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Countercurrent separation assisted identification of two mammalian steroid hormones in *Vitex negundo*

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Abstract

Countercurrent separation (CCS) has been widely used for the separation of high abundance compounds. However, the identification of low abundance compounds, such as mammalian steroid hormones, from natural sources is still a challenging task. A mixture of 14 human steroid hormone reference compounds was prepared for the development of a CCS enrichment strategy. The TLC-based GUESS (Generally Useful Estimate of Solvent Systems) method along with partitioning experiments were implemented to develop a process for the enrichment of these low abundance compounds with CCS. The application of CCS to the steroid hormone enrichment of *Vitex negundo* extracts was demonstrated by the identification of progesterone and estriol. This method provides a CCS-driven strategy to mine plant sources for low abundance compounds.

Keywords

Countercurrent separation; Mammalian steroid hormones; Progesterone; Estriol; GUESS method

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Declaration

The authors have declared that there is no conflict of interest.

1. Introduction

1.1 Previous investigations of mammalian steroid hormones in plants

Steroid hormones are a class of congeneric steroidal compounds with generally high stability, which have important regulatory functions in mammals and other organisms. Pregnane derivatives, such as estrogen, estradiol and diethylstilbestrol, tend to have estrogenic effects. Steroid hormones were first discovered in humans and other mammals. However, these steroids have been found in a variety of organisms, most notably in the plant kingdom. In 1964, Leboeuf, Cavé, and Goutarel reported that progesterone was found in *Holarthra floribunda* leaves [1]. Since then progesterone and its congeners were successively reported to be present in apple seeds [2], loblolly pine [3], common foxglove (*Digitalis purpurea* L.), tobacco (*Nicotiana tabacum* L.), elecampane (*Inula helenium* L.) [4], *Juglans regia* [5], *Adonis aleppica* [5], and wheat [6], mostly using chromatographic methods, but also via isolation [5]. Other mammalian steroid hormones have been reported in plants. For example, estrone (1,3,5(10)-estratrien-3-ol-17-one) has been found in the seeds of the date palm, *Phoenix dactylifera* L. [7]. Estriol was tentatively identified in willow flower extract by UV absorption and melting point in 1933 [8]. Analytical techniques including GC–MS and ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) have been powerful tools to identify these low abundance compounds [4,9].

1.2. Challenges to identifying mammalian steroid hormones in plants

Despite of the current development of highly sensitive MS techniques, the identification of mammalian steroid hormones in natural sources is still a challenging task. For example, Gawienowski and Gibbs estimated the amount of progesterone was 500 ng/g in apple seeds using thin layer and gas chromatography methods [2]. The concentration of progesterone in the leaves of *Prunus virginiana* L. was reported to be 13 ng/g of dry weight [7]. Using GC–MS, Iino et al. reported finding progesterone in the shoots and inflorescences of in *Arabidopsis thaliana* L. in concentrations of 160 and 400 ng/kg of fresh weight, respectively [9].

1.3. Sample preparation and CCS for enrichment of target compounds

Countercurrent separation (CCS) is an advanced liquid–liquid chromatographic separation technique that is performed by instruments such as high-speed countercurrent chromatography (HSCCC) and centrifugal partition chromatography (CPC). CCS has been widely used for the preparative separation and isolation of (typically high-abundance) constituents due to its technical merits, such as low solvent consumption, highly selective solvent systems, and high recoveries [10]. Thus, this technique has been widely used for natural product separation and isolation [11–13]. However, the solvent system (SS) selection process presents a considerable challenge for CCS applications, especially when trying to target low-abundance constituents. Choosing a suitable SS is a critical operation in CCS [14,15]. The Generally Useful Estimate of Solvent Systems (GUESS) method, introduced in 2005, is a one of the few alternatives to SS selection with extensive use of partitioning experiments [16]. The GUESS method employs TLC to identify suitable SSs with minimal labor and has been shown to enable the rapid purification of target compounds [17–19]. The

use of separatory funnel liquid–liquid separations with biphasic SSs of three or more solvents has been used to prepare samples for CCS fractionation [20,21]. A CCS-driven method of systematically fractionating samples with a wide polarity range of target components described as “sample cutting” was used for the isolation of prenylated phenolics from Hops [22].

1.4. The role of CCS x LC–MS in identifying mammalian steroid hormones in plants

CCS, in tandem with LC–MS, is a very useful method for the systematic search for mammalian steroid hormones in plants. There is a precedence for using CCS fractionation in the first chromatographic dimension along with LC–MS in the second chromatographic dimension to identify low abundance compounds from complex matrices. CCS x LC–MS has been used in evaluation of bioactive secondary metabolites [23–28]. CCS x LC–MS has also been applied in determination of *Alternaria mycotoxins* in wine [29] and toxaphene metabolites in polar bear adipose tissue [30].

1.5. A brief introduction to our methodology

A standard mixture of 14 commercially available mammalian steroid hormones and their derivatives was created. With this mixture, a SS selection process utilizing both the TLC-based GUESS method and the liquid-liquid partitioning technique with HPLC-UV detection was followed. Once a SS had been selected, the search for mammalian steroid hormones in plants was initiated by searching for progesterone and other steroid hormones in *Vitex negundo* extract. The liquid-liquid separation protocol used to prepare the CCS sample was designed to enrich the extract in compounds in the polarity range of the target steroid hormones.

2. Experimental

2.1. Materials and reagents

All reagents were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). All analytical grade solvents were purchased from Pharmco-AAPER (Crookfield, CT, USA) and redistilled before use. Analytical TLC was performed at room temperature on Alugram precoated 0.20 mm thick silica gel G/UV254 aluminum plates (10 × 20 cm) from Macherey-Nagel, (Düren, Germany). Steroid reference compounds were purchased from the Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.2. Instrumentation

CCS was performed using a TBE-20A HSCCC (20 mL, Tauto Biotech, China), equipped with a 500 µL sample loop, quaternary pump array, UV detector, phase metering apparatus (PMA) [31], and data processing system (CherryOne, Cherry Instruments, Chicago, IL, USA). HSCCC temperature regulation was managed with a Neslab RTE refrigerated bath circulator. Outflow was collected by a Foxy Jr. fraction collector (Teledyne Isco, Lincoln, NE, USA).

Analytical HPLC was performed on a Waters 2695 HPLC (Waters Corporation, Milford, MA, USA). An analytical Waters Alliance C₁₈ column (5 µm, 4.6 mm × 150 mm) with an

Extend C₁₈-guard column (5 μ m, 4.6 $\times$ 10 mm) was used. A Waters 996 photodiode array detector generated the chromatograms.

LC–MS was performed on a Shimadzu LCMS-2020 HPLC system equipped with a Waters Xterra 2.1 $\times$ 150 mm reversed-phase column coupled to an LCMS-2020 UltraFast ESI/APCI(+/-) spectrometer with a nitrogen gas source.

2.3. GUESS method

TLC solvent systems were prepared by volumetric mixing of the component solvents. Reference steroid (Table 1) solutions in Chloroform/EtOAc (60/40) and prepared at the same concentration of 2 mg/mL were individually spotted on a 20 $\times$ 10 cm TLC plate and developed in a glass chamber. After development to a height of about 80 mm, the plates were visualized under UV (254 nm) light, dipped in vanillin chromogenic agent (95 mL EtOH/1.5 mL H₂SO₄/6 g vanillin), and heated with heat gun to yield multicolor spots. The tests were done in duplicate.

2.4. Partitioning experiments

Liquid-liquid partitioning of reference steroids was used to follow up on the GUESS tests. A 2.00 mg sample of each reference steroid was dissolved in 1 mL of a 1:1 mixture of upper and lower phase. The solution was shaken and allowed to settle. A 40 μ L aliquot from each phase was injected onto an analytical HPLC column. The K value was expressed as the peak area of the target compound in the chromatogram of the upper phase divided by the peak area of the chromatogram of the lower phase. Elution was carried out using gradient elution and the mobile phase consisting of A (water) and B (acetonitrile) at a flow rate of 1.0 mL/min. The gradient was set as follows: 0–26 min (20–40% B), 26–40 min (40% B), 40–66 min (40–50% B), 66–67 min (50–95% B), 67–79 min (95% B), and 79–80 min (95–20% B). PDA acquired chromatograms were extracted at the appropriate wavelength for the steroid under consideration. The tests were done in duplicate.

2.5. Plant material

The leaves of *Vitex negundo* were collected from Xishuangbanna, Yunnan Province, P.R. China, in April 2015, and authenticated by Professor Qi-Shi Song, Xishuangbanna Tropical Botanical Garden. A voucher specimen (no. 20150426) was deposited at the Research Group on Ethnomedicine of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. The air-dried and powdered leaves (3 kg) were extracted with 60 L of 95% aqueous ethanol, filtered at room temperature and concentrated under vacuum to yield 230.2 g of crude extract.

2.6. Liquid-liquid separation

A 2 g sample of the crude extract was dissolved in water (1:30 m/v) and extracted three times with CHCl₃. The CHCl₃ phases were combined and the solvent evaporated on a rotary evaporator. The residue (0.120 g) was dissolved in 2.4 mL in the upper phase of the biphasic system ChMWat 10:5:5. The upper phase was extracted three times with the lower phase. The lower phase extracts were combined and concentrated under vacuum to produce the CCS sample (0.075 g).

2.7. HSCCC fractionation and fraction analysis

The PMA values of each phase were determined. Then the column was rotated at 800 rpm and filled with the upper phase of HEMWat -5 (7:3:7:3) as the stationary phase. The column was then rotated at 2000 rpm and equilibrated with lower phase pumped at 0.5 mL/min in the head-to-tail direction. The CCS sample dissolved in 50 μ L of a 1:1 mixture of upper and lower phases) was injected after the column was equilibrated for 10 min. Throughout the separation, the K values were monitored by the CherryOne system. The stationary phase volume retention ratio (Sf value) was determined to be 0.65. The eluant was collected in 1.0 mL fractions.

LC-MS profiles of each of the 14 reference steroids were generated by dissolving them in acetonitrile to give a final concentration of 1 mg/mL. The CCS fractions were also dissolved in acetonitrile to give a final concentration of 1 mg/mL. LC-MS solvents were 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

3. Results and discussion

3.1. The GUESS method

The generally useful estimate of solvent systems (GUESS) method represents a rapid and practical method to link TLC retention characteristics (R_f values) with CCS K values [18]. Based on previous studies, compounds with R_f values between 0.29 and 0.71 were likely to have K values between 0.4 and 2.5 and be eluted in the region of optimal CCS resolution [32]. A reference standard mixture of 14 steroids (Table 1) was prepared for method development in the present study. These steroids were a representative sample of androgen, estrogen, and progestogen mammalian steroid hormones with LogP values between 1.1 and 4.1. Initially, the TLC-based GUESS method was performed on each steroid separately with hexane-ethyl acetate 7:3, 5:5, 4:6, 3:7, and 1:9 solvent systems (SSE3, SSE5, SSE6, SSE7 and SSE9 respectively). Fig. 1 shows that the R_f values covered a range of 0–0.8 in these solvent systems.

3.2. Partitioning experiments

In order to follow up on the TLC information provided by the GUESS method, a mixture of the 14 standard compounds was used to test the partition coefficients (K values) by using the liquid-liquid partitioning method (Fig. 2) [16].

HEMWat -2 (7:3:5:5), 0 (5:5:5:5), +1 (4:6:5:5), and +2 (3:7:5:5) were tested because they corresponded to TLC hexane-ethyl acetate solvent systems 3 (7:3), 5 (5:5), 6 (4:6), and 7 (3:7), respectively (Table 2).

The partitioning experiment K values (Table 3) suggested that the whole span of steroids could not be targeted with a single solvent system due to the high selectivity of the HEMWat solvent system family. Using a sequence of polarity-adjusted solvent systems to separate a group of compounds is a common CCS strategy.

Given its demonstrated presence in plant material, progesterone (**13**) was our first steroid target. It was one of the most nonpolar steroids, therefore, an adjustment was made to a less

polar HEMWat solvent system. Plotting the Log value of the progesterone K values for HEMWat -2, 0, and +2 gave a straight line ($R^2 = 0.999$) with the $\text{Log}K = 0$ intercept at the HEMWat -5 solvent system. This would give a $K = 1$ value for progesterone. As a result, the HEMWat -5 (7:3:7:3) was chosen to selectively enrich progesterone in *Vitis negundo* extract.

3.3. Sample preparation and HSCCC separation

The leaves of *Vitis negundo* were dried and extracted with aqueous methanol. The crude extract was dissolved in water and extracted three times with chloroform. This extract was selectively enriched in steroids by distributing it between the two phases of ChMWat (chloroform-methanol-water) 10:5:5. The residue from the lower phase was used as the CCS sample. These operations removed high polarity compounds from the extract with a 40,000x enrichment from the dry weight.

The HEMWat 7:3:7:3 solvent system was employed to target the isolation of progesterone from the *V. negundo* sample. With the upper phase stationary and the lower phase mobile, reversed phase mode separation was employed. The collected fractions were analyzed by LC-MS and compared with a progesterone standard (Fig. 3). Progesterone (**13**) was identified in two fractions representing a range of K values from 1.0 to 1.2.

Further analysis of the fractions revealed the presence of another steroid in the fractions representing a range of K values from 0.4 to 0.8 which corresponded to estriol (**4**) (Fig. 4).

4. Conclusion

This investigation featured a strategy for investigating a plant extract for low abundance metabolites that have not already been identified in the particular species and/or genus. This method complements bioactivity guided fractionation and phytochemical driven investigations that are most commonly performed with plant extracts. In bioactivity-guided fractionation, the biological profile of the extracts determines which fractions are investigated and what compounds are discovered. These may or may not be new compounds. In phytochemical driven fractionation, many compounds are identified and dereplicated in order to discover new phytochemical entities. In both these methods, fractionation protocols may determine what types of compounds are investigated but the target compounds are largely unknown until they are identified. In the present investigation, the liquid/liquid partition coefficients of progesterone were selectively used to target this compound in a plant extract. In addition to demonstrating the first use of HSCCC to identify progesterone in plant material, this methodology offers a proof of concept to identify other steroid compounds in plant material. Indeed, this HSCCC application led to the identification of estriol in *V. negundo* for the first time.

An important aspect of this investigation was the rapid identification of a solvent system that gave a partition coefficient near unity for progesterone. Determining the partition coefficients of other steroids gave their hexanes-ethyl acetate-methanol-water solvent system polarity characteristics relative to progesterone that may be exploited to specifically target their identification in a mixture. The scale-up potential of the CCC methodology offers the

possibility that fractionation may be done on a larger scale to obtain large enough quantities of these steroids for full 1D/2D NMR structural elucidation which is considered to be superior to that of LC–MS.

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Abbreviations:

CCS	countercurrent separation
TLC	thin-layer chromatography
K	partition coefficient
R_f	retention factor for TLC
SS	solvent system
HEMWat	hexanes-ethyl acetate-methanol-water biphasic solvent system
ChMWat	chloroform-methanol-water biphasic solvent system
SSE	TLC solvent systems based on ethyl acetate and hexane
GUESS	generally useful estimate of solvent systems
S_f	stationary phase volume retention ratio

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Fig. 1.

TLC Rf values of 14 steroids in 5 different solvent systems. Compounds numbers, names and structures are found in Table 1. The solvent systems SSE3, SSE5, SSE6, SSE7 and SSE9 were hexane-ethyl acetate 7:3, 5:5, 4:6, 3:7, and 1:9 respectively. The area between the dashed lines represents the TLC “sweet spot” interval.

Fig. 2.
Schematic of the partitioning experiments with HPLC-UV analysis.

Fig. 3.

LC-MS of the progesterone (13) standard and the *Vitus negundo* fraction targeting the identification of progesterone. A. HPLC-MS chromatogram of progesterone standard with TIC (APCI positive ion scan mode). B. MS of progesterone peak, $m/z = 315$ corresponding to $C_{21}H_{30}O_2 + H^+$. C. HPLC-MS chromatogram of progesterone-containing HSCCC fraction with TIC (APCI positive ion scan mode). D. HPLC-MS chromatogram of progesterone-containing HSCCC fraction with SIM detection. E. MS of progesterone peak, $m/z = 315$ corresponding to $C_{21}H_{30}O_2 + H^+$.

Fig. 4.

LC-MS of the estriol (**4**) standard and the *Vitus negundo* fraction targeting the identification of estriol. A. HPLC-MS chromatogram of estriol standard with TIC (APCI positive ion scan mode). B. MS of estriol peak, $m/z = 315$ corresponding to $C_{18}H_{24}O_3 + H^+$ C. HPLC-MS chromatogram of estriol-containing HSCCC fraction with TIC (APCI positive ion scan mode). D. HPLC-MS chromatogram of estriol-containing HSCCC fraction with SIM detection. E. MS of estriol peak, $m/z = 315$ corresponding to $C_{18}H_{24}O_3 + H^+$.

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Table 1

A list of 14 reference compounds (mammalian steroid hormones and their derivatives).

no	name	MW	structure	No	name	MW	structure
1	aldosterone	360.44		8	epiandrosterone	290.44	
2	hydrocortisone	362.46		9	17 α -hydroxyl progesterone	330.46	
3	corticosterone	346.46		10	trans-dehydro androsterone	288.42	
4	estriol	288.38		11	medroxyprogesterone acetate	386.52	
5	cortisone acetate	402.48		12	β -estradiol	272.38	
6	hydrocortisone acetate	404.50		13	progesterone	314.46	
7	21-hydroxyprogesterone	330.46		14	estrone	270.37	

Table 2

Equivalence of HEMWat and SSE solvent systems.

HEMWat#	relative proportions				SSE#	relative proportions	
	Hexanes	EtOAc	MeOH	Water		Hexanes	EtOAc
-7	9	1	9	1	1	9	1
-6	8	2	8	2	2	8	2
-5	7	3	7	3	3	7	3
-4	7	3	6	4	3	7	3
-3	6	4	6	4	4	6	4
-2	7	3	5	5	3	7	3
-1	6	4	5	5	4	6	4
0	5	5	5	5	5	5	5
+1	4	6	5	5	6	4	6
+2	3	7	5	5	7	3	7
+3	4	6	4	6	6	4	6
+4	3	7	4	6	7	3	7
+5	3	7	3	7	7	3	7
+6	2	8	2	8	8	2	8
+7	1	9	1	9	9	1	9
+8	0	10	0	10	10	0	10

Table 3

Liquid/Liquid partitioning K values of 13 steroids in 4 different HEMWat solvent systems. Quantitation of steroid concentration was performed with HPLC with UV(PDA) detection.

compound	HEMWat#			
	-2	0	+1	+2
1	0.00	0.06	0.14	0.40
2	0.19	0.64	1.07	1.87
3	0.12	0.53	0.89	1.63
4	3.53	4.55	6.12	6.68
5	0.74	1.42	2.00	2.76
7	0.38	1.00	1.38	2.10
8	0.64	0.94	2.72	8.62
9	0.00	0.00	0.03	0.16
10	1.41	1.50	2.68	3.62
11	4.62	5.19	5.65	6.48
12	0.00	0.05	0.16	0.35
13	1.95	3.06	3.76	6.50
14	0.00	1.59	0.40	0.74