

Short communication

Preparative separation of gambogic acid and its C-2 epimer using recycling high-speed counter-current chromatography

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Abstract

A recycling counter-current chromatographic system was first set up with a high-speed counter-current chromatography instrument coupled with a column switching valve. This system was first successfully applied to the preparative separation of epimers, gambogic acid and epigambogic acid from *Garcinia hanburyi* using *n*-hexane–methanol–water (5:4:1, v/v/v) as the two-phase solvent system. As a result, 28.2 mg gambogic acid and 18.4 mg epigambogic acid were separated from 50 mg of mixture. Their purities were both above 97% as determined by HPLC. The chemical structures were then identified by their ¹H NMR and ¹³C NMR spectra.

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1. Introduction

Gambogic acid (GA, CAS No. 2752-65-0) is the principal active component of gamboge, the resin from various *Garcinia* species including *Garcinia Morella* and *Garcinia hanburyi*. Many modern pharmaceutical studies are focused on its extensive and potent anti-tumor activities [1,2]. In 1970s, it had been developed as an anti-tumor drug for clinical testing via intravenous injection in China. However GA had been believed to be an inseparable C-2 epimeric mixture [3]. The stereochemistry had not been determined until the single crystal of pyridine salt of *R*-epimer was obtained and then analyzed by X-ray diffraction [4]. In our previous report, this epimer pair was separated first by semi-preparative HPLC. The *S*-epimer was found more potent against CYP 2C9 than *R*-epimer [5]. The chemical structures of gambogic acid epimers are very similar, with only one stereochemical difference at C-2 (Fig. 1). This made their ¹H and ¹³C NMR spectra extremely similar to each other (Fig. 2), and also made this epimer pair difficult to separate by common chromatography methods. Moreover, the reverse-phase HPLC separation of these epimers with semi-preparative column was

time-consuming [5]. Therefore, a simpler isolation is necessary to achieve a better separation.

High-speed counter-current chromatography (HSCCC), a special liquid–liquid partition chromatography without solid support matrix, prevents peak tailing and sample loss due to irreversible adsorption [6]. This method has been successfully applied in the preparative separation of natural products and chiral compounds [7–10]. In recent years, multidimensional CCC which effectively extends the separation journey has been successfully applied to natural products separation [11–13]. Accordingly, it is also theoretically feasible to separate any compounds including epimers if the CCC separation course is long enough. This paper describes a successful separation of gambogic acid and its C-2 epimer using a newly built recycling HSCCC system.

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument used in this study was TBE-300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China) with three polytetrafluoroethylene preparative coils (diameter of tube, 2.6 mm, total volume, 300 ml). The revolution radius or the distance between the holder

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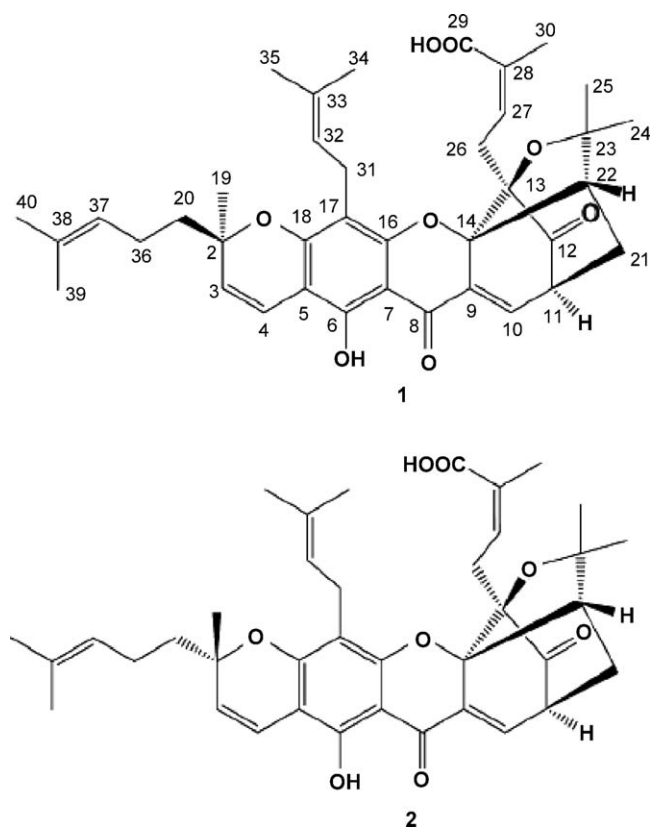


Fig. 1. Chemical structures of gambogic acid (**1**) and its C-2 epimer (**2**).

axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). A HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was used to control the separation temperature. The HSCCC system was equipped with a model S constant-flow pump, a 3725i-038 injector (Rheodyne, USA), the UV-vis G1365B photodiode array detector and Agilent HPLC worksta-

tion of a preparative Agilent 1100 HPLC Series. The recycling HSCCC separation was carried out on this system with a column switching valve.

HPLC analysis was carried out on an analytical Agilent 1100 series and Alltima-C₈ column (4.6 mm \times 250 mm, 5 μ m) at room temperature. The Agilent 1100 HPLC system included a G1311A solvent delivery unit, G1315B UV-vis photodiode array detector, Rheodyne 7725i injection valve with a 20 μ l loop, G1332A degasser and Agilent HPLC workstation. ¹H (400 MHz) and ¹³C (100 MHz) NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as internal standard.

2.2. Reagents

Methanol, acetonitrile, *n*-hexane, 1,4-dioxan anhydrosolan, and acetic acid of HPLC grade were purchased from International Laboratory Ltd., USA. Distilled water was prepared using MILLI-Q SP reagent water system (Nihon Millipore Kogyo K.K., Japan), and was distilled twice before use. The mixture of gambogic acid epimers was isolated as previously reported, from the resin of *G. hanburyi* [5].

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

Several two-phase solvent systems were tested for their partition abilities [7], including *n*-hexane–ethyl acetate–methanol–H₂O, *n*-hexane–methanol–H₂O, *n*-hexane–acetonitrile–H₂O in various ratios. As a result, *n*-hexane–methanol–water (5:4:1, v/v/v) was selected as the separation system, since the pigment GA could equally dissolve in both phases. The two-phase solvent system was prepared by adding the solvents to a separation funnel according to the volume ratios and fully equilibrated by shaking repeatedly at room temperature (20 °C). The upper and lower phases (1:1) were separated shortly before use and degassed by sonication for 30 min. Fifty milligrams of gambogic acid mixture was dissolved in 5 ml lower phase for HSCCC separation.

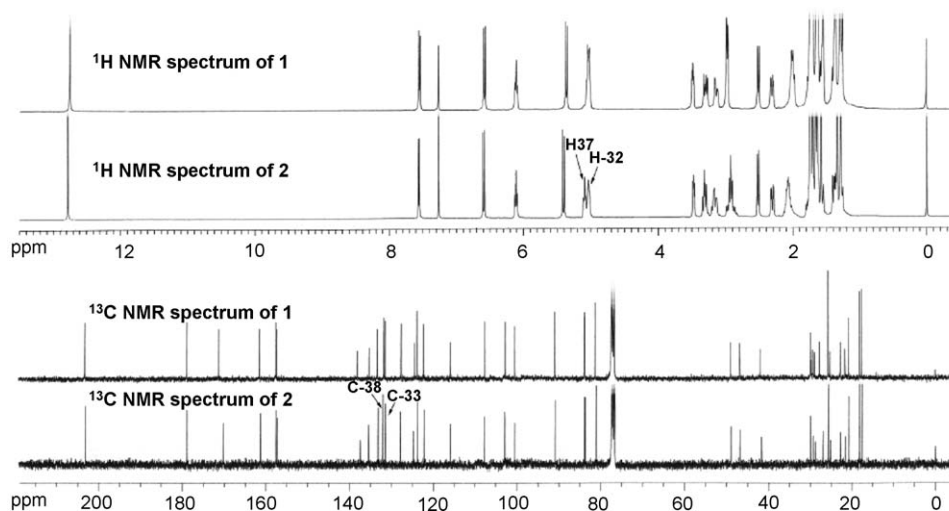


Fig. 2. ¹H (400 MHz) and ¹³C (100 MHz) NMR spectra of **1** and **2** (in CDCl₃, TMS as internal standard).

2.4. HSCCC separation procedure

The whole procedure was carried out in the following three steps. *Step 1:* The coil column was first entirely filled with the upper phase of the solvent system at 10 ml/min. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at 2.0 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 5 ml of sample solution containing 50 mg of crude extract was injected through the injector. The separation temperature was controlled at 20 °C. The effluent from the outlet of the column was continuously monitored at 360 nm by Agilent 1100 HPLC UV–vis detector and ChemStation. *Step 2:* After the sample injection, the switching valve was turned to form a recycling tube. *Step 3:* When the target sample was completely separated after several CCC cycles, the switching valve was returned to its original position to release the separated samples. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure. The residue was dissolved in methanol for subsequent HPLC analysis.

2.5. HPLC analysis and identification of CCC peak fractions

The epimers mixture and each HSCCC peak fraction were analyzed by HPLC on a C₈ column eluted with CH₃CN/0.1% acetic acid/1,4-dioxan (60:30:10, v/v/v). The flow rate was 1.0 ml/min, and the effluents were monitored at 360 nm by a photodiode array detector. The HSCCC peak fraction was then identified by their ¹H (400 MHz) and ¹³C (100 MHz) NMR spectra.

3. Results and discussion

The separation included six cycles as shown in Fig. 3. The first cycle, about 150 min, yielded one single peak. This might suggest these epimers are eluted together. Nevertheless soon after that, the second separation cycle exhibited a fork-like peak. This peak then divided into two in the subsequent cycles. At the same time, the peak range extended from 50 min in the first cycle to 150 min in the sixth cycle. This suggested that the peak would be better separated in the seventh cycle. Meanwhile, the end of

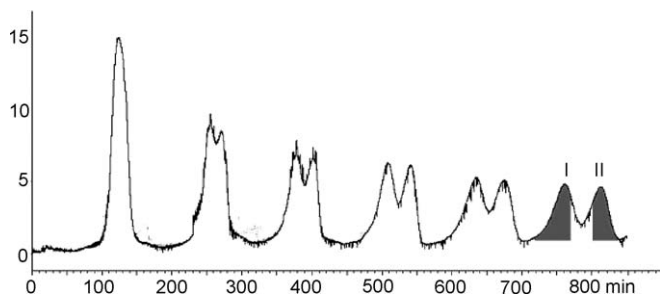


Fig. 3. Chromatogram of gambogic acids by preparative recycling counter-current chromatography. Solvent system: *n*-hexane–methanol–water (5:4:1, v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 50 mg dissolved in 5 ml of lower phase.

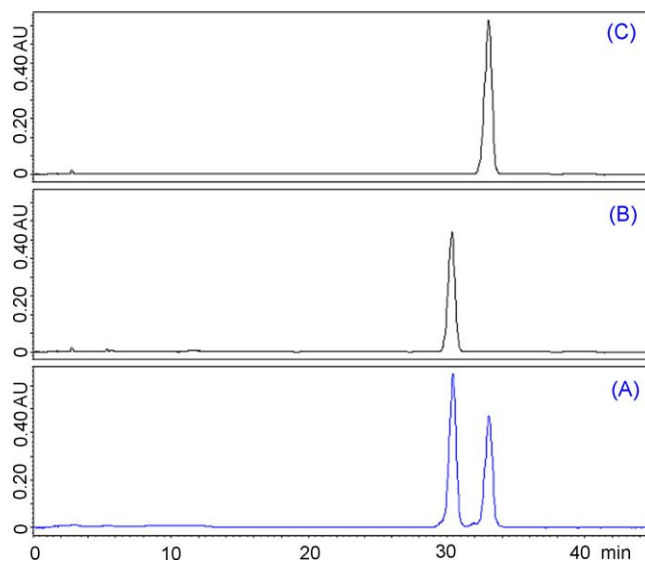


Fig. 4. HPLC analyses of mixed and separated gambogic acid epimers on a C₈ column eluted with CH₃CN/0.1% acetic acid/1,4-dioxan (60:30:10, v/v/v). The flow rate was 1.0 ml/min, and the effluents were monitored at 360 nm by a photodiode array detector. (A) Mixed gambogic acid epimers (reference), (B) *R*-epimer, (C) *S*-epimer.

peak II of the sixth cycle would overlap the front of the next peak I because the column volume was limited. The separated peaks were then collected as shown in Fig. 3 and analyzed by analytical HPLC. The HPLC chromatograms of the separated peaks are displayed in Fig. 4. Their purities were determined to be 97.2% and 97.5% by HPLC analysis with DAD detector, respectively. Peaks I and II were identified to be gambogic acid and epigambogic acid, respectively, by HPLC comparison with the reference compounds and by MS, ¹H and ¹³C NMR spectroscopic analysis as described in our previous paper [5].

As a newly developed method, the recycling CCC showed its unique advantage in achieving effective separations through preventing stationary phase loss. In the case of gambogic acids, when these epimers could be separated by neither the common HSCCC nor two coupled HSCCC, the recycling operation in one HSCCC system would be the best way to do the separation.

The peak extension between separation cycles might be an impact factor for a successful separation. More separation cycles could be carried out with a shorter peak extension. Therefore in order to shorten the peak extension, it might be useful to use a mobile phase in which the target compounds are easily dissolved. Moreover, the initial target peaks must be limited in a narrow range, which made the recycling method unsuitable for the simultaneous separation of several compounds.

The results of our studies demonstrated that recycling counter-current chromatography is an effective method for the preparative separation of gambogic acid and its C-2 epimer, epigambogic acid.

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