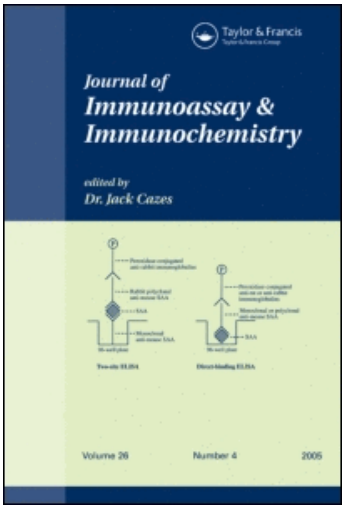

Journal of Immunoassay and Immunochemistry

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

Development of an Homologous Radioimmunoassay for the Synthetic Amino Terminal (1-34) Fragment of Human Parathyroid Hormone Using Egg Yolk-Obtained Antibodies

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To cite this Article Vieira, Jose Gilberto H. , Oliveira, Marli A. D. , Maciel, Rui M. B. , Meaquita, Carlos H. and Russo, Ewaldo M. K.(1986) 'Development of an Homologous Radioimmunoassay for the Synthetic Amino Terminal (1-34) Fragment of Human Parathyroid Hormone Using Egg Yolk-Obtained Antibodies', Journal of Immunoassay and Immunochemistry, 7: 1, 57 — 72

To link to this Article: DOI: 10.1080/01971528608063046

URL: http://dx.doi.org/10.1080/01971528608063046

JOURNAL OF IMMUNOASSAY, 7(1&2), 57-72 (1986)

DEVELOPMENT OF AN HOMOLOGOUS RADIOIMMUNOASSAY FOR THE SYNTHETIC AMINO TERMINAL (1-34) FRAGMENT OF HUMAN PARATHYROID HORMONE USING EGG YOLK-OBTAINED ANTIBODIES

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ABSTRACT

An homologous radioimmunoassay for the synthetic 1-34 amino terminal fragment of human parathyroid hormone (hPTH) was developed using antibodies obtained from yolk of eggs layed by an immunized chicken. The ^{125}I labelled 1-34 hPTH peptide was purified by cation-exchange chromatography which provided a highly stable preparation. The specificity of the assay showed a cross-reactivity of 50% with the 1-34 hPTH (code 81/574) preparation and 27% with the 1-84 hPTH (code 79/500) preparation, when compared with the 1-34 hPTH from Bachem that was used for immunization and labelling. The minimal detectable dose of the assay was 10 pmol/l; in 69 healthy controls the values obtained ranged from <10 to 28

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pmol/l and in 14 patients with surgically proven primary hyperparathyroidism from 10 to 519 pmol/l. (Key words: Parathyroid hormone, Radioimmunoassay, Egg Yolk derived antibodies, Amino terminal specificity.)

INTRODUCTION

The ideal immunoassay is the one that can measure the biologically active molecule, thereby obtaining a high correlation with the bioassay. Concerning human parathyroid hormone (hPTH) the situation is particularly difficult since most of its circulating hormonal forms are biologically inactive mid and carboxyl-terminal fragments (1). The active forms comprise the intact molecule and the amino-terminal fragment; both are short lived (2) rendering the development of an immunoassay a difficult task. The elucidation of the amino acid sequence of the hPTH molecule (3,4) provided a new tool for the development of assays since synthetic peptides can be use for the generation of specific antibodies, and also for labelling and as standard.

The limited number of publications dealing with the development of specific amino-terminal hPTH assays (5,6,7,8,9) are noteworthy. It denotes not only the difficulties in producing high affinity antibodies against the 1-34 hPTH peptide, but also the technical problems in labelling that peptide, and the low serum levels of biologically active hPTH (10).

Chickens have been used as a source of antibodies for the development of radioimmunoassay methods for hPTH (11,12,13) and we have recently reported the possibility of extracting specific antibodies from the yolk of eggs layed by immunized chickens (14).

We report herein the results obtained in the development of a radioimmunoassay (RIA) for the hPTH 1-34 fragment using antibodies obtained from yolk of eggs layed by a chicken immunized with the synthetic peptide, and preliminary results with its application.

MATERIALS AND METHODS

Reagents:

Synthetic 1-34 hPTH used for immunization and iodination was purchased from Bachem Inc., Torrance, California, U.S.A. Synthetic 28-48 hPTH was also obtained from Bachem Inc., and the 1st International Reference Preparation of Parathyroid Hormone, human, for immunoassay (1st IRP, established 1981), code number 79/500, as well as the synthetic 1-34 hPTH, NIBSC reagent 81/574, were obtained from the National Institute for Biological Standards and Control (NIBSC), Holly Hill, Hampstead, London, U.K.. Sheep anti-chicken IgG was obtained in our laboratory using egg yolk DEAE-cellulose purified chicken IgG as immunogen. Unless otherwise stated, all the other reagents were obtained from Merck Indústrias Químicas, Rio de Janeiro, Brasil.

Production of Antisera:

Three chickens were immunized by multiple intradermal injections with 50µg of synthetic 1-34 hPTH emulsified in 1 ml of complete Freund's adjuvant. They were reimmunized at monthly intervals, for three months, with the same dose of 1-34 hPTH, and blood samples (from a wing vein) were obtained monthly after the third injection. Eggs layed by these chickens were also collected during the period of immunization and after that for a period of 18 months. The egg processing for extraction of yolk IgG was performed according to our previously published method (14). Antibodies were produced by the three chickens although only one showed sufficient titer and affinity to be used for the development of the radioimmunoassay. It is noteworthy that this chicken maintained a stable titer during the whole study and curves obtained using serum or egg yolk IgG were almost superimposable (14). As we obtained much more antibodies from the eggs, all the assay standardization studies were done with a pool of egg yolk IgG.

Iodination Procedure:

The peptide was labelled by the chloramine T method (15) used as follows: 50µℓ of phosphate buffer, 500 mmol/l, pH 7.4 and Na ^{125}I (0.5 mCi, Amersham International, Buckinghamshire, UK) were added to 10µℓ of 100 mmol/l acetic acid solution containing 0.6µg of the peptide (1-34 hPTH). Chloramine T (50µg) in 10µℓ phosphate buffer 50 mmol/l, was then added followed by an oxidation time of 30 seconds at room temperature. Sodium metabisulphite (150µg) in 50µℓ of phosphate buffer 50 mmol/l was then used to stop the reaction. The final reaction product was applied to a 0.9x30cm column of CM-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with citrate buffer 10 mmol/l, pH 6.0, and eluted, at room temperature, using a continuous NaCl gradient to 250 mmol/l. Fractions of 1.5 ml were collected and the velocity of elution was calibrated to 0.125 ml per minute. The fractions containing the labelled immunoreactive peptide were pooled, and aliquots of 200µℓ were stored at -20°C.

Serum Samples:

Serum was obtained from 69 normal volunteer out-patients and preoperatively from 14 patients with surgically confirmed primary hyperparathyroidism. Serum samples were kept at - 20°C until assayed.

Assay Procedure:

The buffer used throughout (assay buffer) was phosphate 20 mmol/l, NaCl 140 mmol/l, BSA 5g/l, EDTA 50 mmol/l, Trasylol (Bayer do Brasil S/A, São Paulo) 500 IU/ml and NaN3 1g/l, pH 7.4. Standard curves were prepared by diluting the stock solution of the 1-34 hPTH peptide in assay buffer, to obtain a solution of 250 pmol/l that was thereafter diluted up to 15 pmol/l. 0.1 ml of the appropriate dilutions of the peptide, or serum sample, were incubated with 0.1 ml of an antibody solution calibrated to produce 15-25 percent binding when no unlabelled peptide was present. Following an incubation at 4°C for 2 days, 0.1 ml of the

labelled peptide (4000 to 6000 cpm, approximately 5 fmol) was added and the incubation continued for 2 additional days at 4°C. Separation of bound and free ^{125}I peptides was achieved by the addition of 0.1 ml of sheep anti-chicken IgG serum (final dilution 1/80). After a 60 minutes incubation at 4°C, 0.1 ml of PTH-free serum (charcoal treated outdated serum) was added, followed by immediate addition of 1 ml of a 64 g/l solution of PEG 4000 (Atlas Ind. Quim. Mauá, São Paulo, Brasil), mixing and centrifugation at 2500 rpm for 30 min. at 4°C. After the removal of the supernatants, the pellets were counted in a scintillation spectrophotometer and the results were calculated as described below.

Serum calcium was determined routinely using an atomic absortion spectrophotometry method.

Ria Calculations:

All 1-34 hPTH curve-fitting and dose-interpolation have used the non-linear four-parameter logistic model (16), according the equation

$$B = \frac{BO - BN}{1 + \left(\frac{[hPTH]}{ED_{50}}\right)^{b}} + BN$$

where [hPTH] is the standard or sample hPTH concentration; B is the bound fraction radioactive count rate response; BO and BN are the responses when [hPTH] = 0 and [hPTH] $\to \infty$ ("infinitive" dose), respectively; b = exponent parameter associated with the curve slope; and ED_{50} is the dose when $\% \frac{B - BN}{BO - BN} = 50\%$

RESULTS

Iodination:

The elution profile of a CM-Sephadex C-25 chromatography of a typical iodination is shown in Figure 1. Three peaks of radioactivity are clearly distinguishable: the first (peak 1) is

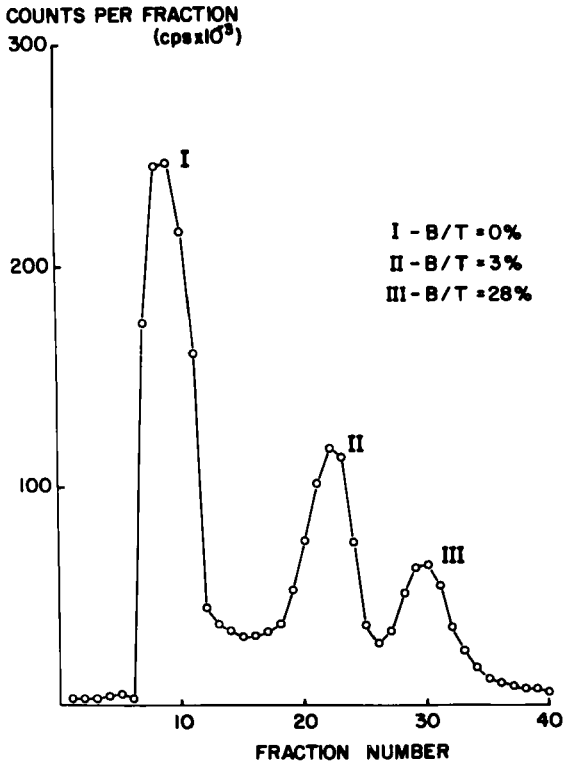

Figure 1. Elution profile of a CM-Sephadex C-25 chromatography of the iodinated products obtained after Chloraminte T radioiodination of hPTH (1-34). B/T indicate the percent binding of the iodinated product obtained under normal assay condition.

composed by the unreacted ^{125}I, the second (peak II) showed a low B/T (3%) ratio being probably composed by damaged 1-34 hPTH peptides that had their structure modified by oxidation and/or overiodination, thus explaining its low immunoreactivity. The third peak (peak III) comprises the 1-34 hPTH with good immunoreactivity that showed excellent stability for at least 4 weeks stored in small aliquots at - 20°C.

Assay Characteristics:

The egg yolk-derived antibody was used in a final dilution of 1:1800. The minimal detectable dose of the assay (table 1), defined as the dose level which results in an expected response which is statistically different from the expected response for a zero dose tube, was 10 pmol/l (41 pg/ml). The study of the specificity of the assay (Figure 2), using the Bachem hPTH 1-34 peptide as reference, showed a cross-reactivity of 50% for the 1-34 hPTH (code 81/574), 27% for the 1-84 hPTH (code 79/500) and undetectable for the 28-48 hPTH. Figure 2 also shows a good parallelism between the different standards and aliquots of serum from a patient with surgically proven primary hyperparathyroidism.

The overall performance of the assay can be analysed by the data contained in Table 1 and by the precision profile shown in Figure 3. We also studied the within-run and between-run precisions with serum pools with mean values of 57, 30 and 14 pmol/l. The within-run coefficients of variation were 8.3, 11.7 and 11.7% and the between-run coefficients of variation were 12.2, 13.4 and 14.9% respectively.

Clinical Use:

The results obtained in the 69 healthy controls and 14 patients with surgically proven primary hyperparathyroidism are shown in Figure 4. In the healthy controls the serum values ranged from <10 to 28 pmol/l, and in hyperparathyroid patients from <10 to 519 pmol/l. There was an overlap between the values of the two groups, since 4 of the 14 patients (29%) with primary hyperparathyroidism had values of 1-34 hPTH within the normal range. This overlap can be completely eliminated if we consider the concomitant serum calcium levels, since all the 4 patients had hypercalcemia. Another remarkable point was that the majority of the normal controls (75%) had undetectable levels of 1-34 hPTH with this assay.

Table 1

Quality Control Parameters of the hPTH (1-34) Assay (n=7)

	B0/T	exponent b parameter	ED_{50} dose	BN/T response at "infinitive" dose	Minimal detectable dose
mean	21%	1,86	40 pmol/l	1,5%	10 pmol/l
range	17 to 24%	1,61 to 2,5	35,5 to 57,1	1 to 2%	7 to 13 pmol/l

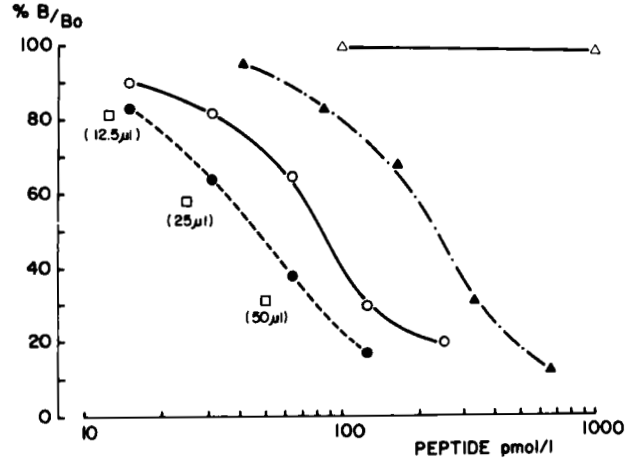

Figure 2. Dose response curves for hPTH 1-34 [Bachem (●), hPTH 1-34 (code 81/574) (o), hPTH 28-48 (Δ), hPTH 1-84 (code 79/500) (▲) and hyperparathyroid serum (=)].

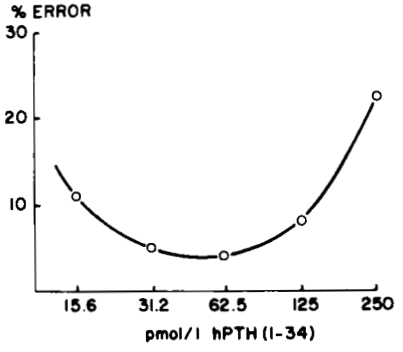

Figure 3. Precision profile obtained with a typical hPTH (1-34) assay using standard assay conditions.

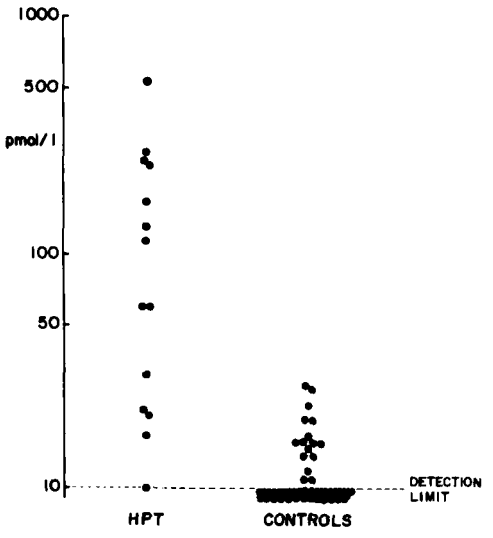

Figure 4. Serum parathyroid hormone measured with the hPTH (1-34) assay system in 69 healthy controls and 14 patients with surgically proven primary hyperparathyroid (HPT).

DISCUSSION

Based on the work of Jensenius et al (17) we have recently reported on the possibility of obtaining specific anti-hPTH Ig from the yolk of eggs of chickens that were good responders (14). In fact, the chosen chicken layed dozens of eggs during the period of study, enabling us to obtain almost 1 liter of IgG preparation with a titer equivalent to half of that obtained in the serum of the same animal. In spite of the relatively low titer (1:1800), the good volume of IgG preparation obtained prompted us to continue with the standardization of an assay using the mentioned antibody source.

The first problem we faced during the development of a 1-34 hPTH specific assay was the labelling of the 1-34 synthetic peptide. This peptide, besides having no tyrosine, has two

methyonine residues that are very prone to oxidation under standard ^{125}I labelling conditions. The transformation of methionines at positions 8 and 18 to methionine sulfoxide or sulfone results in loss of biologic activity (18,19) and possibly in its immunoreactivity. In fact, a simple gel filtration of the iodination reaction products in a Sephadex G-15 column (14) yielded only two peaks of radioactivity; the first one was the labelled peptide, which needed to be rechromatographed weekly in order to obtain a labelled material of acceptable quality. Assuming that some changes in the peptide net charge could have been induced by the oxidation and/or iodination, we tried a cation-exchange column chromatography to purify the iodination products. The results obtained allowed us to get rid of a significant amount of labelled petide (peak II in Figure 1) with almost no immunoreactivity. The ^{125}I-labelled 1-34 hPTH obtained (peak III in Figure 1) could be used during at least four weeks without needing any further purification.

The specificity of the assay (Figure 2) showed interesting characteristics: 1) the cross-reactivity of only 50% for the 1-34 hPTH (code 81/574) demonstrates that this preparation and the one from Bachem are somewhat different; this can be explained by a difference in purity of the preparations or that the Bachem preparation has, for some reason, a different conformational configuration, better recognized by the antibodies; 2) the low cross-reactivity (27%) observed for the 1-84 hPTH (code 79/500) can be explained, at least in part, by the different spacial conformation of the 1-34 hPTH peptide when it forms a part of the complete 1-84 hPTH peptide. This observation can explain the fact that 75% of the normal controls had undetectable hPTH levels.

The presence of amino-terminal fragments with biological activity in circulation, was demonstrated by Goltzman et al (10), using a very sensitive cytochemical bioassay. Nevertheless it accounted for only a small percentage of hPTH bioactivity in normal circunstances, increasing to significant levels only in patients with uremia.

The results obtained in the 14 patients with primary hyperparathyroidism are quite acceptable, especially if one consider that these results were obtained in single samples for all the patients. Multiple sampling could have improved the differentiation between the two groups (20). The necessity of a concomitant analyses of serum hPTH and calcium levels to optimize the diagnoses of primary hyperparathyroidism is common to almost all the hPTH assays (21,22,23,24,25).

The assay described, with its high specificity for the 1-34 hPTH peptide, could be of interest in some especial situation, as the study of parathyroid function in patients with chronic renal failure, where amino-terminal fragments with biological activity are present in the circulation and where carboxyl-terminal assays are misleading because of the retention of carboxyl fragments that occurs in these circunstances (2,26,27).

ACKNOWLEDGEMENTS

We thank Dr. Claudio E. Kater for his help in preparing this manuscript. We also thank the Laboratório Fleury, São Paulo, Brazil, for the technical support.

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