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Note

Separation of withanolides by high-pressure liquid chromatography with coiled columns

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The withanolides are a new class of plant steroids with interesting cytotoxic and chemical properties[1]. High-pressure liquid chromatography (HPLC) has obvious advantages over the older chromatographic methods for analytical and preparative work, especially in biosynthetic studies, but so far only one report on the application of HPLC to withanolides has appeared in the literature. Gustafson *et al.*[2] have separated five synthetic derivatives and two microbial metabolites of withaferin A on a 30-cm μPorasil column with ethyl acetate or ethyl acetate–hexane, using a refractive index detector. We have used Porasil A, which is cheaper and much easier to pack into a column, with hexane–isopropanol mixtures, which permit the use of the more sensitive ultraviolet detector. To compensate for the lower efficiency of our adsorbent, we have increased the column length to 12 ft. (366 cm) and found it convenient to coil the column.

EXPERIMENTAL

The withanolides used in our study are shown in Fig. 1. All of them were isolated from solanaceous plants, except the two hydrogenation products II and IV, which are more stable than the corresponding natural products I and III.

The HPLC apparatus was assembled from commercially available components. The pump was of the single-piston reciprocating type, Altex Model 110 (Altex, Berkeley, Calif., U.S.A.)**. A Hitachi variable-wavelength detector, Altex Model 155-30, equipped with a flowcell having a 10-mm pathlength and a 20-μl volume, was set at

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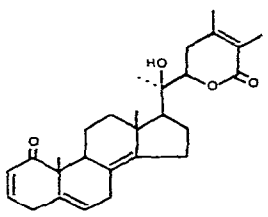
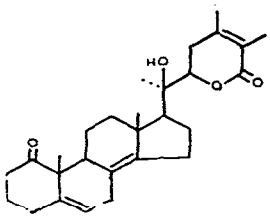
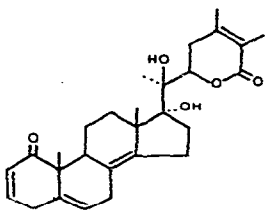
** Reference to a company and/or product named by the Department is only for the purpose of information and does not imply approval or recommendation of the product named to the exclusion of others which may also be suitable.



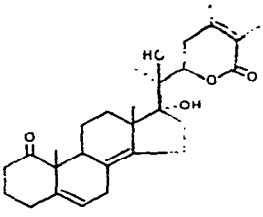
I. Withanolide G



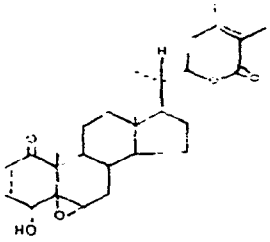
II. 2,3-Dihydrowithanolide G



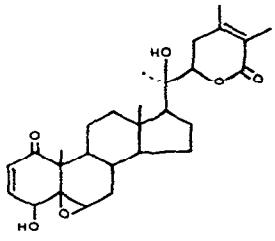
III. Withanolide J



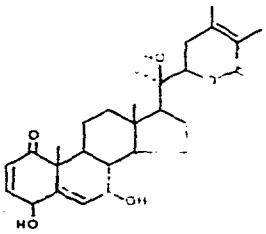
IV. 2,3-Dihydrowithanolide J



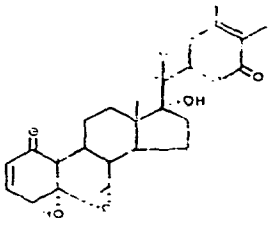
V. 27-Desoxywithanolin A



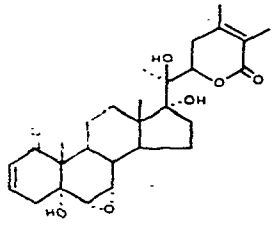
VI Withanolide D



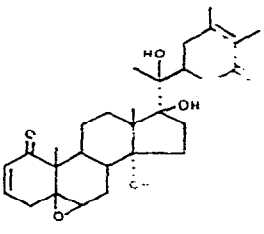
VII. 4β,7β-Dihydroxy-8,14-Dihydrowithanolide G



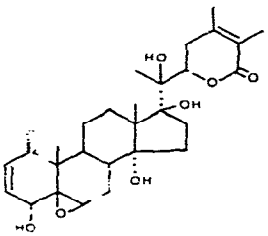
VIII. Withanone



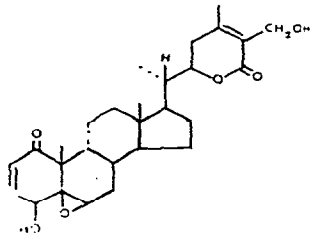
IX. 20-Hydroxywithanone



X. Withanolide E



XI 4β-Hydroxywithanolide E



XII. Withaferin A

Fig. 1. Withanolide structures.

225 nm and attached to a single-channel recorder, Linear Model 335 (Linear, Irvine, Calif., U.S.A.). The column was assembled from two coiled 6-ft sections of $^1/_8$-in. O.D. tubing, made of either copper (1.8 mm I.D.) or stainless steel (2.2 mm I.D.).

The columns were packed with Porasil A (Waters Assoc., Milford, Mass., U.S.A.) by the following procedure. Straight 6-ft. lengths of tubing were first thoroughly cleaned with solvents, detergents and water and then provided with $^1/_8$-in. brass Swageiok female fittings (Crawford Fitting, Salon, Ohio, U.S.A.) on both ends (A in Fig. 2). The bottom end of each tube was fitted with a stainless-steel reducing union (B in Fig. 2), $^1/_8$ to $^1/_{16}$ in., containing a Type 16 stainless-steel removable fritted disk.

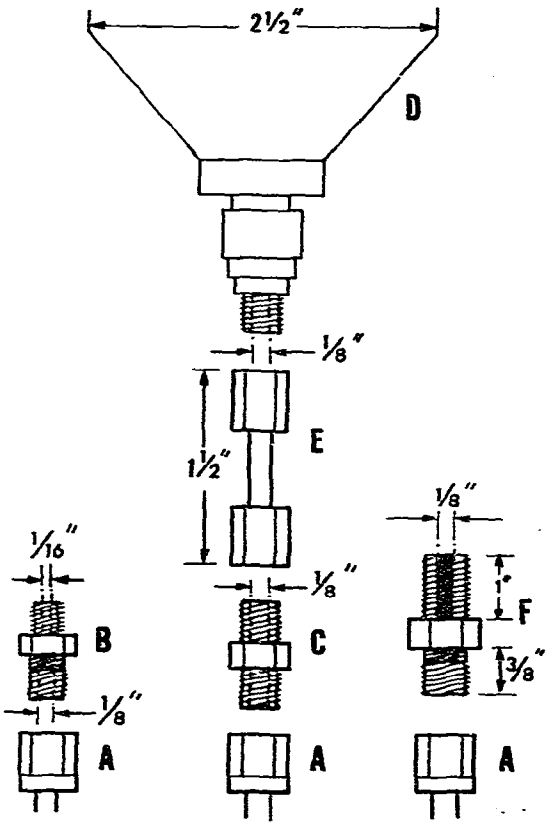


Fig. 2. Column assemblies (exploded view). Left, end fitting. Middle, packing assembly. Right, sample fitting. A = End of 1/8-in. O.D. column with female fitting; B = "zero dead volume" end fitting with removable 2-μm fritted disk; C = male–male union; D = funnel with male thread; E = PTFE tubing with female fittings; F = sample fitting, containing silica gel and a filter paper disk in the top and a fritted disk in the bottom portion.

2-μm pore size (Altex). The top end was fitted with a male–male, $^1/_8$ to $^1/_8$ in., union (Crawford) (C in Fig. 2), which was connected to an aluminum funnel (Altex) (D) through a piece of translucent PTFE (DuPont, Wilmington, Del., U.S.A.) tubing (E), $1^1/_2$ in. long and $^1/_8$ in. O.D., which had $^1/_8$ in. female brass fittings on both ends. Two Chromat-o-Tap IV vibrators (Whatman, Clifton, N.J., U.S.A.) were then attached to the chromatographic tube, 2 ft. from each end. Passing from the funnel into the column under constant vibration, the adsorbent could be observed in the translucent section, and packing was completed when it no longer went down.

The funnel was then removed, and each column was coiled around a 1-l polyethylene bottle (diameter *ca.* 10 cm). The coil was mounted on a sheet-metal tube, 11.5 cm in diameter, and fastened with metal straps. The translucent section was connected to the outlet of the pump, and the eluent, consisting of a 10% solution of isopropanol in *n*-hexane ("Distilled in Glass" quality; Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), was passed through the column at a rate of 2 ml/min for 10 min. Finally, the section containing the PTFE tubing was removed. On one column it

was replaced by the type of fitting used for the bottom end, and that fitting was connected to the bottom end of the other column with a short column coupler (Crawford). The top of that column received a special sample fitting (F in Fig. 2).

The sample fitting was made from a $^1/_8$-in. brass bulkhead union (Crawford). The shorter end of this union was drilled to accept a $^1/_8$-in. stainless-steel fritted disk, and then the longer end was drilled with a $^1/_8$-in. drill to within 1–2 mm of the fritted disk. This well was filled with Porasil A (see F in Fig. 2). Samples were delivered directly onto the well, and then the sorbent was covered with a $^1/_8$-in. disk of filter paper. Finally, the shorter end of the sample fitting was attached to the top of the column and the longer end was connected to the pump. At the end of each analysis, the sample fitting was disconnected and air-dried. After removal of the paper disk, the sample fitting was ready to be reused without changing the sorbent filling.

The eluents used in our experiment were continuously recycled. A 250-ml portion of solvent mixture was kept in a reservoir containing a magnetic stirring bar. After passing through the column and detector the solvent mixture was returned to the reservoir. No appreciable effect on background absorption or chromatographic zones was observed.

RESULTS AND DISCUSSION

Using the absorption at 225 nm for monitoring, we have been able to detect as little as 5 ng of the withanolides. Thus, the UV detector is about 1000 times more sensitive than the refractometer used by Gustafson *et al.*[2]. However, in biochemical work there is a need for fractionating relatively large amounts of crude samples. We have not had sufficient material available to test the load capacity of our columns, but from past experience[3] we believe that it is in the range of several milligrams. As in the past, we have developed our chromatograms slowly and at moderate pressures in order to improve resolution.

The chromatogram presented in Fig. 3 took 20 h. It shows the elution by *n*-hexane–isopropanol (9:1) of the six withanolides: G (I), J (III), 27-desoxywithaferin A (V), D (VI), 20-hydroxywithanone (IX) and E (X). The retention of each compound is reproducible within $\pm$ 10 min. As expected, the elution sequence reflects the nature, number and position of the oxygen functions in the withanolide molecule. Thus, withanolide G (I), which has one tertiary hydroxyl group at C-20, is followed by withanolide J (III), which has an additional tertiary hydroxyl group at C-17, and then by 27-desoxywithaferin A (V), which has a secondary hydroxyl group at C-4, which is followed by withanolide D (VI), having an additional OH at C-20. The trihydroxy steroids 20-hydroxywithanone (IX) and withanolide E (X) have the same kind of oxygen functions, but their positions and orientations are different. The remainder of the peaks is due to impurities in the samples.

As in all isocratic adsorption chromatograms, the early peaks are sharp, while the late peaks are broad and tend to tail. The more polar withanolides, 4β-hydroxywithanolide E (XI) and withaferin A (XII) were therefore chromatographed in a more polar solvent system, *n*-hexane–isopropanol (3:2). Fig. 4 shows that withaferin A (XII), which has one primary and one secondary hydroxyl group, is the most polar of these steroids, being eluted after 4β-hydroxywithanolide E (XI), which has one secondary and three tertiary hydroxyl groups. Withanone (VIII) and 4β,7β-di-

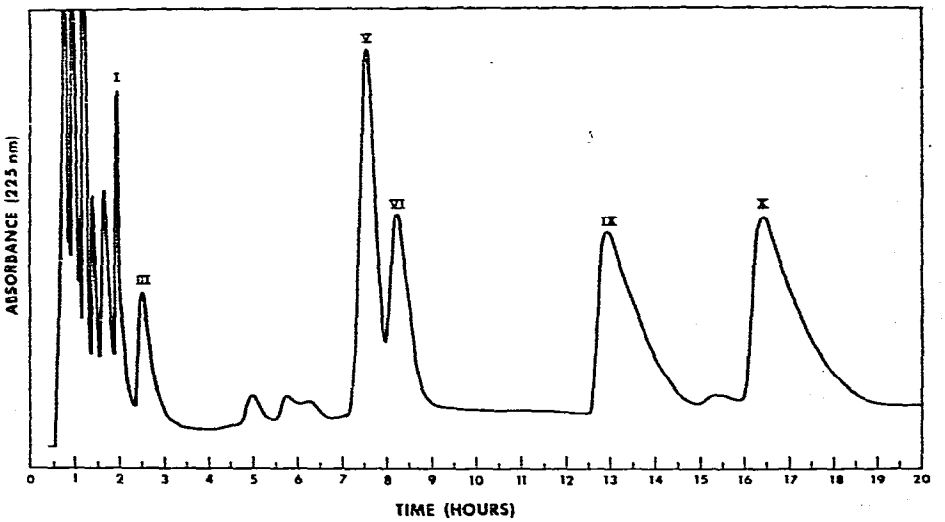
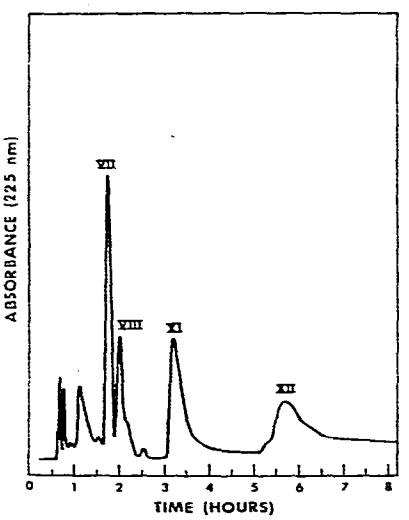


Fig. 3. Elution curve for six withanolides. A mixture of 1.6 μg I, 0.75 μg III, 25 μg V, 2.5 μg VI, 2.5 μg IX and 25 μg X (*cf.* Fig. 1) in 25 μl of chloroform–methanol (1:1) were applied to a 12-ft. 1/8-in. O.D. column of Porasil A. Eluent, *n*-hexane–isopropanol (9:1); flow-rate, 0.2 ml/min; pressure, 1100 p.s.i. Detector at 225 nm; range, 0.2; recorder speed, 2 cm/h; span, 10 mV.

hydroxy-8,14-dihydrowithanolide G (VII) have been included in this chromatogram, because in the one represented by Fig. 3 their peaks tend to overlap those of V and VI.

With the difficult separation of withanolide G (I) and J (III) from their respective dihydro derivatives, II and IV (Fig. 5), we have used the same conditions as



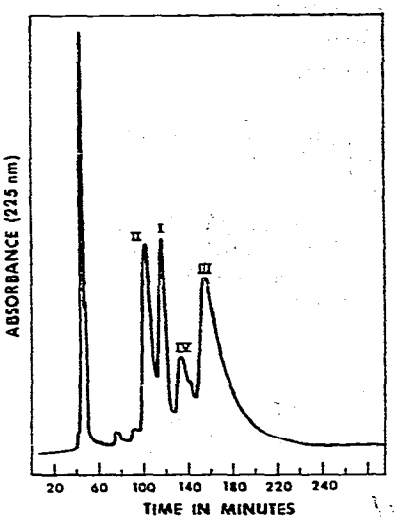


Fig. 4. Elution curve for four withanolides. A mixture of 0.75 μg VII, 7.4 μg VIII, 20 μg XI and 20 μg XII (*cf.* Fig. 1) in 10 μl of chloroform–methanol (1:1) was chromatographed under the conditions given for Fig. 3, except the eluent was *n*-hexane–isopropanol (3:2).

Fig. 5. Elution curve for withanolides G and J and their respective dihydro derivatives. A mixture of 1.96 μg II, 1.60 μg I, 7.40 μg IV and 0.75 μg III (*cf.* Fig. 1) in 5 μl chloroform–methanol (1:1) was chromatographed under the conditions given for Fig. 3, except the recorder speed was 3 cm/h.

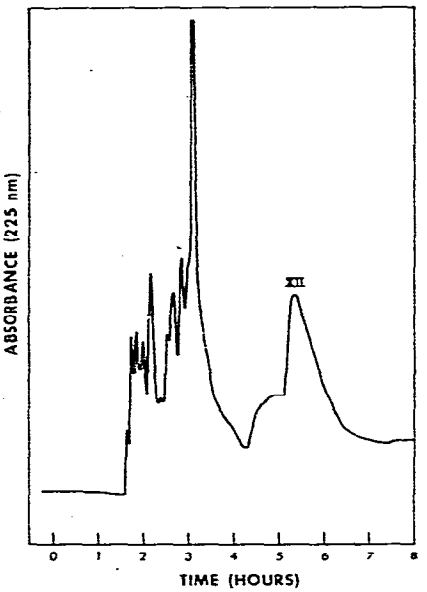


Fig. 6. Elution curve for a crude extract of leaves of *Withania somnifera*, chemotype I. For experimental conditions see Fig. 3.

those represented by Fig. 3, except the recorder speed was increased. In each case, the dihydro derivative was eluted before the corresponding withanolide.

Fig. 6 shows the application of our method to an ether extract of leaves of *Withania somnifera*, chemotype I. This material, which was known to contain withaferin A from previous work[4], showed the expected peak (XII) in the elution curve. The method thus appears to be suitable for biosynthetic studies.

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3 I. R. Hunter, M. K. Walden and E. Heftmann, *J. Chromatogr.*, 153 (1978) 57.
4 A. Abraham, I. Kirson, D. Lavie and E. Glotter, *Phytochemistry*, 14 (1975) 189.