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A Sandwich Transfer Enzyme Immunoassay for Salmon Calcitonin: Determination of the Bioavailability of Intranasal Salmon Calcitonin in Human

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A sandwich transfer enzyme immunoassay for salmon calcitonin (SCT) and its usability for the pharmacokinetic study are described. The assay procedure consisted of the reaction of SCT with 2,4-dinitrophenyl biotinyl anti-SCT IgG and anti-SCT Fab´-β-D-galactosidase conjugate, trapping onto (anti-2,4-dinitrophenyl bovine serum albumin) IgG-coated polystyrene balls, eluting with ε*N*-2,4-dinitrophenyl-L-lysine and transferring to streptavidin-coated polystyrene balls and fluorometric detection of β-D-galactosidase activity. The practical detection limit of SCT was 0.05 pg (15 amol)/50 μl of sample and 1 pg/ml as the concentration. The application of this method has enabled us to directly estimate the bioavailability of SCT dosed intranasally at the therapeutic level (160 IU, 31 μg) for its anti-osteoporotic effect as compared to an intramuscular dose (10 IU, 1.9 μg). The pharmacokinetic parameters of the intranasal SCT (n = 6) thus estimated were as follows: the area under the blood concentration-time curve (AUC) = 9400 ± 5400 (SD) pg·h/ml, and the mean residence time (MRT) = 42 ± 14 (SD) min, when the AUC for the intramuscular SCT (n = 3) = 5600 ± 2000 (SD) pg·h/ml and the MRT = 39 ± 19 (SD) min. J. Clin. Lab. Anal. 11:380–387, 1997.

Key words: pharmacokinetics; osteoporosis; osteomalacia; thyroid; hormone

INTRODUCTION

Calcitonins are a family of natural inhibitors of the mammalian osteoclast (1–3) and salmon calcitonin (SCT), one of the most potent, has been successfully prescribed as the standard therapeutic for osteoporotic diseases caused by pathologically increased osteoclastic activity (4). In spite of the established therapeutic effectiveness of SCT, however, little is known about its pharmacokinetic properties.

We recently have described a highly sensitive enzyme immunoassay (hetero-two-site enzyme immunoassay) for SCT (5,6). The application of the assay method had enabled us to directly estimate the bioavailability of SCT dosed subcutaneously (5) and intranasally (6) at the therapeutic levels in rats. The method in brief proceeds as follows: centrifugal filtration through a polysaccharide membrane to remove plasma proteins, biotinylation, trapping onto an anti-SCT IgG-coated polystyrene ball, acid elution, coupling with affinity-purified anti-SCT Fab´-peroxidase conjugate, final trapping onto streptavidin-coated polystyrene balls and measurement of peroxidase activity bound to the streptavidin-coated polystyrene balls by fluorometry. However, the assay was too tedious to screen a large number of samples emerging from clinical trials in human.

The aims of this study are: 1) to develop a more simple and sensitive enzyme immunoassay technique, and 2) to test the applicability for the pharmacokinetic study of intranasal SCT to be dosed to human.

MATERIALS AND METHODS

Calcitonins

Salmon calcitonin (SCT) (SMC 20–051, 5200 IU/mg) was the gift from Sandoz Pharmaceuticals, Ltd. (Basel, Switzerland). Human calcitonin was purchased from Sigma Chemical Co. (St. Louis, MO). The amount of calcitonin was

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quantified from that of amino groups measured by the fluorescamine method (7) using L-leucine as a standard.

SCT-Ovalbumin Conjugate

SCT was reduced with dithiothreitol and conjugated to ovalbumin using N-succinimidyl-6-maleimidohexanoate as described previously (5). The conjugate was quantified by using the commercial protein assay kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Richmond, CA) using ovalbumin as a standard.

2,4-Dinitrophenyl Bovine Serum Albumin

Thiol groups were introduced into bovine serum albumin molecules using N-succinimidyl-S-acetylthioacetate (8) and were reacted with maleimide groups introduced into εN-2,4-dinitrophenyl-L-lysine molecules using N-succinimidyl-6-maleimidohexanoate (9). The amounts of bovine serum albumin, 2,4-dinitrophenyl groups, and 2,4-dinitrophenyl bovine serum albumin were calculated from the absorbance at 280 and 360 nm (10). The average number of 2,4-dinitrophenyl groups introduced per bovine serum albumin molecule was 6.5 (10).

Antisera

Albino rabbits (New Zealand White, b.w., 2.5–3.0 Kg) were immunized with SCT-ovalbumin conjugate and 2,4-dinitrophenyl bovine serum albumin (5), and the antisera were stored at –20°C.

IgG and Its Fragments

IgG was prepared from serum by fractionation with Na_2SO_4 followed by passage through a column of diethylaminoethyl-cellulose (11). $F(ab')_2$ was prepared by digestion of IgG with pepsin from porcine gastric mucosa (11), and Fab´ was prepared by reduction of $F(ab')_2$ with 2-mercaptoethylamine (11). The amounts of IgG and its fragments were calculated from the absorbance at 280 nm (11).

2,4-Dinitrophenyl Biotinyl Anti-SCT-Ovalbumin IgG

Rabbit anti-SCT-ovalbumin IgG (4.0 mg) in 0.1 mol/l sodium phosphate buffer, pH 7.0 (0.7 ml) was incubated with 22 mmol/l N-succinimidyl-S-acetylthioacetate (Research Organics Inc., Cleveland, O) in N,N-dimethylformamide (0.07 ml) at 30°C for 30 min. The reaction mixture was incubated with 0.04 ml of 0.1 mol/l EDTA, pH 7.0 and 0.08 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 13 (11). The mercaptoacetylated IgG (1.8 mg) in 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA (0.66 ml) was incubated with maleimide-biocytin solution (0.025 ml) at 30°C for 30 min. The maleimide-biocytin solution was prepared by incubating 25 mM biocytin (Sigma Chemical Co.) in 0.1 mol/l sodium phosphate buffer, pH 7.0 (0.15 ml) with 20 mM *N*-succinimidyl-6-maleimidohexanoate (Dojindo Laboratories, Kumamoto, Japan) in *N*,*N*-dimethylformamide (0.0375 ml) at 30°C for 30 min. The average number of biotin residues introduced per the mercaptoacetylated IgG molecule was 8.1, which was calculated from the decrease in the number of thiol groups (11). The mercaptoacetylated biotinyl IgG solution was incubated with the maleimide-εN-2,4-dinitrophenyl-L-lysine solution (9) (0.11 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 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 bovine serum albumin (fraction V, Intergen Co., Purchase, NY). The average number of 2,4-dinitrophenyl groups introduced per IgG molecule was 4.5, which was calculated from the absorbance at 280 and 360 nm (10,11). The amount of the 2,4-dinitrophenyl biotinyl IgG was calculated from the absorbance at 280 and 360 nm (10,11).

SCT-Bovine Thyroglobulin Conjugate

Reduced SCT was conjugated to bovine thyroglobulin using N-succinimidyl-6-maleimidohexanoate as described previously (5). The conjugate was quantified by using the commercial protein assay kit as described above using bovine thyroglobulin as a standard.

Protein-Coupled Sepharose 4B

SCT-bovine thyroglobulin conjugate and 2,4-dinitrophenyl bovine serum albumin were coupled to CNBr-activated Sepharose 4B according to the instructions of Pharmacia LKB Biotechnology (5,10).

Affinity Purification of Antibodies

Anti-SCT-ovalbumin $F(ab')_2$ and 2,4-dinitrophenyl biotinyl anti-SCT-ovalbumin IgG were affinity-purified using columns of SCT-bovine thyroglobulin conjugate-coupled Sepharose 4B (5). Anti-2,4-dinitrophenyl bovine serum albumin IgG was affinity-purified using a column of 2,4-dinitrophenyl bovine serum albumin-coupled Sepharose 4B (10). 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 (12).

Affinity-Purified Anti-SCT Fab´-β-D-Galactosidase Conjugate

Affinity-purified rabbit anti-SCT Fab´ was conjugated to β-D-galactosidase from *E. coli* (Enzyme label for enzyme immunoassay, Boehringer Mannheim GmbH, Mannheim, Germany) using o-phenylenedimaleimide (11). The amount of conjugate was calculated from β-D-galactosidase activity (13).

Biotinyl Nonspecific Rabbit IgG

Thiol groups were introduced into nonspecific rabbit IgG using N-succinimidyl-S-acetylthioacetate as described above and were reacted with the maleimide-biocytin solution. The average number of biotin residues introduced per IgG molecule was 13, which was calculated from the decrease in thiol groups (11).

Protein-Coated Polystyrene Balls

Polystyrene balls (Immuno Chemical, Inc., Okayama, Japan) were coated with affinity-purified rabbit (anti-2,4-dinitrophenyl bovine serum albumin) IgG (0.1 g/l) and biotinyl nonspecific rabbit IgG (0.1 g/l) by physical adsorption (14). Streptavidin-coated polystyrene balls were prepared by incubating biotinyl nonspecific rabbit IgG coated-polystyrene balls with streptavidin (0.1 g/l, GIBCO BRL Life Technologies, Inc., Gaithersburg, MD) (15).

Human EDTA Plasma and Serum

Blood samples were withdrawn into glass tubes (Venoject VT-NA EDTA-2Na and Venoject VT-SA, Termo Corp., Tokyo, Japan), and centrifuged to separate the plasma and serum, respectively. Plasma and serum samples were obtained from eight healthy subjects (four males and four females, aged 23–45 years).

Calcitonin Standards

SCT and human calcitonin 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 a pooled plasma or serum from the eight healthy subjects and used as standards. In some experiments (recovery test), SCT was dissolved 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 bovine serum albumin.

Sandwich Transfer Enzyme Immunoassay for SCT

An assay samples (salmon or human calcitonin standards, plasma samples or serum samples, 0.05 ml) were incubated with 2,4-dinitrophenyl biotinyl affinity-purified anti-SCT IgG (100 fmol) and affinity-purified anti-SCT Fab´-β-D-galactosidase conjugate (10 fmol) in 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.55 mol/l NaCl, 1 mmol/l $MgCl_2$, 1.5 mmol/l 2-mercaptoethylamine, 1 g/l NaN_3 and 1 g/l bovine serum albumin (0.1 ml) at 15°C for 2 h. The reaction mixture was incubated with two colored polystyrene balls coated with affinity-purified (anti-2,4-dinitrophenyl bovine serum albumin) IgG at 15°C overnight. After incubation, the colored 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 bovine serum albumin. The washed polystyrene balls were incubated with 1 mmol/l εN-2,4-dinitrophenyl-L-lysine·HCl (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) 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 bovine serum albumin (0.15 ml) and two white polystyrene balls coated with streptavidin at 20°C for 1 h. The colored polystyrene balls were removed, and the incubation was continued at 20°C for 2 h. The white polystyrene balls were washed as above, and β-D-galactosidase activity bound to the white polystyrene balls was assayed by fluorometry (16) as follows. The white 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 bovine serum albumin (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 30°C for 150 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 spectrofluorophotometer (F-3010, Hitachi, 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 in the same buffer, respectively. The fluorescence intensity was measured relative to the 4-methylumbelliferone solution (16).

Measurement of Bioavailability

All volunteers (nine males, aged 24–35 years) had passed the screening tests to exclude serious defect(s) of liver, kidney, circulation system, respiratory system, digestive system, hematopoietic function, and endocrinological function. Six out of the nine volunteers were administered SCT (160 IU (31 μg)) intranasally. Intranasal SCT formulation was prepared by mixing SCT (Lot No. 314733, 5200 IU/mg, Sandoz Pharmaceuticals, Ltd.) with calcium carbonate solution and lyophilizing. The volunteers loaded the capsule containing the SCT formulation on the novel device (Jetlizer® (UNISIA JECS, Co., Gunnma, Japan)) and puffed it by themselves four times into both their nasal cavities. The detailed procedures for formulation and device will be published elsewhere. Three volunteers were administered SCT (10 IU (1.9 μg), Lot No. YLY002Y, 5190 IU/mg, Salmotonin®, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) intramuscularly. Before and after (5, 10, 15, 20, 25 (only intranasal), 30, 45, 60, 90, 120,

and 180 min) administration, blood samples were withdrawn into glass tubes (Venoject VT-AS), and centrifuged to separate the serum. Serum samples were kept at –80°C until assayed. The SCT concentration was measured in duplicate by the sandwich transfer enzyme immunoassay as described above, and was calculated by curve-fitting using the SCT serum standards. The bioavailability parameters, area under the concentration-time curve (AUC) and mean residence time (MRT), were calculated with a personal computer using a linear trapezoidal equation (17, 18). This study had been approved by Institutional Review Board of St. Marianna University School of Medicine.

RESULTS

Sandwich Transfer Enzyme Immunoassay for Salmon Calcitonin

Concept of the assay procedure

The assay procedure was designed to proceed as follows: 1) formation of immune complex consisting of salmon calcitonin (SCT), 2,4-dinitrophenyl biotinyl anti-SCT IgG and anti-SCT Fab´-β-D-galactosidase conjugate, 2) trapping of the immune complex onto polystyrene balls coated with (anti-2,4-dinitrophenyl group) IgG, 3) elimination of unreacted Fab´-β-D-galactosidase conjugate by specific elution of the immune complex from the polystyrene balls with εN-2,4-dinitrophenyl-L-lysine, 4) the final trapping of the immune complex onto streptavidin-coated polystyrene balls and 5) measurement of the β-D-galactosidase activity bound to the streptavidin-coated polystyrene balls by fluorometry. The whole procedure was completed within 2 days. In terms of the sensitivity, specificity and precision, the present assay could measure SCT not only in EDTA plasma but also in serum.

Sensitivity and Specificity

SCT was diluted with a pooled plasma from eight healthy subjects as described in the Materials and Methods section, and subjected to the present assay. The assay was able to detect 0.05 pg (15 amol)/50 μl of a given sample, that is the minimum concentration for detection being 1 pg/ml (Fig. 1). The detection limit was taken to be the minimal concentration obtained 2SD (n = 4) above the plasma background. The standard curve was linear up to a concentration of 3000 pg/ml (Fig. 1). The assay is specific for SCT and practically no interference occurred by human calcitonin up to 10 ng/ml (Fig. 1). In addition, the variation coefficient (CV) of fluorescence intensity for nonspecifically bound β-D-galactosidase activity using the eight plasma was 9.5% (4.3 ± 0.41 (SD)) (Table 1, left column), indicating that normal human plasma appears to contain no substance detected as SCT.

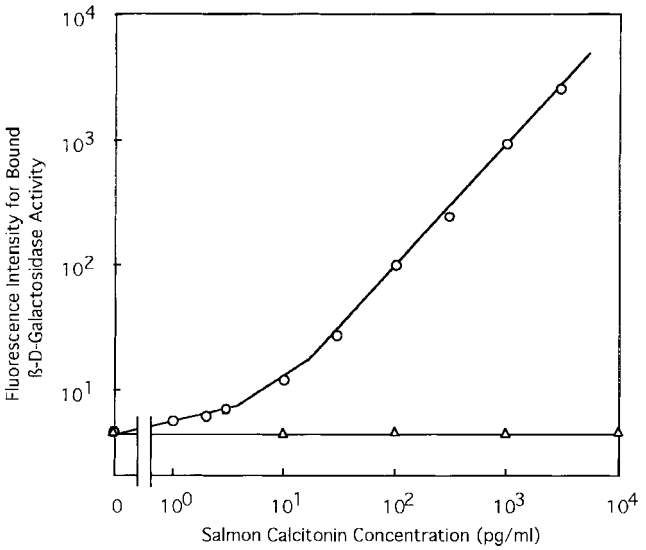

Fig. 1. Dose-response curves for calcitonin by sandwich transfer enzyme immunoassay. Circles and triangles indicate the curves for salmon calcitonin and human calcitonin, respectively, in the presence of a pooled plasma from eight healthy subjects. Each value indicates the mean of 4 determinations (SCT 0–10 pg/ml) and 2 determinations (SCT 30–3000 pg/ml and human calcitonin), and small horizontal bars with vertical bars indicate ± SD (SCT 0–10 pg/ml).

Assay Variation

When examined at three different plasma levels in the range of 10–2000 pg/ml for within-assay and for between-assay variation, the CVs were 6.4–8.5% (n = 8) and 7.4–13.3% (n = 7), respectively (Table 2). The samples using the assay variation were prepared by mixing SCT with the pooled plasma.

The SCT plasma standards (0, 10, 30, 100, 300 and 1000 pg/ml) were subjected to the present assay seven times, the

TABLE 1. The Background in the Presence of Human Plasma and the Recovery Rate of Salmon Calcitonin Mixed with Human Plasma

Plasma No.	Background (Fluorescence intensity for nonspecifically bound β-D-galactosidase activity)	Recovery rate of salmon calcitonin mixed with plasma[a] (%) Salmon calcitonin concentration (pg/ml) 20	200
1	4.3	101	111
2	3.7	92	85
3	4.6	89	107
4	3.8	100	106
5	4.5	109	107
6	4.9	88	93
7	4.2	82	101
8	4.5	86	101

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

TABLE 2. Within-Assay and Between-Assay Imprecision

Assay	SCT Level Added to Plasma (pg/ml)	Number of Determinations	SCT Level Detected mean ± SD (pg/ml)	Coefficient of Variation (%)
Within-assay	10	8	9.4 ± 0.6	6.4
	100	8	104 ± 7.4	7.1
	1000	8	1035 ± 88	8.5
Between-assay	20	7	18 ± 2.4	13.3
	200	7	196 ± 18	9.2
	2000	7	2155 ± 160	7.4

CVs for fluorescence intensity for specifically bound β-D-galactosidase activity were 20–24% (Fig. 2). The between-assay variation and SCT standards variation were run over a period of 12 days.

Recovery

SCT was mixed with eight plasma samples at concentrations of 20 and 200 pg/ml and assayed. The recovery rates of SCT were 93.4 ± 9.1 (SD)% (ranged 82–109%, CV = 9.7%) and 101 ± 8.6 (SD) % (ranged 85–111%, CV = 8.5%) (Table 1, middle and right columns), respectively, which were calculated by curve-fitting of a standard curve in the absence of plasma.

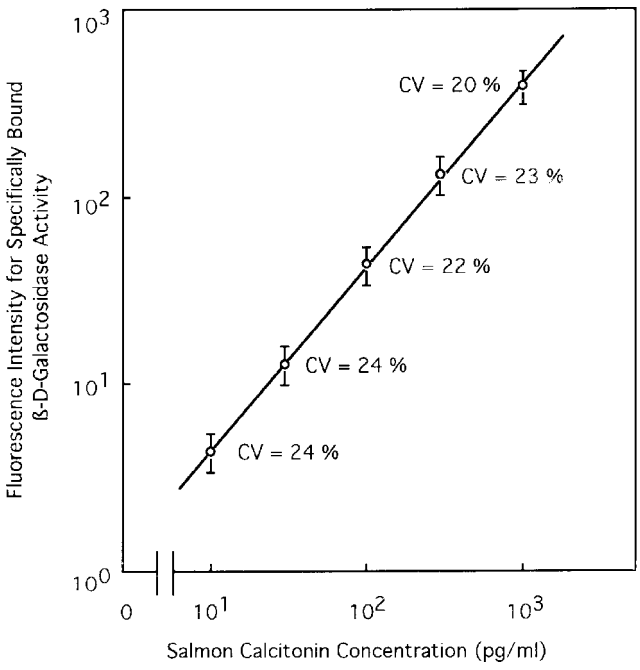

Fig. 2. Intermediate precision of standard curve for SCT. The SCT standards (0, 10, 30, 100, 300 and 1000 pg/ml) were assayed by the present assay seven times, and the variation coefficients for fluorescence intensity for specifically bound β-D-galactosidase activity were calculated.

Stability of SCT in Plasma Matrix

Firstly, SCT was mixed with the pooled plasma at concentrations of 100 and 1000 pg/ml, incubated at 20°C or 37°C for 0–2 h, and subjected to the present assay. The recovery rates of SCT in the plasma matrix were satisfactory until 2 h incubation (Fig. 3-(a)).

Secondly, SCT was mixed with the pooled plasma at concentrations of 20, 200, and 2000 pg/ml, stored at –20°C for 0–4 mon, and assayed. The recovery rates of SCT were satisfactory until 4 mon storage (Fig. 3-(b)).

Thirdly, SCT was mixed with the pooled plasma at concentrations of 100 and 1000 pg/ml, and subjected to freezing (–20°C) and thawing (room temperature) three times. The thawed samples were assayed. The recovery rates were satisfactory until three freeze-thaw cycles (Fig. 3-(c)).

Bioavailability of Intranasal SCT

Figure 4 shows the appearance-disappearance pattern of intranasal SCT (160 IU, 31 μg) and intramuscular SCT (10 IU, 1.9 μg) in human. The area under the blood concentration-time curve (AUCs) were calculated to be 9400 ± 5400 (SD) and 5600 ± 2000 (SD) pg·h/ml, respectively (Table 3). The comparative AUC (bioavailability) of intranasal SCT as compared with intramuscular SCT was 11%. The mean residence time (MRTs) were calculated to be 42 ± 14 min and 39 ± 19 min, respectively (Table 3). The maximal concentration (Cmax) of intranasal SCT was calculated to be 210 ± 94 pg/ml with the maximal time (Tmax) of 10–20 min and the Cmax of intramuscular SCT was calculated to be 140 ± 60 pg/ml with the Tmax of 5–15 min (Table 3).

DISCUSSION

In order to improve patient acceptability, clinical trials to develop parenteral formulations of calcitonin, which is with the ease for self-administration and can replace injections, have been reported. However, no direct study has been attempted to know the pharmacokinetic properties of the formulations in the therapeutic doses. The reason is simple, due to the lack of a sensitive assay method. From such a consideration, Rong, H., Deftos, L.J., Ji, H., Bucht, E. (19) reported a two-site immunofluorometric assay for salmon calcitonin (SCT) using a monoclonal antibody against SCT1–11 and a polyclonal antibody against SCT10–32. The polyclonal antibody was labeled with europium ion and used as a "signaling antibody". This assay is simple and highly specific, furthermore, the sensitivity is almost same as the present assay. An advantage of the use of europium ion as a label is that labeling with europium ion is much simpler than that with enzyme and radioisotope. However, a disadvantage was that the fluorescence intensity for europium ion was enhanced to various degrees due to unknown reason(s), damaging the precision (20,21). Accordingly, variation coefficients (CVs) of within-

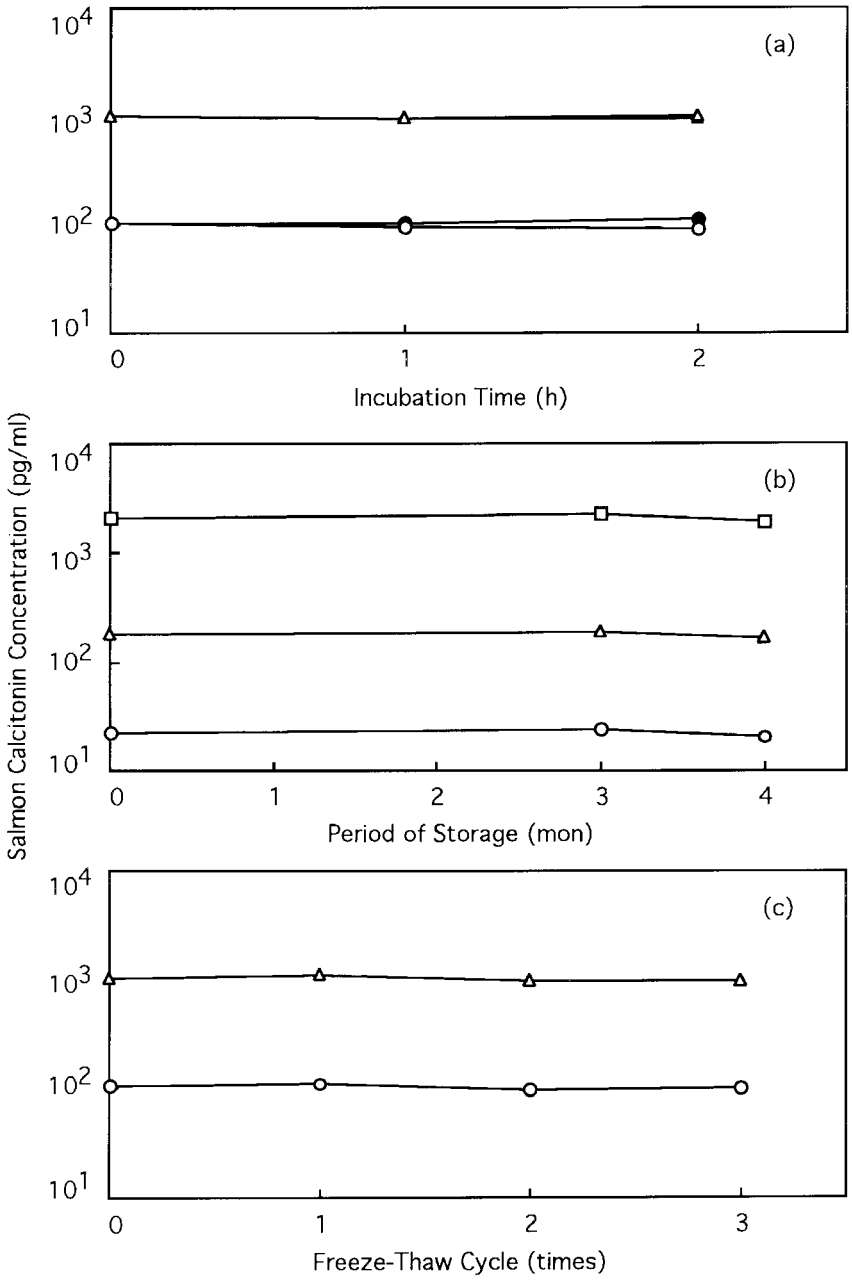

Fig. 3. Stability of SCT in plasma matrix. (a) SCT was mixed with a pooled plasma at concentrations of 100 pg/ml (circles) and 1000 pg/ml (triangles), incubated at 20°C (open symbols) or 37°C (closed symbols), and assayed. (b) SCT was mixed with a pooled plasma at concentrations of 20 (circles) 200 (triangles) and 2000 (squares) pg/ml, stored at –20°C, and assayed. (c) SCT was mixed with a pooled plasma at concentrations of 100 pg/ml (circles) and 1000 pg/ml (triangles), subjected to freezing-thawing, and assayed.

assay and between-assay using europium ion as a label were 13–31%, while those using enzyme as a label were less than 13% (20). Furthermore, the CVs of reagent blank (only the enhancement solution) were 496% (3204 ± 15894 (SD) counts, n = 48) in a conventional room and 13% (230 ± 30 (SD) counts, n = 132) in an isolated room (unpublished observations). The assay precision described in their report were too high (the CVs of within-assay and between-assay were 1.0–7.8%), without comment as to how such a low variation was achieved using europium ion as a label.

We recently had developed a highly sensitive enzyme immunoassay (hetero-two-site enzyme immunoassay) for SCT (5), and made it possible to estimate the bioavailability of nasal SCT given in a therapeutic dose range to rat (6). It was too tedious to screen a large number of clinical trial samples, though the assay was sensitive. In this study, a simpler en-

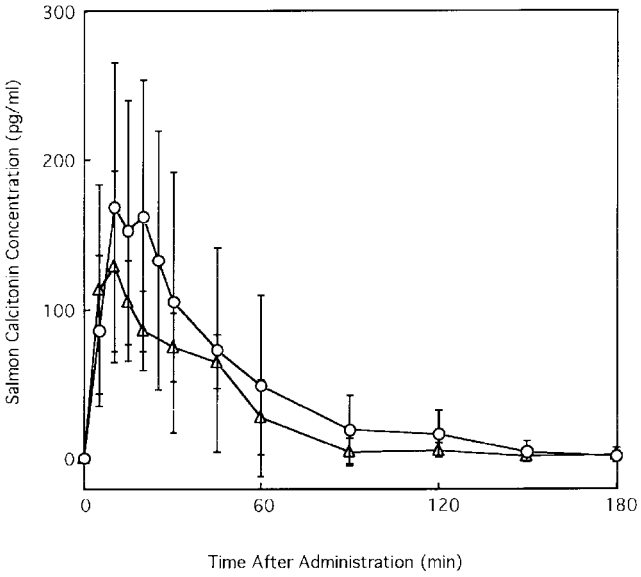

Fig. 4. Concentration of SCT after an intranasal administration of SCT (160 IU (31 μg)/subject, n = 6, circles) and intramuscular administration of SCT (10 IU (1.9 μg)/subject, n = 3, triangles) to human. Each value indicates the mean, and small horizontal bars with vertical bars indicate ± SD.

zyme immunoassay (sandwich transfer enzyme immunoassay), eliminating centrifugal filtration step, biotinylation step, and one incubation step from the previous assay, has been able to be developed without sacrificing the sensitivity and rather raising. By the previous assay, SCT was able to be measured at the detection limit of 4 pg/ml with the recovery rate of 25–37% in the presence of human plasma (data not shown). By the new method, now it became possible to measure 1 pg/ml with the recovery rate of 82–111% in the presence of human plasma (Fig. 1 and Table 1).

The present assay did not recognize SCT fragments (SCT1–7 and SCT1–11), however, recognized SCT10–32 and SCT19–32. The cross-reactivity rates estimated for SCT10–32 and SCT19–32 were 33 and 10%, respectively. In so far as examined by HPLC using a column of COSMOSIL 5C 18-P, no degradation product of SCT has been found in serum samples of volunteers injected with SCT intramuscularly. These results clearly show that the assay developed herein can provide us reliable and sensitive method to estimate the clinical bioavailability of SCT applied in a therapeutic dose range to human.

TABLE 3. Pharmacokinetic Parameters After Salmon Calcitonin Administration

	Dose (IU)	AUC[a] mean ± SD (pg·h/ml)	MRT[b] mean ± SD (min)	C_{max}[c] mean ± SD (pg/ml)
Intranasal	160	9400 ± 5400	42 ± 14	210 ± 94
Intramuscular	10	5600 ± 2000	39 ± 19	140 ± 60

[a]Area under the blood concentration-time curve.
[b]Mean residence time
[c]Maximal concentration

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