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The hTSH Measurement by Solid-Phase Fluoro Immunoassay and Using FITC as Probe

Ali MELLATI[1] Ali NAZARIAN[1] Gasem AHMADI[2] Mohammad MORADI[3*]

[1] Department of Biochemistry, Zanjan Medical School, Zanjan, Iran

[2] Nuclear Medicine, Valiasr Hospital, Zanjan, Iran

[3*]Department of Biology, Zanjan University, Zanjan, Iran

[*]Corresponding Author
E-mail: moradi_g@yahoo.com

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Abstract

Different fluoroimmunoassay methods for thyrotropin (hTSH) measurement have been discussed previously. We describe here fluoroimmunoassay method based on solid-phase system and using antigen-binding FITC to measure hTSH. The monoclonal antibody immobilized on polystyrene tubes were used for competitive binding of serum sample hTSH and F-TSH. The fluorescein-labeled TSH (F-TSH) were prepared by overnight incubation of fluorescein isothiocyanate (FITC) and commercial pure TSH, followed by gel filtration(G-25) purification to obtain pure F-TSH.The constracted standard curve (from F-TSH) was linear from 0.05 to 15 mIU/L. The interassay coefficient of variation was 7.3% , 11.2% , 6.3% , at 0.015 , 0.5 and 15 mIU/L TSH, and intraassay CV was 10.2% , 6.0% and 7.7% at mentioned TSH concentration, respectively. A good correlation was found with RIA ($y = .097 \pm 0.046$, $r = 0.91$, $P < 0.005$, $n = 43$).Our approach assay method has advantages of suitable sensitivity that alow the identification of primary hyperthyroidism, independent of serum effect, fast, technically simple and low cost , which is ideally suited to use in routine and normal clinical laboratories.

Key words: hTSH, FITC, fluoroimmunoassay, antigen binding

INTRODUCTION

The earliest and most sensitive manifestation of the thyroid is a change in serum thyroid-stimulating hormone(TSH) concentration [1,3]. The conventional way of quantifying human serum TSH, radioimmunoassay, have been limited by, among other problems (like health hazard, expensive reagents with limited half life, special permits and laboratories), poor specificity and sensitivity (functional detection limit of 1 to 2 mIU/L)[4], which could not discriminate TSH concentration in serum between hyperthyroid and euthyroid [5]. Increasing need for high sensitive immunometric methods and development of analytical techniques let to four generation TSH methodology [4,6,7] with continuously decreasing detection limits [8,9]. At present, nonisotopic methods (second and third generation) have replaced radioactive tracer methods (first generation) in most central and some routine laboratories. Labeling of samples with fluoroscensing compounds (second generation) [10,11,12], and lanthanide chelates [13,14,15] have about a ten fold (0.1 to 0.2 mIU/L) or more [15,16] improvement in their functional detection limit in comparison with RIA method. In addition fluorometric labeling is relatively inexpensive, safe, stable, rapid and wide applicability. The development of suitable solid-phase separation techniques have facilitated utilization of fluorescence in heterogeneous assays. Recently, to overcome undesired serum's own fluorescence [17], the workers have explored solid-phase in which monoclonal antibodies immobilized on different surface as well as coupled with various fluorescent molecules for detection [18,19]. More recently, reports have drawn attention to the use of smaller tracer antibody fragments [20, 21] and recombinant antibody fragments in solid-phase methods [22,23] for further improvement and increasing specificity in the TSH immunoassay.In considering different fluorescent probes that have been used for TSH measurement [13, 24], and FITC(Fluorescin Isothiocyanate) probe for a number of proteins detection[25,26], we report here our evaluation of TSH measurement by using FITC isomer I, as antigen binding, base on solid-phase separation method, and derermination of precision and its correlation with RIA method.

MATERIAL AND METHOD

Most of the chemical, fluorescein isthocyanante isomers I (FITC), TSH, sephadex G-25, Tween20, NaN3 were produt of sigma chemical company. Palystyrene tubes coated with monoclonal antibodies (against TSH) were purchased from ADDLTIS company Montreal, Cananda. Assay buffer contained 1g of Tween20 surfactant per liter of the 50mM carbonate-bicarbonate buffer (PH 7.4).

Specimens

Sera were obtained from subjects who referred to a clinical laboratory (Ahmadi Nuclear Medicine Center) with and without history of thyroid disease. Serum were separated at room temperature followed by centrifugation, and stored at -20°C until assayed. The TSH of 60 serum samples were tested by RIA method according to the instructions of the manufacturer (commercial kit), and 43 of the same serum samples (17 serum samples were eliminated, which were below and above normal range in RIA method) were used for our fluoroimmunoassay experiment.

Preparation of Fluorescein-labeled TSH(F-TSH)

Fluoresceinthiocarbamy thyrotropins (F-TSH) were prepared as previously described by Nargessi et al [27] with some modifications. 3.9 ml of pure TSH(50 mIU/L) were mixed with 0.1ml fluorescein isothiocyanate isomer 1 (0.05mg/ml) in 50mM carbonate-bicarbonate buffer (PH 9.0) and left overnight at 4°C with continuous shaking. 4 ml of reaction mixture were applied to a 1*20cm column of sephadex G-25 equilibrated with 50 mM phosphate buffer (PH 7.4) and eluted with the same buffer. Each 1 ml fraction were montiroted for absorbance at 280 nm and for fluorescence. The fractions that had highest F-TSH were identified, combined and stored at -20°C until used.

Assay

The assay is based on solid-phase method, employing immobilized antibody and dissolving the fluorescent probe from antigen (13,28) with some modification. 200µl of standards (0, 0.05, 0.1, 1.4, 10, 50 mIU/L) or serum samples in assay buffer were pipetted in douplicate into 12*50 mm coated polystyrene tubes coated with monoclonal antibodies against β-subunit of TSH. Then, it was added 100µl portions of F-TSH solution to each tube, and then cover them with paraffin film and incubated for 4h at room temperature, with continuous horizontal shaking. After aspirating the reagents and washing the tubes two times with 1ml wash buffer, then top the head of each tube firmely against absorbent paper for at least 5 minutes. After that it was added 1 ml (0.1 mol/L) NaOH [28] and left at least for 10 min to dissociate fluorescent probe from the coated tubes.

Fluorescence Measurment

Fluorescence measurment were made in an JENWAY 6200 Model Fluorimeter with xenon arc lamp source, using standard 1 cc glass cuvette, the excitation wavelength was 470 nm and the emission wave length was 520 nm. The background signals due to the buffer, serum sample were corrected in all experiments. The fluorescence intensity was expressed in arbitrary scales.

RESULTS

Purification of F-TSH

Figure 1 demonstrates the result of second time chromatographic purification of F-TSH. The unreacted fluorescein isothiocyanate was well separated from F-TSH by twice purification. The eluted fractions of the first purification had high fluorescence intensity that was out of fluorimeter sensitivity. After washing extensively with wash buffer (until the eluate shows no fluorescence intensily), the column were loaded by combined fractions (6.5 ml) of the first purification. We used the eluate from the second time purification for subsequent analysis and calculation.

Standard Curve

Two standard curves were generated (Figure 2 and 3). Figure 2 is a typical standard curve which demonstrate fluorescence intensity versus fluorescin-Thyrotropin (F-TSH) concentrations ranging from 0 to 15 milli-int.untils/L. Figure 3 illustrate relative fluorescence intensity (F/F0) versus increasing the level of bound calibrrator (unfluorescin standard) TSH solution with a final concentration of 0.015, 0.05, 0.015, 0.5, 1.5, 5.0 and 15.0 milli-int.Units/L. Relative fluorescence intensity (F/F0) is fluorescence intensity of F-TSH relative to that of the fluorescent component alone. The data were fitted to the straight line plot by using Sigma plot 8 software (SPSS science, Chicago, IL).

Table 1. Precission of the assay

Assay standard Concentration(milli.int.U/L)	n	$\bar{X}$: fluorescence intensity (arbitrary unit)	SD	CV%
		Intraassay		
0.015	10	64.2	4.68	7.3
0.5	10	38.6	4.34	11.2
15	10	18.1	1.14	6.3
		Interassay		
0.015	7	65.0	6.6	10.2
0.5	7	39.2	2.37	6.0
15	7	17.6	1.35	7.7

CV=Coefficient of Variation

Precision

A precision profile calculated from several replicates of each assay standard concentration is shown in table 1. The intraassay precision were assessed by measuring each sample 10 times, giving coefficient of variation of 7.3, 11.2 and 6.3% at 0.015, 0.5, 15 miliIU/L TSH concentration. For estimation of interassay precision, each stored assay standard were measured in 7 independent assays, giving coefficient of variation of 10.2, 6.0 and 7.7% at mentioned TSH concentration.

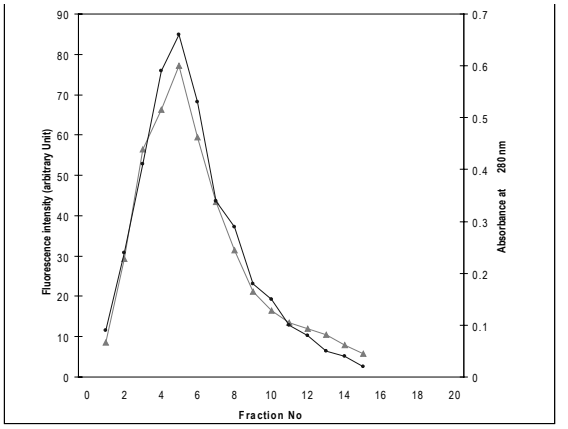

Figure1. Purification of fluorescein thyrotropin(F-TSH) on sephadex G-15 column.

Column fractions were monitored for protein content at 280nm (●-●) and for F-TSH by conventional fluorimetry (▲-▲).

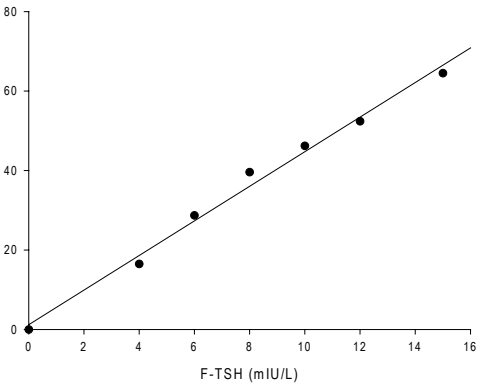

Figure 2. Standard curve showing increasing fluorescence intensity with increasing F-TSH concentration.

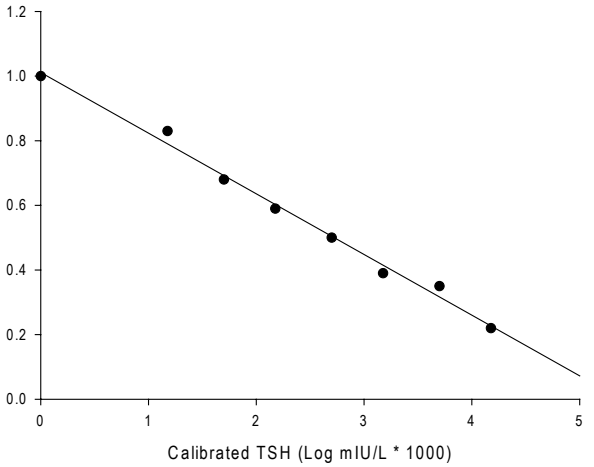

Figure 3. The standard curve showing decreasing relative fluorescence intensity (Ordinate: F/F0) vs unfluoresin TSH solution (Abscissa: log of concentrations of TSH standard multiply 1000).

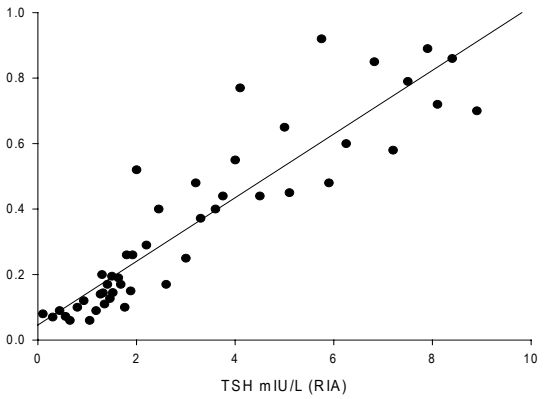

Figure 4. Correlation between TSH levels in 43 patient serum samples determined by RIA and solid-phase fluoroimmunoassay (y=0.097± 0.046, r=0.91, P<0.005, r=43)

Correlation with Radioimmunoassay (RIA) kits

For comparison of the results obtained with our solid-phase fluoroimmunoassay with those obtained with a commercial RIA kit, we measured TSH from 43 referred subjects by fluorimmunoassay which had previously been measured by RIA methods. Figure 4 demonstrates correlation between the two methods. The least squares regression of the fluoroimmunoassay results (y) on the RIA results (Fig.4) are given by the equation y=0.097x+0.046 (units mili. IU/L TSH), and the correlation coeficient (r) was 0.91.

CONCLUSION

The main goal of our study was to evaluate the usefulness of fluorescein isothiocyanate (FITC isomer I) as a proble to label antigen (TSH) in solid-phase-based immunoassay system by washing and dissociation process of FITC for the measurement of human serum TSH.The use of solid-phase immunofluorometric system in sandwich-type assays in which antibodies, are labeled have been investigated for detection of various analytes, present in serum at micromolar and nanomolar concenteation [26, 29, 30]. Kalhala and Torresani etal [13, 24] reported measurment of thyrotropin by using Delfia hTSH (commercial kit) which is based on solid-phase procedure with two monoclonal antibodies against antigenic site of hTSH, one immobilized (captured antibody), the other labeled with europium (lanthanide label). Comparison with published data indicates that our precision is similar to those obtained by antibody labeled by europium process [13,24] and the sensitivity is significantly higher than RIA method [5] and similar or a little better than antibody-labeled by europium for TSH measurement [13, 24]. Good correlation was found between our antigen-labeled fluoroimmunoassy and RIA (r=0.91) (figure 4), which is highly significant (p<005). In conclusion in considering with previous antibody-labeling process by different probes including Lanthanide chelate and FITC [13, 24, 32] and more recent report using smaller tracer antibody fragments and recombinant antibody fragments in solid-phase methods [23], our approached assay method has advantages of suitable sensitivity that allow the identification of primary hyperthyroidism without the need for additional testing. This procedure is independent of serum effect, has long-term stability of reagents, is also fast, technically simple and low cost, which is ideally suited to use in routine and normal clinical laboratories.

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