride–methanol–water         | 2:1:1           | 0.075|
| 2   | Hexyl hydride–ethyl acetate–methanol–water | 1:1:1:1     | 0.21 |
| 3   | Hexyl hydride–ethyl acetate–methanol–water | 0.8:1:2:1:1.2 | 0.56 |
| 4   | Hexyl hydride–ethyl acetate–methanol–water | 1:2:1:1.5 | 1.16 |
| 5   | Hexyl hydride–ethyl acetate–methanol–water | 1:2:1:2       | 2.22 |

*K=solute concentration in the upper mobile phase divided by that in the lower stationary phase.
acetate–methanol–water (1:2:1, v/v/v/v). The retention of the stationary phase was 75.0%, and the separation time was about 2 h in one separation run, and about 8.4 mg of 12,13-dihydroxyeuparin were obtained from 200 mg of crude sample with the purity of 96.71%. The HPLC chromatogram of pure 12,13-dihydroxyeuparin is shown in Fig. 4.

3.3. Identification of 12,13-dihydroxyeuparin

12,13-dihydroxyeuparin was identified based on the retention time and the UV spectrum against the standard because it is a known structure compound typically present in *Radix Eupatorii Chinensis*. 

Figure 3  HSCCC chromatogram of the ether extract of *Radix Eupatorii Chinensis*. Solvent system: hexyl hydride–ethyl acetate–methanol–water (1:2:1:2, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 mL/min; revolution speed: 860 rpm; retention of stationary phase: 75.0%; sample size: 200 mg crude extract; detection at 240 nm.

Figure 4  The HPLC-DAD chromatogram of 12,13-dihydroxyeuparin by HSCCC separation. (A) HPLC chromatogram of 240 nm; (B) ultraviolet spectrogram; and (C) 3D chromatogram of 12,13-dihydroxyeuparin. HPLC conditions: column, Dikma-Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) with a C_{18} guard column (10 mm × 4.6 mm, 5 μm); the binary mobile phase consisted of acetonitrile (B) and ultrapure water (A) 0–20 min: 20%B–30%B, 20–30 min: 30%B, 30–40 min: 30%B–60%B; flow rate: 1 mL/min; DAD detector; injection volume: 10 μL; column temperature: 35 °C.
3.4. Recrystallization of 12,13-dihydroxyeuparin

The purity of 12,13-dihydroxyeuparin could be further reached 99.83% by recrystallization. Light yellow granular crystals were got through the methanol–water recrystallization. The HPLC chromatogram of 12,13-dihydroxyeuparin after methanol–water recrystallization is shown in Fig. 5.

4. Conclusion

An efficient HSCCC method for the preparative isolation and purification of 12,13-dihydroxyeuparin from the medicinal plant *Radix Eupatorii Chinensis* was developed using hexyl hydride–ethyl acetate–methanol–water (1:2:1:2, v/v/v/v) as the two-phase solvent system. 12,13-dihydroxyeuparin at purity of 96.71% was obtained from 200 mg crude extract in a single run and it reached 99.83% through methanol–water recrystallization. The compounds obtained can be used as reference substances for chromatographic purposes as well as for further physiological studies.

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References

[1] Guangdong Food and Drug Administration, Standard of Traditional Chinese Medicinal Materials for Guangdong Province (Version 2004), vol. 1, Guangdong Science and Technology Press, Guangzhou, China, 2004 pp. 21–22.
[2] Q.G. Wu, J.Z. Su, P.M. Liu, Experimental study on the anti-inflammatory analgesia function of *Radix Eupatorii Chinensis* oral liquid, Chin. J. Exp. Trad. Med. Form 11 (3) (2005) 56–57.
[3] Y.F. Sun, Q.W. Xu, Q.X. Mei, et al., Clinic effect of compound *Radix Eupatorii Chinensis* syrup on treating urgent chronic pharyngitis tonsillitis, Chin. Arch. Trad. Chin. Med. 25 (3) (2007) 503–504.
[4] X.Y. Liu, X.C. Zeng, J.D. Jiang, et al., Study on the anti-inflammatory analgesia function of *Radix Eupatorii Chinensis*, Chin. Arch. Trad. Chin. Med. 8 (22) (2004) 1566–1568.
[5] R. Zhang, W.M. Xiong, Experimental study on the anti-bacterial & anti-virus function of *Radix Eupatorii Chinensis* mixture, Anti-Infect. Pharm. 3 (3) (2006) 137–138.
[6] Q.W. Lu, J. Li, P. Xiao, et al., Determination of adenosine in *Radix Eupatorii Chinensis* Linn by RP-HPLC, J. Guangdong Coll. Pharm. 23 (2) (2007) 131–133.
[7] Z.S. Xie, X.J. Xu, C.Y. Xie, et al., Simultaneous determination of 12,13-dihydroxyeuparin and glycyrrhizic acid in Yanyanfang mixture by high performance liquid chromatography, J. Pharm. Anal. 1 (3) (2011) 219–222.
[8] X.L. Xie, X.J. Xu, X. Chen, et al., Preparation of 12,13-dihydroxyeuparin reference substance from *Radix Eupatorii Chinensis* and Yanyanfang mixture, Lishizhen Med. Mater. Med. Res. 21 (7) (2010) 1730–1732.
Preparative isolation and purification of 12,13-dihydroxyeuparin from *Radix Eupatorii Chinensis* by HSCCC

[9] Y. Ito, Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography, J. Chromatogr. A 1065 (2005) 145–168.

[10] Q.F. Tang, C.H. Yang, W.C. Ye, et al., Preparative isolation and purification of bioactive constituents from *Aconitum coreanum* by high-speed counter-current chromatography coupled with evaporative light scattering detection, J. Chromatogr. A 1144 (2007) 203–207.

[11] H.B. Li, F. Chen, Preparative isolation and purification of chuanxiongine from the medicinal plant *Ligusticum chuanxiong* by high-speed counter-current chromatography, J. Chromatogr. A 1047 (2004) 249–253.

[12] Y. Xu, Y.X. Jin, Y.Y. Wu, et al., Isolation and purification of four individual theaflavins using semi-preparative high performance liquid chromatography, J. Liq. Chromatogr. Relat. Technol. 33 (2010) 1791–1801.

[13] Y. Liang, L.X. Wei, Z.R. Zhu, et al., Isolation and purification of kaempferol-3, 7-O-α-L-dirhamnopyranoside from *Siraitia grosvenori* leaves by high-speed counter-current chromatography and its free radical scavenging activity, Sep. Sci. Technol. 46 (2011) 1528–1533.

[14] Q.B. Han, L. Wong, N.Y. Yang, et al., A simple method to optimize the HSCCC two-phase solvent system by predicting the partition coefficient for target compound, J. Sep. Sci. 31 (2008) 1189–1194.

[15] X.F. Guo, D.J. Wang, W.J. Duan, et al., Accelerated solvent extraction of monacolin K from red yeast rice and purification by high-speed counter-current chromatography, J. Chromatogr. B 878 (2010) 2881–2885.