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Preparative Isolation of Six Major Saponins from Platycodi Radix by High-speed Counter-current Chromatography

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ABSTRACT:

Introduction – Platycosides, the primary constituents of Platycodi Radix, are known to have numerous and varied biological activities, exerting anti-inflammation, anti-allergy, anti-tumour, anti-obesity and anti-hyperlipidemia effects. However, effective methods for isolating and purifying platycosides from Platycodi Radix are not currently available.

Objective – To develop an efficient method for the preparative separation of six platycosides from Platycodi Radix by high-speed counter-current chromatography (HSCCC) coupled with an evaporative light scattering detection (ELSD) system.

Methodology – Preparative separation was performed by water extraction using reversed-phase C_{18} column chromatography on an HSCCC-ELSD system. A two-phase solvent system comprised hexane–*n*-butanol–water (1:40:20, v/v) and (1:10:5, v/v) was employed. Two other key parameters, revolution speed of the separation column and flow-rate of the mobile phase, were also investigated for optimum HSCCC performance. Each peak fraction obtained from separation of the platycosides was collected according to the ELSD elution profile and determined by HPLC.

Results – Using the described method, six platycosides, all with purities of over 94%, could be isolated from 300 mg of the platycoside-enriched fraction. Their structures were characterized by electrospray ionisation mass spectrometry (ESI-MS), ^{1}H-NMR and ^{13}C-NMR.

Conclusion – Six of the main bioactive platycosides in Platycodi Radix could be isolated and purified systematically by HSCCC.
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Keywords: High-speed counter-current chromatography; preparative isolation; evaporative light scattering detection; platycosides; Platycodi Radix

Introduction

Platycodi Radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae), is used in traditional oriental medicine as an expectorant and antitussive to treat coughs, colds, upper respiratory tract infections, sore throats, tonsillitis and chest congestion. Platycosides, the primary constituents of Platycodi Radix, are known to have numerous and varied biological activities, exerting anti-inflammation, anti-allergy, anti-tumour, anti-obesity and anti-hyperlipidemia effects, as well as augmenting immune responses and stimulating apoptosis in skin cells (Han *et al*., 2002; Shin *et al*., 2002; Choi *et al*., 2004; Lee *et al*., 2004; Wang *et al*., 2004; Ahn *et al*., 2005; Kim *et al*., 2005, 2006; Zhao *et al*., 2005).

A large amount of pure platycoside is required for pharmacological studies, for 'marker compounds' in the chemical evaluation or standardization of Platycodi Radix, and for producing medicinal products. Thus, an effective method of isolating and purifying platycosides from Platycodi Radix is required. Such saponins are often obtained by conventional protocols of extraction and separation using, for example, organic solvents on column chromatography including silica gel and high-performance liquid chromatography (HPLC). However, organic solvents produce environmental problems, and conventional separation methods are tedious, time-consuming, require multiple steps, can result in irreversible adsorptive loss of sample in the stationary phase, and can entail artefact formation.

High-speed counter-current chromatography (HSCCC) is an all-liquid partition chromatography (Conway and Ito, 1984). HSCCC eliminates the irreversible adsorptive loss of samples onto the solid support matrix used in conventional chromatographic columns, and offers excellent sample recovery compared with some conventional methods. This method has been used successfully for the preparative separation of natural products from traditional herbal medicines (Gu *et al*., 2004; Chu *et al*., 2005; Wei and Ito 2006; Yanagida *et al*., 2006; Ha *et al*., 2007).

Saponins exhibit very weak absorptions, even in the short wavelength range, rendering sensitive detection by ultraviolet (UV) spectroscopy impossible. Some recent papers have shown that substituting evaporative light scattering detection (ELSD) for UV allows for successful monitoring of saponins from HSCCC effluent (Ha *et al*., 2007; Shi *et al*., 2007; Yao *et al*., 2007; Tang *et al*., 2008).

However, there have yet to be any reports on the use of HSCCC for the isolation of platycosides derived from Platycodi Radix. Therefore, in the present study, six polar platycosides

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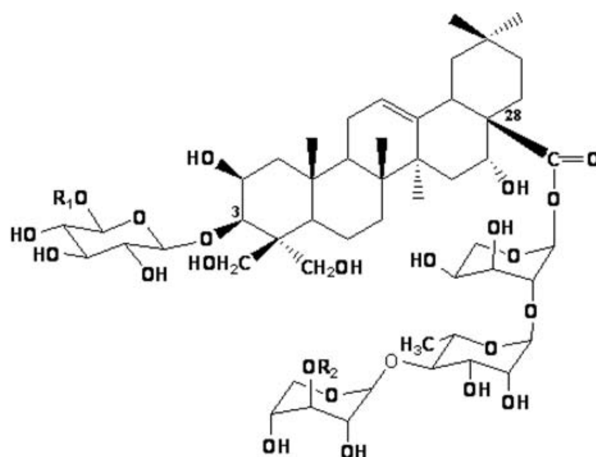

Name	Molecular formula	Molecular weight	R_1	R_2
Deapi-platycoside E	$C_{64}H_{104}O_{34}$	1416	Gen	H
Platycoside E	$C_{69}H_{112}O_{38}$	1548	Gen	Api
Deapi-platycodin D_3	$C_{58}H_{94}O_{29}$	1254	Glc	H
Platycodin D_3	$C_{63}H_{102}O_{33}$	1386	Glc	Api
Deapi-platycodin D	$C_{52}H_{84}O_{24}$	1092	H	H
Platycodin D	$C_{57}H_{94}O_{28}$	1224	H	Api

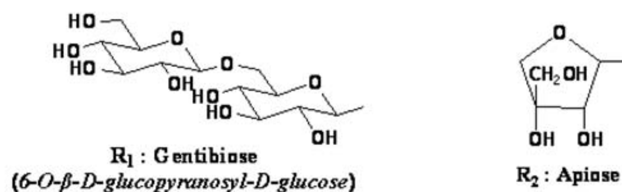

Figure 1. Structures of platycosides.

(platycoside E, platycodin D_3, platycodin D and their deapiose forms; Fig. 1) were isolated from Platycodi Radix by HSCCC-ELSD.

Experimental

Apparatus. A TBE-300A HSCCC (Shanghai Tauto Biotech Co. Ltd, Shanghai, China) with three serially connected multilayer coil separation columns (i.d. of the tubing = 1.6 mm; total volume = 260 mL) and a 20 mL sample loop was used for this study. The revolution radius, or the distance between the holder axis and the central axis of the centrifuge (R), was 5 cm, and the β values of the multilayer coil 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). The speed of revolution of the apparatus coould be regulated with a speed controller within the range 0–1000 rpm. The solvent was infused into the column using a Hitachi model L-6200 HPLC pump (Tokyo, Japan) and the eluent was continuously monitored by connecting the tail outlet of the coiled column to a Sedex 55 (Sedere, Virty-sur-Seine, France) ELSD system through a split valve.

Reagents and materials. Acetonitrile, methanol, *n*-butanol, ethyl acetate, hexane and isopropanol (HPLC-grade) were purchased from Fisher Scientific (Pittsburg, PA, USA) for the preparation of the crude sample and for HSCCC separation. Distilled water (NANO pure Diamond, Barnstead, MA, USA) was used in all solutions and for all dilutions. The reversed-phase C_{18} resin was purchased from Merck (Darmstadt, Germany). Platycodi Radix was obtained from a local market in Seoul, Korea.

Preparation of crude sample. The dried Platycodi Radix was extracted by sonication with water for 3 h at room temperature. The extraction procedure was repeated three times. The extracts were combined and evaporated to dryness under reduced pressure. The crude extract was loaded onto a reversed-phase C_{18} open column (50 × 3 cm i.d.; the volume of the column was 250 mL) and sequentially eluted with water, and 30 and 70% methanol. The enriched platycoside fraction was eluted with 70% methanol from the crude extract of Platycodi Radix. This fraction was evaporated, lyophilised and stored in a refrigerator for the subsequent HSCCC separation.

Measurement of partition coefficients. The composition of the two-phase solvent system was selected according to the partition coefficient (K) of the target compounds in the enriched platycoside fraction. The partition coefficients for the solvent system were determined by HPLC. A suitable amount of the enriched platycoside fraction was dissolved in 2 mL of the aqueous phase of the pre-equilibrated two-phase solvent system, and the peak area was recorded as $A1$. An equal volume of the

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organic phase was then added to the solution and mixed thoroughly. After equilibrium was established, the aqueous phase was determined by HPLC again, and the peak area was recorded as *A*2. The partition coefficient (*K*) was obtained by the following equation: $K = (A1 - A2)/A2$.

Preparation of the two-phase solvent system and sample solution. A solvent system consisting of hexane–*n*-butanol–water (1:40:20, v/v) was used for the separation of platycoside E and deapio-platycoside E. Hexane–*n*-butanol–water (1:10:5, v/v) was used as the solvent system for separation of platycodin D_3, platycodin D and their deapiose forms. The solvent mixtures were thoroughly equilibrated in a separation funnel at room temperature, and the two phases were separated shortly before use. The sample solution was prepared by dissolution in a 20 mL mixture of the lower and upper phases (1:1, v/v) of the solvent system used for the HSCCC separation.

HSCCC separation. In the first separation process for platycoside E and deapio-platycoside E, the lower phase (stationary phase) and the upper phase (mobile phase) were simultaneously pumped (HPLC pump) into the multilayer-coiled column in a 70:30 volumetric ratio. When the column was totally filled with the two phases, the lower phase alone was pumped at a flow rate of 1.3 mL/min. At the same time, the HSCCC apparatus was run at a revolution speed of 850 rpm. After hydrodynamic equilibrium was reached, the sample solution was injected into the separation column.

In the second separation for platycodin D_3, platycodin D and their deapiose forms, the upper phase (stationary phase) and the lower phase (mobile phase) were again pumped simultaneously into the multiplayer-coiled column according to the 70:30 volumetric ratio. When the column was totally filled with the two phases, the upper phase alone was pumped at a flow rate of 1 mL/min for platycodin D_3 and deapio-platycodin D_3, and 1.5 mL/min for platycodin D and deapio-platycodin D. Then, the HSCCC apparatus was run at 850 rpm. The eluent was continuously monitored by connecting the outlet of the coiled column with the ELSD system through a split valve. The ELSD system was set to a probe temperature of 70°C and a gain of 6, and the nebuliser gas (nitrogen) was adjusted to 2.2 bar. Each peak fraction obtained from separation of the platycosides was collected according to the elution profile and determined by HPLC. After each separation was completed, the retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas.

HPLC analysis and identification of HSCCC peak fractions. Analysis of the saponin-enriched fraction for each peak collected from the HSCCC was carried out on a Hitachi L-6200 HPLC equipped with a Sedex 75 ELSD and an SIL-9A auto injector (Shimadzu, Kyoto, Japan). A Zorbax SB-Aq C_{18} column (150 × 4.6 mm i.d.; 5 μm particle size) from Agilent Technologies (Palo Alto, CA, USA) was used for all of the separations. The HPLC conditions for the platycosides were as follows: eluent A, water; eluent B, acetonitrile; gradient, 0–6 min (10–15% B), 6–50 min (15–25% B), 50–60 min (25–47.5% B) and then equilibrated with 10% B for 8 min at a flow of 1 mL/min. The ELSD system was set to a probe temperature of 70°C and a gain of 7, and the nebuliser gas (nitrogen) was adjusted to 2.5 bar.

Identification of the HSCCC peak fractions was carried out by ESI-MS, ^{1}H-NMR and ^{13}C-NMR spectra with references. The ESI-MS experiments were performed in a negative ion mode using an LCQ DECA XP MS (Thermo Finnigan, San Jose, CA, USA) system equipped with an electrospray source. The source was operated at 4 kV ion spray voltage, −15 V capillary voltage, 275°C capillary temperature, and under nitrogen sheath gas set at 35. The sample was analysed by direct injection through a 2 μL loop, and the elution solvent was a mixture of water and methanol (50:50, v/v). For the MS/MS, the $[M - H]^-$ of each purified platycoside was selected as a precursor ion, and the MS/MS product ions were obtained using the condition of 27% collision energy in the ion trap analyser. The NMR spectra were recorded on a Varian (Palo Alto, CA, USA) model UI5000 spectrometer (500 MHz) with TMS (tetramethylsilane) as an internal standard.

Results and Discussion

Selection of the two-phase solvent system and HSCCC conditions

A series of experiments were performed in order to determine the optimum two-phase solvent system for HSCCC separation of each platycoside. The platycoside-enriched fraction, containing all 13 platycosides with three pairs of platycosides and their deapiose forms, was used as a testing material (Ha *et al*., 2006). In this study, HSCCC was employed for the preparative isolation of three pairs of platycosides and their deapiose forms.

The chloroform–methanol–water solvent system is widely used in HSCCC, and is suitable for separation of glycoside compounds such as flavonoid glycosides and many saponins. However, the platycosides, due to their highly polar characters, were tested in an *n*-butanol–water system.

In conventional HSCCC experiments, a multi-layer coiled column is first entirely filled with one phase of the two-phase solvent system as a stationary phase, followed by elution with the other phase. Here, the column was first filled with a mixture of the two phases, thus reducing the amount of time for hydrodynamic equilibrium to be established (Slacanin *et al*., 1989). The ratio of two phases was also optimised at 70:30 (stationary phase–mobile phase, v/v) within the range 90:10 to 60:40 based on the amount of time required to reach hydrodynamic equilibrium owing to retention on the stationary phase.

In the present experiment, several solvent systems based on *n*-butanol–water with added hexane or ethyl acetate were tested, and the results are summarized in Table 1. The table shows that platycoside E and deapio-platycoside E, when analysed in the reverse-mode solvent system composed of *n*-butanol–water at volume ratios of 2:1 (v/v), have appropriate *K* values (0.5–5) for separation. However, the *K* values of platyodin D and deapio-platycodin D, in the same solvent system, were smaller than expected. It was difficult to separate platyodin D and deapio-platycodin D from the other compounds. Also, when *n*-butanol–water (2:1, v/v) was used, the retention of the stationary phase was poor (<30%), and so that system was deemed unsuitable for separation.

The addition of ethyl acetate or hexane to the *n*-butanol–water system could improve retention on the stationary phase (>40%). However, the *K* values of the target compounds became unsatisfactory in the *n*-butanol–water–ethyl acetate solvent systems. When the *n*-butanol–water–hexane system (10:5:1, v/v) was used for HSCCC separation, the *K* values of platycodin D_3, platycodin D and their deapiose forms were found to be suitable, but the *K* values of platycoside E and deapio-platycoside E were too small.

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Table 1. The *K* values of target compounds from Platycodi Radix as measured in the solvent systems

	Solvent system	Volume ratio	Mode	dPE	PE	dPD_3	PD_3	dPD	PD
1	B–W	1:1	Normal	0.4	0.27	1.06	1.11	11.06	8.04
2			Reverse	2.5	3.7	0.94	0.9	0.09	0.12
3	B–W	2:1	Normal	0.4	0.25	1.41	1.12	13.32	9.45
4			Reverse	2.5	4	0.71	0.89	0.08	0.11
5	B–W	1:2	Normal	0.41	0.28	1.05	0.97	8.79	6.79
6			Reverse	2.44	3.57	0.95	1.03	0.11	0.15
7	B–W–E	2:1:2	Normal	<0.01	<0.01	0.12	<0.01	0.26	0.32
8			Reverse	∞	∞	8.33	∞	3.85	3.13
9	B–W–E	1:2:1	Normal	<0.01	<0.01	0.81	0.64	2.32	1.72
10			Reverse	∞	∞	1.23	1.56	0.43	0.58
11	B–W–E	1:1:1	Normal	0.03	0.02	0.7	0.63	2.08	1.95
12			Reverse	33.33	50	1.43	1.59	0.48	0.51
13	B–W–H	10:5:1	Normal	0.09	0.07	1.14	0.69	3.34	2.06
14			Reverse	11.11	14.28	0.88	1.45	0.3	0.49
15	B–W–H	5:5:1	Normal	0.06	0.04	0.88	0.54	1.73	1.14
16			Reverse	16.66	25	1.14	1.85	0.48	0.87
17	B–W–H	40:20:1	Normal	0.38	0.23	1.25	0.96	10.65	8.14
18			Reverse	2.63	4.35	0.8	1.04	0.09	0.12

Normal mode: use of lower phase as a mobile phase. Reverse mode: use of upper phase as a mobile phase. dPE, deapio-platycoside E; PE, platycoside E; dPD_3, deapio-platycodin D_3; PD_3, platycodin D_3; dPD, deapio-platycodin D; PD, platycodin D; B, *n*-butanol; W, water; E, ethyl acetate; H, hexane.

In contrast, when the *n*-butanol–water–hexane system (40:20:1, v/v) was used in reversed mode, the *K* values of platycoside E and deapio-platycoside E were suitable, whereas those of platyodin D and deapio-platycodin D were too small. Thus, HSCCC separation could not be performed using the single two-phase solvent system mentioned above. Therefore, the gradient elution mode was tested for the one-step HSCCC separation with *n*-butanol–water–hexane (40:20:1, v/v) and *n*-butanol–water–hexane (10:5:1, v/v). In the gradient elution mode retention on the stationary phase was extremely low and, consequently, target compounds could not be separated. Therefore, the platycoside-enriched fraction was separated in two stages. First, platycoside E, deapio-platycoside E, mixed fraction I (containing platyodin D and deapio-platycodin D) and mixed fraction II (containing platycodin D_3 and deapio-platycodin D_3) were separated using the *n*-butanol–water–hexane (40:20:1, v/v) reversed-mode system. Second, mixed fractions I and II could be satisfactorily separated with *n*-butanol–water–hexane (10:5:1, v/v) in the normal elution mode. When other solvent systems were used for HSCCC separation, such as *n*-butanol–water and those containing methanol, ethanol, isopropanol, acetonitrile or methyl *tert*-butyl ether, the target compounds could not be successfully separated.

Two other key parameters, revolution speed of the separation column and flow rate of the mobile phase, were also investigated for optimum HSCCC performance. When a revolution speed of 850 rpm and a flow rate of 1.3 mL/min were employed in separation, platycoside E and deapio-platycoside E were isolated and the retention of stationary phase in the coil was about 48%. Platycodin D_3 and deapio-platycodin D_3 were separated and purified under the optimum revolution speed (850 rpm) and flow rate (1 mL/min) with 62% of the stationary phase retention. Finally, a revolution speed of 850 rpm and a flow rate of 1.5 mL/min were employed in separating platyodin D and deapio-platycodin D (Fig. 2). Under these conditions the retention of stationary phase in the coil was about 56%.

When the platycoside-enriched fraction (300 mg) was separated by HSCCC under the above conditions, 21 mg of platycoside E, 14 mg of deapio-platycoside E, 10 mg of platycodin D_3, 6 mg of deapio-platycodin D_3, 28 mg of platycodin D and 6 mg of deapio-platycodin D could be obtained. The purities of platycoside E, deapio-platycoside E, platycodin D_3, deapio-platycodin D_3, platyodin D and deapio-platycodin D were 98.4, 97.3, 99.1, 98.1, 96.3 and 94.2%, respectively, as determined by HPLC-ELSD (Fig. 3).

Structural identification

The chemical structures of components present in each peak fraction purified by HSCCC were identified from ESI-MS, ESI-MS/MS, ^{1}H-NMR and ^{13}C-NMR data. Initially, the ESI-MS experiments were performed in both the negative and positive mode for determining molecular weights. The ESI-MS/MS were obtained in the negative mode for further structural elucidation and to facilitate comparison with a previous study (Ha *et al*., 2006): peak 1, molecular weight (1416), MS/MS product ion (1283.2, 1253.2, 1091.2, 1005.3); peak 2, molecular weight (1548), MS/MS product ion (1415.3, 1385.0, 1283.1, 1223.7); peak 3, molecular weight (1224), MS/MS product ion (1091.0, 959.1, 681.1); peak 4, molecular weight (1092), MS/MS product ion (959.3, 681.1); peak 5, molecular weight (1386), MS/MS product ion (1253.2, 1121.2, 843.1); and peak 6, molecular weight (1253), MS/MS product ion (1121.2, 843.2). The subsequent structural identification of the peak fractions collected from the HSCCC was performed by comparison with previous ^{1}H-NMR and ^{13}C-NMR data (Ishii *et al*., 1984; Nikaido *et al*., 1999; Kim *et al*., 2005; Fu *et al*., 2006a, b). Thereby, the purified peaks were identified as follows: peak 1, deapio-platycoside E;

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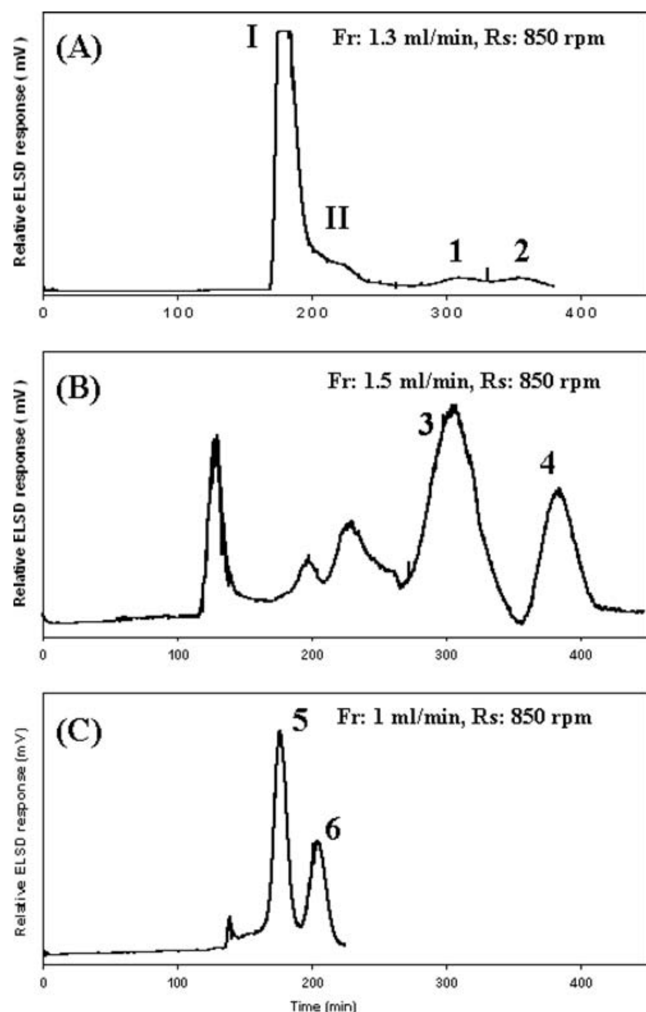

Figure 2. HSCCC chromatograms of the platycoside fractions showing: (A) platycoside-enriched fraction—peak I (fraction containing platycodin D and deapio-platycodin D), peak II (fraction containing platycodin D_3 and deapio-platycodin D_3), peak 1 (deapio-platycoside E), peak 2 (platycoside E); solvent system, hexane–*n*-butanol–water (1:40:20, v/v); partition mode, reverse mode, upper phase was used as the mobile phase; sample, 300 mg dissolved in 20 mL of mixture solution with the lower phase and upper phase (1:1, v/v) of the solvent system; (B) peak I fraction—peak 3 (platycodin D), peak 4 (deapio-platycodin D); solvent system, hexane–*n*-butanol–water (1:10:5, v/v); partition mode, normal mode, lower phase was used as the mobile phase; sample, dried peak I fraction presented in (A) dissolved in 10 mL of mixture solution with the lower phase and upper phase (1:1, v/v) of the solvent system; (C) peak II fraction—peak 5 (platycodin D_3), peak 6 (deapio-platycodin D_3); solvent system, hexane–*n*-butanol–water (1:10:5, v/v); partition mode, normal mode; lower phase was used as the mobile phase; sample, dried peak II fraction presented in (A) dissolved in 10 mL of mixture solution with the lower phase and upper phase (1:1, v/v) of the solvent system. Detection. ELSD; drift tube temperature, 70°C; nitrogen flow rate, 2.5 bar. Fr, flow rate; Rs, revolution speed.

peak 2, platycoside E; peak 3, platycodin D; peak 4, deapio-platycodin D; peak 5, platycodin D_3; and peak 6, deapio-platycodin D_3.

In conclusion, six platycosides, the main bioactive constituents in Platycodi Radix, an important oriental herbal medicine, were isolated and purified systematically by HSCCC.

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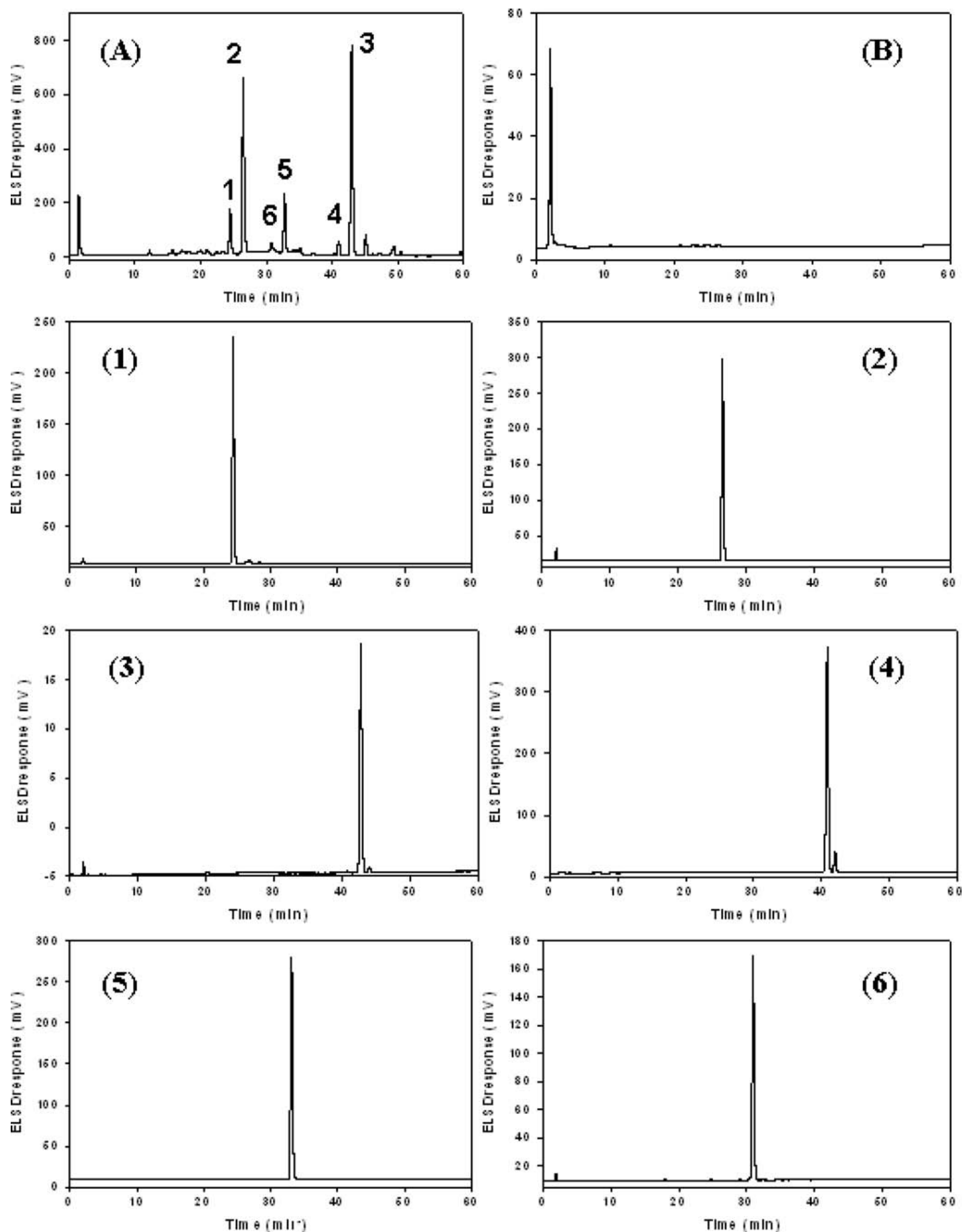

Figure 3. HPLC chromatograms of the platycoside-enriched fraction from Platycodi Radix (A) showing: the residual sample in the coil after the first HSCCC separation (B), and the HSCCC peak fractions (1–6). Peak 1, deapio-platycoside E; peak 2, platycoside E; peak 3, platycodin D; peak 4, deapio-platycodin D; peak 5, platycodin D_3; peak 6, deapio-platycodin D_3.

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