Pak. j. life soc. sci. (2005), 3(1-2): 45-49

Pakistan Journal of
Life and Social Sciences

Temperature and Time Dependent Studies on TSH Tracer Immunoreactivity

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Abstract

The study was designed to evaluate the storage effect on stability of ready-to-use form of TSH tracer (^{125}I labeled mAb). TSH concentration was measured by immunoradiometric assay (IRMA) using fresh tracer stored at –20°C until just before use at room temperature and stored tracer which was kept at different storage conditions i.e. laboratory temperature (25-34 °C) and 37°C initially for five days and then stored at 4°C for a period of 60 days. The two tracers were used for the estimation of TSH concentration of euthyroids (0.35-6 mIU/L), and in patients with hyperthyroidism (TSH <0.35mIU/L) and hypothyroidism (TSH>6 mIU/L). It was concluded that when tracer was stored initially at laboratory conditions (25-34 °C) or at 37°C for five days and then at 4°C up to 60 days, its immunological and antigenic binding properties with anti-TSH were not affected.

Key words: TSH tracer, hyperthyroidism, hypothyroidism, ^{125}I labeled mAb, IRMA

Introduction

The horseshoe shaped thyroid gland produces thyroid hormones. These are peptides containing iodine. The two most important hormones are tetraiodothyronine (thyroxine or T4) and triiodothyronine (T3). These hormones are essential for life and have many effects on body metabolism, growth, and development. The thyroid gland is influenced by hprmones TSH (produced by pituitary gland and TRH produced by hypothalamus. The three glands and the hormones they produce make up the "Hypothalamic - Pituitary - Thyroid axis" (Blumhart and Williams, 1996).

The thyroid stimulating hormone (thyrotropin or TSH) is a glycoprotein with a molecular weight of 28,000 secreted by the adenohypophysis. Like other glycoprotein hormones (FSH, LH and HCG), TSH contains two different subunits, an alpha- and a ß-chain, linked by noncovalent bounds. The primary structure of alpha subunits of TSH and of the gonadotropins is the same, whilst their ß subunits are different. The ß subunits are responsible for the immunological and biological specificity of these hormones.

The determination of TSH by immunoassay methods plays a crucial role in the diagnosis of thyroid disorders and in the evaluation of the functional integrity of the hypothalamic-pituitary axis.

The outstanding sensitivity of the present hTSH IRMA system makes it particularly suitable for the measurement of subnormal hTSH levels, a key to both the diagnosis and treatment follow up of hyperthyroid patients. Immunoradiometric technique has been developed using multiple antibodies to produce a "sandwich" type assay in which one antibody (usually directed against the α subunit) serves to anchor the TSH molecule and another (usually monoclonal antibodies directed against the ß subunit) is radioiodinated (Nicoloff and Spencer, 1990; Hay et al., 1991). In this assay, the signal is directly related to the amount of the ligand present (Kricka, 1991). This results in decreased background "noise" and a greater sensitivity, decreased interference from related compounds as well as an expanded useful range (Nicoloff and Spencer 1990; Hay et al., 1991; Spencer et al., 1993). The significant feature of IRMA is the use of ^{125}I-labeled TSH tracer.

^{125}I is chemically very reactive and can be incorporated into several molecules containing tyrosine (Edwards, 1985). -radiations emitted by ^{125}I have high penetrating power. The specific activity of -emitting ^{125}I is much greater than those of -emitting, so that counting times are reduced and no sample preparation is required for the purpose of counting the radioactivity counts as in liquid scintillation counting techniques, giving a considerable savings in time and materials (Burdon and Kinppenberg, 1987). Hence, crystal scintillation, 60 days half-life which is convenient for storage and its low photon energy which requires less shielding are the most important aspects of ^{125}-I which is used in labeling of tracer in IRMA kits. Severe and variable colour quenching in hemolyzed and hyperbilirubinemic samples does not arise with ^{125}I (-emitters) (Hunter et al., 1975).

^{125}I has a short half-life of 60 days, after a certain time span the radioactive disintegration rate falls to such a level that the count rate is too low for practical counting time. ^{125}I molecules in case of ready-to-use form of tracer undergo radioactive decay with the emission of -rays. These rays might disrupt the proteinaceous structure.

TSH tracer, during transportation may be subjected to different temperature conditions. As the tracer is labeled antibody, hence, hydrogen bonding and Vander Wall's forces involved in the maintenance of a protein structure may be affected because most of the proteins are fragile and heat labile.

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The above-mentioned conditions of storage period and temperature would affect the native structure of proteinaeous TSH tracer (labeled antibody). Therefore, the lock and key relationship of antigen and antibody becomes affected and pH variations may be caused in ready-to-use form of tracer because of any microbial growth. These conditions may change the immunological properties of ready-to-use form of tracer and unreliable results produced by immunoradiometric assay could have serious consequences regarding the diagnostic aspects and health of a patient. Therefore, it is required to evaluate that the ready-to-use form of TSH tracer is in its intact form and its immunological, biochemical and antigen-antibody binding properties are not changed during storage period and at different temperatures encountered in transportation.

Materials and Methods

The present study of ready to use form of tracer (TSH ^{125}I-labeled Ab) storage was based on the specific conditions of temperature and time period with respect to radioactivity loss. The storage time was selected as 8 weeks or two months because the half-life of ^{125}I-radioisotope is 60 days. To choose the most suitable storage temperature (especially for transport) one-day study was made as a result of which 25-34 °C and 37°C temperatures were selected. The tracer was stored at these temperatures for first five days (the maximum time for transport) and then at 4°C upto 60 days. The tracer stability and sensitivity was checked by IRMA technique at days 1, 3, 5, 10, 20, 30, 40 and 60. The obtained results were compared with the fresh tracer assay performed on the same day.

Sample Collection:

Serum of TSH related patients was collected from INMOL Routine Laboratory according to following TSH concentrations:

i Patients with low TSH concentration: TSH < 0.35 mIU/L

ii Patients with normal TSH concentration: TSH 0.35-6 mIU/L

iii Patients with high TSH concentration: TSH > 6 mIU/L

The blood samples after incubation at room temperature were centrifuged at 5000 rpm for 5 minutes. Then serum was separated out and was stored at –20°C in four portions in eppendorf tubes to avoid repeated freezing and thawing for further use.

Tracer Reconstitution:

^{125}I -labeled tracer aliquot prepared by chloramine-T method stored at –20°C or 4°C was reconstituted with 100 ml of freshly prepared assay buffer to give 1,00,000 counts/50Ul/ 60sec., in case of both the temperatures of 25°C-34°C and 37°C selected for the study. After five days at the selected temperatures, it was stored at 4°C for two months. Total activity counts of fresh tracer in case of tracer stored at 25°C-34°C were selected as 60,000 – 70,000 cpm (usually used for IRMA technique) and for tracer stored at 37°C were selected as 100,000 for comparative study.

^{125}I-Anti-TSH:

For ^{125}I-Anti-TSH, ^{125}I–labeled TSH tracer aliquot prepared by chloramine-T method stored at –20°C or 4°C was reconstituted with 5 ml assay buffer to give 60,000-70,000 counts/50ul/60sec and 1,00,000 counts/50ul/60sec for 25°C-34°C and 37°C temperatures study of stored tracer, respectively, in case of preparation of fresh assay tracer.

Assay:

For assay, 100 l sample/standards/quality control, 300 l assay buffer (10 ml 0.5 M phosphate buffer (pH 7.4), 1 g Bovine serum albumin, 0.1 g Sodium azide, 0.5 ml 10% Tween 20 in 100 mL distilled water), 50 l labeled antibody and 50 l solid phase first antibody were vortexed and placed on rotary mixer overnight. Two ml wash buffer (1 ml 10% Triton X 100, 1 g Sodium azide in distilled water) was added and centrifuged for 5 minutes at 1000xg. The washing step was repeated and the supernatants were decanted. The tubes were counted for 120 seconds on -counter. Counts from the detector were analyzed using immunoassay data-analysis programmes (e.g. IAEA software) (IAEA TECDOC-346, 1985).

Results and Discussion

IRMA is more precise and sensitive technique and is based on the use of antigen-antibody reaction in presence of a trace amount of radioactive structure. The immunoradiometric assay is dependent upon time and temperature. The structurally intact molecules of ^{125}I labeled antibody are essential requirements to achieve a stable equilibrium in antigen antibody association (Burdon and Knippenberg, 1987). Any structural change in the binding site on antigen or antibody molecule will affect this equilibrium. Storage of antibody macromolecules at low temperatures often enhances their immunological functional properties by reducing the proteolytic enzymes if present in a medium and by stabilizing hydrogen bonding and Vander Wall's forces involved in the maintenance of a protein structure.

In present study, to determine the effect of selected conditions i.e., temperature and time period on tracer immunoractivity euthyroid samples were used. Table 1 represents TSH concentration of euthyroid samples by IRMA using stored tracer at 25-34°C and at 37°C for initial five days. Day 1 results were considered as reference and fresh tracer assay performed on the same day was taken as control. It was observed that all values of stored and fresh tracers were fall in the provided range of 0.35-6 mIU/L. The data were further analyzed by statistical test of difference between two means and non-significant results were obtained (Swinscow, 1985). Table 2 expresses the behaviour of stored tracer at 4°C from day 10 to 60 that was previously stored at 25-34°C and at 37°C. It was observed that all TSH concentration values fall in normal range of 0.35-6.0 mIU/L and

statistical evaluation has suggested that the trend of stored and fresh tracer was same. Hence, the non-significant difference represents tracer stability in ready-to-use form for two months.

Results of euthyroid samples in Tables 1 and 2 are helpful to provide information about tracer stability during selected storage conditions. It was found that out of 15 samples only in a few cases TSH concentration was higher than the normal range 0.35–6.0 mIU/L (individual data not shown). In spite of these observations, insignificant difference was observed between the stored tracer and fresh tracer in case of both the temperature studies.

Table 1: TSH concentration of euthyorid samples measured by IRMA using stored tracer kept at laboratory conditions (25°C–34°C) or at 37°C for five days in comparison with control assay using fresh tracer stored at –20°C until just before use at room temperature.

Storage Conditions	TSH concentration (mIU/L)			
	Day 3		Day 5	
	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer
25-34°C for 5 days (mean values)	2.004	1.538	2.475	2.298
SE diff.	0.403		0.477	
Probability	P > 0.10		P > 0.5	
37°C for 5 days (mean values)	2.407	2.016	2.344	1.836
SE diff.	0.385		0.354	
Probability	P > 0.10		P > 0.10	

P>0.1= non-significant

Table 2: TSH concentration of euthyroid samples measured by IRMA using stored tracer kept at 4°C for 60 days. The tracer was stored at laboratory conditions (25°C– 34°C) or at 37°C for five days before moving to 4°C. The table also gives results of control assay using fresh tracer stored at –20°C until just before use at room temperature.

Storage Conditions before moving to 4°C	TSH concentration (mIU/L)									
	Day 10		Day 20		Day 30		Day 40		Day 60	
	Fresh tracer	Stored Tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer
25-34°C for 5 days (mean values)	2.464	2.106	2.964	2.651	3.044	2.695	3.209	3.202	2.860	3.093
SE diff.	0.489		0.609		0.642		0.561		0.613	
Probability	0.5 > P > 0.317		P > 0.5		P > 0.5		P > > 0.5		P > 0.5	
37°C for 5 days (mean values)	2.724	2.746	2.878	2.186	2.483	3.088	2.064	2.427	2.263	2.473
SE diff.	0.474		0.424		0.455		0.385		0.402	
Probability	P > > 0.5		0.317 P > 0.10		0.317 P > 0.10		0.5 > P > 0.317		P > 0.5	

P>0.5= non-significant

Table 3: High TSH concentration of patient samples measured by IRMA using stored tracer kept at laboratory conditions (25°C–34°C) or at 37°C for five days in comparison with control assay using fresh tracer stored at –20°C until just before use at room temperature.

Storage Conditions	TSH concentration (mIU/L)			
	Day 3		Day 5	
	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer
25-34°C for 5 days (mean values)	23.519	12.973	19.922	18.579
SE diff.	7.420		6.658	
Probability	P > 0.10		P > 0.5	
37°C for 5 days (mean values)	23.736	20.875	22.599	15.569
SE diff.	9.057		7.784	
Probability	P > 0.5		P > 0.317	

P>0.1= non-significant

Table 4: High TSH concentration of patient samples measured by IRMA using stored tracer kept at 4°C for 60 days. The tracer was stored at laboratory conditions (25°C–34°C) or at 37°C for five days before moving to 4°C. The table also gives results of control assay using fresh tracer stored at –20°C until just before use at room temperature.

Storage Conditions before moving to 4°C	TSH concentration (mIU/L)									
	Day 10		Day 20		Day 30		Day 40		Day 60	
	Fresh tracer	Stored Tracer	Fresh Tracer	Stored tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer
25-34°C for 5 days (mean values)	21.090	21.259	19.956	18.317	18.117	16.121	20.095	18.328	21.724	24.422
SE diff.	8.165		6.756		5.507		4.544		9.631	
Probability	P > 0.5		P > 0.5		P > 0.5		P > 0.5		P > 0.5	
37°C for 5 days (mean values)	18.021	19.038	16.642	15.601	19.641	27.067	11.873	13.325	18.728	23.477
SE diff.	6.349		5.701		6.407		3.133		6.367	
Probability	P > 0.5		P > 0.5		0.317 > P > 0.10		P >> 0.5		P > 0.5	

P>0.5= non-significant

For further confirmation of assay reliability and tracer stability, patient samples with high TSH concentration were used. The results given in Tables 3 and 4 show insignificant probability values in case of study of both the temperature conditions i.e. laboratory temperature (25°C–34°C) and 37°C temperature for high TSH concentration (TSH > 6 mIU/L). By considering Table 3 at day 3 in case of stored tracer, it was found that although values were according to the provided range but are lower than the control assay results (using fresh tracer). This is due to experimental error or due to other reasons involved in batch variation such as incubation time etc.

To analyze the tracer immuoracitivty further study was made on patient samples with low TSH concentration. Results of study of low TSH concentration (TSH < 0.35 mIU/L) for both the temperature conditions are shown in Tables 5 and 6. Table 5 represents that when tracer was stored at laboratory conditions for 5 days there is a significant difference at days 3 and 5 between the behaviour of stored and fresh tracers in case of 25-34°C-temperature condition. Similarly, significant difference was observed in case of day 10 results (Table 6).

Table 5: Low TSH concentration of patient samples measured by IRMA using stored tracer kept at laboratory conditions (25°C–34°C) or at 37°C for five days in comparison with control assay using fresh tracer stored at –20°C until just before use at room temperature.

Storage Conditions	TSH concentration (mIU/L)			
	Day 3		Day 5	
	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer
25-34°C for 5 days (mean values)	0.218	0.071	0.295	0.072
SE diff.	0.026		0.041	
Probability	P > 0.0027		P > 0.0027	
37°C for 5 days (mean values)	0.146	0.133	0.272	0.09
SE diff.	0.057		0.039	
Probability	0.317 > P > 0.10		0.317 > P > 0.10	

P>0.1= non-significant P<0.01= significant

The results of 37°C temperature study for low TSH concentration at the above mentioned days have shown non-significant difference. So, it can be predicted that the variation in results in case of 25-34°C temperature study for initial 10 days may be due to an error in assay handling as these assays were performed at the initial stage of the study and at low TSH concentration measurements IRMA often shows such type of variations. It is noted that Table 6 shows significant probability value (P < 0.001) at day 60. Calculated data indicates that the problem was observed only in fresh tracer assay whereas observed values of stored tracer were in provided range of TSH < 0.35 mIU/L. The reason is that at low TSH concentration measurements immunoradiometric assay precision is poor, also observed at routine assay results and is mainly related to precision of the assays. Such types of results are repeated in routine case and provide meaningful information. It would be logical to conclude that tracer stability in early days i.e. at 3 to 10 days period should also be acceptable as indicated in the data obtained for 20 –60 days period. Therefore, it is concluded that the TSH tracer is stable at least up to 60 days.

Table 6: Low TSH concentration of patient samples measured by IRMA using stored tracer kept at 4°C for 60 days. The tracer was stored at laboratory conditions (25°C–34°C) or at 37°C for five days before moving to 4°C. The table also gives results of control assay using fresh tracer stored at –20°C until just before use at room temperature.

Storage Conditions before moving to 4°C	TSH concentration (mIU/L)									
	Day 10		Day 20		Day 30		Day 40		Day 60	
	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer	Fresh tracer	Stored tracer
25-34°C for 5 days (mean values)	0.191	0.066	0.348	0.149	0.533	0.113	0.270	0.165	0.387	0.141
SE diff.	0.039		0.108		0.122		0.058		0.074	
Probability	0.0027 > P > 0.001		0.317 > P > 0.10		0.50 > P > 0.317		0.10 > P > 0.05		0.10 > P > 0.05	
37°C for 5 days (mean values)	0.466	0.363	0.345	0.275	0.182	0.229	0.251	0.223	0.449	0.245
SE diff.	0.107		0.064		0.049		0.070		0.049	
Probability	P > 0.5		P > 0.5		P > 0.5		P > 0.5		P < 0.001	

P>0.5= non-significant P<0.001= significant

The above-mentioned results provide considerable information about ready-to-use form of TSH tracer stability for IRMA up to 60 days (two months). The detailed study of both temperature conditions i.e. laboratory conditions (25°C–34°C) and 37°C temperature shows that when tracer was stored at these particular temperatures for 5 days and then stored at 4°C up to 60 days its immunological and antigenic binding properties remain constant under these variables. These results are supported from the work of previous workers (Waite et al., 1987; Zaheer, 1992; Simmonet and Simmonet, 1990; Ahsan et al., 1992).

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