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Short communication

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An enzyme-linked immunosorbent assay (ELISA) for measuring prolactin levels in ovine and cervine plasma

L. K. LEWIS
P. A. ELDER[1]
G. K. BARRELL
Animal and Veterinary Sciences Group
P. O. Box 84
Lincoln University
Canterbury, New Zealand

[1] Steroid Unit
Department of Clinical Biochemistry
Christchurch Hospital
Christchurch, New Zealand

Abstract An indirect enzyme-linked immunosorbent assay (ELISA) for ovine plasma prolactin that can be completed in less than 8 h is described and validated for ovine and cervine plasma. Ovine prolactin is covalently bound to bovine thyroglobulin and passively adsorbed in guanidine hydrochloride to a standard 96-well microtitre plate. Addition of standards, samples, and rabbit anti-ovine prolactin is specific for this assay, but subsequent steps which include addition of peroxidase-labelled goat-anti-rabbit IgG, washing, addition of *o*–phenylenediamine substrate with colour development, and reading of plates at 492 nm using an automatic ELISA processor are common to most ELISAs currently being performed in this laboratory. Assay sensitivity is less than 2.5 ng/ml, and intra- and inter-assay coefficients of variation are less than 9 and 16 % respectively. All steps except for sample addition are performed on a Behring Elisa Processor M automatic machine. This ELISA is quick, simple, and cost-effective compared with conventional ^{125}I-labelled radioimmunoassays for ovine prolactin, making it a useful alternative for the measurement of prolactin levels in ovine and cervine plasma samples.

Keywords prolactin; ELISA; ovine; cervine

INTRODUCTION

There is a close association between secretion of prolactin and the occurrence of seasonal or other photoperiodic phenomena in animals such as sheep and deer so studies in this field regularly involve measurement of this hormone (Ravault 1976; Barrell & Lapwood 1978; Kelly et al. 1982; Barrell et al. 1985; Adam et al. 1987). Plasma or serum concentrations of prolactin in ruminant animals have been measured by radioimmunoassays (RIA) (Davis et al. 1971; McNeilly 1976; Chesworth 1977) which can be time-consuming, costly, and sometimes unreliable. Enzyme-linked immunosorbent assays (ELISAs) are useful alternatives to RIA because they are quicker and eliminate the need for radioactive reagents and counting equipment. The assay described can be completed within 8 h as opposed to 2–3 days with RIA. The entire assay is performed in a 96-well microtitre plate. After addition of sample and first antibody, the remainder of the assay is automated and common to most other ELISAs performed in this laboratory. The procedure described here is based on those previously described for steroid hormones such as testosterone (Elder & Lewis 1985), cortisol (Lewis & Elder 1985), and progesterone (Elder et al. 1987).

Prolactin secretion in ruminants can be stimulated by the administration of thyrotrophin-releasing hormone (TRH) (Schulte et al. 1981; Kelly et al. 1982; McNeilly & Baird 1983; Curlewis et al. 1988) and there is a large divergence between summer (high) and winter (low) levels of prolactin in plasma (Buttle 1974; Schams & Reinhardt 1974; Ravault 1976; Thimonier et al. 1978; Schulte et al. 1981; Kelly et al. 1982; Barrell et al. 1985; Curlewis et al. 1988). TRH stimulation and the difference between summer and winter prolactin levels were utilised to

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evaluate the assay for its specificity to prolactin in ovine and cervine plasma.

EXPERIMENTAL

Preparation of prolactin-thyroglobulin conjugate

Prolactin-thyroglobulin conjugate was prepared following a modified method of Skowsky & Fisher (1972).

Ovine prolactin (NIADDK–*o*–prl–16, obtained from National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Baltimore, Maryland, U. S. A.) solution (10 mg in 1 ml distilled water) was mixed with a solution containing bovine thyroglobulin (Sigma Chemical Company, St Louis, Missouri, U. S. A.) (10 mg in 1 ml distilled water). Conjugation was achieved by adding N–ethyl–N'–(3–dimethylaminopropyl)–carbodiimide hydrochloride (Sigma Chemical Company, St Louis, Missouri, U. S. A.) (5 mg in 0.5 ml distilled water) to the stirred prolactin and thyroglobulin solution. This mixture was left stirring overnight at room temperature, then dialysed for 24 h against distilled water at 4°C. The dialysate was freeze-dried and the resultant powder reconstituted when necessary by adding 1 ml of distilled water to 10 mg.

Buffers

Phosphate-buffered saline solution (PBS), pH 7.4, containing 0.1% (w/v) gelatin and Tween 20 (BDH Chemicals Ltd, Poole, U. K.) (0.05% v/v) was used as the assay buffer. PBS containing 0.6% (v/v) Tween 20 was used for washing microtitre plates.

Prolactin-free plasma

Plasma obtained from horses did not react with the anti-ovine-prolactin antiserum and was used as prolactin-free plasma for dilution of standards and samples. New batches of horse plasma were checked against old batches to ensure that they contained no measurable cross-reactivity.

Standards

Ovine prolactin standards were prepared in prolactin-free plasma from a stock solution containing 25 mg (NIADDK–*o*–prl–16, as above in Preparation of prolactin-thyroglobulin conjugate) per ml in 0.9% saline solution, pH 9.5. After aliquotting standards to their final dilution they were stored at –20°C until the day of assay. Standards prepared in this way remained stable for at least 6 months.

Antibodies

Rabbit antiserum to ovine prolactin (provided by Dr D. F. M. van de Wiele, Research Institute For Animal Husbandry, Schoonoord, Netherlands) was diluted 1:500 with assay buffer, aliquotted into 0.1 ml portions, and stored frozen at –20°C until the day of assay. The final dilution used in the assay was 1:50 000.

Affinity-purified, peroxidase-labelled goat anti-rabbit IgG (Tago Immunodiagnostics Inc, Burlingame, California, U. S. A.) was used at a final dilution of 1:2000.

Enzyme substrate was freshly prepared just before use. 40 mg *o*–phenylenediamine (Sigma Chemical Company, St Louis, Missouri, U. S. A.) was added to 100 ml of substrate buffer (50m*M* Na_2HPO_4, 25 m*M* citric acid buffer, pH 5.0). Immediately before use 60 µl of hydrogen peroxide (30% v/v) was added to this mixture.

Plasma samples

Blood was collected by jugular venepuncture into evacuated 10 ml glass tubes containing 125 units sodium heparin (Leo Pharmaceutical Products, Denmark). These were centrifuged at 1500 g for 15 min at 4°C and the plasma from each sample was collected and stored at –20°C.

Adsorption of prolactin-thyroglobulin to the microtitre plates

Conjugate solution was prepared by the addition of stock prolactin thyroglobulin conjugate (7 µl) to 10 ml of aqueous 6*M* guanidine hydrochloride (Sigma Chemical Company, St Louis, Missouri, U. S. A.). ELISA plates (Falcon 3912 Microtest III, Becton Dickinson Co., Oxnard, California, U. S. A.) were activated by the addition of 100 µl of conjugate solution per well and incubation overnight at 4°C. Incubation for 2.5 h at room temperature gave equivalent activation but it was convenient for our daily laboratory schedule to leave the plates overnight at 4°C. A Behring Elisa Processor M (BEPM, Behring, Marburg, Germany) removed unadsorbed conjugate by a 4 × 200 µl wash per well following which plates were inverted and shaken dry. Further active binding sites were blocked by the addition of 150 µl assay buffer per well. Each

microtitre plate was then incubated for at least 1 h at 4°C.

ELISA procedure

After blocking, plates were emptied by inversion, shaken dry, and 50 µl of prolactin standard or plasma sample was dispensed into the appropriate well, followed by 50 µl of rabbit anti-prolactin serum. The plates were incubated at room temperature for at least 2 h then automatically washed as above. Peroxidase labelled goat-anti-rabbit-IgG was added (100 µl per well) and plates were incubated for a further 2 h at room temperature.

Plates were re-washed and dried before the addition of 100 µl of enzyme substrate solution.

Development of colour was allowed to proceed in the dark for c. 20 min then 100 µl of 1.25*M* H_2SO_4 was added to stop the reaction. Absorbance was read at 492 nm, with a reference wavelength of 650 nm using the BEPM. The zero standard was analysed in quadruplicate; all other standards and samples were analysed in duplicate, and the sample values were interpolated from a standard curve.

Specificity

Antibody specificity was determined by assessing potentially cross-reacting ovine pituitary hormones (all provided by National Hormone and Pituitary Program, University of Maryland School of Medicine, Baltimore, Maryland, U.S.A). The corresponding purified cervine pituitary hormones were not available. Solutions containing, 10–1000 ng/ml luteinising hormone (LH) (LH–NIADDK–oLH–26) or follicle stimulating hormone (FSH) (NIAMDD–oFSH–RP–1), and 10–25 mg/ml growth hormone (GH) (NIH–GH–S10), thyroid stimulating hormone (TSH) (NIH–TSH–S8–ovine), or prolactin standard were assayed. A standard curve of cervine prolactin (generously provided by Dr S. R. Haines, Invermay Agricultural Research Centre, Mosgiel, N. Z.) which had been obtained from pituitaries of red deer at slaughter premises in New Zealand (purified by Dr A. F. Parlow, Pituitary Hormones and Antisera Centre, Torrance, California, U. S. A.) was prepared from a 1 mg/ml stock solution and compared with an ovine standard curve. Values obtained from cervine standards and serially diluted cervine plasma were utilised to check parallelism and cross-reactivity.

Recovery

Aliquots of plasma from hypophysectomised sheep (Immuno-Chemical Products, Auckland, N.Z.) to which 25, 50, 75, or 100 ng of ovine prolactin had been added were assayed to determine recovery by comparing the amount of prolactin added with that measured.

Precision

Intra-assay coefficients of variation (CV) were calculated from differences in pairs of duplicates in the low, medium, and high range of the standard curve. Inter-assay coefficients of variation were calculated from results obtained in repeated assays of these same samples. Samples of sheep and deer plasma were assayed at several dilutions to test for parallelism.

Radioimmunoassay

A ^{125}I radioimmunoassay kit (National Institutes of Health, Bethesda, Maryland, U. S. A., with NIDDK–*o*–prl–I–2 as the standard) for measuring ovine plasma prolactin concentration in the range of 0.6–50 ng/ml was utilised for comparison with the ELISA. This RIA was carried out by Vernon Choy, Ruakura Agricultural Research Centre, Hamilton, New Zealand.

Physiological studies

Plasma was collected from domestic sheep (ewes, n = 5) on 7 July (winter) and 12 December (summer) and from farmed red deer hinds (n = 6) on 1 June (winter) and 20 December (summer). Plasma samples were also collected from 10 red deer stags in conjunction with an i.v. injection of 200 µg synthetic TRH (Hoechst New Zealand, Auckland, N. Z.) per animal as follows. An indwelling polyvinyl chloride cannula (0.5 mm i.d.) was inserted into a jugular vein of each stag 45 min before the start of the procedure. Four blood samples were taken at 15-min intervals to determine basal prolactin levels in plasma then TRH was injected via the cannula and subsequent samples were taken at 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300, and 360 min post-injection.

RESULTS

Binding of prolactin to the microtitre plate

To test for even adsorption of conjugate to the microtitre plate, plates were treated as described in the experimental section but without addition of sample or standard. Mean absorbance and standard deviation was 1.406 ± 0.079, giving a CV of 5.6%.

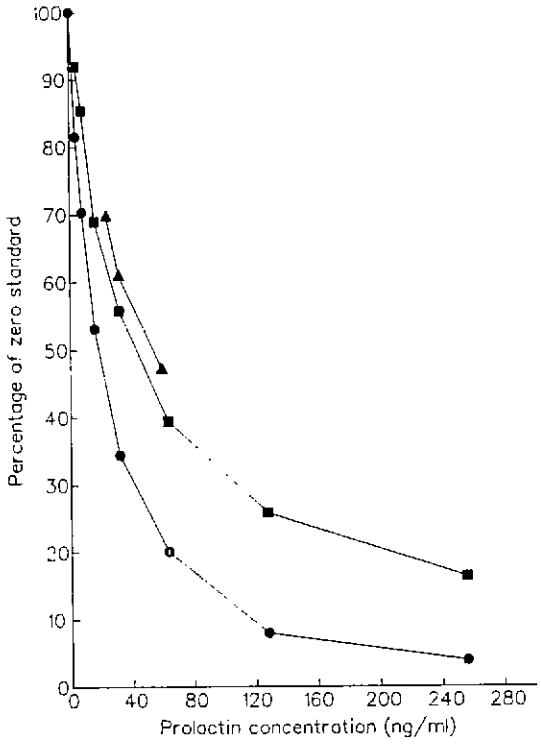

Fig. 1 Typical standard curve for ovine (●) and cervine (■) prolactin, showing parallelism with a cervine plasma sample (▲). Each point is the mean of duplicate estimates.

It was noted that the two outside columns (1 and 12) often had higher absorbance than inner columns, and removal of data from the outer columns decreased the CV to 3.5%. For this reason only the 10 inner columns were used in an assay.

Standard curve

Figure 1 shows typical standard curves for ovine and cervine prolactin prepared in prolactin-free plasma. The zero standard was arbitrarily set at 100% binding. Addition of ovine or cervine prolactin caused the absorbance at 492 nm to decrease in a similar dose-responsive manner for both species although the decrease observed with the cervine standards was lower than that for the ovine standards (Fig. 1). This indicated that both sets of standards could displace the antibody from the solid support antigen quantitatively. All results have been determined using the ovine standards. Sensitivity, calculated at two standard deviations from 0, was less than 2.5 ng/ml for the ovine curve.

Specificity

The specificity of the anti-ovine prolactin serum is summarised in Fig. 2. The cross-reactivity with GH was 0.7%, (determined as the amount required for a 50% reduction in absorbance) whereas cross-reactivity with other ovine pituitary hormones tested was negligible.

Recovery

Recoveries of exogenously added ovine prolactin ranged from 85 to 113% over the range of 25–100 ng/ml.

Precision

Precision was assessed using three pools of plasma, each measured at least 7 times in duplicate. Mean results were 15.8 ± 2.4, 40.7 ± 5.1, and 104.9 ± 10.3 ng/ml with intra-assay coefficients of variation (CV) of 6.4, 7.3, and 8.5% respectively. Inter-assay CV for these samples were 15.4, 12.4, and 9.8% respectively.

Radioimmunoassay

A comparison of plasma prolactin levels determined by RIA versus ELISA is plotted in Fig. 3. The regression line fitted to these data (n = 9) had a correlation coefficient (r) of 0.96 which indicates excellent agreement between the two assay methods.

Physiological studies

Summer/winter comparisons

Mean (± SEM) prolactin levels in plasma were higher ($P < 0.05$, Student's t-test) in summer (157.2 ± 43.9 and 146 ± 42.2 ng/ml) than winter (14.8 ± 5.0 and 15.0 ± 4.8 ng/ml) for both sheep and deer respectively.

TRH response

In all 10 stags there was a marked rise in the prolactin concentration of plasma following a single injection of TRH (Fig. 4). The maximum concentration was observed 10–20 min after injection, with a decline to basal levels within 45 min of injection (Fig. 4).

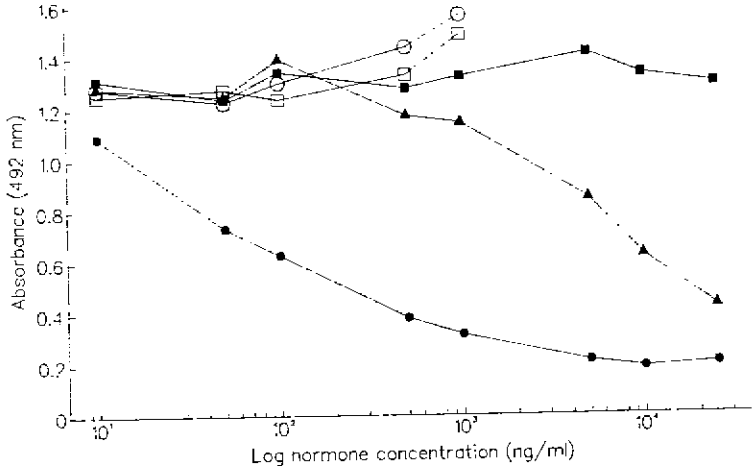

Fig. 2 Cross-reactivity of various ovine pituitary hormones with the anti-ovine prolactin antibody. Each point is the mean of duplicate estimates. (●) prolactin, (■) TSH, (▲) GH, (□) LH, (○) FSH.

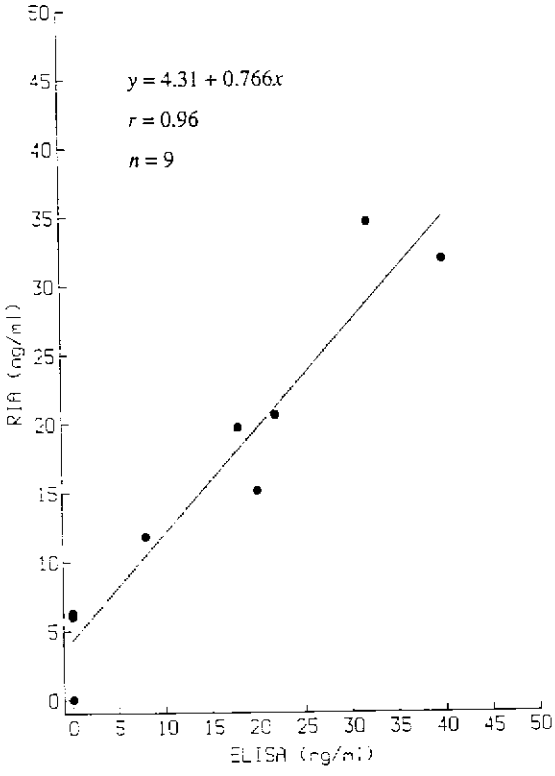

Fig. 3 Relationship between plasma prolactin concentration measured by ELISA and RIA. Each point is the mean of duplicate estimates from both assays.

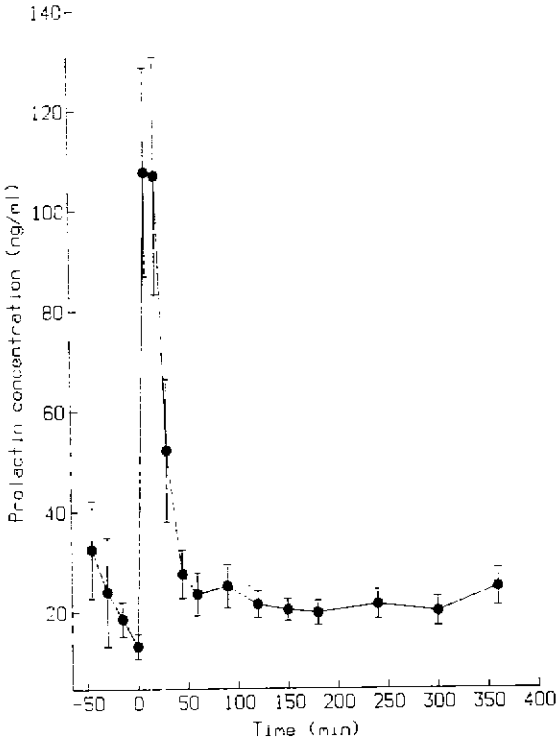

Fig. 4 Mean (± SEM, n = 10) plasma prolactin concentration in red deer stags following administration of 200 μg synthetic TRH i.v. (at Time = 0).

DISCUSSION

Prolactin radioimmunoasays generally involve centrifugation, iodination procedures, and long incubation times. An ovine prolactin ELISA assay has been validated here for the measurement of ovine and cervine prolactin and is specific, accurate, and precise, with measurements for assayed samples comparing favourably with those determined by a RIA for ovine prolactin (Fig. 3). This assay was developed along similar lines to reported assays for progesterone, cortisol, and testosterone for several reasons that have been outlined by Elder & Lewis (1985). The use of an immobilised antigen as opposed to an immobilised antibody means there is no risk of damaging the prolactin molecule by synthesising prolactin-enzyme conjugates and the first antibody can be utilised without wastage. ELISA assays are convenient as after addition of the first antibody, all subsequent steps are common to most other ELISAs currently being performed in this laboratory. Automation, and a high degree of precision in dispensing aliquots of reagents into microtitre wells, is possible (CV < 1 %) using the BEPM.

Studies have shown that plasma prolactin levels are higher in summer than winter in many animal species (*see* Introduction). Our assay results agreed with this, with plasma prolactin levels decreasing about 10-fold between summer and winter. Many studies have shown that intravenous TRH is a potent stimulus for the transient release of prolactin from the pituitary gland (*see* Introduction). Our assay detected an increase in plasma prolactin concentration in stags following administration of TRH (Fig. 4). The considerable variation in the mean prolactin response resulted from some of the animals taking longer (> 10 min) to respond maximally to TRH. TRH also induces TSH release (McNeilly & Baird 1983); however, the lack of any cross-reactivity with TSH in the present assay (Fig. 2) confirms that the response recorded resulted from prolactin.

The inability of GH, TSH, LH, and FSH to inhibit antibody binding to the solid support antigen at the levels tested suggested that these hormones do not influence prolactin estimation in this system. GH showed some cross-reactivity (Fig. 2), but only at supra-physiological levels, e.g. 1000 ng of GH was necessary for the same final absorbance response as 10 ng of prolactin. Exogenous prolactin could be completely recovered after its addition to plasma so plasma proteins per se do not seem to interfere with the estimation of prolactin levels. Taken collectively, these findings indicate that the immunoreactive substance detected in ovine plasma is identical to prolactin. Thus the assay may be considered valid for measurement of ovine prolactin in plasma.

When cervine prolactin standard levels were interpolated from the ovine standard curve, they were found to have approximately half the potency of the ovine standards in their ability to displace the antibody, e.g. 4 ng/ml of cervine prolactin produced the same absorbance as 1.8 ng/ml of NIH ovine prolactin. There are two possible reasons for this. The purity of the cervine prolactin was not known, and impurities could cause the standard curve to overestimate consistently the amount of cervine prolactin in the standard. Alternatively cervine prolactin may not bind as avidly to the ovine antibody, therefore displacement from the solid support antigen would be less, giving an underestimate of plasma prolactin levels. This does not limit the usefulness of the assay as the parallel response of deer plasma (Fig. 1) showed that relative levels of cervine prolactin could be measured using the ovine standard curve. This was confirmed by the physiological results obtained following TRH challenge and in comparison of summer and winter levels.

Our ELISA was developed because of a need for a sensitive, quick, non-radioisotopic assay for the measurement of ovine and cervine plasma prolactin. ELISA assays for prolactin levels in blood of rats (Signorella & Hymer 1984) and humans (Ishikawa et al. 1982) have been reported but these do not meet the criteria of convenience and speed which are achieved in the assay described here.

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