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Note

High-performance liquid chromatography of ecdysteroids and ecdysteroid-22-phosphates

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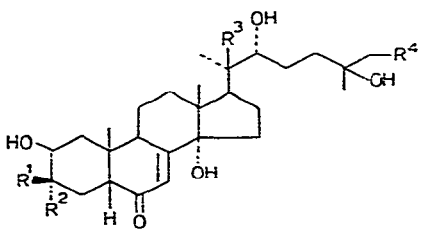
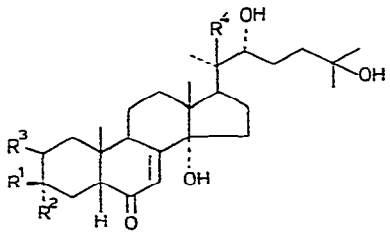
The ecdysteroids (insect moulting hormones) are a group of polyhydroxylated steroids possessing a 5β,7-ene-6-one ring structure. 20-Hydroxyecdysone (II) is generally regarded as the major active hormone[1], although ecdysone (I) may also elicit a hormonal response in certain tissues at some stages in development. 2-Deoxyecdysone[2–4] and 2-deoxy-20-hydroxyecdysone[5,6] have been isolated from the ovaries and eggs of a number of insect species as major ecdysteroid components of largely unidentified highly polar conjugates. These 2-deoxy ecdysteroids may be possible precursors of ecdysteroids I and II. Other ecdysteroids which have been isolated from insects include the 26-hydroxy[7–11], 3-epi[12–14], 3-dehydro[15,16], 3-acetate[17] and 22-phosphate[18] derivatives. The latter compounds are considered to be inactivation products of the hormones. Thus, ecdysteroids varying widely in polarities are encountered in insect metabolism.

Complex mixtures of ecdysteroids are amenable to chromatography on reversed-phase high-performance liquid chromatographic (HPLC) systems[19] and, owing to the wide range of polarities, are best resolved using a methanol–water gradient for elution. In the present paper we describe the chromatography of a wide spectrum of insect ecdysteroids on an efficient, short (15-cm) Ultrasphere-ODS (particle size 5 μm) column. The data presented (Table I) indicate potential pitfalls in the





(I) R^1 = OH, R^2 = R^3 = R^4 = H
(II) R^1 = R^3 = OH, R^2 = R^4 = H
(III) R^1 = R^3 = R^4 = H, R^2 = OH
(IV) R^1 = R^4 = H, R^2 = R^3 = OH
(V) R^1, R^2 = O, R^3 = R^4 = H
(VI) R^1, R^2 = O, R^3 = OH, R^4 = H
(VII) R^1 = R^4 = OH, R^2 = R^3 = H
(VIII) R^1 = R^3 = R^4 = OH, R^2 = H
(IX) R^1 = O-CO-CH_3, R^2 = R^3 = R^4 = H

(X) R^1 = OH, R^2 = R^4 = H, R^3 = O-CO-CH_3
(XI) R^1 = OH, R^2 = R^3 = R^4 = H
(XII) R^1 = R^4 = OH, R^2 = R^3 = H
(XIII) R^1 = R^3 = R^4 = H, R^2 = OH
(XIV) R^1 = R^3 = H, R^2 = R^4 = OH

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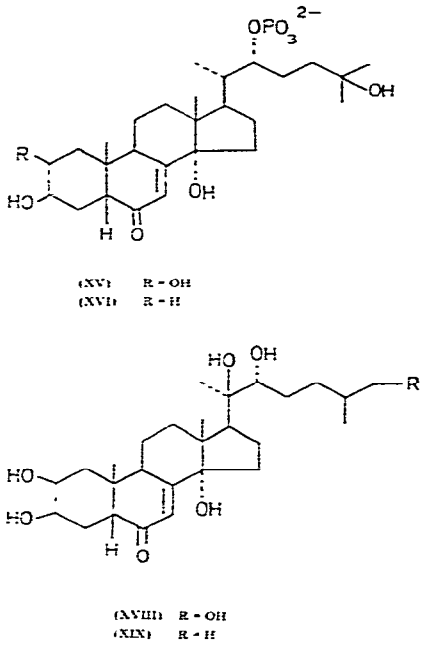

identification of some ecdysteroids and metabolites having similar chromatographic properties, and the value of an efficient reversed-phase column in resolving such steroids. The chromatography of the newly identified ecdysteroid monoacetates and 22-phosphates are also reported. Ecdysone-22-phosphate and 2-deoxyecdysone-22-phosphate have also been analysed by HPLC using reversed-phase paired-ion and anion exchange (Whatman SAX) chromatography.

EXPERIMENTAL

Chemicals

The ecdysteroid samples used in the present study were obtained from various sources: ecdysone (I) (Simes, Milan, Italy); 20-hydroxyecdysone (II) and 3-epi-2-deoxyecdysone (XIII) (Dr. G. B. Russell, D.S.I.R., New Zealand); 26-hydroxyecdysone (VII) (Dr. J. N. Kaplanis, U.S.D.A., Beltsville, MD, U.S.A.); 20,26-dihydroxyecdysone (VIII), 2-deoxy-20-hydroxyecdysone (XII) and 3-epi-2-deoxy-20-hydroxyecdysone (XIV) (Dr. D. H. S. Horn, C.S.I.R.O., Melbourne, Australia), 2-deoxyecdysone (XI) (Professor E. Ohnishi, Nagoya University, Nagoya, Japan); poststerone (XVII) (the late Dr. J. B. Siddall, Zoecon Corporation, CA, U.S.A.); inokosterone (XVIII) (Professor T. Takemoto, Tohoku University, Tohoku, Japan); ponasterone A (XIX) (Professor K. Nakanishi, Columbia University, New York, NY, U.S.A.); 3-dehydroecdysone (V) and 3-dehydro-20-hydroxyecdysone (VI) (prepared by the method of Spindler *et al.*[20]); 3-epiecdysone (III) and 3-epi-20-hydroxyecdysone (IV) (prepared by the method of Dinan and Rees[21]), ecdysone-2- (X) and 3-monoacetates (IX) (prepared by the method of Galbraith and Horn[22]); ecdysone-22-phosphate (XV) and 2-deoxyecdysone-22-phosphate (XVI) (isolated

from eggs of *Schistocerca gregaria*[18]). The mixture of ecdysteroids used in the separation shown in Fig. 1 was obtained from ecdysteroid conjugates, isolated from developing eggs of *Schistocerca gregaria*, by hydrolysis with a crude enzyme preparation from *Helix pomatia*[4].

HPLC grade methanol was purchased from Rathburn Chemicals (Walkerburn, Great Britain) and tetra-*n*-butylammonium hydroxide solution (40% in water) and AnalaR-grade orthophosphoric acid were purchased from BDH (Poole, Great Britain).

High-performance liquid chromatography

HPLC was carried out using a Model ALC/100 liquid chromatograph (Waters Assoc., Northwich, Great Britain) linked to a Perkin-Elmer LC-55 variable wavelength detector set at 250 nm. Linear solvent gradients were formed with the aid of a Model 660 solvent programmer (Waters Assoc.) and samples, dissolved in methanol, were injected via a Model U6K injector (Waters Assoc.).

An Altex Ultrasphere-ODS column (15 cm × 4.6 mm, particle size 5 μm) (Anachem, Luton, Great Britain) was used for reversed-phase chromatography. Some reversed-phase separations of ecdysteroid phosphates involved addition of tetra-*n*-butylammonium hydroxide (BDH) adjusted to pH 7.5 with orthophosphoric acid, to the aqueous methanol solvent to make a 0.6% solution.

RESULTS AND DISCUSSION

Reversed-phase HPLC using a methanol–water gradient on an Ultrasphere-ODS column allowed the separation of ecdysteroids of widely differing polarities in a single run (Table I). The application of such a system to the analysis of biological extracts is illustrated in Fig. 1. The chromatogram shows the separation of a series of ecdysteroids isolated from developing eggs of *S. gregaria*. These ecdysteroids probably form part of a metabolic scheme for the biosynthesis and inactivation of 20-hydroxyecdysone (II).

It is apparent from Table I that several of the ecdysteroids are either not resolved or have very similar chromatographic properties. Thus, potential pitfalls in identification of radioactive ecdysteroid metabolites are clear. However, use of such a reversed-phase column in conjunction with an adsorption-type column, such as APS-Hypersil (aminopropyl) or silica, obviates such problems. The system described in this paper allows good resolution of four ecdysteroids and their 3-epimers on this short column (Figs. 1 and 2). Although separation of ecdysteroids from their 3-epimers has been achieved on some other types of reversed-phase columns (μBondapak C_{18}, 30 cm[14]), this was not always the case[23]. On reversed-phase, the 3-epi-ecdysteroids have very similar retention volumes to the corresponding 3-dehydro compounds (Table I), but these pairs can be easily separated by chromatography on an APS-Hypersil column[23]. It is interesing that the order of elution of some plant ecdysteroids during isocratic reversed-phase HPLC was changed when different organic solvents in admixture with water were used as mobile phases[24].

It is important to note that whereas on silica thin-layer chromatography (TLC) ecdysone-2- and 3-monoacetates co-migrate with 3-dehydroecdysone[17], on a reversed-phase system these three ecdysone derivatives are easily resolved. Therefore, the

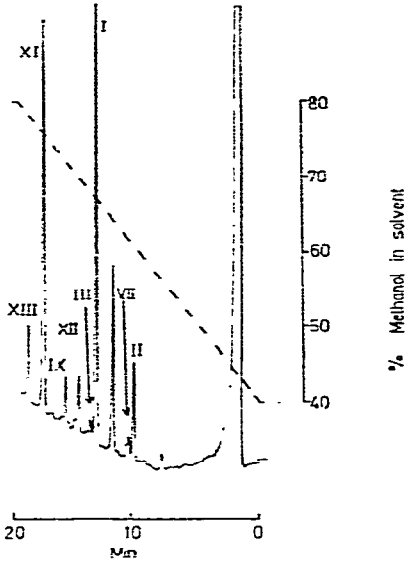

Fig. 1. Separation of ecdysteroids on an Ultrasphere-ODS column (15 cm × 4.6 mm I.D.). Mobile phase: linear gradient of 40% methanol in water increasing to 80% methanol in water over 20 min; flow-rate, 1 ml/min. Detection: UV absorbance at 250 nm. Peaks: II = 20-hydroxyecdysone: VII = 26-hydroxyecdysone; I = ecdysone; III = 3-epiecdysone; XII = 2-deoxy-20-hydroxyecdysone; IX = ecdysone-3-acetate; XI = 2-deoxyecdysone; XIII = 3-epi-2-deoxyecdysone.

identification of 3-dehydroecdysone and ecdysone monoacetates by co-chromatography with authentic compounds must be carried out on both adsorption and reversed-phase systems.

Reversed-phase columns are unsuitable for the chromatography of the highly polar ecdysteroid phosphates unless the anionic group is first neutralised. We have added tetra-*n*-butylammonium hydroxide to the eluting solvent as the anion pairing agent[25], which results in greater retention of the phosphate conjugates on the reversed-phase column (Table I, solvent system B). The reproducible results obtained by this method should facilitate the identification of radiolabelled ecdysteroid metabolites by co-chromatography with authentic steroid conjugate.

We have used chromatography on a strong anion exchange column as a major step in the purification of ecdysone-22-phosphate and 2-deoxyecdysone-22-phosphate from the eggs of *Schistocerca gregaria*[18]. For routine analytical studies, phosphate buffer (0.02 *M*) pH 5.0 was employed as the mobile phase, but when it was necessary to isolate ecdysteroid conjugates free of phosphate salts, this buffer was replaced with ammonium acetate (0.1 *M*). These systems separate ecdysone-22-phosphate, 2-deoxyecdysone-22-phosphate (Table II) and a highly polar 20-hydroxyecdysone conjugate of, as yet, unknown structure (data not presented).

In conclusion, complex mixtures of ecdysteroids can be resolved on an Ultrasphere reversed-phase column using a methanol–water gradient (total elution volume, 20 ml). Small elution volumes and low solvent flow-rates result in greater efficiency in the detection of UV absorbing compounds and in the monitoring of radiolabelled components either by liquid scintillation counting of fractions or by the use of an on-line radioactivity scintillation monitor. Ecdysteroid phosphates are

TABLE I

RETENTION VOLUMES FOR ECDYSTEROIDS ON ULTRASPHERE-ODS

Column: 15 cm × 4.6 mm I.D. Mobile phases: A, linear gradient of 40% methanol in water increasing to 80% methanol in water over 20 min; B, 46% methanol in water containing 0.6% tetra-*n*-butylammonium hydroxide; flow-rate, 1 ml/min.

	Ecdysteroid	*Retention volume (ml)*	
		Solvent system	
		A	*B*
I	Ecdysone	13.1	
II	20-Hydroxyecdysone	9.4	
III	3-Epiecdysone	13.6	
IV	3-Epi-20-hydroxyecdysone	9.9	
V	3-Dehydroecdysone	13.5	
VI	3-Dehydro-20-hydroxyecdysone	9.9	
VII	26-Hydroxyecdysone	9.7	
VIII	20,26-Dihydroxyecdysone	4.9	
IX	Ecdysone-3-acetate	16.0	
X	Ecdysone-2-acetate	18.3	
XI	2-Deoxyecdysone	18.6	
XII	2-Deoxy-20-hydroxyecdysone	14.8	
XIII	3-Epi-2-deoxyecdysone	20.0	
XIV	3-Epi-2-deoxy-20-hydroxyecdysone	16.6	
XV	Ecdysone-22-phosphate	1.3	8.5
XVI	2-Deoxyecdysone-22-phosphate	2.0	21.5
XVII	Poststerone	9.6	
XVIII	Inokosterone	9.9	
XIX	Ponasterone A	17.2	

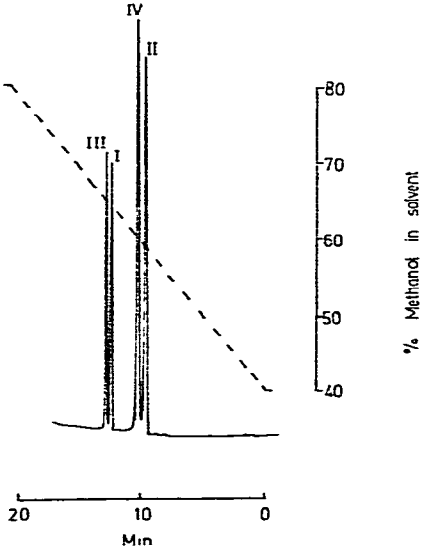

Fig. 2. Separation of ecdysone (I), 20-hydroxyecdysone (II), 3-epiecdysone (III) and 3-epi-20-hydroxyecdysone (IV) on an Ultrasphere-ODS column (15 cm × 4.6 mm I.D.). Chromatographic conditions were the same as those described for Fig. 1.

TABLE II

RETENTION VOLUME FOR ECDYSONE-22-PHOSPHATE AND 2-DEOXYECDYSONE-22-PHOSPHATE ON PARTISIL-SAX

Column: 50 cm × 9.4 mm I.D. Mobile phases: C, 0.02 *M* phosphate buffer, pH 5.0; D, 0.1 *M* ammonium acetate; flow-rate, 5 ml/min.

Ecdysteroid	*Retention volume (ml)*	
	Mobile phase	
	C	*D*
Ecdysone-22-phosphate	92	80
2-Deoxyecdysone-22-phosphate	120	105

amenable to both anion exchange and paired-ion reversed-phase chromatography, although peak shape is much better in the latter method.

ACKNOWLEDGEMENTS

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