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LOCAL DEVELOPMENT OF TSH RADIO IMMUNOASSAY AND IT'S EVALUATION

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Pakistan is an iodine deficient country, Thyroid diseases are very common and their investigation requires heavy foreign exchange to import relevant commercial kits. AEMC, Multan therefore tried to develop adequate and economical protocol for TSH radioimmunoassay. TSH-RIA protocol supplied by the commercial source did not provide the desired quality and evaluation of thyroid diseases. Our method was therefore optimized using precision profile as the criteria of performance. A total of six assay systems were studied, out of which the most precise one was selected. The new system gives results within expected limits. The sensitivity of the system, 0.3 µIU/ml is suitable for low dose measurements. The working range, 0.6-25.1 µlU/ml covers hypo, hyper and euthyroid levels of the hormone. Comparison with other laboratories shows an agreement of values. A low cost, locally developed good quality TSH RIA is therefore in our hand.

Key words: Development, TSH, Evaluation, Thyroid, Thyroid function.

Introduction

Radioimmunoassay is the method of choice for the estimation of trace substances like TSH (Thyroid Stimulating Hormone) in the blood. The method is commonly used in Pakistan. This usually involves heavy cost of foreign exchange. Transportation and storage conditions adversely affect shelf life of commercial kits. Trials were therefore conducted to develop these kits locally [1-3]. Recently we have tried to develop an indigenous technique for TSH assay using raw chemicals purchased from commercial sources. The methodology adopted involves double antibody precipitation, this is more efficient and economical than many other separation techniques [4]. Although methods alternative to RIA are also in use [5], they are not economical under our technical environment. For example immunoradiometeric assay (based on excess antibody principle) will require larger amounts of expensive purified antibodies, ultimately increasing the cost.Methods employing non-isotopic tracer are also very expensive and require retraining of personnel and purchase of new equipment and are therefore usually not feasible. Even in some western countries. RIA is still preferred over other methods [6]. This paper describes a strategy to set up a good quality RIA technique to measure TSH under local conditions.

Materials and Methods

Essential chemicals. (1) Radio-isotope 1125 as NaI, specific activity=16.1mCi/µg, Amersham, U.K. (2) Chloramine-T, BDH (Oxidant). (3) Sodium metabisulphite, BDH (reducing agent). (4) Pure TSH, SIGMA, activity=7Iµ/mg, measured by RIA. (5) Quality control sera (low, medium, high) from Amersham for quality control of locally made reagents. (6) External Quality Assurance Samples (EQAS) for between laboratory comparison received from IAEA.

Immunochemicals. (1) Rabbit anti-TSH serum (first antibody), SIGMA, working dilution recommended by the supplier 1:1000.

The specific antibody had a cross reactivity with LH and FSH of less than 0.001%. The affinity constant (K_a) calculated from Scatchard by the manufacturer was 1 x 10^{10} litres per mole. The assay sensitivity as reported by the manufacturers was 0.2 µU per tube (1 µlU/ml).

(2) Second antibody or anti-rabbit Ig G, SIGMA, working dilution = 1:5.

Reagent prepared locally. (1) Phosphate Buffer, pH7.4, 0.05M, 1%BSA. (2) Calibration standards: These were prepared by diluting SIGMA TSH with phosphate buffer. Following concentrations were prepared to construct dose response curve, 0.5, 1.5, 3.12, 6.25, 12.5, 25, 50, 100 µlU/ml. The expected normal range was 1-6 µlU/ml [7]. (3) Precipitating solution, 6% Polyethylene glycol, (MW=6000) phosphate buffer.

Optimization of dilution of antibody-1. The recommended working dilution of the first antibody was 1:1000. However experience showed that this dilution does not give good sensitivity and the error is high at low concentrations. It was therefore decided to find the optimum dilution by constructing a dilution curve. For this purpose different dilutions of the first antibody (1:250, 1:500, 1:1000, 1:2000, 1:4000 etc.) were processed in two sets of tubes (in duplicate), the first without unlabelled TSH, and the other containing 100 µl of TSH at 1.0 µlU/ml concentration. Briefly the following quantities

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were mixed and incubated as indicated below:

	Set-1(Bo)	Set-2(Bx)
Phosphate buffer	100 µl	—
TSH Solution (1µlU/ml)	—	100
Antibody-1 dilution	100 µl	100

These were vortexed and incubated for 18-20 hrs at room temperature.

I125-TSH	100 µl	100

Vortexed and incubated for 18-20 hrs at room temperature.

Ab-2 (dilution, 1:5)	100µl	100
6%PEG	500 µl	500

The mixtures were again vortexed and allowed to stand for 10 mins, then they were centrifuged, decanted and counted for 1 min.

The count rate (after correction for non-specific binding) of the tubes was plotted against the antibody dilution. Two curves were obtained, one for binding in the absence of hormone and the other for the binding in the presence of fixed quantity of unlabelled TSH (Fig.1). The dilution at which there was maximum displacement of the titration curve in the presence of fixed amount of TSH (Bx), from the titration curve in the absence of unlabelled TSH (Bo) was selected as the optimum dilution of the antibody-1 i.e. 1:4000. This dilution was expected to give highest sensitivity in the assay measurements [8].

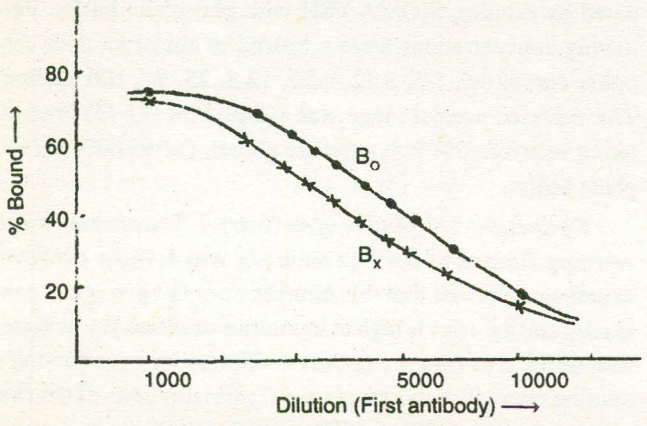

Fig. 1. Dilution Curves. Percentage binding against antibody dilution. (Bo) Binding of the tracer with antibody in the absence of unlabelled TSH. (Bx), Binding of the tracer with the antibody in the presence of unlabelled TSH at concentration 1 µlU/ml.

Preparation of radioactive tracer. Radioiodination of TSH was performed after some modifications in Chloramine-T Method described by Hunter and Greenwood [9]. Buffered solutions of 1 µg TSH/10 µl, 0.5mCi I125/5µl and 5 µg Chloramine-T/5 µl were mixed in a microtitre tube for 30 secs. The reaction was then stopped by adding 5µl of Sodium metabisulfite (5µg), followed by 100 µl KI (100µg). The mixture was then applied onto 1X30 cm Sephadex-G50 column and eluted with phosphate buffer. The radiochromatogram obtained is shown in Fig.2. 40% (SD=5) of added radioactivity was incorporated into the hormone giving a specific activity of approximately 200 µCi/µg. The radiochemical purity checked by paper chromatography using acetone/methanol solvent (1:1, v/v; ref. batch analysis sheet for I-125, code=IMS 30) was >90% (Fig.3). Approximately 20000 CPM(~0.01 µCi) was added per tube for subsequent assays. Stability of the tracer was checked by estimating the binding of tracer with antibody in the absence of TSH (Bo); non-specific binding (%NSB), radiochemical purity (percentage of activity tagged to TSH) on different days after the preparation of TSH-1125 was also checked. Plot of these parameters with respect to time is shown in Fig.4. The time at which the binding is reduced by 50% is more than 5 weeks.

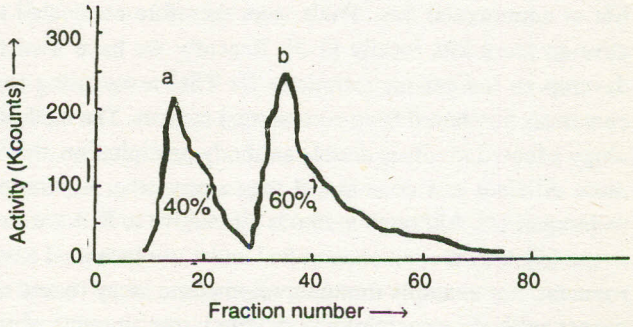

Fig. 2. Radiochromatogram obtained after gel filtration. (a) 1125-TSH peak. (b) Free iodide.

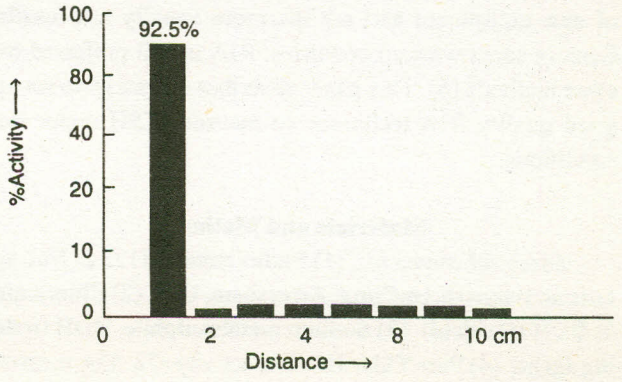

Fig. 3. Radiochromatogram obtained after paper chromatography. More than 90% of activity is tagged to TSH.

Selection of most precise assay protocol. The assay procedure supplied by SIGMA in the literature on immunochemicals [10] involves long incubation steps (total incubation period=4 days). The recommended antibody dilution 1:1000 was also investigated. Statistical analysis of assay data obtained from this procedure revealed poor performance (standard curve Fig. 5 and imprecision profile in Fig.6 for assay protocol-1. The apparent hook effect in protocol-1 (see standard curve) shows that the dilution recommended by the supplier was not appropriate (the antibody is in excess). A modified procedure was therefore desired) to eliminate the long incubation steps and facilitate the early release of results ii) to improve the performance. The new system was also to be evaluated in terms of precision, sensitivity and repro-ducibility.

Six assay protocols were tried. The detail of these protocols is summarized in Table.1. Precision profiles were constructed for each assay protocol to compare the systems [11] and select the assay system giving the most precise results.

Quality control and quality assurance. After selecting the most precise assay protocol, 10 assay batches were performed with 3 internal quality control sera (low, medium, high) to find within batch and between batch variation of the values [12-14] and compare them with values reported by Amersham. External quality assurance samples (EQAS) received from IAEA were also analyzed to find agreement of our values with other laboratories. Three sample distributions were assayed by 8 different laboratories of the country using the strategy described by Bacon *et al.* [15].

Results and Discussion

Standard curves and related data. The standard curves obtained with different assay protocols are shown in Fig.5. The precision profiles relating %CV with the concentration are displayed in Fig.6. The working ranges derived from these profiles are given in Table 2. The observed sensitivities (corresponding to 2SD of zero standard) are given in Table 3. External quality assurance results for comparison with other laboratories are shown in Table 5. In order to find normal TSH levels, 348 blood samples (males: 120, females:228, aged between 22-41 years) of euthyroid university teachers and students were analyzed. The observed normal range was 0.8-6.3 µlU/ml (Mean=2.84, SD=1.15).

TSH is glycoprotein with a molecular weight of, approximately, 28000 daltons and is secreted by anterior pituitary to regulate the secretion of T3 and T4. Estimation of TSH in serum is important to differentiate between primary and secondary hypothyroidism.Level are suppressed in thyrotoxicosis. In blood, TSH concentration is in the range of picograms per ml. Only sensitive technique like RIA can measure

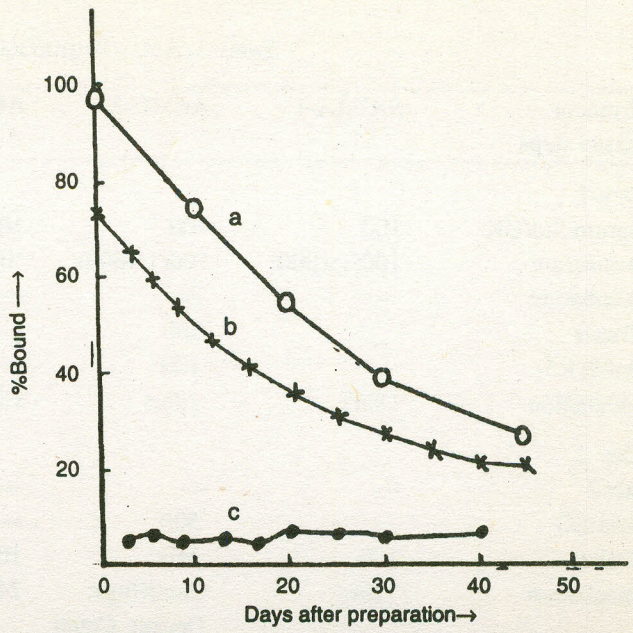

Fig. 4. Bound fractions of the tracer as a function of time after preparation. (a) Percent activity tagged to TSH. (b) %Bo or binding for zero concentration of TSH. (c) %NSB or non specific binding.

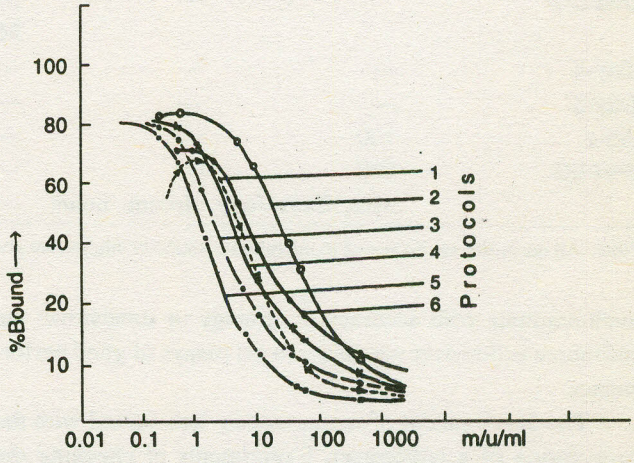

Fig. 5. Standard curves obtained with different assay protocols.

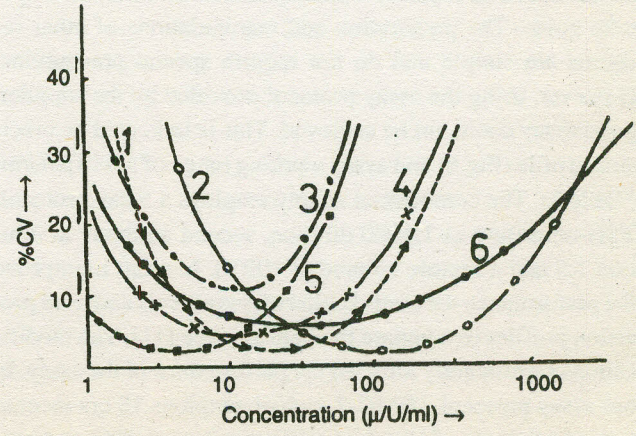

Fig. 6. Precision profiles (%CV versus TSH concentration).

TABLE 1. ASSAY PROTOCOLS EVALUATED AT OUR LABORATORY.

Protocol Assay steps	SIGMA-1	AEMC-2	AEMC-3	AEMC-4	AEMC-5	AEMC-6
Day-1						
Serum Std/UK	100	100	100	100	200	200
Antiserum	100(1:1000)	100(1:1000)	100(1:1000)	100(1:4000)	100(1:4000)	100(1:4000)
Incubation	—	—	—	—	—	4hrs
Tracer	—	100	—	—	—	100
Ab-2(1:5)	—	100	—	—	—	—
Incubation	18hrs	18hrs	18hrs	18hrs	18hrs	18hrs
Day-2						
Ab-2	—	—	—	—	—	100
6%PEG	—	500	—	—	—	500
Tracer	100	Mix,	100	100	100	Mix,
Incubation	72hrs	Centrifuge,	24hrs	24hrs	24hrs	centrifuge,
		Decant, Count				Decant Count
Day-3						
Ab-2	"	—	100	100	100	—
6%PEG	"	—	500	500	500	—
			Mix, Centrifuge, decant, count,			
Day-4	—	—	—	—	—	—
Day-5	—	—	—	—	—	—
Ab-2	100	—	—	—	—	—
6%PEG	500	—	—	—	—	—
	Mix, Centrifuge, decant, count,		—	—	—	—

Note: All quantities are expressed in microlitres. Antibody dilution are given in brackets.

such amounts with accuracy. A strategy to standardize the technique is however necessary to get assays of good performance.

The development of immunoassay was started with the preparation of a radiotracer. Experiments of checking the purity, immunoreactivity and stability of tracer indicate that the product is acceptable in quality with a minimum non-specific noise. The preparation and manipulations of other reagents are simple and do not require special precautions. However, using the assay protocol provided by the supplier, good assay could not be achieved. This is indicated by precision profile (Fig.6) and assay working range of 5-118 µIU/ml (Table 2). The commercial supply employs a 5 day protocol, TSH-antiserum at 1:1000 dilution, second antibody at dilution 1:5 and a sample volume of 100 µl. In order to improve the performance we tried six alternate protocols and used precision profiles to compare the performance [11]. The modifications significantly affected the performance of the assay. In our assay protocol AEMC-2, (which employs 18 hrs incubation of mixtures of standard and patient sera), first and second antiserum and radiotracer followed by addition of PEG for precipitation, a working range of 17.8-1000 indicates that the system is not applicable to thyroid disease as the lower limit crosses the upper limit of the normal range. AEMC-3, differs from SIGMA protocol in the length of second incubation i.e., 24 hrs instead of 72 hrs. Reduced second incubation raises the overall error to a very high extent i.e., more than 10% error is seen at all concentration levels. The system is therefore not applicable. AEMC-4 is similar to AEMC-3 with the difference that here we used first antibody at 1:4000 dilution. This significantly reduced the error in the performance without increasing the incubation period. A working range of 2.5-79 a normal range of 0.8-6.3 µIU/ml shows that the system works well in normal and hypothyroid region. In AEMC-5, we have used increased sample volume i.e., 200 µl instead of 100 µl, the other parameters are the same as in AEMC-4. The error is further lowered at low concentration and a working range of 0.6-25.1 is achieved. The test will complete in 48 hrs. We have also tried to reduce the first incubation but results were not favourable. Hence in AEMC-6, the performance is poor and we get a working range of 5.6-300 µIU/ml. All this suggests that AEMC-5 is giving the best perfor-

mance. The sensitivity of the procedure, 0.3 µlU/ml is also the highest. The observed normal range for this protocol is 0.8-6.3 µlU/ml. The assay working range of 0.6-25.1 µlU/ml suggests that the system is applicable to hypo, hyper and euthyroid patients.

TABLE 2. ASSAY WORKING RANGES DEFINED BY PRECISION PROFILES.

Protocol type	Assays working ranges at 10% error limit
1	5—118
2	17.8—1000
3	More than 10% error at all levels
4	2.5— 79
5	0.6— 25.1
6	5.6— 300

TABLE 3. SENSITIVITIES OF DIFFERENT ASSAY SYSTEMS.

Protocol type	Observed sensitivity at 2SD of 0 standard
1	2.0 µlU/ml
2	4.0 µlU/ml
3	7.0 µlU/ml
4	1.4 µlU/ml
5	0.3 µlU/ml
6	6.5 µlU/ml

TABLE 4. WITHIN AND BETWEEN BATCH QUALITY CONTROL RESULTS (DATA POOLED ON 27 ASSAY BATCHES).

QC Pool	Observed values	Expected values	Within batch %CV	Between batch %CV
High	14.5	13.1-28.9	6.3	11.4
Medium	9.6	8.6-15.8	3.6	9.1
Low	2.9	1.8-3.6	4.8	12.3

TABLE 5. BETWEEN LABORATORY DATA (EXTERNAL QUALITY ASSURANCE SAMPLES ANALYZED BY 8 LABS; DATA ON 3 SAMPLE DISTRIBUTIONS).

Batch	Observed values		ALTM		Lab bias	
	Sample-1	Sample-2	Sample-1	Sample-2	Sample-1	Sample-2
1	2.1	16.3	2.5	15.3	-16.3	6.5
2	2.6	3.3	2.9	4.0	-09.6	-18.8
3	2.6	9.3	2.8	12.5	-7.0	-12.5
				Mean bias	-11	-8.3
				Overall bias=		-9.7

* All Laboratories trim mean.

The results of internal quality control sera (Table 4) obtained with AEMC-5 suggest that the values are within expected limits (reported by Amersham) [16]. The observed between batch variation is better than commercial kits [14]. We hope this will be further controlled when reagents prepared in bulk will be in routine use under similar operating conditions.

Comparison of results obtained with external quality control samples (EQAS) with other laboratories (Table 5) shows that our results are in agreement with other Laboratories. The overall negative bias will be controlled when more reproducible experimental conditions are maintained by the use of reagents prepared in bulk and increase in skill of staff. Further a more reliable estimate of bias will be available when the number of participating laboratories in EQAS scheme is increased. There are only 8 laboratories at present which are using different methods to estimate EQAS samples i.e. commercial RIA kits, commercial IRMA kits, enzyme immunoassays etc.

To summarize, the strategy we adopted to establish TSH methodology is successful and a good inexpensive working TSH assay is in our hand to investigate the patients of our area, in which thyroid disease is endemic. The technique has been established without any external assistance, additional staff and within the centre's strained budgetary provisions. The cost of the test will be very low compared to that using commercial kits i.e., in the range of 20-30 rupees per patient results. A commercial RIA costs as much as 150 rupees per patient result excluding staffing and other charges.

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