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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ECDYSTEROIDS AND THEIR 3-EPI, 3-DEHYDRO AND 26-HYDROXY DERIVATIVES

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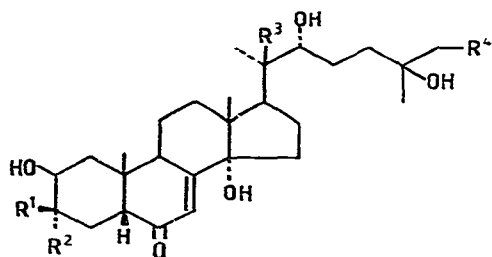
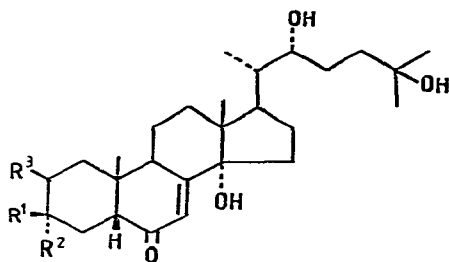
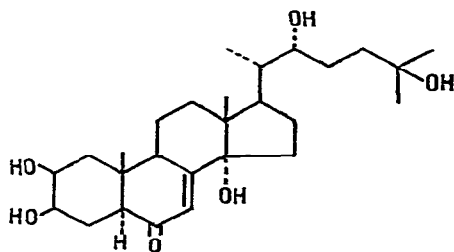
SUMMARY

The separation of ecdysteroids by high-performance liquid chromatography (HPLC) on reversed-phase (C₁₈) and APS-Hypersil (aminopropyl) microparticulate columns and by thin-layer chromatography has been evaluated. Reversed-phase columns with solvent gradient elution are particularly suitable for analysis of ecdysteroids of widely differing polarities since the re-equilibration time is shorter than with adsorption columns. Clear separation of ecdysteroids from the corresponding 3-dehydro and 3-epi derivatives can be achieved on a 25 cm long APS-Hypersil column, but 3-epiecdysone and 20-hydroxyecdysone are incompletely resolved in this system. However, the separation of the latter pair of compounds can be readily achieved on a longer APS-Hypersil column or on a reversed-phase HPLC column or by thin-layer chromatography. APS-Hypersil has advantages over silica gel as column packing material for separation of polar compounds such as ecdysteroids. The mechanism of retention on APS-Hypersil is discussed.

INTRODUCTION

A major moulting hormone (ecdysteroid) in larvae of most investigated insect species is 20-hydroxyecdysone (II), which is usually accompanied by smaller amounts of ecdysone (I) (ref. 1). 3-Dehydro^{2,3}, 3-epi^{4–6}, and 26-hydroxy^{7–11} derivatives of these hormones have also been isolated from insects (III–VIII) by mass and/or as metabolites of radioactive substrates. Furthermore, 2-deoxyecdysone (IX) has been isolated as a major hormone in the ovaries and eggs of some insect species^{12–14}. Since the above ecdysteroids probably form a metabolic scheme in insects, it is frequently necessary to separate the individual compounds.

Although most of these ecdysteroids can be separated by thin-layer chromatography (TLC) with appropriate choice of developing solvent, the complete resolution of ecdysteroids and their corresponding 3-epi derivatives is difficult. Furthermore, when small amounts of ecdysteroids are subjected to TLC losses can be appreciable. High-performance liquid chromatographic (HPLC) methods employing both adsorption (silicic acid) and reversed-phase techniques have been used

(I) $R^1 = \text{OH}, R^2 = R^3 = R^4 = \text{H}$ (II) $R^1 = R^3 = \text{OH}, R^2 = R^4 = \text{H}$ (III) $R^1 = R^3 = R^4 = \text{H}, R^2 = \text{OH}$ (IV) $R^1 = R^4 = \text{H}, R^2 = R^3 = \text{OH}$ (V) $R^1, R^2 = \text{=O}, R^3 = R^4 = \text{H}$ (VI) $R^1, R^2 = \text{=O}, R^3 = \text{OH}, R^4 = \text{H}$ (VII) $R^1 = R^4 = \text{OH}, R^2 = R^3 = \text{H}$ (VIII) $R^1 = R^3 = R^4 = \text{OH}, R^2 = \text{H}$ (IX) $R^1 = \text{OH}, R^2 = R^3 = \text{H}$ (X) $R^1 = R^3 = \text{H}, R^2 = \text{OH}$ 

(XI)

successfully for separation of many ecdysteroids (*e.g.* refs. 15 and 16). The fact that ecdysteroids are very polar compounds may give rise to some difficulties during their HPLC separation by adsorption on silicic acid, since polar eluting solvents (often containing some alcohol) have to be used. This tends to cause progressive changes in retention properties of the column owing to deactivation of the packing material and peak shape is sometimes distorted.

Partial resolution of ecdysone (I) and 3-epiecdysone (III) by HPLC on silicic acid has been reported⁴, whereas complete (but close) separation of such pairs can be achieved on μ Bondapak C_{18} reversed-phase columns (60 cm \times 4.0 mm I.D.)⁶. However, in our experience the separation of the 3-epimers on an analogous system is difficult and can only be achieved under ideal conditions. It is also apparent that ecdysteroids and their corresponding 3-dehydro derivatives cannot be resolved on reversed-phase columns.

To circumvent some of the difficulties outlined above and to develop a satisfactory HPLC separation of ecdysteroids on columns which might complement reversed-phase (C_{18} or C_8) and silica columns, we have examined the separation of these steroids, particularly of ecdysteroids and their 3-epi and 3-dehydro derivatives on AFS-Hypersil. The latter column packing material is synthesised by bonding aminopropyl groups to Hypersil spherical silica gel via siloxane linkage¹⁷.

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 (X; Dr. G. B. Russell, D.S.I.R., New Zealand; isolated from *Blechnum volcanicum* — personal communication), 26-hydroxyecdysone (VII; Dr. J. N. Kaplanis, U.S.D.A., Beltsville, MD, U.S.A.), 20,26-dihydroxyecdysone (VIII; Dr. D. H. S. Horn, C.S.I.R.O., Melbourne, Australia), 2-deoxyecdysone (IX; Professor E. Ohnishi, Nagoya University, Japan), 3-dehydroecdysone (V) and 3-dehydro-20-hydroxyecdysone (VI; prepared from the corresponding ecdysteroids by the method of Spindler *et al.*¹⁸), 3-epiecdysone (III) and 3-epi-20-hydroxyecdysone (IV; prepared from the corresponding 3-dehydro-ecdysteroids by reduction¹⁹) and the 5 α -epimer of ecdysone (XI; prepared from ecdysone by base equilibration²⁰). AnalaR-grade methanol was purchased from BDH and re-distilled prior to use. 1,2-Dichloroethane (HPLC grade) and propan-2-ol (SLR grade) were purchased from Rathburn Chemicals, Walkerburn, Great Britain and Fisons, Loughborough, Great Britain, respectively.

Chromatographic procedures

A Model ALC/100 liquid chromatograph (Waters Assoc., Northwich, Great Britain) connected to a Perkin-Elmer LC-55 variable-wavelength detector set at 242 nm was used. The instrument was fitted with dual M6000 pumping system, a Model 660 solvent programmer (Waters Assoc.) and injection of samples dissolved in methanol was via a Model U6K septumless high-pressure valve injector.

Two types of column material were used: (i) APS-Hypersil, particle size 5 μm (Shandon Southern Products, Runcorn, Great Britain) and (ii) Partisil 10 ODS particle size 7 μm (Whatman, Maidstone, Great Britain). Columns (25 cm \times 4.6 mm I.D.) were prepared by an upward packing procedure²¹. Other details were as described previously²².

RESULTS AND DISCUSSION

From our experience, the best TLC systems for separation of ecdysone (I) and 20-hydroxyecdysone (II) from their corresponding 3-epi derivatives involve multiple elution (four times) in chloroform-methanol (90:15) or utilize continuous elution (*e.g.* Table I). These systems, although not entirely reproducible, can give resolution of small amounts of the ecdysteroid 3-epimers. Of course, in such systems the R_F

TABLE I

TLC OF SOME ECDYSTEROIDS

Kieselgel GF₂₅₄ plates were activated at 110°C for 1 h, allowed to cool to room temperature, pre-washed by development in methanol and allowed to air-dry overnight. Plates were developed by continuous elution for 2.5 h with chloroform-ethanol (9:2).

Compound	R_F
3-Dehydroecdysone (V)	0.88
3-Dehydro-20-hydroxyecdysone (VI)	0.77
3-Epiecdysone (III)	0.53
Ecdysone (I)	0.45
3-Epi-20-hydroxyecdysone (IV)	0.34
20-Hydroxyecdysone (II)	0.28

values are variable. Good resolution of 26-hydroxyecdysone (VII) and 20,26-dihydroxyecdysone (VIII) can be obtained on TLC by using a more polar solvent for single development.

It is apparent from Table II that although ecdysone (I), 20-hydroxyecdysone (II) and their 26-hydroxy-derivatives (VII and VIII) can be clearly resolved on this reversed-phase column, the 3-epi and 3-dehydro derivatives are not separated significantly from the parent ecdysteroids. In a recent publication⁶, 3-epiecdysteroids were separated (albeit close) from the parent ecdysteroids by reversed-phase HPLC. This may be due to the use of a longer column (60 cm) of μ Bondapak C₁₈, which also contains a higher percentage of C₁₈ hydrocarbon (approximately 10%) bound to silica as compared to the Partisil 10 ODS (approximately 5%). Under suitable isocratic conditions for separation of most of the ecdysteroids studied (solvent system A), 2-deoxyecdysone (IX) has a large retention volume. Employing an appropriate isocratic solvent system for elution of 2-deoxyecdysone (*e.g.* solvent system C) would cause too rapid elution of most of the other ecdysteroids. It is clear that a gradient solvent system is most appropriate to elute ecdysteroids covering a wide range of polarities (*e.g.* solvent system D; Fig. 1). Reversed-phase columns are particularly appropriate for solvent gradient elution, since after completion of a chromatographic run, rapid (*ca.* 10 min) re-equilibration to the initial conditions can be achieved. During solvent gradient work, the peak positions are not totally reproducible but usually vary only by approximately 0.5 min.

On the APS-Hypersil column, the 3-epi and 3-dehydro compounds are very well separated from the parent 3 β -hydroxyecdysteroids (Table III). Examining the compounds, 3-epiecdysone (III), ecdysone (I), 3-epi-20-hydroxyecdysone (IV) and 20-hydroxyecdysone (II), which chromatograph fairly closely as a group on TLC, it

TABLE II

RETENTION VOLUMES FOR ECDYSTEROIDS ON PARTISIL-10 ODS

Column 25 cm \times 4.6 mm I.D.; solvents: A, methanol-water (1:3), flow-rate 1-6 ml/min (most often 2 or 2.5 ml/min); B, methanol-water (3:7), flow-rate 4 ml/min; C, methanol-water (2:3), flow-rate 4 ml/min; D, linear gradient from methanol-water (1:3) at $t = 0$ min to methanol-water (3:2) at $t = 40$ min; flow-rate = 2 ml/min.

Ecdysteroid	Retention volume (ml)			
	Solvent system			
	A	B	C	D
Ecdysone (I)	75	38	14	35
20-Hydroxyecdysone (II)	31			22
3-Epiecdysone (III)	75			35
3-Epi-20-hydroxyecdysone (IV)	31			22
3-Dehydroecdysone (V)	75			35
3-Dehydro-20-hydroxyecdysone (VI)	31			22
26-Hydroxyecdysone (VII)	45			28
20,26-Dihydroxyecdysone (VIII)	20			16
2-Deoxyecdysone (IX)	>>120	136	32	54
Ecdysone-2,3-acetonide			75	

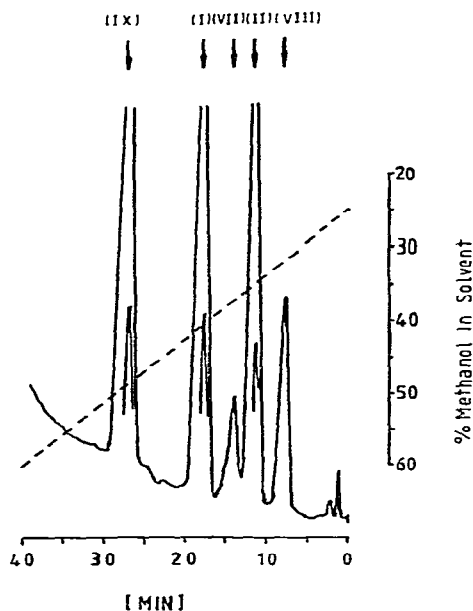


Fig. 1. Separation of ecdysteroids on a Partisil 10 ODS column (25 cm \times 4.6 mm I.D.); mobile phase, linear gradient of 25% methanol in water increasing to 60% methanol in water over 40 min; flow-rate 2 ml/min. Detection, UV absorbance at 242 nm; the recorder range was changed for compounds I, II and IX to obtain the peaks. VIII = 20,26-dihydroxyecdysone; II = 20-hydroxyecdysone; VII = 26-hydroxyecdysone; I = ecdysone; IX = 2-deoxyecdysone.

TABLE III

RETENTION VOLUMES FOR ECDYSTEROIDS ON APS-HYPERSIL

Column, 25 cm \times 4.6 mm I.D.; Solvents: E, F, G and H: dichloroethane-methanol-isopropanol; volume ratios: E, 95:1:4; F, 95:2:3; G, 95:3:2; and H, 95:4:1; flow-rate, 2-4 ml/min. I, dichloroethane-methanol (92:8); flow-rate, 3 ml/min.

Ecdysteroid	Retention volume (ml)				
	Solvent system				
	E	F	G	H	I
Ecdysone (I)	61	44	36	28	
20-Hydroxyecdysone (II)	97	65	54	42	
3-Epicdysone (III)	107	68			
3-Epi-20-hydroxyecdysone (IV)	184	110		52	
3-Dehydroecdysone (V)	18	12			
3-Dehydro-20-hydroxyecdysone (VI)	27				
26-Hydroxyecdysone (VII)	>>		124	103	24
20,26-Dihydroxyecdysone (VIII)	>>	323		166	35
5 α -Ecdysone (XI)	40				
2-Deoxyecdysone (IX)	20		14		
3-Epi-2-deoxyecdysone (X)	27				

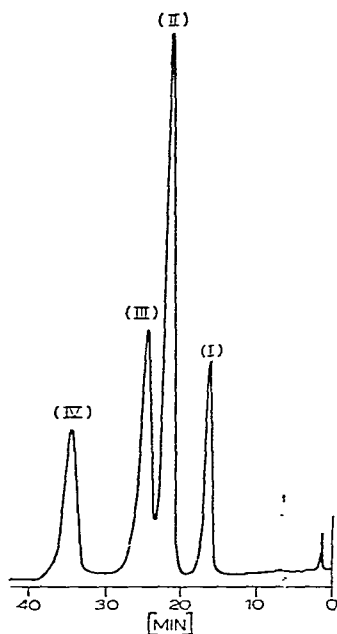


Fig. 2. Separation of ecdysteroids on an APS-Hypersil column (25 cm \times 4.6 mm I.D.); mobile phase, dichloroethane-methanol-propan-2-ol (95:4:1); flow-rate, 3 ml/min. I = ecdysone; II = 20-hydroxyecdysone; III = 3-epiecdysone; IV = 3-epi-20-hydroxyecdysone.

is apparent that 3-epiecdysone (III) and 20-hydroxyecdysone (II) which migrate furthest apart in the latter system, are least well resolved on the APS-Hypersil column. Therefore, it is convenient to separate 3-epiecdysone (III) and 20-hydroxyecdysone (II) on TLC or by HPLC on a reversed-phase column prior to carrying out the HPLC chromatographic fractionation on APS-Hypersil.

It was found that during regular use over several weeks, there was a tendency for APS-Hypersil columns to retain ecdysteroids more strongly, thus necessitating the use of more polar eluting solvents. This did not present a significant problem especially when regular checks were carried out with appropriate standard compounds. Resolution was often improved under the changed conditions (*e.g.* Fig. 2). The reason for this change in column properties is uncertain, but may be due to some change (*e.g.* oxidation) in the NH_2 groups on the column material. Although keto compounds might be expected to form Schiff's bases with the NH_2 groups in the column material, recoveries of ecdysteroids were always good (>90%). Peak shape with the APS-Hypersil columns is not as symmetrical as in the case of reversed-phase chromatography, but this does not present any problem. However, the APS-Hypersil material is less suitable for solvent gradient work than C_{18} reversed-phase columns, since the re-equilibration time is longer.

The mechanism of retention on the APS-Hypersil column is uncertain. There is evidence that this material can act both as a polar adsorbent or as a weak anion exchanger¹⁷. In the case of the ecdysteroids examined, all the compounds with the exception of the 3-epiecdysteroids eluted from the APS-Hypersil with the particular

solvent combinations used in a sequence corresponding to their polarity on silica gel TLC (*cf.* Table I). However, the 3-epi-ecdysteroids eluted in reverse order as compared to the parent compounds. In view of these results it is difficult to explain satisfactorily the mechanism(s) of retention of the ecdysteroids in this chromatographic system.

In summary, the APS-Hypersil system is of particular value for separation of 3-epi- and 3-dehydro-ecdysteroids from their parent compounds. This systems could be used in conjunction with TLC on silica gel or HPLC on reversed-phase (or possibly silicic acid). It is often desirable to use chromatographic procedures based on more than one principle *e.g.* reversed-phase and adsorption. This is the case during identification of unknown compounds by co-chromatography with authentic material or during establishment of the association of radioactivity with particular compounds.

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