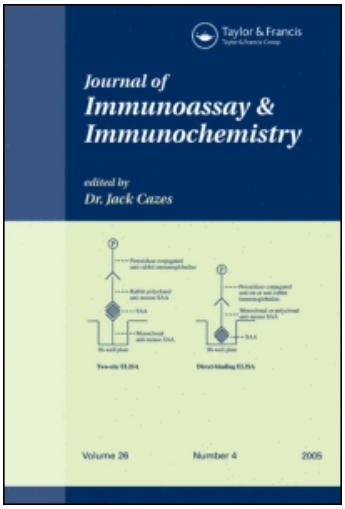

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A NONCOMPETITIVE ENZYME IMMUNOASSAY FOR RAT PROLACTIN

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ABSTRACT

A sensitive and specific noncompetitive rat prolactin (rPRL) enzyme immunoassay (EIA) is described. In this assay, the same rabbit anti-rPRL antibody is both adsorbed to a solid-phase support, i.e. 96-well microtiter plates and conjugated covalently to peroxidase as a tracer. PRL being sandwiched between antibody molecules, the enzymatic activity is thus proportional to the amount of rPRL concentration. This assay was found highly specific for rat PRL and displayed a sensitivity of 12.5 pg/well (0.125 ng/ml) of NIH-RP2 equivalents. The intra-assay and inter-assay coefficients of variation were less than 10% over a wide range of rPRL concentration (0.25-40 ng/ml). This rPRL-EIA permits to quantify PRL in culture media or biological samples containing up to 25% of plasma. Comparison with a radioimmunoassay revealed a good correlation (r=0.984, the slope=1.04). This EIA is rapid, results being obtained within 4h30 or 18h30 depending on the nature of the biological samples. The tracer, easily performed with a low cost enzyme, can be stored for very long durations. Thus, this sensitive and rapid assay provides a valuable method for measuring rPRL. (KEY WORDS: enzyme immunoassay, rat prolactin, rat pituitary, plasma).

INTRODUCTION

Enzyme immunoassays (EIAs) offer a number of advantages compared to the radioimmunoassays (RIAs). Namely, they eliminate handling of radioactive isotopes and permit the storage of labeled-reagents for prolongated periods. Thus, EIAs are more and more frequently used to measure hormone content in biological fluids. In this respect, three EIAs have been already described for quantification of rPRL. All of them were based on competition (1-3). The more recent methods were found suitable only for measurements in plasma samples. In these assays, the labeling enzyme (either acetylcholine or alkaline phosphatase) is conjugated to rPRL. Here, we describe a noncompetitive assay where the labeling enzyme, i.e, horseradish peroxidase was linked to a specific anti-rPRL polyclonal antibody.

MATERIALS AND METHODS

Chemicals

Rat prolactin standard (rPRL) previously calibrated against rPRL-NIH (NIH-RP2), and rabbit anti-rPRL antibodies were purchased from INRA (Nouzilly, France). Rat pituitary hormones were obtained from UCB product (Brussels, Belgium). Microtiter plates (96F immunoplatesI, with certificate) were from NUNC. Horseradish peroxidase was supplied by Boehringer Mannheim (Mannheim, Germany). Bovine serum albumine BSA and o-phenylenediamine were obtained from Sigma (St Louis, MO). All others chemicals and reagents were from Merck (Nogent/Marne, France) or Prolabo (Paris, France).

Stock solutions for EIA

Coating buffer was phosphate buffer saline (PBS), 0.1M, PH 7.2. Blocking buffer (PBS-BSA) was 0.5 % BSA in PBS . The washing buffer (PBS-Tween) was PBS containing 1 % (V/V) Tween 20, 0.9% NaCl . Assay buffer (PBS-assay) was 6 % BSA in PBS. Peroxidase substrate was 3mg/ml 0-phenylenediamine, 0.078% hydrogen peroxide in phosphate-citrate buffer 0.16 M, pH 5.8. The stop solution was 2 N sulfuric acid.

Antibodies purification and antibody-peroxidase conjugates preparation

Polyclonal antibodies were purified according to Steinbuch and Audran (4) and were conjugated with an enzyme peroxidase in compliance with the methodology described by Nakane and Kawaoi (5).

Enzyme immunoassay procedure

Rabbit anti-rPRL antibody was diluted (1:1500) in PBS to a final concentration of 3.5 µg/ml and used for coating the plates. 100 µl were added to each well of 96-well microtiter plates and incubated overnight at room temperature (20°C) for immediate use. Plates may also be stored for 72 h at least at 4°C, the longest duration tested. The solution was then discarded and the plates were washed three times with PBS-Tween. After washings, 150 µl per well of PBS-BSA were incubated for 1h, to block any remaining free binding sites. Thereafter, the wells were washed twice with PBS-Tween and 100 µl of sample containing unknown rPRL or standard rPRL were added to each well and incubated overnight at 4°C when plasma samples were assayed. This incubation was performed for two hours only at room temperature for assaying serum-free culture media. Then, after three washings with PBS-tween, 100

µl of anti-rPRL antibody-peroxidase conjugate at a final dilution of 1:1000 in PBS, were added for 2 h at 20°C. Wells were then washed three times and 100 µl of substrat solution were added. After 30 min of incubation at room temperature in the dark, the enzymatic reaction was arrested with 100 µl of stop solution added into each well. The optical density at 490 nm, was measured using an automated microplate reader (EL 340, Bio-Tek Instruments).

Radioimmunoassay procedure

RIA using [^{125}I]-radiolabeled rPRL was performed using the AMERSHAM determination kit (AMERSHAM, Les Ulis, France) according to the manufacturer's instructions excepted that all dilutions were performed in assay buffer and not in the buffer provided with the kit.

Rat pituitary cell culture

Anterior pituitary cell dispersion was obtained according the methodology described by Hopkins and Farquhar (6). Briefly, 10 female Wistar rats (200-250g, Iffa credo, France) were decapitated and anterior pituitaries were removed under sterile conditions. Small tissue blocks were treated sequentially as follow : 15 min with 0.5 % trypsin (Sigma), 1 min with 2µg/ml DNase (Sigma) , 5 min with 1mg/ml soybean trypsin-inhibitor (Sigma), 5 min with 2mM EDTA, then 15 min with 1mM EDTA in Dulbecco's minimal essential medium (DMEM, Boehringer). Finally, cells were mechanically dispersed. 2.10^5 cells/well were seeded into 24-well plates in DMEM supplemented with 10% (V/V) foetal calf serum (FCS, Boehringer), 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma), 1 mM glutamine (Gibco) for 3 days under a 95% air/ 5% CO_2 atmosphere. At the end of this period, the monolayers were

rinsed with DMEM and preincubated in DMEM alone for 2 h. Incubation in DMEM, Hepes 15mM, pH 7.4, acid ascorbic (10^{-4}M), was then performed with or without dopamine (1μM) in a humidified 37°C atmosphere of 5% CO_2 and 95% air. After incubation for the duration indicated, mediums were collected and frozen at -20°C until rPRL-EIA was performed.

Plasma samples

Adult, pregnant or suckling female and male Wistar rats bred in the laboratory were kept in a temperature controlled room (22 ± 1°C) with free access to food and water. Blood samples were collected from the abdominal aorta of animals under ether anesthesia into heparinized tubes and stored at -20°C until assayed.

Data analysis

Fitting of the standard curves and quantification of rPRL in biological samples were performed with the software Kineticalc 2.03 (Bio-Tek Instruments) using a 4 parameter transformation. All measurements were made in duplicate and prolactin results were expressed in nanograms equivalent of rat PRL NIH-RP2 per ml.

RESULTS

Calibration

The assay was calibrated using serial dilutions of rPRL NIH-RP2 equivalents from 40 ng/ml to 0.125 ng/ml of PBS-BSA and optical densities were measured at 490 nm. As expected, the absorbance increased in a dose-dependent manner with the concentration of rPRL. Maximal absorbance slightly varied from one microplate to

another ranging between 1.4 and 1.6 optical density units. The results from six independent assays were pooled, expressed in % of maximal absorbance and plotted versus the logarithm of increasing amounts of rPRL. The resulting standard curve is illustrated in Fig.1. The limit of quantification of the assay was 0.125 ng/ml of rPRL (i.e. 12.5pg/well) statistically different from the background ($P<0,01$, n=6; mean±S.D.) with an EC_{50} of 1.5 ng/ml.

Specificity

The reactivity of different rat pituitary hormones, previously shown unable to recognize anti-rat PRL antibody in a radioimmunoassay (7), was investigated. Different concentrations were tested : 1000 ng/ml for GH, 5 to 5000 ng/ml for LH and TSH. Results were compared to a standard curve obtained using increasing doses of rPRL standard. As illustrated Fig.2, except PRL, none of the hormone tested was able to absorbe above control, even using very high concentrations. Similary no cross reaction was observed when 10 % (V/V) foetal calf serum were added in control samples (PBS-BSA), in contrast to what observed by RIA (Table.1).

Precision

The inter and intra assay variability (fig.3 and fig.4, respectively), were evaluated in order to assess the reproductibility of this EIA. Eight serial dilutions of rPRL standards (from 0.125 to 40 ng/ml) were assayed in duplicate on 6 consecutive days for the interassay variability and were measured in sextuplates for the intra assay variability. As illustrated, the mean day to day variation (fig.3) and the intra-assay coefficient of variation (fig.4) were below 10 % over a wide range of rPRL concentration (0.250-40 ng/ml).

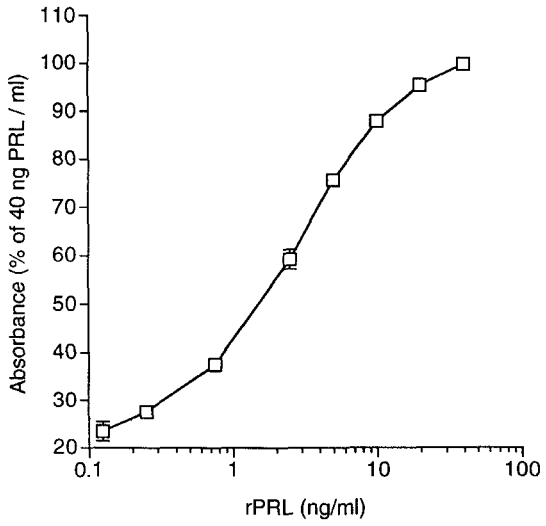

FIGURE 1. : Semi log plot of absorbance as a fonction of PRL concentration. Serial dilutions of PRL standards were submitted to the protocole described in Materials and Methods and absorbance measured at 490nm. Values are expressed as % of the absorbance displayed by a sample containing 40 ng/ml of rPRL. Each value is the mean ± SEM of six independent determinations, each performed in duplicate.

Correlation

The results obtained by this EIA were compared to those obtained using a rPRL RIA. To this aim, aliquotes of same rat plasma samples, known to present very different plasma PRL concentrations (male, female and pregnant female) were submitted to the two assays, in parallel. The results were plotted and yield a linear relationship (fig.5). The correlation observed was good, as illustrated by the parameters of the relation : r=0.9842, y=1.0446 ± 1.9121. The slope was not significantly different from 1.00, indicating that no proportional systematic error occured between the 2 methods.

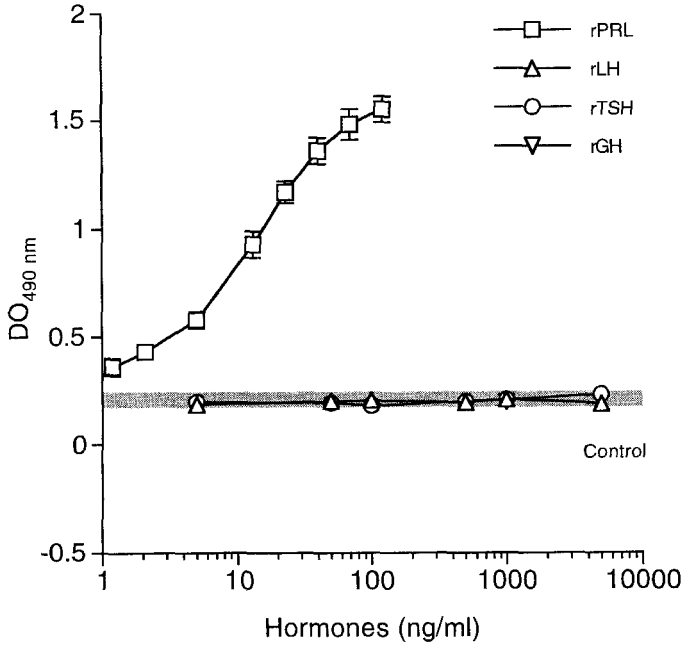

FIGURE 2. : Specificity of the assay for rat PRL. Different concentrations of rLH, rTSH (5 to 5000 ng/ml) and rGH (1000 ng/ml) were assayed in parallel to a calibration curve with rPRL standard (0.125 to 40 ng/ml). Each point is the mean of triplicate determinations ± SEM. Error-bars when non visible are included in the symbols.

Assay of rPRL in plasma samples

Serial dilutions (from 1:4 to 1:16) of 3 different rat plasma samples in assay buffer were submitted to the EIA. As illustrated in fig.6, the rPRL levels increased linearly with the concentration of plasma. However, experiments not illustrated here indicated that the concentration of plasma should not exceed 25 µl/well (1/4) to ensure the absence of nonspecific interferences. When raw data were corrected by the dilution factor, the concentration results obtained, showed a coefficient of variation which did

TABLE 1

Comparison of the specificity of EIA for foetal calf serum (FCS) vs RIA

n° Assay	Assay 10% FCS	
	EIA (ng/ml)	RIA (ng/ml)
1	<0.125	2.76 (± 0.08)
2	<0.125	2.90 (± 0.01)
3	<0.125	3.05 (± 0.06)
4	<0.125	2.67 (± 0.27)
5	<0.125	2.86 (± 0.01)
Mean	<0.125	2.85
SD		0.14
CV (%)		5

FCS 10% (V/V) in PBS-assay was assayed in EIA and RIA.
Each value (ng/ml) is the mean ± SD of duplicate determinations.

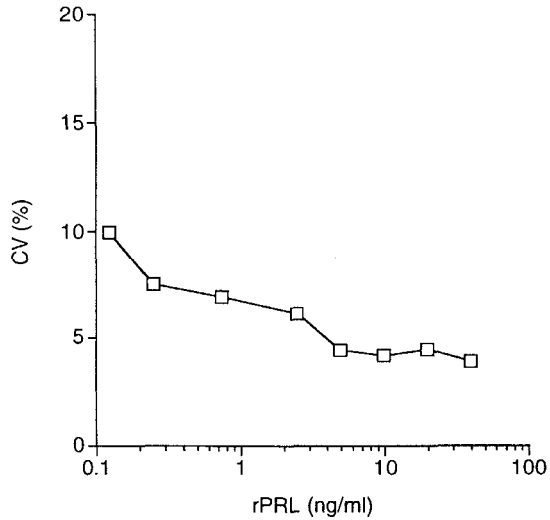

FIGURE 3. : inter-assay precision profile. Results are expressed as the coefficient of variation between values determined in assays performed on 6 consecutive days using duplicate samples of each rPRL standard concentration.

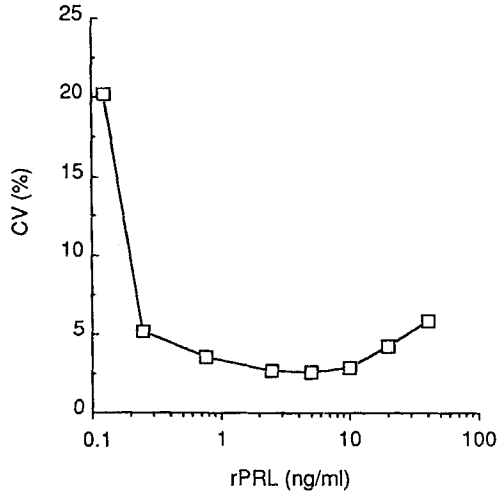

FIGURE 4. : Intra-assay precision profile. Results are expressed as the coefficient of variation between values obtained using 6 replicates for each concentration of PRL standard.

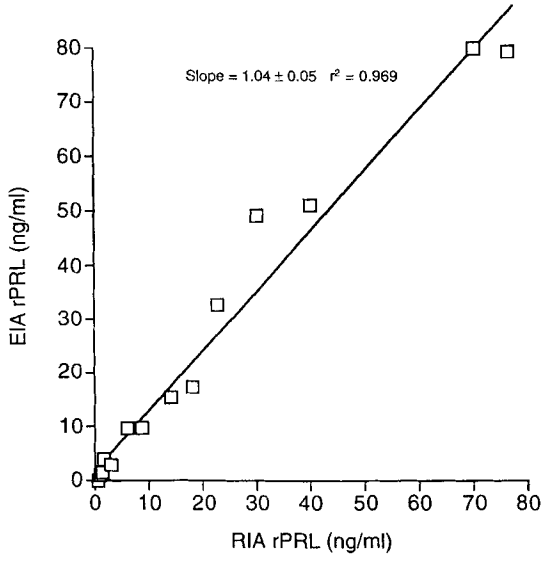

FIGURE 5. : Correlation between enzyme immunoassay (EIA) and radioimmunoassay (RIA). Plasma samples were collected from 15 different animals, males (n=3), females (n=3) and pregnant females (n=9) and assayed in EIA and RIA in duplicate.

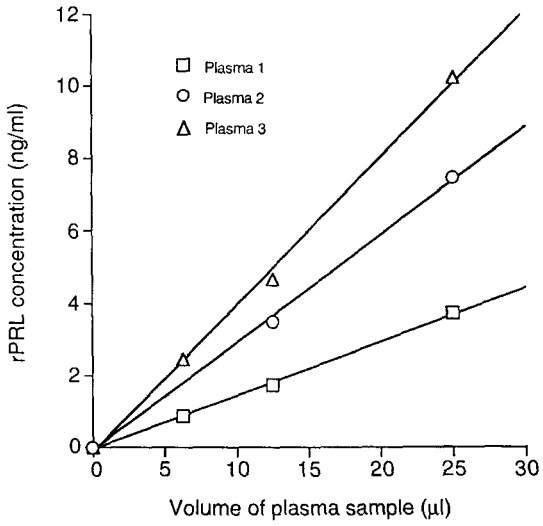

FIGURE 6. : Effect of dilution on the determination of rPRL content in plasma. 3 different rat plasma samples were diluted in assay buffer to a final volume of 100 µl per sample. Each point is the mean of duplicate determinations.

not exceed 5 % (Table.2). Recovery experiments were conducted in order to study possible effects of plasma. Three rat plasma samples with a known endogenous rPRL content, i.e previously assayed, were supplemented with a fixed amount of exogenous standard rPRL (10 ng/ml). A good recovery was observed as far as the final dilution of plasma was ≥ 1/4 (table.3). Recovery indexes were not different from the nominal 100 % value .

Assay of rPRL in cell culture media

PRL secreted by rat pituitary cells in vitro was determined by EIA. Static cell monolayers were exposed or not to dopamine (DA, 1 µM) for 1h, 2h or 4h. In control conditions, as expected, the rate of PRL secretion level was linear versus time and

TABLE 2

Effect of dilution on the determination of rPRL content in plasma

	rPRL (ng/ml) Plasma 1		rPRL (ng/ml) Pasma 2		rPRL (ng/ml) Plasma 3	
Dilutions	observed	(1) calculated	observed	(1) calculated	observed	(1) calculated
1:4	3.77	15.08	7.45	29.8	10.25	41
1:8	1.74	13.92	3.52	28.16	4.73	37.84
1:16	0.89	14.24	1.85	29.6	2.45	39.2
Mean		14.41		29.16		39.35
SD		0.6		0.89		1.58
CV(%)		4		3		4

(1) Calculated : theoretical rPRL concentration / ml of undiluted plasma.

TABLE 3

Test of recovery

	rPRL in plasma diluted to 1:4 ng/ml (endogenous)	Total rPRL measured ng/ml (endogenous + exogenous)	Calculated values (ng/ml) (exogenous)	Recovery index %
Plasma 1	2.03	11.56	9.53	95.3
	2.03	13.88	11.85	118.5
	1.78	11.68	9.9	99
Plasma 2	4.47	15.62	11.15	111.5
	4.43	15.6	11.17	111.7
Plasma 3	2.79	12.76	9.97	99.7
	2.68	11.68	9	90
	2.68	13.52	10.84	108.4
	2.86	12.72	9.86	98.6
	2.86	12.04	9.18	91.8

Three plasma samples were assayed for their endogenous rPRL level (column 1) and then supplemented with exogenous standard PRL (10 ng/ml). The values of rPRL (column 3) were calculated by subtracting column 1 from column 2 (total rPRL assayed). Recovery index is the ratio of calculated values to added quantity of exogenous rPRL. Values are the mean of duplicate determinations.

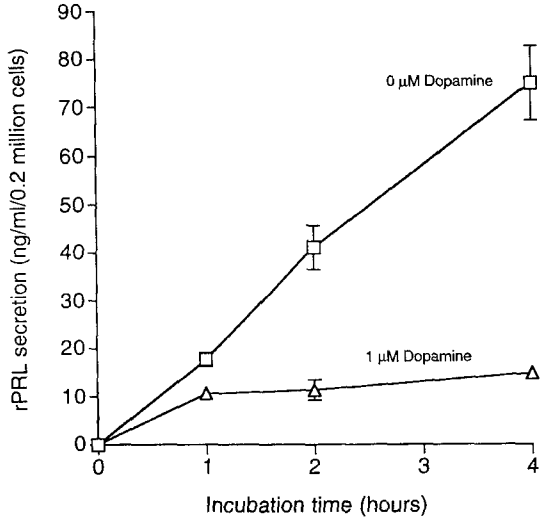

FIGURE 7. : Effect of dopamine (1µM) on prolactin secretion by cultured pituitary cells. Cells plated in multiwell culture were precultured for three days, preincubated in serum-free culture medium for 2h and exposed with or without DA for 1h, 2h or 4h. Values are the mean ±SEM of five independent experiments performed in triplicate wells.

reached 75ng/ml/2.10^5 cells at 4 hours. A marked decrease in rPRL secretion was observed in presence of DA (fig.7). DA inhibited PRL accumulation since the first hour; This inhibition yielded 80 % of control values after 4 hours of incubation.

DISCUSSION

The present study describes a rPRL-EIA that may be used as an alternative to RIA for measuring rPRL. This assay is the third enzyme immunoassay described today to quantify rat PRL (1-3), but it is the first sandwich rPRL-EIA. This assay offers a number of avantages. PRL is quantified within 4h30 for culture medium

samples and within 18h30 for plasma samples .The antibody is linked to peroxidase which means that conjugation is easily carried-out, and the purified enzyme is cheap. The antibody-peroxidase tracer is found to be stable up to 3 years. The limit of quantification of the "sandwich type" assay described here, is 0.25 ng/ml for culture media. For samples containing plasma, the limit of quantification is 25 pg/well i.e 1 ng/ml of plasma. This assay is highly specific for rPRL and the concentrations determined by EIA in rat plasma samples are in agreement with those determined by RIA in different conditions. However, unlike a competitive-RIA, it does not cross-react with foetal calf serum at concentrations currently used in tissue culture media. The absence of cross-reaction in sandwich enzyme immunoassay compared to competitive radio-immunoassay was already reported in the case of LH, FSH and their subunits (8). This increased specificity likely results from the involvement of at least two epitopes of antigen detected. This assay is validated for its use in both rat plasma and in culture media. The rat PRL-EIA described by Signorella and Hymer,1984 was validated for quantification in serum free culture media but not in plasma samples (9). In that EIA, rat PRL and PRL adsorbed to a solid-phase support competed for limited specific rabbit anti-rPRL antiserum sites. The present rPRL-EIA provides a good, rapid, sensitive and specific assay for in vivo and in vitro studies.

ACKNOWLEDGEMENT

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ABBREVIATIONS:

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

PBS : Phosphate Buffer Saline

DMEM : Dulbecco's minimal essential medium

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