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Note

High-pressure liquid chromatography of derivatives and microbial metabolites of withaferin-A

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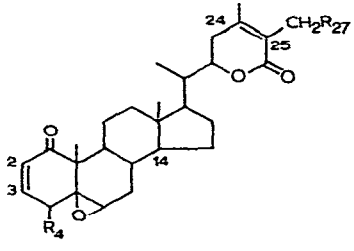
Withaferin-A is the prototype compound for a group of polyfunctional steroid-lactones known as the withanolides. This compound has been isolated from species of *Withania*[1–3] and from the plant *Acnistus arborescens*[4,5]. Interest has been shown in the withanolides since they display activity in a number of tumor test systems[5,6]. In the course of conducting microbial transformation studies with withaferin-A (I), we attempted to devise a simple analytical system which could be adapted to the detection of a variety of potential metabolites and derivatives of I. This report describes the application of high-pressure liquid chromatography (HPLC) to the separation of I, several chemical derivatives (II–VI), and two microbial metabolites (VII and VIII)[7].



		R_4	R_{27}
I	Withaferin-A	OH	OH
II	2,3-Dihydrowithaferin-A	OH	OH
III	Withaferin-A diacetate	$OCOCH_3$	$OCOCH_3$
IV	4-Dehydrowithaferin-A	=O	OH
V	2,3-Dihydro-27-deoxywithaferin-A	OH	H
VI	2,3,24,25-Tetrahydro-27-deoxywithaferin-A	OH	H
VII	14-α-Hydroxywithaferin-A (metabolite)	OH	OH
VIII	Unknown withaferin-A metabolite	–	–

EXPERIMENTAL

Withaferin-A was isolated from the leaves and stems of *Withania somnifera* Dun. (S. B. Penick & Co., New York, N.Y., U.S.A.) and was characterized as described by Nicholas and Rosazza[8]. Treatment of withaferin-A with pyridine–acetic anhydride gave the known diacetate (III)[8]. Compounds II, V and VI were prepared by stepwise catalytic reduction of withaferin-A using palladium on carbon under hydrogen[5]. Compound IV was prepared by MnO_2 oxidation of I[5]. The metabolites VII and VIII were obtained through microbial transformation experiments using cultures of *Cunninghamella elegans*[7]. All compounds gave single spots on examination with thin-layer chromatography[8].

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HPLC experiments were performed with a Waters ALC/GPC 202 instrument equipped with an M6000 solvent delivery system, a U6K universal injector, and an R-401 differential refractometer detector. All separations were best achieved with a μPorasil column (30 × 0.4 cm I.D.) (Waters). Solvents used were of analytical reagent quality. Compositions of solvent mixtures and flow-rates were adjusted as necessary to maximize separations. Samples of the steroids were dissolved in chloroform except for VII which would only dissolve in methanol–chloroform (1:9).

RESULTS AND DISCUSSION

Several types of columns and solvent systems were initially examined for their potential to provide adequate separations of the withanolides. Since reversed-phase HPLC has been employed with other steroids, we tried to obtain separations using C_{18} Porasil, and phenyl-Porasil (Waters) columns. With these columns, no retentions were obtained with water and/or methanol solvent systems. Although adequate retention of the steroids could be obtained when ethyl acetate was employed as solvent, peaks were broad, and resolution was poor. Because withaferin-A and its derivatives are highly functionalized, they do not behave like typical steroids either in solubility properties, or in mobilities on thin-layer chromatograms. Corasil I and Porasil A (61 × 0.2 cm) columns were also tried, but resolutions of mixtures were inferior to those obtained with a μPorasil column.

The best solvent systems for separating mixtures of the withaferin-A derivatives consisted of ethyl acetate and hexanes mixtures. Seven of the eight compounds available could be resolved from one another with ethyl acetate–hexanes (5:1) within 15 min following injection (Fig. 1). Several of the withaferin-A derivatives with similar retention times (Fig. 1, V and VI) were clearly separated by using ethyl acetate–

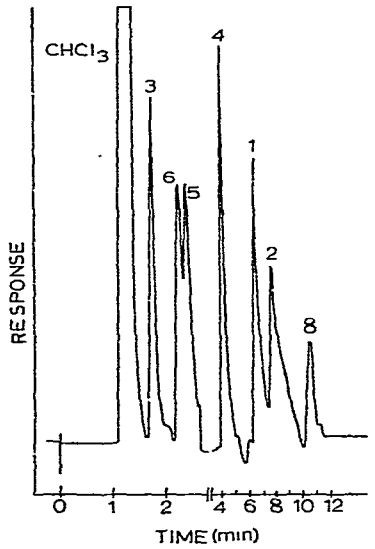

Fig. 1. HPLC separation of seven withanolides with ethyl acetate–hexanes (5:1). Flow-rate, 3 ml/min; pressure, 1800 p.s.i.; temperature, 75°; detection, refractive index; injection 60 μg of each compound. Chart speed and detector sensitivity were adjusted following the elution of V. Peaks 1–8 correspond to compounds I–VIII.

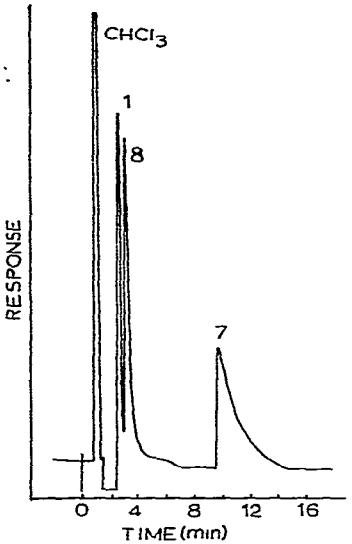

Fig. 2. HPLC separation of withaferin-A (1) and two microbial metabolites (7 and 8) was achieved at 25°, using 100% ethyl acetate. Flow-rate, 3.5 ml/min; pressure, 2100 p.s.i.; detection, refractive index; injection, 50-μg samples.

hexanes (1:1) as the solvent system. Withaferin-A was separated from metabolites VII and VIII using 100% ethyl acetate (Fig. 2). Solvent mixtures of higher polarities were needed to elute VII from the column.

None of the compounds examined exhibit ultraviolet absorption beyond 230 nm, thus necessitating the use of the less sensitive refractive index detector. Limits of detectability of the withanolides with a signal-to-noise ratio of no less than 2:1 were as follows: III, 1 μg; IV–VI, 2 μg; I, 3 μg; II, 5 μg; VIII, 7 μg; and VII, 9 μg. These sensitivities are adequate for use in typical microbial transformation experiments.

The chromatographic systems described will be of value in monitoring microbial transformation studies with withaferin-A, and should prove to be of value in determining withaferin-A and other withanolides in plant extracts.

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