MINIMIZATION OF NONSPECIFIC BINDING TO IMPROVE THE SENSITIVITY OF A MAGNETIC IMMUNORADIOMETRIC ASSAY (IRMA) FOR HUMAN THYROTROPIN (hTSH)

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Abstract

An IRMA of hTSH, based on magnetic solid phase separation, was studied especially in terms of its nonspecific bindings (B_0). These were identified as a product of the interaction between radioiodinated anti-hTSH monoclonal antibody (^{125}I-mAB) and the uncoupled magnetizable cellulose particle (matrix). The negative effects of B_0 on the assay performance were minimized and practically eliminated, in the optimized system, with tracer storage at 4°C, repurification and pre-incubation with the same matrix, serum addition during incubation and solid phase saturation with milk proteins. These findings were used in order to reproducibly decrease non specific binding to values $<0.1\%$ (or <70 cpm), thus increasing the signal-to-noise ratio (B_{60}/B_0) into values of 300-500. This way, hTSH IRMAs were obtained with functional sensitivities of about 0.05 mIU/L and analytical sensitivities of the order of 0.02 mIU/L, which represent an approximate 10-fold increase in sensitivity when compared with non-optimized system. A more optimistic sensitivity calculation, based on Rodbard's definition, provided values down to 0.008 mIU/L. Such sensitivities, moreover, were obtained in a very reproducible way and all over the useful tracer life.

1. INTRODUCTION

The determination of hTSH in serum has been considered the single most sensitive and specific index of thyroid status, being an efficient first-line test of thyroid function and especially useful for the screening for neonatal hypothyroidism [1].

One of the factors that strongly influences sensitivity, together with assay design, antibody affinity, incubation time and specific activity of the labels [2], is the amount of nonspecific binding (B_0), which directly determines the signal-to-noise ratio. Hence, we studied the influence of B_0 on a system particularly sensitive to it, a magnetic solid phase hTSH IRMA, in an attempt to reduce this phenomenon and its negative effects and possibly improve the sensitivity of this type of assay.

2. RESULTS AND DISCUSSIONS

In the present study, we utilized a second reference preparation, derived from human pituitaries, that has been prepared and calibrated in our laboratory [3] or the pituitary hTSH standard (IRP-1) kindly provided by the National Hormone and pituitary program/NIDDK (Rockville, USA). Anti-hTSH monoclonal antibody (mAB) for radio-iodination (detecting antibody) (batch code TSH. 0584 0001) was purchased from Serono Diagnostic, Surrey, UK. The ^{125}I labeling of anti-TSH mAB was carried out by the classical Chloramine-T reaction as shown in previous work [1].

Magnetic anti-TSH solid phase was prepared in our laboratory starting from magnetizable cellulose M-174 (Scipac, Sittingbourne, Kent, UK) and from a polyclonal anti-TSH (pAB) preparation (capture antibody) kindly donated by A. Bulatov from the National Research Center for Endocrinology, Moscow, Russia. The magnetizable cellulose particles were covalently linked to the polyclonal antibody via 1-1'-Carbonyldiimidazole(CDI) coupling reaction according to Scipac and NETRIA (North East Thames Region Immunoassay Unit, London, UK) protocols [4].

The NETRIA magnetic solid phase was used in some experiments, especially as a quality reference reagent. The ideal amount of solid phase to be used in the assay was determined by analyzing the range of 0.25-5.0 mg/tube. Percent radioactivity (^{125}I-mAB) bound at zero (B_0) and at 60 mIU/L (B_{60}) hTSH was used to calculate the signal-to-noise ratio (B_{60}/B_0), a quality parameter already usefully applied by other authors [5].

hTSH IRMA was carried out with the simultaneous addition of all reagents. When not specified, the routine procedure was as follows: 200 μL of reference preparation diluted in 0.05M phosphate buffer pH7.4, plus 1% BSA, 1% sodium azide and 0.5% Tween-20; optimal amount (see below) of non cross reacting horse serum (TSH-free serum from Sera-Lab Ltd., Crawley Down, UK); 50 μL of ^{125}I-mAB (~60,000 cpm) and 80 μL (2.0 mg/tube) of magnetizable solid phase-coupled anti-hTSH polyclonal antibody, in a total volume of 0.5 mL completed with the phosphate buffer (0.05M phosphate pH7.4, with 1% sodium azide and 0.5% Triton-X) were added. After overnight incubation at room temperature on a rotatory mixer, 2 mL of wash buffer (0.05M phosphate, pH7.4 with 1% sodium azide and 0.5% Triton X-100) was added. The preparation was allowed to sediment for 10 minutes on the Amerlex-M (N4001) magnetic separator from Amersham (Aylesbury, Buckinghamshire, UK) and washed twice. Tubes were counted for 5 minutes in a "Cobra Auto-Gamma" counter (Packard, Meriden, CT, USA). B_0 and B_{60} were calculated as a percentage of the total counts added to the assays tubes. Table I summarizes the results of a study carried out to determine the origin of nonspecific binding (B_0), in hTSH IRMA.

TABLE I. ORIGIN OF NONSPECIFIC BINDING IN hTSH MAGNETIC IRMA

Incubation conditions	B_0 (%)	B_{60} (%)	B_{60}/B_0
^{125}I-Mab without solid phase	0.04	0.07	1.7
^{125}I-mAB + uncoupled solid phase	1.42	3.42	2.4
^{125}I + AB bound solid phase	0.06	0.05	0.8
^{125}I-BSA + AB bound solid phase	0.03	0.05	1.7
^{125}I-mAB + AB bound solid phase	1.05	31.3	29.8

It is evident that two components are required for B_0 formation, i.e. ^{125}I-mAB (or something derived from it) and the solid phase which, in this specific case, is magnetite (Fe_3O_4) adsorbed to cellulose. The presence of a coupled antibody for capture does not seem to influence B_0. Radioactivity, or a labeled protein per se, does not produce B_0, as confirmed by the practically zero binding of ^{125}I and ^{125}I-mAB BSA. It is interesting to observe the increase in binding when ^{125}I-mAB was incubated with an uncoupled solid phase in the presence of hTSH (B_{60}). This may be due to the formation of a high molecular weight antigen-antibody complex, with some affinity for the magnetizable cellulose. A similar study carried out on finely dispersed plain magnetite or on silanized magnetite particles has clearly shown that Fe_3O_4 itself, more than cellulose, is responsible for high rates of nonspecific binding.

The variation in the specific (B_{60}) and nonspecific (B_0) binding for a period of up to two months was also studied with anti-hTSH ^{125}I-mAB stored under different conditions: frozen (-20°C), lyophilized (4°C) or in liquid form (4°C), as shown in Table II. In the same study, the correlation between B_{60} or B_0 variation and time was determined, confirming that B_{60} decreases with practically the same kinetics (from 0.33 to 0.38 percent/day) presenting high levels of significance ($P<0.01$) under the three different storage conditions, and that B_0 increases with the same speed in either frozen or lyophilized forms (0.04 and 0.05 percent/day, respectively, $P<0.001$). B_0 at 4°C remains around 0.3-0.6%, irrespective of the period of storage. However, the values of B_{60} decrease with time, indicating the change in quality of the labeled antibody with time.

Along with the experiments, performed to investigate the nature and behavior of the component producing nonspecific binding, a series of other experiments were carried out mainly to decrease the presence and influence of this component as much as possible.

Specific and nonspecific bindings which were present before and after tracer repurification on a high resolution superfine Sephadex G-200 column (1.5 × 80 cm; flow rate = 2 mL/h), can be seen in Table III.

TABLE II. CORRELATION BETWEEN B_0 OR B_{60} VARIATION AND TIME, FOR ^{125}I-mAB STORED UNDER DIFFERENT CONDITIONS

Storage time(days)	-20°C		Lyophilized		4°C	
	B_0(%)	B_{60}(%)	B_0(%)	B_{60}(%)	B_0(%)	B_{60}(%)
7	0.95	39.0	0.74	36.4	0.63	36.8
14	1.12	36.2	1.1	33.9	0.33	32.0
21	1.5	33.3	1.5	31.0	0.51	28.7
36	2.2	26.7	2.3	26.1	0.42	23.1
63	13.4	18.8	3.5	18.1	0.33	14.7

Binding type	Storage condition	Slope (% / day)	Correlation Coefficient	Level of significance
B_0	-20°C	0.044	0.9996	$P < 0.001$
	Lyophilized	0.049	0.9987	$P < 0.001$
	4°C	- 0.003	- 0.6110	n.s.
B_{60}	-20°C	- 0.364	- 0.9955	$P < 0.001$
	Lyophilized	- 0.325	- 0.9983	$P < 0.001$
	4°C	- 0.379	- 0.9896	$P < 0.01$

TABLE III. SPECIFIC AND NONSPECIFIC BINDINGS BEFORE AND AFTER TRACER REPURIFICATION ON SEPHADEX G-200

Tracer condition	B_0 (%)	B_{60} (%)	B_{60}/B_0
Right after labeling	0.20	37.4	187
27 days after the labeling, stored at -20°C	1.54	26.7	17
4 days after repurification	0.67	32.4	48
5 days after repurification	0.70	33.3	48
7 days after repurification	0.80	34.4	43

Chromatographic repurification by gel filtration is an established technique for prolonging the life of the tracer but, as we can observe, it is not so efficient in lowering B_0 although there is some increase in B_{60} due to the elimination of ^{125}I formed during storage.

Another treatment applied to ^{125}I-mAB was pre-incubation with the same matrix as that used for the assay (uncoupled magnetizable cellulose). This procedure, defined as "tracer cleaning", was carried out by 4 hours incubation at room temperature on a rotatory mixer using 50 μL of tracer (corresponding to 5-10 $\times$ 10^6 cpm) and 2.5 mg (50 μL) of magnetic matrix in 400 μL of 0.05M phosphate buffer pH7.4. The solid phase was separated using the magnetic batch separator and the tracer was used in the assay, the results being presented in Table IV.

TABLE IV. EFFECT OF TRACER PRE-INCUBATION WITH THE MAGNETIC PARTICLES (TRACER "CLEANING")

Tracer conditions	B_0 (%)	B_{60} (%)	B_{60}/B_0
Stored at -20°C without cleaning	1.2	34.7	29
Stored at -20°C with cleaning	0.2	33.6	168
Stored at 4°C witout cleaning	0.26	34.1	131
Stored at 4°C with cleaning	0.16	33.7	211

The mechanism related to this procedure of tracer cleaning, one of the most efficient treatments for reducing B_0 with no effect on B_{60}, is self evident. The undesirable components are removed just before the actual assay incubation, exploiting the same phenomenon that occurs during the assay.

Incubation with 20% TSH-free horse serum is very efficient in decreasing B_0 but can decrease B_{60} by about 30%, as shown in Table V. For these reasons, subsequent experiments were carried out in order to choose the best serum concentration that could lower B_0 but still maintain approximately the same B_{60} values. This was found to be 5%, a condition that provided ~0.15% B_0 and only ~5% B_{60} decrease.

TABLE V. EFFECT OF TSH-FREE HORSE SERUM ON THE SPECIFIC AND NON SPECIFIC BINDINGS OF ^{125}I-mAB

Tracer condition	B_0(%)	B_{60} (%)	B_{60}/B_0
Stored at -20°C, incubated without serum	1.20	34.2	28
Stored at -20°C, incubated with 20% serum	0.12	24.6	205
Stored at 4°C ,incubated without serum	0.26	34.1	131
Stored at 4°C, incubated with 1% serum	0.20	32.4	162
Stored at 4°C, incubated with 5% serum	0.14	32.4	231
Stored at 4°C, incubated with 10% serum	0.14	30.5	218
Stored at 4°C, incubated with 20% serum	0.12	27.4	228

Serum is an extremely heterogeneous fluid and it presents the positive effect of lowering B_0 but, at the same time, the negative effect of lowering the specific binding. Some serum proteins, probably not the same, act as competitors in both cases, either occupying nonspecific sites or hindering specific sites. This last possibility had been suggested also by Ho et al. [6] In a study of serum effects on antibody immobilized on different polymeric substrates.

Saturation of the magnetic particle with BSA and milk proteins (Molico non-fat dry milk from Nestlé, São Paulo, Brazil) was carried out adding these components (1%) to the bicarbonate buffer used in the washing procedure, after CDI coupling reaction. A strong positive effect can be seen in Table VI and the data shows that the improvement was mainly due to milk proteins.

TABLE VI. EFFECT OF THE SATURATION WITH BSA AND MILK PROTEINS (MP) AFTER THE ANTIBODY COUPLING REACTION

Coupling reaction	Type of saturation	B_0(%)	B_{60}(%)	B_{60}/B_0
A	without BSA or MP	0.78	29.5	38
	BSA + MP	0.17	28.3	166
B	BSA + MP	0.25	38.2	153
	only BSA	0.67	38.5	57
	only MP	0.24	36.4	152

This positive effect of milk proteins was quite interesting and it has been used, without any comparative study with other blocking agents, also by Bodmer et al. [7]. The properties of milk proteins should be tested in many other systems that always rely only upon the classical blocking agents such as serum proteins.

An example of combination of different treatments, leading an almost complete elimination of B_0 is presented in Table VII.

TABLE VII. COMBINATION OF DIFFERENT B_0 LOWERING EFFECTS

Tracer condition	B_0 (%)	B_{60} (%)	B_{60}/B_0
1. Sephadex G-200 repurification	0.80	34.4	43
2. Repurification + cleaning	0.30	35.9	120
3. Repurification + 20%serum	0.03	25.6	853
4. Repurification + cleaning + 20%serum	0.01	27.1	2710

All or some of the described treatments, including storage at 4°C and solid phase saturation with milk proteins, were then routinely applied according to tracer age or to the particular situation, for the purpose of setting up optimized IRMAs in which a controlled nonspecific binding ($B_0 \leq 0.1\%$ or ≤ 70 cpm) could improve the assay sensitivity.

In Fig.1, a typical optimized dose response curve is compared to a traditional curve carried out without any specific treatment. The same figure also shows two inter-assay precision profiles (n = 4 assays, with duplicate run of each point of the standard curve), referring to the two different conditions and used for the calculation of functional sensitivity. Precision profiles were calculated using the radioimmunoassay programme (PC/RIA - TECDOC-509) kindly donated by the International Atomic Energy Agency.

Finally, Table VIII illustrates the influence that different B_0 levels have on functional and analytical sensitivity, the latter being determined by two well accepted methods. It is also important to emphasizes that assays 1-4 were carried out with the same tracer, after different B_0 lowering procedures, at respective ages of 11, 24, 31 and 60 days, providing, essentially, the same degree of sensitivity.

As a final observation, we should emphasize that, thanks to the described treatments and to the rigid control introduced for the acceptable value of B_0 (better defined as counts instead of percent), it has been possible to obtain, probably the most sensitive hTSH magnetic IRMA reported thus far in literature [9, 10]. Moreover, this level of sensitivity is found to be reproducible over several assays and also over the entire useful life of the tracer.

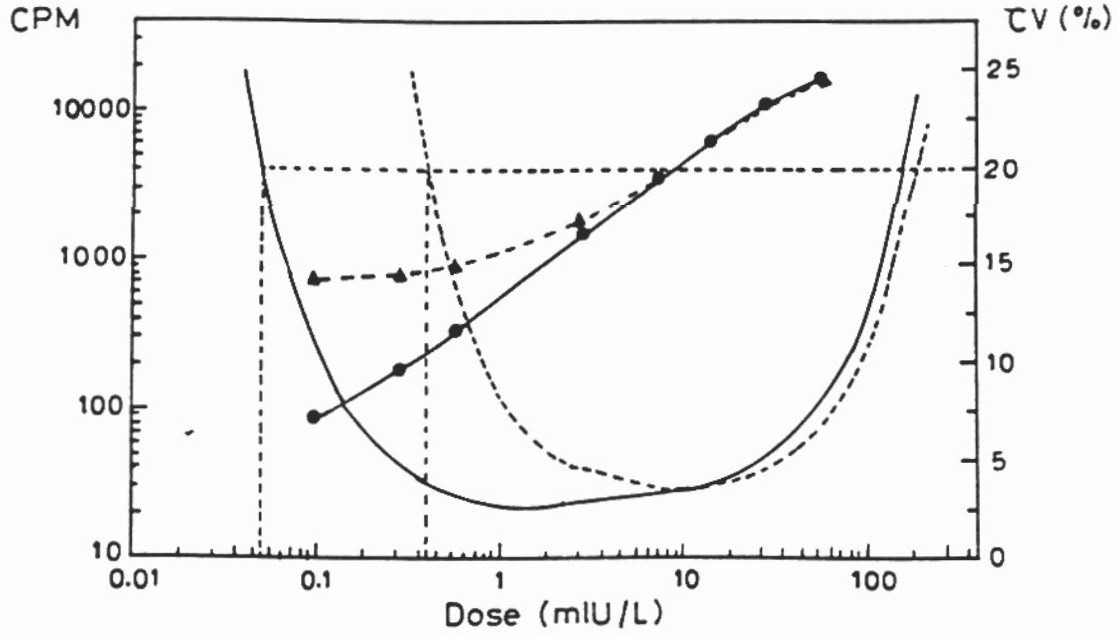

FIG.1. Dose response curves and inter-assay precision profiles of magnetic hTSH IRMA obtained with and without B_0 optimization:
_____ with B_0 optimization, ------ without B_0 optimization.

TABLE VIII. SENSITIVITIES OBTAINED WITH TRACERS PRESENTING DIFFERENT LEVELS OF B_0

Assay number	B_0 (%)	B_0 (cpm)	functional sensitivity # (mIU/L)	Analytical sensitivity (mIU/L)	
				A*	B**
1	0.09	55		0.024	0.008
2	0.10	62		0.018	0.008
3	0.06	39	0.05	0.018	0.009
4	0.09	69		0.030	0.012
5	0.86	714		0.096	O.033
6	1.13	832		0.240	0.079
7	1.11	724	0.48	0.144	0.048
8	1.0	582		0.120	0.034

Determined on the inter-assay precision profile with a CV = 20%.
* Determined using 20 replicates of zero dose + 2SD.
** Rodbard's definition [8].

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