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CONVENIENT METHOD FOR THE DETERMINATION OF PICOMOLE AMOUNTS OF JUVENILE HORMONE

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SUMMARY

From an aqueous methanol extract, both juvenile hormone (JH) and, as an internal standard, its ethyl ester are extracted into isooctane and adsorbed on alumina, washed free from accompanying material and then desorbed with dichloromethane. After structural modification by addition of methanol, 10-hydroxy-11-methoxy-JH is adsorbed on alumina, again washed with dichloromethane to remove all the unchanged material and then desorbed. The next derivative obtained is 10-DMNFHS-11-methoxy-JH, which again is adsorbed on alumina, washed free from accompanying material, desorbed and then determined by combined gas chromatography–selected ion monitoring mass spectrometry. The versatility and high yield of this procedure are demonstrated for a series of insect species with JH contents from less than 1 up to 40 pmol/g fresh weight.

INTRODUCTION

Of the many analytical methods for the determination of juvenile hormone (JH), only two make use of micro-derivatization of the epoxide ring and of an internal standard combined with a final gas chromatographic–mass spectrometric (GC–MS) analysis[1,2]. Both of these techniques, in one of which methanol is added[2] and in the other fluorinated alcohols[1,3], include careful purification of the analytes by high-performance liquid chromatography (HPLC). High specificity and sensitivity were demonstrated first by the establishment of a JH titre curve for queen and worker honeybee castes[4] or *Locusta migratoria* individuals[3], as in the isolation and structural elucidation of JH-0 from developing embryos of *Manduca sexta*[5], and the identification of JH homologues in different insect species. However, their limitations were revealed in the analysis of insects such as *Drosophila hydei* larvae with high fat contents and an extremely low JH titre of less than 1 ng/g fresh weight of JH-III[6].

We describe here a method that avoids liquid chromatographic purification of the analytes before GC–MS analysis. The final sample is sufficiently pure for direct injection of gram aliquots of insect material into a capillary GC column. Application of this technique has shown the absence of JH-I in *D. hydei* larvae and the presence of JH-III as the only homologue[7].

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EXPERIMENTAL

Reagents and materials

Analytical-reagent grade and Nanograde solvents were purchased from E. Merck (Darmstadt, F.R.G.) and Mallinckrodt (Paris, KY, U.S.A.), respectively. Juvenile hormones I, II and III were products of Sigma Chemie (Taufkirchen, F.R.G.). [^{3}H-C_{10}]JH-II, specific activity 11.6 Ci/mmol, was purchased from NEN (Dreieichenhain, F.R.G.). The ethyl ester homologues of JH-I and -III were prepared from the respective synthetic hormones according to Mori *et al.*[8] and purified by HPLC on LiChroprep RP-8 (40–63 μm) (E. Merck) with methanol–water as an isocratic eluent (80% methanol for JH-I-Et and 75% methanol for JH-III-Et). Neutral aluminium oxide for column chromatography, type W 200 with activity III (Woelm Pharma, Eschwege, F.R.G.) was used. The washed glassware was heated at 200°C for several hours and then used without further precautions.

Dimethyl(nonafluorohexyl)silyl chloride (DMNFHSC). This was prepared by analogy with the procedure of Morgan and Poole[9]. A 15-g amount of perfluorobutylethylene (a gift from Dr. K. v. Werner, Hoechst, Werk Gendorf, F.R.G.), 6.6 g of dimethylchlorosilane and 0.6 ml of 0.1 *M* H_2PtCl_6 (in 2-propanol) were heated under nitrogen in a sealed glass tube (160 ml) at 240°C for 15 h. The dark brown reaction product was filtered under nitrogen and distilled through a Spaltrohr HMS 500 column (Fischer, Cologne, F.R.G.). DMNFHSC was redistilled under the same conditions (b.p. 47°C/11 mbar; yield 30%).

Gas chromatography–mass spectrometry

For gas chromatography–selected ion monitoring mass spectrometry (GC–SIMS), a Varian SS 100 computer and a CH-7A-DF mass spectrometer coupled on-line to an IGC 120 DFL gas chromatograph (Intersmat, Courtry, France) were used. For injection a falling needle injector (LKB-Clinicon, Bromma, Sweden) and for separation of the JH derivatives a fused-silica capillary column, stationary phase DB 1, film thickness 0.25 μm (30 m × 0.32 mm I.D.) (J&W, Rancho Cordova, U.S.A.) were used. The injector temperature was set at 300°C and the oven temperature at 245°C (isothermal). For one analysis, 5.5 min are needed under these conditions and the mass spectrometry is started 3 min after injection of the analyte.

Juvenile hormone analysis

Whole insects (up to 3 g) were anaesthetized with carbon dioxide or killed by shock freezing with liquid nitrogen. They were stored at −20°C if not immediately homogenized.

Step 1: methanol extraction of insects. The biological material was homogenized (Ultra-Turrax) in a 50-ml tube with 10 ml of methanol at 0°C, centrifuged (Minifuge Christ, 5 min, 4000 rpm, 3500 *g*, 0°C) and the pellet re-extracted twice under the same conditions; each of the three extracts was collected separately. The first and second methanol extracts were combined, JH-ethyl standard (1 nmol/ml in isooctane) added, depending on the amount of JH present in the extract (for an unknown sample, 5 pmol of the JH-ethyl standard was added) and 20 ml of 2% sodium chloride solution were slowly added to the magnetically stirred methanol extract. After centrifugation (10 min, 4000 rpm, 3500 *g*, 0°C), the third methanol

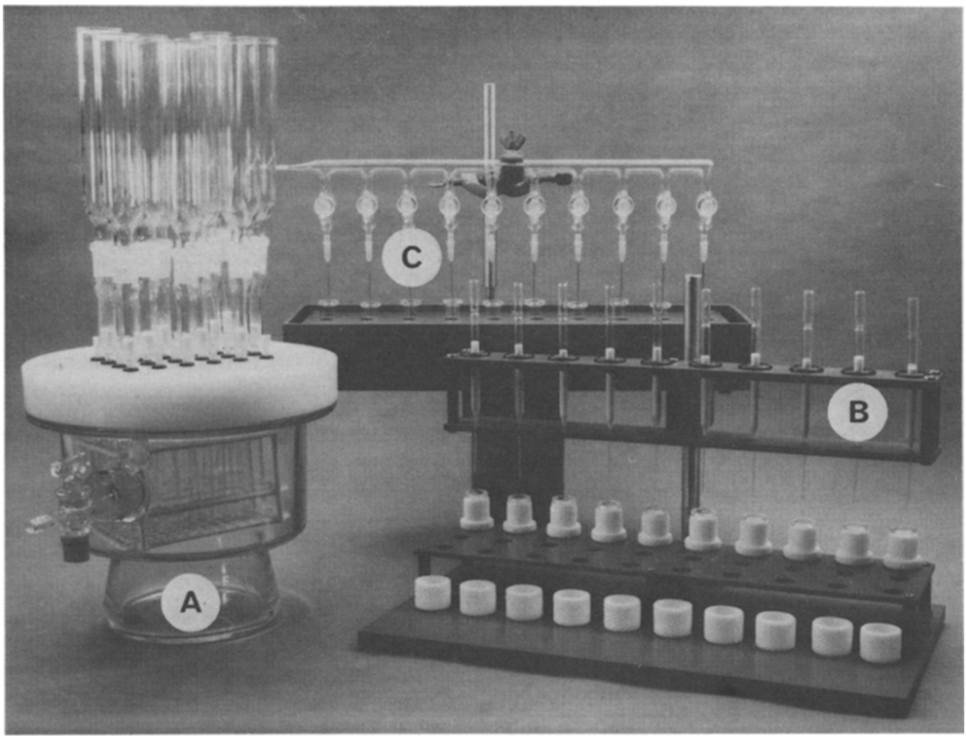


Fig. 1. All-glass equipment for isolation of juvenile hormone from biological material and its stepwise derivatization. A, 4-ml suction filters with LiChroprep RP-8, connected with a 70-ml reservoir and with a vacuum desiccator; B, frame with 2-ml reaction vessels, PTFE screws and PTFE-coated septa; Pasteur pipettes are filled with 1.3 g of alumina–glass-wool; C, manifold with glass taps and steel needles, extending into 2-ml reaction vessels.

extract (10 ml) was added to the precipitate, stirred and again 10 ml of 2% sodium chloride solution were added. After centrifugation the extracts were combined (60 ml).

Step 2: processing of the methanol extracts. In principle the technique using the Bond Elut/Vac Elut System (Analytichem International, Harbor City, CA, U.S.A.) was adopted. However, the whole equipment coming into contact with JH (Fig. 1A) was made of glass. A maximum of 24 samples can be processed concomitantly. In the desiccator was placed either a glass tray for collecting wash fluids or a test-tube rack filled with 15-ml centrifuge tubes. A 4-ml suction filter was packed with LiChroprep RP-8 (0.75 g), washed with ethyl acetate (2 × 2.5 ml), methanol (2 × 2.5 ml) and methanol–2% sodium chloride solution (1:1) (2 × 2.5 ml). The filter tube was then connected with a 70-ml reservoir and the whole extract (60 ml) slowly (4 ml/min) sucked through the LiChroprep RP-8 adsorbent into the waste collection glass tray. The RP-8 was then rinsed with methanol–2% sodium chloride solution (1:1) (2 × 5 ml), aspirated to dryness and the waste tray replaced with a 15-ml centrifuge tube. The JH-containing material was eluted with a total of 3 of methanol and to the methanol solution obtained the same volume of 2% sodium chloride solution was added.

The preceding extraction (step 1 and LiChroprep RP-8 purification of the methanol extract in step 2) is usually not necessary for haemolymph samples. Haemolymph was diluted to 1.5 ml with 2% sodium chloride solution, 1.5 ml of methanol were added and the solvent, including the precipitate, was directly extracted. Depending on the precipitate formed, the total amount of solvent may be increased to 6 ml. If a considerable amount of lipid is present, the haemolymph extraction should start from the beginning of step 2.

JH was extracted with isooctane (3 × 0.5 ml) from either the insect or haemolymph sample. For separation of the phases centrifugation may be necessary before the upper, isooctane phase is layered off. If the phases do not separate, addition of 0.5 ml of dimethylformamide is necessary.

The isooctane extracts (1.5 ml) were collected in a 2-ml reaction vessel (Fig. 1B), washed with 0.25 ml of 2% sodium chloride solution, the aqueous phase was removed with a syringe and the isooctane phase further purified through an aluminium oxide column (Pasteur pipette, 50 mm × 5 mm diameter, corresponding to 1.3 g of alumina–glass-wool). The column was first stirred with 1 ml of isooctane to remove air bubbles. The isooctane phase was then filtered through the column, followed by 1 ml of isooctane (used for rinsing the reaction vessel) and 2 × 1 ml of dichloromethane–*n*-pentane (3:7). After removal of the washings the JH-containing fraction was eluted with 2 ml of dichloromethane and collected in a new reaction vessel. The solvent was removed with a stream of helium or nitrogen (Fig. 1C).

Step 3: 10-hydroxy-11-methoxy-JH derivatization. The residue from the dichloromethane elution was dissolved in 0.075 ml of methanol and, with shaking, 0.075 ml of 5% trifluoroacetic acid in methanol (freshly prepared) was slowly added. The reaction vessel was then closed with a Teflon-coated septum and a Teflon screw, and kept at 60°C for 15 min. After cooling, 1 ml of *n*-pentane was added, as much of the solvent as possible removed with a stream of helium (Fig. 1C), another 1 ml of *n*-pentane added and the fraction evaporated to dryness. The residue was then dissolved in 1 ml of isooctane. A new aluminium oxide column was prepared as for step 2, and the purification sequence was followed: 1 ml of isooctane for rinsing the column, 1 ml of isooctane with the residue from reaction vessel, 1 ml of isooctane, 2 ml of dichloromethane. All of the washings were removed, then the JH derivative was eluted with 2 ml of ethyl acetate–*n*-pentane (1:1), collected in a new reaction vessel and the product evaporated to dryness in a stream of helium.

Step 4: 10-dimethyl(nonafluorohexyl)silyloxy-11-methoxy-JH derivatization (10-DMNFHSO, 11-MeO-JH). The residue from step 3 was dissolved in 0.1 ml of pyridine, 0.025 ml of DMNFHSC was added and the reaction vessel was closed with a Teflon-coated septum (see step 3) and kept at 60°C for 20 min. After cooling to room temperature, 1 ml of *n*-pentane was added. The white precipitate formed was either removed by centrifugation or more conveniently by another aluminium oxide filtration as follows.

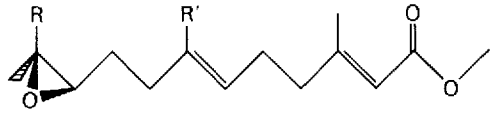
An aluminium oxide column of length about 2 cm was prepared in a Pasteur pipette and washed with 2 ml of dichloromethane, then the reaction product was transferred into the column, the JH derivative eluted and the aluminium oxide washed with 1 ml of dichloromethane–*n*-pentane (2:3), all into a new reaction vessel. The *n*-pentane was removed in a stream of helium, three 1-ml portions of *n*-pentane were added, with evaporation each time in order to remove the pyridine. The residue

Insect material (1 g or less)

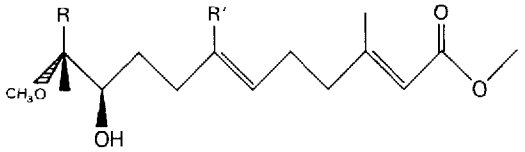
↓ Homogenize (10 ml CH_3OH), centrifuge, re-extract twice (10 ml CH_3OH each), add JH-ethyl standard (5 pmol), dilute with 2% aqueous NaCl (30 ml) and centrifuge

Step 1 extract, CH_3OH–2% aqueous NaCl (1:1, 60 ml)

↓ Filter through LiChroprep RP-8 (0.75 g), rinse with CH_3OH–2% aqueous NaCl (1:1, 10 ml), elute with CH_3OH (3 ml), add 2% aqueous NaCl (3 ml), extract with 3 vol. of 0.5 ml isooctane, wash extract with 2% aqueous NaCl (0.25 ml) and filter isooctane extract through alumina (1.3 g), wash with isooctane (1 ml), CH_2Cl_2–*n*-pentane (3:7, 2 ml). Elute JH + JH-ethyl with CH_2Cl_2 (2 ml) and remove solvent

Step 2 residue, 

↓ CH_3OH/H^+ (0.15 ml), 60°C, twice add and evaporate *n*-pentane (2 ml). Dissolve residue in isooctane (1 ml), filter through alumina (1.3 g) and wash with isooctane (2 ml), CH_2Cl_2 (2 ml). Elute with ethyl acetate–*n*-pentane (1:2, 2 ml) and remove solvent

Step 3 residue, 

↓ Dissolve residue in pyridine (0.1 ml), add DMNFHSC (0.025 ml), 60°C, filter through alumina (0.5 g), wash with CH_2Cl_2–*n*-pentane (2:3, 1 ml) and evaporate three times with *n*-pentane. Dissolve residue in isooctane (1 ml), filter through alumina (1.3 g) and wash with toluene–isooctane (15:85, 2 ml) *n*-pentane (1 ml). Elute with CH_2Cl_2–*n*-pentane (2:3, 2 ml) and remove solvent. Repeat alumina filtration procedure and dissolve residue in isooctane (0.05–0.1 ml) for GC–SIMS

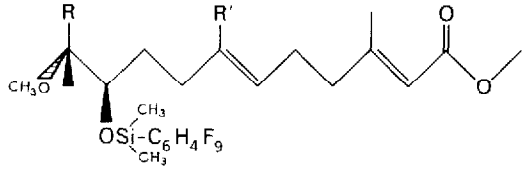
Step 4 residue, 

Fig. 2. Flow diagram for picomole analysis of juvenile hormones in biological samples. JH-I, R = R′ = CH_3; JH-II, R = CH_3, R′ = H; JH-III, R = R′ = H.

was dissolved in 1 ml of isooctane. The remainder of the DMNFHSC has to be removed carefully from the sample by another aluminium oxide treatment. A column (1.3 g) was prepared and washed with 2 ml of dichloromethane and 2 ml of isooctane, then the JH derivative (dissolved in 1 ml of isooctane) was added to the column, the reaction vessel rinsed with 0.5 ml of isooctane, the rinsings were transferred into the column, then 2 ml of toluene–isooctane (15:85) followed by 1 ml of *n*-pentane were used for washing the column. All this eluted material was discarded and finally the JH derivative was eluted into a new reaction vessel with 2 ml of dichloromethane–*n*-pentane (2:3). The eluent was finally removed under a stream of helium. This procedure had to be repeated once again to remove DMNFHSC products, which interfere in the final analysis. The residue was finally dissolved in 0.05–0.1 ml of isooctane and analysed by GC–SIMS[1].

RESULTS AND DISCUSSION

Derivatization and purification

The present procedure follows an analytical approach based exclusively on an adsorption–desorption sequence. Only the polarity of the JH molecule is changed stepwise by derivatization and hence also its adsorption characteristics on alumina. Most of the unchanged material that has been coeluted with JH or with its derivative from alumina can now be easily removed by use of the same eluent as in the preceding purification step.

In the flow diagram in Fig. 2, the above four steps are summarized. The total yield from steps 1 to 4 was followed by use of tritium-labelled JH-II (Table I). Starting with 5 pmol of JH-II, an amount which is equal to that of the internal standard, a recovery of 90–95% was found after extraction of 40 ml of 50% methanol–water solution with 1.5 ml of isooctane. In absolute terms, this means a loss of about 0.1 ng of JH-II if biological material such as *Drosophila* is extracted. For the whole isolation scheme in Fig. 2, from a recovery of 65–70% an absolute loss of about 0.4 ng of JH-II can be calculated. In none of the other purification and derivatization steps was a significant loss of radioactivity detectable.

TABLE I

RECOVERY OF ^{3}H ACTIVITY DURING DERIVATIZATION

To each of the four samples, about 5 pmol ($1.13 \cdot 10^5$ counts per minute) of [^{3}H] JH-II were added after step 1 in the flow diagram in Fig. 2. The recovery rate is indicated as a percentage of the primary radioactivity.

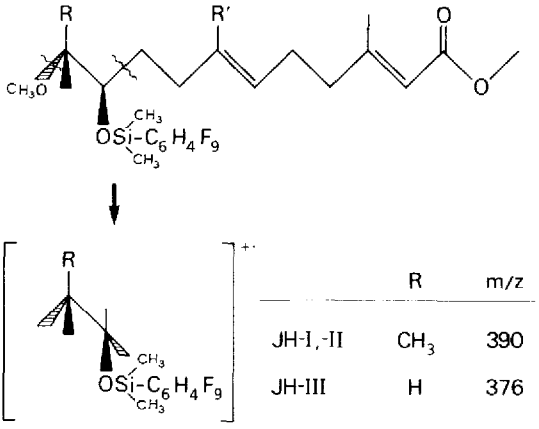
Starting material in 20 ml of methanol	*Step 2, isooctane extract before alumina*	*Step 4, final elution from alumina*
5 pmol of JH-I- and JH-III-ethyl	91.3	67.3
2.1 g of *Drosophila hydei*, 96–108 h	93.5	64.8
2.5 g of *Drosophila hydei*, 108–120 h	90.4	64.7
2.6 g of *Drosophila hydei*, 120–132 h	94.7	69.2

Mass spectrometry

Two analytical signals are present in the electron-impact mass spectra of the JH derivatives, both containing the tail fragment of the molecules and therefore a m/z value higher by 14 for JH-I and -II compared with JH-III (data given in parentheses). Fragmentation between C-10 and C-11 yields a fragment of m/z 87 (73), which contains the methoxy group of the parent molecule (Scheme 1). Another analytical fragment with similar intensity has m/z 390 (376) and is used for our routine measurements.

Application to biological samples

Table II gives some quantitative data for insects obtained with the present technique. The detection limit is *ca.* 0.01 pmol per sample (0.01 pmol/g) fresh insect material. Only in the lepidopterous *Galleria mellonella* was JH-II present, and no



Scheme 1.

TABLE II

OCCURRENCE OF THE THREE HOMOLOGOUS JUVENILE HORMONES (JH-I–JH-III) IN SEVERAL SPECIES OF INSECT ORDERS

The concentrations are given in pmol/g fresh weight of the whole animals.

Species	*Developmental stage*	*Order*	*JH-I*	*JH-II*	*JH-III*
Epilachna varivestis	Adult	Coleoptera	ND*	ND	41.8
Galleria mellonella	L VI (12 h)	Lepidoptera	ND	11.2	ND
Apis mellifera	Adult	Hymenoptera	ND	ND	37.5
Scaptotrigona postica	L V	Hymenoptera	ND	ND	5.9
Melipona quadrifasciata	Adult	Hymenoptera	ND	ND	35.0
Drosophila melanogaster	Pupa	Diptera	ND	ND	9.0
Drosophila hydei	Larva, 120 h	Diptera	ND	ND	0.3
Locusta migratoria	Adult, 10 d	Orthoptera	ND	ND	1.4

* ND = not detectable (amounts of less than 0.01 pmol JH per sample).

JH-I and JH-III were found in this sample of sixth instar larvae. All the other insect species from different orders contained JH-III only. Neither JH-I nor JH-II was detected. JH-0 has also never been found in our insect samples.

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