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Development of a Highly Sensitive and Specific Two-Site Enzyme Immunoassay for Parathyroid Hormone (1-34): Application to Pharmacokinetic Study on Intranasal Parathyroid Hormone (1-34) in Human

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A highly sensitive and specific two-site enzyme immunoassay for parathyroid hormone (1-34) (PTH(1-34)) and its usability for the pharmacokinetic study are described. Plasma samples were incubated simultaneously with 2,4-dinitrophenylated anti-PTH(1-34) IgG and anti-PTH(1-34) Fab´-β-D-galactosidase conjugate. The immune complex formed of the three components was trapped onto (anti-2,4-dinitrophenyl group) IgG-coated polystyrene balls. β-D-Galactosidase activity bound to the polystyrene balls was assayed by fluorometry. The practical detection limit of PTH(1-34) was 50 fg (12 amol)/0.05 ml of sample and 1 pg/ml as the concentration and practically no interference occurred by PTH(1-84) and PTH-related protein (1-34) up to 300 pg/ml and 10 ng/ml, respectively. The application of this method has enabled us to directly estimate the bioavailability of PTH(1-34) dosed intranasaly at the prescribed level (0.090 mg). The pharmacokinetic parameters of the intranasal PTH(1-34) (n = 4) thus estimated were as follows: the area under the plasma concentration-time curve (AUC) = 20,500 ± 15,900(SD) pg·min/ml; the mean residence time (MRT) = 194 ± 16.3(SD) min; and the maximal concentration (Cmax) = 98 ± 51(SD) pg/ml with the maximal time (Tmax) = 35.0 ± 12.2(SD) min. J. Clin. Lab. Anal. 12:268–275, 1998.

Key words: osteoporosis; calcium; fluorometry; bioavailability; bone formation; bone resorption

INTRODUCTION

The principle role of parathyroid hormone (PTH) is in the regulation of mineral (especially calcium) metabolism (1,2). PTH is secreted as intact PTH (PTH(1-84)) and is processed to PTH(1-34) and PTH(35-84) in kidney and/or liver (3). Both PTH(1-84) and PTH(1-34) can act on kidney and increase in calcium absorption by causing an increase in biosynthesis of 1,25-dihydroxy vitamin D3. While, as to bone metabolism, only PTH(1-34) is physiologically active (4,5). PTH(1-34) stimulates the ostoclastic bone resorption mediated by interaction with ostoblast. On the contrary, various studies have demonstrated so far about its anabolic effect (6–8), thus, therapeutic use of PTH(1-34) for osteoporosis has been emphasized.

The immunoassay methods to measure PTH as a diagnostic aid had been developed (9–14), however, these methods could measure PTH(1-84) and physiologically inactive C-terminal portion of PTH but could not PTH(1-34) specifically without interference occurred by PTH (1-84) and PTH fragments.

The aims of this study are: (1) to develop enzyme immunoassay methods to measure PTH(1-34) with sufficiently sensitive and specific; and, (2) to test the applicability of the technique for the pharmacokinetic study of PTH(1-34) in humans.

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MATERIALS AND METHODS

Parathyroid Hormone

Parathyroid hormone (1-34) (PTH(1-34)) and PTH-related protein (1-34) (PTHrp (1-34)) were obtained from UCB Bioproducts, S. A., (Belgium) and Peninsula Laboratories, Inc. (Belmont, CA), respectively. PTH(1-84) was the gift from Suntory Limited (Osaka, Japan).

2,4-Dinitrophenyl Bovine Serum Albumin

Thiol groups were introduced into bovine serum albumin (BSA) molecules using *N*-succinimidyl-*S*-acetylthioacetate (15) and were reacted with maleimide groups introduced into ε*N*-2,4-dinitrophenyl-L-lysine molecules using *N*-succinimidyl-6-maleimidohexanoate (16). The amounts of BSA, 2,4-dinitrophenyl groups and 2,4-dinitrophenyl BSA were calculated from the absorbance at 280 and 360 nm (17). The average number of 2,4-dinitrophenyl groups introduced per BSA molecule was 6.0 (17).

Antisera

Albino rabbits (New Zealand White, b.w., 2.5–3.0 Kg) were first intracutaneously injected with PTH(1-34) (0.1 mg) or dinitrophenylated BSA (0.1 mg) in 1.5 ml of Freund's complete adjuvant (Nacalai Tesque, Inc., Kyoto, Japan) and thereafter twice boosted with PTH(1-34) (0.05 mg) or dinitrophenylated BSA (0.05 mg) in 1.5 ml of Freund's incomplete adjuvant (Nacalai Tesque, Inc.) at three-week intervals. Blood was collected two weeks after the last injection, and the antisera were stored at –80°C.

Epitope Mapping of Rabbit Anti-PTH(1-34) IgGs

The reactivity pattern of anti-PTH(1-34) IgG was determined by using Multipin NCP NON CLEAVABLE KIT according to the instruction of Chiron Mimotopes Pty Ltd. (Victoria, Australia). In brief, overlapping seven amino acid long peptides of PTH(1-34) were synthesized (peptide 1 consists of residues 1–7, peptide 2 consists of residues 4–10, peptide 3 consists 7–13....) on pegs, anti-PTH(1-34) IgG was trapped on the pegs, and was detected by (antirabbit IgG) Fab´-peroxidase conjugate (18).

IgG and Its Fragments

IgG was prepared from serum by fractionation with Na_2SO_4 followed by passage through a column of diethylaminoethylcellulose (19). F(ab´)$_2$ was prepared by digestion of IgG with pepsin from porcine gastric mucosa (19), and Fab´ was prepared by reduction of F(ab´)$_2$ with 2-mercaptoethylamine (19). The amounts of IgG and its fragments were calculated from the absorbance at 280 nm (19).

2,4-Dinitrophenylated Rabbit Anti-PTH(1-34) IgG

Rabbit anti-PTH(1-34) IgG (1.5 mg) in 0.1 mol/l sodium phosphate buffer, pH 7.0 (0.5 ml) was incubated with 16.5 mmol/l *N*-succinimidyl-*S*-acetylthioacetate (Research Organics Inc., Cleveland, OH) in *N*,*N*-dimethylformamide (0.05 ml) at 30°C for 30 min. The reaction mixture was incubated with 0.03 ml of 0.1 mol/l EDTA, pH 7.0, and 0.06 ml of 1 mol/l hydroxylamine-HCl, pH 7.0 at 30°C for 15 min. After incubation, the reaction mixture was subjected to gel filtration on a column (1 × 30 cm) of Sephadex G-25 (Pharmacia LKB Biotechnology, Uppsala, Sweden) using 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA. The average number of thiol groups introduced per IgG molecule was 15 (19). The mercaptoacetylated IgG (1.2 mg) in 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA (0.6 ml) was incubated with maleimide-biocytin solution (20) (0.016 ml) at 30°C for 30 min. The average number of biotin residues introduced per the mercaptoacetylated IgG molecule was 7.0, which was calculated from the decrease in the number of thiol groups (19). The mercaptoacetylated biotinyl IgG solution was incubated with the maleimide-ε*N*-2,4-dinitrophenyl-L-lysine solution (16)(0.134 ml) at 30°C for 30 min. After incubation, the reaction mixture was subjected to gel filtration on a column (1 × 30 cm) of Sephadex G-25 using 0.1 mol/l sodium phosphate buffer, pH 7.0. The average number of 2,4-dinitrophenyl groups introduced per IgG molecule was 6.0, which was calculated from the absorbance at 280 and 360 nm (17,19). The amount of the 2,4-dinitrophenyl biotinyl IgG was calculated from the absorbance at 280 and 360 nm (17,19).

PTH(1-34)-BSA

Maleimide-PTH(1-34)

PTH(1-34) (0.9 mg, 220 nmol) in 0.1 mol/l sodium phosphate buffer, pH 7.0 (0.2 ml) was incubated with 11 mmol/l *N*-succinimidyl-6-maleimidohexanoate (220 nmol)(Dojindo Laboratories, Kumamoto, Japan) in *N*,*N*-dimethylformamide (0.02 ml) at 30°C for 30 min. The average number of maleimide groups introduced per PTH(1-34) molecule was 1.0, which was calculated from the decrease in the number of amino groups (21).

Mercaptosuccinylated BSA

BSA (fraction V, Intergen Co., Purchase, NY, 6.6 mg, 100 nmol) in 0.1 mol/l sodium phosphate buffer, pH 7.0 (0.5 ml) was incubated with 16.5 mmol/l *N*-succinimidyl-*S*-acetylthioacetate in *N*,*N*-dimethylformamide (0.05 ml, 825 nmol) at 30°C for 30 min, and then with 0.1 mol/l EDTA, pH 7.0 (0.03 ml) and 1 mol/l hydroxylamine-HCl, pH 7.0 (0.06 ml) at 30°C for 15 min. After incubation, the reaction mixture was subjected to gel filtration by centrifuged column

procedure (22) using 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA. The average number of thiol groups introduced per BSA molecule was 5.5 (19).

PTH(1-34)-BSA

The maleimide-PTH(1-34) solution (0.13 ml, 130 nmol) was incubated with the mercaptosuccinylated BSA (1.7 mg, 26 nmol) in 0.4 ml of 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA at 4°C overnight. The reaction mixture was further incubated with 0.1 mol/l *N*-ethylmaleimide in 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA (0.03 ml) at 30°C for 30 min, and then with 0.1 mol/l 2-mercaptoethylamine-HCl in 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA (0.06 ml) at 30°C for 30 min. The reaction mixture was subjected to gel filtration on a column (1.5 × 45 cm) of Ultrogel AcA 44 (IBF Biotechnics, Villeneuve-la-Garenne, France) using 0.1 mol/l sodium borate buffer, pH 8.0, containing 0.5 mol/l NaCl. The average number of PTH(1-34) molecules introduced per BSA molecule was 3.8, which was calculated from the decrease in the number of thiol groups (19). The amount of PTH(1-34)-BSA was determined by a commercial protein assay kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Richmond, CA) using BSA as a standard.

Protein-Coupled Sepharose 4B

2,4-Dinitrophenylated BSA (1.0 mg), PTH(1-34)-BSA (1.0 mg) and anti-PTH(1-34) IgG (1.0 mg) were coupled to CN-Br-activated Sepharose 4B (0.1 g, Pharmacia LKB Biotechnology AB) according to the instructions of Pharmacia LKB Biotechnology.

Affinity Purification of Antibodies

Anti-PTH(1-34) $F(ab')_2$ and 2,4-dinitrophenyl biotinyl anti-PTH(1-34) IgG were affinity-purified using columns of PTH(1-34)-BSA-coupled Sepharose 4B (23). Anti-2,4-dinitrophenyl BSA IgG was affinity-purified using a column of 2,4-dinitrophenyl BSA-coupled Sepharose 4B (23). The specific antibodies were eluted from the corresponding columns with 3.2 mmol/l HCl, pH 2.5, and neutralized by the addition of 1 mol/l sodium phosphate buffer, pH 7.0 (23).

Affinity-Purified Anti-PTH(1-34) Fab´-β-D-Galactosidase Conjugate

Affinity-purified rabbit anti-PTH(1-34) Fab´ was conjugated to β-D-galactosidase from *E. coli* (Enzyme label for enzyme immunoassay, Boehringer Mannheim GmbH, Mannheim, Germany) using *o*-phenylenedimaleimide (19). The amount of conjugate was calculated from β-D-galactosidase activity (22).

Protein-Coated Polystyrene Balls

Polystyrene balls (Immuno Chemical, Inc., Okayama, Japan) were coated with affinity-purified rabbit (anti-2,4-dinitrophenyl BSA) IgG (0.1 g/l) by physical adsorption (24).

Human EDTA Plasma, PTH Free Plasma and Pooled Plasma

Blood samples were withdrawn into glass tubes (Venoject VT-NA EDTA-2Na, Termo Corp., Tokyo, Japan), and centrifuged to separate the plasma. Plasma samples were obtained from 19 healthy male subjects, aged 25–51 years. The plasma samples were mixed with aprotinin from bovine lung (Calbiochem-Novabiochem Corporation, La Jolla, CA) at a concentration of 500 KIU/ml, and were stored at –80°C. To prepare PTHs (PTH(1-34) and PTH(1-84)) free plasma, the plasma samples were absorbed with columns (3 × 7 mm) of anti-PTH(1-34) IgG-coupled Sepharose 4B using 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl. The PTHs free plasma samples were mixed and used as a PTHs free pooled plasma.

Standards

PTH(1-34), PTH(1-84) and PTHrp(1-34) were dissolved in 33 mmol/l sodium acetate buffer, pH 4.1, containing 0.1 mol/l NaCl at a concentration of 1 mg/ml and diluted with the PTHs free pooled plasma and used as standards.

Two-Site Enzyme Immunoassay for PTH(1-34)

An assay sample (standards or plasma samples, 0.05 ml) were mixed with nonspecific rabbit IgG (0.6 mg/ml) in 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl, 1 mmol/l $MgCl_2$, 1 g/l NaN_3 and 1 g/l BSA (0.05 ml), subsequently, incubated with 2,4-dinitrophenyl biotinyl affinity-purified anti-PTH(1-34) IgG (100 fmol) and affinity-purified anti-PTH(1-34) Fab´-β-D-galactosidase conjugate (12.5 fmol) in 10 mmol/l sodium phosphate buffer, pH 7.0, containing 1 mol/l NaCl, 1 mmol/l $MgCl_2$, 3 mmol/l 2-mercaptoethylamine, 1 g/l NaN_3 and 1 g/l BSA (0.05 ml) at 4°C overnight. The reaction mixture was then incubated with two polystyrene balls coated with affinity-purified (anti-2,4-dinitrophenyl BSA) IgG at 4°C for 6 h. After incubation, the polystyrene balls were washed twice by addition and aspiration of 2 ml of 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl, 1 mmol/l $MgCl_2$, 1 g/l NaN_3 and 0.1 g/l BSA. β-D-Galactosidase activity bound to the washed balls was assayed by fluorometry (25) as follows. The balls were preincubated with 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl, 1 mmol/l $MgCl_2$, 1 g/l NaN_3 and 0.1 g/l BSA (0.1 ml) at 30°C for 5 min. The enzyme reaction was initiated by addition of 0.3 mmol/l 4-methylumbelliferyl-β-D-galactopyranoside (0.05 ml) (Boehringer Mannheim GmbH) and incubated at 37°C for 30

min. The enzyme reaction was then terminated by the addition of 0.1 mol/l glycine-NaOH buffer, pH 10.3 (2.5 ml) and the fluorescence intensity was measured using a spectrofluorometer (FP-750, JASCO, Tokyo, Japan) at 360 nm for excitation and 450 nm for emission. The scales of 0 and 100 were adjusted by using 0.1 mol/l glycine-NaOH buffer, pH 10.3 and 10^{-8} mol/l 4-methylumbelliferone (Nacalai Tesque, Inc.) in the same buffer, respectively. The fluorescence intensity was measured relative to the 4-methylumbelliferone solution (25).

Measurement of Bioavailability

Young adult male volunteers had passed the screening tests to exclude serious defect(s) of liver, kidney, circulation system, respiratory system, digestive system, hematopoietic function, and endocrinological function. The volunteers were administered PTH(1-34) (0.090 mg, Lot J08009T1, American Peptide Co., Inc., Sunnyvale, CA) intranasaly. Intranasal PTH(1-34) formulation was prepared by mixing PTH(1-34) solution with calcium carbonate powder and freeze-drying. The volunteers loaded the capsule containing the PTH(1-34) formulation on the novel device (Jetlizer® [UNISIA JECS, Co., Gunnma, Japan]) and puffed it in a parallel position along to the central axia of the nose by themselves four times into both of their nasal cavities. The detailed procedures for formulation and device will be published elsewhere. Before and after (5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720 min) administration, blood samples were withdrawn into glass tubes (Venoject VT-NA EDTA-2Na). The blood samples were centrifuged to separate the plasma, and bovine lung aprotinin was added to the plasma samples at a concentration of 500 KIU/ml. The plasma samples were kept at -80°C until assayed. The PTH(1-34) concentration was measured in duplicate by the two-site enzyme immunoassay as described above, and was calculated by curve-fitting using the PTH(1-34) plasma standards. The bioavailability parameters, area under the plasma concentration-time curve (AUC) and the mean residence time (MRT), were calculated with a personal computer using a linear trapezoidal equation (26,27). This study had been approved by Institutional Review Board of St. Marianna University School of Medicine.

RESULTS

Epitope Mapping of Anti-Parathyroid Hormone (1-34) IgG

The epitopes for anti-parathyroid hormone (PTH) (1-34) antibodies used in this study were identified by Geysen method (18). Using simultaneous multiple peptide synthesis, 10 overlapping heptapeptide which corresponded to PTH(1-34) were synthesized on polypropylene pegs. The antibody Nos. 1 and 2 from two rabbits were trapped onto the pegs, after washing, were detected with (antirabbit IgG) Fab´-peroxidase conjugate. Antibody Nos. 1 and 2 recognized epitopes corresponding to residues 4–10 (N-terminal portion of PTH(1-34)) and 16–22 (central portion of PTH(1-34)), respectively (Fig. 1). The antibody No. 1 (N-terminal portion antibody) was labeled with β-D-galactosidase, and the antibody No. 2 (central portion antibody) was labeled with 2,4-dinitrophenyl groups.

Two-Site Enzyme Immunoassay for Parathyroid Hormone (1-34)

Concept of the assay procedure

The assay procedure was designated as follows:

1. Formulation of immune complex consisting of PTH(1-34), 2,4-dinitrophenylated anti-PTH(1-34) IgG and anti-PTH(1-34) Fab´-β-D-galactosidase conjugate;

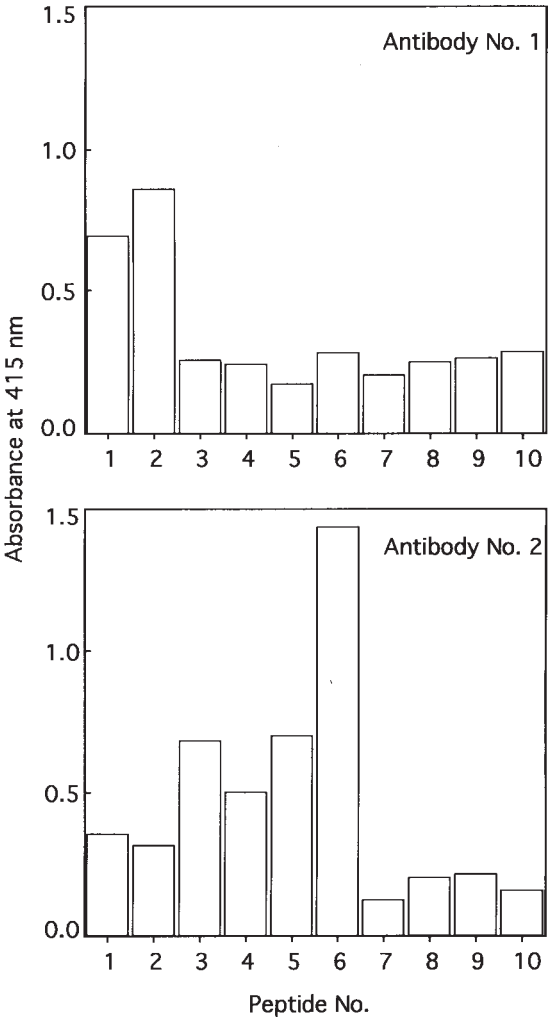

Fig. 1. Epitope mapping of anti-parathyroid hormone (1-34) IgGs. Ten overlapping heptapeptides (X-axis) which corresponded to parathyroid hormone (1-34) were synthesized on polypropylene pegs. The binding of antiparathyroid hormone (1-34) antibody Nos. 1 and 2 was detected by (antirabbit IgG) Fab´-peroxidase conjugate.

2. Trapping of the immune complex onto polystyrene balls coated with (anti-2,4-dinitrophenyl group) IgG;

3. Measurement of the β-D-galactosidase activity bound to the balls by fluorometry. The whole procedure was completed within two days.

Sensitivity and Specificity

PTH(1-34) was diluted with a PTHs (PTH(1-34) and PTH(1-84)) free pooled plasma from 19 healthy subjects, which had been absorbed endogeneous PTHs with anti-PTH(1-34) IgG-coupled Sepharose 4B, and subjected to the present assay. The assay was able to detect 50 fg (12 amol)/0.05 ml of a given sample, that is the minimum concentration for detection being 1 pg/ml (Fig. 2). The detection limit was taken to be the minimal concentration obtained 2SD (n = 8) above the plasma background. In addition, the background PTH(1-34) concentration (0.02 ± 0.41(SD) pg/ml, ranged from –0.37 to 0.73 pg/ml) (Table 1, left column) in eight PTHs free plasma samples was lower than the detection limit (1 pg/ml). The existence of a significant difference of fluorescence intensity for bound β-D-galactosidase activity at 1 pg/ml from the background was also confirmed by student t-test (n = 4, $P < 0.0001$). The standard curve was linear up to a concentration of 1000 pg/ml (Fig. 2). The assay is specific for PTH(1-34) and practically no interference occurred by PTH(1-84) up to 300 pg/ml and by PTH-related protein (1-34) up to 10 ng/ml (Fig. 2).

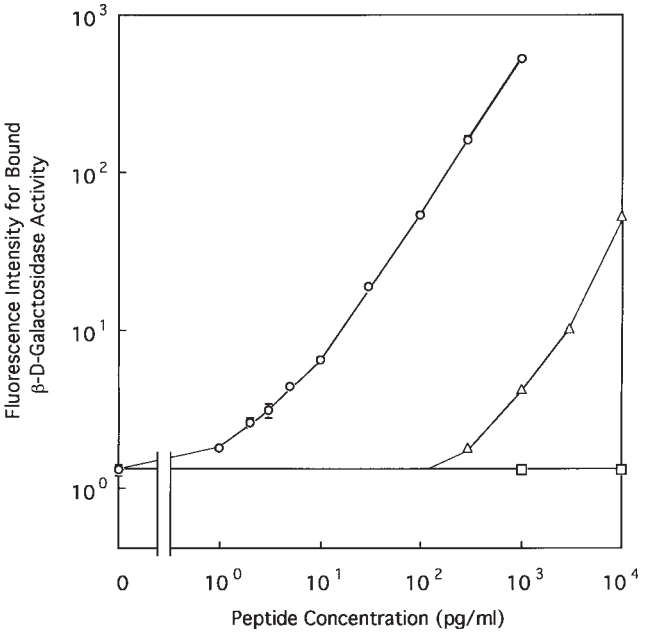

Fig. 2. Dose-response curves for parathyroid hormone and parathyroid hormone related protein by two-site enzyme immunoassay. ○, curve for parathyroid hormone (PTH(1-34)); △, curve for PTH(1-84); □, curve for PTH-related protein (1-34); in the presence of a pooled plasma from 19 healthy subjects. Each value indicates the mean of four determinations (PTH(1-34) 0–5 pg/ml) and two determinations (PTH(1-34) 10–1,000 pg/ml, PTH(1-84), PTH-related protein (1-34)), and small horizontal bars with vertical bars indicate ± SD.

Assay Variation

When examined at three different plasma levels in the range of 10–250 pg/ml for within-assay and for between-assay variation, the CVs were 2.9–7.8 % (n = 8) and 4.5–6.5 % (n = 8), respectively (Table 2). The samples using the assay variation were prepared by mixing PTH(1-34) with the PTHs free pooled plasma.

The PTH(1-34) plasma standards (0, 10, 30, 100, 300, and 1,000 pg/ml) were subjected to the present assay eight times, the CVs for fluorescence intensity for specifically bound β-D-galactosidase activity were 4.6–7.7 % (Fig. 3). The between-assay variation and PTH(1-34) standards variation were run over a period of 30 days.

Recovery

PTH(1-34) was mixed with the PTHs free plasma at concentrations of 20 and 200 pg/ml and assayed. The recovery rates of PTH(1-34) were 93 ± 13.0(SD) % (ranged 80%–

TABLE 1. The Background in the Presence of Human Plasma and the Recovery Rate of Parathyroid Hormone (1-34) Mixed With Human Plasma

Plasma no.	Background (pg/ml as parathyroid hormone (1-34))	Recovery rate of parathyroid hormone (1-34) mixed with plasma[a] (%) parathyroid hormone concentration (pg/ml)	
		20	200
1	0.37	85	81
2	0.73	85	91
3	–0.37	110	118
4	–0.18	80	85
5	0.37	85	84
6	–0.18	85	86
7	–0.18	115	115
8	–0.37	95	97

[a]Recovery rate was calculated by curve-fitting of a standard curve in the presence of a pooled plasma.

TABLE 2. Within-Assay and Between-Assay Imprecision

Assay	PTH[a] level added to plasma (pg/ml)	Number of determinations	PTH[a] level detected means ± SD (pg/ml)	Coefficient of variation (%)
Within-assay	10	8	9.8 ± 0.3	3.1
	50	8	49 ± 3.8	7.8
	250	8	254 ± 7.4	2.9
Between-assay	10	8	11 ± 0.5	4.5
	50	8	54 ± 3.5	6.5
	250	8	263 ± 14	5.3

[a]Parathyroid hormone (1-34).

115%, CV = 14%) and 95 ± 14 (SD)% (ranged 81–118, CV = 15%) (Table 1, middle and right columns), respectively, which were calculated by curve-fitting of a standard curve in the presence of the PTHs free pooled plasma.

Stability of PTH(1-34) in Plasma Matrix

Firstly, PTH(1-34) was mixed with the PTHs free pooled plasma at concentrations of 10, 100, and 1,000 pg/ml, incubated at 20°C or 37°C for 0–2 hr, and subjected to the present assay. The recovery rates of PTH(1-34) in the plasma matrix were satisfactory until 2-hr incubation (Fig. 4a).

Secondly, PTH(1-34) was mixed with the PTHs free pooled plasma at concentrations of 10, 50, and 250 pg/ml, stored at –80°C for 0–3 months, and assayed. The recovery rates of PTH(1-34) were satisfactory until 3 months storage (Fig. 4b).

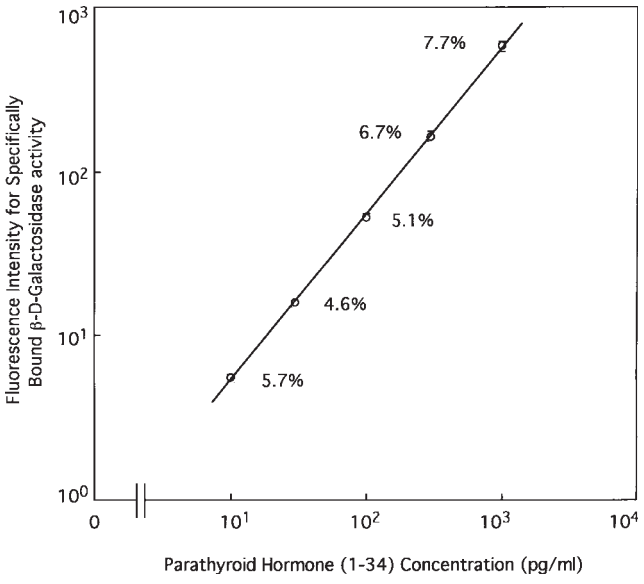

Fig. 3. Intermediate precision of standard curve for parathyroid hormone (1-34). The parathyroid hormone standards (0, 10, 30, 100, 300, and 1,000 pg/ml) were assayed by the present assay eight times, and the variation coefficients for fluorescence intensity for specifically bound β-D-galactosidase activity were calculated.

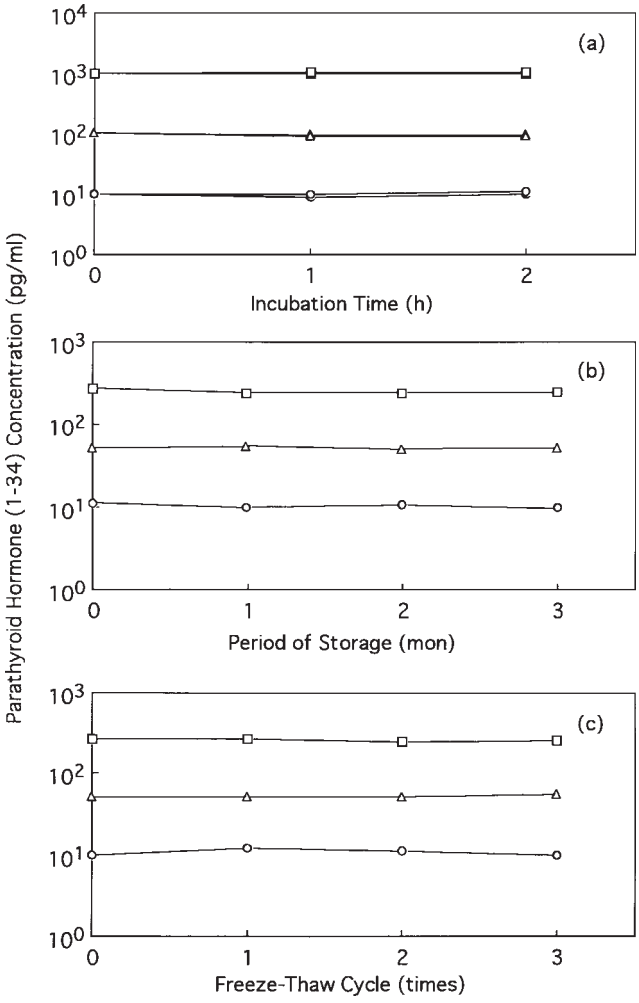

Fig. 4. Stability of parathyroid hormone (1-34) in plasma matrix. **a,** Parathyroid hormone (1-34) was mixed with a pooled plasma at concentrations of 10 (○), 100 (△), and 1,000 pg/ml (□), incubated at 20°C (open symbols) or 37°C (closed symbols), and assayed; **b,** Parathyroid hormone (1-34) was mixed with a pooled plasma at concentrations of 10 (○), 50 (△), and 250 (□) pg/ml, stored at –80°C, and assayed; **c,** Parathyroid hormone (1-34) was mixed with a pooled plasma at concentrations of 10 (○), 50 (△), and 250 (□) pg/ml, subjected to freezing-thawing, and assayed.

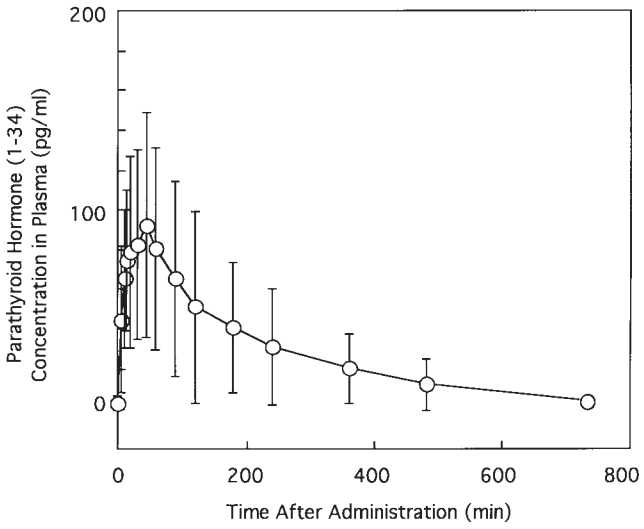

Fig. 5. Concentration of parathyroid hormone (1-34) after an intranasal administration of parathyroid hormone (1-34) (0.090 mg/subject, n = 4) to human. Each value indicates the mean, and small horizontal bars with vertical bars indicate ± SD.

Thirdly, PTH(1-34) was mixed with the pooled plasma at concentrations of 10, 50, and 250 pg/ml, and subjected to freezing (–80°C) and thawing (room temperature) three times. The thawed samples were assayed. The recovery rates of PTH(1-34) were satisfactory until three freeze-thaw cycles (Fig. 4c) .

Bioavailability of Intranasal PTH(1-34)

Figure 5 shows the appearance-disappearance pattern of intranasal PTH(1-34) (0.090 mg) in humans. The area under the plasma concentration-time curve (AUC) was calculated to be 20,500 ± 15,900(SD) pg·min/ml. The mean residence time (MRT) = 194 ± 16.3(SD) min. The maximal concentration (Cmax) of intranasal PTH(1-34) was calculated to be 98 ± 51 pg/ml with the maximal time (Tmax) of 35.0 ± 12.2 min.

DISCUSSION

The sensitive and specific assay method to parathyroid hormone (PTH) (1-34) developed herein was found to specifically measure sub-picomolar levels of PTH(1-34) without interference occurred by PTH(1-84) in the presence of human EDTA plasma. In conventional two-site enzyme immunoassay, an antigen to be measured is reacted sequentially or simultaneously with an antibody-coated solid phase and an enzyme-labeled antibody, accordingly, a reaction of antibodies to antigen occurs on the solid phase (28). As evident from the principle of the two-site enzyme immunoassay, an antigen has to have two or more epitopes, which are sufficiently separated from each other to allow simultaneous binding of two antibodies. The distance between the two epitopes appears to correspond to 12–15 amino acids (28). The epitope-mapping study showed that more than three epitopes (N-terminal portion, central portion, and C-terminal portion, as described below) were located on PTH(1-34) molecule. However, immunoreaction did not occur efficiently by the conventional format, probably due to spatial interference(s). In the present assay, PTH(1-34) was reacted to labeled antibodies within a solution. This might eliminate the spatial interferences and improve the sensitivity. Thus, the use of this format rather than the conventional one is the most important condition for the sensitive assay method (28). Recently, Ping Gao et al (14) reported an immunochemiluminometric assay for PTH(1-38) using two monoclonal antibodies for PTH(1-38). In the Gao's assay, the detection limit for PTH(1-38) was 0.4 pmol/l (1.8 pg/ml) and for PTH(1-34) was probably 0.8 pmol/l (3.3 pg/ml), which was calculated from the cross-reactivity rate described in their report. In terms of sensitivity, the detection limit of the present assay was slightly (3.3-fold) lower than the Gao's assay. In terms of specificity, the Gao's assay detected both PTH(1-34) and PTH(1-84) with the cross-reactivity rate of 200% (PTH(1-84)/PTH(1-34)), thus their assay was not N-terminal PTH specific. In the present assay, the cross-reactivity rate was only 0.5 %. This is significant difference between the present assay and the Gao's assay. The cross reactivity rate of 0.5 % was reconfirmed by using PTH(1-84) standard enclosed in intact PTH assay kit (Allegro, Nichols Institute Diagnostics, San Juan, CA) (data not shown). In order to examine the best combination of antibodies for specific assay method to PTH(1-34), we firstly prepared three antibodies recognizing to epitopes corresponding to N-terminal (residues 4–10, antibody No. 1, Fig. 1), central (residues 16–22, antibody No. 2, Fig. 1) and C-terminal (residue 28–34, antibody No. 3, data not shown) portions by immunizing rabbits with PTH(1-34). The cross-reactivity rate estimated for PTH(1-84) was 0.5% in the combination of antibody Nos. 1 and 2, but more than 5% in the other combinations. Whereas, the appropriate combination of antibodies was essential to develop PTH(1-34) specific method, though no comment was given as to epitope(s) in Gao's report.

In conclusion, this is the first study in which sensitive and specific assay for N-terminal PTH (PTH(1-34)) has been developed. This method was specific for PTH(1-34) with practically no interference by healthy level of PTH(1-84). The application of this method has enabled us to estimate the bioavailability of PTH(1-34) dosed nasally to human. This method is being applied to diagnostic aid for parathyroid, renal, connective tissue, and bone disorders. An extensive investigation of plasma PTH(1-34) level in relation to the disorders will be published elsewhere.

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