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RADIOIMMUNOASSAY OF HUMAN PROLACTIN BASED ON A 13 AMINO ACID SYNTHETIC ANALOG OF THE AMINO TERMINUS

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SUMMARY: A 13 amino acid analog of the human prolactin amino terminus was synthesized, substituting tyrosine for valine at residue 13. The peptide was coupled to crystalline bovine serum albumin for antisera production. The peptide was used for iodination with ^{125}I, and displacement curves were found to be parallel when human prolactin and the synthetic peptide were compared as standards. The radioimmunoassay using the synthetic peptide has the advantages of purity in its roles as hapten in the antigen and as labelled peptide, of ease of iodination of the peptide, of its stability after iodination, and of obviating the need for native human prolactin. The radioimmunoassay is suitable for the measurement of human prolactin concentration in plasma.

The World Health Organization 29th Expert Committee on Biological Standardization authorized a collaborative study of human prolactin (hPRL)[1] in order to establish an International Reference Preparation. Das and Cotes (1) organized the study, and they noted that the estimates of prolactin content of human plasma and serum among the 15 participating laboratories showed good agreement in ranking order but only fair agreement in the numerical value of the estimates. Further, numerical agreement was poor between estimates of identical samples coded differently, illustrating "the difficulty in achieving continuity of estimates when any laboratory calibrates a replacement standard". Our group supplied human prolactin and rabbit antiserum both for that study and for the National Hormone Distribution Program. Although the publications of Kleinberg and Frantz (2), Forsyth and Myres (3) and Hwang et al. (4) clearly preceded that from our group by Sinha et al. (5), the fact that we were suppliers of materials gave us an unusual opportunity to consult with

[1]Abbreviation: hPRL, human prolactin.

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others on the difficulties entailed in measuring hPRL, especially in radioiodination. Some claimed hPRL would not iodinate. The more common complaints, however, indicated a strong tendency for aggregation of hPRL during iodination and problems with binding to antibody or with displacement from it. No complaints were directed to us about standards. The antisera we supplied had anti-hGH antibodies, but no problem from this was encountered.

This paper presents an attempt to solve the major problems of measurement of hPRL in plasma and serum. It entails the use of a synthetic 13 amino acid analog of the amino terminus of hPRL in which tyrosine is substituted for valine at position 13.

Materials and Methods

An hPRL analog, Leu-Pro-Ile-Cys-Pro-Gly-Gly-Ala-Ala-Arg-Cys-Gln-Tyr-OH, [hPRL (1-13*)] was synthesized by solid-phase peptide synthesis methodology of Merrifield (6) as modified by Ling et al. (7). The synthetic product possessed the correct amino acid composition, and the homogeneity was verified by reverse-phase high performance liquid chromatography. The structure of hPRL was taken from Cooke et al. (8). Tyrosine was substituted for valine at position 13 so that the peptide could be prepared for immunization by coupling to a protein carrier, bovine serum albumin, with bis-diazotized benzidine in a modification of the procedure of Guillemin et al. (9). The peptide-protein conjugate was emulsified in Freund's complete adjuvant and, in dosages of 2 mg, was injected into the exposed retropopliteal lymph nodes of the hind limbs of 3 New Zealand male rabbits. Two additional subcutaneous immunizing doses of the same amount of antigen but in incomplete adjuvant were administered fortnightly starting one month later. The immunization procedure was that of Sigel et al. (10).

The tyrosine addition permitted radioiodination of the peptide, and this was performed with lactoperoxidase-glucose oxidase. The enzymobead radioiodination reagent (Bio-Rad Laboratories, Richmond, CA) was employed with slight modification for small peptides. The radioiodinated peptide was purified over a column of Sephadex G-25. The first major radioactive peak was found to bind to antibody. The selection of the antiserum to be used was made by testing binding of the radioiodinated peptide to dilutions of antisera collected from each rabbit two weeks after the third immunization. The antisera initially were tested at 1:100, 1:1000 and 1:10000 dilutions; the diluent consisting of 0.2 g of bovine serum albumin and 10 ml of normal goat serum in veronal buffer (9.2 g of 5,5'-diethylbarbituric acid, sodium salt, 1.5 g of 5,5'-diethylbarbituric acid, and 0.1 g of sodium azide per liter, pH 8.6). Separations of bound and free labelled peptide were made by the charcoal method of Herbert et al. (11) with the current modification that dextran was omitted. The final assay condition which proved practical was a three-day pre-incubation of the combination of 350 µl of antibody at initial dilution of 1:7,000 with 50 µl of standards or plasma. On Day 4 100 µl of ^{125}I hPRL(1-13*) at 10,000 counts per minute was added. Separation of antibody-bound and free labelled peptide was achieved by charcoal adsorption 24 hours later.

Both syn-hPRL(1-13*) and native hPRL, obtained from the National Hormone and Pituitary Program, (NIH-hPRL-VLS$_4$), were used as standards. Each was dissolved in buffer in concentrations ranging from 0.078 to 8.702 picomoles/ml.

In both procedures ^{125}I-hPRL(1-13*) was used as labelled antigen and the rabbit antibody employed was that which had been raised against the synthetic hPRL(1-13*)-BSA complex.

Plasma samples which had been stored frozen were assayed. Data are expressed as picomoles per ml. The plasma values of hPRL in nanograms per ml may be obtained by multiplying the numerical value in picomoles/ml by 23.

Results

The notion that hPRL could be measured in plasma or serum by a radioimmunoassay for the amino terminus of the molecule rested on the assumption that the amino terminus is an exposed portion of the molecule. The results in Fig. 1 of curves in which hPRL itself or the synthetic analog of the amino terminus were employed as standards validate the supposition. The curves show reasonable parallelism, with hPRL being slightly more potent in displacement activity than the synthetic analog. The range of the assay is broad.

In Fig. 2 are shown the results in picomoles/ml of measurement of hPRL in plasma or serum. On the abscissa the estimates for hPRL are derived from the data based on hPRL as standard, and on the ordinate, from synthetic peptide as standards. The estimates using the two standards show good agreement generally both in the

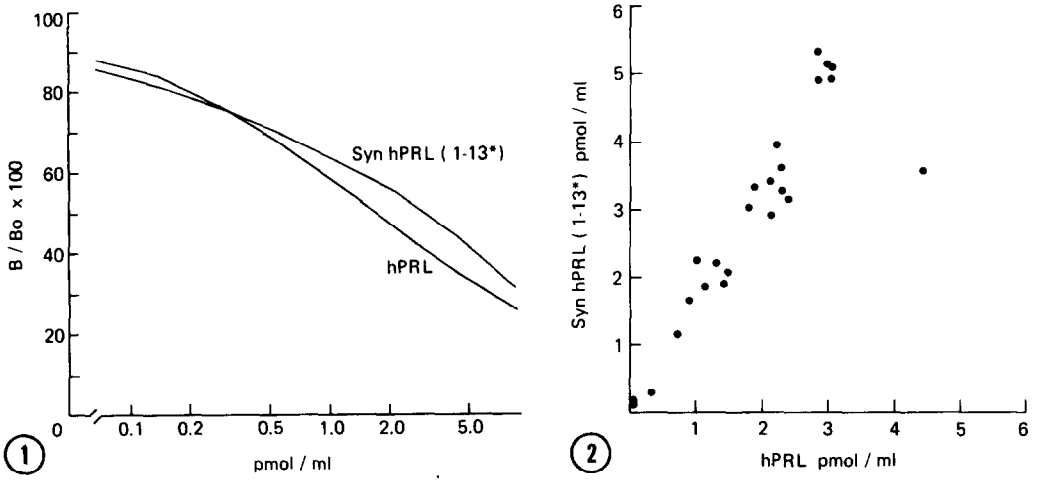

Fig. 1. Two standard curves are shown for displacement of ^{125}-hPRL(1-13*) by the synthetic peptide and by hPRL, plotted as log dose in picomoles/ml on the abscissa and B/Bo on the ordinate. In the expression, B/Bo, B = B-NSB/T-NSB of each test material and Bo = B/T of the zero dose level after correcting for non-specific binding, NSB. Here, B = counts/min. of ^{125}I-synhPRL(1-13*) bound to antibody. NSB = counts/min of bound in absence of antibody. T = total counts per min added per tube. In 4 assays maximum binding corrected for NSB was from 12.86% to 13.96%.

Fig. 2. Plasma values through the range of the assay are based on hPRL standard on the abscissa and on synhPRL(1-13*) on the ordinate. The regression line has the slope of 1.30, y-intercept = 0.50, and correlation = 0.88.

ranking order and in the numerical value. The regression line has the slope of 1.30 and intercepts the y axis at 0.50.

Discussion

A basic hypothesis in the present study is that the amino acid terminus of hPRL is exposed. The data presented here indicate that this is the case, for hPRL displaces the synthetic labelled peptide from antibody raised against the small peptide with roughly equal potency and in parallel.

The advantages in the use of the synthetic peptide include the ease of iodination and the apparent stability of the iodinated product. The analog of the amino terminus was chosen in order to have a tyrosine both for coupling to bovine serum albumin and for radioiodination. It obviates the need for purified pituitary hPRL for measurements of hPRL concentrations in blood, since the synthetic analog is suitable for raising antibody after coupling, for radioiodination, and for standards in the construct of displacement curves. The use of a small synthetic peptide with only a single tyrosine provides uniformity of labelling, which may lessen interassay variation. Variability observed in the usual polyclonal antibody is obviated, lending to the procedure some of the advantage of the monoclonal technique and, in addition, quite precise identification of the antigenic site.

We conclude that the amino terminus in native hPRL is exposed and that the approach to hPRL measurement by radioimmunoassay is successful when a synthetic analog of the amino terminus is employed for raising antibody after the small peptide is coupled to a large protein and when the synthetic peptide is used both for radioiodination and for construction of standard displacement curves.

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