Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1100 (2005) 76–80
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Large-scale isolation and purification of geniposide from the fruit of *Gardenia jasminoides* Ellis by high-speed counter-current chromatography

Tingting Zhou, Guorong Fan*, Zhanying Hong, Yifeng Chai, Yutian Wu

Shanghai Key Laboratory for Pharmaceutical Metabolite Research, School of Pharmacy, Second Military Medical University, No.325 Guohe Road, Shanghai 200433, China

Received 16 June 2005; received in revised form 31 August 2005; accepted 12 September 2005
Available online 3 October 2005

Abstract

High-speed counter-current chromatography (HSCCC) was applied to the isolation and purification of geniposide from *Gardenia jasminoides* Ellis. Analytical HSCCC was used for the preliminary selection of a suitable solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v). According to the above solvent system, preparative HSCCC was successfully performed with the optimal solvent system composed of ethyl acetate–*n*-butanol–water (2:1.5:3, v/v/v) yielding 389 mg of geniposide at over 98% purity from 1 g of the partially purified extract with 38.9% recovery in a one-step separation.

Keywords: Counter-current chromatography; Preparative chromatography; *Gardenia jasminoides* Ellis; Geniposide

1. Introduction

Geniposide is one of the major iridoid glucosides in the fruit of *Gardenia jasminoides* Ellis, which has been reported to possess anti-inflammatory activity [1], activity against tumor-promoting 12-*O*-tetradecanoyl-phorbol-13-acetate (activation of protein kinase C) [2,3], and which has been used for treatment of a liver disorder (inhibition of P450-3A monooxygenase) [4]. Some authors [5–7] also found that geniposide could promote collagen synthesis in false-aged rats, stimulate the proliferation of endothelial cells but, interestingly, also act as a cross-linking agent with low cytotoxicity and biocompatibility. Further studies on pharmacological and clinical effects of geniposide necessitate the development of an efficient preparative separation method of these drugs. Such a method will also facilitate quality control and improvement of the quality of existing *Gardenia jasminoides* Ellis products. The chemical structure of geniposide is shown in Fig. 1.

At present, geniposide is commercially purified from *Gardenia jasminoides* by several steps such as crystallization and chromatography. However, those conventional methods may encounter various problems. For example, the compound of interest is often strongly adsorbed onto the solid support of conventional silica gel column chromatography resulting in low recoveries [8–11]. Existing high-performance liquid chromatography (HPLC) methods are not suitable for large-scale isolation of geniposide.

High-speed countercurrent chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support [12], and has been widely used in preparative separation of natural products [13–15]. However, no report has been published on the use of HSCCC for the isolation and purification of the highly bioactive geniposide, in particular, from *Gardenia jasminoides*.

The present paper describes the successful preparative separation and purification of geniposide from the partially purified

* Corresponding author. Tel.: +86 21 2507 0388; fax: +86 21 2507 0388.
E-mail address: Guorfan@yahoo.com.cn (G. Fan).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.09.026

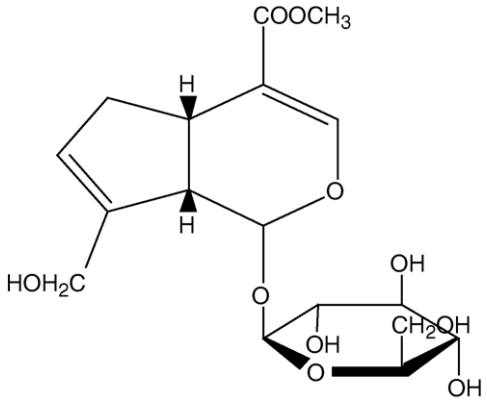

Fig. 1. Chemical structure of geniposide.

extract of *Gardenia jasminoides* by high-speed countercurrent chromatography.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a Model TBE-60A analytical high-speed countercurrent chromatograph (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.6 mm, total volume = 60 ml) and a 10 ml sample loop.

The preparative HSCCC instrument employed in the present study is a Model TBE-1000A preparative high-speed countercurrent chromatograph (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 3.0 mm, total volume = 1000 ml) and a 50 ml sample loop.

These two HSCCC systems are equipped with a Model S constant-flow pump, a Model 8823A UV monitor operating at 254 nm and a Model 3057 recorder.

The HPLC equipment used was a Dionex HPLC system including P680 pump, ASI-100 automated sample injector, thermostatted column compartment and PDA-100 photodiode array detector. Chromeleon software (Version 6.50) was used for evaluation and quantification.

The nuclear magnetic resonance (NMR) spectrometer used here was a Varian Unity Inova-500 NMR system with TMS as internal standard. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan LCQ DecaXP ion trap mass spectrometry (Thermo Finnigan, San Jose, CA, USA). Positive and negative ion modes of ESI were used for structural analyses.

D101 macroporous resin (Tianjin Agricultural Chemical Co. Ltd. Tianjin, China), a kind of milkwhite spherical granule, shows stronger intension, larger adsorption capability and easier activation than some other sorbents.

2.2. Reagents

All solvents used for HSCCC were of analytical grade and purchased from WuLian Chemical Factory, Shanghai, China. Methanol used for HPLC was of chromatographic grade (Merk, Germany). Reverse osmosis Milli-Q water (18 MΩ, Milli-Q, Millipore, Bedford, MA, USA) was used for all solutions and dilutions.

The *Gardenia jasminoides* Eillis was purchased from a local drug store and identified by Dr. Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China).

2.3. Measurement of partition coefficient and separation factor

About 4 ml of each phase of pre-equilibrated two-phase solvent system was mixed with 2 mg of the partially purified extract in a 10 ml test tube. After shaking vigorously for 5 min to equilibrate the sample thoroughly with the two phases, the two phases were separated and an aliquot of each phase was evaporated to dryness under reduced pressure. The residue was diluted with 30% CH_3OH and analyzed by HPLC. The partition coefficient (K) value was expressed as the peak area of geniposide in the upper phase divided by that in the lower phase.

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

For the present study, several two-phase solvent systems with suitable K values were selected. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use. 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.

2.5. Preparation of partially purified sample and sample solution

About 500 g of dried fruit of *Gardenia jasminoides* Eillis was chopped and extracted three times by reflux with 4000 ml volume of 50% ethanol in a haven for 2 h. After filtration, the extract was combined and evaporated to dryness by rotary vaporization at 60 °C under reduced pressure and redissolved in water. The water soluble extract was then chromatographed on D101 macroporous resin by eluting stepwise with water and 20% ethanol. Water was first used to remove some un-target chemicals, which have no or little retention on D101 macroporous resin, 20% ethanol was then used to yield target sample, and 95% ethanol was used to activate the resin for another use. 46.6 g of dried material was obtained, and this partially purified sample of *Gardenia jasminoides* Eillis was subjected to HSCCC.

The sample solutions were prepared by dissolving the partially purified extract in the mobile phase of the solvent system used for separation at suitable concentrations according to the analytical or preparative purpose.

2.6. HSCCC separation procedure

The selection of suitable solvent system is the first and most important step in performing preparative HSCCC. In the present

studies, analytical HSCCC was used for selecting a suitable solvent system from those with suitable *K* values for the separation of target compounds. The above solvent systems composed of ethyl acetate–*n*-butanol–water (2:1:3, 1:4:5, 1:1:2, 1:2:3, v/v/v), *n*-butanol–water (1:1, v/v) and *n*-butanol–acetic acid–water (4:1:5, v/v/v) were examined using analytical HSCCC.

In each analytical separation, the coiled column was first entirely filled with the organic stationary phase, and then the apparatus was rotated at 1000 rpm, while the aqueous mobile phase was pumped into the column at a flow-rate of 0.6 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 5 ml of the sample solution containing 5 mg of the partially purified extract was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile.

The preparative separation was similarly performed with the optimal solvent system composed of ethyl acetate–*n*-butanol–water (2:1.5:3, v/v/v) using a 50 ml sample volume (1 g partially purified extract) at a flow-rate of 5.0 ml min^{-1} at 550 rpm.

2.7. HPLC analysis and identification of HSCCC peak fraction

The partially purified sample and each peak fraction obtained by HSCCC were analyzed by high-performance liquid chromatography. The column used was a reversed-phase Diamonsil C_{18} (4.6 mm × 200 mm i.d. 5 μm) (Dikma Technologies Company, China) with a pre-column filled with the same stationary phase. In the present study, some organic aqueous-based mobile phases were tested on a reversed-phase C_{18} column, including methanol–water and acetonitrile–water in combination with acetic acid, phosphate buffer and phosphoric acid. The flow rate of the mobile phase and the temperature of the column, which might affect the separation, were also tested.

Identification of geniposide was carried out by MS, ^{1}HNMR and ^{13}CNMR spectra.

3. Results and discussion

3.1. Selection of chromatographic conditions

When the partially purified extract was analyzed by HPLC, it was found that an excellent separation was achieved by the following separation conditions: the mobile phase composed of methanol–water–acetic acid (30:70:1, v/v/v) was isocratically eluted at a flow rate of 0.9 ml min^{-1}, and UV detection was set at 238 nm. No complex gradient of mobile phase and no buffer were necessary. The HPLC chromatogram of the partially purified extract is shown in Fig. 2; it contained several compounds including genipin-1-β-D-geniobioside, geniposide (peak1, peak2).

3.2. Optimization of suitable two-phase solvent system

A successful separation of the target compounds using HSCCC requires a careful search for a suitable two-phase solvent system to provide an ideal range of partition coefficients (*K*) for the applied material. In order to achieve efficient separation of geniposide from the partially purified extract, four kinds of solvent systems providing a broad range of hydrophobicity at different volume ratios were tested (Table 1). When ethyl acetate–*n*-butanol–water (10:1:10, v/v/v) was used as the two-phase solvent system, the *K* value was small. It was difficult to separate geniposide from other compounds. When chloroform–methanol–water (4:3:2, v/v/v) was used as the two-phase solvent system, geniposide could be separated from the other compounds. However, its *K* value in the two-phase solvent system was big with which geniposide was eluted in an excessively broad peak with long elution time. Thus, the two solvent systems were not suitable for the isolation of geniposide from the partially purified extract. The other six solvent systems ethyl acetate–*n*-butanol–water at various volume ratios (2:1:3, 1:4:5, 1:2:3, 1:1:2, v/v/v), *n*-butanol–water (1:1, v/v) and *n*-butanol–acetic acid–water (4:1:5, v/v/v) had suitable *K* values.

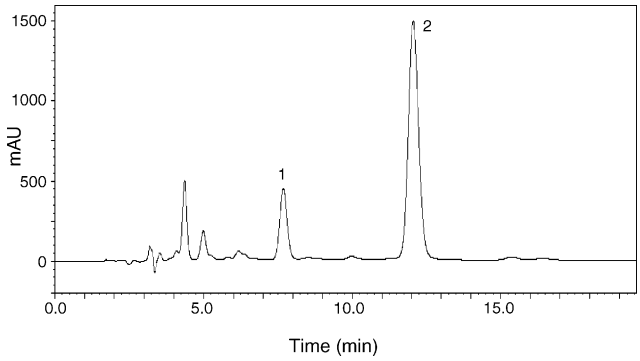

Fig. 2. Chromatogram of partially purified extract from *Gardenia jasminoides* Ellis by HPLC analysis. *Conditions*: column: reversed-phase Diamonsil C_{18} column (4.6 mm × 200 mm i.d. 5 μm); mobile phase: methanol–water–acetic acid (30:70:1, v/v/v); flow-rate: 0.9 ml min^{-1}; detection at 238 nm; injection solvent: mobile phase; inject volume: 20 μl.

Although *K* value can provide useful information for the solvent selection process to a certain extent, sometimes, it gave false information due to some unknown reasons. Analytical HSCCC with its speedy separation and minimum solvent consumption offers a very promising way to carry out rapid solvent system selection and method development for preparative HSCCC separations [16].

Performance of the above six solvent systems with suitable *K* values was evaluated by analytical HSCCC in terms of peak

Table 1
The *K* values (partition coefficient) of geniposide in different two-phase solvent systems

Solvent system	*K* value
Ethyl acetate–*n*-butanol–water (2:1:3)	0.44
Ethyl acetate–*n*-butanol–water (1:4:5)	1.04
Ethyl acetate–*n*-butanol–water (1:1:2)	0.70
Ethyl acetate–*n*-butanol–water (1:2:3)	0.92
Ethyl acetate–*n*-butanol–water (10:1:10)	0.08
n-Butanol–water (1:1)	0.94
Chloroform–methanol–water (4:3:2)	5.05
n-Butanol–acetic acid–water (4:1:5)	1.12

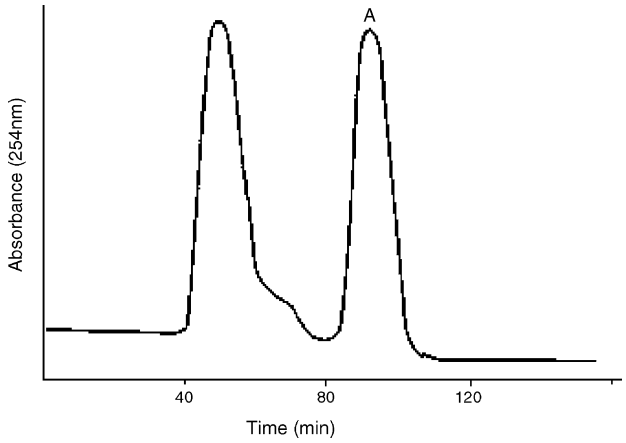

Fig. 3. Chromatogram of partially purified extract from *Gardenia jasminoides* Ellis by analytical HSCCC separation, (A) geniposide. Conditions: column: multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 60 ml; rotary speed: 1000 rpm; solvent system: ethyl acetate–*n*-butanol–water (2:1:3, v/v); mobile phase: lower phase; flow-rate: 0.6 ml ml^{-1}; detection at 254 nm; sample size: 5 mg; injection solvent: mobile phase; inject volume: 5 ml; retention of the stationary phase: 60%.

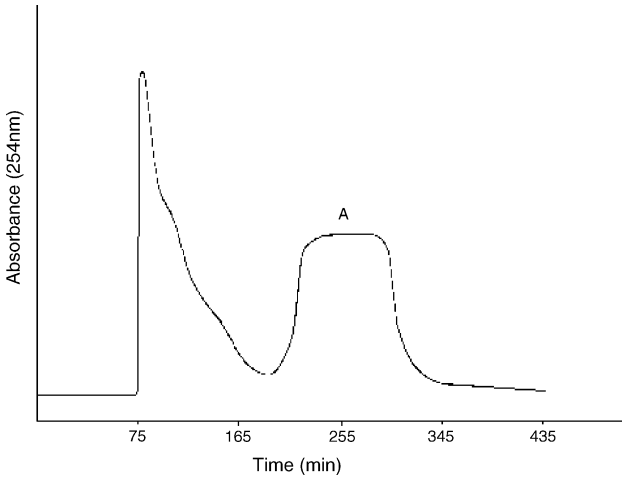

Fig. 4. Chromatogram of partially purified extract from *Gardenia jasminoides* Ellis by preparative HSCCC separation, (A) geniposide. *Conditions*: column: multilayer coil of 3.0 mm i.d. PTFE tube with a total capacity of 1000 ml; rotary speed: 550 rpm; solvent system: acetate–*n*-butanol–water (2:1.5:3, v/v); mobile phase: lower phase; flow-rate: 5 ml min^{-1}; detection at 254 nm; sample size: 1 g; injection solvent: mobile phase; inject volume: 50 ml; retention of the stationary phase: 70%.

resolution. When *n*-butanol–acetic acid–water (4:1:5, v/v/v) was used, the retention of the stationary phase was poor (<20%), so it was not suitable for the separation. When ethyl acetate–*n*-butanol–water (1:4:5, 1:1:2, 1:2:3, v/v/v) and *n*-butanol–water (1:1, v/v) were used, geniposide could be well separated from the other compounds using these solvent systems. However, the settling time was long (>180 min). At last, the two-phase solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v) was found to be satisfactory for the separation of geniposide from the partially purified extract within short retention time. Fig. 3 shows the separation of HSCCC using this solvent system.

3.3. Separation of geniposide by HSCCC

Since the sample size was 200-times as large as that used in the analytical HSCCC, a solvent system which can achieve an equivalent or even better separation was required in preparative HSCCC. Geniposide is not soluble in non-polar solvent, and has little solubility in ethyl acetate, but it has some solubility in *n*-butanol, and is freely soluble in water. Accordingly, since the volume ratio of *n*-butanol has great influence on the polarity of the two phases, the optimal solvent system was modified from ethyl acetate–*n*-butanol–water (2:1:3, v/v) to ethyl acetate–*n*-butanol–water (2:1.5:3, v/v). Fig. 4 shows the preparative HSCCC separation of 1 g of the partially purified sample using the solvent system composed of ethyl acetate–*n*-butanol–water (2:1.5:3, v/v). A total of 389 mg of geniposide with 38.9% recovery was yielded. HPLC analysis of each peak fraction of this preparative HSCCC revealed that the purity of geniposide corresponding to peak A was over 98%. The HPLC chromatogram and UV spectrum of geniposide as purified from the preparative HSCCC is shown in Fig. 5.

The structural identification of geniposide was carried out by MS, ^{1}HNMR and ^{13}CNMR spectra as follows: ESI-MS: 389 (M + 1), 227, 209 (M-glu). ^{1}HNMR (500 MHz, DMSO-d6) δ: 3.6 (3H, s, H-12), 4.0 (1H, d, J = 14.0 Hz, H-10), 4.2 (1H, d, J = 15.0 Hz, H-10), 4.53 (1H, d, J = 8.0 Hz, H-1′), 5.12 (1H, d, J = 7.0 Hz, H-1), 5.6 (1H, brs, H-7), 7.5 (1H, brs, H-3). ^{13}CNMR (500 MHz, DMSO-d6) δ: 34.4 (C-12), 37.9 (C-6), 45.9 (C-5), 51 (C-9), 59.3 (C-10), 61 (C-6′), 70 (C-4′), 73.3 (C-2′), 76.6 (C-5′), 77.2 (C-3′), 95.7 (C-1), 98.6 (C-1′), 110.9 (C-4), 125.4 (C-7), 144.1 (C-8), 151.5 (C-3), 166.9 (C-11).

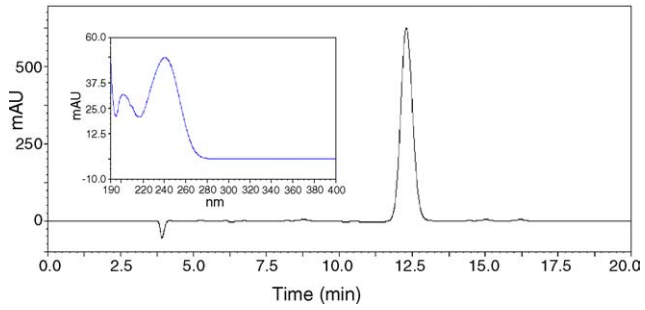

Fig. 5. HPLC chromatogram and UV spectrum of geniposide purified from *Gardenia jasminoides* Ellis by the preparative HSCCC. *Conditions*: column: reversed-phase Diamonsil C_{18} column (4.6 mm × 200 mm i.d. 5 μm); mobile phase: methanol–water–acetic acid (30:70:1, v/v/v); flow-rate: 0.9 ml min^{-1}; detection at 238 nm.

4. Conclusion

The results of our studies clearly demonstrate the potential of HSCCC for the large-scale isolation of geniposide at high purity from *Gardenia jasminoides* Ellis in a single run. In particular, the combination of K value measurement and analytical HSCCC offers a very efficient means to carry out optimization of solvent systems for separation and purification of natural products by preparative HSCCC.

Acknowledgements

This work was supported by Fundamental Research Key Project founded by Science & Technology department of Shanghai, PR China, Grant No. 03JC14005. We thank Li Liu, Jianning Zhou and Chungui Wu from Tauto Biotechnique Company (Shanghai, China) for their excellent technical assistance.

References

[1] M. Nishizawa, R. Izuhara, K. Ksnrko, Y. Koshihara, Y. Fujimoto, Chem. Pharm. Bull. 36 (1988) 87.
[2] S. Ueda, Y. Iwahashi, J. Nat. Prod. 54 (1991) 1677.
[3] M. Lee, J. Hsu, C. Wang, Anticancer Res. 15 (1995) 411.
[4] J.J. Kang, H.W. Wang, T.Y. Liu, Y.C. Chen, T.H. Ueng, Food Chem. Toxicol. 35 (1997) 957.
[5] T. Kaji, N. Miezi, K. Kaga, N. Ejiri, N. Sakuragawa, Planta Med. 56 (1990) 353.
[6] L.L. Huang, H.W. Sung, C.C. Tsai, D.M. Huang, J. Biomed. Mater. Res. 42 (1998) 568.
[7] Y. Li, S. Kamo, K. Metori, K. Koike, Q. Che, S. Takahashi, Biol. Pharm. Bull. 23 (2000) 54.
[8] F. Fen, Chin. J. Nat. Prod. 10 (1998) 60.
[9] J. Pan, J. Lu, Y. Zhang, Zhongguo Zhongyao Zazhi 20 (1995) 614.
[10] X. Jia, J. Wu, Q. Mao, Zhongguo Zhongyao Zazhi 21 (1996) 290.
[11] B. Guo, J. Yu, P. Xiao, Zhongguo Zhongyao Zazhi 23 (1998) 161.
[12] Y. Wei, T.-Y. Zhang, G.-Q. Xu, Y. Ito, J. Chromatogr. A 929 (2001) 169.
[13] F. Chen, H.B. Li, R.N.S. Wong, B. Ji, Y. Jiang, J. Chromatogr. A 1064 (2005) 183.
[14] X. Wang, Y.Q. Wang, Y.L. Geng, F.W. Li, C.C. Zheng, J. Chromatogr. A 1036 (2004) 171.
[15] J.Y. Peng, G.R. Fan, Z.Y. Hong, Y.F. Chai, Y.T. Wu, J. Chromatogr. A 1074 (2005) 111.
[16] D.E. Schaufelberger, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography (Chemical Analysis, vol. 132), Wiley-Interscience, New York, 1996, pp. 45–70, Chapter 2.