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MICRO ANALYSIS OF PROSTAGLANDINS AND ECDYSTEROIDS IN INSECTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE LABELING

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SUMMARY

A method is discussed for the quantitative determination of prostaglandins and ecdysteroids in insects by pre-column derivatization and high-performance liquid chromatography. Prostaglandin and ecdysteroid fractions are first purified from homogenized insect organs by a Sep-Pak C_{18} cartridge and derivatized quantitatively by treatment with 1-anthroyl nitrile. These derivatives are then used for quantitative high-performance liquid chromatography analysis on a C_{18} column equipped with fluorescence detection. The detection limit is presently below 10 pg as an injected amount of the standard derivatives. The foregoing methods are applied for the determination of the prostaglandin and ecdysteroid titers in the various organs of an individual insect.

INTRODUCTION

Recent studies have indicated that prostaglandins (PGs) and ecdysteroids are involved in an insect's reproductive mechanism[1–6]. Some insects are difficult to rear, or to rear with many on the same developmental clock. Thus, the quantitative determination of these compounds from a single insect's individual organs is crucial towards understanding these insects' physiology. The micro analysis of PGs is also important to other fields, especially studies in medicine and pharmacology. PGs have very important roles in the physiology of man. Many analytical methods have been developed for the determination of PGs and ecdysteroids, *e.g.* bioassay[7,8], radioimmunoassay (RIA)[9,10] and gas chromatography–mass spectrometry (GC–MS)[11,12] methods. Bioassays have the possibility of detecting active and unstable compounds and have played an important role in the discovery of previously unidentified compounds. This method, however, can provide only approximate quantitation and often fails to distinguish between chemical species. RIA seems to be more sensitive than the other methods. However, RIA methods require pretreatment such as by thin-layer chromatography[10], column chromatography[13] or high-performance liquid chromatography (HPLC)[6] for the measurement of individual compounds in a sample

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because it is difficult to obtain an antibody with little cross-reactivity and a relatively high affinity[9,10]. GC–MS offers good specificity in species distinction as well as sensitivity in the pg range. However, this method often requires complicated pre-purification and derivatization and can be unsuitable for heat labile compounds. On the other hand, HPLC provides a very sensitive method especially suited for the determination of these heat sensitive compounds.

Several HPLC methods for the determination of PGs[14–18] and ecdysteroids[19–20] have been reported. Since PGs do not show sufficient absorption in the UV region, HPLC methods have used derivatization reagents which react with the carboxyl group to form UV absorbing[15] or fluorescent[16–18] esters. However, previous detection limits can be improved upon. Ecdysteroids have a strong UV absorbance due to the α,β-unsaturated ketone moiety and can be analyzed without derivatization[20]. However, the sensitivity is still insufficient for many types of studies.

Recently a new fluorescence labeling reagent, 1-anthroyl nitrile was developed for use with the HPLC analysis of hydroxy steroids[21]. This reagent is not only extremely fluorescent but it is also reactive with hydroxyl groups under very mild conditions. This method has now been extended for the microanalysis of PGs and ecdysteroids in various individual organs of an individual insect. We describe here the detailed determination of these compounds using the African termite, *Macrotermes subhyalinus*, as an example. This termite queen has enormously developed ovaries and can lay up to 10 000 eggs a day. The termite queen is thus a proper choice for the study of the involvement of PGs and ecdysteroids in insect reproductive mechanisms.

EXPERIMENTAL

Apparatus

A DuPont Model 850 liquid chromatograph equipped with a Kratos Model FS950 fluoromat (excitation 365 nm, emission 418 nm) was employed. Either a prepacked Regis Little Champ (3 μm) column (5 cm × 4.6 mm I.D.) or a DuPont Zorbax ODS C_{18} (5 μm) column (25 cm × 4.6 mm I.D.), each equipped with an Uptight pre-column (Upchurch Scientific; 2 cm × 1 mm I.D.) packed with pellicular Whatman Co:Pell ODS, was used. Samples were injected into the column using a Rheodyne rotary valve 7120 syringe-loading injector.

Materials

Authentic PGE_2 and $PGF_{2\alpha}$ were kindly donated by Ono Pharmaceutical (Osaka, Japan). The ecdysone was purchased from Sigma (St. Louis, MO, U.S.A.), and 20-hydroxyecdysone had previously been isolated from various tropical medicinal plants[22,23]. PGE_2 *anti*-methyloxime[24] and 20-hydroxyecdysone *anti*-methyloxime[25] were prepared for use as internal standards. 1-Anthroyl nitrile was prepared as previously described[21]. All other reagents employed were of reagent grade. Solvents were purified by distillation prior to use. A SEP-PAK C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) was used for the preliminary purification.

Preparation of authentic derivatives

Anthroyl PGE_2. Derivatization was accomplished by the reaction of PGE_2 (2 mg, 5.68 μmole) with 1-anthroyl nitrile (5.2 mg, 22.5 μmole) in the presence of 0.08%

quinuclidine in 2.5 ml of dry acetonitrile. The reaction mixture was stirred for 30 min at 60°C. After addition of methanol (50 μl) for decomposing excess reagent, the mixture was evaporated, *in vacuo*. The residue was applied to preparative TLC on silica gel using chloroform–methanol (90:10) as the eluting solvent, followed by extraction of the fluorescence anthroyl PGE_2 band at R_F 0.6 with ethyl acetate. NMR (C^2HCl_3) δ:0.90 (3H, distorted t, terminal CH_3), 3.32 (1H, m, 11α-H), 5.43 (1H, d, d, d, J = 6, 6, 6 Hz, 15-H), 5.62–5.73 (2H, m, 5,6-H), 5.94 (1H, d, d, J = 13, 7 Hz, 13-H), 6.22 (1H, m, 14-H), 7.50–8.50 (9H, m, aromatic Hs). SIMS *m/z* 557 (M^+ + 1).

Anthroyl 20-hydroxyecdysone. Derivatization was accomplished by the reaction of 20-hydroxyecdysone (4.8 mg, 10 μmole) with 1-anthroyl nitrile (9.2 mg, 40 μmole) in the presence of 0.08% quinuclidine in 4.5 ml of dry acetonitrile. The reaction mixture was stirred for 30 min at 60°C. After addition of water (50 μl) for decomposing excess reagent, the mixture was evaporated, *in vacuo*. The residue was extracted with ethyl acetate (3 × 50 ml) and 5% sodium carbonate (5 ml). The organic layer was washed with water and dried over magnesium sulphate, filtered and evaporated to dryness, *in vacuo*. The residue was applied to preparative TLC on silica gel using chloroform–methanol (85:15) as the eluting solvent, followed by extraction of the fluorescence anthroyl 20-hydroxyecdysone band at R_F 0.7 with ethyl acetate. NMR (C^2HCl_3) δ:0.91 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 1.25 (3H, s, 21-CH_3), 1.27 (6H, s, 25,26-CH_3), 3.48 (1H, m, 22-H), 4.39 (1H, m, 3α-H), 5.44 (1H, m, 2α-H), 5.92 (1H, d, J = 3Hz, 7-H), 7.40–8.50 (9H, m, aromatic Hs). SIMS *m/z* 684 (M^+).

Insect material

A queen of the African termite, *M. subhyalinus* (about 15 g) was collected from a termite mound located near Kajiado, Kenya. The termite queen was immediately put into ethanol near the collecting place and then mailed to University of California, Berkeley, U.S.A.

Procedure for determination of PGs and ecdysteroids in the termite

Extraction procedure. The ovaries of a single termite queen were homogenized in 80% aqueous ethanol (20 ml). After centrifugation, the solid material of the tissue was extracted with three 20-ml portions of ethanol. The pooled supernatant was then devided into two parts.

Determination of PGs. PGE_2 *anti*-methyloxime (500 pmol) was added as an internal standard to one half of the supernatant. This solution was evaporated *in vacuo*. The residue was partitioned between 5 ml of light petroleum (b.p. 35–60°C) and 5 ml of 0.2% potassium phosphate buffer (pH 7.0). The aqueous layer was applied to a Sep-Pak C_{18} cartridge. After washing the cartridge with 5 ml of water and 2 ml of 20% aqueous methanol, the PGs were eluted with 2 ml of 80% aqueous methanol. The PG fraction was evaporated to dryness, added with 0.2% 1-anthroyl nitrile in acetonitrile (50 μl) and 0.16% quinuclidine in acetonitrile (50 μl). The mixture was heated at 60°C for 30 min. After the addition of methanol to decompose any excess reagent, the mixture was evaporated to dryness. The residue was dissolved in 0.5 ml of *n*-hexane–ethyl acetate (4:1) and applied to a silica gel column (2 cm × 6 mm I.D.). After washing this column with 4 ml of *n*-hexane–ethyl acetate (4:1) and 0.5 ml of ethyl acetate, the anthroyl PGs were eluted with 2 ml of ethyl

acetate–methanol (3:1). The eluent was then evaporated and the residue obtained was redissolved in methanol (400 μl). An aliquot (10 μl) of this solution was injected into the HPLC system.

Determination of ecdysteroids. 20-Hydroxyecdysone anti-methyloxime (500 pmol) was added as an internal standard to the other half of the original ethanolic supernatant and evaporated, *in vacuo*. The residue was partitioned between 5 ml of light petroleum (b.p. 35–60°C) and 5 ml of water. The aqueous layer was applied to a Sep-Pak C_{18} cartridge. After washing the cartridge with 15 ml of water and 5 ml of 25% aqueous methanol, the ecdysteroids were eluted with 5 ml of 60% aqueous methanol. The ecdysteroid fraction was evaporated to dryness, added with 0.2% 1-anthroyl nitrile in acetonitrile (50 μl) and 0.16% quinuclidine in acetonitrile (50 μl). The mixture was heated at 60°C for 30 min. After the addition of 10 μl of water to decompose any excess reagent, the mixture was evaporated to dryness. The residue was partitioned between ethyl acetate and 5% sodium carbonate. The organic layer was evaporated and the residue was redissolved in methanol (400 μl). An aliquot (10 μl) of this solution was injected into the HPLC system.

RESULTS AND DISCUSSION

Determination of PGs

1-Anthroyl nitrile was developed as a fluorescence labeling reagent for hydroxy steroids[21], and has already been used for the quantitative determination of bile acids in human serum[26]. Efficient derivatization was provided by using acetonitrile as a reaction solvent in the presence of 0.08% quinuclidine as an organic base. We found PGs were also derivatized quantitatively into fluorescent compounds by treatment under the above conditions (Fig. 1). The reactivity of 1-anthroyl nitrile has been examined for various hydroxy functions[21]. According to these results, 1-anthroyl nitrile did not react with tertiary and sterically hindered secondary hydroxy groups. Most PGs have 2 or 3 secondary hydroxy groups, however, only the C-15 hydroxy group has reactivity for 1-anthroyl nitrile according to the following data of the

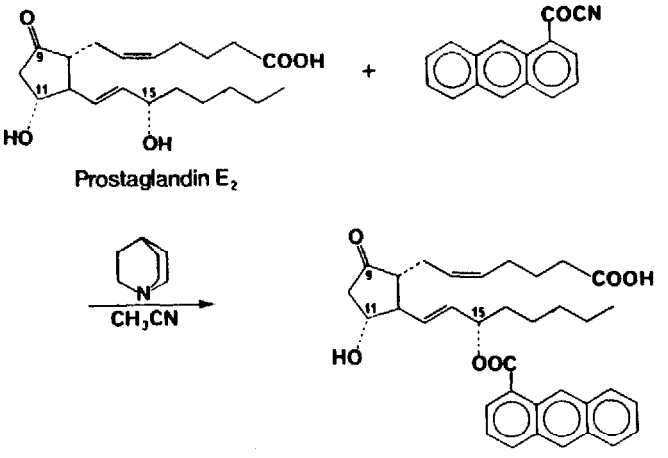

Fig. 1. Reaction of prostaglandin E_2 with 1-anthroyl nitrile.

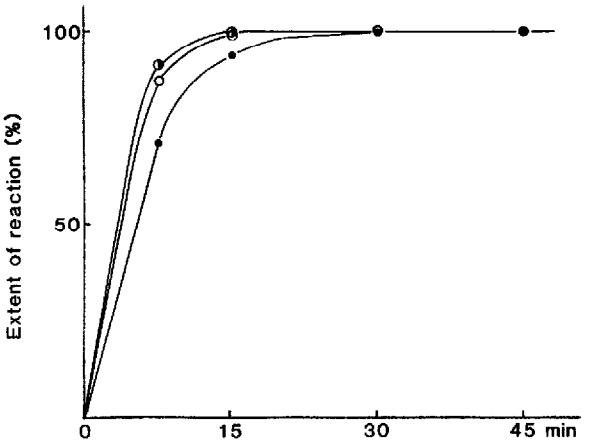

Fig. 2. The derivatization of prostaglandins and ecdysteroids with 1-anthroyl nitrile at 60°C *vs.* time. (○) $PGF_{2\alpha}$; (●) PGE_2; (◑) ecdysteroids.

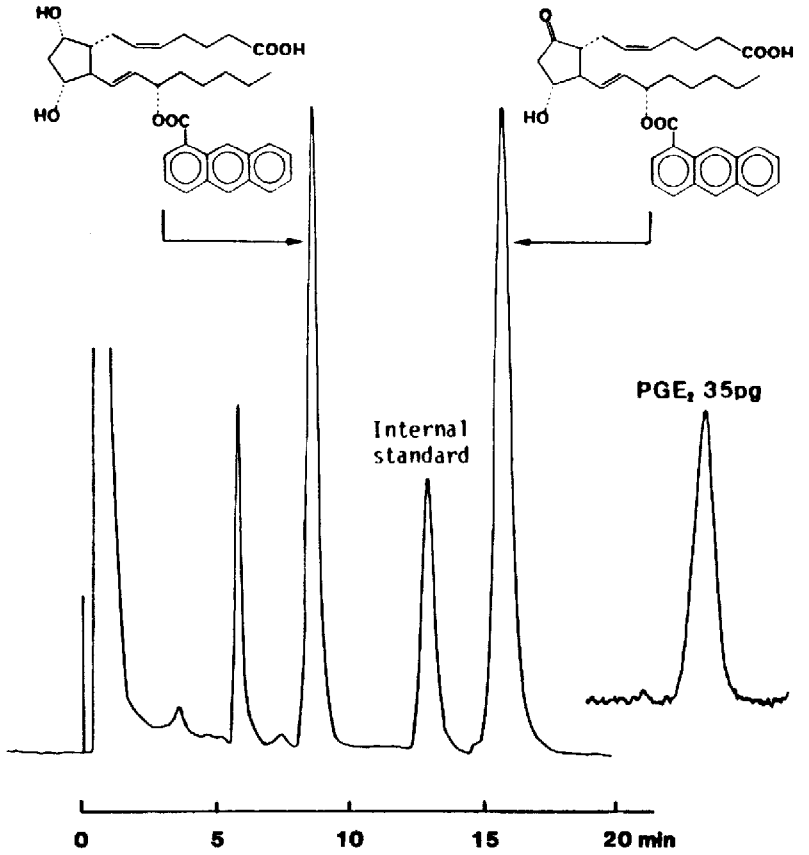

Fig. 3. Separation of anthroyl derivatives of prostaglandins. Column: Regis Little Champ (5 cm × 4.6 mm). Mobile phase: 0.1% potassium phosphate buffer (pH 6.0)–methanol (29:71, v/v), 1 ml/min. Detection, ex: 365 nm, em: 418 nm.

PGE_2 derivative: mass spectrum, m/z 557 ($M^+ + 1$); ^1H NMR spectrum, shifting of C-14 olefinic proton signal from 5.65δ to 6.22δ and C-15 carbinolic proton signal from 4.12δ to 5.43δ. The yields of the anthroyl PGs were calculated by comparison with the peak height of the standard sample. The reaction is completed upon heating at 60°C for 15 min (Fig. 2). The best resolution for PG derivatives was obtained with 0.2% potassium phosphate buffer (pH 6.0)–methanol (29:71, v/v) as the mobile phase on a Little Champ 3-μm particle size ODS column (Fig. 3). A calibration curve was constructed by plotting the peak height of PG derivatives relative to the internal standard, thus allowing the quantitative determination of PGs from the termite queen.

In the past PGs were extracted from aqueous biological solutions with an organic solvent after acidification to pH 3.0–4.0. This method, however, has the disadvantage in that a portion of the sample can be lost to an emulsion layer. In order to eliminate this problem, we decided to use a Sep-Pak C_{18} cartridge to separate the organic compounds from water. Authentic PGE_2 and $PGF_{2\alpha}$ (1 nmol each) were dissolved in a 0.2% potassium phosphate buffer (pH 7.0) and applied to a cartridge already filled with the buffer solution. After washing with 5 ml of water and 1 ml of 20% aqueous methanol, PGE_2 and $PGF_{2\alpha}$ were eluted with 2 ml of 80% aqueous methanol and then quantitatively determined by HPLC with UV detection at 208 nm. PGE_2 and $PGF_{2\alpha}$ were recovered at a rate of more than 95% in the initial 2 ml of the 80% methanol.

A standard procedure for the determination of PGs in insects is shown in Fig. 4. The reaction mixture was initially injected directly into the HPLC column. However, a satisfactory chromatogram could not be obtained due to the interference from excess anthroylating reagent. Therefore, it is necessary to pre-purify the reaction mixture. After the derivatization reaction, methanol was added to decompose any

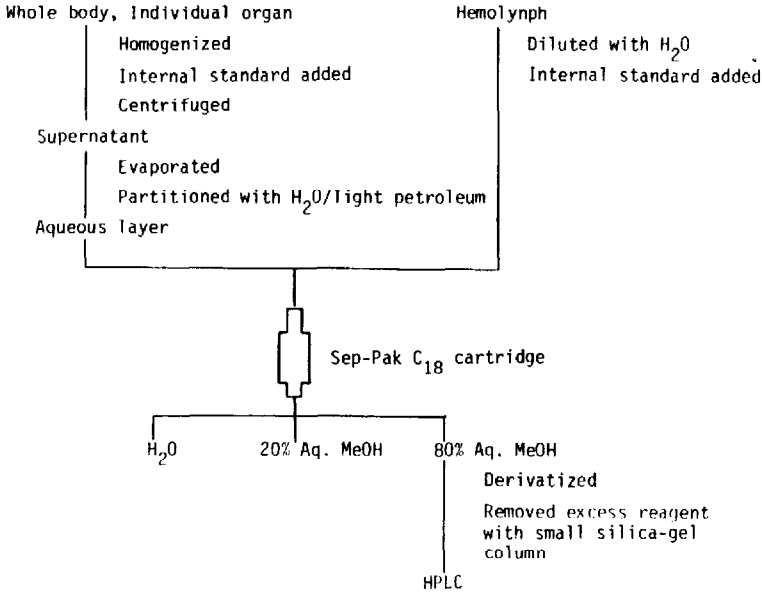

Fig. 4. General scheme for the determination of prostaglandins in insects. MeOH = Methanol.

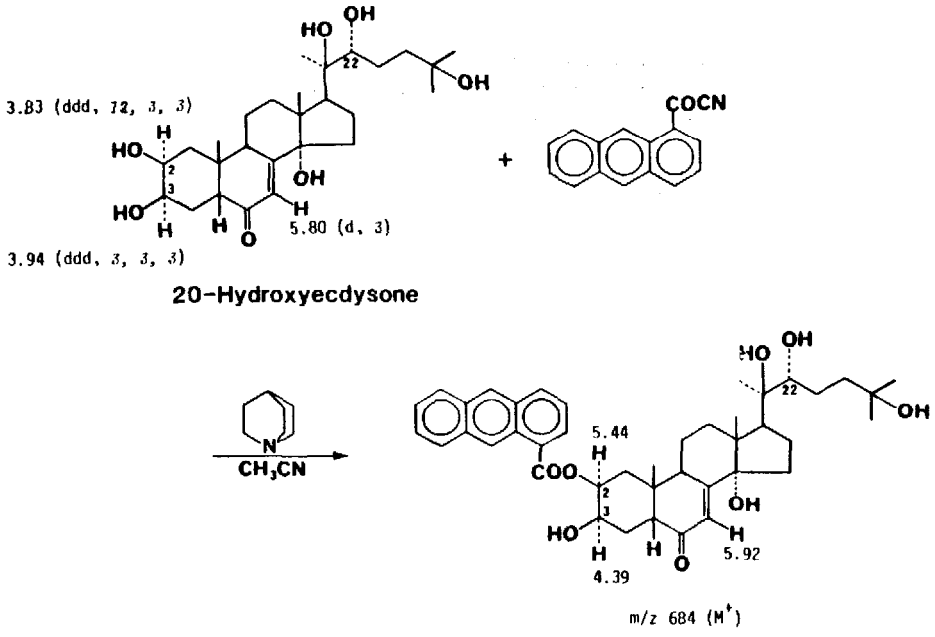

Fig. 5. Reaction of ecdysone with 1-anthroyl nitrile.

excess reagent, and the resulting methyl anthroate was easily removed from the anthroyl PGs with a small silica-gel column (2 cm × 6 mm I.D.).

Determination of ecdysteroids

It seems that both C-2 equatorial and C-22 hydroxy groups of ecdysteroids are reactive for 1-anthroyl nitrile. However, the structure in Fig. 5, 2-(1-anthroyl)-20-hydroxyecdysone, was determined by the following data: mass spectrum, *m/z* 684 (M^+); ^1H NMR spectrum, shifting of C-2 carbinolic proton signal from 3.83δ to 5.44δ. Reaction conditions were quite similar to the case with PGs. The yields of the anthroyl ecdysteroids were calculated by comparison with the peak heights of the standard sample. The reaction was completed with 0.1% 1-anthroyl nitrile in 0.08% quinuclidine in acetonitrile and was heated at 60°C for 15 min (Fig. 2). A chromatogram of standard ecdysteroids is shown in Fig. 6. The optimal mobile phase was methanol–water (8:2). The sensitivity obtained with the fluorescence detector (excitation 365 nm, emission 418 nm) was 10 pg per injection. A calibration curve was obtained by plotting the peak height ratio against the amount of ecdysteroids. Satisfactory linearity was obtained in the range 5 pmol–1 nmol of ecdysteroids.

In a previous study, a Sep-Pak C_{18} cartridge was employed for pre-purification of ecdysteroids in insects for HPLC determination[20]. This method was combined with our derivatization technique. For comparison, the standard procedure for the determination of ecdysteroids in insects is shown in Fig. 7. The procedure is similar to that for PGs, and requires the removal of any excess reagent which can cause interfering absorptions in the chromatogram. The excess reagent is readily removed from the reaction mixture by conversion to its acid with water and subsequent extraction using 5% aqueous sodium carbonate and ethyl acetate.

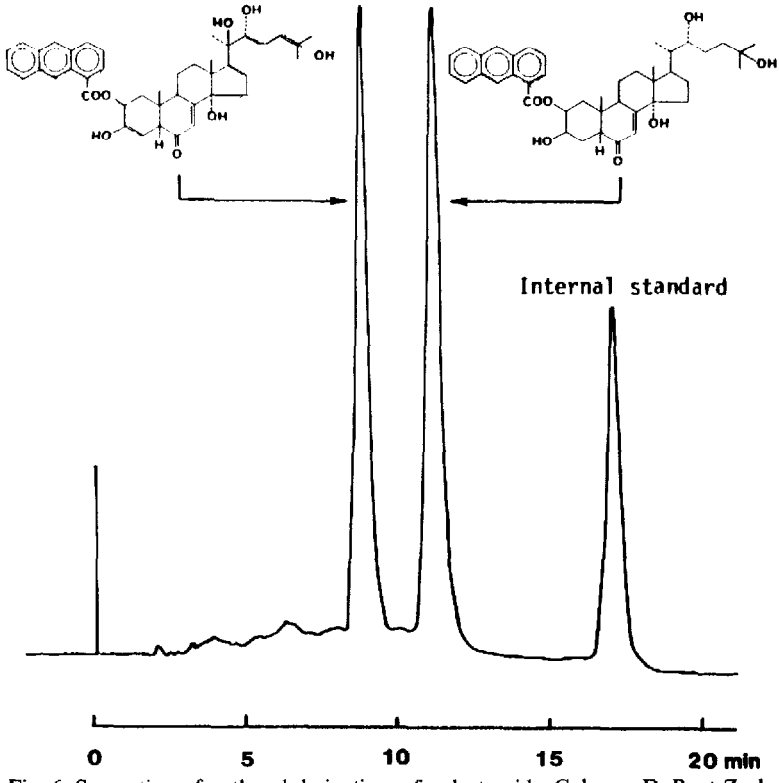

Fig. 6. Separation of anthroyl derivatives of ecdysteroids. Column: DuPont Zorbax ODS (25 cm × 4.6 mm). Mobile phase: water–methanol (20:80, v/v), 1 ml/min. Detection, ex: 365 nm, em: 418 nm.

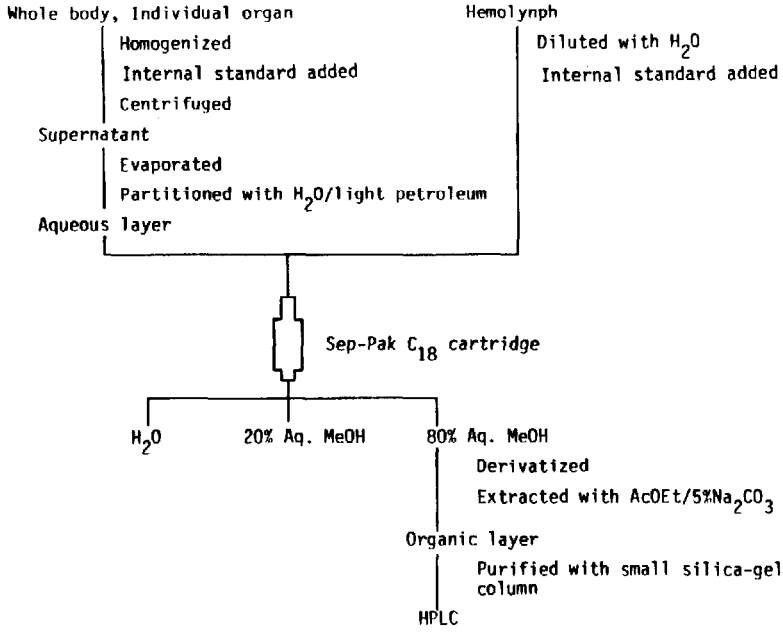

Fig. 7. General scheme for the determination of ecdysteroids in insects. MeOH = Methanol; AcOEt = ethyl acetate.

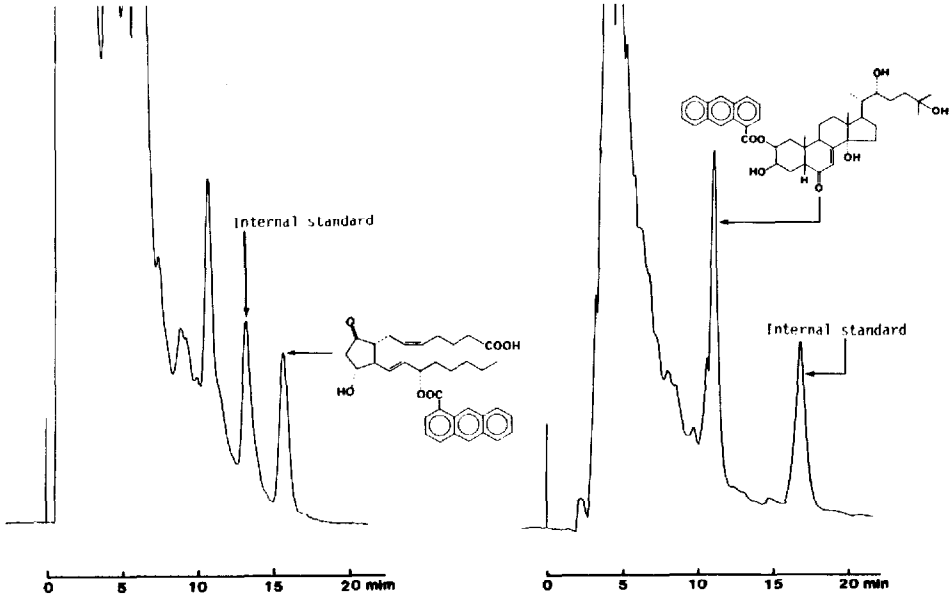

Fig. 8. HPLC of prostaglandins (left) and ecdysteroids (right) in the termite queen, *Macrotermes subhyalinus*.

The above procedure was applied to quantify the PGs and ecdysteroids in the queen of the African termite, *M. subhyalinus*. The resulting chromatograms are illustrated in Fig. 8. A total of 350 ng of PGE_2 and 930 ng of ecdysone in the ovaries of this individual termite queen were determined using these chromatograms. A large amount of ecdysteroid in the termite queen *M. subhyalinus*, has already been reported using an RIA method[27]. This is the first report of PGE_2 from this termite species.

In conclusion, HPLC using 1-anthroyl nitrile as a derivitization reagent was very useful for the determination of PGs and ecdysteroids, and offers a significant improvement in sensitivity over previously reported HPLC methods for the determination of these compounds. We believe that the availability of this new method, with its increased reliability and sensitivity, will be very useful to the study of insect physiology and may prove to be useful in studies of PGs in man as well.

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