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A sensitive solid-phase micro-spot radio-tracer assay for the simultaneous estimation of thyroid stimulating hormone and thyroglobulin.

KEYWORDS: Multianalyte immunoassays, Track-etched membranes, TSH, Thyroglobulin.

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ABSTRACT *Hormones and tumour-markers in blood are assayed using immunoassays since this technique offers high sensitivity and specificity. Clinical diagnosis usually requires the blood concentration of several related analytes. Since immunoassays for each analyte is performed individually, estimating multiple analytes take time and are costly. This need can be fulfilled by use of multi-analyte immunoassay (MAIA). Using this concept, we have developed immunoradiometric assays for the simultaneous estimation of thyroid stimulating hormone (TSH) and thyroglobulin (Tg) in serum samples. Highly micro-porous polycarbonate (PC) track-etched membrane (TEM) was used as a solid support for immobilization of the anti-TSH and anti-Tg antibodies. ^{125}I labeled antibodies were used for detection. The assays were validated and compared to established methods. Assays are found to be sensitive, with wide range and with acceptable precision. Significant correlation was seen r=0.98, p < 0.001 for TSH and r= 0.91, p < 0.001 for Tg when compared to established methods*

INTRODUCTION

Immunoassays play a prominent role in the analysis of many clinical laboratory analytes and have been widely in use for the last half a century (Wood, 2007). However, immunoassays, are currently performed for a single analyte per assay with low through-put and consume relatively large amount of expensive antibodies and also patients' sample. The need for rapid and reliable measurement of multiple analytes in clinical samples has encouraged the development of multi-analyte immunoassays (MAIA), the concept of which was put forth by Ekins in the 1980s, in his "ambient analyte theory" (Ekins, 1989, Ekins & Chu 1991). MAIA consists of "antibody chip" which can have as many antibodies as required, each specific to a different analyte, spotted in minute quantities on spatially isolated and predetermined sites on an inert solid support. Substantial savings could be made in terms of assay cost, assayist time and sample volume by using MAIA. Till date, MAIAs have been demonstrated mostly for assaying cytokines or immunoglobulins (Tighe, Ryder, Todd and Fairclough, 2015). In this work, we describe the indigenous use of the concept of MAIA for developing a ^{125}I-tracer based immunoassay for two important analytes, viz., TSH and Tg, which are important analytes in thyroid-cancer patients. Most of the MAIA reported utilizes fluorescence and chemiluminescence for detection of bound analytes (Espina et al., 2004). Very few publications have described the use of radioisotopes (Ptacek et al., 2005; Zhu et al., 2001; Zhu et al., 2000), however, ^{125}I was used as the tracer in this work using MAIA because of the several advantages of using radio-tracers. MAIA requires the immobilization of the antibodies in fully functional form. Based on our previous experiments, we have used thin (~20 microns) polycarbonate (PC) track-etched membranes (TEM) with a pore size of 0.4 micron and pore density of 10^8 pores/cm^2 as the support, for the immobilization of the antibodies. PC-TEM is highly microporous that are prepared through a combination of charged particle bombardment (or irradiation) and chemical etching. Being highly microporous, it provides high antibody binding capacity and low background support for MAIA (Gupta, Rajan, Iyer, & Nair, 2006, Jain & Rajan,2013, Rajan & Gupta,2003). The MAIA for TSH and Tg was validated using several parameters like sensitivity and working range, intra and inter day precision and recovery and linearity studies for matrix evaluation. The results of the developed MAIA indicates the scope for multiplexing several assays.

MATERIALS AND METHODS

PC-TEM of 25 mm diameter and pore size of 0.4 micron, was procured from Millipore, USA. Bovine serum albumin (BSA) was purchased from Sigma, USA. Purified recombinant human-TSH was purchased from Genzyme Corporation, USA. ^{125}I-labeled monoclonal anti-Tg antibodies and Tg standards were obtained from Izotop, Budapest, Hungary. Monoclonal antibodies to TSH were purchased from Biodesign International, USA. ^{125}I-labeled monoclonal anti-TSH antibodies were from Board of Radiation and Isotope Technology (BRIT), Vashi, Navi Mumbai. Polyclonal antibodies against Tg were produced in-house. All other chemicals and reagents required for this study were purchased locally and were of analytical or equivalent grade. PhosphorImager (Typhoon Trio$^+$) from GE Healthcare Biosciences, India, was used to image the radioactivity by autoradiography, which was analysed with ImageJ v.1.49 open-source software from imagej.en.softonic.com. Commercial TSH IRMA kit from BRIT and Tg IRMA kit from M/s Izotop were procured for comparison studies.

The required numbers of PC-TEM were activated by soaking in 2.5% glutaraldehyde (v/v) in phosphate buffer saline (PBS) (0.025M, pH-7.4, 0.9% NaCl) for two hours with gentle agitation. The PC-TEMs were washed thrice with PBS. Using a micropipette, anti-TSH and anti-Tg antibody in

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PBS were spotted (0.5 µl volume, concentration of 1mg/ml) on the activated PC-TEM at pre-determined locations. The required number of antibody spots for standards and samples in duplicate was spotted. The membranes were rinsed with PBS, soaked briefly in 4% (w/v) bovine serum albumin (BSA) solution in PBS to block the remaining activated sites, and rinsed thoroughly with PBS. Standards with both TSH and Tg were prepared in serum using the recombinant human TSH and Tg standards from Izotop. Seven standards (S1 –S7) were prepared having TSH concentration of 0, 0.15, 0.5, 1.5, 5, 15 and 50µIU/ml and Tg concentration of 0, 0.3, 1, 4, 20, 100 and 250 ng/ml respectively. The ^{125}I-anti-TSH monoclonal antibodies and ^{125}I-anti-Tg monoclonal antibodies were mixed to give a cocktail tracer with 100,000 cpm/100 µl of each. The PC-TEM were reacted with 50 µl of standards / QC samples / test samples and 200 µl of tracer cocktail for overnight with gentle agitation and washed thrice with PBS containing 0.1%Tween-20. The radioactivity from the ^{125}I-labeled antibodies bound to the capture antibody spots on the PC-TEM were analyzed using the PhosphorImager. Intensity of each spot was quantified with ImageJ v.1.49. Local background was subtracted from the mean spot intensities. Background-subtracted mean spot intensity of the duplicate spots was plotted against the analyte concentration and standard curve was obtained. RIA analysis software Mgamma in-vitro test (available with Stractec Gamma Counter) was used for standard curve plotting and intrapolating the concentration of the sample.

Cross-reactivity studies were carried out by reacting both anti-TSH and anti-Tg antibody spots with the highest concentration of standards (S7) and with either individual tracer or tracer cocktail. Analytical sensitivity of the assays was determined with ten replicates of zero calibrator and appropriate statistics. The intra-assay coefficients of variation were determined with ten replicates of two serum samples with low and high concentrations of analytes in the same assay. Another three serum samples were assayed in ten consecutive assays and the mean concentration of duplicates in each run were used to calculate the inter-assay CV. Recovery, expressed as a percentage of the expected values, was determined in three serum samples. Linearity was determined in serum sample containing high concentration of both TSH and Tg which were serially diluted in analyte-free serum. TSH and Tg results were compared to those obtained with the commercial kits for TSH and Tg.

RESULTS

Calibration curves were constructed for both TSH and Tg. Typical standard curves for TSH and Tg were generated using MAIA as shown in Figure 1. The analytical sensitivity of the TSH was 0.03 µIU/ml and that of Tg was 0.1 ng/ml. The upper limit of the working range of assay was 50 µIU/ml for TSH and 250 ng/ml for Tg.

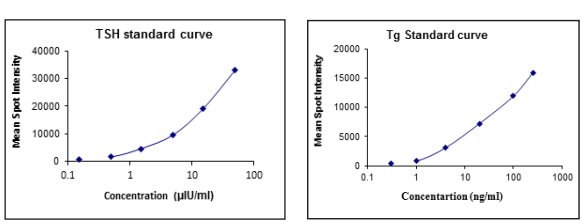

Figure 1 Standard curve for TSH and Tg obtained using MAIA.

When reacted with the individual tracers, only the spots of anti-TSH antibodies produced a signal with TSH and ^{125}I-labeled anti-TSH antibodies, and no signal was obtained with Tg and ^{125}I-labeled-anti-Tg. Similarly, anti-Tg antibody spots did not produce a signal with TSH and ^{125}I-labeled anti-TSH showing there was no-cross-reactivity among them in the assay. The results of cross-reactivity are summarized in Figure 2.

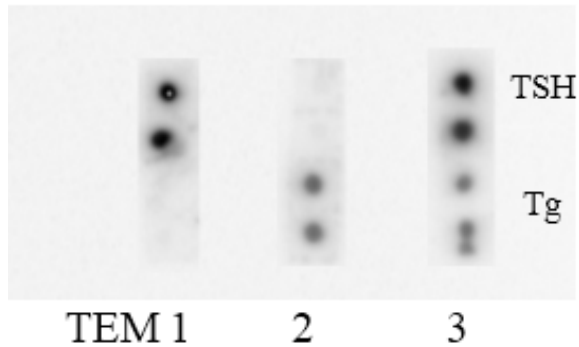

Figure 2 Image showing the results of cross-reactivity study in MAIA. TEM1 was reacted with ^{125}I-TSH antibodies. TEM 2 was reacted with ^{125}I-Tg antibodies. TEM 3 was reacted with tracer cocktail of ^{125}I-TSH and ^{125}I-Tg antibodies. When reacted with the individual tracers, only the spots of anti-TSH antibodies produced a signal with TSH and ^{125}I-labeled anti-TSH antibodies, and no signal was obtained with Tg and ^{125}I-labeled-anti-Tg. Similarly, anti-Tg antibody spots did not produce a signal with TSH and ^{125}I-labeled anti-TSH showing there was no-cross-reactivity among them

The intra-assay CVs for TSH for samples with TSH concentration of 2 and 16.8 µIU/ml were 9.1% and 10.3% respectively, and for Tg concentration of 20 and 67ng/ml the intra-assay CV was 9.9% and 11.6% respectively. The inter-assay CV for TSH for samples with TSH concentration of 1.8, 4 and 14 µIU/ml was 20%, 5% and 12% respectively. For Tg, the inter-assay CV for concentrations of 2, 19.2 and 56 ng/ml was 18%, 13% and 20% respectively. Recovery was determined in serum samples having TSH concentration of 0.03 and 19.5 µIU/ml and Tg concentration of 33 and 10.2 ng/ml spiked with standard S4 in 1:1 ratio. The mean recovery for two samples spiked with S4 was 90.7 % for TSH and 99.1 % for Tg. Observed to expected ratio (O/E) was determined for linearity studies with sample having TSH concentration of 100µIU/ml and Tg concentration of 50 ng/ml which was serially diluted from 1:1 – 1:32 in hormone free serum. O/E for linearity varies from 69.3% to 116 %. 41 human serum samples measured by MAIA for TSH and Tg. The results obtained correlated well with the TSH results obtained by conventional IRMA. (MAIA=0.95IRMA+0.32, r = 0.98, p < 0.001, n = 41). Results of the Tg MAIA also showed a good correlation with conventional Tg IRMA (MAIA =1.03IRMA+10.1, r = 0.91, p < 0.001, n = 41).

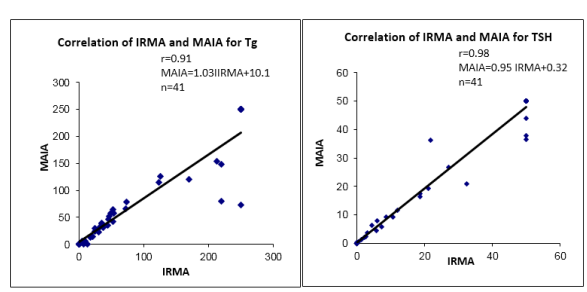

Figure 3 Graph showing the comparison of Tg and TSH concentration in forty one human serum samples measured by MAIA and conventional immunoassays.

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DISCUSSION

The main goal of this research was the development of MAIA for simultaneous estimation of TSH and Tg and to compare the MAIA results to IRMA, a clinically well-known and established assay. Capture antibodies were immobilized on PC TEM and ^{125}I labeled antibodies were used for detection. Although fluorescence and chemiluminescence are most common means of detection for MAIA, we have used isotopic labels for our experiments. Radiolabeling has several advantages over non-isotopic detection for MAIA including no interference from dust, no photo bleaching effects, no autofluorescence and not dependent on substrates, triggering solutions or attenuation of the emitted signal. Although fluorescence and chemiluminescence are reported to provide higher sensitivity compared to radioactivity, the sensitivity of the MAIA we have developed is not compromised by using radiotracer. Sensitivity obtained for TSH MAIA was 0.03µIU/ml and Tg MAIA was 0.1ng/ml. Working range obtained for TSH MAIA was 0.03-50 µIU/ml and Tg MAIA was 0.1-250 ng/ml. Both the sensitivity and linear ranges observed are satisfactory for clinical use.

The developed MAIA assay for TSH and Tg compares very well with commercial kits for both TSH and Tg. The precision and reproducibility of MAIA is acceptable with both intra-assay and inter-assay CV less than 20%. Linearity, recovery and cross-reactivity experiments show that the antigen in the sample is behaving as expected and there is no scope for errors in estimates.

CONCLUSION

In summary, we have developed for simultaneous detection of two antigens, TSH and Tg using the MAIA concept. This method utilizes only 50 µl of serum sample, whereas conventional IRMA would require about 500 µl of sample. Similarly,the tracer and buffer volumes are lower. This is especially critical in samples that may come from small babies, biopsy samples, very sick individuals or small experimental animals.

By using MAIA, we can conveniently detect TSH and Tg with acceptable sensitivity, accuracy and reproducibility. There is a clear benefit of reduction in sample volume, cost and assayists time.

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