Process Biochemistry 45 (2010) 799–804

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

Isolation and purification of silychristin, silydianin and taxifolin in the co-products of the silybin refined process from the silymarin by high-speed counter-current chromatography

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ARTICLE INFO

Article history:
Received 16 October 2009
Received in revised form 8 December 2009
Accepted 5 January 2010

Keywords:
Silychristin
Silydianin
Taxifolin
Silymarin
HSCCC
Pre-RPLC

ABSTRACT

A preparative high-speed counter-current chromatography (HSCCC) method for isolation and purification of silychristin, silydianin and taxifolin in the co-products of the silybin refined process from the silymarin was successfully established by n-hexane–chloroform–methanol–water (0.5:11:10:6 (0.5 acetic acid), v/v/v/v) as the two-phase solvent system. 146 mg silydianin, 280 mg silychristin and 63 mg taxifolin from 1.463 g co-products sample in one separation were obtained with the purities of 95.1%, 99.3% and 98.2%, respectively, determined by HPLC. The structures of the compounds were identified by means of ESI-MS-MS, TOF-MS, ^{1}H NMR, ^{13}C NMR and 2DNMR-HMBC. Silychristin, silydianin and taxifolin had been separated as standards by HSCCC for the first time. A comparative study between HSCCC and RPLC for separation and isolation of taxifolin, silychristin and silydianin was investigated. The differences between the two preparative chromatographic methods were all discussed. The results demonstrated that HSCCC was a powerful separation tool and could contribute to identifying and quantifying plant ingredients.

1. Introduction

Silymarin, derived from the milk thistle plant *Silybum marianum*, has been used widely for centuries for the protection of the liver from toxic substances [1]. Silymarin [2–4] primarily consists of an isomeric mixture of active flavonolignans: silychristin, silydianin and two diastereoisomers of silybin (silybin A and silybin B) and isosilybin (isosilybin A and isosilybin B) and a flavonoid: taxifolin. Silybin is the principal active component of silymarin [4,5]. There were many other different biological active components [6–9] such as silychristin, silydianin and taxifolin. There were lots of these active components in the co-products in the refined process of silybin. In order to increase revenues from a given feedstock, these co-products should be further isolated and purified.

There were several reports about respective preparative methods for the major active constituents from silymarin [3,5,10,11]. However, these methods were usually complex, time consuming and many steps, involving repetitive precipitation, column chromatographies [3] and TLC [5]. And more studies focusing on the compounds (silybin A, silybin B, isosilybin A and isosilybin B) were described subsequently [5,10].

High-speed counter-current chromatography (HSCCC), being 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 [13,14]. The separation and purification fruit extract of *Silybum marianum* by HSCCC [15] and fast centrifugal partition chromatography (FCPC) [16] were recently reported with a two-phase solvent system consisting of heptane–ethyl acetate–methanol–water (1:4:3:4, v/v/v/v). However, the purities of silychristin and silydianin were very low and the diastereoisomers of silybin and isosilybin were overlapped.

In this paper, analytical HSCCC was used for the systematic selection and optimization of the two-phase solvent system for the separation of taxifolin, silychristin and silydianin in co-products of the silybin refined process from the silymarin. Using the optimized solvent system, the preparative HSCCC separation of taxifolin, silychristin and silydianin with high purity was performed. At the same time, a comparative study between HSCCC and RPLC for separation and isolation of taxifolin, silychristin and silydianin was investigated. The differences between the two preparative chromatographic methods were all discussed.

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doi:10.1016/j.procbio.2010.01.004

2. Materials and methods

2.1. Materials

Silymarin extract was kindly provided by Wagott Pharmaceutical Co. Ltd. (Sichuan, China). Taxifolin, silychristin and silydianin standard over 98% purity were prepared by pre-LC in our laboratory in another paper as an unpublished data. All organic solvents used for sample preparation or HSCCC separation were of analytical grade and purchased from Beijing Reagent Co. (Beijing, China). Solvents used for HPLC and MS analyses were of chromatographic grade and purchased from Fisher Scientific. $CDCl_3$ used for NMR experiments was also purchased from Fisher Scientific.

2.2. Pre-separation of the crude extract by recrystallization

The sample of co-products was obtained from the residue of the recrystallization of silybin using methanol according to Kim et al. [3]. 20 g silymarin extract was first dissolved in hot methanol. The mixture was filtered, and the filtrate was collected. Then the mixture was concentrated to 1/3 volume by rotary evaporator (RE-52A, Shanghai Yarong Instrument, China) until the crystals were precipitated. Then add a small amount of methanol to dissolve the precipitation at 79 °C. Then, the mixture was stored at 4 °C for 48 h. Silybin crystals were collected by vacuum filter, washed with hot methanol and petroleum ether. The filtrate was collected, concentrated and crystallized. Then, all the obtained crystals were combined, recrystallized and vacumm-dried. The filtrate was collected, concentrated and then used for the samples of the HSCCC.

2.3. Preparation of two-phase solvent system

The two-phase solvent system (v/v/v/v) used in the paper was prepared by adding the solvents to a separation funnel according to the volume ratios (Table 1) and thoroughly equilibrated at room temperature, and the two phases were separated shortly before use. The stationary phase was the upper aqueous phase and the mobile phase was the lower organic phase.

2.4. Systematic selection of the two-phase solvent system by analytical HSCCC

Analytical HSCCC was first used for the systematic selection of the two-phase solvent system. The analytical HSCCC instrument was performed on a Model TBE-20 A (Tauto Biotech, Shanghai, China) equipped with three preparative multiplayer coils (total volume 20 mL, wound with 2.6 mm I.D. PTFE tubing). A manual sample injection valve with a 20 μL loop was used.

The whole procedure was as follows: the upper (stationary) phase was pumped into the coil to completely fill it. The apparatus was then rotated at 1700 rpm, and the lower phase was pumped into the column at a flow rate of 0.5 mL/min. The temperature was set at 25 °C. After the lower (mobile) phase emerged and hydrodynamic equilibrium was established in the column, 20 μL sample solution (containing 3 mg/mL co-products) was injected through the injection valve. The effluent of the column was continuously monitored with an UV detector at 280 nm.

2.5. PRE-HSCCC separation procedure

The HSCCC instrument in the present study was TBE-300A high-speed counter-current chromatography (Tauto Biotechnique, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.5 mm, total volume = 260 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the values β ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge) of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 1000 rpm. The system was also equipped with one TBP-50A constant flow pump, a TBD-UV detector and a constant temperature circulator (Tauto Biotechnique, Shanghai, China). The data were collected with the model N2000 chromatography workstation (Zhejiang University, Hangzhou, China). The HSCCC tubing was first filled with the (aqueous) stationary phase with no rotation at a flow rate of 10 mL/min. Then, the coils were being rotated at 850 rpm as the (organic) mobile phase was pumped at a flow rate of 2 mL/min from head-to-tail. In order to observe the stationary phase retention volume ratio in the column, the resulting effluent was collected in a graduated cylinder. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column. After hydrodynamic equilibrium was kept for 30 min, the sample solution by dissolving 1463 mg co-products in 20 mL the lower phase was injected into the separation column through the injection valve. The effluent from the tail end of the separation column was continuously monitored at 280 nm. After the sample injection, the data were collected immediately. The fractions were collected manually according to the obtained chromatogram and then evaporated under reduced pressure. The residuals were dissolved in methanol for subsequent purity analysis by HPLC.

2.6. Preparative RPLC separation procedure

Preparative LC was performed on a Waters DP 4000 and a reversed phase C18 column (Symmetry prep™ C18, 7 μm, 19 mm × 300 mm). The pre-LC system was controlled by Waters Empower software (Waters, Milford, MA, USA). The mobile phase was solvent A (methanol in water (70:30, v/v)) and solvent B (methanol in water (30:70 (1‰ formic acid), v/v)). Gradient A% in B was as follows: initial, 30% A; 45 min, 60% A; 80 min, 60% A; 80.01 min, 30% A; 120 min, stop. Flow rate was 8 mL/min. Injection volume was 3 mL. Compound separation was monitored with a Waters 2487 dual λ absorbance detector set at 254 and 288 nm. Column temperature was room temperature. The production peaks were collected manually at 288 nm, respectively. Samples for pre-LC were dissolved in a mixture of CH_3OH:Water (70:30, v/v). The samples were filtered quickly through a 0.45 μm membrane filter, before they were used for pre-LC. The optimum chromatography operating parameters were discussed in detail in another paper as unpublished data.

2.7. HPLC analysis and identification of CCC peak fractions

The co-products sample and each peak fraction were analyzed by HPLC on a Shimadzu LC-20AT and a reversed phase C18 column (250 mm × 4.6 mm, 5 μm, Diamodsil™). The mobile phase was solvent A: methanol and solvent B: water (1‰ formic acid) in the gradient mode as follows modified according to the paper [17]: 0–3 min, 43% A; 3–13 min, 43–50% A; 13–25 min, 50–60% A; 25–35 min, kept at 60% A; 35.01 min 43% A, 45 min stop. Flow rate was 1 mL/min; injection volume was 10 μL; wavelength was 288 nm and column temperature was 30 °C. Routine sample calculations were made by comparison of the peak area with that of the standard. Chromatographic peaks of taxifolin, silychristin and silydianin were confirmed by comparing their retention time and UV spectrum with those of the reference compounds.

Identifications of the target compounds (silydianin, silychristin and taxifolin) were based on ESI-MS, ESI-MS-MS, TOF-MS, ^{1}H NMR, ^{13}C NMR and 2DNMR-HMBC spectra. ESI-MS/MS-MS analyses were performed using a Waters Acquity Ultra-Performance Liquid Chromatograph (UPLC) coupled to a Waters Acquity Triple Quad Detector operated in the ESI mode. UPLC-TOF-MS analysis were performed using a Agilent Series 1200 Ultra-Performance Liquid Chromatograph (UPLC) coupled to a Agilent MSD TOF 6210 equipped with an Dual electrospray interface operating in positive ion. The instrument performed the accurate-mass internal mass calibration automatically using a nebulizer ion source combined with an automated calibrant delivery system, which introduced the internal reference masses (121.0509, 149.0233, 322.0581, 922.0098, 1221.9906, 1521.9714 and 1241.9140). ^{1}H NMR, ^{13}C NMR and 2DNMR-HMBC spectra were performed using a Brucker AV600 NMR Spectrometer.

3. Results and discussion

3.1. Pre-separation of the crude extract of silymarin by recrystallization

The HPLC analyses of the co-products after the crystallization of silybin from the crude silymarin extract in our laboratory indicated that it contained several compounds (Fig. 1). The purities of the taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B in the co-products were 8.96%,

Table 1
The partition coefficient of main active compounds in silymarin.

Solvent system (v/v/v/v)	$K_{taxifolin}$	$K_{silychristin}$	$K_{silydianin}$	$K_{silybin\ A}$	$K_{silybin\ B}$	$K_{isosilybin\ A}$	$K_{isosilybin\ B}$
n-Hexane–ethyl acetate–methanol–water (1:4:3:4)	0.48	1.07	1.23	1.54	1.57	1.61	1.64
Petroleum ether–ethyl acetate–methanol–water (0.6:2:1:1)	0.49	0.62	1.16	1.42	1.43	1.56	1.62
n-Hexane–dichloromethane–methanol–water (0.8:6.4:6:3)	0.53	0.72	1.21	2.49	2.49	2.55	2.57
n-Hexane–trichloromethane–methanol–water (0.5:12:10:5)	2.23	1.65	1.28	0.56	0.55	0.51	0.51
n-Hexane–trichloromethane–methanol–water (0.5:12:10:5 (2% acetic acid))	1.82	1.36	1.01	0.65	0.62	0.53	0.51
n-Hexane–trichloromethane–methanol–water (0.5:11:10:6 (0.5% acetic acid))	1.94	1.49	1.11	0.72	0.70	0.56	0.53

K = CU/CL = Peak area of analyte in upper phase/peak area of analyte in lower phase.