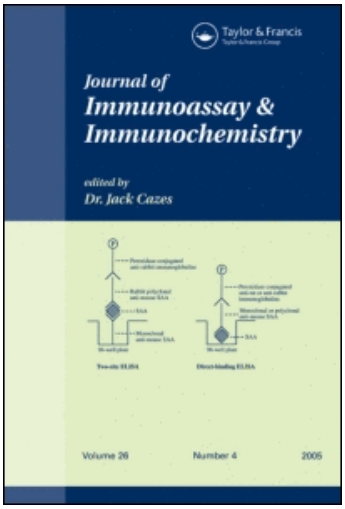

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An Homologous and Sensitive Radioimmunoassay for the Synthetic Amino-Terminal (1-34) Fragment of Human Parathyroid Hormone: Application to the Clearance of This Peptide Administered in Vivo

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AN HOMOLOGOUS AND SENSITIVE RADIOIMMUNOASSAY FOR THE SYNTHETIC AMINO-TERMINAL (1-34) FRAGMENT OF HUMAN PARATHYROID HORMONE: APPLICATION TO THE CLEARANCE OF THIS PEPTIDE ADMINISTERED IN VIVO

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ABSTRACT

The synthetic 1-34 amino-terminal fragment of human parathyroid hormone (hPTH 1-34) is undergoing multicentre clinical trials to assess its long term therapeutic potential in the treatment of osteoporosis. An homologous radioimmunoassay (reagents prepared from the synthetic hPTH 1-34 peptide) has been developed to monitor the pharmacokinetics of hPTH 1-34 in man and in a dog model. The assay is rugged, sensitive (detection limit 1.75×10^{-11} moles/litre) and precise (coefficient of variation 6%). Three different ampouled preparations of the native intact hPTH 1-84, of different degrees of purity (approximately 3%-90% pure) gave complete log dose response curves parallel to that of the ampouled synthetic hPTH 1-34 peptide, and were equipotent on a molar basis. Native intact bovine PTH 1-84 showed an incomplete non-parallel displacement curve; there was no recognition of synthetic hPTH 44-68 and 53-84 peptides. Preliminary application of the assay to the determination of the plasma disappearance of hPTH 1-34 in man and dog gave half-times ($t_{\frac{1}{2}}$) of 3-8 minutes for a first exponential component and 12-18 minutes for the second; in the dog, metabolic clearance rate was calculated to be 9ml/kg/minute and the distribution space 160ml/kg.

INTRODUCTION

It is generally accepted that the structural requirements for all known biological activities of the parathyroid hormone molecule reside in the amino-terminal 1-34 fragment (1,2). The synthetic 1-34 fragments of bovine and human parathyroid hormones have been prepared by commercial firms for experimental use (Beckman, Palo Alto, California, USA; Armour Pharmaceutical Co., Kankakee, Illinois, USA; Bachem Fine Chemicals, Torrance, California; Peninsula Chemicals, Palo Alto, California; Ciba-Geigy Ltd, Basle, Switzerland; Protein Research Institute, Osaka, Japan).

Human parathyroid hormone 1-34 (hPTH 1-34), synthesized according to the sequence proposed by Niall et al (3) by Armour Pharmaceutical Co., has been administered to man a) in a diagnostic provocation test in the differential diagnosis of hypocalcaemia (4) and b) in a multicentre clinical trial as an anabolic agent in the treatment of involutional osteoporosis (5,6, 7,8).

Peptide hormones are metabolically less stable than most classes of drugs due to their susceptibility to peptidase degradation, for example, at the subcutaneous site of injection (9,10). Thus maintenance of circulating bioactive concentrations within the therapeutic range necessitates a detailed knowledge of their absorption, distribution and clearance (11). An homologous immunoassay was required to investigate the pharmacokinetics of exogenously administered hPTH 1-34 in an experimental animal model (12,13) set up in conjuction with the clinical trials (8).

This paper describes the development of a radioimmunoassay (RIA) for the hPTH 1-34 fragment and preliminary results of its application.

MATERIALS AND METHODS

Tracer labelled ligand

Human PTH 1-34 (Batch No. K744-167) was synthesized by Armour Pharmaceutical Co., Kankakee Illinois. A working solution of this material was made by dissolving a weighed amount in 0.1M acetic acid to a concentration of 2.5×10^{-5} moles/L (100μg/ml); 20μl aliquots (5×10^{-10} moles ≡ 2μg) were snap frozen and stored at -40°C for radioiodination.

Radioiodination

The peptide was labelled with 37kBq (1mCi) ^{125}I (Radiochemical Centre, Amersham, Bucks, UK) by the chloramine T method (14) using 53.2μg chloramine T and 180μg sodium metabisulphite in 0.2M phosphate buffer, pH7.4 (reagents from BDH Chemicals Ltd, Poole, Dorset, UK) and an oxidation time of 30 seconds at room temperature. The reactants were then diluted with human plasma protein (Lister Institute, Elstree, Herts, UK) and unreacted iodide removed with AG1-X10 (100-200 mesh, Bio-Rad Laboratories, Bromley, Kent, UK). Radioiodinated peptide was retrieved either by separation on Biogel P2 (50-100 mesh, Bio-Rad) or by adsorption onto Quso (Quso G32, Philadelphia Quartz Co., Philadelphia, PA, USA) and subsequent elution according to a published method (15). The solution of ^{125}I hPTH 1-34 was snap frozen in 100μl aliquots. Before addition

to the assay, it was further purified on Biogel P10 (200-400 mesh, Bic-Rad) at +4°C and eluted with barbitone buffer at pH 8.6 containing 0.2% human plasma protein, previously shown to be free from peptidase activity (16).

Standard preparation of hPTH 1-34

An ampouled preparation of hPTH 1-34 (coded 75/596) of the Niall sequence (3), synthesized by Armour Pharmaceutical Co., was used as a standard. Each ampoule has a nominal content of 100µg of peptide, and was assigned a biological potency of 500 units on the basis of in vivo bioassays in the chick (17) against a preparation of highly purified native bovine PTH in ampoules coded 72/286. The hPTH 1-34, in ampoules coded 75/596, is used as the house standard for calibration, by bioassay, of all preparations of hPTH 1-34 used in the multicentre clinical trials (8).

It was assumed that each ampoule of 75/596 contained 2.5×10^{-8} moles hPTH 1-34, and for immunoassay purposes a working stock solution was made by initially dissolving the contents of an ampoule in 100µl 0.1M acetic acid with subsequent dilution using assay diluent to 5×10^{-6} moles/L (20µg/ml). This solution was further diluted to 1×10^{-7} moles/L (400ng/ml) and 50µl aliquots (5×10^{-12} moles ≡ 20ng) snap-frozen in glass tubes and stored at $-40^{\circ}C$

Antiserum

Antiserum G10/017 was generously provided by Dr C. Desplan and Professor M.S. Moukhtar, Hopital Saint Antoine, Paris, France, and was diluted, freeze-dried in ampoules at NIBSC with the code

number 79/533. The antiserum was raised in the goat against hPTH 1-34 (Niall sequence) and its properties and use in assays of endogenous human PTH have been previously described (18,19). In our laboratory, the antiserum was routinely used at a final dilution of 1/600,000.

Radioimmunoassay

A 3+3 day delayed tracer addition system was used; all manipulations were carried out on a cold tray or in a +4°C cold room, with vortex-mixing of reactants at all addition steps.

Assay diluent

All dilutions of working stocks of standard and antiserum were carried out in 0.05M barbitone buffer, pH 8.6, containing 0.2% human plasma protein, 0.01M ethylenediamine tetra-acetic acid, 0.01% Thiomersal (both from BDH Chemical Ltd, Poole, Dorset, UK) and 100 K.I.U/ml aprotinin (Trasylol) (Bayer UK Ltd., Haywards Heath, Sussex, UK).

Incubation

Human PTH 1-34 standard was serially diluted in 2-fold steps between the range 2.5×10^{-9} to 5×10^{-12} moles/L (10ng-0.019ng/ml) and 100µl of each of the 10 doses were incubated at +4°C for 3 days with 100µl of antiserum (1:600,000 final dilution) and 100µl of assay diluent containing outdated blood bank plasma (10% final concentration) previously tested for absence of hPTH 1-34 immunoreactivity. Purified tracer diluted in assay diluent to approximately 3000cpm/100µl (5×10^{-15} moles or 20pg) was then added to

give a final volume of 400µl and the mixture incubated for a further 3 days. Experimental plasma samples were incorporated into the assay at several dilutions to check for parallelism. Controls for the assessment of non-specific binding were set up for each sample. Each determination was carried out in triplicate in neutral borosilicate glass tubes (Radley and Co.Ltd., Sawbridgeworth, Herts, UK).

Separation

Separation of antibody-bound and free peptide was assessed by two different systems:

a) Dextran-coated charcoal was prepared by mixing equal volumes of 1.4% Norit SXL charcoal (Hopkins and Williams, Chadwell Heath, Essex, UK) and 0.1% dextran T70 (Pharmacia (Great Britain) Ltd., Hounslow, UK) both in 0.05M barbitone buffer (20) with subsequent removal of charcoal fines and excess dextran by centrifugation and resuspension in barbitone buffer to the original volume. Separation of bound and free fractions was achieved by addition of 1ml of dextran-coated charcoal (containing approx. 7mg charcoal and 0.5mg dextran) to each assay tube, standing for 10 minutes followed by centrifugation at 1000g for 30 minutes. The supernatants (containing bound fraction) were decanted and counted with reference to tubes containing 100µl tracer made up to a similar volume to equalize counting geometry.

b) The second antibody used was a donkey anti-goat IgG (Batch No. HP/0/7/-IVC, generously provided by Dr B. Morris, Guildhay

Antisera, Guildford, Surrey, UK) diluted with barbitone buffer to a working concentration of 1:5. This was mixed in equal volumes with normal goat serum at a working concentration of 1:120 in barbitone buffer immediately prior to addition of 100µl of the mixture to each of the assay tubes which were then vortex-mixed and left to incubate at +4°C for a minimum of 3 hours. Subsequent addition of 1ml of 3% polyethylene glycol 6000 (BDH Chemicals Ltd) for 10 minutes helped to accelerate flocculation of the precipitating antigen-antibody complex and also served as a diluting wash (procedure recommended by Dr B. Morris). Tubes were centrifuged for 30 mins at 1000g and supernatants (containing free fraction) removed by aspiration, leaving a discrete pellet (bound fraction) at the bottom of the tube.

Counting and data processing

Assay tubes were counted in an LKB Multigamma counter with integral data processing (spline function) and results were expressed as B/Bo and/or concentration after automatic correction for non-specific binding. Comparison of calibration dose-response curves was computed using a four parameter logistic transformation (21). Scatchard analysis (22) was used to determine the affinity constant.

In vivo blood sampling

Blood samples were taken from freely mobile male Beagle dogs, weight 13-18kg, via indwelling venous cannulae as previously described (23) and calcium levels determined by atomic absorption

spectrophotometry (24); samples from human subjects were obtained via a cannula in the antecubital vein after administration of hPTH 1-34 either by intravenous infusion or by intravenous or subcutaneous injection. The human subjects were 2 normal healthy males (aged 26 and 29) and one osteoporotic female (aged 55) on the clinical trial programme, all of whom had given informed consent according to a procedure laid down by Northwick Park Hospital Ethical Committee. The blood was collected in chilled heparinized tubes and plasma was separated by centrifugation within 10 minutes of sampling after which it was snap-frozen and stored at -40°C for not longer than 4 weeks before assay. Estimates of the concentrations of immunoreactive hPTH 1-34 (ihPTH 1-34) in samples taken at intervals after a 2-hour infusion of the fragment into a dog, were used for calculations of the metabolic clearance rate (25) and a distribution space (26) of the peptide.

RESULTS

Tracer labelled ligand

The specific activity was estimated to be 3.7kBq/2.5 x 10^{-10} moles (100μCi/μg) based on gel chromatography and on precipitation of radioactive protein at concentrations of 6.6 and 15% trichloroacetic acid carried out before and after purification on Biogel P10. This estimate suggests a product monoiodinated at one of the histidine residues at position 9,14 or 32 (there are no tyrosines in the hPTH 1-34 fragment). The elution profile of the labelled tracer on

Biogel P10 showed only one sharp peak. The labelled peptide was usable for 8 weeks and there was little evidence of aggregation or "damage" even after 3 months storage.

Assay characteristics

Antiserum dilution curves, consisting of 7 two-fold dilutions, from 1/30,000-1/2,000,000 (final), and separated by either dextran coated charcoal or second antibody, were virtually superimposable (Figure 1). The dose-response calibration curve, 10 two-fold dilutions in the range 2.5×10^{-9} to 5×10^{-12} moles/L (10ng to 0.019 ng/ml), antiserum dilution 1/600,000 (Final) is shown in Figure 2. The detection limit of the assay, defined as the smallest concentration of analyte at 2 standard deviations from zero dose, was 1.75×10^{-11} moles/L (70pg/ml). The fraction of labelled ligand

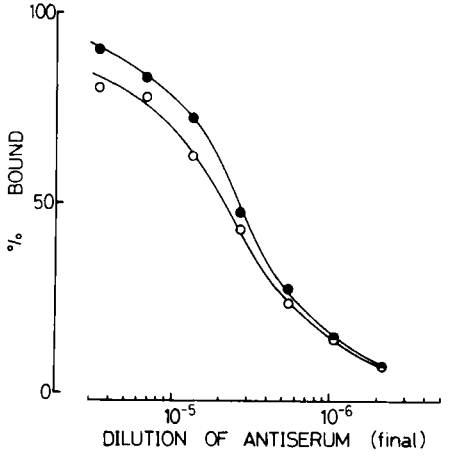

FIGURE 1. Antibody dilution curves (1:30,000 - 1:2,000,000) separated by either charcoal (○) or second antibody (●).

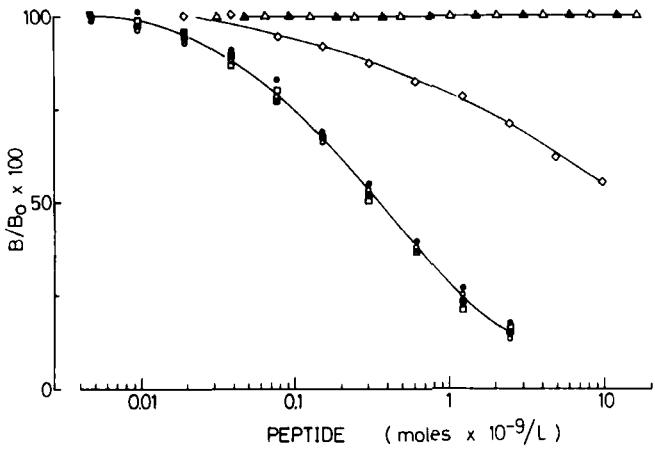

FIGURE 2. Dose-response curves for hPTH 1-34 (Code 75/596) (■), hPTH 1-84 (Code 75/549) (●), hPTH 1-84 (Code 79/500) (○), hPTH 1-84 (Code 78/551) (□), hPTH 44-68 (△), hPTH 53-84 (▲) and bPTH 1-84 (Code 71/324) (◇).

bound to the antiserum (approx. 5×10^{-15} moles, 20pg/ml added) was 25-30%, depending on the age of the tracer. The non-specific binding was between 2 and 4% even in the presence of 25% plasma or serum and irrespective of the separation procedure used. Scatchard analysis of the hPTH 1-34 dose-response curve with the antiserum at a final dilution of 1/600,000 gave a Ka of 1.18×10^{11} L/mole. Intra-assay coefficient of variation, assessed by comparing the complete dose-response curves of two independently diluted aliquots of hPTH 1-34 standard using a four parameter logistic transformation (21) was 6% which was similar to the variation found within several determinations (n=12) of a plasma sample to which 1.25×10^{-10} moles/L (0.5ng/ml) hPTH 1-34 had been added for assessment of recovery. Inter-assay coefficient of variation and accuracy were assessed by

measurements of the same recovery plasma. These gave a mean value of 1.25×10^{-10} moles/L (0.5ng/ml) with a standard deviation of 7.5×10^{-15} moles/L (0.03ng/ml), and a coefficient of variation of 6%. Slight deterioration of the hPTH 1-34 liquid standard, stored at -40°C for two years, was detected when assayed directly against a dilution prepared from freshly opened ampoules (75/596). Transformation of the resulting dose-response curves gave a weighted mean relative potency estimate of 0.90, limits 0.80-1.02 at the 95% confidence level.

Three different preparations of native intact 1-84 human parathyroid hormone, extracted from pooled human parathyroid adenomata and processed to different degrees of purity were assayed. i) A trichloroacetic acid precipitated extract, estimated to be approximately 3% pure in ampoules coded 78/551, ii) NIBSC Research Standard, extracted hPTH partially purified by gel chromatography and estimated to be approximately 10% pure in ampoules coded 75/549 (27), iii) extracted hPTH highly purified by chromatography and estimated to be approximately 90% pure, in ampoules coded 79/500. All native hPTH 1-84 preparations showed complete dose response curves, parallel to that of the hPTH 1-34 synthetic fragment and were equipotent on a molar basis (Figure 2). The International Reference Preparation for Bovine Parathyroid Hormone 71/324 gave incomplete displacement and was non-parallel. The synthetic fragments hPTH 44-68 and hPTH 53-84 (28) tested at a 5-7-fold molar excess showed no cross-reactivity in this system.

Assay application

Serial dilution of plasma samples taken from dog and man at intervals up to one hour after receiving hPTH 1-34, showed displacement curves parallel to that of the standard hPTH 1-34.

The results in Figure 3 show the clearance of ihPTH 1-34 from plasma from three human subjects after a bolus intravenous

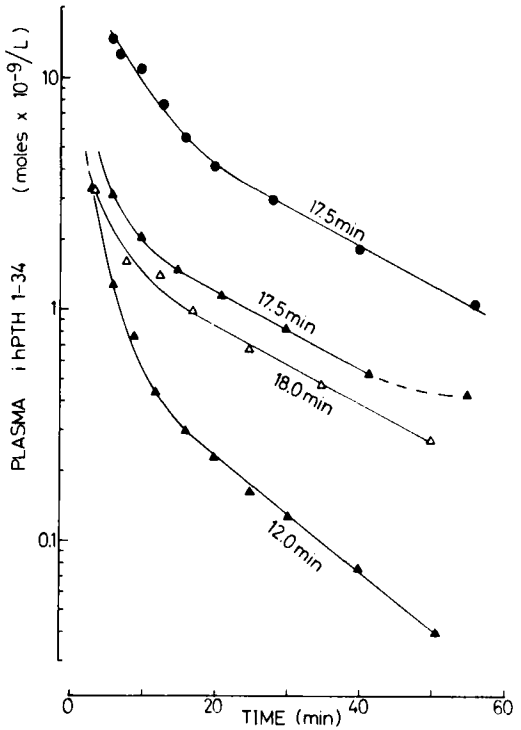

FIGURE 3. Disappearance curves of plasma ihPTH 1-34 after bolus intravenous injection of (i) 1.25 x 10^{-8} moles (50μg) hPTH 1-34 to two normal male subjects (▲) and one female subject with involutional osteoporosis (△) and (ii) 7.5 x 10^{-10} moles (3μg)/kg to a normal dog (●). The $t_{\frac{1}{2}}$ values for the second slower exponential component are shown on each curve.

injection of 1.25×10^{-8}moles (50μg) hPTH 1-34. Clearance appears biphasic with a first fast exponential component ($t_{\frac{1}{2}}$ = 3-8 minutes) followed by a slower phase ($t_{\frac{1}{2}}$, 12-18 minutes). A single intravenous bolus given to a dog produced a similar clearance profile (Figure 3).

Constant infusion of hPTH 1-34 at a rate of 6×10^{-10} moles (2.4μg)/kg/h to a dog for a period of 2 hours produced plateau blood concentrations of ihPTH 1-34 of 1.125×10^{-9}moles/L (4.5ng/ml). On cessation of infusion, clearance of the fragment followed a biexponential curve over the subsequent 60 minutes with $t_{\frac{1}{2}}$ estimates of 12.5 and 30 minutes resepctively. The metabolic clearance rate was calculated to be 9ml/kg/minute and the apparent volume of distribution 160ml/kg (Figure 4).

DISCUSSION

The homologous radioimmunoassay described has proved in this laboratory to be a rugged, precise and sensitive assay system. Its limit of detection is similar to that of other hPTH 1-34 assay systems (18,19,29,30) which have been applied to the study of endogenous human PTH in normal and pathological states.

Although the exact nature of the circulating levels of endogenous amino-region immunoreactivity remains unresolved, the assay system described here is able to estimate the amino-terminal immunoreactive synthetic fragment on an equimolar basis with amino-region immunoreactivity in the uncleaved intact native human PTH extracted and purified from human parathyroid adenomata. There is no

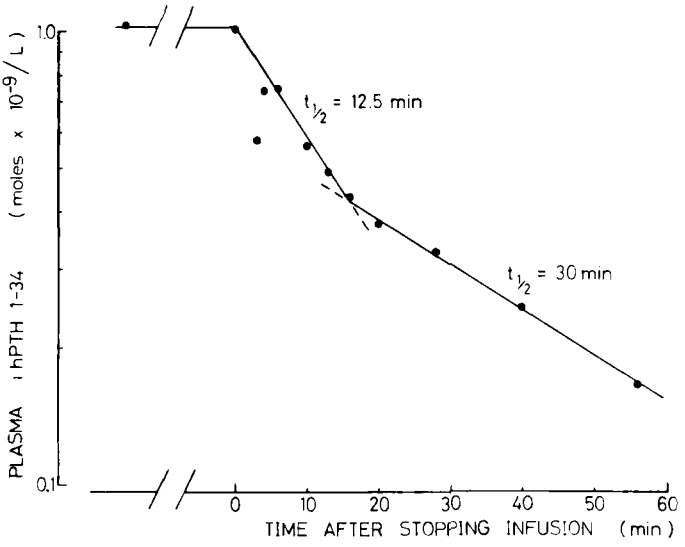

FIGURE 4. Plasma levels of ihPTH 1-34 in a normal dog during and after cessation of a 2-hour intravenous infusion of hPTH 1-34 at 6×10^{-10} moles (2.4μg)/kg/h.

recognition of other sequences within the intact PTH as confirmed by lack of cross-reactivity with the 44-68 and 53-84 synthetic fragments of human PTH.

Even homologous PTH 1-34 assay systems cannot distinguish immunoreactivity circulating as cleaved amino-region fragments from the intact hormone or a heterogeneous mixture unless gel chromatography is carried out on plasma prior to assay. We have therefore applied the present system to a pharmacokinetic study of the synthetic hPTH 1-34 peptide administered in vivo.

The disappearance of ihPTH 1-34 from the circulation of three human subjects and one dog after single intravenous injection typically followed multiexponential curves (Figure 3).

Allowing for a mixing time of at least 3 minutes, the fastest discernible components corresponded to circulating half lives between 3 and 8 minutes, followed in each case by a slower component ($t_{\frac{1}{2}}$, 12-18 min) which remained exponential for at least 20 minutes. It is not clear which of these components corresponds to the true biological half-life of the synthetic peptide. Some published estimates of half-life of bPTH have been in the same range as our second component (31-35) but others correspond more closely with the first component (for references see 2 and 36). In one experiment in which infusion of hPTH 1-34 to a dog was stopped after achieving equilibrium, the first exponential component appeared to have a $t_{\frac{1}{2}}$ of 12.5 min. The metabolic clearance of hPTH 1-34 (9ml/kg/min) in the present study is within the ranges reported for the bovine amino terminal iPTH in man (36) and dog (31).

Comparison of the time courses of circulating levels of ihPTH 1-34 measured in plasma and the hypercalcaemic response after subcutaneous injection of hPTH 1-34 into a normal dog, have shown that the peak hypercalcaemia occurred approximately 4 hours after injection, ie. 3 hours after the ihPTH 1-34 levels had ceased to be detectable (37). However in the interpretation of data obtained after administration of hPTH 1-34, the absence of non-parallelism does not exclude the possibility that smaller fragments resulting from cleavage of the synthetic peptide may contribute to the measured levels of ihPTH 1-34 and yet be devoid of biological activity.

Since metabolism of PTH is presumed to take place in extravascular sites (35), it is not surprising that the calculated distribution space of hPTH 1-34 in the dog of 16% of body weight is greater than the theoretical blood volume (38) but less than total extracellular body water of the dog (39). A more detailed study of the pharmacokinetics of hPTH 1-34 in the experimental dog model will be the subject of a separate report.

In summary, we have developed and validated an homologous hPTH 1-34 radioimmunoassay which has application to the study of the pharmacology and therapeutic potential of the hPTH 1-34 peptide.

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