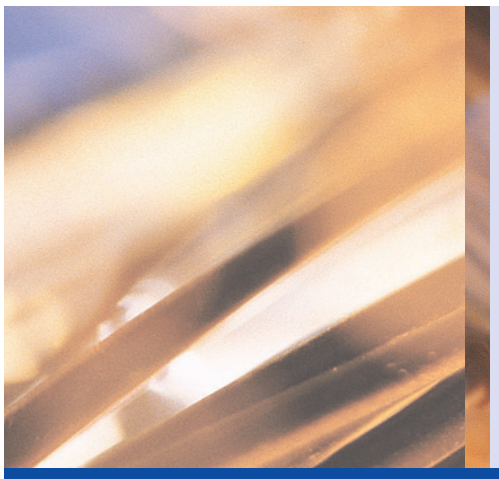


Chromatography Focus

PURIFICATION, REDUCTION AND IDENTIFICATION OF THE ANTI-HUMAN THYROID STIMULATING HORMONE IN DEVELOPMENT OF A HIGHLY SENSITIVE IMMUNO-PCR METHOD

Sargazi M (1), McCreavy D (2), Roberts NB (1), Dutton J (1), Gallagher JA (2) Fraser WD (1)

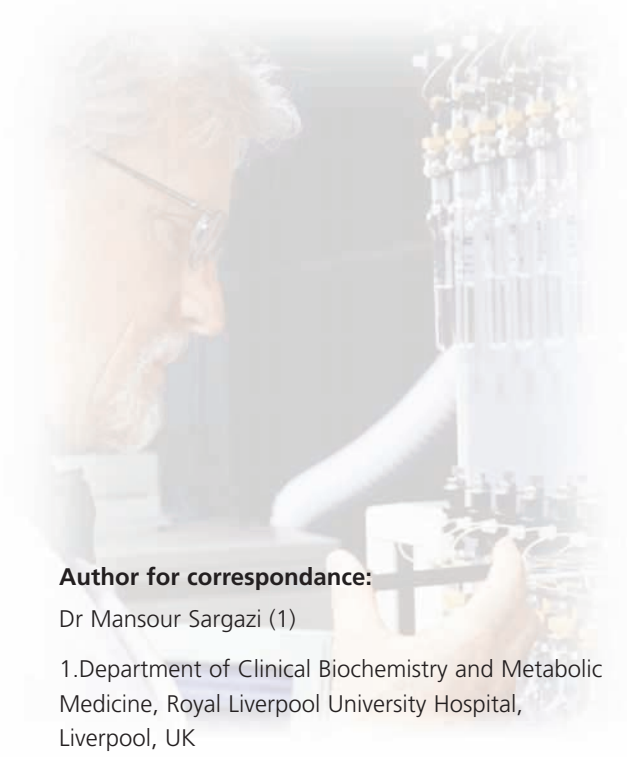
Measuring circulatory level of thyroid stimulating hormone (TSH) is the first line test recommended for the early detection of thyroid disorders in particular primary hyperthyroidism. Although the functional sensitivity of the current 3rd generation TSH assays have improved, in practical terms, medical laboratories usually are unable to report levels below 0.05μIU/mL.

The immuno-PCR has given hope for lowering the detection limit and improving the functional sensitivity as recommended by the Department of Health. In this study, we optimised the conditions for an ELISA method which is the first part of an immuno-PCR method for detection of low levels of TSH and established the optimum conditions for purification, reduction and identification of the detector human anti-TSH antibody.

The antibody was reduced and we identified it's peak at 14 mins of the HPLC run, whereas the retention time for intact antibodies was 18 minutes.

The incubation period after reduction of the antibody was the crucial part of the experiment. It should be kept as short as possible to prevent reduced fragments from reassociation.

The reduced antibody is required for conjugation to the single strand oligonucleotide (ssDNA). The lowest functional sensitivity of the ELISA assay was found to be 0.02μIU/mL TSH and the assay was linear at the TSH concentration at 0-2.6μIU/mL.



Author for correspondance:

Dr Mansour Sargazi (1)

1.Department of Clinical Biochemistry and Metabolic Medicine, Royal Liverpool University Hospital, Liverpool, UK

2.Department of Human Anatomy and Cell Biology, The University of Liverpool, Liverpool, UK

INTRODUCTION

Thyroid disorders are very common worldwide. Based on statistics published by the UK Department of Health, 0.09% of hospital episodes were for thyroid disorders in 2002-2003 [1]. TSH determination is the first line test in diagnosis of thyroid disorders. It is sensitive and specific for assessment of thyroid function and is particularly suitable for early detection or exclusion of disorders of the hypothalamus-pituitary-thyroid axes [2]. The National Academy of Clinical Biochemistry current guidelines recommend a minimum functional sensitivity of less than 0.02 μIU/mL for TSH assays [3,4].

Although the functional sensitivity of the current 3rd generation assays may meet this target, in practical terms, medical laboratories usually are unable to report levels below 0.05μIU/mL. This is due to unacceptable high imprecision at levels below 5 μIU/mL for the majority of commercial assays.

The immuno-polymerase chain reaction (IPCR) has given hope for lowering the detection limit for some analytes including TSH. In general, a linker molecule with bi-specific binding affinity for DNA and antibody (Ab) is used to attach a marker DNA molecule specifically to an Ag-Ab complex, resulting in the formation of a specific Ag-Ab-DNA conjugate. The attached marker DNA can be amplified by PCR with appropriate primers which can be quantified [5].

Our ultimate aim is to develop a highly sensitive Immuno-PCR assay for measuring low concentrations of TSH. Initially we have focused on the optimisation of a TSH ELISA assay and optimisation of the conditions for reduction, purification and detection of the anti-TSH detector antibody/fragments using an appropriate protein HPLC separation assay.

MATERIALS AND METHODS

Enzyme-linked immunoassay: An ELISA system on a 96-well plate (Nunc Maxisorp) coated with anti-human TSH was optimised for detection of very low levels of TSH.

Coating

Briefly, coating solution was prepared by dilution of anti-hTSH (1.02 mg/mL, Medix Biochemica, Asematie, Denmark) in binding buffer ($NaHCO_3$ 5 mmol/L) 1 in 200. The prepared solution was used for coating 96-well plates.

TSH calibration curve

Linearity, detection limit and functional sensitivity of the ELISA assay at TSH concentrations of 0.02 to 83.2 μIU/mL were investigated. Briefly, 120 μL of each TSH solution (Scipac, Kent, UK) was placed into the coated wells in triplicate and incubated for 2 hrs at room temperature (RT).

After washing (Trizma 25 mmol/L, NaCl 50 mmol/L, pH 7.4 and 500 μL/L of Tween-20), 100 μL of the biotinylated human monoclonal anti TSH antibody (5000 times diluted, Medix Biochemica, Asematie, Denmark) was added to each well and the plate incubated for 2 hrs. The wells were washed and 100 μL avidin D-HRP (25 000-fold dilution) was added and further incubated for 45 min. 100 μL TMB substrate was added to each well and the reaction was stopped by addition of 100μL HCl 0.5 mol/L after 30 min and the absorption measured at 450 nm on a multiwell-plate reader (Molecular Devices, Hampshire, UK) after gentle agitation for 30 sec on a Milenia Micromix 4, plate agitator (ADC, Gwynedd, UK). Each experiment was carried out in triplicate and a blank (diluent with no TSH) was used.

Purification of TSH antibody; Buffer exchange; Desalting

Buffer exchange/desalting/purification of the anti-human TSH antibody was carried out on a PD-10 column (Amersham, Bucks, UK) after equilibration with 25 mL Trizma buffer (10 mmol/L). 500 μL intact IgG1 (Medix Biochemica, Asematie, Denmark) was placed on the equilibrated column. The column was washed with 1 mL of buffer twice. Fractions 1- and 2 were discarded and fractions 3-6 were collected.

BCA Protein Assay

The protein content of each fraction collected in a previous stage was determined using bicinchoninic acid (BCA) assay based on the reduction of Cu^{2+} to Cu+ by protein in an alkaline pH (the biuret reaction) at 540 nm [6].

Reduction (cleavage) of the IgG1

Intact IgG1 was reduced after buffer exchange using three different reducing agents; a strong reducing agents (2-mercaptoethanol; 2-ME), Tri-n-butylphosphin (TBP) and a mild reducing agent (mercaptoethylamine; MEA). Combinations of different temperatures and time were applied to optimise the reduction process.

1. Reduction with 2-mercaptoethanol

Twenty-five μL of 2-ME (Sigma, Poole, UK) was mixed with 500 μL of the desalted IgG1 and incubated at 37°C for 2 min.

2. Reduction with mercaptoethanolamine (MEA)

One mL of desalted IgG1 and 50 μL EDTA (Sigma, Poole, UK) at 0.1 mol/L was mixed with 6 mg MEA (Pierce, Kent, UK) and incubated at 37°C for 90 mins.

3. Reduction with Tri-n-butylphosphine (TBP):

TBP (Sigma, Poole, UK) in 10% dimethylformamide (Sigma, Poole, UK), which is currently used for the cleavage of disulfide bonds, was mixed with desalted IgG1.

After incubation of reducing agent with antibody, the final solution was desalted on an equilibrated column and the fractions were collected as described previously.

HPLC system

A SP Thermo Separation Products HPLC system, Solvent delivery module and autosampler with injection system, a 200 μL sample loop, a Gilson dual pump (Gilson, Luton, Bedfordshire, UK) with adjusted flow rate to 1.5 mL/min, an anion exchange column; TSK DEAE-5PW (TOSOH Bioscience, Stuttgart, Germany); 75 mm length, an ID of 7.5 mm and 5 μm packing material, a KRATOS, spectroflow 773 detector at 280 nm, a Gilson autosampler model 234 Autoinjector (Gilson, Lutton, Bedfordshire, UK) and a Gilson model FC 204 programmable fraction collector with a 60 positions rack were used. Two mobile phases A and B containing a Trizma buffered solution at concentration of 10 mmol/L at adjusted pH to 8.4 were used (mobile phase B contained 500 mmol/L NaCL). The run time of mobile phases is presented in *Table 1.*

Table 1. Time course of a HPLC mobile phase run for 35 min

Time (min)	Mobile phase A %	Mobile phase B %
0	100	0
3	100	0
10	90	10
20	70	30
25	0	100
27	0	100
30	100	0
35	100	0

Figure 1 shows the HPLC system used for analysis consists of a pump, a detector, a fraction collector, assisted with a Gilson software.

200 µl of sample was introduced to the HPLC system by the autosampler on a 35 mins gradient run. Up to 60 fractions were collected at 30 second interval time.

Confirmation of Intact IgG1/fragments in HPLC fractions

The next step was to confirm that the peaks obtained from the HPLC system after introduction of desalted and/or reduced IgG1 was the actual peak and not an artifact. To do this, an ELISA method was used on wells of a plate coated with immobilised goat anti-mouse antibody.

IgG1 or cleaved IgG1 present in the fractions collected by HPLC fraction collector was incubated with immobilised goat anti-mouse antibody for 90 min. Addition of the second detector goat anti-mouse antibody attached to the Avidin-HRP, createed a sandwich ELISA. After addition of the substrate (TMB), the enzyme catalysed the substrate and produced colour which was proportional to the concentration of the intact/cleaved IgG1 present in the fraction.

RESULTS

1. TSH calibration curve

The TSH calibration curve and the assay characteristics including detection limit, functional sensitivity and the linear range were investigated using known concentrations of TSH (0-83.2 µIU/mL, *Figure 2*).

The calibration curve was linear at a range of 0 to 2.6µIU/mL. The results obtained from the TSH ELISA, showed that the assay was sensitive at low concentration with the functional sensitivity of 0.02 µIU/mL.

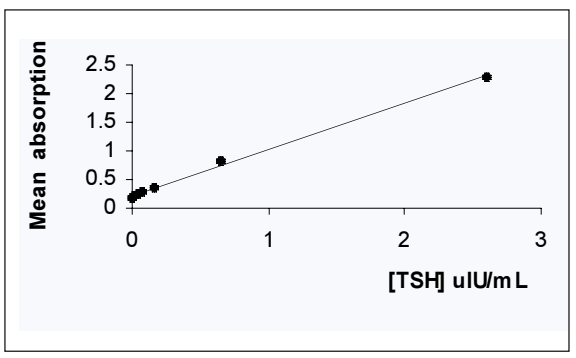


Figure 2. Calibration curve for TSH at concentrations from 0-2.6 µIU/mL.

2. Characteristics of the BCA assay

The results obtained for the BCA assay at different incubation periods confirmed that the assay was linear up to 2000 µg/L albumin at all incubation period from 30 to 210 min correlation > 0.99). The detection limit was dependent on the incubation period and varied from 12 to 50µg/mL for the incubation period of 30 to 210 min. The detection limit was 37µg/mL for 60 min incubation period.

3. Identification of albumin, intact IgG1, cleaved IgG1 peaks by the HPLC system

Albumin (Alb) solutions with known concentration were introduced to HPLC to identify the retention time as well as establishing a dose-response curve. The chromatograms obtained

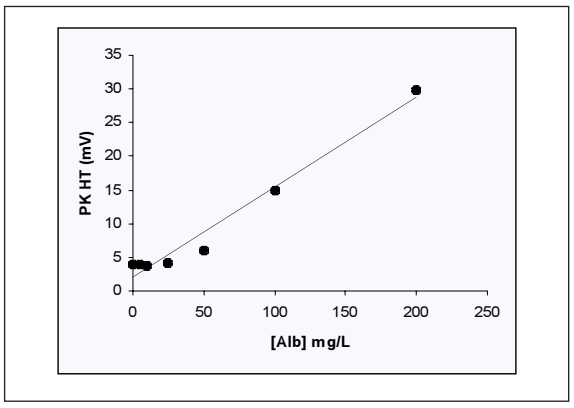


Figure 3. Albumin calibration curve

for the human serum albumin solutions showed a retention time of 24 min, a linearity up to 200 mg/L and a functional sensitivity of 25 mg/L (*Figure 3*).

Similar conditions were employed to identify the relevant peak for intact IgG1 and to plot the calibration curve (*Figure 4*). Solutions with different concentrations of IgG1 were analysed on the same HPLC system. The retention time for intact desalted IgG1 was 18 min. HPLC analysis showed that the assay sensitivity was 25 mg/L IgG1.

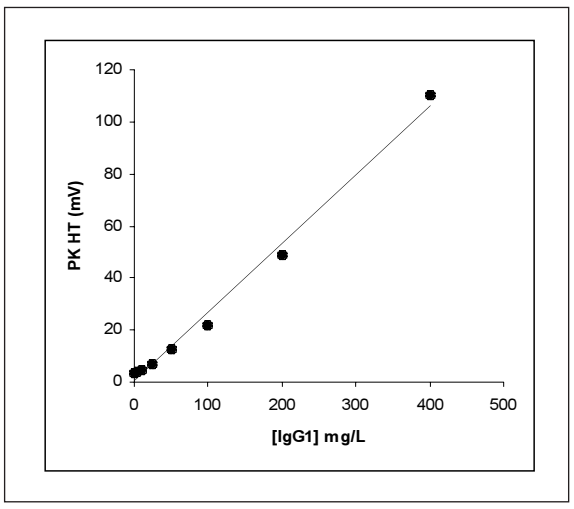


Figure 4. IgG1 calibration curve

Consistency in the retention time obtained for the Alb and IgG1 showed that the assay was capable of separation of these molecules with good resolution based on a dose response curve. (*Figure 5*).

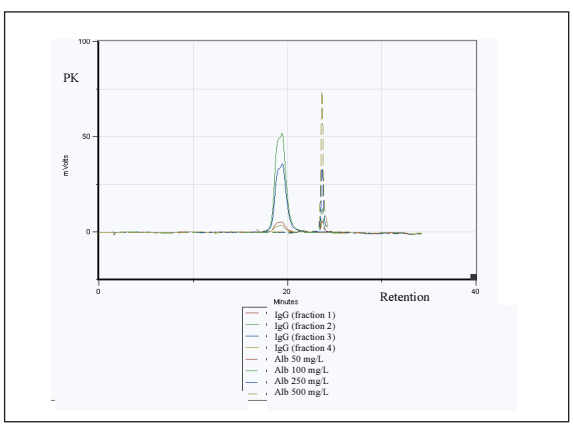


Figure 5. HPLC chromatograms of the PD-10 fractions of IgG1 compared with albumin

The next step was to identify the cleaved IgG1 after reduction with various agents. For identification of the cleaved IgG1, the same method was used.

Samples were analysed after reduction to prevent reassociation of the reduced IgG1 fragments. The peaks obtained for the cleaved IgG1 using various reducing agents are shown in *Figure 6* (a-c). After reduction of antibodies using MEA, mixture was desalted and fractions collected. *Figure 7* illustrates the peaks obtained for fractions 1-5.

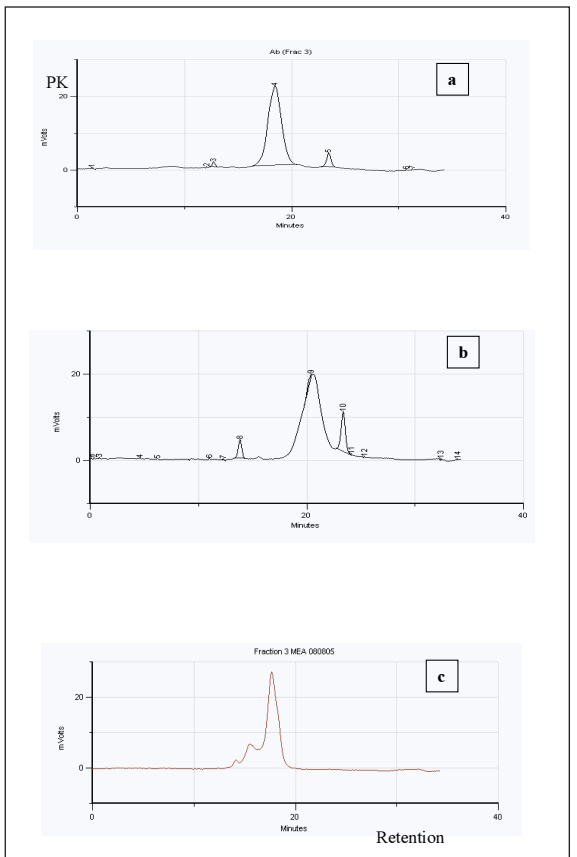


Figure 6. Chromatogram of the reduced IgG1 by 2-ME (a), TBP (b) and MEA (c)

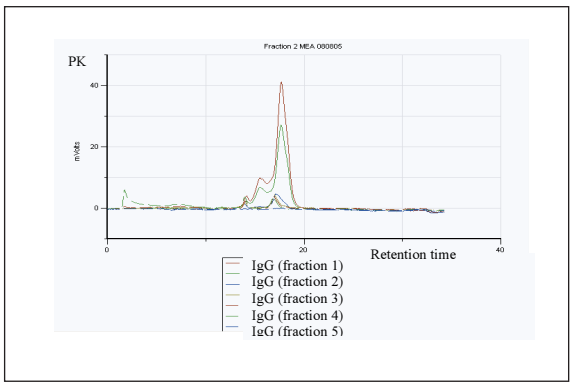


Figure 7. HPLC chromatogram of the reduced IgG1 with MEA for 90 min at 37°C with EDTA (fractions collected by a PD-10 column)

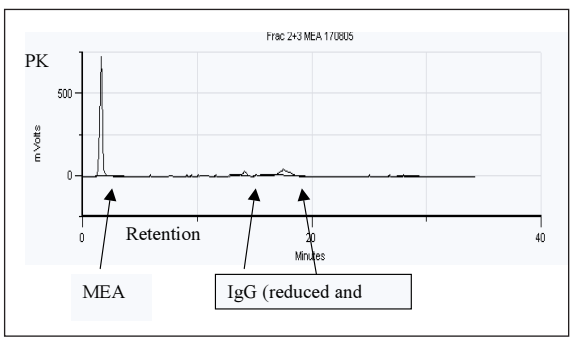


Figure 8. HPLC chromatogram of reduced IgG1 in mixed fractions of 2 and 3

A very big response (peak) due to the presence of the reducing agent in 2-3 min of the HPLC *(Figure 8)* run was observed, which probably undermined the peaks for the IgG1 (intact and cleaved). *Figure 9* shows the IgG1 (intact and cleaved) in different fractions after reducing agent was removed.

After this step, seven fractions were collected and the protein concentration for each fraction was determined.

The experiment also showed that fractions 2 and 3 had the highest protein content with the total recovery of 75% for IgG1. These fractions were then analysed by HPLC on a 35 min HPLC run as described.

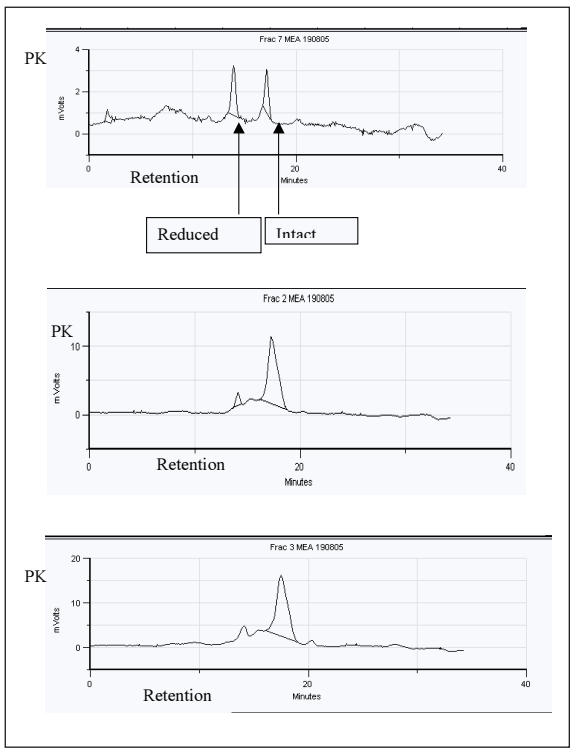


Figure 9. Intact and cleaved IgG1 after reduction and removing the reducing agent

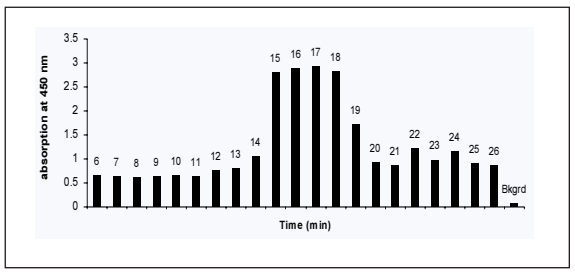


Figure 10. Confirmation of the presence of IgG1/reduced IgG1 in fractions collected from HPLC

These peaks were confirmed to be either intact IgG1 (18 min retention time) or reduced form (retention time 14 min) using goat anti mouse monoclonal IgG1 *(Figure 10).*

DISCUSSION

Although the functional sensitivity of the current 3rd generation TSH assay may meet the target cut off value set by Department of Health (0.02 µIU/mL), in practical terms, medical laboratories are unable to report levels below 0.05µIU/mL probably due to unacceptable high imprecision at these low TSH concentrations. At TSH concentrations lower than 0.08 µIU/mL, there is the lack of agreement between methods due to differences in the specificities of the anti-TSH monoclonal antibodies used [8]. Rawlins et al., (2004) reviewed 6 current third generation methods (Access 2, Advia Centaur, Architect i2000, E170, Immunolite 2000 and Vitros Eci) and showed a good agreement between these methods at TSH concentrations greater than the functional sensitivity, but disagreement by up to 10 folds was observed when the TSH was less than 0.02 µIU/mL [3].

The intermethod discrepancies at low TSH suggest that the clinical usefulness of some TSH assays is impaired. Diagnosis of patients with hyperthyroidism and improved assessment of response to treatment for thyroid carcinoma and other conditions require accurate TSH measurement at TSH concentrations of below 0.01µIU/mL .

Using biotinylated detector antibody in the first part of an immuno-PCR which is ELISA, we were able to measure concentrations of TSH as low as 0.02 µIU/mL (functional sensitivity). In our biotinylated immunoassay experiments, where high concentrations of TSH were used, the absorption signal was suppressed significantly in ELISA. Increasing concentration of the detector antibody to double, showed no change in TSH linearity of the standard curve. This clarified that the 5000 fold dilution of the 2nd antibody was an optimum concentration for ELISA reaction to take place.

The most common interference in sandwich immunoassays is as a result of heterophilic antibodies. At present, there is no simple technique that can completely eliminate interference from heterophilic antibodies. Use of blocking agents such as nonspecific immunoglobulins in the assay buffer was the most common practice as reported by Boscato et al., (1988) [12]. Warren et al (2005) eliminated the heterophilic interference by producing a single chain antibody fragment (scFv). Use of a biotinylated F(ab')2 capture reagent and the inclusion of a heat-aggregated irrelevant mouse mAb in the assay buffer protected the assay from interference, which is used routinely [13]. He also reported that the assay was less affected by this interference when single chain antibody was used compared with the parent antibody.

Biotinylation of antibody is also effective in reducing the interference. Random chemical biotinylation can lead to either destruction of the antigen binding site or the production of antibody fragments that binds to streptavidin in an orientation that is sterically unfavorable for antigen binding. However, site specific biotinylation has shown to guarantee not only each antibody fragment which is labeled with only one molecule of biotin, but also that this residue is placed distally to the antigen binding site [13]. At concentrations of more than 5.2 µIU/mL, a nonlinear curve was obtained for TSH solutions, however, at concentration up to 2.6 µIU/mL, a linear curve with a correlation of 0.99 between was obtained. The linear curve at low concentration of TSH showed that we were able to use this optimised procedure at lower concentrations of THS as 0.02 µIU/mL. Functional sensitivity of TSH assay with Roche reagents based on biotinylated sandwich immunoassay was reported to be 0.014 µIU/mL with 8.6% CV for TSH level of 0.034 with Elecsys 1010/2910 and 3.0% for Modular analyser E170 [14]. Enhancers have been shown to improve functional sensitivity of the assays. Kricka et al (1996) has reported a sensitivity as low as 1.5 and 5 µIU/mL when enhancers i.e 1,1-biphenyl-4-yl boronic acid and 4-iodophenol, which reduces the background and increases signal and hence improves signal/background ratio were used in an endpoint chemiluminescent assay [15]. Detection limit for TSH was reported to be 0.1 µIU/mL when analysed with radioimmunoassay and/or enzyme immunoassay [16], however, Zoha et al., (1998) has reported 10 times lower detection limit for TSH immunoassay using stabiliser phycobilisomes of red algae (0.01 µIU/mL) compared with Yuan group [17]. He also reported that the assay was linear up to 10 µIU/mL using 5-point calibration curve (r = 0.99).

Immuno-PCR is a powerful analytical technique for detection of very low concentrations of analytes such as TSH, where lower functional sensitivity is required for early diagnosis and improving the patient management. Conventional PCR/immuno-PCR has shown post analytical-associated errors, however, real-time immuno-PCR is more sensitive for quantification of DNA-conjugate without the problems associated with post-analysis i.e quantification of the final product as associated with conventional immuno-PCR method.

Lind and Kubista (2005) were able to measure PSA as low as $5.7 \times 10^{7} - 2.8 \times 10^{10}$ molecules with a sandwich immunoassay, whereas the sensitivity was improved 100 times when real-time immuno-PCR was used ($4.8 \times 10^{5} - 5.6 \times 10^{9}$) [9]. Alder et al., (2005) reported that immuno-PCR was able to distinguish as few as 100 Rotravirus/mL whereas ELISA had a detection limit of 100,000 virus particles per mL [10]. Joerger et al., (1995) established an immuno-PCR for measuring very low levels of human TSH and hCG. Using various optimised conditions, they detected as low as 1×10^{-19} mole/L [11].

Immuno-PCR requires optimisation of many variables some are common to all types of immunoassays and some are unique to the use of DNA labels and PCR for signal amplification. Jeorger et al., (1995) established an immuno-PCR method for detection of human TSH and hCG reported that the antibody affinity and non-specific binding of the conjugates were more important in affecting assay detection limit than was the efficiency of DNA label amplification [11]. In our protein measurement using BCA assay, there was significant interference with all reducing agents used. This has happened usually in fraction 5 and above after buffer exchange. Usually fractions 2 and 3 had the highest protein content which was reduced in fraction 5 and above, but a signal belonging to reducing agent gradually increased from fractions 5 and above with a retention time of about 3min. This was confirmed with HPLC chromatography. It has been shown (Brown et al., 1988) that the BCA assay is extremely sensitive to the presence of thiol-containing reducing agents like dithiothreitol (DTT), 2-mercaptoethanol (2-ME) and mercaptoethylamine (MEA). They reported that the interference could be eliminated by selectively precipitating protein with deoxycolate and trichloroacetic acid [18,19]. All reducing agents including 2-mercaptoethanol, mercaptoethylamine and tri-n-butyl-phosphine cleaved the IgG molecule. We observed that analysis time was a critical issue in keeping the fragments in reduced form as the fragments were prone to oxidisation and therefore reassociation.

In summary, a biotinylated ELISA which is the initial part of an immuno-PCR method was developed. Biotin could be a component of the immuno-PCR method and/or the method could be designed based on the direct conjugation of DNA to detector antibody, similar to Lind et al (2005) study [9] with a better functional sensitivity.

We optimized the conditions for reduction of IgG1. This means that the thiol group of the antibody can directly attach to DNA. This would be a preferred method as there is no need for more chemicals e.g biotin molecule for immuno-PCR reaction, as it has been shown that biotin increased background noise in the immuno-PCR assay.

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