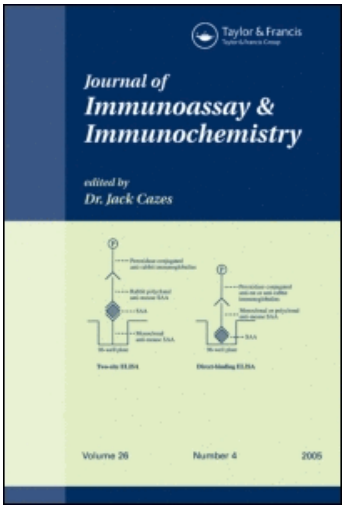

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

An Enzyme Immunoassay for Rat Prolactin: Application to the Determination of Plasma Levels

Laurent Duhau[a]; Jacques Grassi[a]; Dominique Grouselle[b]; Alain Enjalbert[c]; Jean-Marc Grognet[a]
[a] Section de Pharmacologie et d'Immunologie, Département de Biologie, CEN/Saclay, GIF sur YVETTE Cedex, France [b] Groupe de Neuroendocrinologie Cellulaire, College de France, Place M. Berthelot, PARIS Cedex 06, France [c] INSERM 159, PARIS, France

To cite this Article Duhau, Laurent , Grassi, Jacques , Grouselle, Dominique , Enjalbert, Alain and Grognet, Jean-Marc(1991) 'An Enzyme Immunoassay for Rat Prolactin: Application to the Determination of Plasma Levels', Journal of Immunoassay and Immunochemistry, 12: 2, 233 — 250
To link to this Article: DOI: 10.1080/01971529108055069
URL: http://dx.doi.org/10.1080/01971529108055069

JOURNAL OF IMMUNOASSAY, 12(2), 233-250 (1991)

AN ENZYME IMMUNOASSAY FOR RAT PROLACTIN : APPLICATION TO THE DETERMINATION OF PLASMA LEVELS

Laurent DUHAU[1], Jacques GRASSI[1], Dominique GROUSELLE[2], Alain ENJALBERT[3], and Jean-Marc GROGNET[1].

[1]Section de Pharmacologie et d'Immunologie, Département de Biologie, CEN/Saclay, 91191 - GIF sur YVETTE Cedex, France.
[2]Groupe de Neuroendocrinologie Cellulaire, Collège de France, Place M. Berthelot, 75231 - PARIS Cedex 06, France.
[3]INSERM 159, Centre Paul Broca, 2ter rue d'Alésia, 75014 - PARIS, France.

ABSTRACT

Pure acetylcholinesterase (EC 3.1.1.7) from Electrophorus electricus has been covalently coupled to rat prolactin using the heterobifunctional reagent : N-succinimidyl-4 (N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). This conjugate was used as a tracer in a competitive enzyme imunoassay using a rabbit antiserum, raised against rat prolactin, as first antibody. The assay was performed in 96-well microtiter plates coated with a mouse monoclonal anti-rabbit immunoglobulin antibody. This second antibody solid phase ensured separation of bound and free moieties of the tracer during the specific immunoreaction. The total reaction volume was 150 µl. Each component (tracer, antiserum and standard) was added in a volume of 50 µl. The sensitivity of the assay was good since calculation indicated a detection threshold of 25 pg (0.5 ng/ml) and a B/Bo 50 % value of 220 pg (4.4 ng/ml). Intra-assay variation was better than 10 % over a wide range (135 to 2500 pg) with an optimum of 4 % at 300 pg. The inter-assay coefficient of variation was less than 15 % for rat plasma samples in the concentration range of 8 to 1000 ng/ml. The good parallelism observed between the standard curve and sample dilution curves, and recovery experiments, indicated that direct assay is possible. This was confirmed by molecular sieve fractionation of plasma samples. The reliability of the assay was confirmed by good correlation with conventional radioimmunoassay (r = 0.996, slope = 0.978). (KEY WORDS : Acetylcholinesterase, rat prolactin, enzyme immunoassay, plasma).

INTRODUCTION

Radioimmunological methods (RIAs) provide valuable assays of plasma pituitary hormones due to their high specificity and sensitivity (for review see 1). However, these methods are subjected to constraints imposed by the stringent regulations in force concerning the handling of radioactive substances and the elimination of radioactive wastes. In addition, RIAs involve use of short-lived radionuclides and this constitutes an inherent limitation. For these reasons, a number of non-isotopic immunoassays, generally enzyme immunoassays (EIAs), have been recently developed for animal and human hormones (for review see 2 and 3).

We have shown that the use of enzymatic conjugates comprising haptens coupled to acetylcholinesterase (AChE) from Electrophorus electricus can be used to develop immunoassays that are more sensitive and precise than the corresponding radioimmunoassays using ^{125}I-labelled antigen (4, 11, 12 and 18). We describe here a solid phase competitive enzyme immunoassay for rat prolactin, a pituitary hormone involved in lactation (5). The assay is based on the use of a specific rabbit polyclonal antiserum together with a conjugate obtained by coupling rat prolactin to AChE. The assay is performed in 96-well microtiter plates coated with a second antibody so that separation of bound and free tracer fractions is concomitant with the specific immunoreaction.

MATERIALS AND METHODS

Chemicals

Rat prolactin standard R.P.3 and rat prolactin for iodination I-5 were gifts from the National Institute of Arthritis, Diabetes, Digestive Diseases and Kidney (NIADDK, USA). Microtiter plates (96F immunoplates I, with certificate) were from NUNC (Denmark). Biogel A-0.5M, A-1.5M and A 15M were supplied by BIORAD (Richmond, CA, USA). Unless otherwise stated, all reagents were from SIGMA (St Louis, MO, USA). SMCC was supplied by CALBIOCHEM (San Diego, CA, USA), and Sephadex G 25 was from PHARMACIA (Uppsala, Sweden).

Animals

Sprague Dawley male or female rats (IFFA-CREDO, Les Oncins, France) were housed under controlled temperature conditions (22°C ± 2) in an artificially illuminated room (12 h on and 12 h off). Food and water were available ad libitum.

Apparatus

Enzyme immunoassays were performed using specialized TITERTEK microtitration equipment, including an automatic plate washer (Microplate Washer S 8/12), an automatic dispenser (Autodrop), and a spectrophotometer (Multiskan MC) from FLOW Laboratories (Helsinki, Finland).

Radioactive counting was performed with an LKB Multigamma solid scintillation counter (Finland). Centrifugation was performed using a JOUAN K110 centrifuge (JOUAN, Paris, France).

Production of Polyclonal Antibodies

Rabbits (Blancs du Bouscat, EVIC, Blanquefort, France) were immunized by subcutaneous injections with 500 μg of purified rat prolactin (NIADDK R.P.3) in Freund's complete adjuvant. Rabbits were injected into the axillar and crural regions (6). Booster injections were given after 3 weeks. Rabbits were bled weekly after booster injection for 3 weeks. After blood coagulation, serum was collected and stored at 4°C in the presence of 0.01 % sodium azide.

Purification and Assay of AChE

AChE (EC 3.1.1.7) was purified from the electric organ of the electric eel Electrophorus electricus by one-step affinity chromatography as described by Massoulié et al. (7). The characteristics of this preparation are detailed elsewhere (4, 8). The tetrameric form of the enzyme (G4 form) was prepared from the purified preparation by incubation with trypsin (1 μg/ml) for 18 hours at 25°C in 0.1 M phosphate buffer pH 7.0 (trypsin/AChE (W/W), 1/2000). This mixture, which contained mainly G4 forms, was used without further purification for the preparation of conjugates. Details of the molecular forms of AChE are reviewed in Massoulié et al. (9). AChE activity was measured using the method of Ellman et al. (10) as described in Pradelles et al. (4). Ellman's reagent consists of 20 mg acetylthiocholine iodide and 21.5 mg 5,5'-dithio-bis(2-nitrobenzoic acid)

(DTNB), in 100 ml of 10^{-2} M phosphate buffer (pH 7.4). One Ellman Unit is defined as the amount of enzyme inducing an absorbance increase of 1 during 1 min in 1 ml of Ellman's medium, with an optical pathlength of 1 cm at 412 nm. It corresponds to about 8 ng of enzyme.

Preparation of Rat Prolactin-AChE Conjugates

Rat PRL (NIADDK I-5) was coupled to AChE with the heterobifunctional reagent N-succinimidyl-4 (N-maleimidomethyl) cyclohexane 1-carboxylate (SMCC) as already described for the conjugation of substance P, atriopeptin or interleukines (11, 12 and 13). This method involves the reaction of a thiol group (previously introduced into rat PRL) with a maleimido group incorporated into the enzyme after reaction with SMCC.

Thiol groups were first introduced into rat PRL by reaction of primary amino groups of the protein with S-succinimidyl-S-acetyl thioacetate (SATA). Briefly, 17 μl of a 17 mg/ml SATA solution in anhydrous dimethylformamide was added to 250 μg of rat PRL dissolved in 250 μl of 0.1 M borate buffer (pH 8.5). The mixture was allowed to react for 30 min at 25°C, before addition of 125 μl of 1 M hydroxylamine (pH 7.0), in order to hydrolyze the thioester group of SATA. Thiolated rat PRL was isolated from excess thiol reagent by chromatography on a Sephadex G 25 column (1 x 25 cm). Elution was performed with 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. Before and during chromatography the eluent was kept under a continuous stream of nitrogen in order to eliminate dissolved oxygen and avoid any oxidation of thiol groups. Fractions containing rat PRL were pooled. The concentration of rat PRL was evaluated by UV spectrophotometry at 280 nm (assuming a value of 10 for 1 %) and its thiol content was determined colorimetrically (at 412 nm) after reaction with 0.5 mM 5-5'-(dithiobis)nitrobenzoic acid (DTNB) as described by Ellman (10). These measurements revealed that 0.8 thiol groups were incorporated per molecule of rat PRL.

Before the incorporation of maleimido groups into AChE, the enzyme was first treated with N-ethyl-maleimide in order to block any thiol groups. This was achieved by adding 20 μl of a 125 mg/ml solution of N-ethyl-maleimide dissolved in 0.1 M phosphate buffer (pH 7.0) to 100 μl of G4-AChE preparation (1.84 mg/ml). After 30 min at room temperature, the N-hydroxy succinimide moiety of SMCC was reacted with the primary amino groups of the enzyme by addition

of 12.5 μl of SMCC (11.2 mg/ml in anhydrous dimethylformamide). The mixture was then left for a further 30 min at 30°C. The various molecular forms of AChE were then isolated by chromatography on a Biogel A 1.5M column (30 x 1 cm) equilibrated in 0.1 M phosphate buffer pH 6.0. Fractions corresponding to the G4-form of the enzyme were pooled and AChE concentrations were determined enzymatically.

Conjugation of thiolated rat PRL with maleimido-AChE was carried out by mixing activated enzyme (G4-form) immediately after its isolation on the Biogel A 1.5M column with an excess of rat PRL-SH (rat PRL-SH/AChE molecular ratio 3/1). After incubation for 3 hours at 30°C, unreacted rat PRL was removed by molecular sieve chromatography on a Biogel A 15M column (1.6 x 90 cm) as described previously (4). The conjugate was stored frozen at -20°C. No loss of enzymatic activity was observed during the coupling procedure. No significant modification of the immunological binding properties of the conjugate was noted under these storage conditions over a one-year period. The rat PRL content of the AChE conjugate was determined using a RIA for rat prolactin (see below). A value of 0.16 mol of prolactin per mol of G4-form of AChE was noted, indicating that most AChE was not coupled to rat PRL during the reaction. This point will be discussed below.

Enzyme ImmunoAssay

EIA was performed in the following assay buffer : phosphate buffer (0.1 M, pH 7.4) containing 0.4 M NaCl, 1 mM EDTA, 0.1 % bovine serum albumin, and 0.01 % sodium azide. EIA for rat PRL was performed using the procedure already described for eicosanoids (4). The total incubation volume was 150 μl, with each component (standard or sample, primary antibody and conjugate) being added in a 50 μl volume. A standard curve was generated using purified rat PRL (NIADDK R.P.3). The primary antibody was used at a final dilution of 1/30 000 and rat PRL-AChE conjugate was introduced at a dilution corresponding to 1 Ellman unit per ml (see purification and assay of AChE). Immunoreaction was performed at 4°C or at 22°C. After incubation (ranging from 1 night to 5 days depending on the sensitivity needed for the assay), the plates were washed, before addition of 200 μl of Ellman's reagent to each well. The enzymatic reaction was allowed to proceed with gentle agitation at room temperature (generally for 1-2 hours) and the absorbance of each well was then measured with a spectrophotometer at 412 nm.

RadioImmunoAssay

RIA measurements were performed according to a previously described procedure, with slight modifications (14). Radiolabeled rat prolactin was prepared by iodination using the chloramine T method. Five μg of rat prolactin (NIADDK I-5), in 40 μl of phosphate buffer 0.05 M pH 7.5, and 400 μCi (14.8 MBq) of Na ^{125}I (Compagnie ORIS Industrie, CEA, France) were mixed. This mixture was allowed to react with 15 μl of chloramine T (3 mg/ml) for 20 seconds. Oxidation was stopped by addition of 50 μl of sodium metabisulfite. Non-covalent binding of ^{125}I to proteins was prevented by addition of 10 μl of 1 M NaI. Free iodine and radiolabelled protein were separated by molecular sieve chromatography (Sephadex G 25, column 25 x 1 cm). RIA was performed in (13 x 75 mm) polystyrene test tubes in a total volume of 300 μl. Each component of the immunoassay was added in a volume of 100 μl. For the iodinated tracer, this corresponded to about 16 000 dpm. The final dilution of specific antiserum was 1/30 000. The reaction was allowed to proceed overnight in a controlled temperature room (22 $\pm$ 2°C). The buffer used was the same as in the EIA experiments. Separation of antigen-antibody complexes was achieved by second-antibody immunoprecipitation using a swine anti-rabbit IgG antiserum as described elsewhere (15). Under these conditions, non-specific binding was lower than 5 %.

Cross-Reactivity Studies

The specificity of the enzyme immunoassay was checked by comparing standard curves obtained with rat PRL and various rat pituitary hormones (LH, TSH, GH). Results were expressed in terms of per cent cross-reactivity using the Abraham criterion (16). IC50 is the mass of the hormone that induces a 50 % decrease of the tracer binding to antibodies. Cross-reactivity is defined as the ratio of the IC50 of prolactin on the IC50 of the tested hormone.

Imprecision Profiles

Imprecision profiles for EIA were established on the basis of standard curves for which eight replicates of each standard concentration were used. Results are expressed in terms of the coefficient of variation (CV %) versus the logarithm of the dose (17).

Characterization of PRL Immunoreactive Material

Three ml of rat plasma (pool of samples from 3 rats) were analysed by molecular sieve chromatography using a Biogel A 0.5M column (100 x 3 cm). Elution was performed with 0.05 M phosphate buffer pH 7.0. Fractions of 1 ml were collected and assayed for PRL immunoreactivity as described above. Absorbance at 278 nm was recorded for each fraction.

Data Analysis

Results were expressed in terms of B/Bo x 100 where B and Bo represent the radioactivity or the absorbance measured on the bound fraction in the presence or absence of hormone, respectively. Fitting of the standard curves and calculations of the quantity of hormone in biological samples were performed using a microcomputer (IBM/PC) and software developed in the laboratory, using a linear log-logit transformation. Unless otherwise stated, all measurements were made in duplicate.

The detection limit was defined as the PRL concentration inducing significant reduction (three standard deviations) in the Bo value.

Animal study

Male rats are injected intraperitonealy by haloperidol (1 mg/kg) in 1 ml of 0.9 % NaCl or by bromocriptine mesylate (4 mg/kg) in 1 ml ethanol. Rats are killed by decapitation 0.5 or 1 hour after injection. Heparinized blood samples are obtained and centrifuged at 1 600 *g* for 20 min. Plasma are collected and kept at -20°C until assay.

RESULTS

The effectiveness of the coupling between rat PRL and AChE, as well as the preserving of rat PRL immunoreactivity in the conjugate, were checked in two ways. First, radioimmunological measurements were made on the enzymatic conjugate. In these experiments, PRL-AChE conjugate provided dilution curves parallel to the standard curves obtained with rat prolactin. These experiments (see methods) demonstrated the presence of immunoreactive rat PRL-like material in the enzymatic tracer preparation. They allowed us to determine a rat PRL/AChE (G4) ratio of 0.16. These data were confirmed by performing anti-

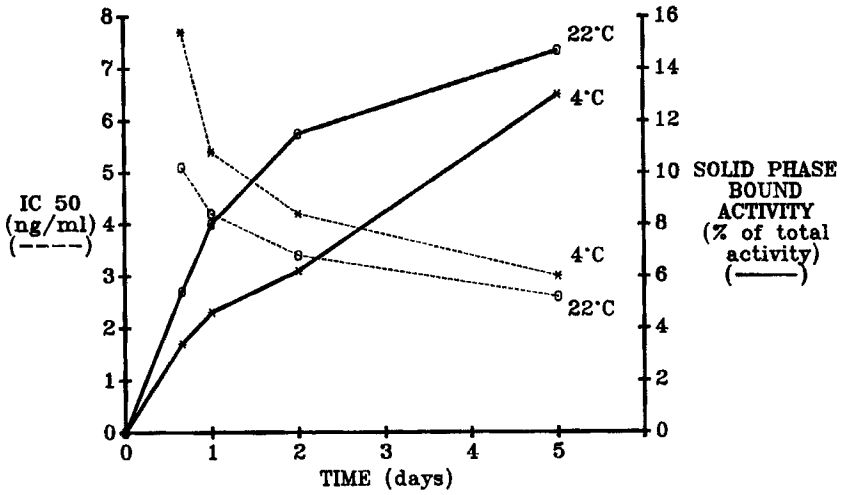

FIGURE 1.: Effect of temperature and time of incubation on binding parameters of PRL-AChE.
Binding kinetics were measured at an antiserum dilution of 1/1000 (full lines) at 4°C (*____*) or 22°C (O____O).
IC 50 were measured at an antiserum dilution of 1/30000. IC 50 values indicate the PRL concentration at which the binding of PRL-AChE conjugate was inhibited by 50 %. IC 50 (dotted lines) were measured at 4°C (*---*) or 22°C (O---O).

body dilution curves. Different curves obtained using various reaction periods (from 1 to 5 days) are presented in figure 1. Under equilibrium conditions (5-day reaction), the maximum bound activity observed at a 1/1 000 dilution of first antibody did not exceed 15 % (0.15) of the total enzyme activity introduced in the assay. It is worth noting that this value very closely corresponds to that expected from radioimmunological measurements performed on the conjugates (see above). This is clearly linked to the fact that most of the AChE (near 85 %) was not coupled to rat PRL during conjugate preparation. As discussed in detail elsewhere (4, 18), the presence of excess free enzyme is not problematic because of very low non-specific binding (0.01 % of total activity). This guarantees that not more than one PRL molecule is coupled to each enzyme molecule, thus ensuring maximum specific activity for the conjugate. The curves in figure 1 also show that binding on the solid phase is rather slow since at least five days were

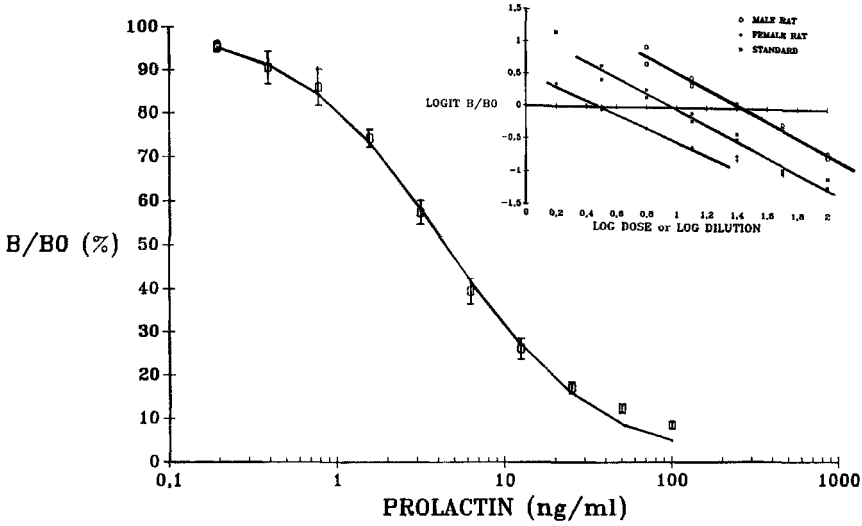

FIGURE 2. : Calibration curve of the enzyme immunoassay of rat prolactin. Each point is the mean ± standard deviation of 6 independent experiments. A log-logit transform was used for curve-fitting.
Insert. Comparison of male (o) and female (+) rat plasma dilution curves and the rat prolactin standard curve. The logarithm of the prolactin concentration (ng/ml) or the logarithm of sample volume (µl) are plotted against logit B/Bo.
Slopes were determined after linear regression (slope ± S.E.).
Male rat : - 1,2335 ± 0,0597 (df = 8)
Female rat : - 1,1840 ± 0,0207 (df = 8)
Standard : - 1,2872 ± 0,0876 (df = 12)

necessary before equilibrium was reached. This was observed in all our previous EIA studies (18), as well as with anti-AChE antibodies (15). It is likely that these slow kinetics are correlated with the considerable size of AChE, since equilibrium was reached in about 24 hours when ^{125}I iodinated rat PRL was used as tracer (data not shown).

In order to determine the optimum assay conditions, the binding kinetics were studied. From the curves in figure 1 it is clear that prolonged incubation results in a marked gain in sensitivity. A good compromise between sensitivity and the length of the experiment was reached by using a 24-hour reaction period at 22°C. Figure 2 shows the standard curve obtained under these conditions. A mean IC 50 value of 4.4 ng/ml of prolactin (*i.e.* 220 pg/well or 220 pM) was

observed. The detection limit was 0.5 ng/ml (*i.e.* 25 pg/well or 25 pM). These values are equivalent to those found by other workers for the RIA of rat PRL (14) and squirrel PRL (19), or EIA of human PRL (20) using competitive methods.

The cross-reactivities of three pituitary hormones (rat LH, rat TSH and rat GH) are shown in table I. No cross-reactivity coefficient exceeded 1 %. These values are in good agreement with those previously found for RIA using the same primary antibody (Grouselle D., unpublished results). They show that the use of an AChE-PRL conjugate does not modify the specificity of the assay.

Whatever the specificity of the antibodies used, the validity of an immunoassay as well as its applicability to biological fluids must be verified carefully. This was first done by comparing dilution curves and performing recovery experiments. Figure 2 (inset) shows the classical log-logit transformation of the standard curve as well as a series of sample dilution curves for male and female rats. The corresponding slopes were respectively -1.2872 ± 0.0876, -1.2335 ± 0.0597 and -1.1840 ± 0.0207 (slope $\pm$ S.E.). A statistical analysis based on Student's discriminant function indicated that there were no statistical differences between the standard curve and the plasma dilution curves at a threshold of 5 %.

Table II presents the results of the recovery experiments performed on rat plasmas spiked with different concentrations of standard PRL. Good recovery was observed, and taken with the results of the dilution curve comparisons, these results strongly indicate that the assay is not significantly influenced by non-specific components present in the plasma samples. This conclusion was supported by molecular sieve fractionation of plasma samples. Figure 3 shows the immunoreactivity profile observed for a plasma sample chromatographed on a Biogel A 0.5M column. Only one peak of immunoreactive material was observed in an exclusion volume identical to that measured for standard PRL under the same conditions. This confirms the specificity of the assay and the lack of non-specific interference due to the plasma.

The imprecision profile obtained in the range 2 500 pg/well (50 ng/ml) to 40 pg/well (0.80 ng/ml) is presented in figure 4. An intra-assay coefficient of variation of less than 10 % was observed above 145 pg/well (2.9 ng/ml). The coefficient of variation exceeded 20 % for prolactin concentrations of less than 55 pg/well (1.1 ng/ml).

TABLE I : Cross-reactivity of pituitary hormones as measured by enzyme immunoassay.
IC 50 values indicate the hormone concentration which causes 50 % inhibition of the binding of PRL-AChE conjugate.

Hormone	IC 50	Cross-reactivity index
rat PRL	4.2 ng/ml	100 %
rat GH	> 200 ng/ml	< 0.5 %
rat TSH	> 400 ng/ml	< 1 %
rat LH	> 400 ng/ml	< 1 %

TABLE II : Assay of rat plasma spiked with standard prolactin. Recovery index (expressed in %) is taken as the ratio of measured to added quantities.

Prolactin added ng/ml	Prolactin measured ng/ml (n=4)	Recovery ng/ml	Recovery %
0 (basal level)	26 ± 4	-	-
10	37 ± 4	11	110
20	51 ± 5	25	125
40	75 ± 8	49	123
60	95 ± 9	69	115

Linear regression : $Y_{found} = 1.17Y_{added} + 0.47$

$r^2 = 0.9986$

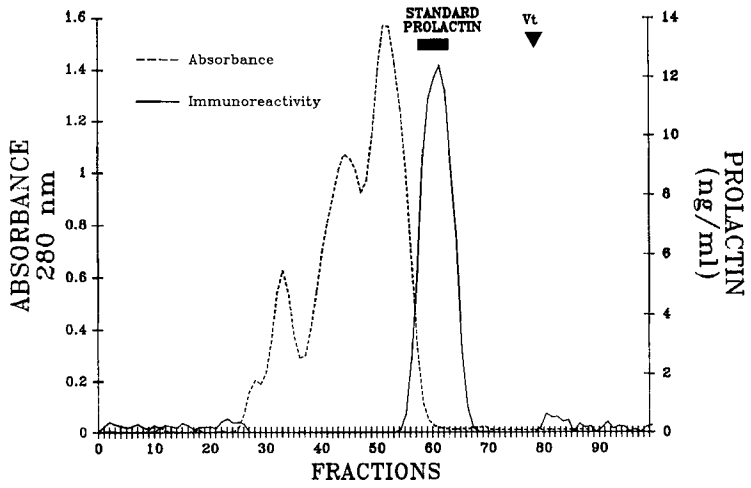

FIGURE 3. : Elution profile of prolactin immunoreactive material from a plasma sample in molecular sieve chromatography (Biogel A 0.5 M, 100 x 3 cm). Comparison with standard prolactin (NIADDK I-5). Vt (total volume of the column) was taken as the retention volume of ferrycyanide sodium (1 ml fraction).

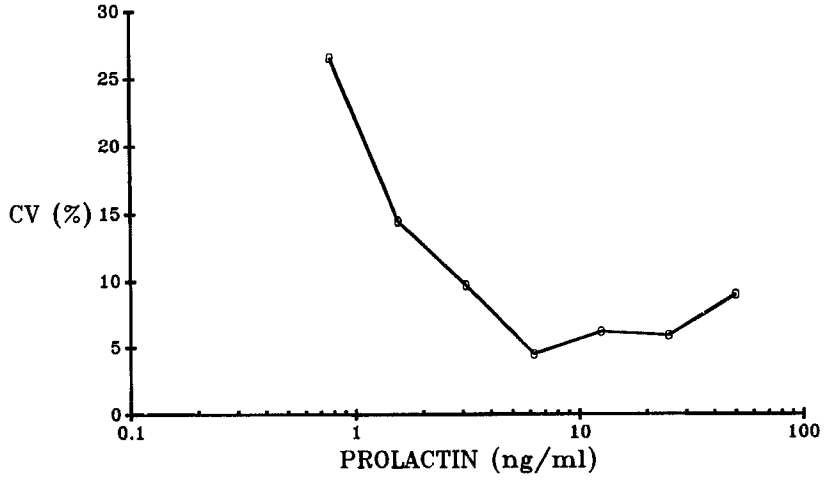

FIGURE 4. : Imprecision profile of rat prolactin enzymoassay (intra-assay variation, n = 8).

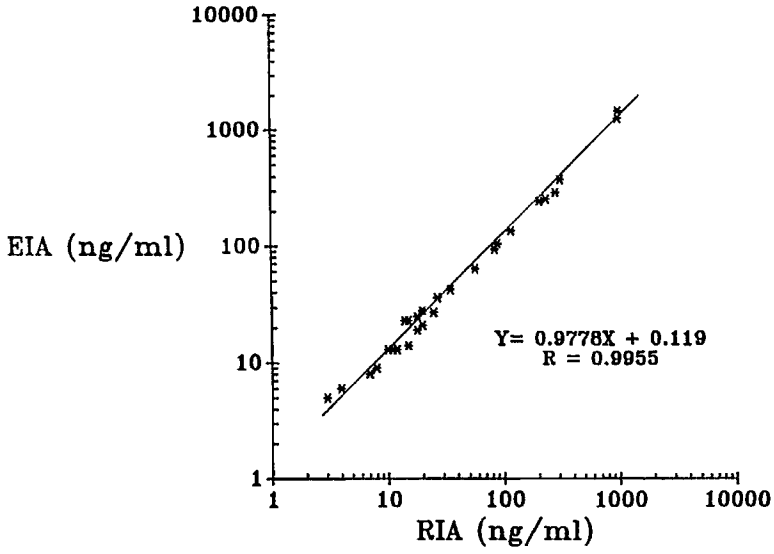

FIGURE 5. : Correlation between enzyme immunoassay (EIA) and radioimmunoassay (RIA) of rat prolactin in 28 rat (male and female) plasma samples.

Eighteen rat (male and female) plasma samples ranging from 8 to 977 ng/ml were assayed in 7 daily experiments. Plasma was diluted to give a decrease in tracer binding close to B/Bo = 50 %. The mean day-to-day variation was close to 14 %. Prolactin values in male rat range from 8 to 33 ng/ml (n=8). Mean variation is equal to 14 %. Prolactin values in female rat range from 43 to 977 ng/ml (n=10). Mean variation is equal to 13 %.

Good correspondence was observed between prolactin values obtained using EIA and those obtained using RIA. This is shown in figure 5, which gives the results for a series of 29 rat plasma samples. High correlation was observed ($r = 0.9955$, $y = 0.9778\ x + 0.119$).

Figure 6 shows the changes in plasma prolactin concentrations in rats after intraperitoneal injection of two drugs. Important increase in prolactin levels is observed after haloperidol injection as expected for a butyrophenone drug (5). On the contrary, bromocriptine induced a rapid decrease in the prolactin levels. One hour after administration, plasma levels are under the limit of detection of the assay.

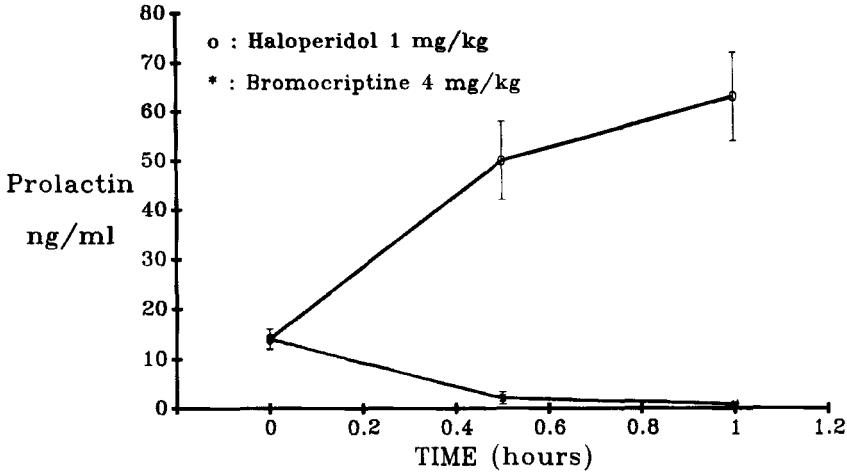

FIGURE 6. : Effect of acute intraperitoneal administration of drugs on prolactin plasma levels in rats (mean +/- S.E.M.; haloperidol :n = 10 for each time ; bromocriptine n=4 for each time).

DISCUSSION

In previous papers (4, 11, 12, 18), we have shown that acetylcholinesterase from Electrophorus electricus can be efficiently used to prepare enzyme-hapten conjugates suitable for competitive enzyme immunoassay. Because of its very high turnover number, AChE can be detected in the attomole range (2), thus allowing enzyme immunoassays of greater sensitivity than the corresponding radioimmunoassay using ^{125}I iodinated tracer. In addition, the combined use of microtiter plates and second-antibody solid phase allows relatively complete assay automation, rendering the method highly suitable for routine analysis.

In this paper, we have shown that AChE can also be used to prepare enzyme-protein antigen conjugates, as has been already shown with acidic fibroblast Growth Factor (21) or interleukins (13). In the case of rat prolactin we have developed an enzyme immunoassay for determination of the hormone in plasma. The sensitivity of the assay is comparable to that of the corresponding RIA and the conventional criterias for precision and specificity are satisfactory.

In practical terms, the assay has all the advantageous characteristic (ease of use, high degree of automation) previously described for the assay of haptens (4). In addition, prolactin-AChE conjugates are very stable since they can be kept frozen (-20°C) for at least one year without loosing their enzymatic or immunological properties.

When compared with other non-isotopic competitive immunoassays for rat PRL (22), our method is more sensitive and benefits from shorter incubation times. It is worth noting, however, that the sensitivity of this assay is inferior to that observed with enzyme immunometric assays using enzyme antibody conjugate . It is very likely that this difference is not due to the use of AChE but rather the increased sensitivity allowed by immunometric methods which partially circumvent insufficient antibody affinities by using highly concentrated reagents (23). In a recent paper (13), it has been shown that AChE could also be used to label antibodies providing very sensitive assays. In the case of rat prolactin, the development of an immunometric assay was not immediately possible because of the limited availability of a polyclonal antiserum. The production of large amounts of specific antibodies (either polyclonal or monoclonal) would have been work-intensive and time-consuming. This is why we chose to develop a competitive assay involving labelled antigen. As demonstrated above, this strategy was justified, since the advantageous properties of AChE, allowed development of an assay of sufficient sensitivity for expeditious achievement of our aims.

The assay is suitable for monitoring prolactin secretion in rat plasma, under both physiological and pharmacological conditions.

ACKNOWLEDGEMENTS

This work was supported by the Commissariat à l'Energie Atomique (CEA), the Centre National de la Recherche Scientifique (CNRS) and the Institut National de la Statistique et de la Recherche Médicale (INSERM).

Authors are indebted to Pr TIXIER-VIDAL for generous gift of rat PRL and specific antisera.

We warmly thank Dr M. ISTIN and Dr P. PRADELLES for their interest and constructive criticisms.

Abbreviations used :

AChE : Acetylcholinesterase (E.C.3.1.1.7.)
PRL : Prolactin
LH : Luteinizing hormone
TSH : Thyrotropin
GH : Growth hormone
EIA : Enzyme Immunoassay
RIA : Radio Immunoassay
NIADDK : National Institute of Arthritis, Diabetes, Digestive Diseases and Kidney
BSA : Bovine Serum Albumin
SMCC : N-Succinimidyl-4 (N-maleimido methyl cyclohexane-1 carboxylate)
SATA : S-Succinimidyl-S-acetyl thioacetate
DTNB : 5,5'-dithio-bis(2-nitrobenzoic acid)

REFERENCES

1 Cocchi D. Radioimmunoassay of pituitary and hypothalamic Hormones In Handbook of Experimental Pharmacology (Patrono C. and Peskar B.A., eds) Springer Verlag, Berlin, Vol. 82, 1987 : 255-280.

2 Grassi J., Maclouf J. and Pradelles P., Radiodination and Other Labelling Techniques. In Handbook of Experimental Pharmacology (Patrono C. and Peskar B.A., eds) Springer Verlag, Berlin, Vol. 82, 1987 : 91-142.

3 Schall R.F. and Tenoso M.J. Alternatives to RadioImmunoAssays : Labels and Methods. Clin. Chem. 1981 ; 27 : 1157-1164.

4 Pradelles P., Grassi J. and Maclouf J. Enzyme Immunoassay of Eicosanoids Using Acetylcholinesterase as Tracer : an Alternative to Radioimmunoassay. J. Anal. Chem. 1985 ; 57 : 1170-1173.

5 Jaffe R. B. Physiologic and pathologic aspects of prolactin production in humans. In Prolactin, (Jaffe R.B. ed), Elsevier, N.Y., 1981 : 181-217.

6 Grouselle D., Faivre-Bauman A. and Tixier-Vidal A.. A radioimmunoassay for thyroliberin (TRH). Comparison with a TRH radioreceptor assay. Neurosciences Letters 1978 ; 7 : 7-15

7 Massoulié J. and Bon S. Affinity Chromatography of Acetylcholinesterase. The Importance of Hydrophobic Interactions. Eur. J. Biochem. 1976 ; 68 : 531-539.

8 Grassi J., Frobert Y., Lamourette P. and Lagoutte B. Screening of Monoclonal Antibodies Using Antigens Labeled with Acetylcholinesterase : Application to the Peripheral Proteins of Photosystem I. Anal. Biochem. 1988 ; 168 : 436-450.

9 Massoulié J. and Bon S. The Molecular Forms of the Cholinesterase and Acetylcholinesterase in Vertebrates. Annual Rev. Neurosc. 1982 ; 5 : 57-106.

10 Ellman G. L., Courtney K. D., Andres V., Featherstone R. M.. A new and Rapid Colorimetric Determination of Acetylcholinesterase Activity. Biochem. Pharmacol. 1961 ; 7 : 88-95.

11 Renzi D., Couraud J. Y., Frobert Y., Nevers M. C., Geppetti P., Pradelles P. and Grassi J. Enzyme Immunoassay for Substance P using Acetylcholinesterase as label. In Trends in Cluster Headache, (F. Sicuteri et al. eds.), Elsevier Science Publishers. 1987 ; 125-134.

12 Mc Laughlin L. L., Wei Y., Stockmann P. T., Leahy K. M., Needleman P., Grassi J. and Pradelles P. Development, Validation and Application of an Enzyme Immunoassay (EIA) of Atriopeptin. Biochem. Biophys. Res. Commun. 1987 ; 144 : 469-476.

13 Grassi J., Frobert Y., Pradelles P., Chercuitte F., Gruaz D., Dayer J. M. and Poubelle P. E. Production of Monoclonal Antibodies against Interleukins 1α and 1β : development of two Enzyme Immunometric Assays (EIA) using Acetylcholinesterase and Application to Biological Media. J. Immunol. Methods 1989 ; 123 : 193-210.

14 Neill J. D. and Reichert L. E.. Development of a Radioimmunoassay for Rat Prolactin and Evaluation of the NIAMD Rat Prolactin Radioimmunoassay. Endocrinology. 1971 ; 88 : 548-555.

15 Marsh D., Grassi J., Vigny M. and Massoulié J.. An Immunological Study of Rat Acetylcholinesterase : Comparison with Acetylcholinesterases from Other Vertabrates. J. Neurochem. 1984 ; 43 : 204-213.

16 Abraham E.G. Radioimmunoassay of Steroids in Biological Materials. Acta Endocrinol. 1974 ; (Suppl) 75 : 7-42.

17 Ekins R.P. and Newman G.B. (1970). Theoretical aspects of saturation analysis. Acta Endocrinol., 64, 11-30.

18 Pradelles P., Grassi J., Charbades D. and Guiso N. Enzyme Immunoassays of Adenosine Cyclic 3',5'-Monophosphate and Guanosine Cyclic 3',5'-Monophosphate Using Acetylcholinesterase. Anal. Chem. 1989 ; 61 : 447-453.

19 Thordarson G., Holekamp K. E. and Talamantes F. Development of an Homologous Radioimmunoassay for Secreted Prolactin from the California Ground Squirrel (Spermophilus beecjey). Biol. Reprod. 1987 ; 36 : 1186-1190.

20 Ishikawa K., Narita O., Noguchi M. and Kato K. Practical Enzyme Immunoassay for Prolactin in Human Serum. Clin. Chim. Acta. 1982 ; 121 : 181-187.

21 Caruelle D., Grassi J., Courty J., Groux-Muscatelli B., Pradelles P., Barritault D. and Caruelle J.P. Development and Testing of Radio and Enzyme Immunoassays for Acidic Fibroblast Growth Factor (aFGF). Anal. Biochem. 1988 ; 173 : 328-339.

22 Signorella A. P. and Hymer W. C.. An Enzyme-Linked ImmunoSorbent Assay for Rat Prolactin. Anal. Biochem.. 1984 ; 136 : 372-381.

23 Ekins R. and Jackson T. Non-Isotopic Immunoassay : An Overview. In Monoclonal Antibodies and New Trends in Immunoassays, (Bizollon C., ed.), Elsevier Science Pub., Amsterdam, 1984 : 149-163.