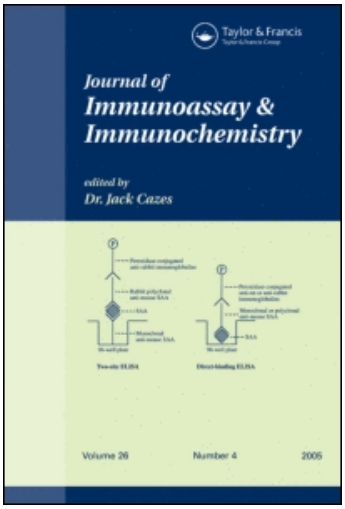

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Calcitonin Determination by a Fast and Highly Sensitive Enzyme Amplified Immunoassay

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CALCITONIN DETERMINATION BY A FAST AND HIGHLY SENSITIVE ENZYME AMPLIFIED IMMUNOASSAY

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ABSTRACT

A colorimetric enzyme amplification system was used to develop an immunoassay for human calcitonin (hCT) with a sensitivity of 6 pmol/l, and intra- and inter-assay CVs of 12% and 11.8% respectively for the low pool, and 10% and 11.2% for the high pool. The mean recovery of added synthetic hCT (58.5 pmol) from the plasma of 10 patients was 110% (64.4 pmol). The correlation coefficient between radioimmunoassay (RIA) and amplified enzymo-immunoassay was found to be 0.96 (p 0.001). The assay was successfully applied to the measurement of elevated

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calcitonin levels in plasma from patients with medullary carcinoma of the thyroid (MCT). AEIA offered a reliable and sensitive alternative to RIA for calcitonin determination with the added advantage of convenience as the label employed was much more stable.

INTRODUCTION

Initially bioassays were used to measure human calcitonin in plasma from patients with medullary carcinoma of the thyroid (MCT) (1). However, the extraction of very large volumes of plasma was required to show the presence of hCT in normal adult plasma (2). Later, a radioimmunoassay (RIA) was developed (3) which was more specific and less laborious. Through these approaches the diagnostic value of hCT determinations is now well established and high risk individuals are screened for MCT and the progress of the disease followed by such a method (4). In addition, a variety of non-thyroid malignancies have also been associated with elevated levels of plasma hCT (5,6,7).

Whilst RIA has been indispensable in demonstrating the clinical value of hCT measurements, the disadvantages of using RIAs for hCT estimations are that long incubation times are necessary for the sensitive assays which are required to measure normal levels. Freshly labelled reagents have to be prepared frequently owing to the relatively fast decay of the radioactive label. There is, therefore, a need for a robust, sensitive and fast assay with reproducibly high precision utilising a stable label. We have developed such a method for calcitonin by

application of enzyme amplification as previously described (8,9).

The assay format was established as being competitive to provide a direct comparison to our competitive RIA. In such assays it is widely recognised that sensitivity is directly related to the affinity of the antibody used and inversely related to the amount of conjugated label which has to be employed to provide a quantifiable signal. Our intention has been to exploit the high signal generating power of enzyme amplification, thereby reducing the amount of enzyme conjugate employed in the assay and thus increase the sensitivity of the assay. The RIA used for comparison was set up to provide the highest sensitivity possible with the same antibody.

The particular amplification scheme employed here uses the enzyme alkaline phosphatase which is conjugated to synthetic hCT and competes with hCT in the sample for a surface bound antibody against hCT. The conjugate which successfully competes and becomes surface bound is then determined by its ability to dephosphorylate NADP to NAD which then activates a highly NAD-specific redox cycle to produce an intense colour (Fig. 1).

MATERIALS AND METHODS

Diaphorase (NADH:Lipoamide oxidoreductase (E.C. 1.6.4.3)) was obtained from Boehringer Corporation London; alcohol

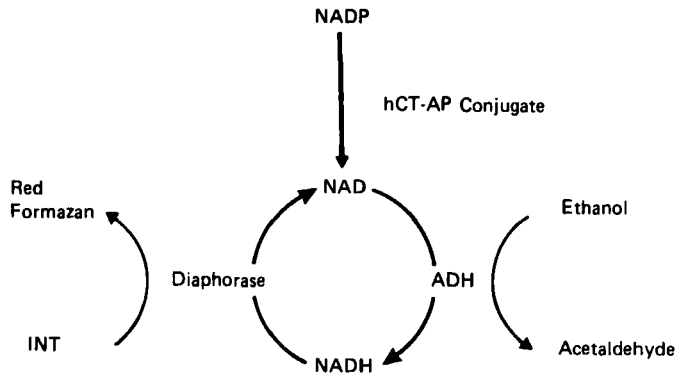

Figure 1

Diagram of enzyme amplification cycle triggered by alkaline phosphatase-calcitonin (AP-hCT) conjugate.

dehydrogenase (Alcohol NAD oxidoreductase (E.C. 1.1.1.1; ADH)) and p-iodonitrotetrazolium violet (INT) were obtained from Sigma Chemical Company; Chloramine-T was obtained from BDH Chemicals Limited. Sheep anti-rabbit γ-globulin was obtained from IRE-UK Ltd.; ^{125}I was obtained from Amersham and synthetic human calcitonin (hCT) was a generous gift from Dr. W. Rittel, Ciba-Geigy Ltd., Basle, Switzerland. Trasylol (aprotinin proteinase inhibitor) 20,000 kIU l^{-1}, was obtained from Bayer, Berks., UK and human plasma protein fraction B.P. from the Blood Products Laboratory, Herts.,U.K. All other reagents were of the highest grade available.

Antiserum

Antiserum to human calcitonin (hCT) was raised by repeated immunization of 3 rabbits with an hCT-ovalbumin conjugate. A primary injection of 100 µg/ rabbit CT in Freund's complete adjuvant was administered into the hind footpads of each rabbit. dorsal booster injections of 25 g hCT conjugate in Freund's incomplete adjuvant were given subcutaneously six weeks after the primary injections and then at monthly intervals until a satisfactory titre of anti-calcitonin antiserum was obtained (designated OC 31).

Plate-coating

Micro-titre plates from Nunc, Denmark, were coated with anti-calcitonin antiserum in 20 mM carbonate buffer, pH 9.6. To each well was added 120 µl of a 1:40,000 dilution of antiserum. The plates were sealed using Titertek Plate Sealers (Flow Laboratories Ltd) and incubated overnight at 22°C. The antibody solution was then shaken out and the wells washed four times with a buffer containing 5% lactose, 0.5% bovine serum albumin (BSA) and 0.02% Tween 20 (pH 7.4). The plates were dried, covered with plate sealer and stored in a desiccator at room temperature.

The CT-alkaline phosphatase conjugate was prepared by reducing the N-terminal disulphide bridge of hCT and coupling the resulting thiols to maleimide-activated alkaline phosphatase as follows. Human CT was dissolved in 0.1M triethanolamine-HCl (pH 8.5) containing 0.1M NaCl and 1mM EDTA. Dithiothreitol, 10 µl of

a 50 mM solution in water, was then added and the reaction incubated at 37°C for 1 hour. The reduced peptide was recovered by gel-filtration on a Sephadex G-10 column in 0.1M triethanolamine-HCl (pH 7.3) containg 0.1M NaCl and 1 mM EDTA. Calf-intestinal alkaline phosphatase (2mg) was reacted with succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate for 15 minutes and the reduced peptide and activated enzyme coupled and recovered as described previously (10).

Amplified Enzyme Immunoassay (AEIA)

Calcitonin standards were made by serial dilution of a concentrated standard in plasma protein fraction B.P. Samples and standards (80 µl) were placed in the wells of a micro-titre plate coated with anti-hCT antibody. The plate was covered and incubated overnight at room temperature. Twenty µl of conjugate (1:5000 initial dilution) was added to each well and incubated for 6 hours at room temperature. The samples were then shaken out and the wells washed four times with 50 mM Tris (pH 7.4) containing 0.15 M NaCl and 0.02% Tween 20.

A substrate solution (100 µl of 0.2 mM NADP in 50 mM diethanolamine at pH 9.5) was then added to each well and incubated at room temperature for 15 minutes. Following this, 200 µl of an amplifier solution consisting of 60 U/ml ADH, 0.1 U/ml Diaphorase, 0.55 mM INT and 4% v/v ethanol in 25 mM phosphate buffer at pH 7.0 was mixed into the contents of each well. The

reaction was allowed to proceed until the colour of the zero standard wells reached about 2 absorbance units at 492nm (approximately 20 mins). Sulphuric acid (50 µl of 0.2 M) was then added to each well and the absorbance of the wells read at 492 nm in a Titertek Multiskan MCC (Flow Laboratories).

Radioimmunoassay

Radio-iodination of synthetic hCT was performed by the Chloramine-T method (11) and the resulting label purified on HPLC using a linear acetonitrile/water/0.1% TFA gradient, from 25% to 45% ACN. The RIA method used was a modification of the previously published method (12) utilising antiserum OC31. The same antiserum was used in the amplified enzyme immunoassay. Synthetic hCT house standard was standardised against human calcitonin (Ref. 70/234) from NIBSC, Holly Hill, London. Serial dilutions of hCT were prepared in PPF and duplicate 100 µl of sample or standard were then dispensed with 100 µl of phosphate buffer (0.05 M, pH 7.4, containing 0.025% HSA and Trasylol). Antiserum OC 31 (50 µl in phosphate buffer) was added to each tube to give a final dilution of 1 in 180 000 in the assay tube. Fifty µl of ^{125}I hCT were then added to each sample to give 10 000 counts in 100 seconds. Following an overnight incubation at 4°C the assay was separated using sheep anti-rabbit second antibody bound to cellulose.

Coefficient of Variation

To determine the precision of the AEIA two quality control

pools were prepared at approximately 73.1 pmol/l and 292.6 pmol/l hCT. Each pool was assayed several times using different wells on single plates and then repeatedly in 10 separate assays using different plates to obtain intra-plate and inter-plate coefficient of variation (CV).

Recovery

Plasma samples were obtained from 10 patients with non-thyroid malignancies. Synthetic hCT was added to a 1 ml volume of plasma from each patient to give a final concentration of 58.5 pmol/l. The plasmas, before and after addition of synthetic hCT, were then assayed using the AEIA and the mean percentage recovery of hCT determined.

Correlation with clinical samples

Samples of plasma from various patients with MCT and non-thyroid malignancies were assayed by both RIA and AEIA. The results obtained were used to determine the correlation between RIA and AEIA.

RESULTS

A standard curve for the competitive calcitonin AEIA is shown in Fig. 2, which shows the overnight assay to have a sensitivity of 6 pmol/l. For comparison the figure also shows a standard curve for calcitonin measured by RIA using an overnight incubation and employing the same antibody. The AEIA is more sensitive.

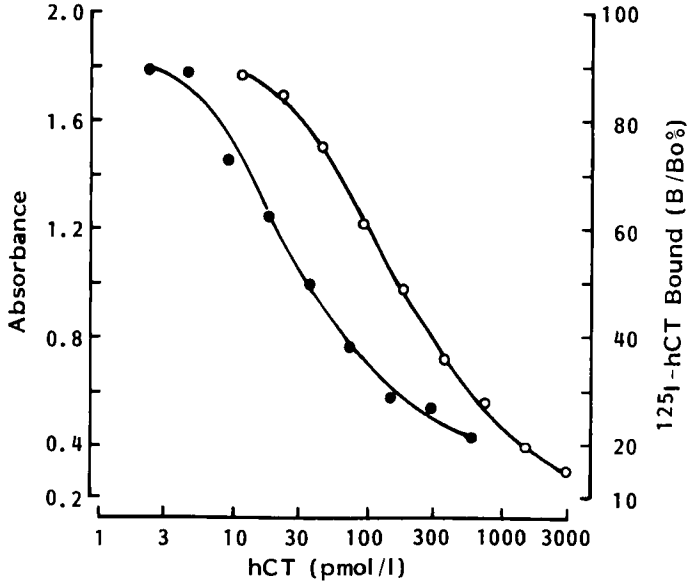

Figure 2

Calcitonin standard curves determined by AEIA (●) and RIA (○).

Coefficient of Variation

The within assay CV of the lower pool (mean 61.4 pmol/l) was found to be 12% (for 16 replicates). For the higher pool (mean 307.2 pmol/l) the intra-plate CV was 10%. Using the same pools the inter-plate CV for 10 assays was 11.8% for the low pool and 11.2% for the high pool.

Recovery

The mean recovery of 58.5 pmol/l added synthetic hCT from the plasma of 10 patients was 64.4 pmol/l (110%).

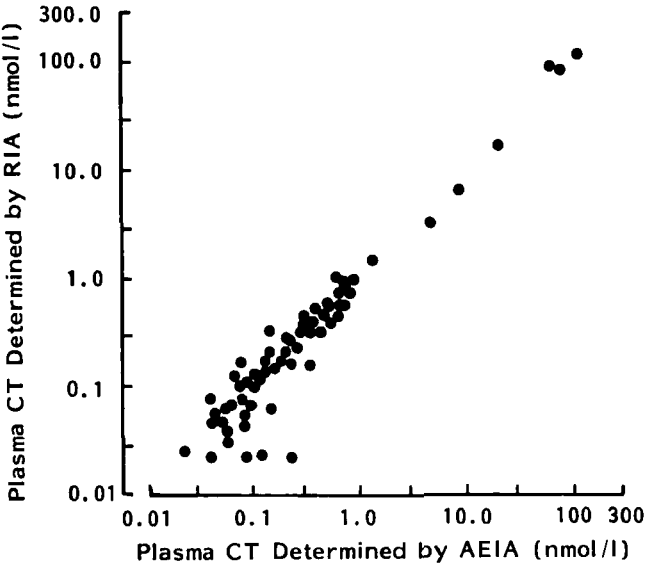

Figure 3

Correlation of plasma samples determined by RIA and amplified enzyme immunoassay. Samples were obtained from patients with MCT and non-thyroid malignancies.

Correlation with clinical samples

The correlation between the RIA and AEIA is shown in Figure 3. The correlation coefficient (v) was found to be 0.96 (p 0.001) and the regression equation was y= −0.0776 + 1.02x. Fig. 4 shows the dilution of hCT in plasma from patients with medullary thyroid carcinoma to be parallel to the standard curve.

DISCUSSION

The method of choice for hCT measurement has long been RIA.

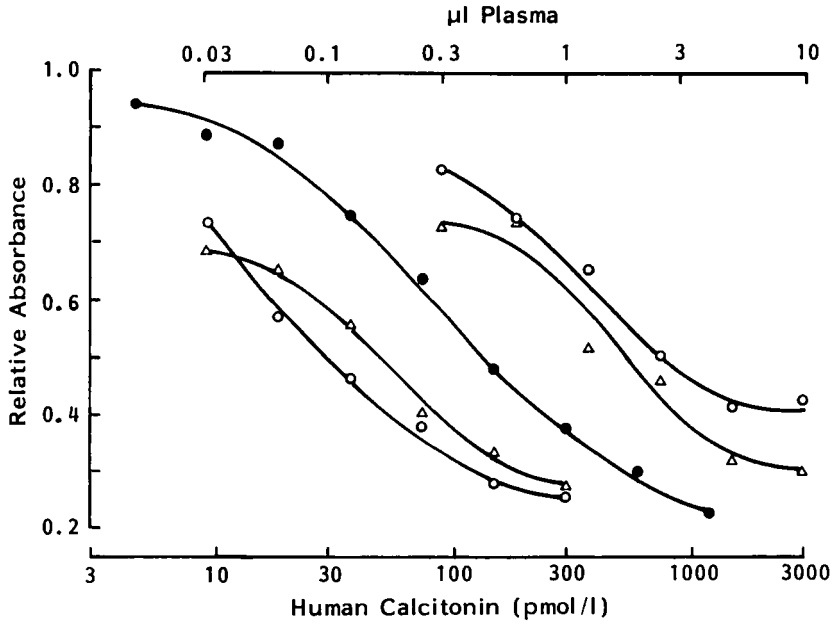

Figure 4

Calcitonin standard curve (●) (n = 6) showing parallel dilutions of immunoreactive calcitonin in plasma from patients with MCT (○, △).

The limitations of RIAs are the instability of the radiolabel, long counting times, the fact that a scintillation counter is required and the health hazards and disposal problems of ionizing radiation.

We have found amplified enzyme immunoassay to offer a reliable and sensitive alternative to RIA in the determination of calcitonin. The increased signal produced in the assay by enzyme

amplification has allowed us to employ very low concentrations of conjugate and increase the sensitivity of the assay to a point where it outperforms the best comparable RIA we have been able to develop using the same antiserum. The assay is also more convenient in that the enzyme label is much more stable than an ^{125}I label, particularly when stored at -20°C. The microtitre plate format we have used adds to the convenience in that samples may be easily loaded onto the plates and the developed plates read quickly using readily available plate readers. It must, however, be emphasised that such amplified assays depend greatly on a good separation of specific signal from noise. In this respect it is important to achieve good antibody plate coating and in particular care must be exercised with respect to obtaining an even coating of wells on the edge of the plate.

In a clinical sense the speed and sensitivity of the assay should prove useful. In screening for medullary carcinoma of the thyroid where the expected levels of calcitonin are high (4) the results may be obtained in a few hours. Conversely, in the assay of plasma from patients with non-thyroidal malignancies, and expected calcitionin levels near the normal range, the fact that the assay is capable of determining calcitonin levels within the normal range without long incubation periods or extraction procedures is important. In addition to these clinical uses we hope that enzyme amplified immunoassay will be of value in the further elucidation of the physiological role of calcitonin and

thereby remove some of the controversy which presently exists (13).

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