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Note

Determination of prostaglandin E_2 in the cricket, *Teleogryllus commodus*, by reversed-phase high-performance liquid chromatography

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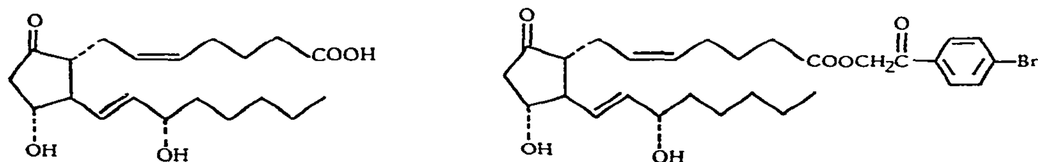
Previous studies have shown that prostaglandins (PGs) release oviposition in several insect species[1–3]. The micro-quantitative identification of these C_{20} fatty acids, especially PGE_2, is therefore crucial to our physiological and behavioral studies. Many methods have been reported for the separation and identification of naturally occurring PGs, but they are not completely satisfactory for the following reasons. (1) Identification of PGs by gas chromatography requires derivatization of both hydroxyl and carboxyl groups to endow thermal stability, and in the case of the PGE series, the derivatization is problematic[4]. (2) The radioimmunoassay method is sensitive, but lacks specificity[5]. (3) Open-column reversed-phase partition chromatography has been applied for isolation and purification of PGs and their metabolites, but this technique is limited to rather large-scale separations[6]. On the other hand, high-performance liquid chromatography (HPLC) seems very useful for identification of PGs from natural sources. A number of PGs were separated by HPLC on a microparticulate silica gel column as the *p*-nitrophenacyl ester. The lowest limit of detection of PGE_2 ester was about 1 ng, but this technique has not been applied to living organisms which are complex matrices[7].



We recently reported a micro-quantitative identification of several *Rabdosia* diterpenes in a single leaf by HPLC on a reversed-phase column[8]. This HPLC method has now been extended for detecting PGE_2 in the cricket, *Teleogryllus commodus*. This HPLC method, which is both specific and sensitive, proved to be a useful tool for the micro-quantitative identification of PGs in living organisms.

MATERIALS AND METHODS

The separations were performed on a DuPont Model 850 liquid chromatograph. A prepacked DuPont Zorbax ODS C_{18} (particle size 5–6 μm) stainless-steel

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column (25 cm × 4.6 mm I.D.) equipped with a stainless-steel guard column (7 cm × 2.1 mm I.D.) packed with pellicular Co:Pell ODS was used. The compounds were detected by a DuPont variable-wavelength ultraviolet (UV) spectrophotometer. The samples were injected into the column using a Rheodyne rotary valve 7120 syringe-loading injector. UV spectra were recorded on a Hitachi UV-100-80 spectrophotometer with 10-mm cells. Preparative thin-layer chromatography (TLC) on silica gel GF plates (Analtech, Newark, DE, U.S.A.) was used for the preliminary purification. HPLC solvents were of high quality (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

PGE_2 p-bromophenacyl ester[9]

PGE_2 was provided by Upjohn (Kalamazoo, MI, U.S.A.). Solvents used for the reactions were of high purity, distilled in glass, and dried prior to derivatization. *p*-Bromophenacyl bromide (MC/B, Norwood, OH, U.S.A.) and diisopropylethylamine (Aldrich, Milwaukee, WI, U.S.A.) were used as received.

Derivatization was accomplished by the reaction of reference PGE_2 (1 mg, 2.8 μmole) with *p*-bromophenacyl bromide (2.3 mg, 8.4 μmole) in the presence of diisopropylethylamine (1 μl, 5.6 μmole) in 0.5 ml of dry acetonitrile under dry nitrogen gas. After 2 h at room temperature, the solvent was removed, *in vacuo*, and 3 ml water was added to the residue. Then, it was extracted with diethyl ether (4 × 3 ml) and the solution was dried over $MgSO_4$, filtered, and evaporated to dryness, *in vacuo*. The purity of PGE_2 ester was confirmed by HPLC, showing only one peak with a retention time of 11.8 min. UV, $\lambda_{max.}^{C_2H_5OH}$ 254 nm (ε = 11,000 l mol^{-1} cm^{-1})[10].

Insect materials

The adults of the cricket, *T. commodus*, were taken from a laboratory colony.

The following steps, outlined in Fig. 1, were generally applicable to all the extraction procedures.

(a) Sixty-seven virgin (thirty-five mated) crickets were homogenized in 80% cold aqueous ethanol (80 ml), followed by centrifugation and a triple ethanol extraction of the remainder of tissue. The ethanol was evaporated, *in vacuo*, leaving an aqueous solution (15 ml), which was acidified to pH 3 with 0.2 *M* citric acid, thus converting PGs to the water-immiscible compounds, and extracted with ethyl acetate (5 × 20 ml). After evaporation of the organic phase, the residue was partitioned between light petroleum (b.p. 35–60°C) and 65% aqueous ethanol. The freeze-dried crude residue, obtained from the aqueous phase, was subjected to further purification.

(b) This partially purified residue was applied to preparative TLC on silica gel. A pure PGE_2 sample was chromatographed separately on a reference plate and both plates were developed together in chloroform–methanol (85:15). The reference plate was exposed to iodine vapor and a band at R_F 0.5 was detected. Therefore, from the plate containing the crude extract, a region corresponding to R_F 0.5 was extracted with methanol, evaporated, and the resulting residue was subjected to derivatization.

(c) The partially purified fraction was derivatized according to the above procedure mentioned for the reference PGE_2. For example, in the case of virgin female bodies, after purification by preparative TLC, 93 mg of crude extract was recovered which was converted to the corresponding ester by reacting with 218 mg of *p*-bromo-

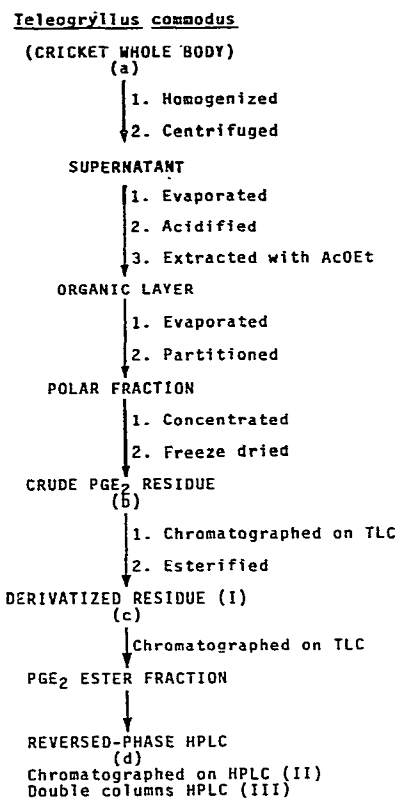

Fig. 1. Extraction, purification and detection of PGE_2. a–d correspond to the procedures for extraction, purification, derivatization and HPLC detection, respectively (see text). I, II, and III refer to the HPLC chromatograms in Fig. 2.

phenacyl bromide in the presence of 90 μl diisopropylethylamine in 0.5 ml of acetonitrile as solvent. To detect PGE_2 as its *p*-bromophenacyl ester by HPLC, it was necessary to further purify the ester, and remove excess amount of the reagent, on preparative TLC on silica gel using methylene chloride–acetonitrile (70:30) as the eluting solvent[7], followed by extraction of the UV-active PGE_2 ester band at R_F 0.3 with methanol.

(d) HPLC detection of PGE_2, as the *p*-bromophenacyl ester derivative, was performed on a reversed-phase column using methanol–water (78:22) as mobile phase with a flow-rate of 1 ml/min. The compound was monitored by a UV detector at 254 nm. Under these chromatographic conditions, the retention time was 11.8 min for PGE_2 ester. The pure compound, resulting from derivatization of commercial PGE_2, was applied as internal reference and co-injected with the sample preparation.

The purification and quantitative measurements of PGE_2 ester were accomplished by re-chromatography on two reversed-phase analytical columns, using the same flow-rate and mobile phase. The resulting peak, with a retention time of 23.6 min, was easily separated from the other peaks. This peak was collected and the amount was confirmed to be 8.35 μg (for virgin female bodies) of PGE_2 ester, or 5.36 μg PGE_2, by UV absorption spectroscopy. This quantitation was also confirmed by comparison of

the peak height, using an aliquot of the solution, with the standard curve. The calibration curve was linear within the range of 20 ng to 180 ng.

RESULTS AND DISCUSSION

Fig. 1 illustrates the steps that are generally applicable to the complete extraction procedure. Our preliminary study indicates that detection of PGE_2 itself by UV absorption at 215 nm by HPLC on a reversed-phase column is possible, but impractical, especially for trace amounts of the sample in, *e.g.*, insects*. The *p*-bromophenacyl ester of PGE_2 was therefore employed as a means of providing a UV-absorbing tag. This ester chromophore is responsible for the UV absorption around 254 nm. However, it should be noted that since the quantity of PGE_2 in *T. commodus* was unknown, excess *p*-bromophenacyl bromide reagent was used for esterification. Since this reagent itself has a strong UV absorption in the same region of the desired PGE_2 ester as well as a retention time close to that of the ester, the unreacted reagent had to be removed before HPLC injection in order to prevent swamping of the UV detector (Fig. 2, I and II).

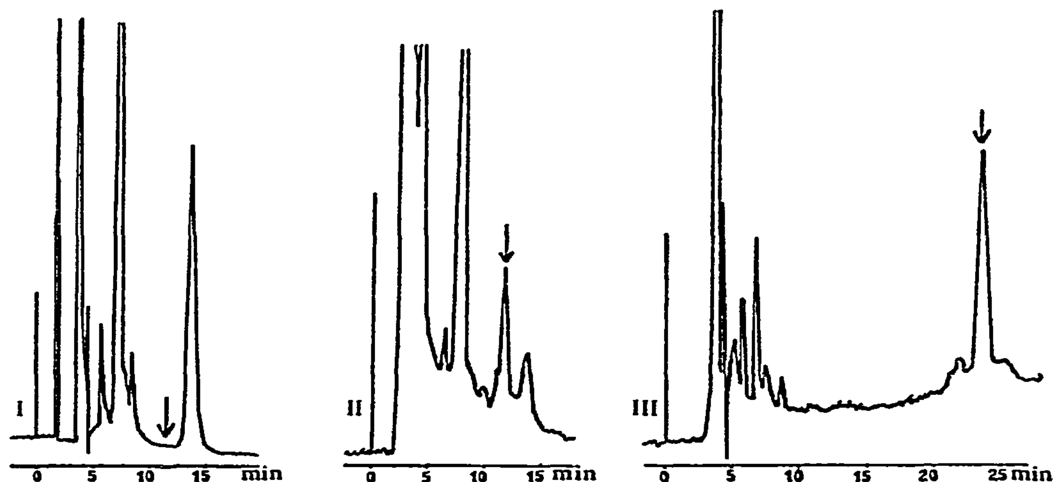

Fig. 2. I, II: Reversed-phase HPLC chromatograms of PGE_2 ester on one analytical column before and after TLC purification, respectively. Mobile phase: methanol–water (78:22); flow-rate 1 ml/min; detection at 254 nm. III: Separation and final purification of PGE_2 ester on two analytical columns, using the same mobile phase and flow-rate. The arrow is an indication of PGE_2 ester.

The free carboxyl group of PGE_2 is useful not only to form the ester, but also to separate it from other non-acidic substances in the crude extract.

The best resolution for PGE_2 ester was obtained with methanol–water (78:22) as mobile phase on a reversed-phase C_{18} column; under these conditions the retention

* A preliminary attempt was made to separate natural PGE_2 on a reversed-phase column, at a wavelength of 215 nm. Using methanol–water (90:10), at a flow-rate of 1 ml/min, the PGE_2 retention time was 5.6 min, as shown in Fig. 3. Due to the following difficulties, this approach was not applicable to our study: (1) the appearance of two ghost peaks, one of which appeared exactly at the same retention time as PGE_2; and (2) problems associated with the detection of the minute quantities of PGE_2 in the living bodies.

time of the ester was 11.8 min. The final purification was carried out by HPLC on a system consisting of two reversed-phase C_{18} columns (directly connected) using the same solvent mixture and flow-rate (Fig. 2, III). The pure PGE_2 ester was applied as the internal reference. The identity and amount of the purified PGE_2 ester was also supported by UV spectral data. The calibration curve was also made with the pure PGE_2 ester to determine the amount of PGE_2 in living organisms. In order to prevent column contamination and to obtain reproducibility a reversed-phase C_{18} guard-column was employed.

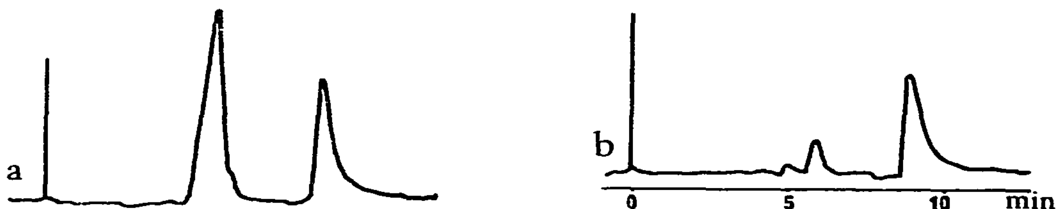

Fig. 3. Chromatograms of (a) natural PGE_2 dissolved in methanol; (b) pure methanol.

The quantity of PGE_2 in headless crickets of *T. commodus*, determined by this HPLC method was 80 ng/virgin female and 140 ng/mated female. Thus, the amount of PGE_2 found in a mated female was almost twice as much as that of a virgin. Further analysis showed that the lowest detection limit for PGE_2 using this HPLC method is 0.5 ng. Therefore, this procedure can not only be employed on a single cricket, but even on part of it, such as a reproductive organ. For instance, by the same procedure and using 100 spermatheca of mated female crickets the amount of PGE_2 was determined to be 0.5 ng/spermatheca. This method was extended to various organs of *T. commodus*. The biological significance of these findings will be published in detail elsewhere[11].

ACKNOWLEDGEMENT

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