Two-Site Immunoradiometric Assay of Intact Salmon Calcitonin

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We developed a two-site immunoradiometric assay (IRMA) of salmon calcitonin (SCT) that detects intact SCT(1–32) and not peptide fragments of the hormone. This was accomplished by using monoclonal antibodies prepared against the peptide fragments SCT(1–11) and SCT(11–32). Two antibodies with specificity for each of the peptides were purified from their respective ascites and evaluated in a two-site format wherein one of the antibodies was adsorbed to polystyrene beads and the other was radioiodinated. In this assay format, the antibody pair detected intact SCT(1–32) but did not react with either SCT(1–11) or SCT(11–32). The sensitivity of the assay could be increased by exchanging the antibodies with respect to bead adsorption and radioiodination. Furthermore, by increasing the incubation time and volume of incubation of sample with the polystyrene-bead-adsorbed antibody, the effective detection limit of the assay could be improved. This assay system can be used to detect intact SCT when the presence of fragments of the hormone might otherwise complicate interpretation of assay data.

Radioimmunoassays have been used to monitor the serum concentrations of salmon calcitonin (SCT) after its administration to humans and experimental animals (1–6). Although such studies have provided useful information about the metabolism of administered SCT, they have been limited by the detection limits of the assays and the presence in serum of hormone fragments with unclear biological activity (2). To overcome these limitations, we developed a two-site immunoradiometric assay (IRMA) of SCT that improves assay sensitivity and performance and detects only the intact peptide hormone (7).

Materials and Methods

We prepared monoclonal antibodies in mice against synthetic SCT(1–11) and SCT(11–32), kindly provided by Sandoz Pharmaceutical. The peptides were dissolved in 0.01 mol/L acetic acid for all protocols. The procedures for immunization, fusion, and hybridoma and ascites production were standard and have been previously described (7, 8). We screened the antibodies with radioiodinated peptides for their reactivity and specificity for SCT peptides. On the basis of results from these screening procedures, we selected two antibodies, one against each of the two peptides, for further evaluation in a two-site format (8). Each of the antibodies was purified from its ascites by Staphylococcus Protein A (Calbiochem, San Diego, CA) chromatography. Purified antibody was radioiodinated and adsorbed to 8-mm-diameter polystyrene beads (Hoover, Cumming, GA) as previously described (8). In brief, the antibodies were adsorbed to the beads at a concentration of 1 mg/L in sodium bicarbonate buffer, pH 9.6, for 24 h at 4 °C. The beads were washed three times with 50 mmol/L phosphate-buffered saline buffer (PBS), pH 7.2, and stored in this at 4 °C for ≤4 weeks in the presence of sodium azide, 0.1 g/L, until use. The two antibodies were evaluated for their reactivity against SCT(1–32), SCT(1–11), SCT(11–32), and control peptides in two-site and standard immunoassay formats. For measurements of SCT in samples, peptide was added to a pooled human serum that otherwise did not react in the salmon assay. These serum samples were then added to the assay incubation in different volumes for quantification. All incubations were performed at 4 °C for up to 36 h in PBS, pH 7.4, with bovine serum albumin (5 g/L; Pentex, Kankakee, IL) at the volumes indicated later, in Figure 3 (1, 8).

Results

Figure 1 demonstrates the sensitivity and specificity for SCT peptides of the two antibodies in a standard RIA format. Both antibodies detect SCT(1–32) and their respective peptides, and Ab1 is more sensitive than Ab2. The sensitivity of Ab1 for intact SCT(1–32) and peptide SCT(11–32) is similar, whereas the sensitivity of Ab2 for SCT(1–32) is considerably greater than for peptide SCT(1–11). The intra- and interassay variations for all assay procedures were 7% and 12%, respectively. Neither antibody reacted with the other SCT peptide, or with human calcitonin (CT) or CT gene-related peptide.

Figure 2 demonstrates Ab1 and Ab2 in a two-site format and the differences in sensitivity achieved by reversing which antibody is adsorbed to beads and which one is radiolabeled. A much more sensitive assay is achieved by having Ab1 on the solid phase and Ab2 as the radiolabeled antibody.

Figure 3 demonstrates the effects of incubation conditions on effective assay sensitivity and detection limits when we used human serum samples with added SCT(1–32). For these serum samples, recovery was >90%. There is a progressive increase in detection from a sample incubation volume of 0.1–4.0 mL. Furthermore, detection is also increased by increasing the time of incubation with the adsorbed antibody from 2 to 18 h.

Discussion

Immunoassays of SCT were originally developed and used to measure the hormone in the blood and tissue of
Fig. 1. Detection by radioimmunoassay of SCT peptides by two monoclonal antibodies

(Top) Ab1, directed against SCT(1-11), detects SCT(1-32) and SCT(1-11) but does not detect SCT(11-32). (Bottom) Ab2, directed against SCT(11-32), detects SCT(1-32) and SCT(11-32), but does not detect SCT(1-11). These assays were performed in a standard immunoassay format as previously described with radiodinated SCT(1-32) as tracer along with the indicated peptides (1).

Fig. 2. Two-site IRMA of SCT peptides with the antibodies characterized in the text and Fig. 1.

Fig. 3. Effect of incubation conditions on the detection of SCT(1-32) by the IRMA format in which Ab1 (5G9) is on the solid phase and Ab2 (3G4) is in solution.

Different volumes of samples containing the same concentration of SCT(1-32), 100 ng/ml, were incubated with the polystyrene bead containing antibody for either 2 or 18 h at the indicated volumes. After this incubation, radiodinated Ab2 was added to the incubation for the corresponding period of time, after which the assay was terminated. The horizontal axis represents the concentration of SCT in the added standard sample. Increases in both the time and volume of incubation increase the detectability of the SCT(1-32).

Fig. 3. Effect of incubation conditions on the detection of SCT(1-32) by the IRMA format in which Ab1 (5G9) is on the solid phase and Ab2 (3G4) is in solution.

These assays were specific for SCT and also detected other related teleost CTs, but they did not cross-react with mammalian CTs (1, 7). The sensitivity of these assays was generally adequate to measure the circulating amounts of this hormone in fish and to elucidate its secretory patterns (9-11).

More recently, SCT assays have been used to study the clinical pharmacology of the hormone during its use in the treatment of human skeletal diseases (2, 4-6). Extant immunoassays applied to such studies could readily measure the high concentrations of SCT achieved after the parenteral administration of the drug (2). Furthermore, the lack of cross-reactivity with endogenous human CT made it possible to specifically measure the exogenous SCT (7). However, the recent development of transmucosal routes of administration of peptide require more sensitive assays to measure the lower concentrations of SCT achieved (4-6, 12, 13). Accordingly, we developed a two-site assay for SCT that should permit enhanced sensitivity for the peptide (Figure 1). Our results indicate that increased assay sensitivity can be achieved with the appropriate format manipulations, including an increase in sample volume that exposes the capture antibody to more SCT molecules (Figure 3). In addition, the two-site format measures only the intact form of the molecule and does not detect its component peptides (Figure 2). Measurements of the intact molecule should allow a closer correlation of immunoreactivity with bioactivity, because the entire CT molecule is necessary for its antiresorptive effect, and fragments of the molecule are devoid of biological activity (14, 15). Another potential application of the two-site assay described here is to attempt detection of the low concentrations of SCT-like immunoreactivity...
described in some, but not all, studies in human tissues (15). The use of even more sensitive detection methods such as chemiluminescence may enhance applications of the SCT immunoassays we developed (16).

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References

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Investigation of N-Methyl-D-Glucamine Buffer for Assay of Alkaline Phosphatase in Serum

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We evaluated N-methyl-d-glucamine (MEG) as a buffer for assay of alkaline phosphatase (ALP; EC 3.1.3.1) and compared the MEG-based assay with the current International Federation of Clinical Chemistry Reference Method for ALP (IFCC/RM/ALP), in which 2-amino-2-methyl-1-propanol (AMP) is the pH buffer. The ALP assay in MEG at 30 and 37 °C shows excellent correlation with the IFCC/RM/ALP at 30 °C, but yields proportionately higher ALP activities (8.2% at 30 °C and 57% at 37 °C). ALP is unstable in both MEG and AMP at 37 °C. Serum incubated in MEG undergoes a pH-dependent biphasic loss of ALP activity: an initial rapid 5% loss after 1 min of incubation and a 10% loss per hour thereafter. A similar pattern was seen for incubation with AMP. The use of a serum-initiated reaction (no preincubation of enzyme with buffer) eliminated the early loss in activity. The addition of the metal ion buffer N-hydroxymethylaminozametacetic acid, along with low concentrations of Zn and Mg, as used in the IFCC/RM/ALP, reduced the slow loss in activity over time, as did decreasing the reaction temperature to 30 °C, but had no effect on the early rapid decay in activity seen in the first minute. Moderate transephorylation (45%) and nonenzymatic hydrolysis (3.3 U/L) were observed with MEG under the conditions of the assay (37 °C). A comparison of different lots of MEG from two manufacturers showed no significant difference in ALP activities.

Several buffers have been used to assay the catalytic concentrations of alkaline phosphatase (ALP; EC 3.1.3.1).4 All of these present some drawbacks, the most serious being inactivation or inhibition of the enzyme by

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