Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1076 (2005) 198–201
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

Preparative isolation and purification of rupestonic acid from the Chinese medicinal plant *Artemisia rupestris* L. by high-speed counter-current chromatography

Yanming Ma [a,b], Haji Akber Aisha [a], Lixin Liao [a], Sirafil Aibai [c], Tianyou Zhang [d], Yoichiro Ito [e,*]

[a] *Xinjiang Technical Institute of Physics & Chemistry, CAS, Urumqi 830011, China*
[b] *Graduate School of the Chinese Academy of Science, Beijing 100039, China*
[c] *Institute of Xinjiang Uyghur Medicine, Urumqi 83001 China*
[d] *Beijing Institute of New Technology Application, Beijing 100035, China*
[e] *Center of Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 50, Room 3334, 50 South Drive MSC 8014, Bethesda, MD 20892-8014, USA*

Received 2 February 2005; received in revised form 7 April 2005; accepted 8 April 2005

Abstract

Rupestonic acid was purified for the first time by high-speed counter-current chromatography from a dichloromethane extract of the traditional Chinese medicinal plant *Artemisia rupestris* L. The separation was performed in two steps with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (6:4:3.5:6.5, v/v) with 0.5% acetic acid in stationary-phase. From 200 mg of the crude extract, 27.9 mg of rupestonic acid was obtained at over 98% purity as determined by HPLC analysis, and its chemical structure was confirmed by MS, ^{1}H and ^{13}C nuclear magnetic resonance.

Keywords: *Artemisia rupestris* L.; Counter-current chromatography; Rupestonic acid

1. Introduction

Artemisia rupestris L., is a well-known traditional Chinese medicinal plant in Xinjiang used for detoxification, anti-hypersusceptibility, antitumor, antibacterial, antivirus and protecting liver [1,2]. Rupestonic acid is the main active ingredient of *A. rupestris* L., and some research work has shown its healing hepatitis. Therefore, rupestonic acid has been chosen as a "maker compound" for the chemical evaluation or standardization of *A. rupestris* L. and its products [3]. The chemical structure of rupestonic acid is given in Fig. 1.

The separation and purification of rupestonic acid using conventional methods, such as column chromatography and high-performance liquid chromatography (HPLC) requires multiple steps resulting in low recovery of the product. High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatographic technique, can eliminate irreversible adsorption of the sample onto the solid support. This technique has the maximum capacity with an excellent sample recovery. Furthermore, it permits introduction of crude samples directly into the hollow column. Therefore, the method has been used successfully for the preparative separation of natural products [4–9].

The present paper describes a method for the purification of rupestonic acid from the crude extract of the traditional Chinese medicinal plant *A. rupestris* L. by HSCCC.

* Corresponding author. Tel.: +1 301 496 1201; fax: +1 301 402 3404.
E-mail address: itoy2@mail.nih.gov (Y. Ito).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.04.040

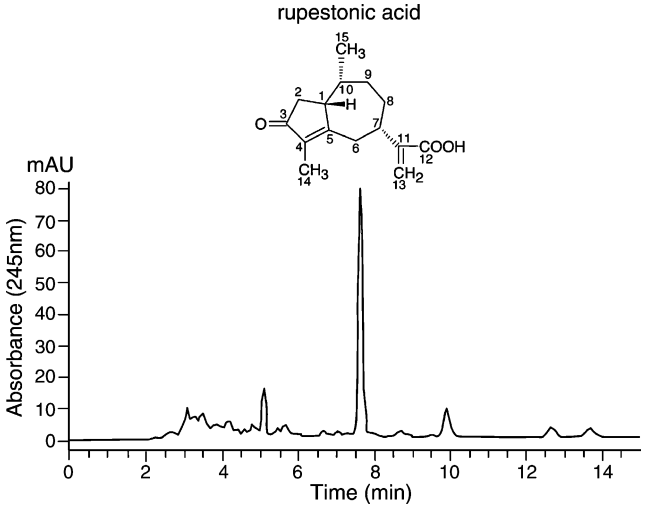

Fig. 1. HPLC analysis of the crude dichloromethane extract of *A. rupestris* L.

2. Experimental

2.1. Apparatus

A Model GS 20A analytical high-speed counter-current chromatography designed and constructed in Beijing Institute of New Technology Application (Beijing, China) was used in the present study. The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multiplayer coil separation column was prepared by winding a 50 m × 0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 ml. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 2000 rpm, an optimum speed of 1800 rpm was used in the present studies.

Preparative HSCCC was performed using a Model GS10A2 multiplayer coil of 110 m × 1.6 mm I.D. with a total capacity of 230 ml. The β values of this preparative column range from 0.5 to 0.8.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application). Continuous monitoring of the effluent was achieved with a Model 8823A-UV monitor (Beijing Institute of New Technology Application) at 254 nm. A manual sample injection valve with a 2.0-ml loop (for the analytical HSCCC) or a 20-ml loop (for the preparative HSCCC) (Tianjin High New Science Technology, Tianjin, China) was used to introduce the sample into the column, respectively. A Portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The Agilent 1100 HPLC consisted of a reversed-phase C18 column (4.6 mm I.D. × 250 mm, 5 μm; YMC, Japan), an Agilent quatpump, a sample injector (Rheodyne, USA), an Agilent temperature control module, and an Agilent VWD. Evaluation and quantification were made on Chemstation Rev A 0901.

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Tianjing chemical Factory, Tianjing, China. Methanol used for HPLC analysis was of chromatographic grade and purchased from Fisher Reagent Factory.

2.3. Preparation of crude sample

About 10 kg raw roots of *A. rupestris* L. were extracted three times with 95% ethanol. The extracts were combined and evaporated to dryness under reduced pressure, which yield 1.8 kg of dry power. Three-hundred grams of dry power was extracted with dichloromethane completely, then the dichloromethane was extracted with 5% $NaHCO_3$ three times. The $NaHCO_3$ fractions were combined and extracted with dichloromethane again after neutralizing its pH with HCl to 3. Finally, the dichloromethane was evaporated yielding 10 g of dried crude sample.

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

The two-phase solvent system used was composed of *n*-hexane–ethyl acetate–methanol–water (6:4:3.5:6.5). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. The stationary phase is upper organic phase with 0.5% acetic acid; the mobile phase is the lower phase.

The sample solutions were prepared by dissolving the crude extract in the upper phase at suitable concentrations according to the analytical or the preparative purpose.

2.5. Separation procedure

The analytical HSCCC separation was performed with a Model GS 20A HSCCC instrument as follows: the multiplayer-coiled column was first entirely filled with the stationary phase. The lower phase was then pumped into the head end of the column at a flow-rate of 1.0 ml/min, while the apparatus was run at a revolution speed of 1800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (20 mg above preparation dissolved in 2 ml of the upper aqueous phase) was injected through the sample port. The effluent from the tail end of the column was continuously

monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram.

Preparative HSCCC was similarly performed with a Model GS-10A2 HSCCC instrument as follows: the multiplayer coiled column was first entirely filled with the upper phase with 0.5% acetic acid as stationary phase. Then the sample solution (200 mg above preparation dissolved in 20 ml of the upper organic phase) was injected through the sample port and the lower organic phase was pumped into the column at a flow-rate of 2.0 ml/min while the column was rotated at 800 rpm. The effluent from the outlet of the column was monitored with a UV detector at 254 nm. Peak fractions were manually collected according to the chromatogram.

2.6. HPLC analysis and identification of CCC peak fractions

The crude dichloromethane extract of *A. rupestris* L. and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a YMC-Pack ODS-A column (4.6 mm I.D. × 250 mm, 5 μm) at column temperature of 30 °C. The mobile phase composed of methanol–0.2% formic acid aqua (67:33, v/v) was eluted at a flow-rate of 1.0 ml/min, and the effluent monitored at 245 nm.

Identification of the HSCCC peak fraction was carried out by mass spectrometry (MS), ^{1}H and ^{13}C nuclear magnetic resonance (NMR).

3. Results and discussion

The crude dichloromethane extract of *A. rupestris* L. was first analyzed by HPLC. The result indicated that it contained several compounds including rupestonic acid (about 30%) and some unknown compounds as shown in Fig. 1.

In order to achieve an efficient resolution of target compounds, the two-phase solvent system of *n*-hexane–ethyl acetate–methanol–water was examined using analytical HSCCC by varying the mutual volume ratios. The result indicated that the volume ratio of 6:4:3.5:6.5 and 0.5% acetic acid with the stationary phase could separate rupestonic acid well.

Fig. 2A shows the result obtained from 200 mg of the crude dichloromethane extract of *A. rupestris* L. by preparative HSCCC. After this separation the fractions containing rupestonic acid (shade peak) was collected. The HPLC analysis of this fraction indicated that it contained rupestonic acid at over 75% purity (Fig. 2A). The recovery of rupestonic acid in the target peak (shaded) was about 72.5%. This partially purified fraction (shaded portion of the peak from Fig. 2A) was dried, redissolved in the organic phase and purified by HSCCC with the same solvent system (Fig. 2B). This second separation yielded 27.9 mg of rupestonic acid at over 98% purity as determined by HPLC analysis as indicated. The recovery of rupestonic acid in this second separation was 46%. The structural identification of the fraction was carried out by MS, ^{1}H and ^{13}C NMR [10,11].

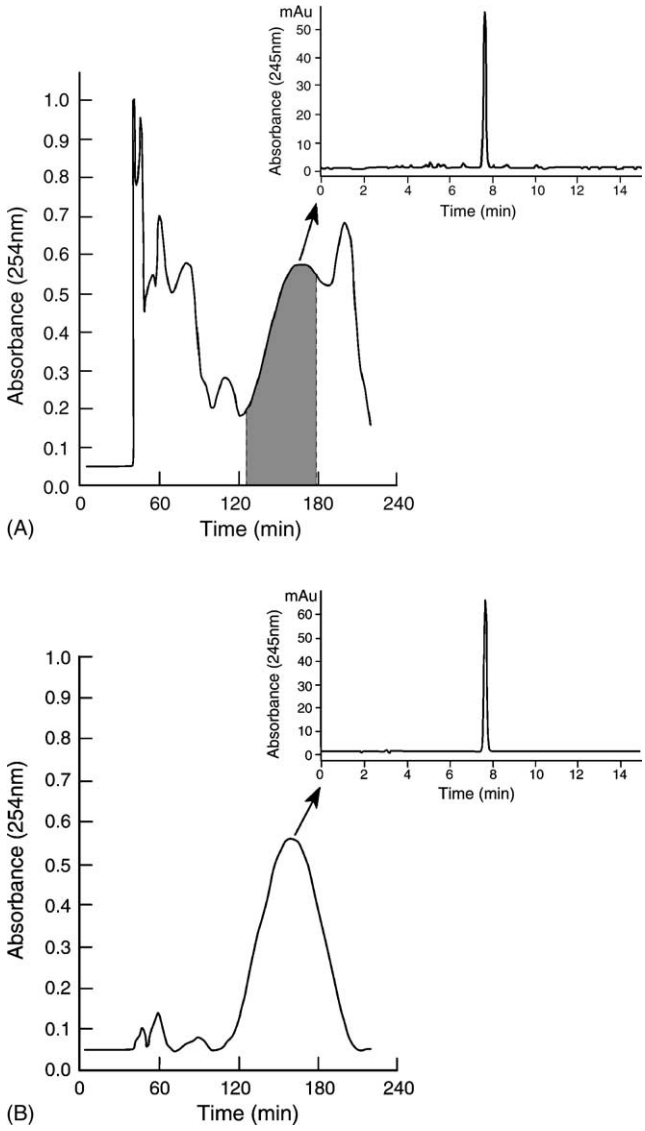

Fig. 2. HSCCC separations of the crude extract of *A. rupestris* L. (A) The first separation and HPLC analysis of the peak fractions (shaded); (B) the second separation of the shaded portion of HSCCC fractions in (A) and HPLC analysis of the peak fractions. Experimental conditions: apparatus: preparative HSCCC centrifuge with a multilayer coil separation column with 230 ml capacity; sample: (A) 200 mg of crude dichloromethane extract of *A. rupestris* L. dissolved in 20 ml of upper organic phase, (B) HSCCC fraction corresponding to the rupestonic acid peak (shaded) of (A) dried and redissolved in the lower phase; solvent system: *n*-hexane–ethyl acetate–methanol–water (6:4:3.5:6.5, v/v); mobile phase: lower organic phase; stationary phase: upper organic phase with 0.5% acetic acid; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; detection at 254 nm; retention of stationary phase: 60.8%.

The characteristic peaks in HR-EI-MS appeared at *m/z* 248[M^+] (base peak), 233[$M-CH_3$], 230[$M-H_2O$], 202[$M-H_2O$–CO], 175(28.95), 145(37.59), 109(47.01). ^{1}H NMR(400 MHz, DMSO-d6): δ0.60 (3H, d, J = 7.2 Hz, H-15′, 15″, 15‴), 1.55(3H, s, H-14′, 14″, 14‴), 3.18(2H, m, J = 2.4, 2.0Hz, H-2′, 2″), 2.07(1H, m, J = 2.8, 4.4, 3.6Hz, H-7), 3.71(1H, s, H-13′), 6.11(1H, s, H-13″), 1.70(1H, m, H-6′), 1.80(1H, m, H-6″), 2.07(1H, d, J = 18.8Hz, H-8′),

2.62(1H, dd, $J = 6.4$, 18.8Hz, H-8″), 1.84(2H, m, H-9′, 9″), 2.12(1H, m, H-10), 2.90(2H, q, $J = 10.4$, 11.6, 20.0Hz, H-2′, 2″), ^{13}C NMR(400 MHz, DMSO-d6): δ45.12(C-1), 37.58(C-2), 206.65(C-3), 136.50(C-4), 174.41(C-5), 40.85(C-6), 37.12(C-7), 31.32(C-8), 36.09(C-9), 34.78(C-10), 146.75(C-11), 167.87(C-12), 122.85(C-13), 7.72(C-14), 11.94(C-15).

The results of our studies clearly demonstrated that HSCCC is very useful for the preparative separation of rupestonic acid from a crude extract of *A. rupestris* L. And it's also a successful method for preparation of high purity rupestonic acid by HSCCC.

Acknowledgements

The authors would like to thank senior engineer Xining Li, and Dr. Yun Wei for their excellent technical assistance in the research work.

References

[1] Y.M. Liu, Pharmacography of Uighur, Part I, Xinjiang Uighur Autonomous Region, Urumqi, 1986, p. 3.
[2] X.Y. Chen, S.H. Wang, J. Chin. Tradit. Herb. Drugs 12 (4) (1981) 25.
[3] Sirafil, Askar, Ilhamjan et al., Chin. J. Biochem. Molec. Biol., 17 (2001) 226.
[4] Y. Ito, CRC Crit. Rev. Anal. Chem. 17 (1986) 65.
[5] X.F. Ma, T.Y. Zhang, Y. Wei, P.F. Tu, Y.J. Chem, Y. Ito, J. Chromatogr. A 962 (2002) 243.
[6] H.T. Lu, Y. Jiang, F. Chen, J. Chromatogr. A 1023 (2004) 159.
[7] X. Han, T.Y. Zhang, Y. Ito, J. Liq. Chromatogr. Rel. Technol. 26 (2003) 1268.
[8] L. Lei, F.Q. Yang, T.Y. Zhang, P.F. Tu, L.J. Wu, Y. Ito, J. Chromatogr. A 912 (2001) 181.
[9] G.L. Tian, Y.B. Zhang, T.Y. Zhang, F.Q. Yang, Y. Ito, J. Chromatogr. A 904 (2000) 107.
[10] G.S. Xu, X.Y. Chen, D.Q. Yu, J. Acta Pharm Sin. 23 (1988) 122.
[11] G.S. Xu, W. Zhao, D. Wu, et al., J. Acta Pharm Sin. 26 (1991) 505.