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A C-Terminal Radioimmunoassay for Human Parathyrin

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Several features concerning the parathyrin (PTH) radioimmunoassay (RIA) in human serum have been considered. The bovine hormone (bPTH) was used as standard and to obtain the radioiodinated tracer and the antisera employed exhibited a C-terminal specificity. A modification of the classical Greenwood and Hunter method was used to label bPTH, based on the sequential addition of small amounts of Chloramine-T to the solution containing the hormone to prevent ovariodination. The purification by Quso G32 followed by a gel-filtration step, produced a suitable tracer for a highly sensitive RIA. Due to unespecific effects from serum in the incubation medium, serum concentration was rendered uniform in all the assay tubes prior to the addition of the separation mixture (charcoal-dextran). The greatest sensitivity reached was 16 pg of bPTH in the incubation mixture with Ch 4M antiserum. This antiserum allows detection of immunoreactive PTH in the majority of normal subjects and has applicability to a wide range of bone disturbances.

Since BERSON *et al.* (2) described a radioimmunoassay (RIA) for detection of parathyrin (PTH) in human serum, many modifications have been reported. The presence of interferent serum substances, the lack of adequate standards, the poor cross-reactivity between commonly heterologus antisera and human PTH(hPTH), the lability of radioiodinated tracer and the immunological heterogeneity of PTH in serum and glands are among the most common difficulties encountered (1, 6, 9, 14). The present paper describes a modified RIA method to measure immunoreactive PTH(iPTH) in human serum by using different antisera against the C-terminal region of bovine PTH(bPTH).

Materials and Methods

Radioiodination of bPTH. Highly purified bPTH (Inolex, Chicago) kept in 10 mM acetic acid and ^{125}I as $Na^{125}I$ (Radiochemical Centre, Amersham), were used for radioiodination. A modification

of the classical GREENWOOD and HUNTER method of labeling (10) has been employed. The labeling reagents were sucked in a polyethylene tube (Portex 1, Portland Plastics, Hythe Kent, England) in the following sequence: 5 μl air; 25 μl 0.2 M phosphate buffer, pH 7.5 (labeling buffer); 5 μl air; 5 μl Chloramine-T solution (0.5 μg dissolved in labeling buffer); 5 μl air; 0.8 mCi ^{125}I (7-10 μl) and 10 μl air. Subsequently they were expelled into the reaction vial containing 2 μg of bPTH diluted with 50 μl of labeling buffer. After 20 seconds, an aliquot of the reaction mixture was taken out and the percentage of iodine incorporation was determined by precipitation with trichloroacetic acid (TCA).

The procedure was repeated by adding a new amount of Chloramine-T until about 40 % of incorporation was reached. Normally, two sequential additions of 0.5 μg of the oxidizing reagent were needed, in no more than 30 minutes. During labeling, the reaction mixture was maintained in an ice bath.

Purification of ^{125}I-bPTH. The labeled hormone was purified by adding 10 mg of precipitated silica (Quso G32, Philadelphia Quartz) to the content of the reaction vial (16). The mixture was shaken and centrifuged at 3,000 rpm for 5 min at 4° C and the supernatant discarded. The pellet was washed with 1 ml of distilled water and a small amount of an anion-exchange resin (AG 1-X 10, 100-200 mesh, Bio-Rad, Richmond, Calif.) was added to remove free iodide. The mixture was centrifuged thereafter and the tracer was dissolved in 1 ml of an aqueous solution of acetone/acetic acid (20 %/1 %, v/v). After a new centrifugation, the organic supernatant was diluted with assay diluent (see below) and fractionated in aliquots which were snap-frozen in dry ice/acetone and kept at —20° C.

Before use, the tracer was usually further purified through a 50 × 1 cm Sephadex G-100 (Pharmacia, Uppsala) column eluted with 50 mM sodium veronal buffer, pH 8.6.

Titration and specificity of antibodies. Cb 9 and Ch 4M antisera were generously supplied by Dr. E. SLATOPOLSKY (Washington U. Sch. Med.) and Dr. C. ARNAUD (Veterans Ad. Hosp., San Francisco). These antisera were found to show C-terminal specificity.

Serial dilutions of every antiserum were incubated with various amounts of tracer in the usual conditions of the assay (see below). A dilution allowing a tracer binding between 35 and 50 % was selected for each antiserum.

Conditions of the assay. The RIA scheme is shown in table I. The following amounts of cold hormone were used for the standard curve: 6.25; 12.5; 25; 50; 75; 100; 150; 200; 400; 750 and 1,000 pg *per* tube. They were freshly prepared from a stock solution containing 2,000 μg in 100 μl of 10 mM acetic acid. The assay diluent was 50 mM sodium veronal buffer, pH 8.6 with 10 mM EDTA, 10 % hypoparathyroid serum and 500 U/ml trasylol.

After 3 days of preincubation at 4° C, about 5,000 cpm (or 30 pg) of tracer were added and the reaction continued for another 3 days at 4° C.

Separation of bound and free fractions. A modification of the dextran-coated charcoal method described by HERBERT *et al.* (11) was employed. After the incubation period, 1 ml of a cold (4° C) charcoal (Sigma)-dextran T-70 (Pharmacia) suspension (2.5 %-0.5 %) was added to the content of each assay tube. After centrifugation at 3,000 rpm for 15 min at 4° C, supernatants were sucked off and the pellets counted in a Nuclear Chicago γ counter with an approximate efficiency of 70 %.

Table I. *General scheme for PTH radioimmunoassay.*

	C_o (µl)+	B_o (µl)+	St (µl)+	C_{St} (µl)+	S (µl)++	C_S (µl)++
Assay diluent *	400	300	200	300	200	300
PTH standard	—	—	100	100	—	—
Serum sample **	—	—	—	—	100	100
Antiserum	—	100	100	—	100	—
Tracer	100	100	100	100	100	100

+ Triplicates; ++ duplicates; * see text; ** undiluted or conveniently diluted.

Calculations. Standard plots were commonly done representing % B (% B= 100 — % F) *versus* cold hormone concentration, where B and F are counts bound and free respectively. Counts were corrected for unspecific binding in the absence of antibody.

The following aspects of the method were considered: Parallelism between serum samples and standard curve, recovery, sensitivity and precision.

Results

A stable tracer for 15-20 days after iodination with a specific activity of 150-200 mCi/mg, was usually obtained by using the method of labeling described above.

The limited capacity of Quso G32 for completely cleaning the tracer required an additional purification step by gel-filtration (fig. 1). Uniform immunoreactivity through the ^{125}I-bPTH chromatographic peak was found with several C-terminal antisera when Sephadex G-100 was used. Tracer adsorption to the gel matrix was found to be independent of the presence of proteins in the eluting buffer but it was dependent on pH, being lower in an acidic medium.

The effect of serum concentration on the RIA system with Ch 9 antiserum is showed in figure 2. Increasing amounts of serum in the incubation mixture produce a corresponding increase on tracer bin-

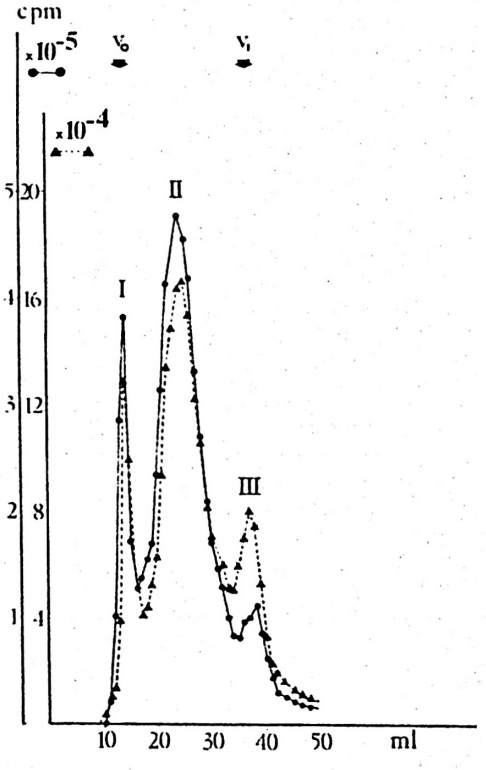

Fig. 1. *Sephadex G-100 purification of ^{125}I-bPTH, two days (continuous lines) and seventeen days (dashed line) after labelling.*

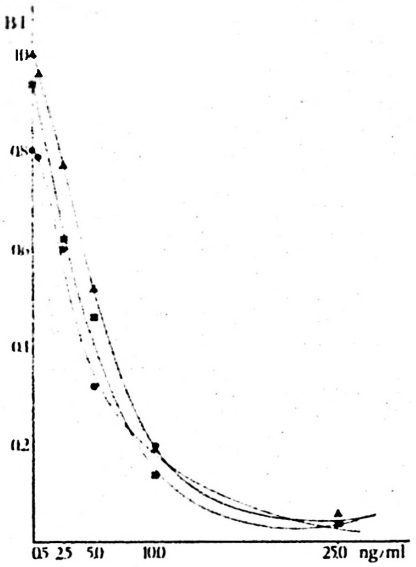

Fig. 2. *Unspecific effects of serum on ^{125}I-bPTH binding to Ch 9 antiserum, 1/600 initial dilution.*
Standard curves were performed at three different serum concentrations: ▲ 46 %; ■ 28 % and ● 10 % *per* tube.

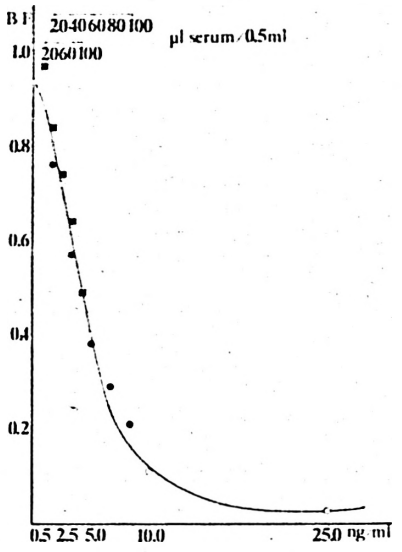

Fig. 3. *Parallelism between serial dilutions of serum with high IPTH content and standard curve.*
● ■: Serum samples of patients with primary hyperparathyroidism, ○: cold bPTH. Ch 9 antiserum, 1/600 initial dilution.

ding. Figure 3 shows the parallelism between different dilutions of sera from patients with primary hyperparathyroidism (containing high amounts of iPTH) and a standard curve, with the same Ch 9 antiserum.

Neither Ch 9 nor Ch 4M antisera recognize the synthetic N-terminal peptide hPTH 1-34 (Ciba-Geigy, Basel) and thus their C-terminal specificity in our RIA system was confirmed.

Ch 4M antiserum was the most widely

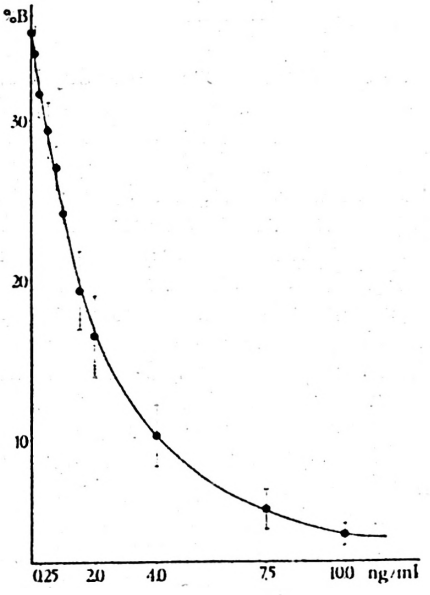

Fig. 4. *Typical standard curve obtained by poltting % B versus cold hormone concentration.*
^{125}I-bPTH was the tracer and Ch 4M antiserum was used at an initial dilution of 1/750. Points and bars are respectively the mean and the standard deviation (n = 5).

used for clinical purposes. The high affinity of this antiserum (apparent affinity constant, $K_{ap} = 2.3 \times 10^9$ l/mol) allows to detect an amount as 16 pg of unlabeled bPTH in the incubation mixture. This sensitivity was calculated from the error in the «zero» point (in the absence of cold hormone) of the standard curve (12).

The percentage of added cold hormone recovered was between 75 and 125 % along the linear zone of the standard curve in different assays. Intra-assay coefficient of variation (V) was ≤ 10 % for hormone concentrations corresponding to the linear part of the standard curve. V values for standard curves made on different days depended on cold hormone concentration, being 3 % for «zero» dose and 22 % for 750 pg (fig. 4). V values for serum samples with high and normal iPTH content never exceeded 16 % in different assays.

Discussion

The stoichiometric technique previously described for the iodination of other polypeptide hormones (13) was adopted for bPTH after considering its kinetics of labeling (8). Furthermore, the classical Greenwood and Hunter method of labeling led to great damage when applied to this hormone (7). In this way it has been possible to control iodine incorporation to the hormone molecule and thus avoiding overiodination of the single tyrosine residue present in its primary structure, which is known to produce immunoreactivity loss (3).

Among the different methods used to purify the tracer, adsorption to silica (Quso G32) followed by repurification through a gel column was found to be the best. The observed pH effect on tracer adsorption to Sephadex G-100 was found to be independent of eluting buffer protein composition. This effect might be related to ionic changes in the primary structure of the hormone molecule (5). In our experience chromatoelectrophoresis does not appear to be a suitable method to purify the tracer, in agreement with previously reports (4, 8).

In spite of the relatively low titer of the C-terminal antisera Ch 9 and Ch 4M, their affinity and specificity make them useful to detect a few picograms of iPTH in serum.

The high serum concentration in the reaction mixture, the relatively low titer of the antiserum and the sensitivity of the charcoal-dextran method for separation of bound and free fractions, are possible reasons for the unspecific effects observed when serum or plasma samples are tested by RIA (15). The most reliable method for overcoming these problems lies in ensuring that protein concentration is the same in all the assay tubes before adding the separation mixture.

A non-equilibrium assay consisting of 3 days of preincubation without tracer followed by an incubation of another 3 days with tracer, both at 4° C, was found to increase sensitivity to detect iPTH not only in different bone disturbances but also in most of the normal subjects (8).

Nonetheless, interlaboratory comparison studies performed by the EPSG (European PTH Study Group), a team in which our laboratory participates, has unequivocally shown the impossibility to compare PTH values from different laboratories using similar RIA techniques but different standard reagents (9). Therefore, use of the same basic components such as standard hormone, antiserum and tracer is needed for clarification of the controversial PTH values found by different groups in several pathological conditions.

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Resumen

Se consideran diversos aspectos concernientes al radioinmunoensayo (RIA) de parathormona (PTH) en suero humano utilizando la hormona de origen bovino (bPTH) como estándar y para la obtención del trazador iodado, y un antisuero de especificidad C-terminal. El marcaje de bPTH utilizando una modificación del método clásico de Greenwood y Hunter, consistente en la adición de pequeñas cantidades del agente oxidante Cloramina-T a la solución que contiene la hormona, permite controlar la incorporación del iodo y evitar la sobreiodación del péptido. La purificación con Quso G32 seguida de repurificación a través de una columna de gel, conduce a un trazador adecuado para conseguir un RIA de gran sensibilidad. La consideración de los efectos inespecíficos del suero en el medio de incubación, hace necesario igualar el contenido sérico en todos los tubos del ensayo previamente a la adición del medio de separación, carbón-dextrano. Utilizando el antisuero Ch 4M, la sensibilidad máxima alcanzada es de 16 pg de bPTH en la mezcla de incubación. Este antisuero permite la detección de PTH inmunoreactiva en la mayoría de los sujetos normales y es de gran aplicabilidad en la fisiopatología ósea.

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