Published in final edited form as:

J Liq Chromatogr Relat Technol. 2015 ; 38(4): 423–429. doi:10.1080/10826076.2014.913518.

Isolation of the Predominant Cycloartane Glycoside, Sutherlandioside B, from *Sutherlandia frutescens* R.Br. by Spiral Countercurrent Chromatography

Korey J. Brownstein^a, George E. Rottinghaus^b, Martha Knight^c, Yoichiro Ito^d, and William Folk^{a,*}

^aDepartment of Biochemistry, University of Missouri, Columbia, MO 65211, USA

^bVeterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO 65211, USA

^cCC Biotech LLC., 9700 Great Seneca Highway, Rockville, MD 20850, USA

^dLaboratory of Bioseparations Technology, Center of Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

The introduction of spiral countercurrent chromatography in the last few years using new separation columns such as the spiral tubing support rotor has enabled the application of more polar volatile solvent systems for natural products separation. This method can be applied to water soluble compounds and their metabolites. We have used spiral countercurrent chromatography with the spiral tubing support rotor to fractionate *n*-butanol extracts of an African plant *Sutherlandia frutescens* and have determined conditions by which the predominant cycloartane glycoside (sutherlandioside B) can be purified in good yield. A solvent system of ethyl acetate, methanol, and water was modified by adding *n*-butanol to separate sutherlandioside B from other compounds. With the optimal amount of *n*-butanol in the two-phase solvent system with the lower aqueous phase mobile, the target compound was eluted well separated from the other components. The purity of sutherlandioside B was determined by high performance liquid chromatography/mass spectrometry analysis and the yield compares favorably with the content in bulk material.

Keywords

spiral countercurrent chromatography; spiral tubing support rotor; *n*-butanol-aqueous solvent systems; *Sutherlandia frutescens*; sutherlandioside; cycloartane glycoside

Introduction

New spiral design separation column-coils or rotors were introduced for countercurrent chromatography that were a modification of the flow pathway from tubing wound in a spool to layers of flat spiral loops either carved in disks [1] or formed by tubing held in a frame [2]. The former is the spiral disk assembly, the first example of spiral countercurrent

*Corresponding author. Tel.: +1 573 884 2921; fax: +1 573 884 4597, folkw@missouri.edu (W. Folk).

chromatography (spCCC), and the second is the spiral tubing support (STS). This spiral configuration more efficiently utilizes the radial acting centrifugal force which serves to improve the retention of the stationary phase (S_F) [3]. The previously available multi-layer coil used in high-speed CCC had the problem of not retaining well the heavy alcohol-containing solvent systems. We found that in these new rotors, the *n*-butanol/0.1M K_2HPO_4 , KH_2PO_4 (1:1) solvent system had a S_F of 70% and *sec*-butanol/0.5% aq. trifluoroacetic acid (1:1) had a S_F of 66% [4,5] compared to 40% or less in the multi-layer coil. Higher S_F results in greater separation efficiency. The heavy alcohols being more polar are useful for partitioning small water soluble molecules. Here we investigated the addition of *n*-butanol to an organic-aqueous solvent system to modify the partitioning and achieve separation of various triterpenoid glycosides in an extract. Previously, we had studied the operation of the spiral disk rotor in some detail with non-polar to polar organic-aqueous solvent systems [4] and later studied the STS rotor and its applicability to small and larger molecules using the polar organic-aqueous solvent systems. Here we are applying this rotor for the first time to small molecule natural products separation.

Sutherlandia frutescens (L.) R. Br. (Fabaceae) is a shrub native to southern Africa that is widely used in traditional medicines [6,7]. Significant secondary metabolites within the leaves and stems include D-pinitol, L-canavanine, γ -amino butyric acid, and multiple cycloartane glycosides (sutherlandiosides A-D) and flavonols (sutherlandins A-D) [6–9]. The sutherlandiosides may be inhibitors of cytochrome P450 enzymes involved in adrenocorticosteroid metabolism [10] and thus could be responsible for the capacity of *S. frutescens* to reduce corticosterone levels in rats subjected to chronic stress [11] and for some of the claimed stress-reducing benefits in humans. Which of these novel compounds is responsible for the claimed benefits is unknown. In previous procedures the sutherlandiosides have been purified from the *n*-butanol soluble portion of methanol leaf extracts by chromatography on silica and reverse-phase silica gel [7], with low yields due to irreversible adsorption and limited capacity. Sutherlandioside B is 3R,7S,24S,25-tetrahydroxycycloartan-1-one 25-*O*- β -D-glucopyranoside [2] (Fig. 1). Thus, we explore the use of high-speed CCC [12,13] using the new STS rotor [5] to fractionate *n*-butanol extracts of *S. frutescens* to determine conditions by which sutherlandioside B can be substantially purified in good yields.

Experimental

Instruments

A Conway Centri Chrom (Williamsville, NY, USA) planetary centrifuge [4] was mounted with a STS rotor (17-cm OD, CC Biotech LLC, Rockville, MD, USA) [5] (Fig. 2). The STS rotor was filled with 1.6 mm ID FEP tubing, in 4 spiral loops per layer and pressed in at radial channels with a total volume of 110 mL. The solvent was delivered by an SSI pump (SSI Instruments, State College PA, USA) and sample loaded through a 10-ml loop (Valco VICI Instruments, Houston TX, USA). The effluent passed into a LKB fraction collector (GE Healthcare, Piscataway, NJ, USA). Absorbance of fractions was determined manually in a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The spiral CCC fractions were freeze dried in a Heto system consisting of a -60°C freeze dryer with a

–90°C condenser (Heto Vac, ATR, Laurel, MD, USA). The upper phase or organic solvent containing fractions were dried in the centrifugal evaporator (Savant, Holbrook, NY, USA) connected to the Heto system. The spiral CCC fractions were further analyzed by thin-layer chromatography (TLC) on a 10 × 20 cm Analtech Inc. (Newark, DE, USA) Silica Gel HL plate. The highperformance liquid chromatography (HPLC) analysis was performed on a Hitachi Model L-7100 pump/Model L-7200 autosampler equipped with a Hitachi D-7000 data acquisition interface and ConcertChrom (Naperville, IL, USA) software on a microcomputer. This system was coupled with an ESA Model 301 (Chelmsford, MA, USA) evaporative light scattering detector (ELSD). Liquid chromatography-mass spectrometry (LC-MS) was conducted with a Thermo-Fisher Scientific Accela HPLC system with diode array detection using a Discovery column (HS F5, 5 µm, 150 × 4.6 mm, Supelco) with a mobile phase of water-acetonitrile (60:40) with 0.1 % formic acid at a flow rate of 350 microliters/min. A Thermo-Fisher Surveyor MSQ plus single quadrupole MS with electrospray ionization (ESI, San Jose, CA, USA) was operated in the ESI positive mode, probe temperature 350 °C, cone voltage 75, nebulizer pressure 55 psi, with scan range from 100 to 1000.

Chemicals and plant material

All organic solvents used for extraction, spiral CCC separation, and HPLC-ELSD/TLC/MS analyses were of analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). The reagent *p*-anisaldehyde for the TLC analysis was purchased from Eastman-Kodak Co. (Rochester, NY, USA). The sutherlandioside standards were prepared as described in Fu *et al.* [8] and provided by Dr. Troy Smillie at the National Center for Natural Products Research, University of Mississippi (Oxford, MS, USA). Freeze-dried milled leaves of *S. frutescens* were purchased from Big Tree Nutraceutical (Fish Hoek, South Africa).

Extraction of *Sutherlandia frutescens*

Milled *Sutherlandia frutescens* leaves (50 g) were extracted with 500 mL of methanol at room temperature on a rotating shaker for an hour under constant agitation [8]. The sample was vacuum filtered and the solids were returned to the flask and twice more extracted with methanol while agitating. The combined filtered methanol solution was evaporated to dryness under a vacuum and resuspended in water and extracted with hexanes, chloroform, and *n*-butanol, successively. The *n*-butanol extract was reduced under a vacuum to an oil or syrup.

Spiral CCC separation

The solvent system of ethyl acetate-*n*-butanol-methanol-water in the volume ratios listed in Table 1 was mixed in a separatory funnel with the phases separated after equilibration. The rotor was filled with the upper phase. Amounts of 0.6 g to 1.25 g each of *S. frutescens n*-butanol extract were dissolved in 2–5 mL of each phase and loaded into the sample loop. After the start of centrifugation at 830 rpm, the sample was injected. The lower phase was pumped at a flow of 1 mL/min and 4 or 5-min fractions were collected. The elution mode was L-i-H, meaning the mobile lower phase was introduced in the inner (top) entry which is

head to tail in the CW direction of rotation used [4]. After elution of mobile phase, around 200 ml, the rotation was ceased and contents pushed out with He gas pressure. The spiral coil was washed with water and acetone and dried with He gas before another run. In the various separation experiments, the S_F was measured and the results included in Table 1. Aliquots of the fractions were diluted in 50% aq. ethanol and the absorbance read. Three wavelengths close to the peak maximas seen in the spectrum of the extract were plotted. The fractions were qualitatively and quantitatively analyzed by TLC and HPLC-ELSD, respectively.

TLC and HPLC-ELSD analyses

A small amount of sample from individual spCCC fractions was dissolved in 0.5 mL of methanol and 4 μ L was spotted with micro-capillaries in 1 cm bands on a 10 $\times$ 20 cm TLC plate. The plate was developed in a mobile phase (chloroform-toluene-methanol, 3:2:1). The plate was dried and then sprayed with methanol-acetic acid-sulfuric acid-*p*-anisaldehyde (17:3:1:0.1) and heated for 4 min at 120°C to visualize the sutherlandiosides, which stained violet. These were identified with highly purified sutherlandiosides A, B, and D and a mixture of sutherlandiosides A–D as standards [7].

After the TLC analysis, the spCCC fractions with similar components were combined lyophilized, and then subjected to HPLC-ELSD analysis. The photomultiplier of the ELSD was adjusted to 850 and T_1 and T_2 were 35°C and 55°C, respectively. The nebulizer gas (nitrogen) was set to 20 psi. Twenty μ L of samples were injected and the flow rate through a C₁₈ Hyperclone Phenomenex 150 $\times$ 4.6 mm column fitted with a C₁₈ ODS SecurityGuard Phenomenex (Torrance, CA, USA) 4.0 $\times$ 3.0 mm guard column, was 1 mL/min with 0.1% aqueous acetic acid (A) and 0.1% acetic acid/acetonitrile (B) in a slightly concave gradient elution as described in Avula *et al.* [14] with modifications. The initial condition was 15% B for 2 min. Afterwards B was increased to 55% between 2–40 min; B was then increased to 100% between 40–42 min. This was followed by a 100% B wash for 3 min, and then between 45–50 min, B was returned to the initial conditions for ten min (50–60 min) before the next injection. Under these conditions, sutherlandioside B eluted at 20 min.

Results and discussion

Initial estimations of K (distribution coefficients) of the extract were made in TLC experiments. The upper phase and lower phase contents were submitted to TLC and ethyl acetate-water showed a high value of K of 1–5 and hexane-ethyl acetate-methanol-water showed a low value <0.3. Ethyl acetate-*n*-butanol-water had a very high K of 2–5. It was decided to try an ethyl acetate-methanol-water solvent system in the volume ratios (8:1:7) A sample load of 2.5 g of the extract (syrup) was run in the spCCC with the lower phase mobile. The results are not shown, but sutherlandioside B eluted close to the solvent front and continued in smaller amounts spread out over many fractions. The early fractions were mixed with sutherlandins and sutherlandioside A, D. It was decided to run a smaller sample mass and to add *n*-butanol to the solvent system.

A solvent system of the same components but including a small volume of *n*-butanol that was used for centrifugal partition chromatography of saponins [15] was tried. The

fractionation of 0.6 g *S. frutescens* extract in ethyl acetate-*n*-butanol-methanol-water (15:1:3:15) is shown in Fig. 3. The solvent front was at fraction 12 and sutherlandioside B came out after fraction 45 well behind the solvent front. In the TLC, sutherlandioside B (violet band with an R_f of approximately 0.33), A, C, & D (violet bands as marked in Fig. 5) were resolved in the middle to last fractions, well separated from other components. The analysis by TLC localized the target compound which was combined in 2 pools, F₁ (fractions 47–60, 15.7 mg yield) and F₂ (fractions 61–74, 13.3 mg).

An amount of 2.1 mg from F₁ and 1.3 mg from F₂ were dissolved in 2.1 and 1.3 mL of methanol, respectively and analyzed by HPLC-ELSD. The results shown in Fig. 6 showed sutherlandioside B separated from various impurities. The spiral CCC separation was repeated with the sample load doubled to 1.2 g in the same conditions as shown in Fig. 4. Sutherlandioside B was identified in fractions 42–63, similar to the previous run. Thus the capacity is high for these types of samples. Previous mass loadings have not exceeded 100 mg peptides [5] and 500 mg of a seaweed extract [16]. Increasing the content of *n*-butanol in the last solvent system of Table 1 (EBMW, 19:5:4:22), for a sample size of 1 g caused the sutherlandiosides to elute later (fractions 53–74) with less resolution from other components in the later fractions (not shown). The conditions of the solvent system EBMW (15:1:3:15) provided the best resolution of sutherlandioside B and the amount recovered was 0.92% of the extract mass input, which compares favorably with the content in the bulk material as previously reported [3]. The purity of sutherlandioside B in pooled fractions F₁ and F₂ were approximately 89.2% and 52.3%, respectively (Fig. 6). Loading of twice the amount of sample in spiral CCC utilizing the same solvent system produced comparable results and recoveries. However, the stationary phase retentions (Table 1) were lower than 60% which is probably due to the high sample loads.

HPLC/MS in the positive ion full scan mode was utilized to compare a standard of sutherlandioside B to the material isolated by HSCCC. The spectra are presented in Fig. 7 where (A) is the standard purified as described by Fu *et al.* [8] provided by the University of Mississippi, and spectra (B) is the material isolated from the spiral CCC runs. The mass spectrums showed the major ion m/z 670 $[M+H_2O]^+$ along with m/z 490.6, 472.7, and 454.7 in both spectra. The fragment ions include the aglycone $[M+Na]^+$ and $[M-H_2O]$.

As a result of these studies we were able to develop an antibody to the sutherlandioside B purified here so that we can detect consumption and metabolism in subjects in a future clinical trial for.

Conclusions

The new spiral CCC method allows the use of polar solvent systems to separate water soluble complex phytochemicals. The solvent system used here was modified with the polar component *n*-butanol which helped the resolution and improved recoveries. The mass loading of the extract was considerable, over 1 gram for a 100-ml volume column (rotor). The efficiency and scalability of spiral CCC for the isolation and purification of sutherlandioside B (and other sutherlandiosides) will facilitate the examination of the biological and biochemical activities of these novel secondary metabolites.

Acknowledgements

The STS CCC rotor is manufactured by CC Biotech LLC under exclusive license (L-144-2007/1) from the United States Public Health Service, National Institutes of Health (NIH) and protected by PCT/US2009/064976 and patents filed in Korea, Republic of China, Japan, and Europe. This research was supported by NIH/National Center for Complementary and Alternative Medicine/Office of Dietary Supplements grants 5U19AT003264 and 1P50AT600273. We thank Dr. Troy Smillie for samples of sutherlandiosides and Deborah and Michael Brownstein for their editorial assistance.

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Figure 1.

The chemical structures of 1; sutherlandioside A, 2; sutherlandioside B, 3; sutherlandioside C, 4; sutherlandioside D [8].

Figure 2.

A) The spiral tubing support CCC rotor mounted in a planetary centrifuge. B) With the cover removed a cross-section view of the circular and radial channels that hold the tubing being inserted [5].

Figure 3.

Separation of 0.59 g extract extract in the solvent system EBMW (15:1:3:15) in the L-i-H elution mode. Four minute fractions were collected. Solvent front came out at fraction 15 and the pump out of contents started at fraction 56. The absorbance at the three wavelengths shown for the fractions is plotted. Sutherlandioside B was identified in the fractions between the marks.

Figure 4.

The chromatogram of the separation of 1.2 g extract where the sample was loaded as described in Fig. 3. The solvent front was at fraction 13 (49.5 ml) and the pump out of contents at fraction 61. The location of sutherlandioside B is marked.

Figure 5. TLC profile (run shown in Fig. 3) of fractions with sutherlandioside standards. The method is described in the Experimental.

Figure 6.
HPLC-ELSD chromatograms of unpurified extract (A) fractions F₁ (B) and F₂ (C) of the run in Fig. 3 are shown. Methods are described in the Experimental.

Figure 7.

HPLC/MS spectra in full scan mode of standard (A) and the isolated sutherlandioside B (B).

Both spectra have the major ion m/z 670 [M+H₂O]⁺ along with m/z 490.6, 472.7, and 454.7.

Table 1

Solvent systems used in spiral CCC

Composition (volume ratios)	S _F
Ethanol-Methanol-Water EMW (8:1:7)	50.5%
Ethanol-n-Butanol-Methanol-Water EBMW (15:1:3:15)	55%
EBMW (19:5:4:22)	48%