IN-HOUSE REAGENT PREPARATION FOR THE SETTING UP OF A MAGNETIC IMMUNORADIOMETRIC ASSAY (IRMA) FOR hTSH*

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Abstract

IN-HOUSE REAGENT PREPARATION FOR THE SETTING UP OF A MAGNETIC IMMUNORADIOMETRIC ASSAY (IRMA) FOR hTSH.

A local production of reagent for the immunoradiometric assay of human thyrotropin (hTSH) was set up and a comparison with good quality established preparations was carried out. The three basic reagents that are necessary for this type of assay were prepared in the laboratory of the Instituto de Pesquisas Energéticas e Nucleares (IPEN): reference preparation, tracer and solid phase coupled antibody. hTSH reference preparation was obtained via extraction and purification of frozen pituitaries, which were lyophilized, ampouled, sealed under nitrogen and distributed to 20 laboratories of the fourteen ARCAL VIII (Regional Co-operative Arrangements for Promotion of Science and Technology in Latin America) participating countries. An intra-laboratory calibration, against NETRIA (North Thames Region Immunoassay Unit, London, United Kingdom) and NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA) reagents, provided a value of 58.8 ± 6.6 μIU/ampoule for $n = 6$ assays. Anti-hTSH monoclonal anatibody, obtained from NETRIA in a purified form, was radioiodinated and compared, in an inter-laboratory study, with imported tracer: the two preparations showed identical behaviour. Finally, a magnetic solid phase was prepared by coupling, through the metaperiodate reaction, an anti-hTSH polyclonal antibody to a commercial magnetizable cellulose. The product, when compared to the cellulose coupled antibody (non-magnetic), used by NETRIA, presented practically equivalent maximum binding (20–35% of the total added radioactivity) but some higher non-specific binding which affects, to a certain extent, the assay sensitivity and precision. However, unknown sample determination ($n = 18$) carried out with the two different solid phases, presented a highly significant correlation: $r = 0.985$ ($p < 0.001$) and practically no bias (slope = 0.997).

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1. INTRODUCTION

Human thyrotropin, hTSH, is one of the hormones most frequently determined in clinical assay laboratories and is now the most widely accepted analyte being determined in the screening for neonatal hypothyroidism. For these reasons organizations such as the North East Thames Region Immunoassay Unit, London, United Kingdom (NETRIA) [1] and the IAEA organized programme ARCAL VIII (Regional Co-operative Arrangements for Promotion of Science and Technology in Latin America), have been dedicating great efforts to the setting up and improvement of this assay, whose more modern and efficient design is represented by an immunoradiometric assay (IRMA) [2].

As the Brazilian reference laboratory within the ARCAL VIII programme, whose main goal is the setting up in Latin America of an autochthonous production of reagents for the radioimmunoassay of thyroid related hormones, we also dedicated all our efforts to the preparation of a good quality set of hTSH–IRMA reagents. Since the programme has been working basically on NETRIA reagent distribution, these reagents have been our main reference preparations for evaluating the quality of our products. Thanks to the experience we had already acquired in pituitary hormone extraction and purification, we could prepare an ampouled 'reference preparation' (or 'secondary standard') of hTSH for distribution to the other participating laboratories. Considering that radioiodinated monoclonal antibody ('detecting Ab') is probably the most critical reagent especially as regards assay specificity, we decided to use the same NETRIA purified antibody for the preparation of our tracers. Finally, also considering the extremely high cost represented by the solid phase coupled antibody ('capturing Ab'), we decided to develop the reagent in a completely independent way, utilizing a methodology already set up in our laboratory for the preparation of magnetic phase second antibodies.

2. METHODS

2.1. hTSH reference preparation

This secondary standard of hTSH was prepared by extraction and purification of a small number of frozen human pituitaries (38 glands, weighing approximately 18 g), following the method of McLean et al. [3]. The scheme of this purification process using solvent precipitation, gel filtration and ion exchange chromatography is presented in Fig. 1. The immunological activity of hTSH bulk preparation was first determined by radioimmunoassay (RIA) against NIDDK–hTSH-RP-1, kindly donated by the National Hormone and Pituitary Program (Baltimore, MD, USA), while the protein concentration was determined reading the A_{280}, assuming 0.1% $E_{280,1\,cm} = 1$. The presence of contaminant luteinizing hormone (hLH) and follicle

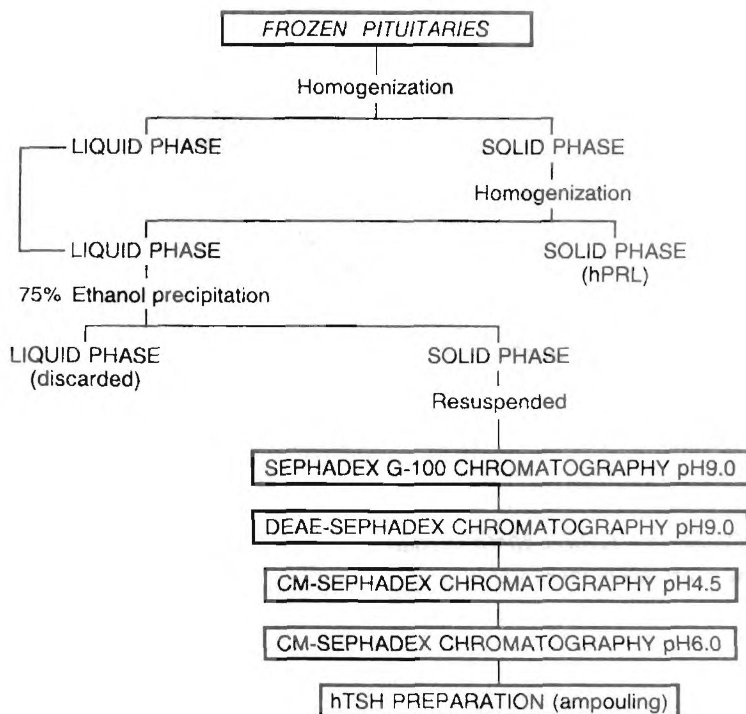

FIG. 1. Schematic presentation of the hTSH extraction and purification method.

stimulating hormone (hFSH) was also determined by sensitive RIA, still utilizing NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA) reagents. Details of the preparation of the ampoules and of the international collaborative study are presented in Paper IAEA-SM-324/62.

2.2. Monoclonal antibody radioiodination

50 μg of a purified monoclonal antibody (MAb) preparation, kindly donated by R. Edwards (NETRIA) were labelled by the classical chloramine-T reaction using 10 μg of chloramine-T and 1 mCi[1] of ^{125}I in a reaction volume of 30 μL, reducing then with 10 μg of sodium metabisulphite. The reaction mixture was purified on a 60 $\times$ 0.9 cm Sephadex G-150 column. The quality of the tracer was tested by seven different laboratories in comparison with NETRIA ^{125}I-MAb. All the assays (using 2 ng of tracers per tube) were run using four different batches of both tracers (A,B,C,D).

[1] 1 Ci = 37 GBq.

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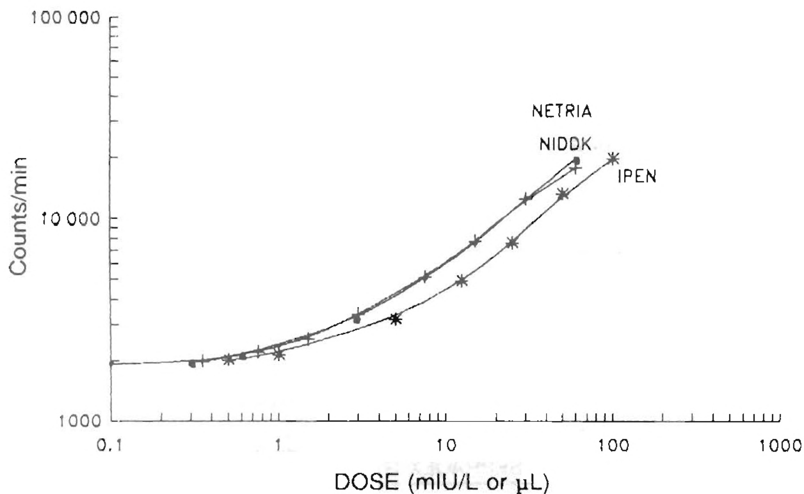

FIG. 2. Comparison between IRMA standard curves of hTSH-NIDDK (+), NETRIA (•) in mIU/L and hTSH-IPEN (*) in µL.

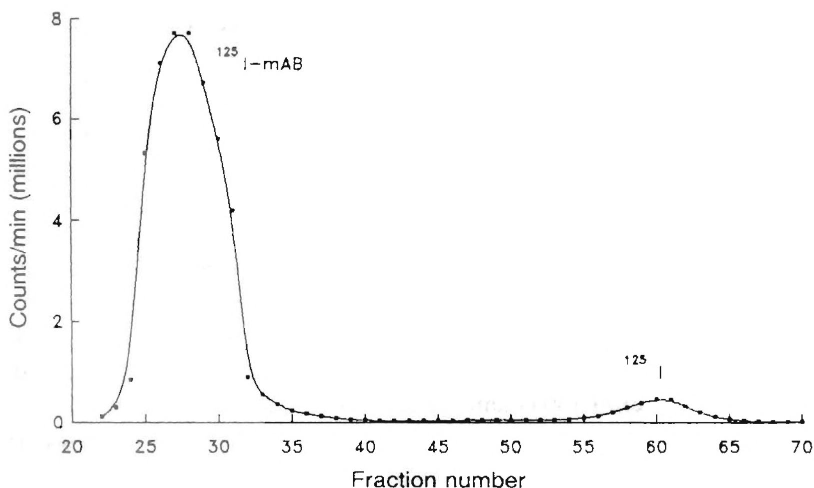

FIG. 3. Typical purification of radioiodinated monoclonal antibody (MAb) on Sephadex G-150; column size 60 × 0.9 cm; flow rate, 3 mL/h.

2.3. Magnetic solid phase

Polyclonal anti-hTSH antibody, kindly donated by G. Vieira (Fleury Laboratory, São Paulo, Brazil) was coupled to magnetizable cellulose M-100 (50% cellulose + 50% Fe_3O_4) and M-104 (75% cellulose + 25% Fe_3O_4) type, purchased from SCIPAC Ltd, Sittingbourne, Kent, United Kingdom. The metaperiodate method was used for coupling reaction, according to the manufacturer protocol, introducing the following main modification: instead of 3 mL/L, 30 mL/L of ethanolamine, pH9.5, were used together with 20% TSH free horse serum in the blocking reaction, after antibody coupling. The ideal solid phase concentration was determined by analysing the range 0.4–2.0 mg/tube while its quality was tested in comparison with NETRIA non-magnetizable cellulose. The per cent of radioactivity (^{125}I-MAb) bound at zero (non-specific binding, B_0) and at 60 mIU/L hTSH (maximum binding, B_{60}) were used to calculate the signal to noise ratio (B_{60}/B_0), a quality parameter already usefully applied by other authors [4].

Incubations were carried out according to NETRIA protocol with simultaneous additions of all the reagents, including the solid phase. After overnight incubation at room temperature on a rotary mixer, NETRIA solid phase was centrifuged for 5 minutes at 1000g, washing once, while IPEN magnetic solid phase was sedimented for 10 minutes on a magnetic separator (IPEN), washing three times.

Automatic data processing was carried out with the PC/RIA program kindly donated by the IAEA.

3. RESULTS AND DISCUSSION

The bulk hTSH preparation presented a specific activity of 1.76 IU/mg, determined by RIA against NIDDK–hTSH-RP-1, 1.3% of contaminant hLH and an undetectable level of hFSH. The ampoule content, determined by IRMA, against NETRIA and NIDDK standards, was 58.8 ± 6.6 μIU/ampoule (inter-assay CV = 11.2%). In Fig. 2 we can see an example of one of these assays and observe the significant paralellism ($p > 0.1$) of the three different curves.

A typical purification of radioiodinated MAb is presented in Fig. 3. Labelling yields in general have been of the order of 85–93% and specific radioactivities 15–20 μCi/μg[1].

In Table I the bindings of our lots of ^{125}I-mAB to NETRIA reagents, at maximum dose (B_{60}), are presented, their values being perfectly comparable to those of the imported tracer. In the same Table we can observe the good correlation coefficient and unbiased results obtained by the seven laboratories taking part in this study.

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[1] 1 Ci = 37 GBq.

TABLE I. INTER-LABORATORY DATA OBTAINED WITH BRAZILIAN AND NETRIA TRACERS IN TSH-IRMA ASSAYS

In-house tracer	B_{60} (%)	Testing laboratory	No. of samples analysed	Slope of regression curve	Correlation coefficient
A	43	1	50	0.820	0.980
B	38	1	117	0.929	0.993
		2	6	0.991	0.999
C	41	3	8	1.030	0.999
		4	4	0.823	0.999
		5	16	1.031	0.988
		6	3	1.151	0.998
D	43	1	3	0.930	0.999
		3	4	1.009	1.000
		4	4	1.034	1.000
		5	8	1.082	0.999
		6	3	0.874	0.999
		7	3	0.333	1.000

TABLE II. DETERMINATION OF THE IDEAL AMOUNT OF IPEN MAGNETIC SOLID PHASE TO BE USED PER ASSAY TUBE

Amount of solid phase (mg/tube)	B_0 (%)	B_{60} (%)	B_{60}/B_0
0.4	0.45	21.6	48.0
0.6	0.53	26.1	49.2
0.8	0.76	28.1	37.0
1.0	0.81	29.5	36.4
1.5	1.2	31.7	26.4
2.0	1.2	31.7	26.4

TABLE III. INTRA-ASSAY COMPARISON BETWEEN MAGNETIC SOLID PHASES PREPARED AT IPEN (BRAZIL) AND NON-MAGNETIC CELLULOSE SOLID PHASES FROM NETRIA (UNITED KINGDOM)

IPEN solid phase						NETRIA solid phase		
Preparation[a]	Final yield (%)	Ideal amount (mg/tube)	B_{60} (%)	B_0 (%)	B_{60}/B_0	B_{60} (%)	B_0 (%)	B_{60}/B_0
1 A (M-100)	62	0.5	27.3	1.3	21	20.9	0.49	43
B (M-100)	71	0.5	19.3	1.3	15			
C (M-100)	64	0.5	19.3	1.8	11			
2 A	60	0.6	21.4	0.95	22	25.9	0.57	45
B	85	1.0	29.6	0.81	36			
C (M-100)	82	0.6	20.1	0.7	29			
3	71	0.8	19.1	1.2	16	26.6	0.57	47
4 A	73	0.8	17.1	0.33	52	38.5	0.18	214
B	79	0.8	22.1	0.42	53			
5	43	1.9	15.1	0.28	54	29.8	0.17	179
6	60	0.6	36.2	0.66	55	36.9	0.40	92
7	54	0.9	29.0	0.63	46	30.0	0.33	91

[a] A, B, C represent different coupling reactions carried out simultaneously and tested the same day. If not otherwise indicated the coupling was done using magnetizable cellulose particle M-104.

In Table II and example of magnetic solid phase, prepared in our laboratory, is analysed especially with regard to the ideal amount to be used per assay tube. We can observe that 0.6 mg/tube was, in this case, the amount that offered the highest signal to noise ratio. Usually, quantities of 0.6–1.0 mg/tube have been used. The results of different coupling reactions are presented in Table III, always using magnetizable cellulose M-100 or M-104. Our preparations were compared each time, in an intra-assay, with NETRIA non-magnetic solid phases routinely used in the same period, considering B_0, B_{60} and B_{60}/B_0. We can observe that, in general, our signal to noise ratio is clearly worse than that presented by NETRIA reagent. This is clearly

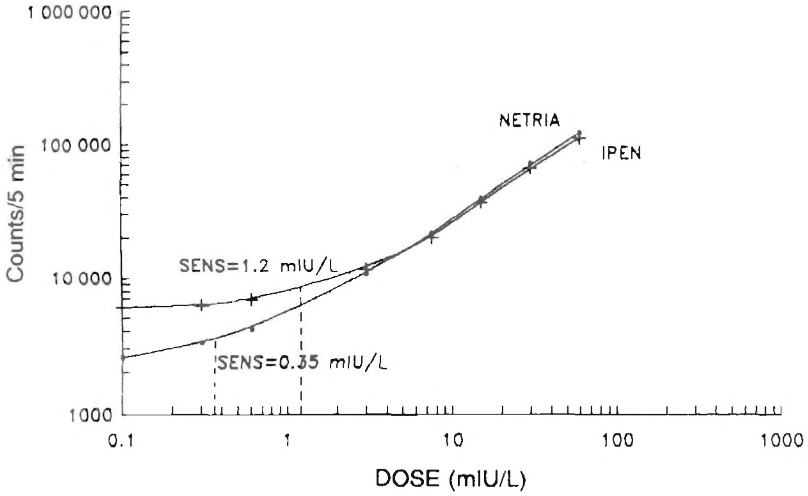

FIG. 4. Comparison between two IRMA standard curves carried out with non-magnetic NETRIA (·) and IPEN magnetic (+) solid phases. The respective sensitivities are indicated.

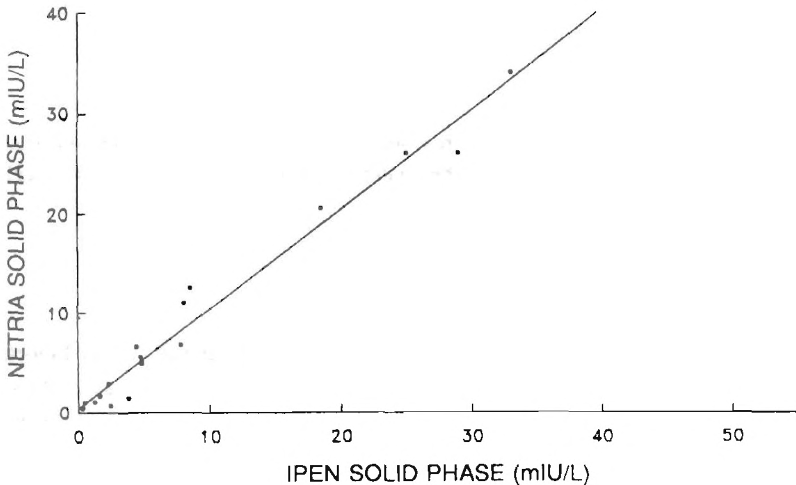

FIG. 5. Correlation curve comparing IPEN and NETRIA solid phases in the IRMA-hTSH determination of 18 unknown serum samples. Six different IPEN solid phases were analysed in six assays, always against the same NETRIA preparation. Correlation coefficient $r = 0.985$ ($p < 0.001$); slope = 0.997.

due to the higher value of B_0 usually presented by our preparations. A certain fluctuation in B_{60}/B_0, affecting both solid phases, was also due to the different age of the tracers. Two IRMA curves, comparing our solid phase with NETRIA's, are presented in Fig. 4, together with the sensitivity calculation [5]. Again we can observe that out lower sensitivity must be related to the higher non-specific binding. An intra-laboratory comparison between the two different solid phases carried out in the determination of $n = 18$ unknown sera (Fig. 5) presented a quite satisfactory correlation coefficient, and practically no bias.

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