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Note

Reversed-phase chromatographic separation of withanolides from *Acnistus breviflorus*

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Various plants of the Solanaceae family contain a group of C_{28} steroidal lactones known as withanolides. A wide variety of these compounds are present in plants of the genera *Withania, Physalis, Acnistus* (*Dunalia*), *Nicandra*, etc., and differ in the position and number of hydroxy groups, chlorine atoms and the presence or absence of double bonds and/or an epoxide ring[1–6]. Also, artifacts are sometimes isolated due to reaction of these compounds with the extraction solvent, *e.g.*, methanol or ethanol[6]. The structural similarities and the presence of pigments usually result in tedious and complicated separations with the concomitant loss of minor constituents.

In a previous paper[7], we described an improved high-performance liquid chromatographic (HPLC) separation method for the isolation of these compounds from crude extracts of *Acnistus breviflorus* leaves on silica gel columns. The use of reversed-phase (RP) columns with these compounds had been tried only once and it was reported that no retention was observed in methanol or methanol–water solvent systems[8]. However, due to progress in the development of RP columns for HPLC and the availability of silica gel with a high percentage of chemically bonded non-polar groups, *e.g.*, octadecylsilane (ODS), as well as RP thin-layer chromatographic plates, we decided to reinvestigate this system for the separation of withanolides.

EXPERIMENTAL

All withanolides were isolated from *A. breviflorus* plants from Argentina, as previously described[4]. Crude extracts were fractionated by column chromatography on silica gel 60 (Merck) eluting with hexane–ethyl acetate mixtures of increasing polarity. Fractions containing withanolide mixtures were evaporated to dryness, redissolved in methanol (*ca.* 15 mg/ml) and used for HPLC separations.

Analytical HPLC experiments were performed with a Hewlett-Packard 1084B liquid chromatograph equipped with a variable-wavelength detector operating at 225 nm, a variable-volume injector with automatic sampling system and an automatic fraction collector. Separations were carried out with a R-Sil C_{18} HL 10-μm column (250 × 3.6 mm I.D.) packed in our laboratory with a Micromeritics 705 stirred slurry column packer), with methanol–water (65:35) as eluent at a flow-rate of 2 ml/min.

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Preparative HPLC experiments were performed with a Micromeritics liquid chromatograph equipped with a 750 solvent delivery system, a 730 manual injector with a 1-ml loop and a refractive index detector. Separations were carried out with an Alltech R-Sil C_{18} HL 10-μm column (500 × 10 mm I.D.) or with an Altex Ultrasphere ODS 5-μm column (250 × 10 mm I.D.) and methanol–water (70:30) as eluent at a flow-rate of 4 ml/min.

Thin-layer chromatography (TLC) was carried out on HPTLC RP-18 plates for nano TLC (Merck) developed with methanol–water (70:30). Spots were visualized by spraying with 50% sulphuric acid and heating at 70°C.

RESULTS AND DISCUSSION

The R_F values obtained with nine withanolides from *A. breviflorus* when chromatographed on HPTLC RP-18 plates are listed in Table I. Our initial results with the more common withanolides encouraged us to attempt HPLC separations on RP columns. However, crude extracts from *A. breviflorus* leaves contained low polarity components that had R_F = 0 under the conditions used; these components could be largely eliminated (as determined by HPTLC RP-18) by fractionation on silica gel as described in Experimental.

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF WITHANOLIDES IN REVERSED-PHASE HPTLC AND ANALYTICAL HPLC

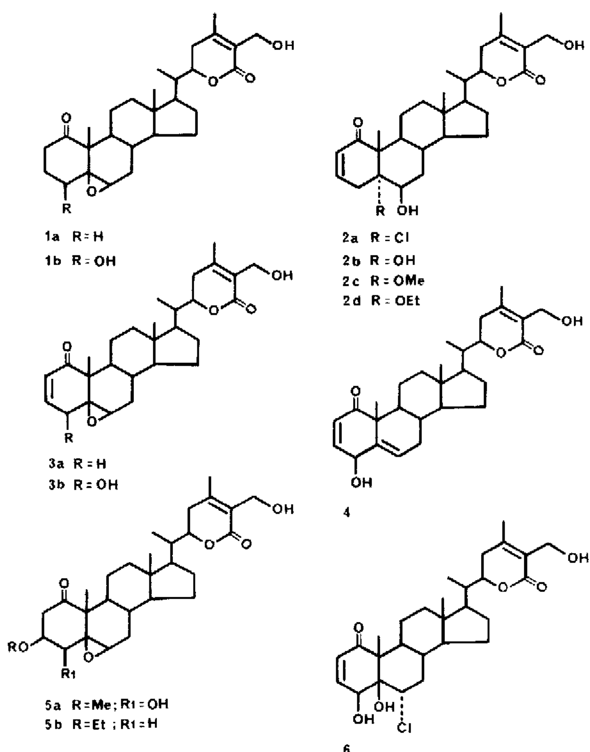
Withanolide	*R_F in HPTLC**	*RT (min) in HPLC***
2,3-Dihydrojaborosalactone A (1a)	0.18	11.47
Jaborosalactone E (2a)	0.20	9.47
Jaborosalactone D (2b)	0.40	3.27
5α-Methoxy-4,5-dihydrojaborosalactone B (2c)	0.31	6.13
5α-Ethoxy-4,5-dihydrojaborosalactone B (2d)	0.25	–
Jaborosalactone A (3a)	0.14	13.12
Withaferin A (3b)	0.34	4.48
3β-Methoxy-2,3-dihydrowithaferin A (5a)	0.28	4.78
6α-Chloro-5β-hydroxy-5,6-deoxywithaferin A (6)	0.34	4.48

* HPTLC RP-18 plates, methanol–water (70:30).

** R-Sil C_{18} HL 10-μm column (250 × 3.6 mm), methanol–water (65:35) at 2 ml/min.

For analytical HPLC separations, we used high sensitivity detection at 225 nm as described by Hunter *et al.*[9]. The use of a packing containing a high percentage of ODS groups resulted in adequate retention times even for the more polar withanolides such as jaborosalactone D (2b) using methanol–water (65:35) as eluent. An artificial mixture of eight withanolides could mostly be resolved within 15 min after injection (Table I); only withaferin A (3b) and 6α-chloro-5β-hydroxy-5,6-deoxywithaferin A (6) were overlapped on the chromatogram.

We then successfully applied these conditions to the preparative scale using a 500 × 10 mm column packed with the same material, and methanol–water (70:30) as eluent. Figs. 1 and 2 show separations of fractions of an ethanolic and a methanolic



extract respectively, partially purified by silica gel chromatography. The isolated pure compounds were identified by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. Table II lists the ^{1}H and ^{13} C NMR data for the hitherto unknown artifact 3-ethoxy-2,3-dihydrojaborosalactone A (5b) formed by a Michael addition of ethanol to jaborosalactone A (3a) during the extraction process. Crude extracts as well as partially purified fractions containing withanolides obtained from large scale separations on silica gel may, however, still contain pigments as indicated above. These pigments have long retention times under the conditions described and hence after several successive separations the column performance was affected. As these remaining pigments had R_F values higher than 0.5 on HPTLC RP-18 plates when developed with methanol, the problem could be overcome by washing the column with methanol once every five to seven injections until a colourless eluate was obtained. Alternatively, highly coloured samples could be freed from pigments using the same column and methanol–water (80:20) as eluent; under these conditions the withanolide mixture was eluted almost with the void volume, while pigments with longer retention times could be separated.

An unexpected result was that, although the preparative separation obtained was almost identical to the analytical one, compound 6 overlapped with 3-methoxy-2,3-dihydrowithaferin A (5a) instead of with withaferin A (3b). Nevertheless, the isolated mixture could be separated on a preparative scale using an Altex Ultrasphere ODS 5-μm column with methanol–water (70:30) as eluent (Fig. 3). The iso-

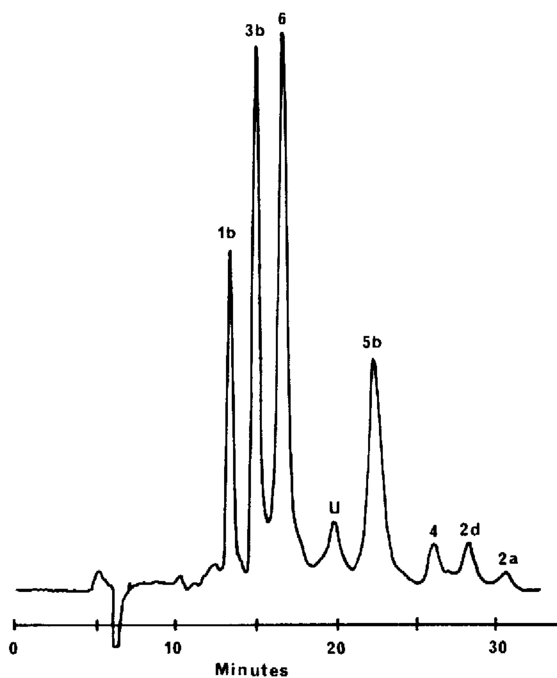


Fig. 1. Preparative HPLC separation of a partially purified fraction (10 mg) from an ethanolic plant extract. Column: Alltech R-Sil C_{18} HL 10-μm (500 × 10 mm). Eluent: methanol–water (70:30), flow-rate 4 ml/min. Numbers correspond to displayed structures; U = unidentified compound.

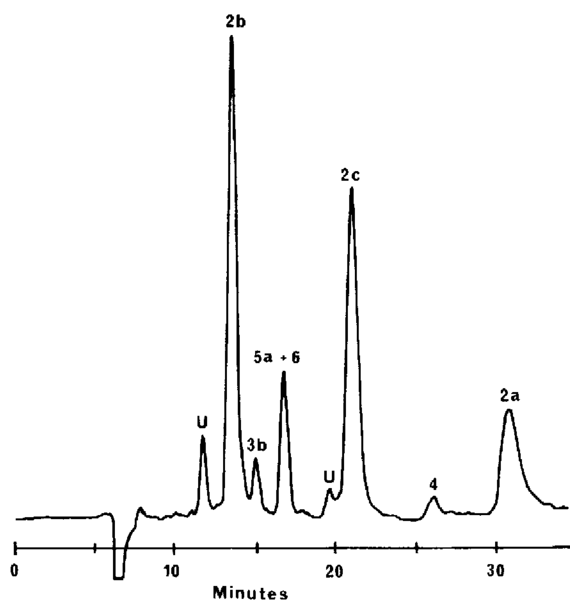


Fig. 2. Preparative HPLC separation of a partially purified fraction from a methanolic plant extract. Details as in Fig. 1.

TABLE II

NMR SPECTRAL DATA OF 3-ETHOXY-2,3-DIHYDROJABOROSALACTONE A (5b)

Solvent: deuterochloroform–tetramethylsilane. s = Singlet; d = doublet; t = triplet; q = quartet; m = multiplet; bs = broad singlet.

^{13}C (25.2 MHz)

Carbon	*δ (ppm)*	*Carbon*	*δ (ppm)*
1	209.62	16	27.26
2	39.14	17	51.96
3	75.34	18	11.53
4	29.62	19	15.36
5	64.80	20	38.76
6	60.18	21	13.31
7	29.89	22	78.66
8	31.23	23	29.44
9	42.81	24	152.50
10	50.38	25	125.66
11	21.56	26	166.70
12	39.14	27	57.36
13	42.65	28	19.93
14	56.12	$\mathit{C}H_2$–CH_3	64.36
15	24.27	CH_2–$\mathit{C}H_3$	15.54

^{1}H (100 MHz)

Proton	*δ (ppm)*	*J (Hz)*
CH_3-18	0.68 s	
CH_3-21	0.99 d	7
CH_3-19	1.31 s	
CH_3-28	2.05 s	
H-2	2.80 ddd	15, 6, 4
H-6	3.24 bs	
H-3	3.80 m	
H-22	4.42 dt	12, 3
H-27	4.38 bs	
CH_2–CH_3 (italic H_2)	3.54 q	7
CH_2–CH_3 (italic H_3)	1.16 t	7

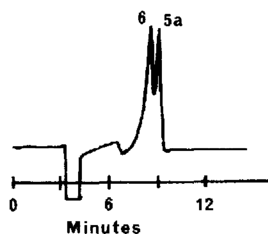


Fig. 3. Preparative HPLC separation of a mixture of compounds 5a and 6 (5 mg). Column: Altex Ultrasphere ODS 5-μm (250 × 10 mm). Eluent: methanol–water (70:30), flow-rate 4 ml/min.

lated fractions were identified by their ^{1}H NMR spectra. Interestingly, an inversion in elution order was also observed between HPTLC and analytical HPLC, as in the former compound 5a was less retained than 5α-methoxy-4,5-dihydrojaborosalactone B (2c) while the opposite situation was obtained in the latter case (see Table I).

We are now using these separation methods for routine work on the separation and identification of new withanolides from *Acnistus breviflorus*[6].

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