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SAMPLE PROCESSING FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ECDYSTEROIDS

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SUMMARY

An improved purification procedure is described for high-performance liquid chromatography (HPLC) analysis of ecdysteroids. It can be used for both quantitative analyses of ecdysone and 20-hydroxyecdysone, and for various metabolic studies.

The procedure comprises two steps only: chloroform–water partition and adsorption/purification of the water phase solutes on a SEP-PAK® C_{18} cartridge. The 60% methanol eluate can be used after evaporation for direct HPLC quantitative analysis of ecdysteroids on a C_{18}-bonded column. The detection limit is about 10 ng of hormones for biological extracts. Makisterone A added before extraction is used as internal standard.

Application of this procedure to metabolic studies requires all the metabolites to be retained on the SEP-PAK cartridge, as is the case for the insect system investigated, *i.e.*, *Pieris brassicae* (Lepidoptera) pupae. Examples of separations are given and the limits of the procedure are discussed.

INTRODUCTION

Since the early work of Hori[1], the use of high-performance liquid chromatography (HPLC) for ecdysteroid studies, has become widespread. It is mostly used to separate compounds that are further characterized or quantified by off-line procedures[2,3]. These procedures include radioimmunoassays[4,5], bioassays[6], mass spectrometry[7] or sulphuric acid-induced fluorescence[8]. To our knowledge, there have been only two reports on the use of HPLC for direct quantification of ecdysteroids by UV absorbance monitoring[9,10], as a routine procedure[9] or for further analysis of fractions previously separated by thin-layer chromatography (TLC)[10]. Another recent attempt with rather crude biological extracts proved much less effective than gas–liquid chromatography (GLC) with electron capture detection[11].

The problem of using HPLC for on-line quantification of ecdysteroids appears

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a priori solvable, since it involves favourable factors: first, the strong UV absorbance of the unsaturated ketone ($E_{243} \approx 12{,}000\ cm^2/mole$); secondly, the relative abundance of ecdysteroids in arthropods, compared to vertebrate steroid hormones. The use of a suitable HPLC system allows easy detection of pure compounds in the nanogram range[2,3], and the problem to be solved is connected with the poor specificity of UV detection, which must be counteracted by extremely efficient chromatographic systems and purification procedures. Adequate purification is of course possible by a multi-step procedure, but this is generally too time-consuming for routine analyses. The aim of the present paper is to describe a new purification procedure, which is both efficient and rapid, and allows many analyses per day. Several chromatographic systems will also be compared.

MATERIALS AND METHODS

Animals

Most analyses were performed with extracts of the white cabbage butterfly, *Pieris brassicae* L., using either hemolymph or whole animals (pupae). A few comparative studies were made with other materials, to assess the general applicability of the procedure. These materials included *Locusta migratoria* hemolymph and eggs, and *Carcinus maenas* eggs.

Chemicals

Reference ecdysteroids (ecdysone, 20-hydroxyecdysone and makisterone A) were from Simes SA (Milan, Italy) or Schering (Berlin, G.F.R.). Ponasterone A was a gift from Dr. Courgeon (Paris, France).

High-purity solvents (HPLC grade) were from Fluka (Buchs, Switzerland), Fisons (Loughborough, Great Britain) or E. Merck (Darmstadt, G.F.R.). Water was twice distilled in a quartz apparatus, and all solvents were filtered on a regenerated cellulose filter (pore size 0.45 μm) from Schleicher & Schüll (Dassel, G.F.R.). All solvents were degassed for at least 20 min in an ultrasonic bath, Bransonic B220 (Branson, Stamford, CT, U.S.A.).

Tritiated ecdysone (specific activity 60–68 Ci/mmole) was purchased from New England Nuclear (Dreieich, G.F.R.).

HPLC

The HPLC system (Waters Assoc.) comprised a 6000A module pump, a U6K septumless injector and a M440 UV detector (254 nm). Certain analyses were performed outside the laboratory, by courtesy of Waters Assoc. and DuPont Instruments, in order to test automated injectors, integrators and temperature control systems.

A Flo-One/DR™ radioactivity monitor (Radiomatic Instruments & Chemical Co., Tampa, FL, U.S.A.) was used for metabolic studies.

The solvent systems and columns chosen are described in the Results section.

RESULTS

Choice of HPLC procedure

A primary consideration was the need for isocratic conditions. The use of

gradient elution would cause baseline disturbances which would impede the use of high sensitivity of detection[11]. On the other hand, isocratic conditions and UV detection at 0.01 a.u.f.s. allow ready quantification of $\leqslant$ 10 ng of pure ecdysteroids.

Very efficient separations of pure ecdysteroids have been obtained with both normal-phase and reversed-phase systems[12,13]. However, when biological extracts are injected, various impurities accumulate at the top of the columns. Consequently, periodical elution of the column with a stronger solvent is necessary. Because of their long equilibration time, silica columns cannot be used in series, although they provide very good separations, even with biological extracts.

The column efficiency must be high enough to separate the ecdysteroids from all interfering substances. This necessitates the use of columns filled with 5-μm rather than 10-μm particles and, at least under the chromatographic conditions used in our laboratory[3], eliminates diol-bonded columns because of their low efficiency. Consequently, the choice is limited to reversed-phase columns. Several C_{18}-bonded columns were tested under various chromatographic conditions chosen to give complete and rapid analyses, and the results are summarized in Table I. A flow-rate of 1.5 ml/min can generally be used, except for methanol–water mixtures which have a high viscosity.

The different mobile phase systems (methanol–water, acetonitrile–water and acetonitrile–buffer) and running temperatures allowed the definition of the most suitable conditions. The best results were obtained with acetonitrile rather than methanol, and at 50°C rather than at ambient temperature. The use of acetonitrile–water mixtures was rejected, because it led to very bad results with certain columns (*e.g.*, Ultrasphere-ODS), and water was replaced by a buffer in order to obtain symmetrical peaks. Columns filled with spherical particles were the most efficient ones (Ultrasphere-ODS and Zorbax-ODS). Ecdysone is generally eluted in less than 20 min, allowing three analyses per hour, provided that no other compound is eluted after this hormone.

Sample processing

A large number of purification procedures have been used to prepare biological samples. They usually involve solvent partitioning between hexane–aqueous methanol, hexane–acetonitrile[9] and chloroform–water[8] for lipid removal, and butanol–water partitioning[14] to eliminate polar substances (for further details, see refs. 13, 15). These steps are then followed by chromatography on a silicic acid column or by TLC on silica plates[13,15]. Holman and Meola[9] recently suggested replacing this second step by HPLC purification on Poragel PN, a cheap large-size reversed-phase support. The whole procedure thus comprised two successive partitions, two HPLC injections on Poragel PN and the evaporation of 50 ml acetonitrile for each sample[9]. Our procedure was designed to involve fewer steps and the evaporation of a small volume of solvent. The principle is to obtain ecdysteroids in a water phase, and then to purify this crude extract on a reversed-phase column.

(a) Extraction of ecdysteroids. These compounds can be extracted with hot water[14], a method that eliminates proteins by thermal coagulation, but another possibility is to mix the biological sample with water and an equal volume of an organic solvent not miscible with water, which can extract the non-polar substances. The solvent chosen should: (i) denature proteins and render them insoluble in water; (ii)

TABLE I

COMPARISON OF VARIOUS CHROMATOGRAPHIC SYSTEMS

Suppliers: 1 = Serva (Heidelberg, G.F.R.); 2 = E. Merck (Darmstadt, G.F.R.); 3 = Waters Assoc. (Milford, MA, U.S.A.); 4 = DuPont (Wilmington, DE, U.S.A.); 5 = Beckman Instruments (Berkeley, CA, U.S.A.). Retention time and plate number are given for ecdysone (see Fig. 4A).

Column	*Supplier*	*Length (cm)*	*Particle size (μm)*	*Solvent*	*Temperature (°C)*	*Flow-rate (ml/min)*	*Retention time (min)*	*Plate number*
Servachrom RP-18	1	25	5	Acetonitrile–water (18:82)	Ambient	1.5	20	3700
LiChrosorb RP-18	2	25	5	Acetonitrile–water (18:82)	Ambient	1.5	20	8700
μBondapak C_{18}*	3	30	10	Acetonitrile–buffer (18:82)	Ambient	1.5	19	4600
Radialpak C_{18}*	3	10	10	Acetonitrile–buffer (18:82)	Ambient	1.5	13	2300
Zorbax-ODS**	4	25	5.5	Acetonitrile–buffer (18:82)	50	1.5	19	11,000
Ultrasphere-ODS	5	25	5	Methanol–water (40:60)	Ambient	1	30	8500
					50	1	15	8000
				Acetonitrile–buffer (20:80)	Ambient	1.5	16	10,500
					50	1.5	16	15,500
				Acetonitrile–buffer (25:75)	Ambient	0.5	15	19,000

* Performed with R. Barbès, Waters Associates.

** Performed with J.-C. Chambet, DuPont Instruments.

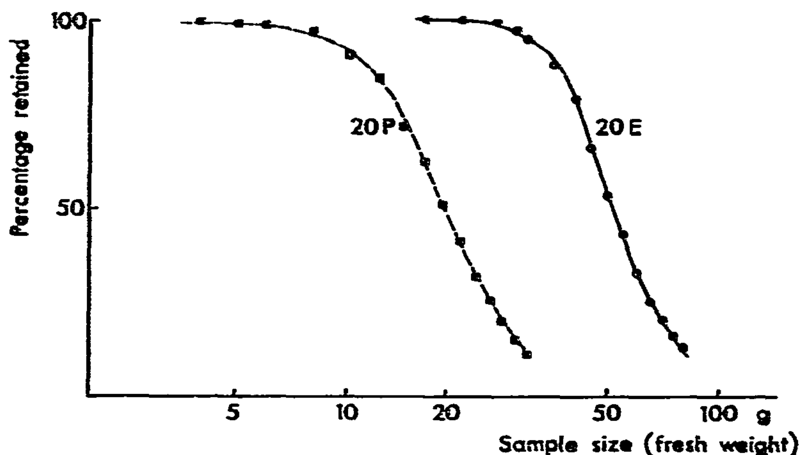

Fig. 1. Saturation experiments with a SEP-PAK cartridge, illustrating the combined retention percentages of 20-hydroxyecdysone (20E) and a polar metabolite (20P) when sample size is increased.

not extract the ecdysteroids, but be as polar as possible in order to extract almost all compounds less polar than ecdysone; (iii) not form unduly stable emulsions with water, so that the two phases can readily be separated by brief centrifugation; (iv) be denser than water, to allow collection of supernatant water phases.

In accordance with these properties, we have chosen chloroform as the organic solvent. The chloroform–water partition coefficient of ecdysone is 7:93, and two successive partitions (v/v) thus allow recovery of more than 99% of the ecdysone in the water phase. This system has an obvious limitation if less polar ecdysteroids are to be recovered. For instance, the partition coefficient of ponasterone A is about 50:50, and this compound is at the limit of the application of the present partition system.

With some samples of whole insects or with crabb eggs, a stable gel appeared in the water phase in our procedure, and this necessitated prior extraction with acetone–ethanol (1:1) and evaporation of the extract to dryness before water–chloroform partition. An alternative possibility would have been to replace chloroform by ethyl acetate, and then to collect the underlying water phase.

(b) Adsorption on a C_{18} phase. When ecdysteroids are contained in a water phase, even in the presence of a few percent methanol or acetonitrile, they are efficiently adsorbed on reversed-phase supports. This was previously shown using a Poragel PN column[9], and we obtained similar results by using a short column (50 × 4.6 mm I.D.) filled with Zorbax-BP-C_8. However, to save time and avoid the need for an HPLC system, we decided to use disposable SEP-PAK C_{18} cartridges (Waters Assoc.). These are currently used to concentrate organic compounds from water[16], but their use in the case of ecdysteroids had been limited to the elimination of non-polar contaminants from methanolic extractions[11]. These cartridges adsorb very efficiently the ecdysteroids contained in the water phase prepared as above. Not only ecdysone and 20-hydroxyecdysone, but also their polar metabolites are retained (Fig. 1). Thus, one SEP-PAK can retain 100% of the 20-hydroxyecdysone in an extract of 20 g *Pieris* pupae. The capacity for the polar metabolites is of course lower, due to the competition by less polar substances in the water extracts. Adsorption was completed by

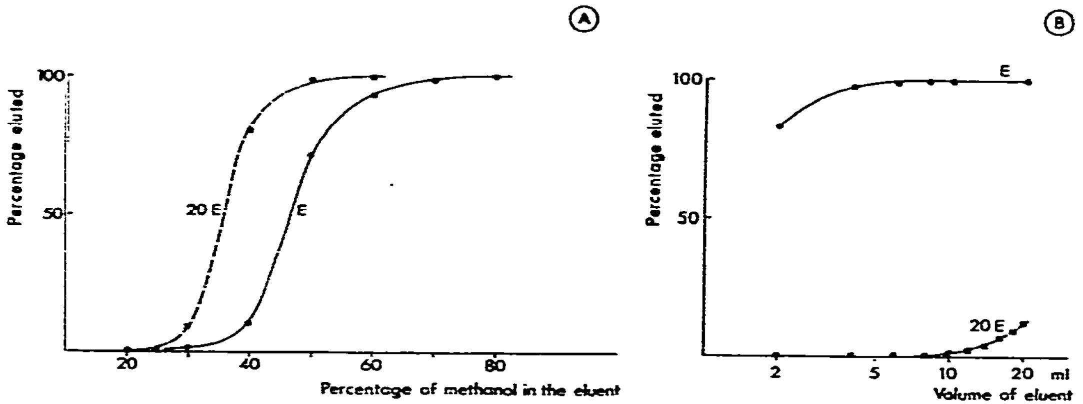

Fig. 2. Elution of ecdysone (E) and 20-hydroxyecdysone (20E) previously adsorbed on a SEP-PAK cartridge. A, Percentage of E and 20E eluted with 3 ml of various methanol–water mixtures; B, percentage of E and 20E eluted with increasing volumes of 60 % methanol (for E) or 25 % methanol (for 20E). See text for further details.

means of a water pump at a flow-rate of *ca.* 2 ml/min. Higher flow-rates would reduce the adsorption efficiency[17].

(c) Elution of ecdysteroids from the SEP-PAK cartridge. We determined standard conditions corresponding to the best use of the SEP-PAK cartridge, by eliminating all substances more polar than 20-hydroxyecdysone, and by retaining all substances less polar than ecdysone. This allowed the selection of compounds of similar polarity, as required for further isocratic HPLC analysis.

Standardized conditions were selected by adsorbing ecdysteroids from four *Pieris* pupae (*ca.* 1.7 g wet weight) on different SEP-PAK cartridges. Tritiated ecdysone or 20-hydroxyecdysone was added as marker. In the first step, 3 ml of various methanol–water mixtures were used, and the percentage of hormone eluted was determined (Fig. 2A). We found that 3 ml of 25 % methanol eluted less than 1 % of 20-hydroxyecdysone, and that 3 ml of 60 % methanol eluted 94 % of ecdysone. In the second step, increasing volumes of 25 % and 60 % methanol were employed (Fig. 2B), and it was found that up to 8 ml of 25 % methanol eluted less than 1 % of the 20-hydroxyecdysone, and that 5–6 ml of 60 % methanol eluted more than 99 % of the ecdysone. These conditions selected were rinsing with 5 ml of 25 % methanol (to elute the polar metabolites of ecdysone) and elution of 20-hydroxyecdysone and ecdysone with 5 ml of 60 % methanol.

(d) Overall design of the procedure. The procedure is shown schematically in Fig. 3. Extraction was performed twice [chloroform–water (1:1)] when ecdysone and 20-hydroxyecdysone were to be analyzed, and three to four times [chloroform–water (1:2)] when samples also contained less polar ecdysteroids (*e.g.*, ponasterone A or 2-deoxyecdysone).

The extraction yield was studied with *Pieris* diapausing pupae (endogenous hormone levels < 5 ng/g) injected with [^{3}H]ecdysone and variable amounts (10–10^3 ng) of unlabelled ecdysone prior to extraction. By using groups of four pupae (*ca.* 1.7 g wet weight), 98.7 ± 0.4 % of the radioactivity in the two first water extracts (10 ml each) was recovered. Recovery was almost independent of biological sample size between 0.2 and 5 g wet weight.

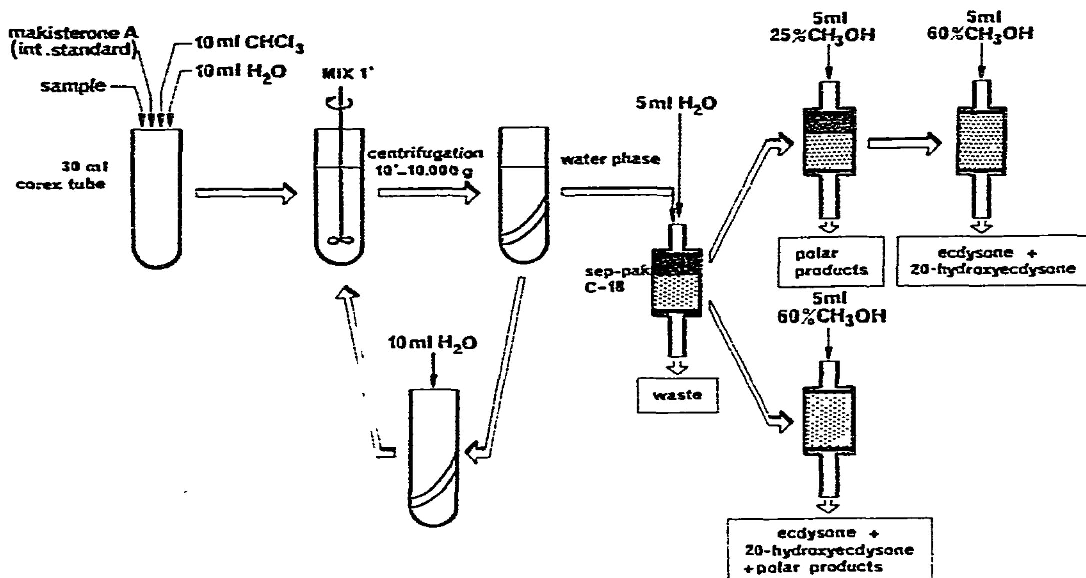

Fig. 3. Purification procedure used for biological samples. The adsorbed ecdysteroids can be eluted in two different ways, depending on the experiment. If ecdysone and 20-hydroxyecdysone are to be quantified, the most polar metabolites are eliminated with 5 ml of 25% methanol. For metabolic experiments in which the entire spectrum of metabolites is analyzed, elution is performed directly with 5 ml of 60% methanol.

The ecdysteroid recovery after extraction and purification may of course vary slightly for each sample, and it is advisable to use an internal standard. Makisterone A, a C_{28} ecdysteroid only found in *Oncopeltus fasciatus* embryos[18], has chromatographic properties intermediate between ecdysone and 20-hydroxyecdysone (Fig. 4A), and appeared suitable for this purpose, if added at the first step of extraction. The ecdysone and makisterone A recovery was checked with diapausing pupae extracts containing 10^3 ng of makisterone A, [^{3}H]ecdysone and 10–10^4 ng unlabelled ecdysone. In all cases, subsequent HPLC analysis showed that the final recovery was the same for both hormones ($80 \pm 5\%$) independently of sample size (0.2–4 g) or ecdysone content.

Easy quantification of hormones was performed on HPLC traces by comparing hormone and internal standard peaks.

Application to various biological materials

The above procedures have essentially been used with *Pieris* pupae (hemolymph or whole bodies) without any special difficulty. From the extract of four pupae or 0.5 ml hemolymph, an aliquot corresponding to one pupa or 0.1–0.2 ml hemolymph was injected. The results mostly agreed with those previously reported using radioimmunoassays[19] or mass fragmentography[20]. Similar experiments with *Locusta migratoria* also gave results consistent with available data[21–23]. In addition, it was possible to quantify ponasterone A in *Carcinus maenas* egg extracts, in which this ecdysteroid was recently reported to occur[24]. Examples of such separations are given in Figs. 4–7.

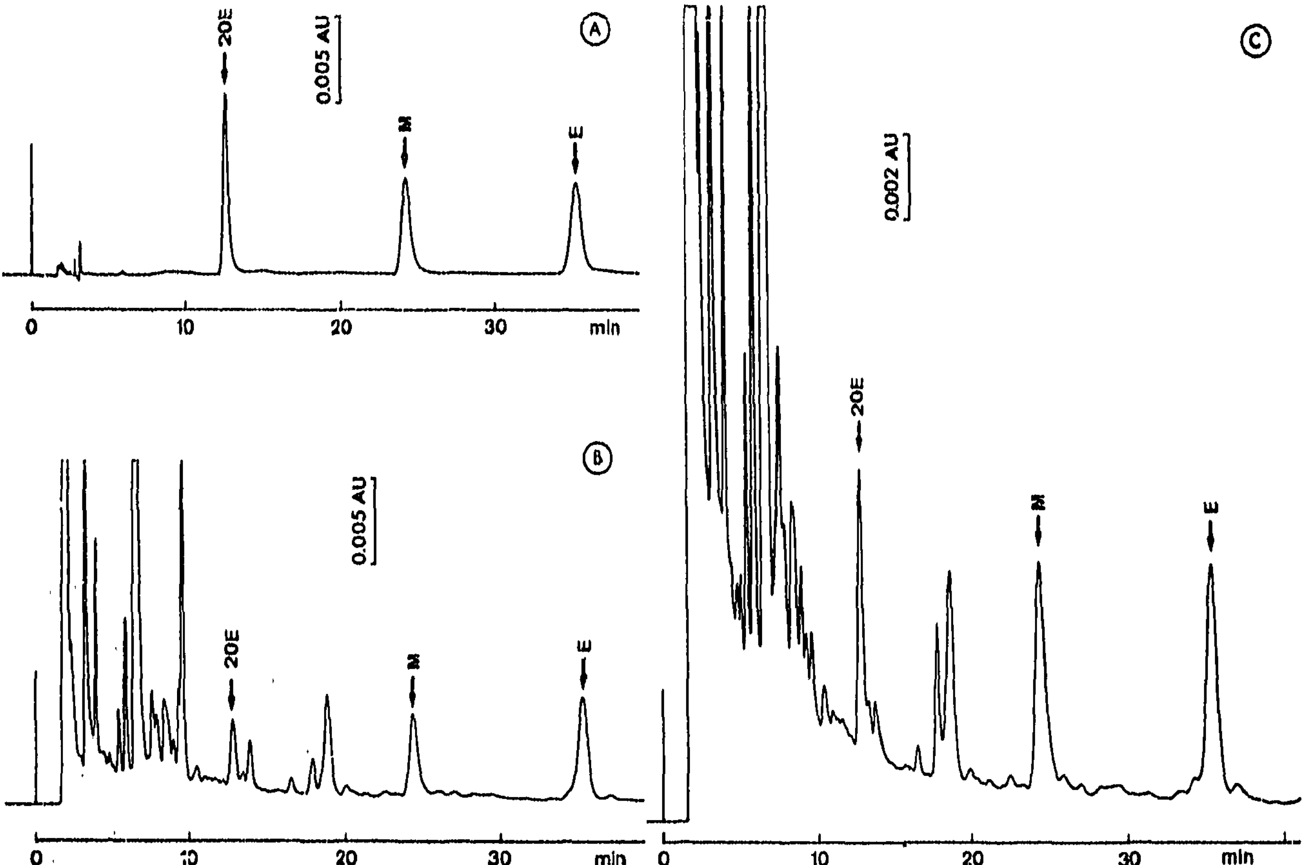

Fig. 4. Analysis of ecdysteroids on an Ultrasphere-ODS column (250 × 4.6 mm I.D.). Solvent: 17% acetonitrile in 20 m*M* Tris–$HClO_4$ buffer, pH 8.5. Flow-rate: 1 ml/min. Temperature: ambient. A, Standard mixture used for calibration (330 ng per compound); B, *Pieris* hemolymph (96 h-old pupae) containing 20E (0.6 μg/ml) and E (2.5 μg/ml); C, *Pieris* hemolymph (108 h-old pupae) containing 20E (1.2 μg/ml) and E (2 μg/ml). M = Makisterone A (internal standard).

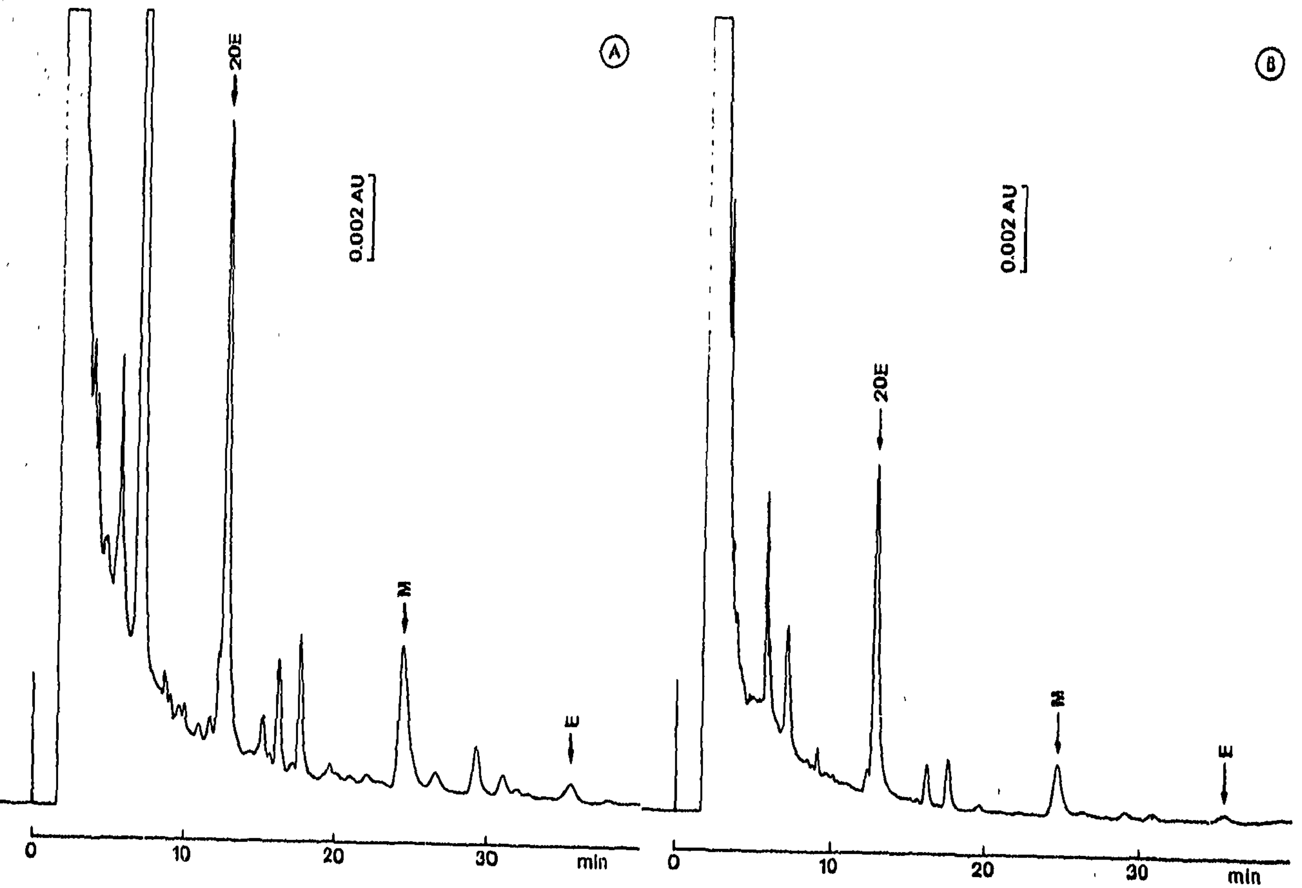

Fig. 5. Analysis of ecdysteroids in last instar larvae *Locusta* hemolymph. Chromatographic conditions as in Fig. 4. A, 7 day-old male larvae; 1.6 ml hemolymph with 1 μg makisterone A added, with injection of a small aliquot. Hormone concentrations: E (0.08 μg/ml) and 20E (1.4 μg/ml). B, 6 day-old female larvae; 0.6 ml hemolymph with 1 μg makisterone A. Hormone concentrations: E (0.24 μg/ml) and 20E (5.8 μg/ml). M = Makisterone A (internal standard).

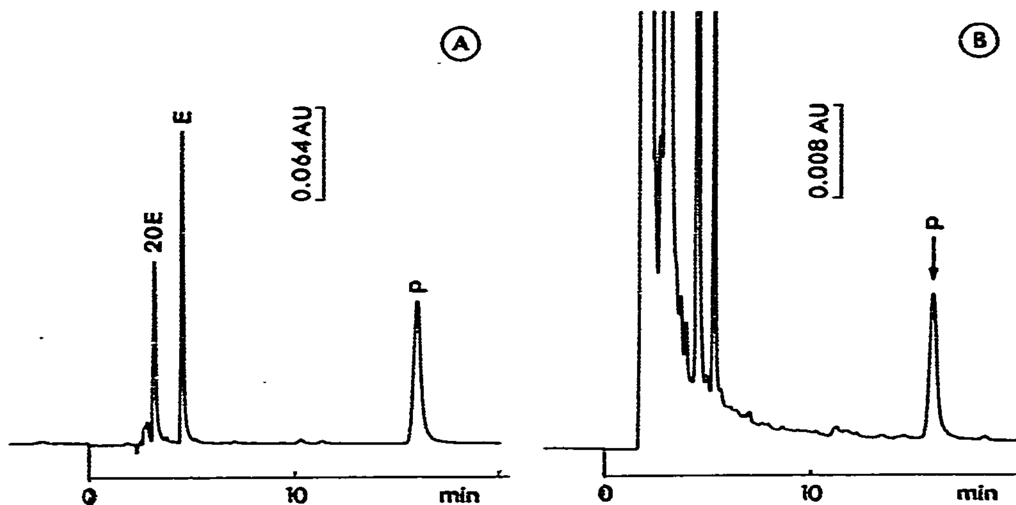

Fig. 6. Analysis of ponasterone A in *Carcinus maenas* eggs. Chromatographic conditions as in Fig. 4 except for the solvent: 26 % acetonitrile in buffer. A, Standard ecdysteroid mixture containing 2 μg of ponasterone A (P); B, egg extract (1.5 g wet weight) containing 170 ng/g of ponasterone A.

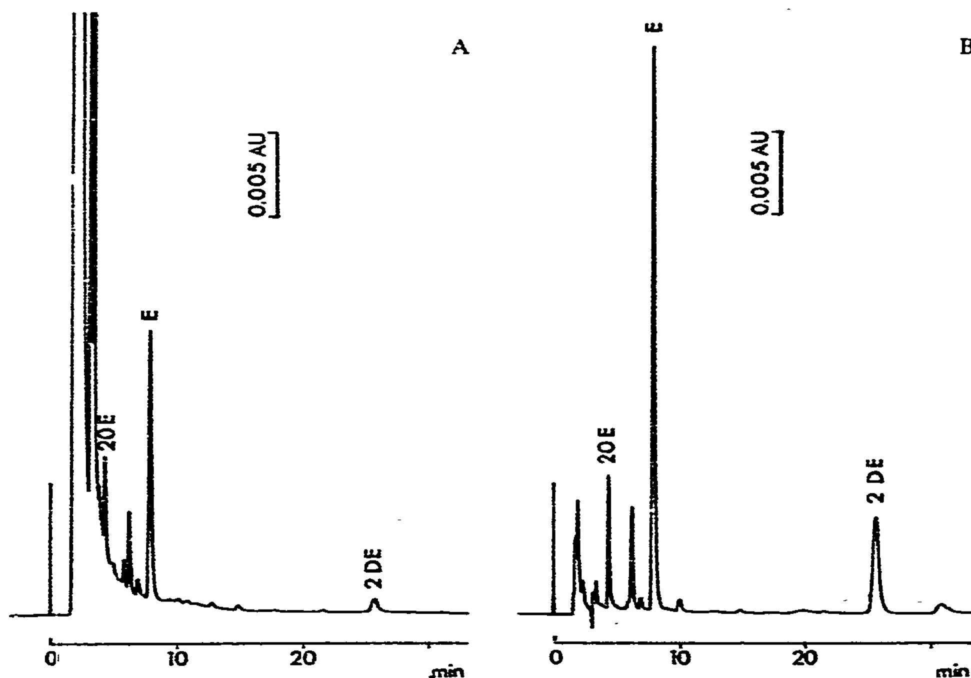

Fig. 7. Analysis of ecdysteroids in *Locusta migratoria* eggs. Conditions as in Fig. 4 except for the solvent: 23 % acetonitrile in buffer. A, Free hormones eluted in the 60 % methanol fraction (injection of eight eggs); B, hormones released from hydrolysed conjugate fraction (water phase and 25 % methanol eluate hydrolysed according to ref. 10 and again processed as in Fig. 3), injection corresponding to one egg. 2DE = 2-Deoxyecdysone.

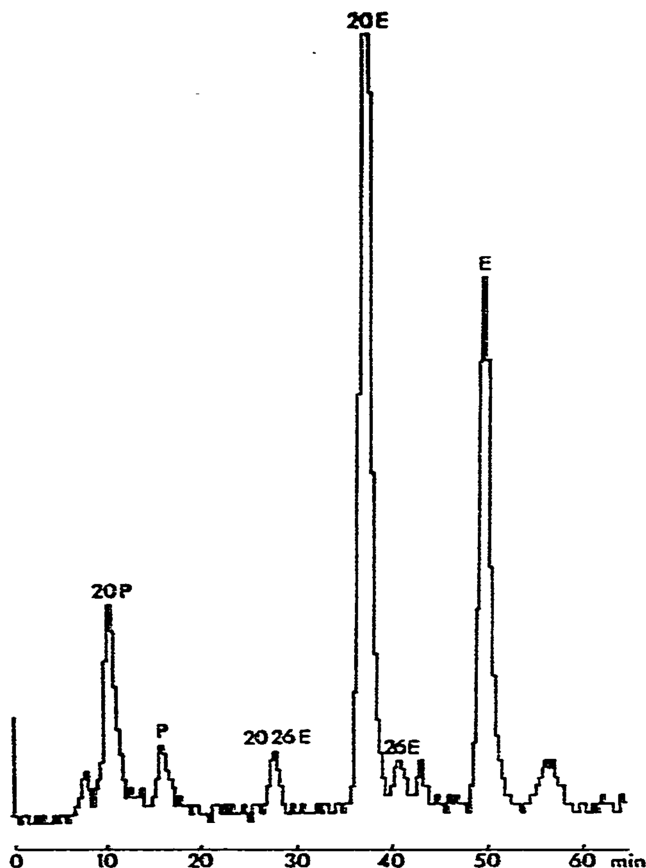

Fig. 8. Analysis of ecdysone metabolites in *Pieris* by on-line monitoring of radioactivity. Column: Zorbax-BP-ODS, 200 × 4.6 mm I.D. (laboratory-made). Solvent: linear gradient (in 85 min) from 13% to 40% acetonitrile in Tris buffer. Flow-rate: 0.3 ml/min. Scintillation cocktail (2,5-diphenyloxazole/1,4-bis(5-phenyloxazolyl-2)benzene/Triton/toluene), flow-rate 1.2 ml/min. Integration time: 20 sec. Major metabolites are ecdysone (E), 26-hydroxyecdysone (26E), 20-hydroxyecdysone (20E) and 20,26-dihydroxyecdysone (2026E); P and 20P refer to the acidic metabolites arising from 26E and 2026E, respectively.

The use of a radioactivity monitor allowed direct analysis of the ecdysone metabolites in *Pieris* pupae (Fig. 8). A conventional column (4.6 mm I.D.) was employed at a reduced flow-rate (0.3 ml/min), and the analysis required 1 h. The use of a small bore column or a splitting device would considerably shorten this period.

DISCUSSION

The purification–HPLC procedure described can be used for the direct quantification of ecdysteroids by monitoring of UV absorbance, or only in combination with an off-line procedure, like radioimmunoassay or fluorometry. This will depend on the relative abundance of ecdysteroids and interfering impurities in biological extracts. When hormone levels are >50 ng/g, direct quantification is generally feasible. Between 10 and 50 ng/g, a control by another method may be needed. Below 10 ng/g, radioimmunoassay of collected fractions is the only solution.

The present procedure is not more efficient than the previous one of Holman and Meola[9], but is simpler and faster. It is suitable for large series of samples and requires no HPLC system for sample preparation, but only a 10-ml glass syringe. From our experience with this procedure, we can draw the following conclusions.

With 1–2 g samples, one SEP-PAK can be used at least four times without noticeable loss of efficiency; rinsing with methanol and reconditioning with water allows repeated use, even if coloured substances such as ommochromes and melanins remain adsorbed on the cartridge.

The procedure may be simplified for studies of organ culture media: even if these contain up to 20% calf serum, they can be directly placed on the SEP-PAK without preliminary chloroform–water partition, without noticeable loss of ecdysteroids (less than 5%). Such treatment is not possible for insect hemolymph, as in this case the major part of the ecdysteroids is not retained on the SEP-PAK. Whether this procedure could be used to study ecdysone-binding compounds in hemolymph remains to be investigated; it could perhaps constitute a faster method than the usual Sephadex G-25 column[25].

The procedure can of course be used for metabolic studies. The catabolism of ecdysone essentially results in the formation of more polar compounds, found in the water phase after partitioning. We obtained evidence (Fig. 1) that, at least in the case of *Pieris*, the acidic metabolites were retained on the SEP-PAK, but this need not be true of all polar metabolites including conjugates. By eluting the SEP-PAK directly with 60% methanol, all the metabolites are eluted together (Fig. 3) and can be analyzed by HPLC in the gradient mode (Fig. 8). If the metabolic study is restricted to one reaction, *e.g.*, conversion of ecdysone into 20-hydroxyecdysone, the mixture eluted from the SEP-PAK can be analyzed isocratically on a short C_{18}-bonded column, with 25% acetonitrile in the mobile phase and on-line radioactivity monitoring. This allows injections every 10 min and easy automation.

By using an integrator and an automated injector, it would be possible to quantify ecdysteroids in 30–50 samples per day, even if a short step-gradient to 60% acetonitrile is needed between successive injections. The number of samples analyzed per day could even be increased by using the recently designed "boxcar" procedure[26], which would eliminate the need for step-gradient cleaning.

There remains, however, the physical impossibility of preparing samples at this rate: in our experience, one person can only process 20 samples a day. As a consequence, sample processing is the limiting factor in this method, and more highly automated sample preparation procedures are being tried, so as to restrict manual operations to the chloroform–water partition step.

CONCLUSIONS

The procedure described appears cheap, rapid and sensitive enough for most samples containing > 30 ng/g ecdysteroids. This study was restricted to a few species, but we believe the method could be extended to other cases.

The same procedure can be applied to metabolic studies, and is used in our laboratory for routine analysis of ecdysone 20-mono-oxygenase activity in various *Pieris* tissues during development.

Further experiments are in progress to improve the method's sensitivity and

specificity (by the use of fluorescence detection) and rapidity (by the automation of certain steps).

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