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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ECDYSTEROID TITERS IN THE HOUSE FLY

G. D. PIMPRIKAR, M. J. COIGN, H. SAKURAI and J. R. HEITZ*
Department of Biochemistry, Mississippi Agricultural and Forest Experiment Station, Mississippi State, MS 39762 (U.S.A.)

SUMMARY

An efficient, rapid and sensitive method has been developed for extraction, clean-up, and analysis of ecdysteroid titers in the larval stage of the house fly. A methanolic extract was filtered, defatted with hexane, and then passed through a C_{18} reversed-phase Sep-Pak cartridge, which retained the ecdysteroids. Polar interfering materials were removed by eluting the cartridge with water, followed by 5% acetonitrile and 20% methanol. The methanol eluate was used for quantitative analysis of ecdysteroids by high-performance liquid chromatography on a C_{18} μBondapak reversed-phase column with 16% aqueous acetonitrile. The detection limits for 20-hydroxyecdysone and ecdysone were 10 and 20 ng, respectively.

INTRODUCTION

In order to study the effect of xanthene dyes on ecdysteroid titers in insects, it was necessary to develop a rapid, efficient and sensitive quantitation method for molting hormones in the larval stage of the house fly, *Musca domestica*. Among the molting hormones, ecdysone, the apparent secretory product of the prothoracic gland, and ecdysterone, a 20-hydroxy metabolite, are of primary interest in the process of molting. The titers of these molting hormones are related to the sequence of developmental events such as molting, pupation, adult development and oogenesis[1].

Several methods have been used in the past for the analysis of ecdysteroids in insects. Of these methods, radioimmunoassay (RIA) is the most convenient and is capable of detecting picogram quantities. However, because of cross-reactions, the method does not differentiate between 20-hydroxyecdysone and ecdysone. Further sample purification by gas–liquid chromatography[2] (GLC) or thin-layer chromatography (TLC) was necessary for qualitative and quantitative analysis. Coupled GLC–mass spectroscopy (MS) offers sensitivity and specificity. However, it requires derivatization[3]. Koolman[4] recently developed a fluorometric method for ecdysteroid analysis. In all of these methods, sample preparation is lengthy and tedious and recoveries are moderately low.

High-performance liquid chromatography (HPLC) can be used to advantage

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for the analysis of ecdysteroids because they absorb strongly at 254 nm. There is no need for derivatization, and ecdysone as well as 20-hydroxyecdysone may be analyzed in one step[5]. Because the method is non-destructive, fractions may be collected and subjected to bioassay, MS, or RIA for verification. One of the major limitations of HPLC quantitation is that polar compounds which appear before and in conjunction with the 20-hydroxyecdysone peak may falsify quantitation of 20-hydroxyecdysone. Adequate purification of extracts is possible by a multistep procedure but this is generally too time-consuming for routine analysis. Holman and Meola[6] used two successive passes through a Poragel column to remove the polar interfering materials. Watson and Spaziani[7] used Sep-Pak cartridges in the clean-up of crab samples for ecdysteroid analysis.

In the present paper we report an efficient and rapid method of extraction, Sep-Pak clean-up, and a reversed-phase HPLC system for quantitation of ecdysone and 20-hydroxyecdysone.

MATERIALS AND METHODS

Insects

Insects used were from the house fly (*M. domestica*) colony maintained at the Biochemistry Department of Mississippi State University. At daily intervals, larvae and pupae of known ages (from 3 to 12 days old) were collected, weighed and frozen immediately.

Chemicals and supplies

Ecdysteroids standards (ecdysone and 20-hydroxyecdysone) were obtained from Sigma. High-purity solvents were from Waters Assoc. (Milford, MA, U.S.A.), Fisher Scientific, and Sargent-Welch. Sep-Pak C_{18} cartridges were purchased from Waters Assoc.

Extraction

A modification of the ecdysteroid extraction procedure of Hsiao and Hsiao[1] was used. In a Tekmar Tissumizer insects (10–20 g) were homogenized in 75 ml of methanol for 5 min. The homogenate was kept at 7°C overnight and then filtered through a Buchner funnel. The filter cake was again homogenized and filtered. The filtrates were pooled.

Clean-up

The combined filtrates were transferred to a separatory funnel and extracted twice with an equal volume of hexane. The methanol phase was evaporated to dryness at 40°C under reduced pressure. The residue was transferred to a separatory funnel with hexane and 10% aq. methanol and after mixing and separation the hexane layer was discarded. The methanol layer was extracted twice with 15-ml portions of hexane, which were discarded.

A Sep-Pak cartridge was prepared by connecting it to a 10-ml glass syringe and washing it with 5 ml of methanol, followed by 5 ml of water. The methanol extract was then passed through the cartridge and eluted, in succession, with water, 5% acetonitrile, 20% methanol, and 100% methanol (20 ml each). The final methanol

eluate was collected and evaporated at 40°C under reduced pressure. The residue, dissolved in acetonitrile, was used for HPLC analysis.

HPLC

A Waters Assoc. M6000A solvent delivery system, coupled with an automatic injector (WISP 710) were used for the HPLC analysis. The quantitation of ecdysteroids was carried out on a μBondapak C_{18} reversed-phase column (Waters Assoc.) under isocratic conditions (16% aq. acetonitrile) at a flow-rate of 1.5 ml/min. Peaks were detected at 254 nm. The samples were dissolved in acetonitrile and a 50-μl injection volume was used for all samples.

Quantitation

Chromatograms of 20-hydroxyecdysone and ecdysone are shown in Fig. 1. Ecdysteroids were estimated from the peak areas by using a Waters Assoc. Data Module. The lowest measurable amounts were 10 and 20 ng for 20-hydroxyecdysone and ecdysone, respectively. Known amounts of standards were also added to the samples just before injection in order to confirm the identity of 20-hydroxyecdysone

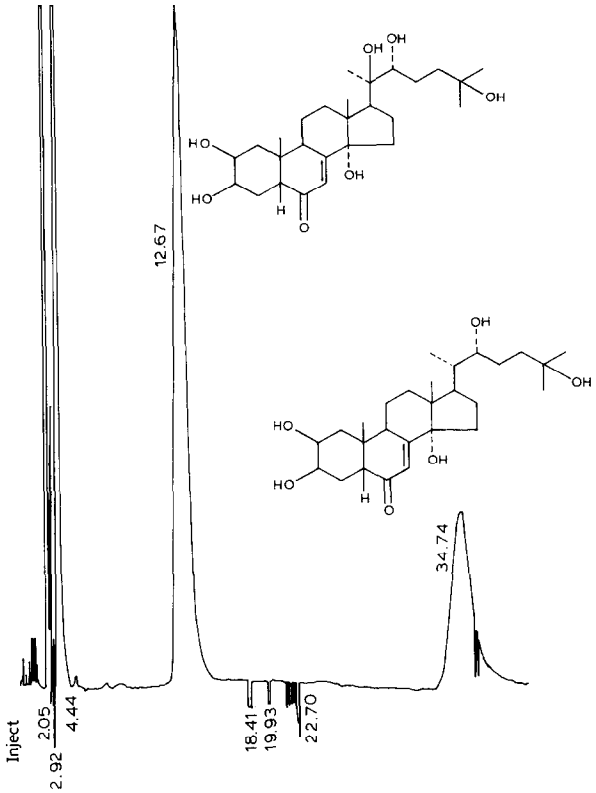

Fig. 1. Chromatogram of 20-hydroxyecdysone and ecdysone. Conditions: μBondapak C_{18} reversed-phase column, detection at 254 nm, 0.005 a.u.f.s.; solvent system: 16% aq. acetonitrile, flow-rate 1.5 ml/min, injection volume 50 μl.

and ecdysone peaks. Each insect sample was extracted in triplicate and each extract was analyzed three times.

Bioassay

The biological activity of 20-hydroxyecdysone and ecdysone in the extracts was confirmed by ligation experiments with a *Musca* bioassay procedure[8]. Standards were used for comparison and the activity of the extract was expressed in *Musca* units.

RESULTS AND DISCUSSION

Earlier, several solvents were used for extraction of ecdysteroids from house fly larvae. These included chloroform–water (1:1), 75% acetonitrile, 50% methanol, acetonitrile and methanol. The aqueous solvents were troublesome, forming emulsions which were not easily broken. Holman and Meola[6] recommended the use of acetonitrile to avoid this problem. Since we were using an acetonitrile-based solvent system for HPLC analysis, we selected a different solvent for extraction. Methanol seems to be as effective as acetonitrile in the extraction of ecdysteroids. Keeping the homogenized samples in methanol when kept overnight at 7°C, as suggested by Hsiao and Hsiao[1], gave better ecdysteroid recoveries. Partitioning between hexane and methanol gave a minimum of emulsions and the residue remaining after evaporation of the methanol was readily soluble in the 10% aq. methanol–hexane partition system.

Very efficient separations of ecdysteroids have been obtained by HPLC with both normal-phase and reversed-phase systems[9–11]. Lafont *et al.*[5] tested several commercially available HPLC columns with different mobile phase systems under various chromatographic conditions. A C_{18} reversed-phase column eluted with an acetonitrile–water system was found to give the best results. Gradient elution caused baseline disturbances, which limited the use of highly sensitive detection. However, isocratic condition and UV detection allow ready quantification of less than 10 ng of ecdysteroids[5].

A chromatogram of standard solutions of 20-hydroxyecdysone and ecdysone obtained with a C_{18} reversed-phase μBondapak column and 16% aq. acetonitrile is shown in Fig. 1. The retention time for 20-hydroxyecdysone was 10 to 12 min and for ecdysone 31 to 35 min. The reverse-phase system is more stable than the normal-phase systems and none of the impurities present in the samples are eluted after ecdysone. This is essential for the use of the automatic WISP injection system. Impurities in larval samples which accumulate in the pre-column and also on the top of the analytical column must be removed by periodic cleaning of the pre-column and elution of the analytical column with a stronger solvent.

The crude extract showed a large number of polar impurities, eluted before the 20-hydroxyecdysone peak, which interfered with the analysis of 20-hydroxyecdysone (Fig. 2). A different clean-up procedure was derived to remove them by a combination of hexane extractions and Sep-Pak elution.

Many purification procedures have been used earlier to prepare biological samples for quantitation of ecdysteroids. They usually involve partitioning between hexane- and aqueous methanol, hexane and acetonitrile[6], chloroform and water[4], or

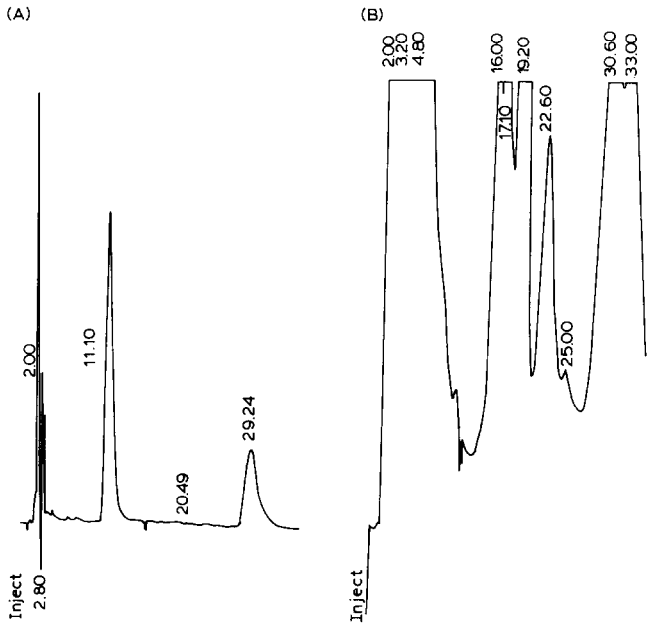

Fig. 2. Chromatogram of ecdysteroid standards (A) and crude extract of house fly larvae (B) showing the polar interfering materials before 20-hydroxyecdysone peak; chromatographic conditions same as Fig. 1.

butanol and water[12]. In our experience, partitioning, first between hexane and methanol and then between hexane and methanol–water (10:90), removed most of the lipid materials.

Chromatography on silicic acid columns or TLC on silica plates have been used to isolate the ecdysteroids[9]. In other chromatographic procedures, Amberlite XAD-2[13,14], silica[15,16] or reversed-phase columns[17,18] have been used. The procedure of Holman and Meola[6] comprised two successive partions and two HPLC separations on Poragel PN. In order to save time and improve recovery, Lafont *et al.*[5] extracted ecdysteroids with chloroform–methanol (1:1) and passed the extract through a C_{18} Sep-Pak cartridge. Emulsion formation in this extraction system as well as the overloading of the Sep-Pak cartridge prevented us from using this system for sample preparation. Moreover, since house fly larvae contain a large amount of lipid material, partitioning with hexane prior to the use of C_{18} Sep-Pak was found to be essential. Watson and Spaziani[7] used Sep-Pak cartridges very effectively for isolating ecdysteroids from crabs. These cartridges very efficiently adsorb the ecdysteroids from the aqueous phase. Not only ecdysone and 20-hydroxyecdysone, but also their polar metabolites are retained on the cartridge[5].

HPLC traces of crude extract before and after clean-up are shown in Fig. 3. Some of the samples seemed to contain large amounts of polar materials that made it impossible to detect and quantify the peak of 20-hydroxyecdysone. The Sep-Pak clean-up removed most of these materials (Fig. 3B). Occasionally, one Sep-Pak clean-up was not enough to remove all of the polar compounds. In such cases, it was necessary to pass the sample through a Sep-Pak cartridge twice. Since most of the

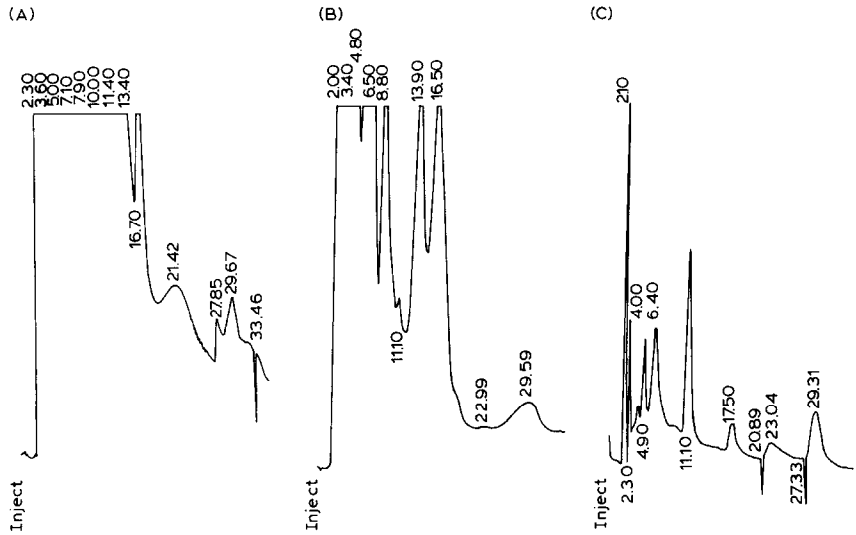

Fig. 3. Chromatogram of (A) crude extract, (B) after one pass through a Sep-Pak cartridge (C) after two passes through a Sep-Pak cartridge, showing the removal of polar interfering materials; chromatographic conditions same as Fig. 1.

interfering materials were removed by elution from Sep-Pak cartridge (Fig. 3c), the extract was suitable for analysis on the μBondapak system.

The recoveries for 20-hydroxyecdysone and ecdysone by this extraction and clean-up scheme were 93% and 86%, respectively. The Sep-Pak cartridge could be re-used after washing with methanol and equilibrating with water.

In conclusion, methanol extraction, hexane partition, Sep-Pak clean-up, and HPLC quantitation systems described in this report provide a convenient, efficient, and rapid determination of 20-hydroxyecdysone and ecdysone in the larvae of the house fly, *M. domestica.*

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