Two-site immunofluorometric assay of intact salmon calcitonin with improved sensitivity

Haiqin Rong, Leonard J Deftos, Hong Ji, and Elisabet Bucht*

We recently developed a two-site immunofluorometric assay (IFMA) of salmon calcitonin (SCT) by DELFIA (dissociation enhancement lanthanide fluoroimmunoassay) technique using the same polyclonal antibodies both for “catching” the antigen and for signaling. In the present study we used a monoclonal antibody to SCT 1–11 as the capture antibody. This antibody was biotinylated before use in streptavidin-coated microtitration plates. The polyclonal antibody labeled with Eu chelate was used as a signaling marker. This combination of antibodies resulted in an assay that was three to four times more sensitive than the previous IFMA, with a detection limit of 0.3 pmol/L serum. Intact SCT 1–32 was detected by the assay (recoveries 94–96%), but not the fragments SCT 1–11 and SCT 10–32 or human calcitonin. Dilutions of plasma samples containing SCT were parallel to the calibration curve of SCT 1–32. Pharmacokinetic studies of SCT, 100 IU administered intramuscularly to 10 men, indicated peak serum concentrations of 32–128 pmol/L within 10–20 min with apparent half-life of 56 ± 18 min (mean ± SD). This new assay will allow study of the pharmacokinetics of new calcitonin preparations that do not require injection.

INDEXING TERMS: fluorometry • lanthanide

Synthetic salmon calcitonin (SCT), a peptide hormone of 32 amino acids, is used in the treatment of diseases with increased bone resorption [1–3] and hypercalcemia of different origins [4, 5]. Many laboratories including ours have developed immunoassays in various formats for SCT [6–8]. We have recently developed a two-site immunofluorometric assay (IFMA) with DELFIA (dissociation and enhancement lanthanide fluoroimmunoassay) technique [8]. In the present study, we have improved the sensitivity of this assay by introducing a biotinylated monoclonal antibody against SCT fragment 1–11 [7] immobilized on streptavidin-coated plates as “catching” antibody. For signaling, a Eu-labeled affinity-purified polyclonal antibody was used as in the former study [8]. The Eu chelates are ideal alternatives to radioactive labels because of their long half-life and because they can be measured with high sensitivity by time-resolved fluorometry [9–11].

Materials and Methods

ANTIBODY PREPARATION

A polyclonal antibody (309#) raised in rabbit against synthetic SCT coupled to ovalbumin was used as a signaling antibody [8]. Before labeling with the Eu chelate the antibody was affinity-purified by means of SCT 1–32 covalently coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and dialyzed against labeling buffer (50 mmol/L NaHCO3, pH 8.5, containing 9 g/L NaCl). Finally the dialyzed antibody solution was concentrated by use of Centrifen membrane cones CF 50A (Amicon Scientific Systems Division, Danvers, MA) to ~1 mg in 500 μL of labeling buffer for Eu labeling.

Three monoclonal antibodies were raised against SCT peptides, 5G9 and 8A4 against SCT 1–11 and 3G4 against SCT 10–32 [7]. They were purified from ascites by Staphylococcus protein A (SPA) chromatography before biotinylation and (or) Eu labeling.

ANTIBODY LABELING

The affinity-purified polyclonal antibody (309#) was labeled with a Eu chelate as follows: 1 mg of the antibodies was incubated at room temperature overnight with 0.2 mg of Eu-labeling reagent (Wallac, Turku, Finland) at a final volume of 0.5 mL of labeling buffer. The Eu-labeled antibodies were purified from excess Eu-labeling reagent and aggregated antibodies on a Sephadex G50 on the top as earlier described [8]. The
labeled antibody was subaliquoted and kept frozen at –80 °C. Before use, diluted labeled antibody solution was passed through a 0.2-μm filter unit Minisart® N (Sartorius, Göttingen, Germany) to exclude any aggregated antibodies. The monoclonal antibody (3G4) was also labeled in the same way.

ANTIBODY BIOTINYLLATION
One mg of antibody in PBS was reacted with biotin-7-NHS (d-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester) for 2 h at room temperature by using a biotin labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany). The amount of biotin-7-NHS added was 30 μg for the monoclonal antibodies 5G9, 8A4, and 3G4 and 150 μg for the polyclonal 309#; this gave a molar ratio of biotin-7-NHS to antibody of 10 for the monoclonal and 50 for the polyclonal. Nonbound biotin-7-NHS was removed by gel filtration. The biotinylated antibodies were divided into 100-μg portions in PBS and lyophilized. Before use, the antibodies were dissolved in 100 μL of water and diluted 1:1000 in assay buffer (50 mmol of Tris-HCl, 0.15 mmol of NaCl, 0.5 g of NaN₃, 5 g of bovine serum albumin, 10 mg of diethylenetriaminepentaacetic acid, and 0.1 mL of Tween 40 per liter).

IFMA
Biotinylated antibody solution (1 mg/L) was incubated in streptavidin-coated microtitration plates (Wallac) (200 ng/well) overnight at 4 °C. The plate was washed three times with 0.3 mL of washing solution [25-fold dilution of 225 g/L NaCl, 125 mmol/L Tris-HCl, 25 g/L Germall II (Sutton Labs., Chatham, NJ), 1.25 g/L Tween 20, pH 7.75]. Calibrator (synthetic SCT 1–32) (Sandoz, E. Hanover, NJ) or samples (0.2 mL) were incubated at 4 °C for 24 h. For dilution studies, plasma samples were diluted with plasma without SCT 1:1, 1:2, and 1:4. After the plate was washed three times, 0.2 mL of the Eu-labeled antibodies was added. After shaking for 2 h at room temperature, the plate was washed six times and 0.2 mL of enhancement solution (Wallac) was added; and after another 5 min of shaking, the fluorescence was measured with a DELFIA 1234 plate fluorometer (Wallac).

SUBJECTS AND SAMPLING
By use of the IFMA we have studied the pharmacokinetics of SCT in 10 healthy men, ages 19–75, within 15% of ideal body weight for height and body frame (Metropolitan Insurance Co.). After an overnight fast, they were given 100 IU of SCT (Miacalcin®, Sandoz) intramuscularly. Blood samples were collected at the indicated time points and the serum kept frozen for SCT assay. The protocol was approved by the Institutional Review Board of the University of California, San Diego. The individual elimination half-life, i.e., the time when the serum concentration of SCT attains half of the peak values after injection, was determined by their respective concentration–time curves.

RESULTS
TESTS OF ANTIBODY COMBINATIONS
Biotinylated antibodies 309#, 8A4, 5G9, and 3G4 (B-309#, B-8A4, B-5G9, and B-3G4) and Eu-labeled antibodies 309# and 3G4 (Eu-309# and Eu-3G4) were respectively tested to determine the best combination (Fig. 1). The combination B-5G9/Eu-309# resulted in the best sensitivity, and the background was low. The fluorescence responses of various SCT concentrations in the calibration curves assayed by B-309#/Eu-309# and B-8A4/Eu-309# increased gradually but not linearly. No significant responses were found in the assays with B-309#/Eu-3G4, B-5G9/Eu-3G4, and B-3G4/Eu-309#. In addition, the assays with Eu-3G4 as a signal had a high background.

EFFECTS OF COATING CONDITIONS
The incubation time, temperature after addition of biotinylated antibody solution, and concentration of biotinylated antibodies were compared (Fig. 2). The coating periods of 2, 15, and 72 h had no obvious effect on the calibration curves. Overnight incubation at 4 °C, 25 °C, and 37 °C showed different fluorescence responses, but the sensitivity was not affected. Two hours of incubation...
at 4°C and 25°C resulted in similar curves. At higher concentrations of SCT, the wells coated with 400 ng of biotinylated antibody showed a slightly higher fluorescence response than those coated with 200 ng of antibody.

**EFFECTS OF INCUBATION TIME**

Incubation of SCT with the immobilized antibodies for 24 and 72 h at 4°C is demonstrated in Fig. 3. The prolonged incubation resulted in lower fluorescence responses, and the sensitivity was not improved.

**B-5G9/EU-309# ASSAY VALIDATION**

Polyclonal antibody 309# and monoclonal antibody 5G9 gave the best assay results. The sensitivity and specificity of the assay when using the polyclonal antibody 309# for signaling and the monoclonal antibody 5G9 for catching are demonstrated in Fig. 4. The fluorescence responses were linear to various SCT concentrations. The dilutions...
of human serum samples supplemented with SCT were parallel to the calibration curve. There was no cross-reaction with the synthetic SCT fragments 1–11 and 10–32 or with human synthetic calcitonin (Peninsula, Belmont, CA) up to a concentration of 9.4 nmol/L. The detection limit based on 2 SD above the background was 0.15 pmol/L in assay buffer and 0.3 pmol/L in serum. The recovery of SCT added to serum was 93.9 ± 5.6% (n = 4), 94.2 ± 7.3% (n = 12), and 95.5 ± 10% (n = 8) at 2.9, 5.9, and 23.5 pmol/L, respectively. The intraassay variations were 7.8%, 4.9%, 2.6%, and 1.0% (n = 12) for SCT concentrations of 0.6, 1.2, 2.4, and 4.7 pmol/L, respectively. The interassay variations were 5.9% (n = 4), 6.9% (n = 8), and 3.7% (n = 8) for 2.9, 5.9, and 23.5 pmol/L, respectively. The binding capacity of the plate when coated with 200 ng of biotinylated antibody was up to 300 pmol/L. The calibration curve is linear until at least 300 pmol/L (data not shown).

**Pharmacokinetic Studies**

Figure 5 shows the individual pharmacokinetic profiles of serum SCT in 10 healthy men. In most of the subjects, serum SCT attained peak concentrations at 10 min and in some at 20 min. The range of peak values was 32–128 pmol/L. At ~1 h, the serum SCT concentration declined to half of the peak values. The mean (± SD) elimination half-life was 56 ± 18 min (range 34–85 min). By 4 h, the serum SCT concentrations were 1.4 ± 0.9 pmol/L (mean ± SD, range 0.3–3.1 pmol/L) in seven subjects and the remaining three subjects had undetectable concentrations (<0.3 pmol/L).

**Discussion**

Imunoassays for SCT were first developed to study the physiology of the hormone in fish [6, 12]. When SCT became an injectable drug [13], immunoassays were used to study its pharmacokinetics. The development of transmucosal preparations of SCT such as the nasal form has required assays of improved specificity and sensitivity for pharmacokinetic studies, since lower serum concentrations are achieved [14]. To improve assay sensitivity, we have modified the two-site IFMA by use of a SPA-purified monoclonal antibody for solid immobilization and an affinity-purified polyclonal antibody for signaling. In addition, for antibody immobilization, biotin conjugation technique and streptavidin-coated microtiteration plates have been used. Compared with a previously developed IFMA [8] in which only one affinity-purified polyclonal antibody was used both for catching and for signaling and the antibody was immobilized by physical adsorption on the plate, the sensitivity of the current assay has been increased 3–4 times. The assay detects only intact SCT 1–32. However, when a monoclonal antibody for SCT 10–32 was substituted for the monoclonal antibody to SCT 1–11, there was no reactivity of SCT 1–32, or any of the other SCT peptides. This result indicates that the polyclonal antibody recognizes primarily the carboxyl terminus of the peptide.

It was not possible to use SPA-purified antibodies for Eu labeling, because that resulted in an extremely high background fluorescence. However, for biotinylation, SPA purification was sufficient. On the basis of tests of various coating conditions, we chose the concentration of 200 ng of biotinylated antibody per well, which had sufficient binding capacity and could be incubated either overnight at 4 °C or 2 h at room temperature to produce similar assay results.

Our studies (Fig. 5) demonstrate that the assay has clinical applications. We can readily monitor the serum concentrations of SCT in patients who have received the drug. Since the assay is linear over the wide range of 0.3 to 300 pmol/L, high and low concentrations can be measured in the same assay and without the need for sample dilution. Both of these assay characteristics improve its accuracy.

In earlier pharmacokinetic studies, synthetic SCT was administered subcutaneously to healthy volunteers [15] and patients [16] and measured with RIA. The mean elimination half-lives were 87 and 88.2 min respectively and the peak-reaching time was ~1 h, which was similar to our previous study [8] in which SCT was also administered subcutaneously. However, when administered intramuscularly in the present study, the peak was reached within 10–20 min and the elimination half-life was diminished to 56 min, which was similar to the data of Beveridge et al., who injected SCT intramuscularly to patients and measured by RIA [16].

Compared with our previous RIAs [6], IRMAs [7], and IFMAs [8], this two-site immunoaassay has several advantages. It avoids the use of radioactivity and produces results in hours rather than days. More importantly, the assay can be used for measuring low concentrations of intact SCT. Thus, this new assay should be useful for studying the pharmacokinetics of the relatively low concentration of serum SCT that occurs with new, noninjectable preparations of the drug.
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References