PRODUCTION OF MONOCLONAL ANTIBODIES FOR USE IN IMMUNOASSAYS BASED ON THE MAGNETIZABLE SOLID PHASE SEPARATION TECHNIQUE

W. CHAROENSIRIWATANA, N. JANEJAI, P.KRASAO
Department of Medical Sciences,
National Institute of Health, Thailand

Abstract

Monoclonal antibodies to TSH were produced by using mouse-ascites technique. Various methods for purifying the antibody from the ascitic fluid have been tried in order to obtain an appropriate TSH kit production protocol. The purified antibodies were then immobilized on magnetisable cellulose for developing an IRMA for TSH. A detailed study of the assay system, including the stability of the magnetic adsorbent was made, which showed that the SCIPAC magnetisable cellulose is suitable for the production of TSH - Blood spot IRMA kits for use in the Neonatal hypothyroid screening programme to be launched in Thailand in the near future.

1. INTRODUCTION

Recently, Immunoradiometric assay (IRMA) of TSH has come to play an important role in the diagnosis of thyroid disorders. In the developing countries this requires an assay system which is reliable, simple and preferably produced locally. The magnetic particle separation technique has been selected as a method of choice in this study due to the elimination of a high cost refrigerated centrifuge.

In this study, the monoclonal antibodies to TSH (Thyroid Stimulating Hormone) have been selected as a model for solid phase immobilization due to the need of the TSH reagent for neonatal screening programme in the country.

2. MATERIALS AND METHODS

- 2nd IRP hTSH, for immunoassay, code no. 80/558 from NIBSC, UK
- Human pituitary TSH from WHO, UK
- Recombinant TSH Standard and human pituitary TSH from IPEN, Brazil Cellulose/Iron oxide particles M-174 from SCIPAC, UK
- Silanized magnetic particles from Institute of Atomic Energy,(IAE) Beijing, China
- Horse Serum from Thai Army Animal Center, Thailand
- DEAE Cellulose (DE52) from Whatman, USA
- 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDC) from Sigma Chemical,Co.,USA
- 1,1'-Carbonyldiimidazole, (CDI),from Sigma Chemical Co., USA
- Protein -A Sepharose from Pharmacia, Sweden
- Na ^{125}I from Du Pont NEN, USA
- Immunosorbent plate from Nunc, Denmark
- Ammonium Sulphate from Merck, Germany

The monoclonal antibodies for TSH were prepared using mouse-ascites technique [1]. Two Monoclonal Antibodies [2] specific to α chain and β chain were utilized as a matching pair in this study.

2.1. Purification of monoclonal antibodies

The following procedures were used in the purification process of mouse ascites.

2.1.1. Purification of ascitic fluid by salt fractionation

29.1 g of ammonium sulphate was added to 100 mL of mouse ascites with continuous stirring at room temperature for 1 hour, followed by centrifugation at 2,500 rpm for 30 minutes. The precipitate was then redissolved in Phosphate Buffer Saline, dialyzed overnight and stored frozen.

2.1.2. Purification of IgG by anion exchange DEAE column

DEAE Cellulose was equilibrate with 0.07M phosphate buffer, pH6.3 and packed into 2.5 cm × 40 cm glass column. 200 mg of the IgG from ammonium sulphate precipitation was then loaded onto the equilibrated column and the IgG was eluted using the same buffer. The eluted fractions were monitored of spectrophotometrically at 280 nm.

2.1.3. Purification of IgG by protein A column

500 μL of ascitic fluid was dissolved in 0.1M phosphate buffer, pH8, and loaded onto protein A column. The elution was performed by using 0.1M citrate buffer, pH6 and dialyzed overnight in 0.05M phosphate buffer, pH7.4. The solution was then concentrated with Ficoll 400 and store at 4°C until further use.

2.2. Selection of the capture and detection antibodies

2.2.1. Preparation of antibody coated immunosorbent plate

The partially purified antibodies (by ammonium sulphate precipitation) were used in this experiment. 50 μL(approximately 5 μg) of protein in 0.05M phosphate buffer pH7.4 was coated onto immunosorbent plate overnight at 4°C. The plates were washed 3 times with the same buffer and then soaked with 50 μL 0.5% bovine serum albumin in 0.05M phosphate buffer, pH7.4 and stored at 4°C until use.

2.2.2. Preparation of ^{125}I labelled antibodies

50 μg of the partially purified antibodies (by ammonium sulphate precipitation) were iodinated by using the iodogen method [3].

2.2.3. Assay procedures for matching pair experiment

50 μL of 0 mIU/L and 60 mIU/L TSH standard were pipetted into each antibody coated well of the immunosorbent plates, followed by 50 μL of ^{125}I antibody and incubated overnight. The plates were then aspirated, washed with 0.05M phosphate buffer three times and then counted in a gamma counter.

2.3. Comparison of purified monoclonal anti β-TSHs from various purification procedures

The purity of antibodies was checked by SDS-PAGE electrophoresis. About 5 μg of specific antibodies from various purification procedures was mixed with SDS-sample buffer and boiled for 2 minutes. The samples were then loaded onto the gel (10% separation gel, 3% concentration gel, 10×8 cm) and the gel electrophoresis was carried out using 10 volt/cm for approximately 100 minutes. The gel was then stained with coomassie brilliant blue.

2.4. Test for RIA binding capacity of various purified antibodies

The purified α specific TSH antibodies were coated onto immunosorbent plates according to the procedure in 3.2.1. 50 μL of ^{125}I -TSH was added into each well and incubated at 4°C overnight. The wells were then aspirated and washed 3 times with 0.05M phosphate buffer pH7.4. The radioactivity in the wells was then counted and the percentage binding determined.

2.5. Coupling of antibody to magnetisable cellulose

TSH monoclonal antibody was coupled to magnetisable cellulose, M-174, from SCIPAC, UK, using two concentrations of CDI.

2.5.1. Method 1

For the activation process, 1 g of magnetic particles was washed with 40 mL distilled water and followed by 25 mL acetone for 4 times. 120 mg of CDI was added to the particles suspended in acetone and rotated for 1 h at room temperature. Then, the particles were washed 4 times with 25 mL acetone in each washing followed by 4 times with 0.1 M sodium bicarbonate, pH8.

In the coupling process, 50 mg of the partially purified antibody was added to 1 g of activated magnetic particles and the volume adjusted to 20 mL with 0.1M sodium bicarbonate, pH8. The suspension was rotated overnight at room temperature. Then, the particles were washed twice with 20 mL of 0.1M of sodium bicarbonate buffer followed by 0.6% ethanolamine in sodium bicarbonate buffer. The suspension was rotated for 30 minutes, resuspended in 0.1M acetate buffer, pH4 and then rotated for another 30 minutes. The particles were washed twice with phosphate buffer pH7.4 containing 0.1% bovine serum albumin and adjusted the total volume to 40 mL with the same buffer.

2.5.2. Method 2

The same procedure as described in method 1 was applied for the antibody coupling except that 800 mg of CDI was used in activation process (instead of 120 mg of CDI) and 30 mg of the antibody.

2.6. Coupling of antibody to silanized magnetic particles

The silanized magnetic particles from China, lot no. 93-21 and 94-7, were used for the antibody immobilization process. In this study, the effect of pH on the efficiency of coupling was studied using phosphate buffer pH5.5 and pH7.4. 200 mg of magnetic particles were washed with 20 mL of washing buffer (0.05M phosphate buffer containing 0.1% bovine serum albumin and 1% Tween 20) for 3 times. The antibody solution in coupling buffer (0.05M phosphate buffer pH5.5 and 7.4) was added and the volume was adjusted to 10 mL with the same buffer. The suspension was rotated end over end for 30 minutes at room temperature, then 20 mg of EDC were added and the suspension was rotated overnight at room temperature. The particles were then washed 10 times with washing buffer and adjusted to 10 mL with 0.05M phosphate buffer pH7.4.

2.7. The stability study of the antibody coated magnetic particles

The stability of the immunoadsorbents was studied over a period of two months. The CDI coated methods using SCIPAC magnetic particles and cellulose were tested in this study for the period of 4 months. The assay protocol is summarized below:

200 μL Standard or Sample

↓

50 μL ^{125}I -Anti TSH

↓

Incubate 3 hr. at room temp.

↓

1.25 mg/ 50 mL Antibody-Magnetic particles

↓

Rotate 1 hr. at room temp.

↓

Wash with 2×1 mL 0.05M phosphate buffer with 0.4% Tween 20

↓

Decant

↓

Count the precipitate

The change of buffer in antibody coated solid phase matrix before the assay performance was also been tested in the stability study.

2.8. Precision profile of TSH-blood spot IRMA assay

The antibody coated magnetic particle have also been tested in the NIH-blood spot IRMA kit [4] using the assay protocol shown above. The precision profile of 10 assay batches was calculated using WHO data processing programme.

2.9. Testing of recombinant TSH

TSH IRMA standard curves were set up using IPEN recombinant TSH, authentic pituitary TSH extract and WHO TSH standards prepared in horse serum and diluted serially to give concentrations ranging from 0 to 75 mIU/L.

3. RESULTS

Three procedures have been used to purifiy the monoclonal antibodies from ascitic fluid, namely, ammonium sulphate precipitation, anion exchange DEAE Column and affinity chromatography over protein A column. The elution profiles are shown in Fig. 1 and Fig. 2 respectively.

The matching pair experiment of the antibodies showed that the suitable combination is the α-specific antibody as the capture antibody, and the β-specific antibody as the ^{125}I labelled antibody, as shown in Table I.

TABLE I. SELECTION OF THE CAPTURE AND DETECTION ANTIBODY PAIR FOR TSH IRMA

Coating	Counts	
	α-specific anti TSH	β-specific anti TSH
Standard 0 mIU/L	160	107
	232	244
Standard 60 mIU/L	11978	179
	11991	249

The SDS-electrphoresis of the β-TSH antibody purified by the Protein A method yielded only two sharp bands corresponding to the light chain and heavy chain of the IgG molecule, while the other two methods of purification showed the presence of impurities. Hence, the antibody for labelling is routinely purified by the Protein A method.

The protein A purified β-specific Antibody was tested for it's binding capacity to the α-specific antibody, purified by the three methods and immobilized on immunosorbent plates.The results showed that the ammonium sulphate precipitation yielded 100% binding whereas the anion exchanger and Protein A were 91.4 and 98.0% respectively as in Table II. Thus, the ammonium sulphate purification procedure was selected as the purification process for the capture antibody.

TABLE II. BINDING CAPACITIES OF THE PURIFIED α-SPECIFIC ANTIBODY FRACTIONS

Methods	% B/T (RIA)
Ammonium sulphate precipitation	100
Anion exchanger (DEAE cellulose)	91.4
Protein A	98.0
Blank	0.4

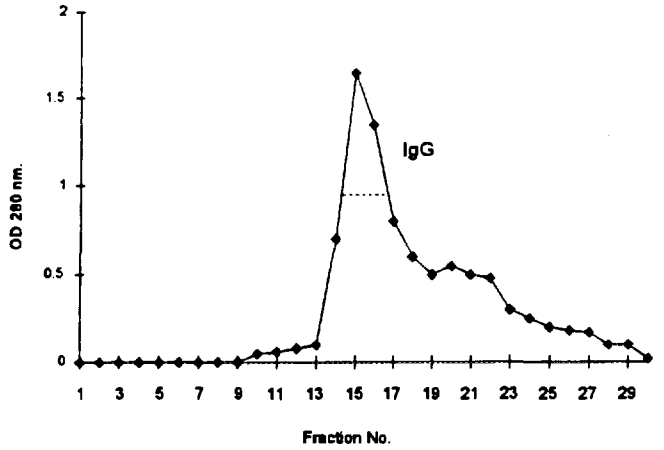


Fig.1 Purification of TSH monoclonal antibody by DEAE column.

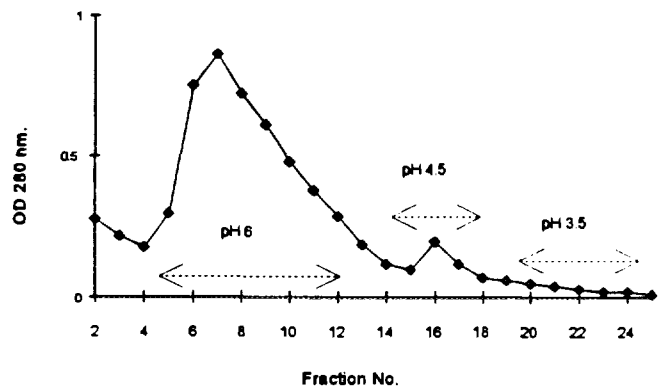


Fig.2 Purification of TSH monoclonal antibody by Protein A column.

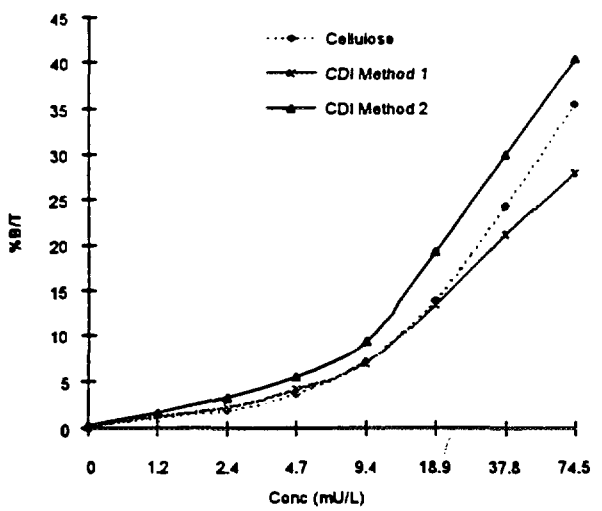


Fig.3 Comparison of CDI activation methods 1&2.

For CDI-antibody coupling procedures, method 2 consumed 40% less antibody for coating onto the SCIPAC magnetic particles and the binding at TSH concentration of 75.5 mIU/L was 40.4% whereas in method 1, the binding is only 27.9%. The reference CDI cellulose procedure also yielded the binding 35.4% as shown in Fig. 3.

TSH antibody was coupled to silanized magnetic particle lot no. 93-21 and 94-7 from IAE, China using the EDAC method. A comparison of the amount of IgG coated onto the magnetic particles of IAE lot 93-21 and 94-7 showed that for IAE lot no. 93-21, the binding at 75.5 mIU/L, for 2.5 mg, 5.0 mg and 10.0 mg of IgG for coating were 5.55, 7.39 and 10.94% respectively at coating pH of 5.5. At coating pH of 7.4, the binding was 11.26, 12.65 and 16.53%respectively. It is observed, that the preparation IAE 93-21 shows a consistently lower binding than IAE 94-7. For the lot IAE 94-7, the change in the coupling pH did not affect the binding. The binding for 75.5 mIU of TSH at pH5.5 were 24.37, 29.79, and 33.14 respectively and for pH7.4 these values were unchanged. This difference in the two particles is also observed in the dose-response curves (Figs 4 and 5). From these studies we have selected an IgG concentration of 10 mg for coupling.

The stability of the above mentioned immunoadsorbents has been studied over a period of 4-6 months. For the SCIPAC M-174 adsorbent prepared by method 1, the binding at 75.5 mIU/L drastically dropped from about 28% to about 13% in the first month and stayed at the range of 8-10% in the following three months as in Fig. 6. In the case of the adsorbent prepared by method 2, the binding dropped from 40.40 to 31.43 in the first month and stayed at the range of 20-24% in the following period as in Fig.7. In both methods, when the particles were resuspended in fresh buffer just prior to the assay, there is an increase in the binding (Figs 6 and 7) and in the standard curves shown in Fig. 8. This indicates the accumulation of free antibody in the supernatant with time, which could be removed by decanting off the old supernatant. As mentioned earlier, method 2 yielded approximately 12% higher binding than the method 1 and the amount of IgG consumed in coating process is about 60% of that in method 1.

For the IAE 94-7 based immunoadsorbent the binding at 75.5 mIU/L of TSH dropped from 32.37 to 20.70 for the first month and stayed in the range of 13-16% in the following five months as shown in Fig. 9.

The magnetisable cellulose based immunoadsorbent has been tested for use in the immunoradiometric assay of TSH in blood spot. Fig. 10 shows the precision profile derived from 10 assays, from a single batch of kits. The WHO data processing programme has been used. The variation, which is <10% for TSH concentrations ranging from1.5 to 100 mIU/L, is acceptable in the neonatal screening for hypothyroidism where the cut off level of TSH is about 25 mIU/L.

The study has also been extended to the recombinant TSH standard from IPEN Brazil and the result showed that the IPEN recombinant standard yielded an equivalent standard curve to the WHO TSH standard and the pituitary TSH extract with the correlation coefficient of 0.9987 and 0.9964 respectively as seen in Fig.11 and could be used as a standard in the TSH - IRMA assay (Fig.12).

4. CONCLUSION

Two types of monoclonal antibodies specific to α-and β-chain TSH were produced by the mouse-ascites technique. The SCIPAC M -174 magnetisable cellulose has been selected as the material for coupling to antibody using theCDI activation method 2. The antibody specific to α-chain was purified by ammonium sulphate precipitation and was selected as the captured antibody, while, the β-specific was purified by protein A affinity column and used as the detection antibody.

The stability of the SCIPAC coated particles is more than 6 months, provided the suspension buffer is changed before assay.For the IAE silanized Magnetic particles, the batch IAE 94-7 is better than 93-21. A comparison of the amount of antibody consumed, showed that the SCIPAC material consumed 25%less than the IAE particles. Hence, the SCIPAC material has been selected for routine use.

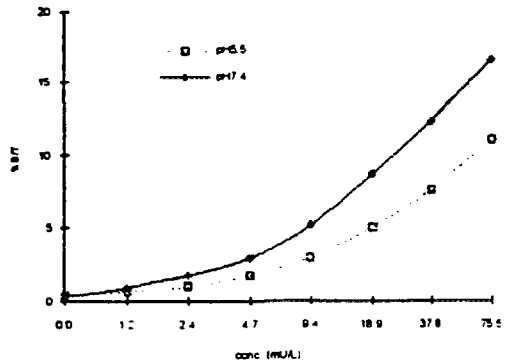


Fig.4 The effect of coating pH on the standard curves with IAE 93-21 magnetic particles.

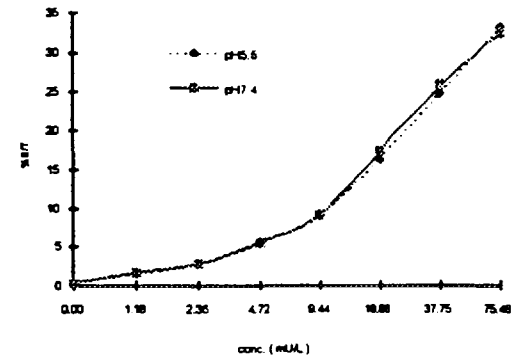


Fig.5 The effect of coating pH on the standard curves with IAE 94-7 magnetic particles.

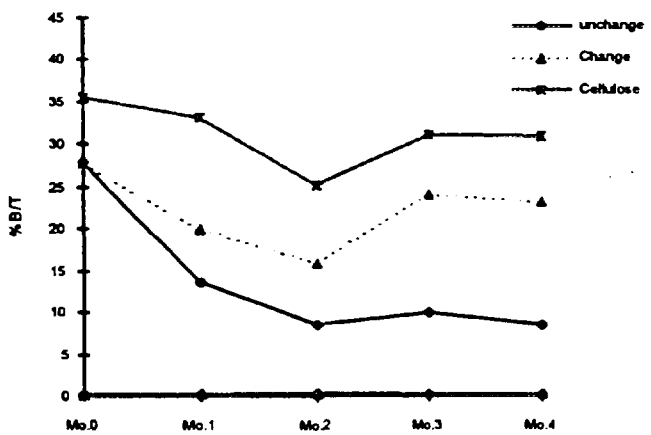


Fig.6 Stability of SCIPAC M-174 adsorbent prepared by method 1.

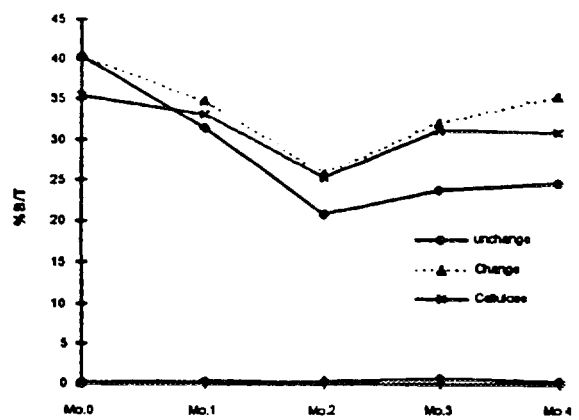


Fig.7 Stability of SCIPAC M-174 adsorbent prepared by method 2.

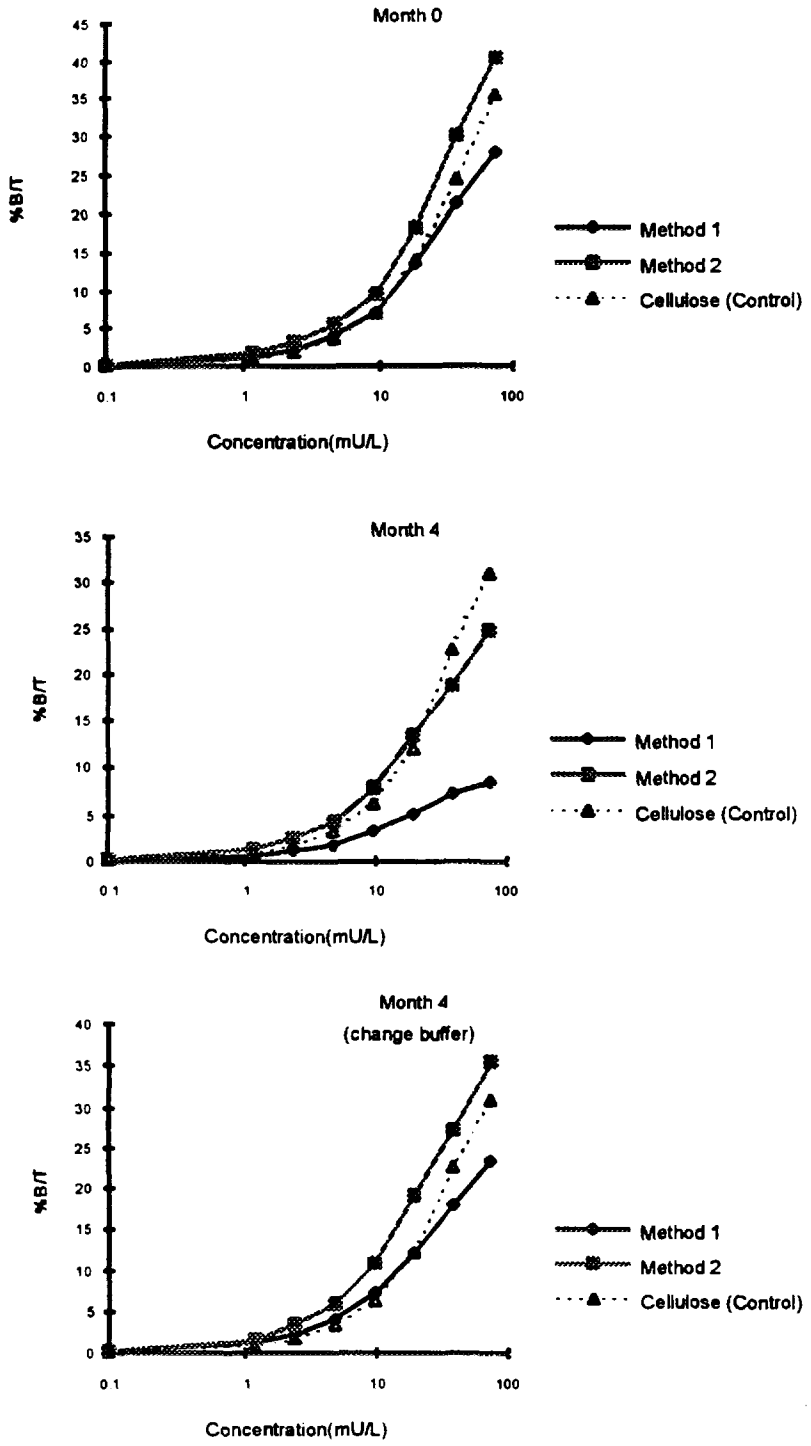


Fig.8 Effect of the age of SCIPAC M-174 adsorbent on the TSH IRMA standard curves.

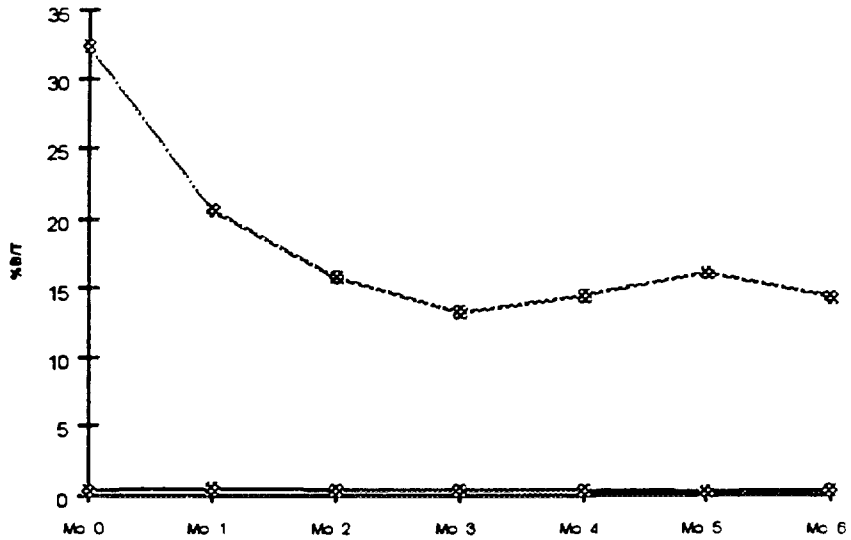


Fig.9 Stability of the IAE 94-7 adsorbent.

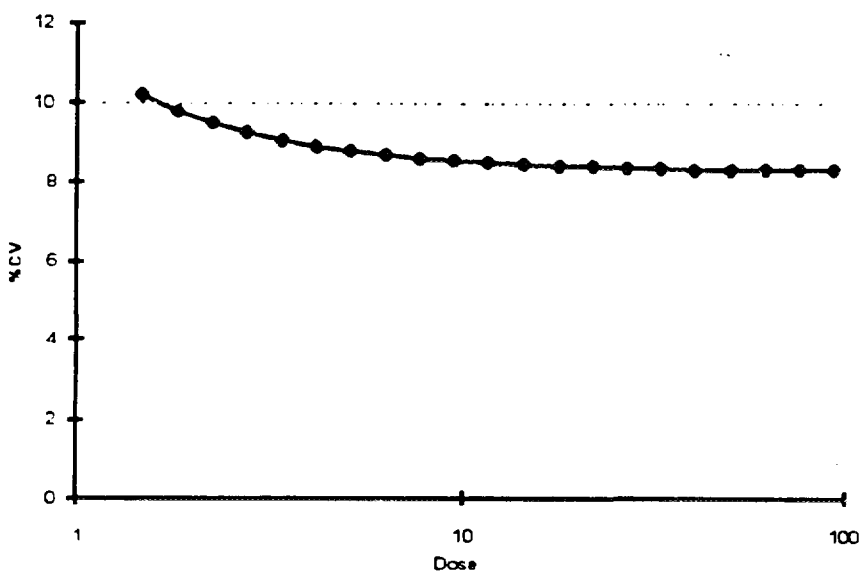
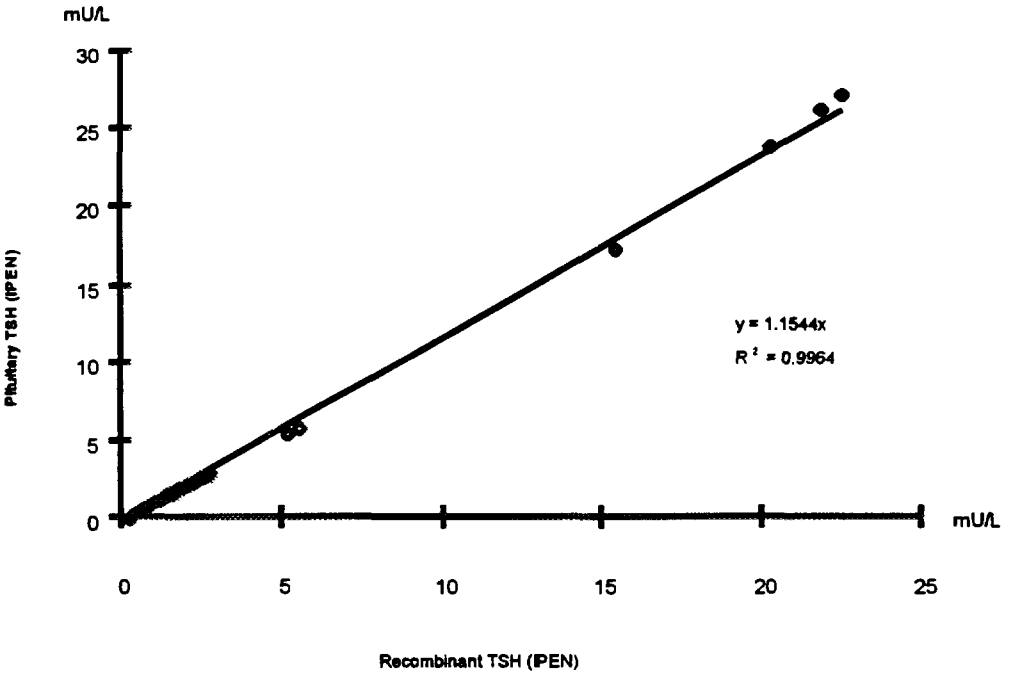


Fig.10 Precision profile of 10 bloodspot TSH IRMAs with magnetizable cellulose as the solid phase.



(b)

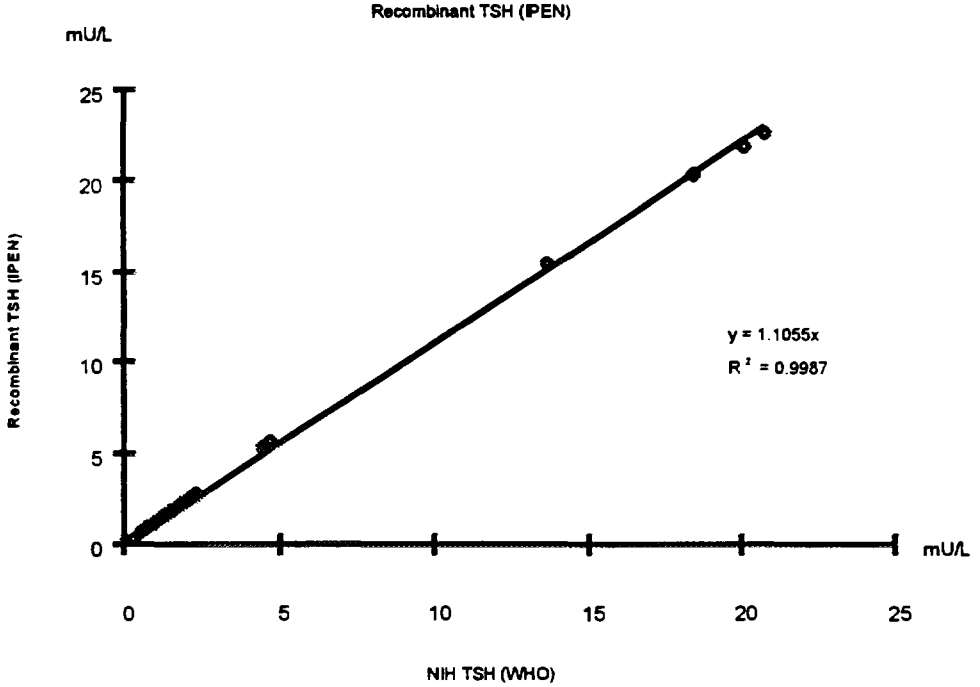


Fig.11 (a) Comparison of pituitary and recombinant TSH standards (IPEN)
(b) Comparison of recombinant TSH standard (IPEN) and pituitary TSH Standard, WHO.

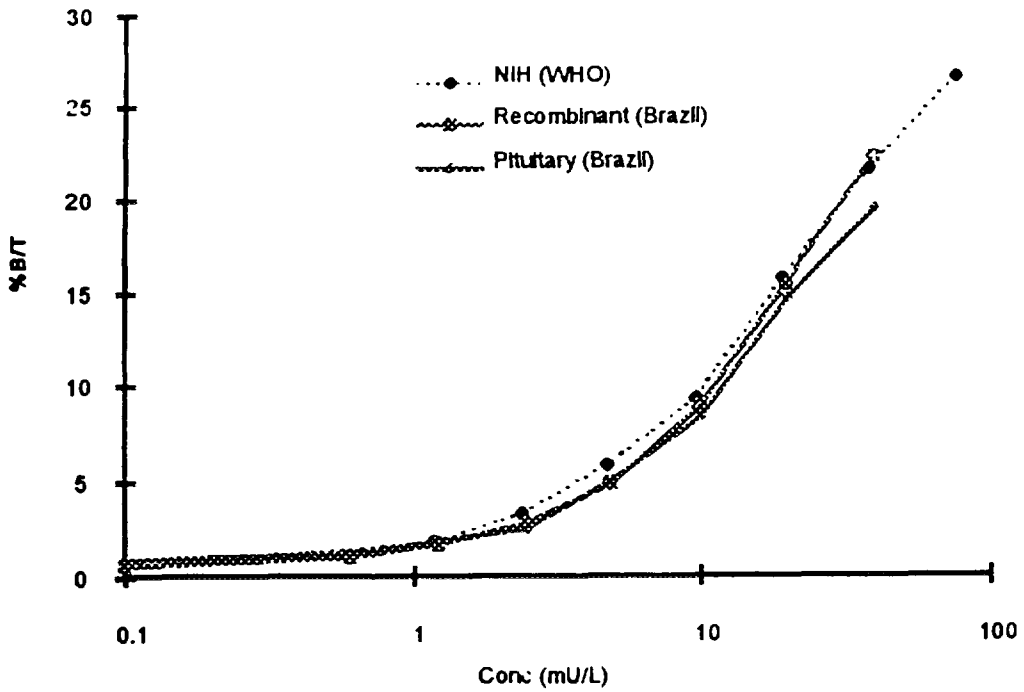


Fig.12 TSH IRMA standard curves obtained with TSH standards from the three sources.

The recombinant TSH standard from IPEN showed a good correlation with the standard TSH from pituitary extract and WHO TSH standard, indicating that it could be used as a standard in immunoassays.

Summarizing, the locally made monoclonal antibody specific to TSH could be used for the production of TSH-blood spot-IRMA kit in combination with the magnetic particle separation technique, which avoids the using of a refrigerated centrifuge. It is apparent that such an assay system can be locally produced and being technically simple, cheap and reliable, it can be used in the neonatal hypothyroid screening programme to be launched in Thailand soon.

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