

Quantification of Ecdysteroids by Immunoassay: Comparison of Enzyme Immunoassay and Radioimmunoassay

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The performance of enzyme immunoassay (EIA) and radioimmunoassay (RIA) in the quantitative analysis of ecdysteroids was compared. The EIA was found to be at least equivalent to the RIA with respect to analytical range and sensitivity and to be more comfortable with respect to safety and time saving. When biological samples were analyzed by both assays a good correlation ($r = 0.83$) was found. Since the EIA has certain advantages over the RIA, we now recommend the use of the former assay for the quantification of ecdysteroids.

Introduction

Ecdysteroids are a family of steroid compounds occurring in plants and animals, in particular in invertebrates. In Arthropods, specific ecdysteroids, notably 20-hydroxyecdysone, serve as hormones controlling central biological processes such as growth, development, metamorphosis and reproduction (see Koolman, 1989, 1990; Rees, 1995, for reviews). Unfortunately little is known about the role of ecdysteroids in other invertebrates or in plants.

Because of the central role of ecdysteroids for arthropod development, their quantitative analysis is a major task in invertebrate endocrinology. Radioimmunoassay (RIA) became the method of choice to quantify ecdysteroids in biological extracts (Warren and Gilbert, 1988; Reum and Koolman, 1989), after the technique was introduced by Borst and O'Connor (1972). More recently, en-

zyme immunoassays (EIA) have been designed for ecdysteroid quantification (Porcheron *et al.*, 1989; De Reggi *et al.*, 1992). Here we report a comparison of EIA and RIA under specified conditions, in particular by the analysis of biological samples with both assays.

Materials and Methods

Animals

Larvae of the blue blowfly, *Calliphora vicina*, were reared on minced beef under controlled conditions (23°C and 55 % RH, light:dark cycle 12h:12h; see Käuser *et al.*, 1988). Third instar larvae were timed from oviposition, the third instar lasting from day 3 to day 8 in our colony.

Reagents

A [23,24–3H]-2-deoxyecdysone with a specific radioactivity of 108 Ci/mmol was gift of Prof. Jules A. Hoffmann and Dr. Charles Hetru (Strasbourg, France). The tritiated 2-deoxyecdysone was purified prior to use by HPLC (Budd *et al.*, 1993). The 20-OH-ecdysone-peroxidase tracer for EIA was gift of Dr. Max De Reggi (Marseille, France) and used without further purification. Unlabelled ecdysone and 20-hydroxyecdysone were obtained

Abbreviations: A_r, activation ratio; BSA, bovine serum albumin, E-CMO, ecdysone 6-carboxymethoxime; 20E-CMO, 20-hydroxyecdysone 6-carboxymethoxime; EIA, enzyme immunoassay; RH, relative humidity; RIA, radioimmunoassay.

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from Simes (Milano). Standard chemical reagents were from Merck (Darmstadt).

Radioimmunoassay (RIA)

For determination by RIA, antiserum WHITE was used. This polyclonal antiserum was generated in a rabbit against ecdysone 6-carboxymethoxime (E-CMO) conjugated to thyroglobulin (Reum and Koolman, 1989). The antiserum showed sufficient specificity for ecdysone, the crossreaction factor for 20-hydroxyecdysone being 15.6. Samples dissolved in methanol contained either reference compound (ecdysone) or immunoreactive ecdysteroids secreted by ring gland tissue during incubation *in vitro* (see below). The solvent was removed from the extract by vacuum centrifugation. The samples were dissolved in 200 μ l RIA buffer (100 mM $\text{Na}_2\text{B}_4\text{O}_7/\text{H}_3\text{BO}_3$, pH 8.4; 75 mM NaCl). For RIA, 50 μ l aliquots were placed in polystyrene vials and mixed with 50 μ l radiotracer ([23,24- ^3H]-2-deoxyecdysone, 188 fmol, 12.5 nCi, dissolved in RIA buffer) and 100 μ l antiserum (diluted 1:3,000 with 5 % normal rabbit serum or 0.2 % bovine serum albumin in RIA buffer). The mixture was incubated for 12 h at 4°C under constant slow shaking. Then charcoal suspension (100 μ l; preparation see below) was added to absorb unbound ecdysteroids. The mixture was agitated vigorously for 10 min before the charcoal was pelleted by centrifugation. An aliquot of the supernatant (150 μ l) was taken for determination of radioactivity by scintillation counting. The calibration curve for RIA was generated with crystalline ecdysone. All samples were measured in triplicate (calibration curve: duplicate) and evaluated by the software RIA-Calc (Pharmacia, Freiburg). The means of these determinations were finally expressed in equivalents of immunoreactive ecdysone.

The charcoal suspension was prepared from charcoal (1.5 g) and water (100 ml). For coating dextran T40 (150 mg; Pharmacia, Freiburg) was added and incubated overnight. The charcoal was separated from the supernatant by centrifugation and mixed with bovine serum albumin (180 mg) and rabbit immune globulin (60 mg) dissolved in RIA buffer (40 ml). This suspension was incubated for 2 h at room temperature. Finally, the charcoal was recovered by centrifugation, resuspended in

fresh RIA buffer (75 ml), and stored (for up to three weeks) at 4°C until use.

Enzyme immunoassay (EIA)

The protocol is based on the EIA described by Porcheron *et al.* (1989), adapted by De Reggi *et al.* (1992) and Delbecque *et al.* (unpublished) for the use of a peroxidase tracer (conjugate of 2-succinyl 20-hydroxyecdysone with peroxidase). Goat anti-rabbit IgG (Sigma, England) was immobilized on 96-well microtiter plates from Nunc (Model 96F, Denmark). All assays were performed in EIA buffer (0.1 M phosphate, pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.1% bovine serum albumin) in a total volume of 150 μ l. Standards, samples, enzymatic tracer and the main antiserum were added at the appropriate dilution in a volume of 50 μ l. The following ecdysteroid specific antisera were tested: DUL1–4 (all against a conjugate of E-CMO with BSA), DBL1, DBL2, RB13–16, AS4919 (all against a conjugate of 20E-CMO with BSA), BLACK, WHITE (against a conjugate of E-CMO with thyroglobulin) and L2 (against a C2/C3 succinyl derivative of ecdysone). After 3h incubation, plates were washed in phosphate buffer (10mM, pH 7.4) containing 0.5% Tween 20. Then, each well was filled with 100 μ l of substrate solution. This solution was prepared with 200 μ l of 0.6% 3,3',5,5'-tetramethylbenzidine in dimethyl sulfoxide and 100 μ l of 1% H_2O_2 in 12.5 ml 0.1 M citrate buffer, pH 5. After appropriate incubation, reaction was stopped by adding 75 ml of 2N H_2SO_4 to each well. Absorbance was read at 450 nm with a Titertek Multiscan Plus II Spectrophotometer (Flow). Since the standard curve was obtained with calibrated solutions of ecdysone, results are expressed as nanogram of ecdysone equivalents per sample. The standard curve was expressed by using a linear log-logit transformation (Tijssen, 1986).

To find the binding affinity and the optimal dilution of enzyme-labelled tracer and antibody, a two-dimensional titer determination was performed on the microtiter plate. After performing displacement experiments using the optimum combination of antibody and enzymatic tracer, sensitivity was defined as the concentration of ecdysone inducing a 50% inhibition of binding of the tracer (50% b/b_0).

Samples

In the course of a search for compounds affecting the biosynthesis of ecdysone in blowfly larvae *Calliphora vicina* (Hua *et al.*, 1994), 114 independent samples were prepared. From blowfly larvae, at day seven after egg laying (1 day before pupariation, = 80 % of third instar) ring glands were dissected. These glands were cut into a left and right half and incubated separately in wells of microtitre plates in 50 μ l *Calliphora* Ringer (Budd *et al.*, 1993) for 4 hours. The incubation was stopped by removal of the tissue and addition of 200 μ l methanol. The samples were dried by vacuum centrifugation and extracted with 1,000 μ l methanol. After removal of solid material by centrifugation, the extract was split into two equal aliquots for RIA and EIA and dried by vacuum centrifugation.

The activation ratio (A_r) was calculated by dividing the ecdysteroid contents of medium from the experimental part of the gland by the contents of the control part. The effectors tested were a juvenile hormone analogue and an ecdysteroid agonist.

Results

In initial experiments, the binding of the EIA tracer (2-succinyl 20-hydroxyecdysone coupled to peroxidase) to various ecdysteroid-specific antisera was tested. Because of steric hindrance the following antisera did not bind the tracer: DUL1, DUL2, DUL3, DUL4, RB13, RB16, RB17, BLACK and WHITE. The antisera RB14, RB15 and AS4919 revealed a weak binding. Only antibodies of the antisera L2, DBL1, and DBL2 bound the enzyme labeled tracer sufficiently. Antiserum DBL2 was used for further EIA measurements.

To characterize the performance of EIA, a standard curve with ecdysone was plotted (Fig. 1). The working range of the assay was 10–1,500 pg per well. With a volume of 50 μ l sample per well, this was equivalent to an ecdysone concentration of 0.4–65 nM in the sample to be assayed. Within these concentration limits, a linear dose response was found. The coefficient of variation of duplicate determinations of 8 standard samples of ecdysone was between 0.2 and 5.7 %.

The ecdysteroid-specific antiserum WHITE and the tritium labelled tracer 2-deoxyecdysone were used for the RIA because antibodies of this antiserum bound the radioactive ecdysteroid sufficiently. The working range of the RIA (defined as $0.8 > b/b_0 > 0.2$) was 20–500 pg per tube. With a sample volume of 50 μ l per assay, this translates into a sample concentration of 0.9–20 nM.

Biological samples were prepared to determine whether the EIA gave the same results as RIA. To this end, ring glands were explanted from blowfly larvae, cut into left and right halves and incubated in *Calliphora* Ringer in the presence of different effectors which potentially could activate or inhibit ecdysone formation. The amount of ecdysone secreted by individual gland halves was determined in parallel by EIA and RIA analysis of the

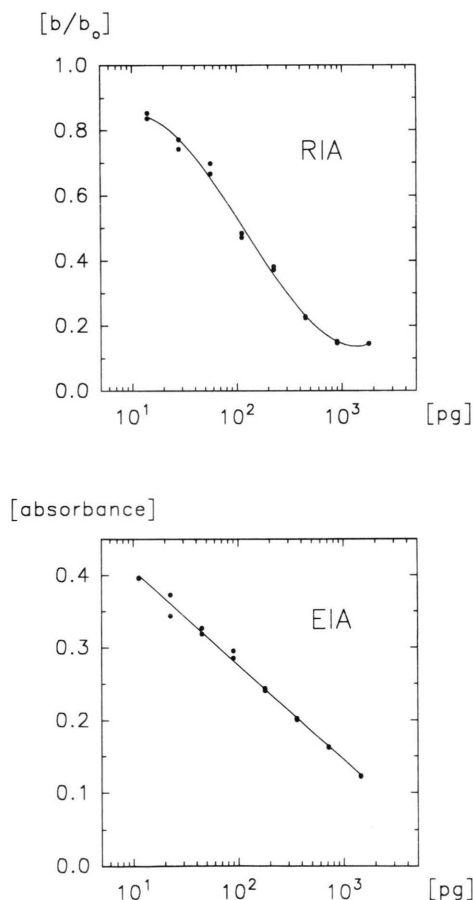


Fig. 1. Standard curves of RIA and EIA with ecdysone. The amount of ecdysone is given in pg per tube. Each assay was run in duplicate. Symbols partly overlap.

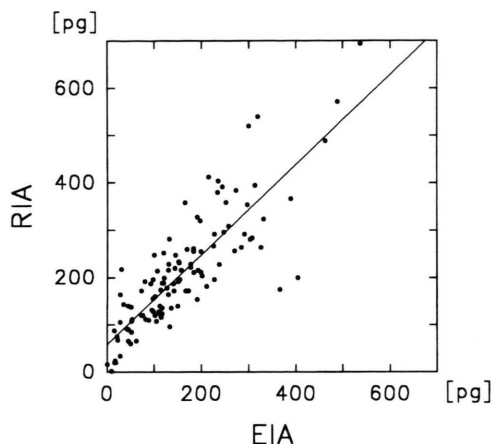


Fig. 2. Correlation of 114 biological samples determined by RIA and EIA. The correlation factor r is 0.833, the slope is 0.949.

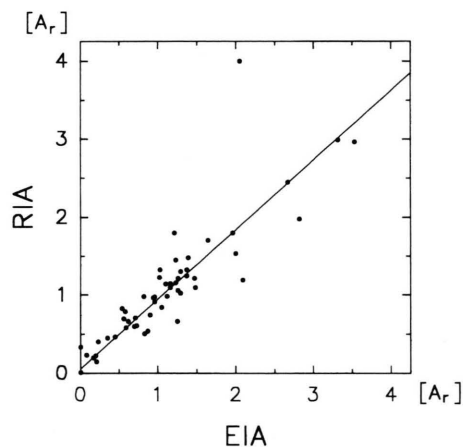


Fig. 3. Correlation of activation ratio (A_r) from 56 pairs of ring glands determined by RIA and EIA. The correlation factor r is 0.928, the slope is 0.892.

media from 114 different incubations. A regression analysis of the quantitative results obtained by the two methods resulted in $Y=0.94X+58.90$, $r = 0.833$ (Fig. 2). When the data were used to calculate activation ratios (A_r) this correlation was even better ($r = 0.928$; Fig. 3).

Discussion

Fifteen different antisera that had been generated against ecdysteroid conjugates were tested in the EIA. While each of them was found to bind radiolabeled 2-deoxyecdysone (unpublished), only

three were able to bind the tracer, i.e. 20-OH-ecdysone labeled with peroxidase. This fact is undoubtedly due to the steric hindrance of the enzyme. As the EIA tracer was formerly designed to be used with L2 antiserum, following the same coupling strategy as the immunogenic derivative (De Reggi *et al.*, 1992 and Delbecque *et al.*, unpublished), binding of tracer to L2 antibodies was observed as expected. However, antisera DBL1 and DBL2 were both generated with an antigen in which the protein was coupled to ring B of 20-OH-ecdysone (instead of ring A). Interestingly, this did not appear to affect binding, though other antisera made according the same strategy, but eventually with another ecdysteroid, were affected (e.g. DUL1, DUL2, DUL3). Apparently, the choice of a good antiserum still requires an element of good luck.

A comparison of the assay performance of RIA and EIA should concentrate on criteria such as specificity (selectivity), sensitivity, precision, and accuracy of the measurements, as well as their costs, practicability and potential health risks.

We did not test here the *specificity* profiles of RIA and EIA by analyzing the crossreaction factors of various ecdysteroids. Significant differences are to be expected because different tracers and antisera were used, which reveal characteristic specificity profiles (Reum and Koolman, 1989). This may be even more relevant for heterologous assays, such as the two used here, in which tracer and reference compound were not identical.

From the calibration curves shown in Fig. 1, the *sensitivity* and the working range can be read. It is obvious that the EIA and RIA had similar sensitivity, with a lower threshold of 10 or 20 pg ecdysone per assay and a maximum of 1500 or 500 pg respectively. These values appear only slightly in favor of the EIA here, but it is undoubted that EIA has a far greater potential than RIA for an increase of sensitivity: thresholds near 1 pg have been already obtained with EIA (Porcheron *et al.*, 1989; Delbecque *et al.*, unpublished) and several strategies are presently under investigation which should still improve these performances in the future.

The *precision* of the assays can be read from the duplicate analyses of calibration curves. When unknown concentrations were determined, the precision was in the same range.

The *accuracy* of the assays can be determined by the method of internal standards or by comparison of data obtained by different analytical methods. Using the latter method, we compared the ecdysteroid content of 114 samples by RIA and EIA and found a good correlation (Fig. 2) indicating that the accuracy of the assays was sufficient. Of special importance was the fact that the processing of data for the calculation of 'activation ratios' of ring glands showed an even better correlation between RIA and EIA. Both methods proved to be reliable for the determination of immunoreactive ecdysteroids secreted by ring glands of blowfly larvae.

Our *costs* were difficult to calculate because the key reagents used were generous gifts from other laboratories. However, the prices of the two key analytical instruments (liquid scintillation counter versus microtitre plate reader) strongly argue for the EIA.

Practicability is an important aspect if many samples have to be processed. The EIA has a further potential for time saving if automatic pipettes and plate washers are used. The limitations of the RIA were due to the 12 h incubation and the time-consuming scintillation counting. Incubation and measurements were much faster with the EIA. An important aspect also concerns the stability of the tracers. Radioactive tracers are fundamentally unstable and thus have to be synthesized and/or purified regularly. On the contrary, enzymatic tracers, though not completely stable, can be used during several years without purification, which is a substantial advantage. This and the administrative paper work related to the purchase, storage, handling and disposal of radioactivity strongly argue in favor of the EIA, against the RIA.

Health risks are also very important to consider in the laboratory and for the environment. The risks of the RIA are mainly due to the radioactivity of tracer and also to the carcinogenic properties of chemicals contained in scintillation cocktails. Such risks are completely absent in the EIA, which is a considerable advantage. A risk can nevertheless exist in EIA, with the use of some substrates of peroxidase having a carcinogenic potential (e.g. *o*-phenylenediamine and some other benzenediamine derivatives). However, this risk can be eliminated by the use of 3,3',5,5'-tetramethylbenzidine, as recommended here, because it is a safe substrate for peroxidase (Bos *et al.*, 1981) or by the use of other enzymes (e.g. acetylcholinesterase, Porcheron *et al.*, 1989).

In summary, it is obvious that EIA presents certain advantages over RIA, without any disadvantage, and we are convinced that it will soon become the method of choice for the determination of ecdysteroids, as also observed in the case of various vertebrate hormones and other molecules of interest.

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