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RAPID AND DIRECT METHOD FOR THE DETERMINATION OF ECDYSTEROID CONJUGATES BY LIQUID CHROMATOGRAPHY

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SUMMARY

The ionic conjugates of ecdysteroids (insect moulting hormones) found in insect ovaries and eggs can be determined quantitatively and quickly by high-pressure liquid chromatography on a reversed-phase system with ultraviolet detection. The mobile phase consists of a gradient of methanol–water containing 0.4 *M* ammonium acetate and tetra-*n*-butylammonium acetate. The conjugates can be determined directly in this way after methanol extraction and two solvent partitions.

INTRODUCTION

The ecdysteroids (insect moulting hormones) are present in large quantities in the ovaries and eggs of insects, both in the free state and as highly polar derivatives[1–5], the latter accounting for 80–90% of the total amount of the hormones in the embryos of several species[2,4–8]. In spite of the relatively large amount of these polar derivatives, their chemical identification has not yet been established. They have been tentatively identified in larvae and eggs as conjugates (sulphates, glycosides, glucuronides or phosphates) on the basis of their electrophoretic behaviour and by enzymic hydrolysis[5–7,9–12]. From our own work[4,5] and that of others[8] it is evident that the conjugates in locust eggs (*Schistocerca gregaria*) consist of anionic derivatives of three ecdysteroids: ecdysone, 2-deoxyecdysone and 20-hydroxyecdysone, which from their enzymic hydrolysis and UV spectra appear to be simple sulphate or phosphate derivatives[5], linked through one of the hydroxyl groups to the ecdysteroids.

A separate group of highly polar ecdysteroid derivatives, not split enzymically, has been reported during late embryogenesis when radioactive cholesterol was injected into adult females *Schistocerca americana*[13]. This group has not been further characterized and is not included in the present study.

The determination of the amounts of the polar ecdysteroid conjugates in ovaries and embryos of different stages of growth is important in the understanding of the physiology of development of insects. At present this is carried out indirectly and laboriously by collecting a polar fraction containing the conjugates by solvent partition or chromatography, then hydrolysing the conjugates with the crude enzyme preparation of the snail *Helix pomatia*, collecting the released ecdysteroids and de-

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termining them directly by high-performance liquid chromatography (HPLC) with UV detection[5,8] or by electron-capture gas chromatography (GC) after derivatisation (refs. 4 and 14).

Because of their ionic character, the conjugates are difficult to analyse directly by liquid chromatography. They are either strongly retained on normal-phase (NP) columns or eluted rapidly in reversed-phase (RP) systems, as broad peaks and poorly separated[15]. Several approaches have been described for separating them as a group from other biological compounds; these include TLC[12,16], reversed-phase chromatography on Amberlite XAD-2[7], column chromatography on DEAE-Sephadex[17] and on silicic acid[8]. Lafont and co-workers have improved their retention and resolution in RP-HPLC systems by buffering the aqueous part of the mobile phase to acid[15] or alkaline[15,16] pH. Another useful technique in handling very polar compounds in reversed phase is to use an aqueous buffered medium containing a large counter-ion (ion-pair chromatography[18,19]). Although all these techniques have been applied in the separation of conjugates, there have been no reports as yet on their use for direct separation, identification and quantification of conjugates, present in insect material.

We have devised a method for the rapid qualitative and quantitative analysis of the conjugates in the eggs of the desert locust (*Schistocerca gregaria*). A methanol extract of the eggs is subjected to two solvent partitions (methanol–water–hexane, and *n*-butanol–water), followed by concentration of the sample and direct ion-paired reversed-phase high-performance liquid chromatography (briefly RP-IPC), on ODS Spherisorb, eluting with a buffered aqueous–methanol gradient. The conjugates, baseline separated, are then quantified by UV monitoring of the effluent.

EXPERIMENTAL

Eggs

Egg pods were obtained from a colony of *Schistocerca gregaria* Forskal reared as previously described[20]. Under the conditions chosen for the incubation of the eggs it required 13 days from oviposition to the hatching of the first instar *Schistocerca* larvae.

Sample preparation

Each batch of eggs (2 g) was ground in methanol in a glazed mortar and the resulting slurry filtered through a sintered glass filter (porosity 3). The filtrate was partitioned between hexane and methanol–water (8:2, v/v), to remove non-polar impurities. The residue from the aqueous methanol phase was then partitioned between countersaturated *n*-butanol and water. Each fraction was backwashed with a small volume of the appropriate counter phase which was added to the main fraction. The free ecdysteroids are partitioned into the butanol phase and the polar conjugates into the aqueous phase which is reduced to dryness *in vacuo* at 40°C. The residue obtained was redissolved in a known volume of water (500 μl) and a portion of this solution (5 μl) was injected into a ODS Spherisorb HPLC column and the eluate monitored by UV detection at 244 nm.

HPLC analyses

The HPLC equipment consists of a LC3XP liquid chromatograph pump (Pye

Unicam, Cambridge, Great Britain) connected to a Pye Unicam gradient elution system, a sample injection valve (Rheodyne, Cotati, U.S.A.) and a Pye Unicam LC-UV variable-wavelength absorbance detector set at 244 nm.

HPLC-grade methanol (Fisons, Loughborough, Great Britain) and glass distilled water were used throughout and degassed by a constant stream of helium.

The analysis was carried out on a 10 cm × 5 mm I.D. column (Shandon Southern, Runcorn, Great Britain) packed with 5-μm particles of ODS Spherisorb (Phase Separations, Clwyd, Great Britain) and eluted with a linear gradient (4% per minute) from 25% to 50% methanol in 0.4 *M* ammonium acetate buffer (pH 7) at a flow-rate of 1 ml/min. An amount of tetrabutylammonium hydroxide (TBA, 0.8 mg/ml) was added to the buffer solution to obtain a concentration of 0.003 *M* of counter ion in the mobile phase. The separation was performed at room temperature (21 ± 1°C).

Quantification was obtained by comparing peak area with a standard curve of peak area against quantity in ng of pure ecdysone (Simes, Milan, Italy), 20-hydroxyecdysone (Simes) and 2-deoxyecdysone (D.H.S. Horn, Melbourne, Australia). For accuracy, comparison must be made between standard and unknown at similar retention volumes, since sensitivity decreases with increasing retention.

For the analysis of the free ecdysteroids two different types of columns were used:

(a) a reversed-phase column 25 cm × 5 mm I.D. packed with 5-μm particles of Hypersil ODS (Shandon Southern) was eluted under isocratic conditions (methanol–water, 60:40, v/v) at a flow-rate of 0.8 ml/min, and

(b) a normal-phase column 25 cm × 5 mm I.D. packed with 5-μm particles of Hypersil (Shandon Southern) was run under isocratic conditions (methylene chloride–isopropanol–water, 125:25:2) at a flow-rate of 1 ml/min. The amount of free ecdysteroids was calculated from standard curves as above.

Comparison of the direct RP-IPC and enzymic hydrolysis methods

The aqueous phase containing ecdysteroid conjugates from eggs after the butanol–water partition, was divided in two equal portions, which were evaporated to dryness. One of them was redissolved in water and analysed for conjugates by RP-IPC, the other portion was taken up in 5 ml of 100 m*M* acetate buffer (pH 5.2) and subjected to overnight enzymatic hydrolysis at 37°C in the presence of sufficient amount of the digestive juice of the snail *Helix pomatia* (10 μl/ml) (Koch-Light Labs., Colnbrook, Great Britain) to give 8000 Roy units per ml of aryl sulphatase, 1000 Fishman units per ml of β-glucuronidase and 200 Sigma units per ml of acid phosphatase. The ecdysteroids, freed by the enzymatic hydrolysis, were purified as follows: the incubation medium was injected into a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) and eluted successively with 5 ml of methanol–water (20:80, v/v) and 7 ml of methanol–water (80:20, v/v). All the different ecdysteroids present in the egg extract were eluted in the methanol–water (80:20) fraction, which was directly analysed for the quantification of the hormones by RP-HPLC with UV monitoring of the eluent from the column.

Identification of the ecdysteroids released from conjugates

The column eluate corresponding to each of the conjugate peaks in RP-IPC

was collected separately under isocratic conditions. The solvent was removed by freeze-drying and the residue redissolved in 100 m*M* acetate buffer (5 ml, pH 5.2), hydrolysed with *Helix pomatia* juice, purified, as described in the previous section, and analysed for ecdysteroids by RP-HPLC and NP-HPLC.

The identification of the individual hormones obtained in this way was confirmed by co-chromatography with the authentic compound on the two different chromatographic systems used, *i.e.* RP-HPLC and NP-HPLC.

RESULTS

The RP-IPC analysis of the hydrolysable conjugates present during embryonic development of *S. gregaria*, was carried out on 1 day old eggs and 11 day old eggs (*i.e.* towards the end of embryogenesis). Each batch of egg pods was extracted and subjected to two successive solvent partitions (as described in the Experimental section). The aqueous layer from the latter partition system was suitable for HPLC analysis without further purification. The conjugates present were separated by RP-IPC and quantified by recording the UV absorbance of the effluent from the column. A typical RP-IPC trace of 1 day old egg extract is shown in Fig. 1.

To verify that this method for the rapid determination of the levels of conjugates in the eggs of *S. gregaria* gave results consistent with the previously used

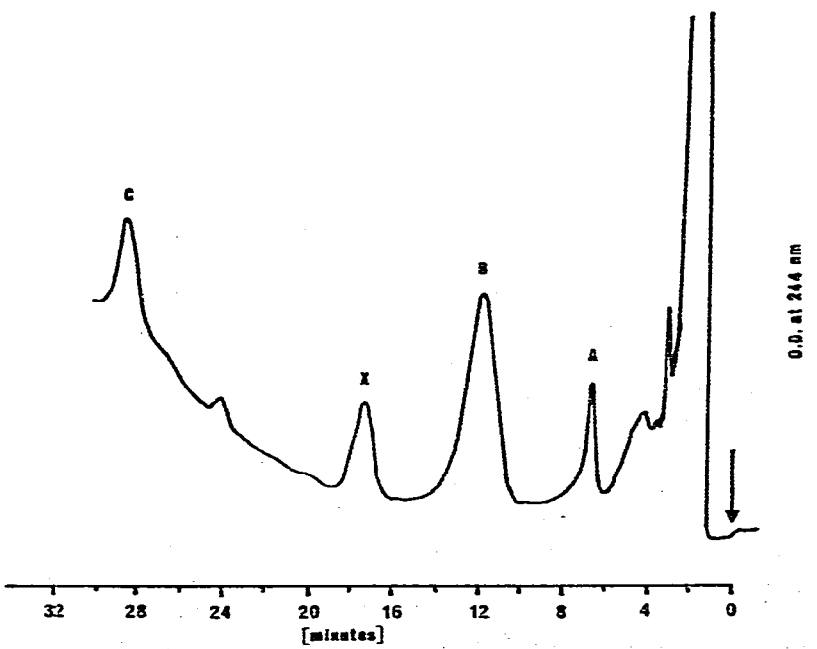

Fig. 1. Typical RP-IPC separation of conjugated ecdysteroids from 1 day old eggs (2 g), using TBA (0.003 *M*). Operating conditions: 10 cm × 5 mm I.D. column of Spherisorb-ODS. Primary solvent: 25% methanol in 40 m*M* ammonium acetate buffer (pH 7). Secondary solvent: 50% methanol in 40 m*M* ammonium acetate buffer (pH 7). Linear gradient: 0 to 100% secondary solvent in 25 min. Flow-rate: 1 ml/min. Peaks: A = conjugated 20-hydroxyecdysone; B = conjugated ecdysone; C = conjugated 2-deoxyecdysone; X = unknown compound, not ecdysteroidal.

TABLE I

COMPARISON OF RESULTS OBTAINED ON 1-DAY-OLD *SCHISTOCERCA* EGGS BY DIRECT RP-IPC METHOD AND BY CHROMATOGRAPHY AFTER HYDROLYSIS

Quantity of ecdysteroids present as conjugates.

Compound	*Direct chromatography (ng/egg)*	*After hydrolysis (ng/egg)*
Ecdysone	382	384
2-Deoxyecdysone	230	218
20-Hydroxyecdysone	33	40

enzymic hydrolysis, coupled with RP-HPLC analysis[5,8], the two methods were compared on the same sample of eggs. The values obtained are given in Table I. The results shown give good agreement between the methods and prove the validity of the RP-IPC titre determination of the hydrolysable conjugates. The limit of detection of the RP-IPC method is the conjugate equivalent to approximately 10 ng of ecdysteroid at a sensitivity of 0.005 absorbance units.

The identity of the compounds separated by RP-IPC analysis was established by collecting the different UV absorbing peaks from the column and then hydrolysing and analysing them on RP-HPLC and NP-HPLC, as described in the Experimental section.

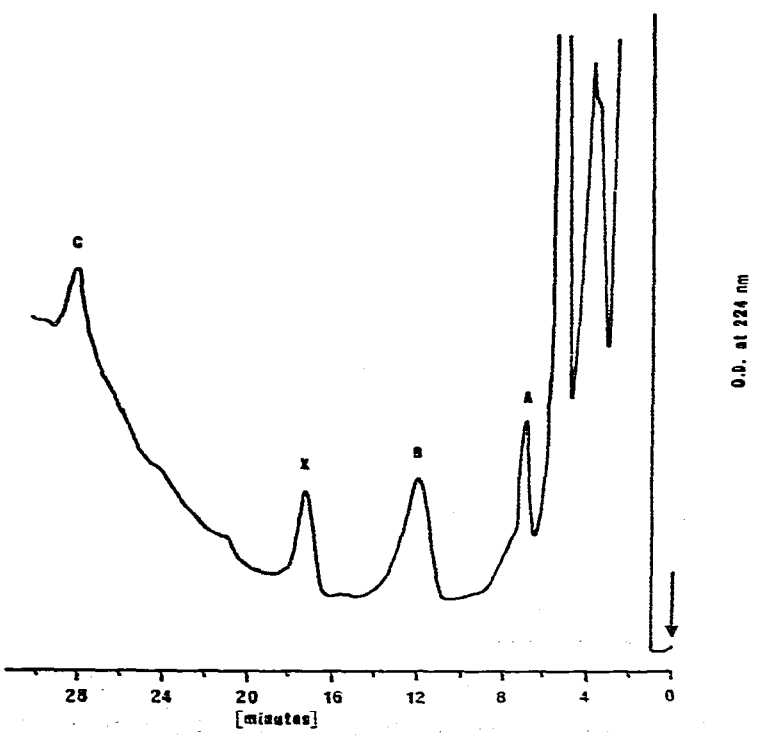

Fig. 2. Typical RP-IPC separation of conjugated ecdysteroids from 11-day-old eggs (2 g). All conditions as in Fig. 1.

After hydrolysis with *Helix pomatia* digestive juice, peak A (Fig. 1) gave 20-hydroxyecdysone, peak B ecdysone, and peak C 2-deoxyecdysone. The amount of ecdysteroid obtained by collecting the effluent, corresponding to each peak in Fig. 1, and then hydrolysing it, corresponded to an efficiency of 82–91% of the amount expected from the same sample of eggs, when the sample was directly hydrolysed and ecdysteroids determined by HPLC with UV detection.

When a sample of 11 day old eggs was subjected to the RP-IPC analysis, the chromatographic pattern obtained was similar to the 1 day old eggs, with peaks at the same retention volume (Fig. 2), showing that the same hydrolysable conjugates are present at the beginning and at the end of embryogenesis. Moreover, at this time too, good agreement was found between titre determination by our method of RP-IPC and by enzymic hydrolysis coupled with RP-HPLC analysis.

The separation and analysis can also be carried out without added TBA, in which case the peaks are less well resolved (Fig. 3). No deterioration of the column due to TBA was noticed over several weeks of continuous use at pH 7. At more basic pH other authors have found the column performance deteriorated[23,24]. The concentration of buffer and TBA were not found to be critical. The appearance of the chromatogram was not altered if the buffer was changed from 0.4 *M* to 0.04 *M* and the TBA from 0.003 *M* to 0.0015 *M*.

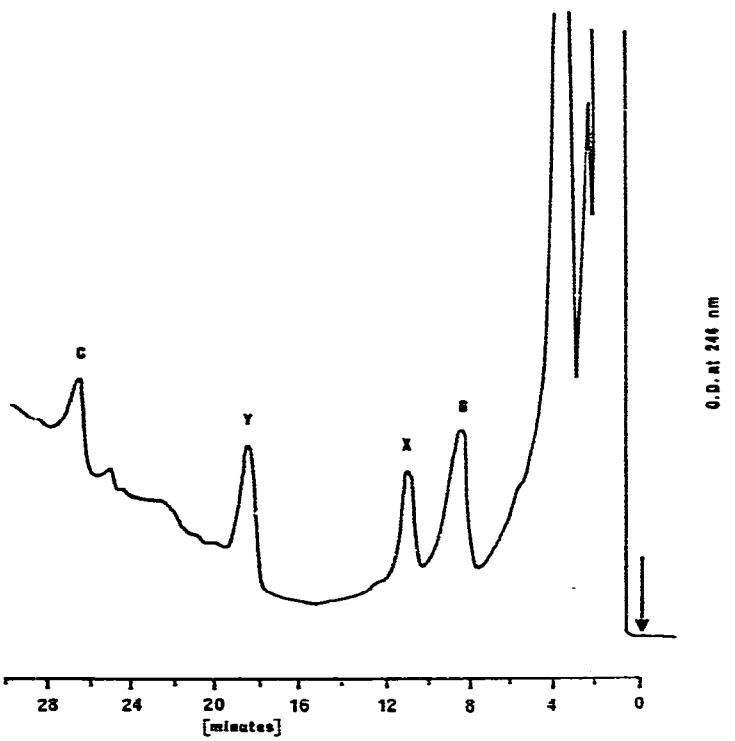

Fig. 3. Separation of conjugated ecdysteroids from 11-day-old eggs (2 g), without TBA in the mobile phase. All other conditions as in Fig. 1. 20-Hydroxyecdysone is not resolved with these conditions. Y = an unidentified substance only appearing in the absence of TBA.

DISCUSSION

The analytical methods used, up to now, for the micro-determination of the ecdysteroids in biological material cannot be applied directly to measure the titre of the polar conjugates of ecdysteroids; they are difficult to analyse by liquid chromatography, inactive in bioassays, and also poorly immunoreactive[21]. Furthermore the radio-immunoassay cannot discriminate between different compounds and it has produced inconsistent results for the determination of the amount of conjugates in 1 day old eggs of *Locusta migratoria*[2,22]. Therefore, quantitative analysis of these substances has been mainly performed on the free hormones released from the conjugates by enzymic hydrolysis. The hydrolysis step not only adds to the time per analysis, but also it causes longer sample manipulation and represents a further source of possible errors as shown in the investigation by Gande and Morgan[4], on the levels of conjugates in eggs of *S. gregaria*. The values reported in that paper were much lower than the quantities present *in vivo*, because of incomplete hydrolysis of the conjugated ecdysteroids[5].

In order to overcome the difficulties connected with the previous analysis of conjugates, a new and rapid method for the separation and quantification of these substances in the eggs of *S. gregaria* has been devised. After the extraction from the eggs and a preliminary purification involving two solvent partition steps, the ionized conjugates were separated as ion-pairs in a single pass through a C_{18} bonded phase column with a solvent gradient. The compounds were detected and quantified by UV monitoring of the eluent. Because UV absorbing impurities are usually present in the biological extracts, UV detection is poorly specific. The 20-hydroxyecdysone peak is associated with, and the ecdysone peak followed by, UV-absorbing substances which are not ecdysteroids. Nevertheless, the method permits accurate and rapid quantification of the substances, suitable for routine analysis.

The level of the conjugates ecdysteroids have been measured (as in the Experimental section) by a standard curve obtained with known amounts of free ecdysteroids. This indicates that the conjugating ionic group itself does not absorb in the ultraviolet region, nor does its attachment involve the unsaturated ketone chromophore.

Furthermore the analysis of eggs of different age suggests that the same hydrolysable conjugates are present at the beginning and at the end of the embryogenesis.

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