# STUDY OF ANTIBODY IMMOBILIZATION ON DIFFERENT MAGNETIC PARTICLES UTILIZED FOR THE RADIOIMMUNOASSAY (RIA) AND IMMUNORADIOMETRIC ASSAY (IRMA) OF HORMONES



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**Abstract**

A study was carried out on antibody immobilization on three different types of magnetic particles: plain magnetite (Institute of Isotopes, Hungary), silanized magnetite (Institute of Atomic Energy, China) and magnetizable cellulose (SCIPAC, UK). For radioimmunoassay (RIA) applications an efficient 2nd antibody (AB)-coupled magnetic solid phase, utilizing plain magnetite and a purified anti-rabbit IgG antibody (Trilab, Brazil), was prepared. A consistent bias, detected in comparison with a well known commercial magnetic solid phase kit, was practically eliminated by modifying the coupling and saturation procedure. Concerning two-site IRMA application, an extensive study was carried out on the matching and selection of anti-hTSH antibodies that could be used for capture and detection. Very satisfactory results were obtained with the three types of magnetic particles using different monoclonal and polyclonal antibodies and in particular, two partners anti-hTSH mABs from the National Institute of Health of Thailand. Utilizing also a recombinant hTSH standard preparation, calibrated and distributed by our laboratory (IPEN-CNEN/SP, Brazil), it was possible to obtain a complete set of in-house reagents for hTSH IRMA, prepared and tested under IAEA support.

## 1. INTRODUCTION

An IAEA organized co-ordinated research programme (CRP) has set special emphasis on the utilization of three different magnetic matrices that can be used for antibody coupling in RIA and IRMA of hormones. These are:

(a) Plain magnetite ($Fe_3O_4$), fine particles, from the Institute of Isotopes (Budapest, Hungary)
(b) Silanized magnetite, type 94-7 from the Institute of Atomic Energy (Peking, China)
(c) Magnetizable cellulose, type M-174, from SCIPAC, (Sittingbourne, UK).

Our laboratory placed special emphasis on their utilization for the preparation of a solid phase 2nd Antibody, to be used in the RIA of different hormones, and of an anti-hTSH solid phase capture antibody for IRMA. Since the preparation of good quality solid phases for use in two-site IRMA techniques involves the matching, in a proper way, of matrix, capture antibody, coupling reaction, antigen and detecting radioiodinated antibody, an extensive study was carried out on these aspects. In the present work, consequently, we analysed the behaviour of different sets of monoclonal and polyclonal antibodies, utilizing two coupling reactions [1,2] and two different reference preparations of pituitary and recombinant hTSH [3,4].

## 2. RESULTS AND DISCUSSIONS

### 2.1. preparation of a second antibody-coupled magnetic solid phase for the radioimmunoassay of $T_4$ and TSH

This reagent was prepared utilizing the plain magnetite particles from Hungary [5] and an anti-rabbit IgG antiserum produced in goat (Trilab, São Paulo, Brazil). The caprylic acid purified IgG was coupled to the matrix utilizing 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and following the manufacturer's protocol. Table I shows that the ideal amount of IgG to be coupled per milligram of matrix was found to be of the order of 0.5 mg.

TABLE I. DETERMINATION OF THE AMOUNT OF IgG 2nd ANTIBODY TO BE COUPLED TO PLAIN MAGNETITE PARTICLES (HUNGARY)

| mg of IgG per mg of matrix | NSB (%) | $B_0$ (%) | $B_0$/NSB |
|---|---|---|---|
| 0.25 | 1.1 | 24 | 22 |
| 0.5 | 0.9 | 42 | 47 |
| 1.0 | 1.1 | 46 | 42 |

This preparation was tested in $T_4$ assays in comparison with a commercial magnetic preparation from Amersham (Aylesbury, UK). By linear regression analysis a limited bias (approximately 8%) was detected.

$$Y = 0.915X + 0.1573 \quad (r = 0.998)$$

When these particles was tested in hTSH RIA, a much higher bias ($\sim$ 65%) in opposite direction was observed.

$$Y = 1.646X + 12.350 \quad (r = 0.971)$$

Analysing these data, the bias seemed to be releated to the amount of serum present,which is 100 $\mu$Lfor hTSH and 10 $\mu$L for $T_4$. This hypothesis was later confirmed by the following two experiments.

(a) When the same sample was analysed for hTSH under different dilutions, (up to 1:20), the bias, which was of the order of 50% in the undiluted sample, practically disappeared at the highest dilutions, i.e. more the amount of serum present in the reaction, higher was the value obtained with the in-house magnetic reagent.

(b) Three standard curves were run, one in plain phosphate buffer, and the two other curves with the addition of equivalent amounts of human or horse serum. The results can be seen in Table II.

TABLE II. SERUM EFFECTS ON THE SPECIFIC BINDINGS OBTAINED IN hTSH RIA UTILIZING AN IN-HOUSE AND A COMMERCIAL 2nd ANTIBODY MAGNETIC PREPARATION

| Particle | Plain buffer | | Human serum | | Horse serum | |
|---|---|---|---|---|---|---|
| | $B_0$ | | $B_0$ | | $B_0$ | |
| | (cpm) | (%) | (cpm) | (%) | (cpm) | (%) |
| in-house | 14409 | 46 | 6690 | 22 | 3930 | 13 |
| commercial | 14497 | 47 | 11019 | 36 | 12159 | 39 |

It is clear that while using the commercial preparation we have a certain limited decrease in binding when using serum instead of buffer. Such decrease becomes dramatic with the in-house reagent. Moreover, the only unknown sample that did not present a bias in this experiment, was a pituitary extract that could be analysed in the serum-free system. We speculated therefore that some serum components might bind to the 2nd antibody coupled particle, greatly decreasing its binding capacity and, consequently, producing the observed bias.

An additional saturation step was therefore introduced in the original coupling procedure, utilizing a buffer containing 1% milk proteins and 1% BSA. The comparison between in-house and commercial reagent was now repeated, running the standard curves in horse serum, the one that presented the highest influence (Table III).

TABLE III. CURVE PARAMETERS AND QUALITY CONTROL SAMPLES (QCS) OBTAINED AFTER MILK / BSA SATURATION OF THE IN-HOUSE 2nd ANTIBODY MAGNETIC REAGENT

| Preparation | $B_o$ (cpm) | $B_o$ (%) | $ED_{80}$ | $ED_{50}$ (mIU/L) | $ED_{20}$ | QCS (mIU/L) Low | Medium | High |
|---|---|---|---|---|---|---|---|---|
| in-house | 8649 | 33 | 6 | 27 | 115 | 4.8 | 11.5 | 30 |
| commercial | 7339 | 28 | 5 | 30 | 175 | 4.4 | 10.5 | 31 |

Previous QCS statistics:

Low QCS X = 4.58 ± 0.72
Medium QCS X = 10.48 ± 2.36
High QCS X = 33.9 ± 4.2

Linear regression analysis:

Y = 0.936x + 1.113 (r = 0.9992)

We conclude that, after saturation, the serum effects on the matrix are practically eliminated and so is the bias between the two magnetic preparations.

## 2.2. Preparation of a complete set of reagents for hTSH IRMA

Knowing that the matching of different capture and detecting antibodies is particularly critical for the success of a two-site IRMA design, a first study was carried out on all possible antibody combinations, for "partners selection". For this purpose the silanized magnetite matrix from China was utilized, carrying out the EDC coupling protocol as recommended by the manufacturer and using four different antibody preparations: two polyclonal antibodies (pAB), one from the Scottish Antibody Production Unit (SAPU, Carluke, Scotland, UK) and the other kindly donated by the National Research Center for Endocrinology (Moscow, Russia) and two monoclonal antibodies (mAB), both kindly distributed by the National Institute of Health (Bangkok, Thailand). For radioiodination and detection also, four different anti-hTSH antibodies (all monoclonal) were tested: the two mentioned preparations from Thailand and two commercial preparations, respectively from SAPU (Product NS 095-110, Batch N.61871) and from Serono Diagnostic, Woking, UK (TSH-0182-0002). The results of this study are reported in Table IV.

TABLE IV. MATCHING DIFFERENT CAPTURE AND DETECTING ANTIBODIES FOR hTSH IRMA

| Capture antibody | Detecting Antibody ($^{125}$I -mAB) Serono* Bo(%) | Serono* $B_{60}$(%) | SAPU* $B_o$(%) | SAPU* $B_{60}$(%) | Thai 1* $B_o$(%) | Thai 1* $B_{60}$(%) | Thai 2* $B_o$(%) | Thai 2* $B_{60}$(%) |
|---|---|---|---|---|---|---|---|---|
| Russian pAB | 0.44 | 33.9 | 0.30 | 13.9 | *No binding* | | *0.29* | *12.7* |
| SAPU pAB | 0.40 | 22.8 | 0.31 | 18.2 | No binding | | 0.37 | 17.1 |
| Thai 1 mAB | 0.33 | 4.5 | 0.11 | 10.7 | No binding | | 0.76 | 16.5 |
| Thai 2 mAB | 0.27 | 31.4 | 0.29 | 0.73 | No binding | | 0.16 | 0.34 |

The best partners clearly are the Russian pAB and Thai-2 mAB for capture, when matched to radioiodinated Serono mAB, which also seems to be the best preparation for detection and performs well also in partnership with coupled pAB from SAPU. This last preparation performs quite well for capture and shows about the same degree of binding with all detecting antibodies being tested, except Thai-1, which, in our hands, had no use for detection at all. An interesting and useful consequence of the efficient matching of the two Thailand antibodies is that, they have been extremely useful for this project and being monoclonals, they could be produced in large quantities and distributed to interested laboratories in the future. It is also observed, that the Russian pAB is probably the best antibody for capture and also matches well with SAPU and Thai-2 as detecting mABs, and the two SAPU antibodies are also efficient partners.

Having identified the best partners, we found it interesting to carry out a study on the ideal amount of antibody to be used in a certain coupling reaction. According to our experience, in fact, there is an optimal amount of antiserum, or purified IgG, to be used per gram of each type of particles. Surprisingly, using an excess of antibody we are not only wasting money and material, but we end up decreasing the binding efficiency. An example of one of this study is shown in Fig. 1 and refers to the utilization of magnetizable cellulose from SCIPAC, to which different amounts of Thai-1 mAB were coupled, using 1-1'carbonyldiimidazole (CDI) as described by Edwards et al. [6] and carrying out four parallel reactions. The same type of analysis, performed on the two other matrices being considered in this study, provided the data presented in Table V. In this table, mL or mg are indicated according to the use of either whole antiserum or purified IgG.

TABLE V. OPTIMAL AMOUNT OF ANTIBODY USED IN THE COUPLING REACTION PER GRAM OF DIFFERENT MAGNETIC MATRICES

| Antibody in the coupling | Plain magnetite (Hungary) | Silanized magnetite (China) | Magnetizable cellulose (UK) |
|---|---|---|---|
| pAB Russian | 60.8 mL | 5 mL | 0.6 mL |
| pAB SAPU | 5.0 mL | 2 mL | 0.4 mL |
| mAB Thai 1 or 2 | 20.0 mg | 5 mg | 2.0 mg |

We can observe a great difference in the ideal amount of antibody to be used, depending on the antiserum, but an even greater difference depending on the type of matrix. So the same antiserum can be used, for example, as the ideal amount of either 60 mL or 0.6 mL/g, for its coupling to plain magnetite or to magnetizable cellulose respectively. It is also interesting to observe the extremely small amount of mAB (2 mg/g) that can be used, for example with magnetizable cellulose. These differences, however, were partly explained when the ideal amount of solid phase per assay tube was determined, as exemplified in Fig. 2 for the case of silanized magnetite, and reported in Table VI for the three different matrices.

TABLE VI. OPTIMAL AMOUNT OF DIFFERENT SOLID PHASES TO BE USED IN hTSH-IRMA (mg/tube)

| Capture antibody | Plain magnetite | Silanized magnetite | Magnetizable cellulose |
|---|---|---|---|
| pAB Russian | 0.026 | 0.340 | 2.50 |
| pAB SAPU | 0.090 | 0.408 | 1.50 |
| mAB Thai 1 or 2 | 0.130 | 0.430 | 1.14 |

It is evident now that very small amounts of solid phase are necessary for those particles to which large amounts of antibodies have been coupled. Each matrix, having a different chemical composition and size, obviously presents different coupling capacity, which determines the optimal amount of reacting antibody and, consequently, the ideal amount of particle to be used in the assay. If this were true, we should be able to run the same number of tubes with approximately the same amount of antibody, coupled to different amounts of each particle. This is shown in Tables VII-IX, where one can see that 2000 tubes are run with approximatelly 1-1.5 mL of SAPU pAB, 3-3.5 mL of Russian pAB and 4-5 mg of Thai-1 and Thai-2 mABs. In each case we need very small amounts of plain magnetite, about 3-14 times more silanized magnetite and up to 100 times more magnetizable cellulose. The Russian pAB seems less effective than SAPU pAB in terms of number of assayed tubes while the coupling and binding efficiency of the two mABs from Thailand are confirmed.

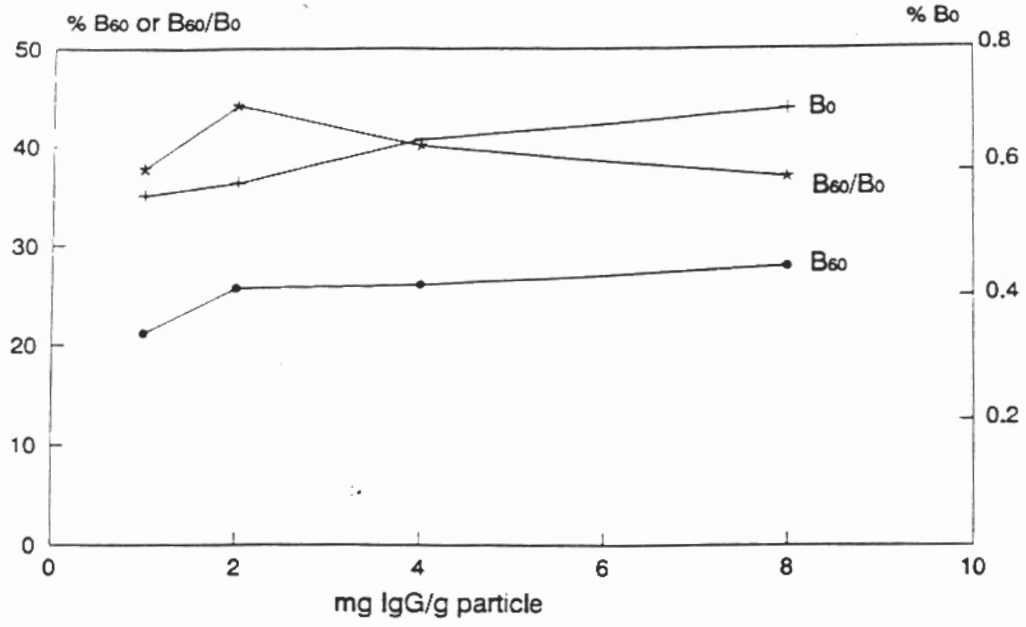


*Fig. 1: Determination of the optimal amount of mAB (Thai 1) per gram of matrix (magnetizable cellulose M-174, SCIPAC) to be used in the CDI coupling reaction.*
*Detecting antibody: Thai 2 mAB.*

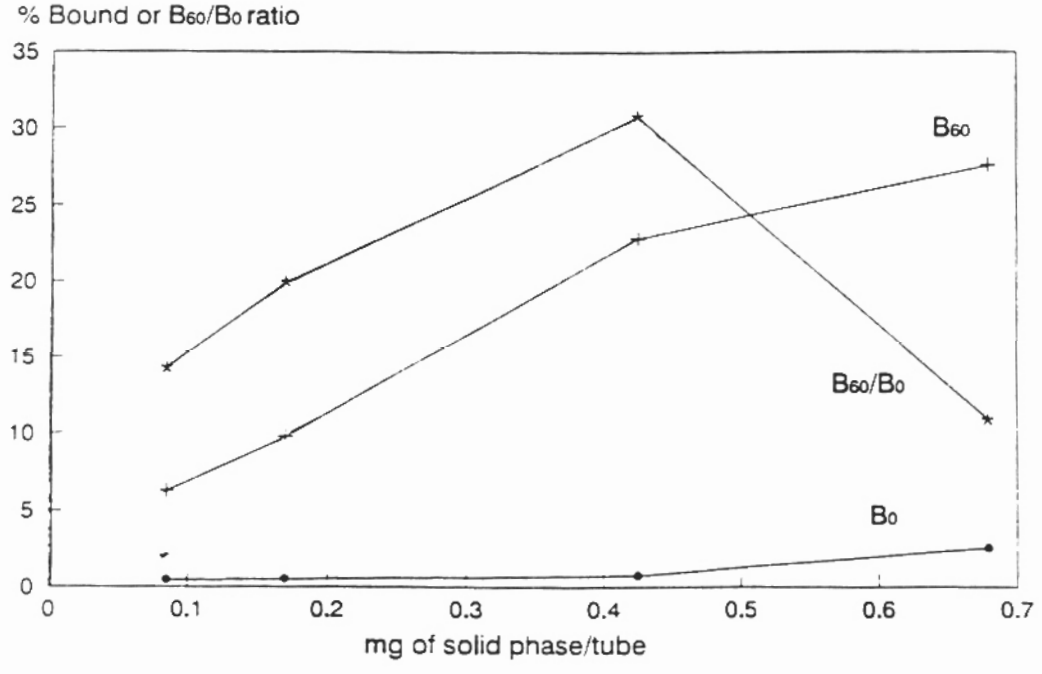


*Fig. 2: Determination of ideal amount of solid phase (anti-hTSH SAPU pAB coupled to silanized magnetite) per assay tube of hTSH IRMA.*
*Detecting antibody: Serono mAB.*

TABLE VII. AMOUNT (g) OF DIFFERENT MAGNETIC PARTICLES AND OF THAI 1 AND 2 ANTIBODIES (mg) NECESSARY FOR 2000 TUBES OF hTSH IRMA

| Matrix | amount of particle for 2000 tubes (g) | amount of antibody for 2000 tubes (mg) |
|---|---|---|
| plain magnetite | 0.26 | 5.2 |
| silanized magnetite | 0.86 | 4.3 |
| magnetizable cellulose | 2.30 | 4.6 |

TABLE VIII. AMOUNT (g) OF DIFFERENT MAGNETIC PARTICLES AND OF RUSSIAN ANTIBODY (mL) NECESSARY FOR 2000 TUBES OF hTSH IRMA

| Matrix | amount of particle for 2000 tubes (g) | amount of antibody for 2000 tubes (mL) |
|---|---|---|
| plain magnetite | 0.05 | 3.16 |
| silanized magnetite | 0.68 | 3.40 |
| magnetizable cellulose | 5.00 | 3.00 |

TABLE IX. AMOUNT (g) OF DIFFERENT MAGNETIC PARTICLES AND OF SAPU ANTIBODY (mL) NECESSARY FOR 2000 TUBES OF hTSH IRMA

| Matrix | amount of particle for 2000 tubes (g) | amount of antibody for 2000 tubes (mL) |
|---|---|---|
| plain magnetite | 0.18 | 0.90 |
| silanized magnetite | 0.82 | 1.64 |
| magnetizable cellulose | 3.00 | 1.20 |

We found this study quite important, to evaluate the quality of each starting reagent, of the final products and also for cost evaluation.

We had now all the elements for running the proper standard curves, and this was done for each matrix after it had been coupled to the same antibody (Russian pAB) (Fig. 3). The assays were carried out as described elsewhere [7]. We can observe that all curves are practically equivalent, with slightly higher bindings presented by magnetizable cellulose. In Table X the quality and statistical parameters relative to each standard curve are presented, showing comparable sensitivities, calculated according to Rodbard [8]. The QCS values confirm an acceptable accuracy for all solid phases, considering that the average inter-laboratory inter-design values (n=21) for Baxter QCS are: low QCS = 0.45 ± 30.9%; medium QCS = 5.7 ± 15.9%; high QCS = 24.2 ± 18.8%.

In Fig. 4 the calculated precision profiles also indicate a comparable and acceptable performance for the three different IRMA systems. These profiles, even with a limited statistics, are indicating a functional sensitivity of ~0.1 mIU/L.

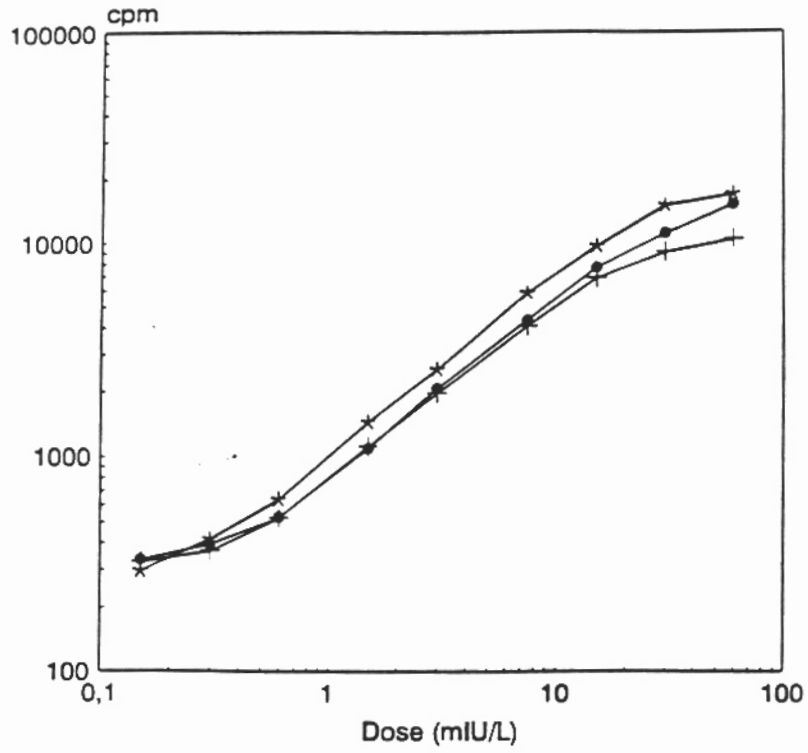


*Fig. 3: Example of hTSH IRMA standard curves obtained with: silanized magnetite ---- . ----, plain magnetite ---- + ----, magnetizable cellulose ---- * ----.*
*Capture antibody: Russian pAB, Detecting antibody: Serono mAB, Standard preparation: rec-hTSH, (BRP-3)*

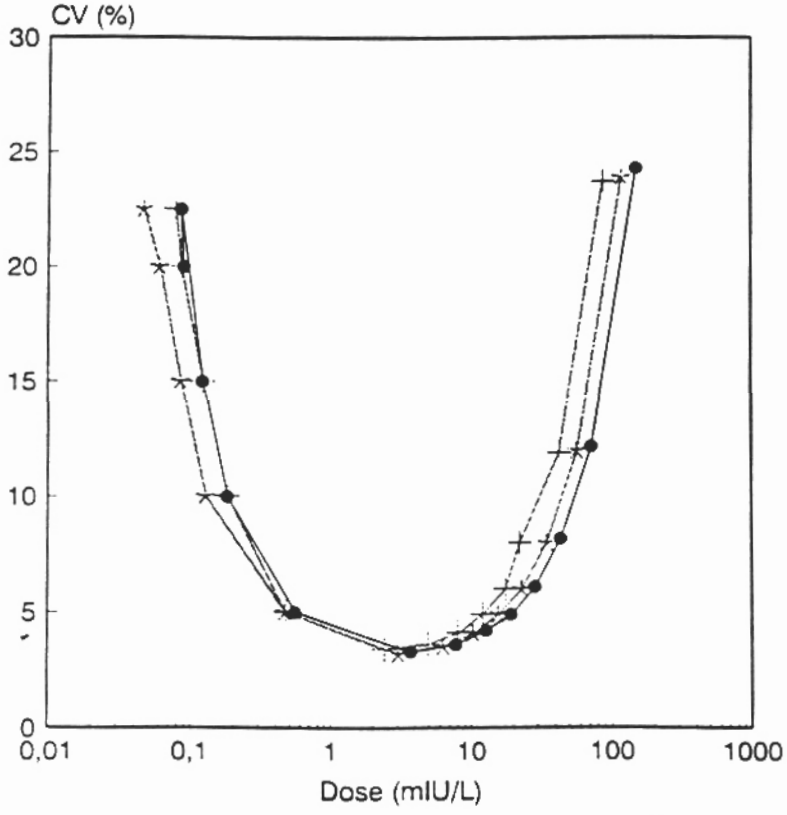


*Fig. 4: Intra-assay precision profiles obtained with the three standard curves presented in Fig. 3 and where each point was run in duplicate: silanized magnetite ---- . ---- , plain magnetite ---- + ----, magnetizable cellulose ---- * ----.*

TABLE X. PARAMETERS RELATED TO hTSH IRMA STANDARD CURVES CARRIED OUT WITH RUSSIAN pAB COUPLED TO THREE DIFFERENT MATRICES

| Parameter | silanized magnetite | plain magnetite | magnetizable cellulose |
|---|---|---|---|
| $B_0$ (%) | 0.38 | 0.35 | 0.35 |
| $B_{60}$ (%) | 22.0 | 15.1 | 24.6 |
| $B_{60}/B_0$ | 57.9 | 43.1 | 70.3 |
| initial SD (cpm) | 30.7 | 76.8 | 43.1 |
| $Y_0$ (cpm) | 264 | 238 | 244 |
| $Y_{min}$ (cpm) | 289 | 284 | 268 |
| initial slope (cpm/L/mIU) | 463 | 587 | 345 |
| $X_{min}$ (mIU/L) | 0.054 | 0.078 | 0.070 |
| low QCS (mIU/L) | 0.48 | 0.64 | 0.44 |
| medium QCS (mIU/L) | 4.7 | 4.9 | 4.4 |
| high QCS (mIU/L) | 23 | 21 | 23 |

Considering that the existence of a certain bias between plain and magnetizable cellulose has been reported [6], a comparison in this respect was carried out (Fig. 5 and Table XI). We can appreciate indeed the existence, even in our hands, of a certain bias that, however, does not have a great significance when compared with the variability of Baxter's data.

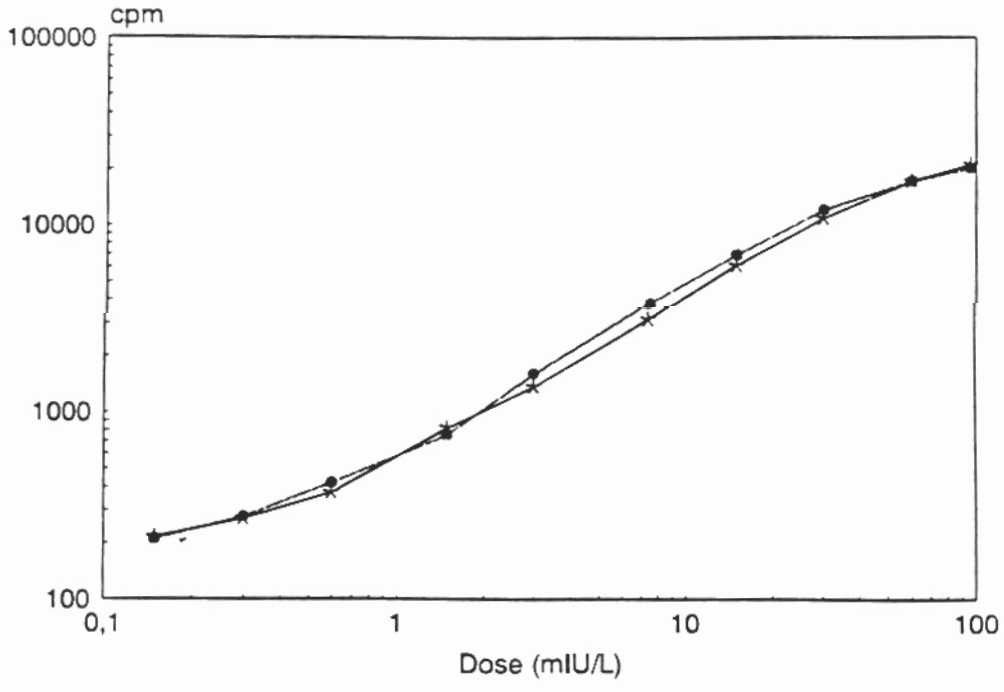


*Fig. 5: Example of hTSH IRMA standard curves obtained with: plain cellulose solid phase (NETRIA) -- * --, in-house magnetizable cellulose based on SCIPAC M-174, using Russian pAB for capture --- . ---, Detecting antibody: Serono mAB ,Standard preparation: pit-hTSH, (BRP-2)*

TABLE XI. COMPARISON. BETWEEN THE PARAMETERS OF THE STANDARD CURVES CARRIED OUT WITH NON MAGNETIC (NETRIA) AND MAGNETIC (IN-HOUSE) SOLID PHASES

| Parameter | magnetic | non magnetic |
|---|---|---|
| $B_0$ (%) | 0.22 | 0.24 |
| $B_{60}$ (%) | 33.7 | 38.6 |
| $B_{60}/B_0$ | 153 | 161 |
| initial SD (cpm) | 11.9 | 3.8 |
| $Y_0$ (cpm) | 130 | 145 |
| $Y_{min}$ (cpm) | 160 | 164 |
| initial slope (cpm/L/mIU) | 611 | 439 |
| $X_{min}$ (mIU/L) | 0.049 | 0.043 |
| low QCS (mIU/L) | 0.40 | 0.44 |
| medium QCS (mIU/L) | 5.1 | 5.9 |
| high QCS (mIU/L) | 20 | 25 |

The hTSH reference preparations utilized to construct all the IRMA curves of this work were also in-house preparations calibrated in our laboatory (IPEN-CNEN/SP-Brazil). First a pituitary (BRP-2) [3] and then a recently validated [4] recombinant hTSH standard (BRP-3) were used, being also distributed to participating laboratories of ARCAL VIII and of the present CRP.

In Table XII we are finally presenting the quality and statistical parameters which are relative to an hTSH IRMA carried out with a complete set of reagents prepared under the two mentioned IAEA-organized programmes. These are:

1- standard hTSH: rec-hTSH BRA-3 (Brazil);
2- capture antibody: mAB Thai-1 (Thailand);
3- detecting antibody: mAB Thai-2 (Thailand);
4- matrix: silanized magnetite (China).

TABLE XII. PARAMETERS RELATED TO hTSH IRMA STANDARD CURVES CARRIED OUT WITH IN-HOUSE REAGENTS

| | |
|---|---|
| $B_0$ (%) | 0.53 |
| $B_{60}$ (%) | 11.5 |
| $B_{60}/B_0$ | 21.7 |
| initial SD (cpm) | 13.3 |
| $Y_0$ (cpm) | 354 |
| $Y_{min}$ (cpm) | 370 |
| initial slope (cpm/L/mIU) | 152 |
| $X_{min}$ (mIU/L) | 0.105 |
| low QCS (mIU/L) | 0.64 |
| medium QCS (mIU/L) | 4.8 |
| high QCS (mIU/L) | 20.5 |

Unfortunately the existence of a certain storage instability of our coupled preparations, together with a limited availability of mAB for capture, led us to run a curve under very low binding conditions, which resulted in a lower sensitivity. The QCS however, still were within the acceptable ranges and we are quite confident that these reagents are perfectly capable of providing very high quality assays.

## 3. CONCLUSIONS

We have shown that the three different magnetic matrixes can be used for the preparation of sensitive, precise and accurate hTSH IRMAs, perfectly comparable and even superior to analogous systems reported in the literature [9-11]. One of them, the plain magnetite matrix, has been also successfully used for the preparation of a 2nd AB magnetizable solid phase, whose initial bias was eliminated through the addition of an extra saturation step during the coupling procedures. This reagent has wide application possibilities for the RIAs of different analytes.

An extensive study on different types of commercial and in-house monoclonal and polyclonal antibodies has identified several efficient partners, to be used in two-site hTSH IRMA. Particularly interesting are the two partners mABs, distributed within the present CRP.

It is also important to stress the successful utilization of a recombinant hTSH reference preparation, also prepared and distributed thanks to IAEA support.

A complete and detailed study on prices could not be carried out yet, but considering the type of manufacturing laboratories, we are quite confident that they should be highly competitive.

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