Failure of an Indirect Competitive Enzyme-Linked Immunosorbent Assay to Measure Prolactin in Rat Plasma Samples (42904)

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Abstract. **An indirect competitive enzyme-linked immunosorbent assay (ELISA) for rat prolactin was applied to the measurement of prolactin (PRL) in plasma. The assay involved PRL adsorbed to a 96-well plate competing with soluble PRL in the sample for rabbit anti-rat PRL antibody-binding sites. Antibody bound by the PRL on the well was detected by a goat anti-rabbit immunoglobulin antibody conjugated to the enzyme horseradish peroxidase. Dilutions of PRL in plasma-free assay buffer produced accurate and reliable standard curves over a range of 1–500 ng/ml. Plasma or serum samples, however, gave very low absorbance values, suggesting falsely high levels of PRL in the samples. For example, hypophysectomized rat plasma and fetal calf serum gave readings equivalent to over 250 ng/ml rat PRL by ELISA, whereas radioimmunoassay confirmed the expected PRL values of less than 5 ng/ml. This result indicated considerable nonspecific interference by the plasma. Inclusion of 20% or 50% hypophysectomized rat plasma in the assay significantly lowered the absorbance values and the slope of the standard curve ($P < 0.05$). Dilution of plasma samples 1:1 and 1:4 with assay buffer caused nonlinear changes in absorbance and resulted in inaccurate ELISA estimates of plasma PRL concentrations compared with radioimmunoassay.**

Several treatments were attempted to remove the interference from plasma. Preexposure of plasma to 56°C for 30 min, dialysis for 24 hr, fractional precipitation with 5% polyethylene glycol, increased ionic strength of the buffer, preadsorption of plasma with rabbit immunoglobulins bound to a solid phase support, and covalent attachment of the PRL to the wells with 2% glutaraldehyde all failed to remove the interference from the plasma samples in the ELISA.

Thus, while the assay worked effectively for plasma-free applications, it was unsuitable for measurement of PRL in plasma or serum samples.

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Enzyme immunoassays (EIA) are now frequently used in endocrinology to measure concentrations of hormones in blood as an alternative to the more common radioimmunoassay (RIA) (1–5). EIA and RIA are similar in performance in terms of sensitivity and specificity (6), but EIA offers several advantages. Assay procedures are rapid and simple, stable reagents with relatively long shelf lives are involved, and the use of radioactive isotopes is avoided. A competitive enzyme-linked immunosorbent assay (ELISA) for rat prolactin (PRL) was developed in 1984 by Signorella and Hymer (7) and used for analysis of cell culture media and electrophoresis gels. In view of the advantages of the EIA, we attempted to apply this method to the measurement of PRL in rat plasma samples.

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Materials and Methods

Materials. Rat PRL (RP-1) and rabbit anti-rat PRL (S-9) were provided by the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP), rabbit peroxidase antiperoxidase (PAP), Tween 20, and *o*-phenylene diamine hydrochloride were purchased from Sigma Chemical Co. Bovine serum albumin (BSA) was obtained from Immunochemical Products Ltd. (New Zealand), polyethylene glycol (PEG) 6000 from BDH Chemicals Ltd. (New Zealand), and gluteraldyhyde (25% aqueous solution) from Polaron Equipment Ltd., Watford England. Microtitration

plates (Nunc Immunoplate II, 96-well) were purchased from GIBCO Ltd. (New Zealand).

Animals. Adult female Wistar rats bred in our laboratory were kept in a temperature controlled room (22 ± 1°C) with free access to food and water. Blood samples were collected from indwelling jugular cannulae, and the plasma samples were stored at −20°C. To provide plasma free of pituitary prolactin, several animals were hypophysectomized by the parapharyngeal approach. Hypophysectomy was confirmed by postmortem examination and later by RIA. High prolactin plasma samples were provided by grafting anterior pituitary tissue under the kidney capsules of several animals.

ELISA Procedure. The method of Signorella and Hymer (7) was followed initially without any modification. The basic procedure was as follows: Rat PRL was dissolved in phosphate-buffered saline (PBS) and coated onto the wells of a 96-well plate by incubating 125 ng of PRL in each well for 12 to 18 hr at 4°C. PRL was diluted in PBS containing 1% BSA to provide assay standards in the range of 1–500 ng/ml. The standards and the plasma unknown samples were incubated undiluted with equal volumes of the primary antibody (rabbit anti-rat PRL) diluted 1:4000 in assay buffer consisting of 0.1% BSA in PBS for 12 to 18 hr at 4°C. After washing the plate with PBS containing 0.05% Tween 20, the PRL/primary antibody solution was added to the PRL-coated wells in 200-μl aliquots and incubated for 4 hr at room temperature. Thus, the PRL that was adsorbed to the wells competed with the soluble PRL in the sample for primary antibody binding sites. Consequently, a high concentration of PRL in the sample resulted in low levels of primary antibody binding to the PRL on the wells. The bound primary antibody was then detected by a second antibody/PAP system.

The plates were washed with 0.05% Tween 20 in PBS, and 200 μl of secondary antibody (goat anti-rabbit immunoglobulins conjugated to HRP) diluted 1:1000 in assay buffer were added to the wells and incubated for 2 hr. The plates were then washed, and 200 μl of PAP complex diluted 1:1000 in assay buffer were added to the wells and incubated for 2 hr. Bound enzyme was detected by adding 200 μl of the substrate for HRP (1 mg/ml *o*-phenylene diamine hydrochloride in citrate (pH 5) buffer containing 0.003% H_2O_2) to each well. After 1-hr incubation in the dark at room temperature, the reaction was stopped by adding 50 μl of 2.5 *M* sulfuric acid to each well, and absorbances were read at 490 nm on a micro-ELISA plate reader (Minireader II; Dynatech). High concentrations of PRL in the samples led to low amounts of HRP attaching to the PRL on the wells, resulting in low absorbance.

In later assays, this procedure of Signorella and Hymer (7) was modified by diluting the reference PRL with PBS containing 20% plasma from hypophysectomized rats (HYPOX). Plasma samples were diluted 1:4 with PBS, so that the plasma content of the samples and the standards was the same.

RIA. The standard double-antibody technique of Neill and Reichert (8) was used to compare the plasma PRL concentrations obtained by RIA and by ELISA. The RIA used NIDDK reagents with rat PRL (RP-1) as the reference preparation. Intra- and interassay coefficients of variation for the RIA were 2.7% and 19.2%, respectively.

Statistical Analysis. Statistical significance of the data at the 95% confidence level was determined by ANOVA using the Statview 512+ program for the Apple Macintosh computer.

Results

Reference PRL was dissolved in PBS with 1% BSA at concentrations from 1 to 500 ng/ml. When measured by ELISA, these standards gave accurate and repeatable absorbance values. Changes in the concentration of PRL could be detected as low as 1–5 ng/ml. Within a single assay, the differences between duplicate wells were usually less than 5% of the mean. All plasma samples assayed by ELISA, however, produced very low absorbance readings compared with the standards, suggesting high concentrations of PRL in the samples. Thus, HYPOX plasma gave readings equivalent to PRL at greater than 250 ng/ml, while radioimmunoassay of this same plasma confirmed the expected PRL concentrations of less than 5 ng/ml. Rat serum samples had the same effect, giving very low absorbance values by ELISA. This effect was not specific to rat plasma or serum, since fetal calf serum, both undiluted and diluted 1:4 in PBS, produced very low absorbance values as well, although not as low as the rat samples.

To examine the effect of plasma on the standard curve, the reference PRL was dissolved in PBS containing 20% or 50% HYPOX plasma. The presence of plasma significantly decreased the absorbance values (Fig. 1). Dilution of standards in both 20% and 50% HYPOX plasma significantly flattened the slope of the standard curve relative to the control ($P < 0.05$). Dilution of the plasma samples 1:1 and 1:4 with PBS produced variable, nonlinear changes in the absorbance readings (Table I). Estimates of plasma PRL obtained from plasma samples that were diluted 1:4 in PBS, using a standard curve in 20% HYPOX plasma, gave incorrect values for PRL when compared with the results obtained using RIA (Table I).

Preliminary attempts to improve the performance of the assay by treating plasma samples prior to the ELISA in order to remove any interference present were not successful. These pretreatments included exposure to 56°C for 30 min, dialysis against PBS for 24 hr at 4°C, fractional precipitation with 5% PEG 6000,

increased ionic strength of the buffer from the original 0.15 *M* PBS to 0.3 and 0.6 *M* PBS, and preadsorption of the plasma samples with rabbit immunoglobulins bound to a solid phase support. Representative results from these experiments are given in Table II. Covalent binding of the PRL to the wells by pretreating the wells for 2 hr with 2% glutaraldehyde in pH 5 PBS (9) to prevent possible plasma-induced loss of PRL from the wells also failed to improve the assay.

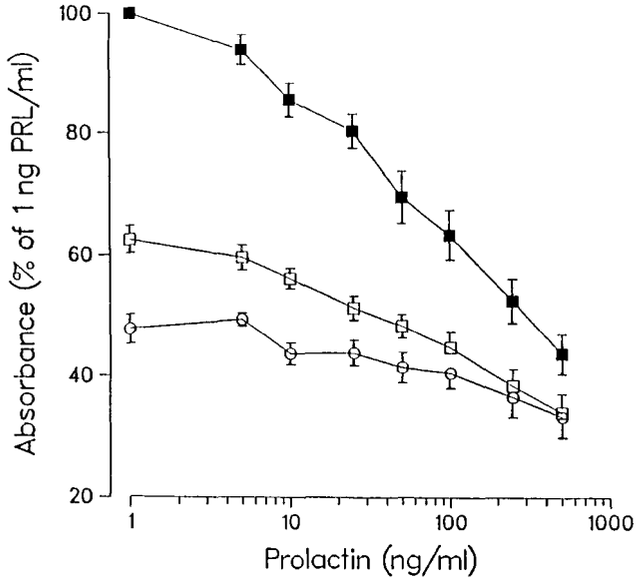

Figure 1. Effect of plasma on absorbance in the ELISA standard curve for PRL. All values are shown as a mean percentage (±SEM) of the absorbance at 490 nm produced by 1 ng/ml of PRL in PBS containing 1% BSA. This corrects for variation in absorbance between ELISA plates. In general the actual absorbance readings for 1 ng/ml of PRL ranged between 0.7 and 1.3 optical density units in different experiments. Standards were diluted with PBS containing: ■, 1% BSA ($n = 8$); □, 20% HYPOX plasma ($n = 8$); ○, 50% HYPOX plasma ($n = 4$).

Table I. Absorbance Changes from Dilution of Plasma Samples

Plasma sample	Absorbance of plasma samples[a]			PRL by ELISA (ng/ml)[b]	PRL by RIA (ng/ml)[c]
	Undiluted	1:1	1:4		
1	0.34	0.42	0.61	150	10
2	0.41	0.44	0.57	200	84
3	0.54	0.66	0.67	75	65
4	0.56	0.67	0.71	40	2
5	0.60	0.62	0.67	75	5
6	0.69	0.85	0.86	10	<1
7	0.71	0.81	0.71	40	47
8	0.75	0.84	0.92	5	<1

[a] Plasma was diluted with PBS.
[b] ELISA prolactin (ng/ml) is estimated from a 1:4 dilution of the plasma samples in PBS, read off a standard curve in PBS containing 20% HYPOX plasma.
[c] RIA prolactin (ng/ml) shows the radioimmunoassay values for the same plasma samples.

Table II. Effect of Treating Plasma Samples on Absorbance Readings in Prolactin ELISA

Treatment[a]	Low PRL plasma[b]	High PRL plasma[c]	Absorbance range of PRL standards from 1–100 ng/ml[d]	
			1% BSA	20% HYPOX
Untreated	0.29	0.15	0.89–0.45	0.56–0.24
Heated	0.74	0.73	0.83–0.49	0.76–0.65
Untreated	0.11	0.08	0.72–0.55	0.41–0.18
Dialyzed	0.33	0.20	0.70–0.58	0.52–0.38
Untreated	0.41	0.27	1.23–0.79	ND[e]
0.3 *M* PBS	0.37	0.26	ND	ND
0.6 *M* PBS	0.41	0.26	ND	ND
Untreated	0.46	0.35	1.34–0.80	ND
PEG	0.62	0.56	ND	ND
Untreated	0.13	0.05	0.34–0.22	ND
IgG adsorbed	0.12	0.04	ND	ND

[a] Plasma samples were treated prior to assay by ELISA (see text for details).
[b] Plasma from HYPOX animals, containing less than 5 ng/ml PRL by RIA.
[c] Plasma from animals with anterior pituitary grafts under the kidney capsule, containing 60–80 ng/ml PRL by RIA.
[d] Range of absorbances obtained from ELISA of reference PRL dissolved in PBS containing either 1% BSA or 20% HYPOX plasma.
[e] ND = no data.

Discussion

The ELISA as described by Signorella and Hymer (7) was simple to use and reproducible in samples free of plasma. Reference PRL diluted in PBS with 1% BSA gave standard curves for rat PRL which were sensitive enough to permit distinctions over a working range of 1–500 ng/ml (see Fig. 1). Thus, the assay was sensitive enough to be used for studies over the entire physiologic range of rat PRL (from less than 5 ng/ml basal to suckling-induced peaks of approximately 300 ng/ml (10)). Because of interference by some component of the plasma, however, we have been unable to obtain satisfactory or reproducible estimates of the PRL concentration in plasma samples. The ELISA produced falsely high estimates of plasma PRL concentrations compared with those from parallel RIA, suggesting that some substance in the samples other than PRL was either binding to the primary antibody or preventing it from binding to the PRL adsorbed to the wells.

Increasing the ionic strength of the assay buffer to reduce nonspecific binding of antibodies (11) did not alter the interference by plasma. Preadsorption of the plasma against rabbit immunoglobulins bound to a solid phase support also had no effect on the assay (Table II). This indicates that the interfering agent was not attaching to the primary antibody but rather was preventing it from binding to the PRL adsorbed to the plate.

Inclusion of HYPOX plasma with the standard dilutions of PRL significantly flattened the slope of the

standard curve. Thus, the effect of the interfering agent was more prominent at low PRL concentrations than at high PRL concentrations. This was inconsistent with simple binding interference which would be expected to lower the values of the standard curve without changing the slope. Serial dilutions of the plasma samples also failed to produce a linear increase in absorbance. These nonlinear dilution effects are presumably the cause of the incorrect PRL values estimated by ELISA (Table I).

Plasma has been shown to cause a 40% reduction in the antibody bound to the wells in an ELISA for hepatitis B surface antigen (9). It is possible that plasma caused a nonspecific loss of PRL from the wells of the microtitration plates in the prolactin ELISA. Such a nonspecific effect is supported by the fact that fetal calf serum reduced primary antibody binding in a similar manner to rat plasma. Preliminary attempts to attach the PRL covalently to the plates, however, were not successful in preventing the interference (data not shown).

Other efforts to remove an interfering substance by pretreating the plasma samples also failed. Heating at 56°C for 30 min to deactivate complement has been shown to improve some assays of this type (1). In the prolactin ELISA, heat treatment increased absorbance values considerably but also appeared to reduce immunoreactivity of PRL in the samples, resulting in a loss of sensitivity in the assay so that there was very little distinction between 1 and 100 ng/ml PRL (Table II). This loss of PRL immunoreactivity after heating did not occur to the same extent in the absence of plasma (Table II).

Dialysis of plasma samples and fractional precipitation with PEG were other treatments used to try to remove interfering plasma components without affecting plasma PRL concentrations. The PEG treatment has been used to remove unspecified interfering factors in an assay for inhibin (12). In the prolactin ELISA, both treatments partially removed the interference from the plasma samples but the absorbance values from these samples were still well below the range of the standards without plasma (Table II). This suggests that the interference may not be due to a single substance in the plasma, since dialysis would remove only low molecular weight substances, whereas PEG would precipitate larger molecules. Combinations of these treatments were not attempted as the time involved in preparation of samples would be impractical in the design of a simple and rapid assay method.

Several other enzyme immunoassays for protein hormones in plasma have been described (1–4). Ishikawa *et al.* (4) developed an assay for human PRL in plasma which was more complicated than Signorella and Hymer's (7) in that it involved labeling the PRL with an enzyme and separation of antibody-bound and free PRL by column chromatography. These differences in basic procedure may account for the success of this method, in contrast to Signorella and Hymer's, in measuring PRL in plasma. Thus, while the ELISA for rat PRL is a simple, accurate procedure for measuring PRL in cell culture media and other plasma-free solutions, in its present form, it is not suitable for measuring PRL in plasma samples.

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