Sensitive Time-Resolved Fluoroimmunoassay of Salmon Calcitonin

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A two-site assay was developed by use of the “dissociation and enhancement lanthanide fluoroimmunoassay” (DELFlIA) technique for determination of salmon calcitonin (SCT) in serum after administration to osteoporotic patients. Polyclonal antibodies were produced in rabbits immunized with SCT coupled to ovalbumin. After affinity purification, the antibodies were used both as immobilized capture antibodies and as Eu-chelate-labeled signal antibodies. A sensitive assay with a detection limit of 1.1 pmol/L was achieved, and no cross-reaction with human calcitonin was observed. The intra- and interassay CVs were <12% (n = 10) and <15% (n = 4), respectively. Analytical recovery of SCT added to serum was 91% ± 3% (mean ± SD, n = 4). SCT was measurable in all the samples from eight osteoporotic patients after subcutaneous SCT administration. We conclude that this new sensitive and specific two-site DELFlIA can reliably measure SCT in serum.

Indexing Terms: osteoporosis/intraindividual variation/europium chelate

Administration of salmon calcitonin (SCT) increases bone mass in a dose-dependent manner and reduces the rate of new fractures by two-thirds in elderly women with moderate osteoporosis (1). However, many patients do not tolerate SCT because of nausea, flushing, and other side effects; in other patients, the drug may be ineffective because of inadequate dosage. Accurate determination of SCT in serum is needed for optimizing individual doses. A reliable and sensitive SCT assay is therefore a prerequisite for pharmacokinetic and experimental studies.

For more than two decades, radioimmunoassay (RIA) of SCT and radioactivity measurements of serum after in vivo injection of radiolabeled calcitonin (CT) have been the main methods for determination of this drug. However, because of the low concentrations of the hormone and the various sensitivities of the assays, the physiological and pharmacokinetic data derived from immunoreactive and radioactive CT data show large variations. The values obtained by RIA tend to be low, whereas those based on radioactivity of the injected labeled CT tend to be high (2).

Europium chelate has been used to label the reagents in many immunoassays (3–5). The dissociated and rechelated europium ions serve as excellent nonradioactive markers that can be measured with high sensitivity by time-resolved fluorometry. Here we describe a two-site immunoreactive assay for determining SCT in blood, based on the use of europium-labeled antibodies. Use of a sandwich antigen–antibody complex provides specificity, and the fluorescence intensity of the europium chelate accounts for the sensitivity. Our aim was to develop a sensitive immunoassay for determination of SCT in serum. The lack of sensitive and specific assays to date has meant few attempts to correlate SCT effects to its concentration in blood.

Materials and Methods

Clinical samples. Serum samples were collected from eight patients—ages 82 ± 4 years (mean ± SD, range 77–86), body weight 56 ± 10 kg (range 41–73), three men and five women—with x-ray-verified vertebral compression fracture due to osteoporosis. None of the subjects had previously been treated with CT of any form, and all had normal kidney and liver functions. The patients were injected subcutaneously in the abdomen with SCT (100 IU/L, Micalsec®, Sandoz, Basel, Switzerland), 50 IU for subjects <60 kg and 100 IU for those >60 kg. For each subject 10 mL of blood was drawn from a cubital vein at various times before and after injection: −10, 0, 30 min and 1, 2, 8, and 24 h. All the serum samples were kept at −20°C until analysis. The study was approved by the Committee of Ethics at the Karolinska Hospital and was performed according to the Declaration of Helsinki of 1975, as revised in 1983.

Antibody production and labeling. Synthetic SCT (Sandoz) was covalently coupled to ovalbumin (OA) with use of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma, St. Louis, MO) as described previously (6). After dialysis against 9 g/L NaCl, the SCT-OA complex was lyophilized, dissolved in 10 mmol/L HCl and emulsified in an equal volume of Freund’s complete adjuvant (Difco Lab., Detroit, MI). Three New Zealand White rabbits (3–4 kg) were immunized with 300–400 µg of coupled SCT, injected subcutaneously on the neck. Subsequent immunizations were performed with ~300 µg of SCT-OA emulsified in Freund’s incomplete adjuvant. The rabbits were bled 10 days after the final boost (n = 3 boosts) and each subsequent injection. Two rabbits produced high amounts of SCT antibodies, designated 309# and 246#. The polyclonal rabbit antibodies were purified by two-step affinity chromatography, as follows. The antiserum was mixed with phosphate-buffered saline (PBS)-washed preswollen Protein A–Sepharose CL-4B gel (Pharmacia, Uppsala, Sweden) in a tube and rotated overnight at 4°C. The gel and serum mixture was thoroughly rinsed.
with PBS in a column. IgG was eluted with 0.2 mol/L glycine-HCl buffer (pH 2.8) and neutralized immediately with 0.1 mol/L NaOH. The fractions containing IgG were pooled and dialyzed against 0.1 mol/L NaHCO₃ in 0.5 mol/L NaCl and then against PBS. SCT ligand was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The SCT antibodies in the dialyzed IgG product were extracted by SCT coupled to Sepharose in a tube and rotated for 2 h at room temperature. The unbound IgG was washed away with PBS in a 1 x 15 cm Econo-column (Bio-Rad, Richmond, CA), and the antibodies directed against SCT were eluted with 1 mol/L acetic acid. After dialysis against PBS, the antibodies were divided into two portions, frozen, and lyophilized.

One portion of the freeze-dried product was dissolved in 0.5 mL of distilled water, and the amount of SCT antibodies was determined by being used as capture antibody, and the other was labeled with europium chelate for use as signal antibodies. The possibility of using the same batch of affinity-purified polyclonal antibodies for both sites in a two-site antibody-sandwich assay has been described earlier (7). Testing the two affinity-purified antibodies 309# and 246# in four possible combination "pairs" showed that the most sensitive assay was achieved with 309# as both capture and signal antibody.

We used a DELFIA Eu-labeling kit (Wallac Oy, Turku, Finland) to label the purified SCT antibody. The freeze-dried antibodies were dissolved in labeling buffer (50 mmol/L NaHCO₃, pH 8.5, containing 9 g/L NaCl), and the Eu-labeling reagent was reconstituted in labeling buffer. The antibody solution was gently mixed with the reconstituted Eu-labeling reagent in an IgG:Eu³⁺ molar ratio of 1:50. After incubation overnight at room temperature, the reaction solution was applied to a column of Sepharose 6B (2 x 40 cm) with Sephadex G50 (2 x 15 cm) on the top. The elution buffer was 50 mmol/L Tris-HCl, pH 7.8, containing 9 g of NaCl and 0.5 g of NaNO₃ per liter. Fractions (2 mL) were collected, and 10 µL of each was diluted serially by 1:1000 to 1:10 000 with elution buffer. We added 10 µL of the diluted fractions and 90 µL of enhancement solution (Wallac Oy) to the microtiter plate wells and, after shaking for 5 min, determined the Eu³⁺ concentration in the fractions by measuring their fluorescence with a time-resolved fluorometer (DELFIA 1232; Wallac Oy). The fractions containing the labeled SCT antibodies were pooled, and stabilizer (purified bovine serum albumin; Wallac Oy) was added at a final concentration of 1 g/L. The labeled antibodies were stored at 4°C. The average labeling yield was 15 mol of Eu³⁺ per mole of IgG, which gives out a specific activity of 5 x 10⁶ fluorescence counts per second per 1 ng of Eu-labeled antibody.

Plate preparation. One volume of SCT antibody stock solution (1 g/L) was incubated with six volumes of 0.1 mol/L glycine-HCl, pH 2.5, for 10 min at room temperature. These acid-treated antibodies were poured into an appropriate volume of 0.2 mol/L NaH₂PO₄, to give a concentration of 1 mg/L, and mixed gently; 150 µL of this capture-antibody coating solution was dispensed into each well of a microtiter plate, and the plate was then covered with Parafilm to avoid evaporation.

After incubation at 37°C for 2 h, the plate was washed once, 350 µL/well, with washing solution (25-fold dilution of NaCl 225 g/L, Tris-HCl 125 mmol/L, Germall® II (Sutton Laboratories, Chatham, NJ) 25 g/L, Tween 20 1.25 g/L, pH 7.75). Then 200 µL of blocking buffer (50 mmol/L Tris-HCl, 30 g/L trehalose, 1 g/L bovine serum albumin, 1 g/L Germall II, pH 7.2) was dispensed into each well and incubated at room temperature for 2 h.

DELFIA procedure. The plate was washed once (with the above diluted washing solution), and 150 µL of the synthetic standard in pH 7.8 assay buffer (per liter, 50 mmol of Tris-HCl, 0.15 mol of NaCl, 0.5 g of NaNO₃, 5 g of bovine serum albumin, 10 mg of diethylenetriamine pentaacetic acid, and 0.1 mL of Tween 40), the serum samples, or the blanks were added. The plate was incubated with shaking at 4°C overnight and washed three times. Then 150 µL of the labeled antibody solution, with 0.2 g/L bovine IgG added, was dispensed into the wells. After 2 h of shaking at room temperature, the plate was washed six times and 150 µL of the enhancement solution was added. After another 5 min of shaking, the fluorescence was measured with a DELFIA 1232 plate fluorometer.

Serum calcium measurement. Total calcium in serum was measured on a Kodak Ektachem 700 Analyzer with a Clinical Chemistry Slide [Ca] (Eastman Kodak, Rochester, NY) at different times after SCT administration.

Results
Methodological aspects. In this SCT DELFIA, the fluorescence counts of serially diluted SCT demonstrated linearity, and the dilutions of serum samples as well as of SCT added to serum showed parallelism to the SCT calibration curve (Fig. 1). The detection limit, based on 2 SD (n = 6) above the blank fluorescence, was 1.1 pmol/L (3.9 ng/L). Given that only 150 µL of standard or

![Fig. 1. Representative calibration curve of synthetic SCT (○), dilutions of SCT added to serum (●), and dilutions of serum samples (●) from osteoporotic patients after SCT administration.](Image)
sample was added to each well, amounts as small as 0.6 pg of SCT can be detected in a well. The intrasay CVs were 11% (n = 10) and the interassay CVs for 5, 15 and 25 pmol/L were 15%, 5%, and 8% (n = 4), respectively. There was no cross-reaction with human CT. The binding capacity of the antibodies for SCT is > 2 nmol/L with ~150 ng of antibodies coated per well. Analytical recovery of 5, 10, 15, and 20 pmol/L SCT added to serum was 91% ± 3% (mean ± SD, n = 4). Recovery of 50 pmol/L SCT added to serum at 1:2, 1:4, 1:8, and 1:16 dilutions was 94% ± 9% (n = 4).

Pharmacokinetics of SCT in osteoporotic patients. The mean serum concentration of SCT in eight osteoporotic patients receiving an average dose of 1.20 ± 0.29 IU/kg of body wt. is shown in Fig. 2A; the individual values for each subject are shown in Fig. 2B. SCT reached its highest concentration in all subjects by 30–60 min after injection; after 120 min, it had decreased by 20–50%. The higher the peak concentrations, the more they decreased. By 8 h SCT was undetectable in most subjects. Because of the differences in the doses given (IU/kg body wt.) and the individual variations in the absorption and metabolism, the peak concentrations varied considerably. The mean (SD) total calcium concentrations in the eight subjects were 2.50 (0.24) mmol/L before the administration of SCT and 2.41 (0.22), 2.31 (0.10), and 2.34 (0.12) mmol/L at 2, 8, and 24 h afterwards, respectively. Total serum calcium concentrations decreased significantly (P < 0.05, paired Student's t-test) by 2 h after SCT administration and reached their lowest values by 8 h.

Absorption and metabolism of SCT. The correlation and linear regression analyses between doses (IU/kg) and peak concentrations and between doses and areas below the pharmacokinetic curves yielded correlation coefficients of 0.41 and 0.36, respectively, neither of which was significant (P > 0.05). The relation between the dosage and the peak value was well as the area below each curve demonstrated a great individual variation in drug absorption and metabolism (Fig. 3).

Discussion

Treatment of postmenopausal osteoporosis with subcutaneously or intranasally administered SCT has become widely used in recent years, now that the hormone has been registered for this indication in increasing number of countries. The treatment effectively inhibits the bone resorption and decreases the rate of bone loss in the majority of patients. However, the effect is dose-dependent; moreover, SCT has little therapeutic effect in some patients and includes side effects such as flushing and gastrointestinal disturbance in many patients. The bioavailability and pharmacokinetics of SCT vary considerably between patients and depend on the mode of administration (8, 9). We believe that a sensitive two-site assay, such as was presented here, can be a valuable tool for pharmacokinetic studies in the near future.

SCT was first determined by immunossay in fish (10, 11); a sensitive assay was not necessary because of the high SCT concentrations. Later, immunossays were used to study the pharmacokinetics of SCT in humans who were using the drug for treatment of skeletal diseases (12, 13). When SCT is administered subcutaneously in relatively low doses, as in our study, a sensitive assay is needed. The DELFIA method could measure SCT in every sample from subjects administered 50 or 100 IU of SCT. The lower dose in patients weighing <60 kg was chosen to avoid side effects (e.g., nausea and vomiting), which are not uncommon and sometimes pronounced.

A two-site assay has a high specificity for the antigen to be measured, because of the requirement that two parts of the antigen have to be recognized by the antibodies. This two-site assay with a lanthanide as the signal substance obtained 10 times greater sensitivity than an earlier two-site IRMA (14) and has the advantage of using nonradioactive markers with long stability. In our hands the europium-labeled SCT antibodies were stable for >1 year.
Several different ways of administration of SCT have been studied. Nasal sprays and suppositories of the hormone appear to exert the known biological effects in healthy subjects (8, 15). In patients with primary hyperparathyroidism, intramuscular SCT injection was more potent than the intranasal administration (9). Subcutaneously administered SCT in combination with etidronate lowers serum calcium in patients with humoral hypercalcemia of malignancy (16). Intravenous infusion of SCT had a potent analgesic effect on patients with phantom limb pain after amputation (17).

Only weak correlations were found between the given doses of SCT and the observed peak values or the calculated areas below the pharmacokinetic curves. This illustrates the large interindividual variation of SCT absorption and metabolism, even though the peptide was administered subcutaneously in the abdomen, a location known for reliable absorption of, for example, insulin (18).

In conclusion, this DELFIA is a highly sensitive two-site immunoassay for determination of SCT in serum. Because the absorption and metabolism of SCT seem to be quite variable in these elderly patients, individual pharmacokinetic observations are of great importance to optimize the dosage in clinical treatment.

H.R. was a recipient of the fellowship from the Wenner-Gren Center Foundation. The study was supported by grants from the Swedish Cancer Society (3129), the Swedish Medical Research Council (5992), the Fundus of Karolinska Institute, the Magn. Bergvall Foundation, the Tore Nilson’s Fund, the Novo Nordisk Foundation, the Swedish Society of Medicine, the Loo and Hans Osterman Foundation, the Foundation of “Syskonen” Svensson, and the Memory Foundation of C. Groshinsky and of S. and E. Golje.

References

CLINICAL CHEMISTRY, Vol. 40, No. 9, 1994 1777