Journal of Chromatography, 346 (1985) 301–308
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 947

SIMULTANEOUS DETERMINATION OF FREE AND CONJUGATED ECDYSTEROIDS BY LIQUID CHROMATOGRAPHY

S. SCALIA*
Institute of Pharmaceutical Chemistry, University of Ferrara, Via Scandiana 21, 44100 Ferrara (Italy)
and
E. D. MORGAN
Department of Chemistry, University of Keele, Keele, Staffordshire ST5 5BG (U.K.)
(Received June 5th, 1985)

SUMMARY

A high-performance liquid chromatographic procedure has been developed for the simultaneous assay of the major free ecdysteroids (insect moulting hormones) and the corresponding ecdysteroid 22-phosphates found in *Schistocerca gregaria* eggs. No preliminary fractionation into the free and conjugated hormones was required. An Ultrasphere ODS column with ultraviolet detection and a methanol–phosphate buffer gradient elution were used. The retention time of the conjugates was dependent on the sodium phosphate concentration in the mobile phase. The unconjugated and conjugated ecdysteroids were determined directly after aqueous methanol extraction and Sep-Pak C_{18} cartridge purification.

INTRODUCTION

The occurrence of ecdysteroids (insect moulting hormones) in the eggs of a number of insect species has been demonstrated by several authors[1–5]. In most cases it has been shown that the hormones were synthesized in the adult ovaries and then transferred to the eggs[2,6–8].

The major ecdysteroids identified in newly laid eggs of the desert locust (*Schistocerca gregaria*) are ecdysone, 2-deoxyecdysone and 20-hydroxyecdysone[4,5,9], all present in the free form, but also and chiefly (90% of the total) as polar conjugates hydrolysable with a crude enzyme preparation from the snail *Helix pomatia*. The conjugated ecdysteroids have been chemically identified[10,11] as ecdysteroid 22-phosphate esters by fast atom bombardment mass spectrometry and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy.

Towards the end of *S. gregaria* embryogenesis a variety of ecdysteroid conjugates and metabolites have been identified[12] in addition to the maternal free and conjugated hormones. This group is not included in the present study.

During egg development in *S. gregaria* a fluctuation of the titre of both the free and conjugated ecdysteroids has been reported[4,5,7,13], and the variation of hor-

0021-9673/85/$03.30

mone levels correlated with distinct stages of embryogenesis[5,13]. Hence the assay of ecdysteroids in the maturing eggs is important for understanding the physiology of the insect embryo development.

Previously the chromatographic resolution of free and conjugated ecdysteroids from insect material has required a preliminary separation of the two classes of steroids, by solvent partition[4,5,14], thin-layer chromatography (TLC)[2,3,15], Sep-Pak C_{18} cartridge[16], C_{18} bonded phase[2] or silicic acid[9] columns. The conjugates were then hydrolysed with *Helix pomatia* enzymes to release the ecdysteroid moieties which were determined by high-performance liquid chromatography (HPLC) with UV detection[5,9,16] or by electron-capture gas chromatography after derivatization[4,17].

From our work[18] and that of others[11,12] it has been shown that the conjugated ecdysteroids can be directly resolved by HPLC without prior enzymatic hydrolysis, after removal of the free hormones.

However, none of these methods can resolve the free and conjugated ecdysteroids by a single HPLC injection.

A method is described here for the simultaneous separation of ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and of the corresponding 22-phosphate esters by reversed-phase HPLC (RP-HPLC). The application of this new chromatographic system to the quantitation of the unconjugated and conjugated hormones in the maturing eggs of the desert locust is also reported.

EXPERIMENTAL

Materials

Eggs were obtained from a colony of *S. gregaria* Forskal reared as described previously[5]. Under the conditions chosen for the incubation of the eggs, they hatched at day 15.

HPLC grade methanol and water were purchased from Farmitalia Carlo Erba (Milano, Italy). All other chemicals were of analytical grade (Farmitalia). *Helix pomatia* aryl-sulphatase preparation was obtained from Sigma (St. Louis, MO, U.S.A.).

Ecdysone and 20-hydroxyecdysone were gifts from Dr. C. Casagrande (Simes, Milano, Italy), 2-deoxyecdysone from Dr. D. H. S. Horn (C.S.I.R.O., Melbourne, Australia). Conjugated ecdysone, 20-hydroxyecdysone and 2-deoxyecdysone were isolated from newly laid eggs of *S. gregaria* as described previously[18].

Methods

Sample processing. Each batch of eggs (2 g) was homogenized in methanol–water (8:2) with a blender. The first three supernatants after centrifugation (1000 *g* for 10 min, at 20°C) were combined and evaporated *in vacuo* at 40°C. The residue obtained was suspended in 7 ml of methanol–water (80:20) and injected into a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) to remove non-polar contaminants. The eluate from the Sep-Pak was reduced to dryness, redissolved in a known volume of the initial mobile phase (500 μl) and a portion of this solution (5 μl) was injected into the HPLC column.

Chromatography. The HPLC analyses were performed with a Jasco apparatus (Model BIP-I pump, Model GP-A40 solvent programmer and Model UVIDEC-100-V variable-wavelength detector; Jasco, Tokyo, Japan) linked to a sample-injec-

tion valve (Rheodyne, Cotati, U.S.A.) and a chromatographic data processor (Chromatopac C-R3A; Shimadzu, Kyoto, Japan). The detector was set to 244 nm.

Free and conjugated ecdysteroids were separated on a 150 × 4.6 mm I.D. stainless-steel column pre-packed with 5-μm particles of Ultrasphere ODS (Alltech, Eke, Belgium) and eluted with a linear gradient, in two steps, of methanol–aqueous buffer at a flow-rate of 0.9 ml/min. Solvent A was 5 m*M* sodium phosphate buffer (pH 6.8) and solvent B was methanol. The gradient programme used was according to the following scheme:

$$15\% \xrightarrow{\text{linear (8 min)}} 40\% \xrightarrow{\text{isocratic (9 min)}} 40\% \xrightarrow{\text{linear (6 min)}} 60\%\ \text{B}$$

The elution programme was begun 0.5 min before sample injection. The eluents were filtered and degassed with helium before use. The chromatograph was operated at room temperature.

The ecdysteroid peaks were quantified using the integrator which was calibrated with standard solutions of pure ecdysone, 2-deoxyecdysone and 20-hydroxyecdysone.

The analysis of the free ecdysteroids was carried out on two different types of HPLC systems: (a) a reversed-phase column (Servachrom RP-18; 250 × 4.5 mm I.D., particle size 5 μm; Serva Feinbiochemica, Heidelberg, F.R.G.) run under isocratic conditions (methanol–water, 60:40) at a flow-rate of 0.8 ml/min, and (b) an adsorption column (LiChrosorb Si 60, 250 × 4.5 mm I.D., particle size 5 μm; Merck, Darmstadt, F.R.G.) eluted with methylene chloride–methanol–water (150:25:2) at a flow-rate of 1.2 ml/min.

Trimethylsilyl ether derivatives of the free ecdysteroids were obtained as described elsewhere[4] and chromatographed with a Fractovap 4200 gas chromatograph (Carlo Erba) fitted with a flame ionization detector. The glass column, 1.8 m × 2 mm I.D., was packed with 1.5% OV-101 on Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The operating conditions were: column temperature, 285°C; injector port temperature, 295°C; detector temperature, 300°C; carrier gas (nitrogen) flow-rate, 30 ml/min.

Identification of ecdysteroids. The free ecdysteroids were identified by co-chromatography with authentic compounds on two different chromatographic systems (reversed-phase and adsorption columns) and by comparison of the gas chromatographic retention times of their trimethylsilyl ether derivatives with those of silylated standard compounds.

The conjugated ecdysteroid fractions were desalted on Sep-Pak C_{18}[10], hydrolysed with *H. pomatia* enzymes and the released ecdysteroids purified as described previously[18]. The identity of the individual hormones obtained in this way was determined as described above.

RESULTS

RP-HPLC

A chromatogram of a typical separation of 20-hydroxyecdysone, ecdysone, 2-deoxyecdysone and the corresponding 22-phosphate ester conjugates using the sys-

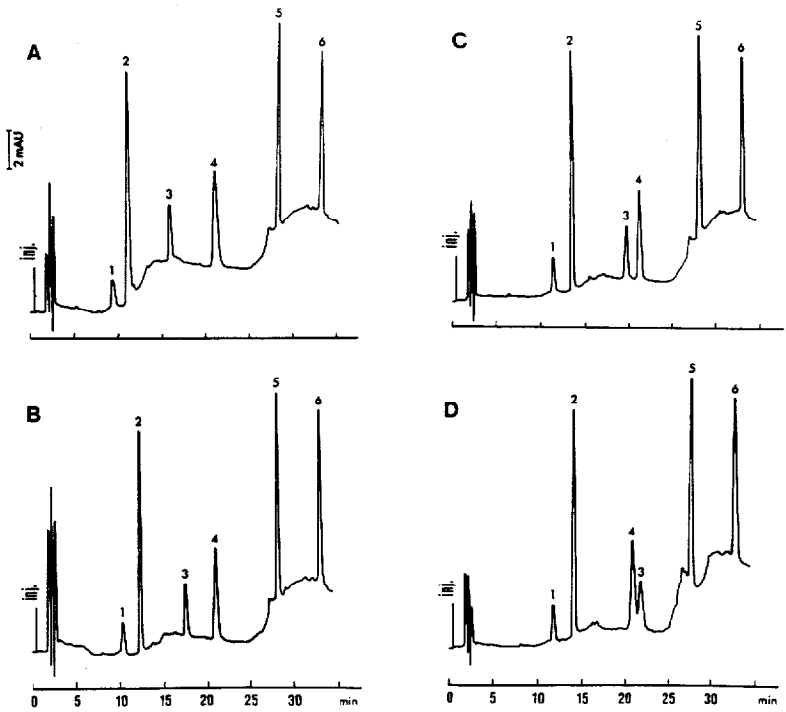


Fig. 1. Elution profiles of ecdysteroids and their phosphate conjugates on an Ultrasphere ODS column (150 × 4.6 mm I.D.). The separations were performed at a flow-rate of 0.9 ml/min with a linear gradient of methanol in 5 m*M* (A), 12.5 m*M* (B), 25 m*M* (C) and 50 m*M* (D) sodium phosphate buffer (pH 6.8). Solvent programme: 0 min, 15% methanol; 8 min, 40% methanol; 17 min, 40% methanol; and 23 min, 60% methanol. Detection: UV absorbance at 244 nm. Peaks: 1 = 20-hydroxyecdysone phosphate; 2 = ecdysone phosphate; 3 = 2-deoxyecdysone phosphate; 4 = 20-hydroxyecdysone; 5 = ecdysone; 6 = 2-deoxyecdysone.

tem described here is shown in Fig. 1A. Each of the three conjugates was resolved one from the other and from each of the corresponding unconjugated ecdysteroids on an Ultrasphere ODS column using a linear gradient in two steps of methanol in sodium phosphate buffer. When sodium phosphate was replaced by water in the mobile phase, the conjugates were eluted with the unretained solvent peak, whereas the free ecdysteroids showed the same retention times with either water or sodium phosphate (not shown).

The influence of the buffer concentration on the chromatography of the free ecdysteroids and of the conjugated pairs has been examined. The range of sodium phosphate concentrations studied was 5–50 m*M*. With increasing salt concentration in the mobile phase the retention volume for each conjugate increased while the retention of the unconjugated ecdysteroids was unaffected over the entire range of mobile phase salt concentrations (Fig. 1A–D). This is further illustrated in Table I where, for each sodium phosphate concentration, the retention volumes measured from the chromatograms in Fig. 1 are listed. The reversal in elution order for conjugated 2-deoxyecdysone and free 20-hydroxyecdysone occurring at a salt concentration of 50 m*M* reflects the increase in retention of the conjugated ecdysteroids.

TABLE I

EFFECT OF MOBILE PHASE SALT CONCENTRATION ON RP-HPLC OF FREE AND CONJUGATED ECDYSTEROIDS

Chromatographic conditions as in Fig. 1.

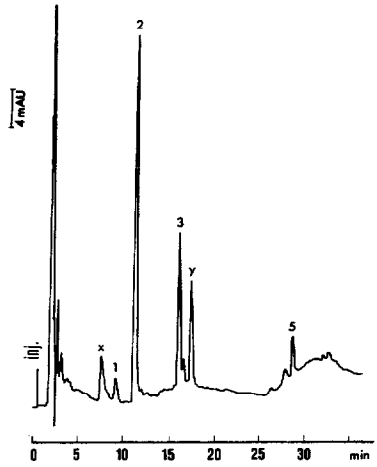
Sodium phosphate concentration (mM)	*Retention volume (ml)*					
	*Ecdysteroid**					
	1	*2*	*3*	*4*	*5*	*6*
5 (Fig. 1A)	8.2	9.6	13.8	18.6	25.1	29.2
12.5 (Fig. 1B)	9.2	11.0	15.5	18.6	25.0	29.3
25 (Fig. 1C)	9.9	11.9	17.4	18.8	25.1	29.2
50 (Fig. 1D)	10.5	12.4	19.4	18.5	24.6	29.2

* Compounds 1–6 refer to the numbered peaks in Fig. 1.

The resolution of conjugated 2-deoxyecdysone and free 20-hydroxyecdysone decreased with increasing buffer concentration and the two hormones were not baseline separated at 50 m*M* sodium phosphate.

Application

We have used the 5 m*M* sodium phosphate–methanol gradient RP-HPLC system for the assay of the major free and conjugated hormones in eggs of *S. gregaria* at different stages of development, *i.e.*, 1-, 3- and 10-day old eggs. Each batch of egg pods was extracted as described in the Experimental section. The resulting solution, after passage through a Sep-Pak, was suitable for HPLC analysis without further purification. The conjugated and unconjugated hormones present were separated by RP-HPLC in a single pass and quantified by recording the UV absorbance of the column effluent. Representative chromatograms of extracts from 1- and 3-day old eggs are shown in Figs. 2 and 3, respectively.



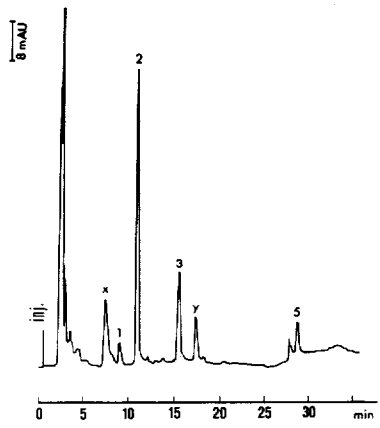


Fig. 2. Typical RP-HPLC separation of ecdysteroids from 1-day old eggs (2 g). Conditions as in Fig. 1A; peaks as in Fig. 1. X, Y = Unknown compounds, not ecdysteroids.

Fig. 3. Typical RP-HPLC separation of ecdysteroids from 3-day old eggs (3 g). Conditions as in Fig. 1A; peaks as in Figs. 1 and 2.

TABLE II

COMPARISON OF AMOUNTS OF ECDYSTEROIDS FOUND IN 1-DAY OLD *S. GREGARIA* EGGS BY THE PRESENT RP-HPLC METHOD AND BY THE PREVIOUS PROCEDURE[5]

Results are the average of two determinations.

Compound	*Amount (ng per egg)*	
	RP-HPLC	*Previous procedure*
Conjugated ecdysone	419	402
Conjugated 2-deoxyecdysone	168	155
Conjugated 20-hydroxyecdysone	32	36
Free ecdysone	31	29

The present method for the rapid and simultaneous determination of the levels of free and conjugated ecdysteroids in the eggs of *S. gregaria* was validated by comparison with the previously adopted procedure[5] on the same sample of eggs. The two methods produced consistent results (see Table II), proving the validity of the RP-HPLC titre determination of the hormones.

In accordance with earlier reports[5,13], the amount of unconjugated 20-hydroxyecdysone and 2-deoxyecdysone was below the sensitivity of the method for the sample size available. The detection limit was 10 ng of ecdysteroid.

The identity of the compounds separated by gradient elution RP-HPLC was assigned by co-chromatography with authentic material and confirmed by collecting the different UV-absorbing peaks from the column. After hydrolysis of the conjugates, they were analysed isocratically on a reversed-phase column, on an adsorption column and after silylation by gas chromatography on an OV-101 column.

The simultaneous separation of ecdysteroids and of their conjugated forms was also accomplished for samples of 10-day old eggs (Fig. 4). Good agreement was again found between the results obtained by the method described here and by the

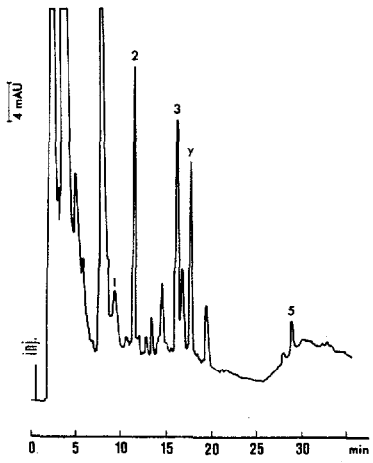


Fig. 4. Typical RP-HPLC separation of ecdysteroids from 10-day old eggs (2 g). Conditions as in Fig. 1A; peaks as in Figs. 1 and 2.

previously reported procedure[5]. The appearance of UV-absorbing substances in the elution profile of 10-day old eggs, in addition to those present in the chromatograms for 1- and 3-day old eggs, is consistent with the more complex pattern of ecdysteroid conjugates and metabolites[1,2] found in late-stage embryos. The necessary reference compounds were not available for further identifications.

DISCUSSION

Until now the assay of unconjugated and conjugated ecdysteroids in biological material required separation of the two forms of the hormones by TLC[2,3], column chromatography[2,9] or solvent partition[4,5]. These methods are time-consuming in routine analysis and require several manipulations which represent a source of possible errors.

An HPLC method has been designed for the simultaneous determination of the free ecdysteroids and of the corresponding conjugates in *S. gregaria* eggs, which does not require prior group separation. The major ecdysteroids and their conjugated forms present in the eggs have been resolved in a single analysis by gradient elution RP-HPLC with the addition of sodium phosphate to the aqueous portion of the mobile phase. The absence of salt did not affect the chromatography of the free ecdysteroids, whereas poor retention was observed for the conjugates. The increase in retention of the conjugated ecdysteroids when sodium phosphate was present in the mobile phase can probably be traced to a neutralization of the ecdysteroid phosphate charge which increases the interaction of the hydrophobic portion of the steroid conjugates with the bonded phase.

The advantages offered by this new RP-HPLC procedure are the minimum sample preparation, circumventing preliminary separation and hydrolysis of the conjugates.

A number of UV-absorbing substances, which are not ecdysteroids, are present in the elution profile. Nevertheless the well separated peaks permit unequivocal identification, accurate and rapid quantification of the hormones, suitable for routine analysis.

ACKNOWLEDGEMENTS

We thank Drs. C. Casagrande and D. H. S. Horn for gifts of ecdysteroids, Drs. A. Sbrenna and G. Sbrenna (Institute of Zoology, Ferrara University, Italy) for providing *S. gregaria* eggs and Mrs. K. Geiger for assistance in preparing the manuscript.

REFERENCES

1 E. Ohnishi, T. Ohtaki and S. Fukuda, *Proc. Jpn. Acad.*, 47 (1971) 413.
2 T. H. Hsiao and C. Hsiao, *J. Insect Physiol.*, 25 (1979) 45.
3 M. Lagueux, C. Hetru, F. Goltzene, C. Kappler and J. A. Hoffmann, *J. Insect Physiol.*, 25 (1979) 709.
4 A. R. Gande and E. D. Morgan, *J. Insect Physiol.*, 25 (1979) 289.
5 S. Scalia and E. D. Morgan, *J. Insect Physiol.*, 28 (1982) 647.
6 A. R. Gande, E. D. Morgan and I. D. Wilson, *J. Insect Physiol.*, 25 (1979) 669.
7 L. N. Dinan and H. H. Rees, *Insect Biochem.*, 11 (1981) 255.

8 M. Lagueux, M. Hirn and J. A. Hoffmann, *J. Insect Physiol.*, 23 (1977) 109.
9 L. N. Dinan and H. H. Rees, *J. Insect Physiol.*, 27 (1981) 51.
10 R. E. Isaac, M. E. Rose, H. H. Rees and T. W. Goodwin, *J. Chem. Soc. Chem. Commun.*, (1982) 249.
11 R. E. Isaac, M. E. Rose, H. H. Rees and T. W. Goodwin, *Biochem. J.*, 213 (1983) 533.
12 R. E. Isaac and H. H. Rees, *Biochem. J.*, 221 (1984) 459.
13 S. Scalia, N. Gorini, A. Sbrenna-Micciarelli, G. Sbrenna and E. D. Morgan, *Acta Embryol. Morphol. Exper.*, 5 (1984) 131.
14 T. Mizuno and E. Ohnishi, *Devel. Growth Differ.*, 17 (1975) 219.
15 J. A. Hoffmann, J. Koolman, P. Karlson and P. Joly, *Gen. Comp. Endocrinol.*, 22 (1974) 90.
16 R. Lafont, J. L. Pennetier, M. Andrianjafintrimo, J. Claret, J. F. Modde and C. Blais, *J. Chromatogr.*, 236 (1982) 137.
17 E. D. Morgan and I. D. Wilson, in J. A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier, Amsterdam, 1980, p. 29.
18 S. Scalia and E. D. Morgan, *J. Chromatogr.*, 238 (1982) 457.