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Note

Analytical and preparative separation of withanolides from crude extracts of *Acnistus breviflorus* leaves by high-performance liquid chromatography

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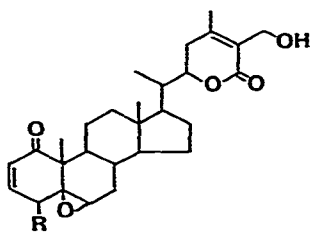
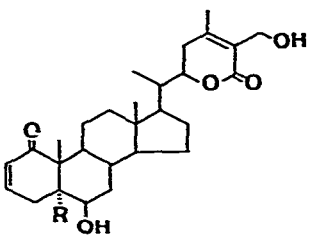
The withanolides are a group of oxygenated C-28 steroidal lactones which occur in various plants of the Solanaceae family[1,2]. Under different growing conditions the withanolides isolated from a plant may differ in the position and number of substituent hydroxy groups and double bonds. Thus, Nittala and Lavie[3] have isolated only 4β-hydroxywithanolides and a few containing also 20-hydroxy groups from *Acnistus breviflorus* plants grown in Israel, while Bukovits and Gros[2] have reported, from the same plant grown in Argentina, jaborosalactone A (I), D (II) and E (III) which are not hydroxylated at position 4, and did not detect any 20-hydroxywithanolides.



I, R=H
IV, R=OH



II, R=OH
III, R=Cl

In connection with our studies of withanolide biosynthesis in *A. breviflorus* plants, we required an efficient method for the analytical and preparative separation of small amounts of the three most abundant withanolides in these plants, *i.e.*, jaborosalactone A and D and withaferin A (IV).

Several solvent systems have been used for the high-performance liquid chromatographic (HPLC) separation of withanolides on silica columns (Porasil A, μPorasil, LiChrosorb Si 100): hexane–2-propanol mixtures[4]; ethyl acetate–hexane mixtures[5] and methylene chloride–acetonitrile–2-propanol mixtures[6]. The ethyl acetate-containing systems, however, have been used with a differential refractometer detector with the concomitant loss of sensitivity. As little as 5 ng of withanolide could be detected with a UV detector operating at 225 nm[4], while 1–9 μg were the lower limits for the differential refractometer[5].

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EXPERIMENTAL

All withanolides used were isolated from *A. breviflorus* plants from Argentina as previously described[2], and characterized by ^{1}H and/or ^{13}C NMR spectroscopy.

Crude extracts of leaves from young *A. breviflorus* plants were made according to Lockley *et al.*[7] by allowing the leaves cut into small pieces to stand in diethyl ether for 24 h at room temperature. After filtration and evaporation, the residue was dissolved in ethyl acetate and filtered through a short silica gel 60 (Merck) column. The less polar pigments and the withanolides could be recovered by elution with more ethyl acetate. (This treatment eliminated highly polar pigments that were retained in the silica, which otherwise might interfere when carrying out closely spaced successive HPLC separations.) Finally the filtered extract was evaporated and redissolved in ethyl acetate (1 ml per leaf).

HPLC experiments were performed with a Hewlett-Packard 1084B liquid chromatograph equipped with a variable wavelength detector operating at 260 nm, a variable volume injector with automatic sampling system and an automatic fraction collector. All separations were carried out with a 10-μm LiChrosorb Si 100 column (250 × 4.6 mm I.D.) (Hewlett-Packard) and ethyl acetate–hexane–2-propanol (30:3:2) at a flow-rate of 0.4 ml/min. Solvents were of analytical reagent quality. Samples were dissolved in ethyl acetate or in the elution solvent.

RESULTS AND DISCUSSION

Owing to the wide polarity range of *A. breviflorus* withanolides, the only system that allowed a separation in a reasonable length of time (*ca.* 30 min) was 100% ethyl acetate. However, this solvent had the disadvantage of producing broad tailing peaks for the more polar withanolides [withaferin A (IV) and especially jaborosalactone D (II)] that affected resolution and sensitivity. This problem could be overcome by adding to the ethyl acetate a hydroxylic solvent mixture of similar eluting strength [as determined by thin-layer chromatography (TLC)], namely, hexane–2-propanol (3:2). Thus, a ternary solvent mixture, ethyl acetate–hexane–2-propanol (30:3:2), proved to be ideal for the separation of withanolides from *A. breviflorus* at low flow-rates (0.4 ml/min).

The detection problem was solved by using a variable wavelength detector operating at 260 nm, as the chosen solvent mixture was optically transparent from *ca.* 256 nm onwards. In this way 50–100 ng of withanolide may easily be detected. (A less sensitive alternative for detectors that lack sufficient dynamic range at this wavelength is to use the weak absorption of these withanolides at *ca.* 340 nm.)

An artificial mixture of six withanolides isolated from *A. breviflorus* could be resolved within 20 min after injection (Fig. 1). A crude extract from one leaf of a young *A. breviflorus* plant may easily be assayed for withanolides, as can be seen in Fig. 2. The less polar jaborosalactone E (III) and A (I) are somewhat superposed with green pigments, but are easily identifiable.

For preparative work, a single column as described in Experimental could be loaded with 100–150 μg of withanolide mixture without affecting resolution. Thus, in ten successive runs the withanolides in a crude extract from one leaf could be separated (total time 200 min, *i.e.*, 80 ml of elution solvent). As even the peak of the more polar jaborosalactone D (II) is symmetrical and fairly sharp, the collected fraction volumes are *ca.* 1 ml or less in each run.

The collected fractions containing the withanolides from a single preparative

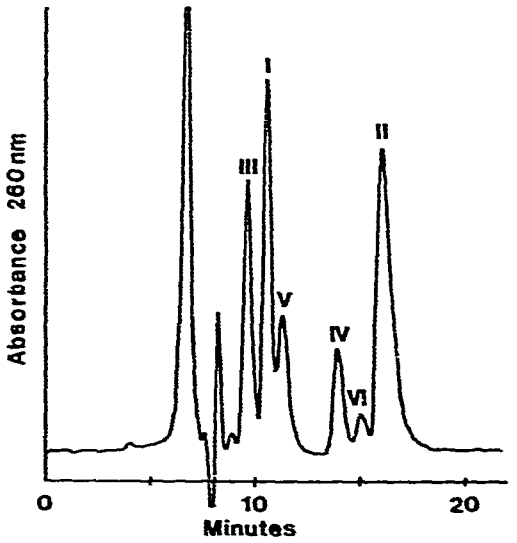

Fig. 1. Chromatogram of an artificial mixture of withanolides from *A. breviflorus*. Peaks I–IV correspond to structures in the text; V and VI are unknown withanolides. Approximate amounts (in parentheses) and retention times were: I (2 μg), 10.56 min; II (3 μg), 16.01 min; III (2 μg), 9.60 min; IV (1 μg), 13.93 min; V (1 μg), 11.34 min; VI (0.4 μg), 15.10 min.

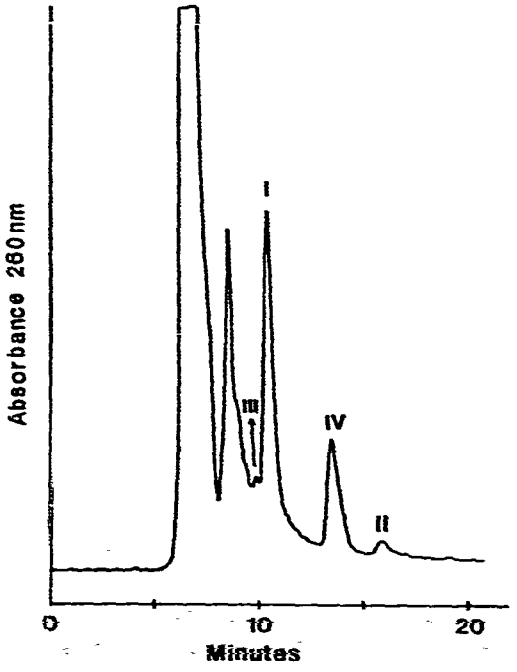

Fig. 2. Analytical separation of a crude extract from one leaf of a young *A. breviflorus* plant containing *ca.* 1.5 mg of withanolides. Injection volume: 1 μl.

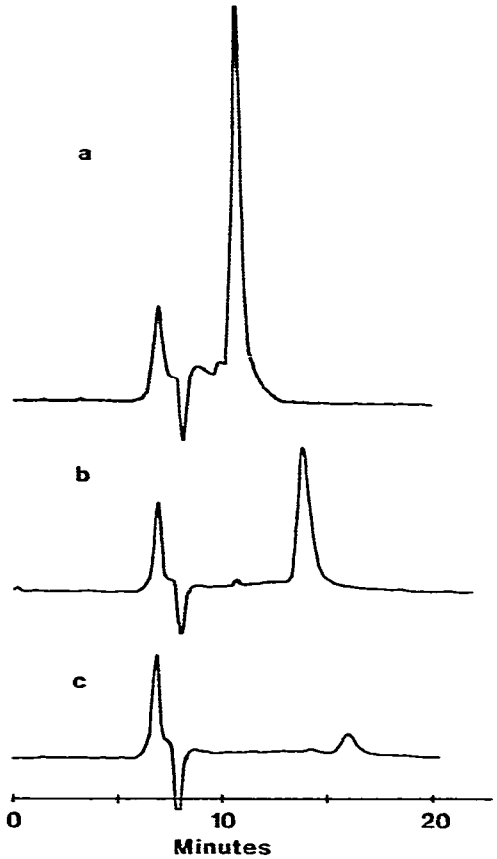

Fig. 3. Chromatograms of the collected fractions from a single preparative run of the extract in Fig. 2, containing withanolides I (a), IV (b) and II (c). Injection volume: 1/20 of fraction volume.

run of the crude extract from one leaf (1/10 of extract injected) were rechromatographed (1/20 of fraction volume injected) to verify the separation achieved; the elution curves are shown in Fig. 3.

We have successfully used this separation method for biosynthetic studies using isolated *A. breviflorus* leaves with different radioactive precursors. The results of these experiments will be published elsewhere.

ACKNOWLEDGEMENTS

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