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RESEARCH ARTICLE

Immunoradiometric Assay for Determination of Thyroid Stimulating Hormone in Human Serum using Solid Phase Anti-TSH Magnetic Particles

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Abstract

The preparation and development of primary reagents of thyroid stimulating hormone (TSH) Immunoradiometric (IRMA) technique using solid phase magnetic particles with low cost is considered to be the main objective of the present study. The production of polyclonal antibodies was undertaken by immunizing four male New-Zealand rabbits. The preparation of ^{125}I-TSH antigen and ^{125}I-TSH MoAb tracers was carried out using iodogen. The preparation of ^{125}I-TSH MoAb radiotracers were carried out using Iodogen as oxidizing agent. The preparation of standard solution of TSH was carried out. Activation and coupling of magnetizable particles with purified polyclonal anti-TSH was carried out. Optimization and validation of the assay were carried out. The results obtained provide a highly sensitive specific and accurate IRMA system of TSH. In conclusion this assay could be used in diagnosis of hypothyroidism.

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INTRODUCTION

Thyroid stimulating hormone (TSH) (also known as thyrotropin TSH or hTSH for human TSH) is a pituitary hormone that stimulates the thyroid gland to produce thyroxine (T_4) and then triiodothyronine (T_3) which stimulates the metabolism of most every tissue in the body (Chan and Sell, 2001). It is a glycoprotein hormone synthesized and secreted by thyrotrope cells in the anterior pituitary gland. Which regulates the endocrine function of the thyroid (Mandel, 1993 & Klee and Hay, 1987). TSH is a glycoprotein of approximate molecular weight 3000 D and consists of two subunits, the alpha and the beta subunit. The alpha subunit is nearly identical to that of human chronic gonadotropin (hCG), luteinizing hormone (LH) and follicle. Stimulating hormone (FSH) the α subunit is thought to be the effectors region responsible for stimulation of adenylase cyclase (Klee and Hay, 1987). The α chain has 92 amino acid sequence the β-subunit is unique to TSH and therefore determines the receptor specificity and it has 118 amino acid sequence.

TSH stimulates the thyroxine (T_4) which has only slight effect on metabolism T_4 is converted to T_3 which is the active hormone that stimulate metabolism.

TSH is secreted through life but particulary reaches high levels during the periods of rapid growth and development (Martin, 1981).

Measurement of circulating TSH has been used as a primary test for differential diagnosis of hypothyroidism and an aid in monitoring the adequacy of thyroid hormone replacement therapy. Estimation of polypeptide hormone concentration (TSH) in biological fluid are changing from limited reagents RIA methods to excess reagents IRMA methods. Two site immunoradiometric assay require two antibody one attached to a solid phase matrix by simple physical adsorption or covalent bond and the other antibody labeled with radioiodine ^{125}I (Ekins, 1981 & Hunter and Budd, 1981).

As the basis for this study, we used s single monoclonal antibody labeled with radioiodine (^{125}I) to optimal specific activity with a polyclonal rabbit antiserum linked to solid phase magnetic particles and devised a two site IRMA for TSH as a model system.

MATERIALS AND METHODS

For establishment of magnetic particles IRMA assays for TSH a number of different reagents were used. These reagents were as follow: Highly purified TSH antigen, TSH monoclonal antibody (MoAb) from Scottich antibody production unit Scotland. Magnetic particles were provided by NETRIA (North East Thomas Region Immunoassay) Na^{125}I (5 mCi/50 µl) pH 7-11 (Institute of Izotopes Co. Ltd. Budapest), 1,1`-carbonyldiimidazole (CDI), sodium metabisulphite. Complete and incomplete Freund's adjuvant were purchased from Sigma Chemical Co. St. Louis Mo. USA. Iodogen was obtained from Pierceard Warriner Ltd. Chester, England. All other chemical reagents were analytical (AR) grad obtained from reputed manufactures.
This study was achieved through the preparation of the following:

Production of TSH-polyclonal antibodies:

Production of polyclonal anti-TSH was carried out according to the immunization schedule described by (Mehany *et al*., 2009 and Shafik, 2009) through immunization of four male mature white New-Zealand rabbits weighing 2.5-3 Kg with highly purified human TSH. Rabbit were kept under the same hygienic conditions fed on standard laboratory diet and water supplied ad libitum. The production of polyclonal antibody was undertaken by immunizing the four rabbits with 50 µg TSH for each rabbit through primary immunization and four boosters. The immunogen preparation was performed by dissolving 0.2 mg highly purified TSH antigen in phosphate buffer 0.05M pH 7.4 (0.2 mg/1ml). Then the solution was emulsified with 3 ml of complete Freund's adjuvant each rabbit was injected by 1 ml of emulsion at 8 sites subcutaneously over the shoulder and two sites intramuscularly. The first booster was given after 3 weeks interval from the primary immunization at the same way except that complete Freund's adjuvant was replaced by incomplete Freund's adjuvant.

Antisera was assessed in terms of titer, displacement and immunoresponse curve. The titer of the collected antisera for TSH was assessed using double antibody RIA technique. The technique can be summarized as follow: 100 µl of different dilution of antisera (1/100, 1/500, 1/1000/ 1/1500/ 1/2000/ 1/1500) were dispersed into the corresponding tubes followed by addition of 100 µl of phosphate buffer (0.05M, pH 7.4) and 100 µl of ^{125}I-TSH into all tubes. After 3h incubation at 4°C, separating reagents were added in the following sequence 100 µl of non-immune rabbit serum (1:200), followed by addition of each of 100 µ Donkey anti-rabbit serum (1:20), and 500 µl of polyethylene glycol 800 (PEG) into all assay tubes. The tubes were allowed to stand for 30 min at room temperature and centrifuged for 20 min at 3000 rpm. The supernatant was aspirated and the sediment containing the precipitated bound antibody was counted in a multichannel gamma counter. The binding percentage for each bleeding for each rabbit was calculated. The dilution of antiserum which gave 50% binding with labeled antigen was considered as the antibody titre (Persoon *et al*. 1981). A collected pool of anti-TSH with highest titer and displacement was subjected to purification using caprylic acid precipitation method according to steinbuch and Audran (1969). The purification step was started by adding 0.85 ml N-octanic acid drop wise drop to 10 ml of rabbit antiserum (anti-TSH) to precipitate protein and the pH was adjusted to 5. The content was stirred continuously for 30 min and centrifuged at 10000 rpm for 30 min at 4°C. The clear supernatant contains IgG was collected and dialyzed against sodium carbonate (0.01 mol/L pH 8). IgG content of TSH was determined using UV spectrophotometer at 280nm.

Preparation of TSH standards:

In this study the preparation of standards TSH was done by diluting of a second reference preparation derived from human pituitaries provided by NETRIA using an assay buffer. The assay buffer contained per 100 ml distilled water. 5 ml phosphate buffer (0.5M pH 7.4), 5 ml NaCl (3M), 0.1M 10% triton, 0.1g sodium azide and 10 ml protein solution Bovine Serum Albumin (BSA). After that the TSH standards ranged from (0.5 – 60 µIU/ml) were validated against International Reference Preparation (IRP).

Preparation of tracers:

Iodination of TSH antigen

The preparation of radiolabeled TSH tracer for estimation of antibody was carried out using Iodogen (1,3,4,5-tetrachloro-3α-diphenylglycoluril) is essentially insoluble in water and so it can be used as a thin film plated onto the bottom of glass vials (Fraker and Speck, 1978). To an iodogen coated tube, 10 µl 0.5M phosphate buffer pH

7.4 was added. Then (6 µg/ 6µl) TSH antigen was added and mixed gently. The reaction was started by addition of 5µl (400µCi) of ^{125}I. The reaction was allowed to proceed for 10 min and then stopped by transfer of the reaction mixture to another vial. The radiochemical yield was calculated by paper electrophoresis. The product was purified by gel filtration using Sephadex G-25. Mougy and Mason (1963).

Iodination of monoclonal antibody for TSH (MoAb-TSH).

Iodination of TSH MoAb TSH was carried out using iodogen as oxidizing agent:

To an iodogen coated tube, 20 µl 0.5M phosphate buffer pH 7.4 was added. Followed by addition of 6µl MoAb-TSH (8 µg) and mixed gently. The reaction was started by addition of 6 µl (600 µCi, 22.2 MBq) of ^{125}I. The reaction was allowed to proceed for 15 min and stopped by transfer of the reaction mixture to another vial. The radiochemical yield was calculated by paper electrophoresis. The product was purified by gel filtration using Sephadex G-25 (Mehany *et al*., 2005).

Preparation of solid phase particles:

Black ferric or ferrous oxide may be readily incorporated into cellulose matrix. Cellulose provide perfect matrix, it is inexpensive, easy to dissolve prior to incorporation of iron oxide, has low density and high surface capacity and above all can bind antibodies to provide a stable reagents with low non-specific binding. Magnetic separation is being widely used in radioimmunoassay techniques due to its simplicity and also considered to be cost effective since the use of centrifuge can be avoided (Forrest and Rattle, 1983).

In the present study, the magnetic particles were applied as a solid phase for IRMA system for determination of TSH. This was achieved due to that the purified anti-TSH polyclonal antibodies were immobilized to the activated magnetic particles preparation of solid phase system of TSH-IRMA performed through carbonyl-diimidazol (CDI) activation followed by coupling of purified antibody to the activated particles according to the procedure of Forrest and Rattle (1983) and El-Kolaly *et al*., (2006).

1. Activation method:

200 mg/4 ml as supplied magnetic particles were separated by using the magnetic separator for 5 min. The supernatant was decanted and the recovered particles were washed once with 4 ml double distilled water followed by 4 ml acetone three times. The particles were recovered after each wash using the magnetic separator for 5 min. Finally, the particle were re-suspended in a conical flask in 2 ml acetone then 0.1 g 1,1`-carbonyl diimidazole was added. The flask was again recovered and washed three times with 4 ml acetone as previously described. The particles were then recovered by filtration over GF/A glass fibrous filter and allowed to air dry overnight. 200 mg of the activated magnetic particles were stored in a tightly scaled container at -20°C unit required for coupling with rabbit anti-TSH.

2. Coupling method:

In a 5 ml glass centrifuge tube, 50 mg of activated magnetic particles were homogenized in 750 µl of 0.05M borate buffer at pH 8 then 50 µl rabbit anti-TSH was added and the tube was incubated for 48 hours on a rotator. The antibody-coupled particles were recovered using the magnetic separator for 5 min. The supernatant was decanted and 1 ml of 0.5M diethanolamine buffer at pH 9 was added to the particles and rotated for one hour then the particles were washed three times with 2 ml of each of the following buffers: 0.5M sodium bicarbonate pH 8, 0.1M sodium acetate pH 4, and 0.05M phosphate buffer containing 0.1% BSA. The particles were then re-suspended in 2 ml of 0.5M phosphate buffer containing 0.1% sodium azide. The solid phase anti-magnetic particles were stored at 4°C until required for testing by IRMA technique.

Assay design:

Assay has been designed to give optimum sensitivity and minimum error. IRMA was set as follow: 25 µl f solid phase anti-TSH magnetic particles were pipette into all tubes except total count (TC) tubes. 50 µl of TSH standards, 100 µl of ^{125}I-TSH (MoAb) were pipette into all tubes. The tubes were mixed and incubated under continuous shaking for 3 hr. The separation of the bound and free components of magnetic particles was carried out on magnetic separator for 5 min. The bound components were washed twice by 1 ml 0.05 phosphate buffer containing 0.1% sodium azide. All tubes were counted using gamma counter and the results were calculated using log-logit graph paper.

Performance characteristics of the IRMA assay for TSH:

To assure the validity of the proposed assay, some performance characteristics studies including sensitivity, precision, accuracy and method comparison were carried out.

Numerous studies were performed to test some effective factors on the assay optimization. In addition validation tests of this TSH-IRMA assay were done.

RESULTS AND DISCUSSION

The determination of TSH in serum has been considered the single most sensitive and specific index of etiology of thyroid disorder.

The results and observation of the present work are summarized to prepare a valid assay to detect TSH concentration in human serum.

With respect to the production of polyclonal anti-TSH. The data showed that R_1, R_2, R_3 and R_4 gave successful immunoresponse and high displacement. The results obtained revealed that R_2 showed the highest displacement (60%) and maximum immunoresponse after three weeks from the third booster at dilution 1/2000 while R_1 and R_3 showed displacement percent 51% and 47% at dilution 1/2000 while R_4 gave low immunoresponse.

The data of displacement and immunoresponse are in good agreement with other reports (Baner 1982 & Pillai and Bhandarkar, 1998). The collected antiserum from R_2 was selected and purified. The results obtained of the IgG of the purified anti-TSH were 9.6 mg/ml. The concentration of total IgG in serum ranged from 5 to 20 mg/ml and very strong polyclonal antiserum might contain 1-3 mg/ml of specific antibody (Goding, 1986).

Radioiodinated hormones are essential for the development of sensitive, precise and accurate immunoassays (RIA or IRMA).

Many investigators stated that the reducing agents often cause some damage to the protein, thereby reducing its immunereactivity. Therefore, the solid phase iodination techniques in which the oxidizing agent is coated to the reaction vessel or coupled to beads have been introduced to avoid the use of reducing agent-Iodogen has been reported to give quality tracers for immunoassays (RIA and IRMA) (Jyotsna *et al*. 1986 & Pillai *et al*. 1985).

The elution pattern of ^{125}I-TSH using gel chromatography on a sephadex G-25 fine column is shown in Fig. (1).

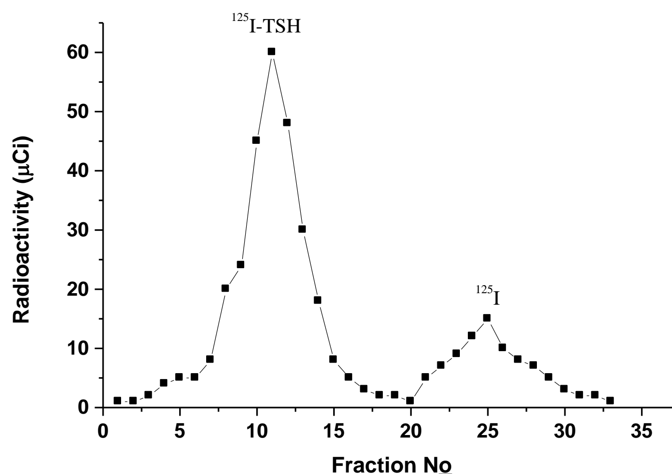

Fig. (1): Purification profile of ^{125}I-TSH using Sephadex G-25.

The figure shows two peaks corresponding to ^{125}I-TSH (70%) and free ^{125}I (13.4%) and the elution of ^{125}I-TSH MoAb is shown in Fig. (2). The figure show two peaks corresponding to ^{125}I-TSH MoAb (72%) and free ^{125}I (17.8%). These results are in good agreement with Mehany *et al*. (2005).

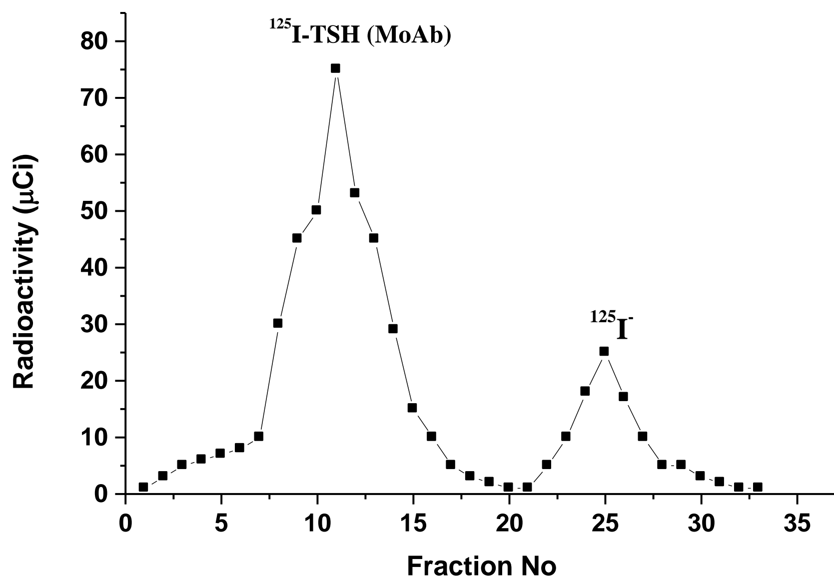

Fig. (2): Purification profile of ^{125}I-TSH MoAb using Sephadex G-25.

For achieving sensitive and reliable assay, general principles of the assay optimization must be followed. After getting the solid phase anti-TSH coated magnetic particles, the development of Immunoradiometric assay (IRMA) was carried out by studying the solid phase volume, incubation time and sample volume.

Solid phase volume:

To obtain the most sensitive IRMA procedure, different volumes of solid phase anti-TSH magnetic particles were used. These volumes ranged from 5µl–50µl. The data obtained revealed that the highest difference in binding between two concentrations of TSH (0.5 and 60 µIU/ml) were demonstrated using 25 µl magnetic particles while using an excess of antibody resulted in decrease in binding efficiency, shown in Table (1).

Table (1): Effect of solid phase volume of anti-TSH magnetic particles on TSH-IRMA.

Solid phase anti-TSH volume µl / Standard	5	10	25	50
Low 0.5 µIU/ml	0.33	0.43	0.7	1.2
High 60.0 µIU/ml	4.1	6.1	16.5	16.1

Incubation time:

The effect of the incubation time on magnetic particles IRMA assay was studied at room temperature at time intervals ranged from 1 to 24 h. The results showed that the highest difference in binding between the two concentrations of TSH used (0.5 and 60 µIU/ml) was increased with increasing incubation time and reached the optimum after 3 hrs. it can be concluded that the optimum incubation time was 3 hours to get a valid assay for TSH, shown in Table (2).

Table (2): Effect of incubation on time on TSH-IRMA.

Time / Standard	1 hr	2 hr	3 hr	24 hr
Low 0.5 µIU/ml	0.25	0.54	0.72	0.75
High 60.0 µIU/ml	7.3	10.9	16.4	17.0

Sample volume:

The results of the sample volume showed that the highest difference in binding between the two levels of (0.5 and 60 µIU/ml) was 50 µl of standards while higher volumes of standards gave low counts. This observation is in good agreement with the data of Pillai and Bhandarkar (1998), shown in Table (3).

Table (3): Effect of sample volume on TSH-IRMA.

Sample volume µl / Standard conc.	25	50	100
Low 0.5 µIU/ml	0.44	0.74	0.77
High 60.0 µIU/ml	6.7	16.3	17.5

Performance characteristics of the proposed IRMA assay for TSH:

To assure the validity and reliability of the assay suggested, some performance characteristics were extensively studied.

Sensitively:

Ultra-sensitive assay is required to provide parallel improvement in biochemical discrimination between TSH levels observed in thyrodism symptoms. The sensitivity or minimum detection limit was calculated by interpolation of the mean plus two standard deviation of 20 replicates of the zero µIU/ml standard TSH. Hence, an efficient separation method (wash procedure) was vital to achieve a high level of sensitivity, so, the ensitivity of TSH-IRMA assay was 0.08 µIU/ml in case of using the magnetic particles.

Precision:

The reliability of the present procedure was assessed by examining its reproducibility on human serum samples selected to represent a wide range of TSH. The data of the intra-assay (within run) and inter-assay (run to run), as illustrated in Table (4), show the consistency of the results obtained by the methods of the present study. These results are in good agreement with the results of Pillai & Bhandarkar (1998) and El-Bayoumy (2012) who stated that the intra-assay coefficient of variation (CV) should be less than 10% while in case of inter-assay, the CV of the measured concentration should be less than 15%.

Table (4): Intra-assay and inter-assay precision for TSH-IRMA.

Serum	Intra-assay			Inter-assay		
	Mean	SD	CV%	Mean	SD	CV%
Pool 1	0.4	0.037	9.2	0.45	0.047	10.3
Pool 2	12.0	1.04	8.7	12.8	1.2	9.4
Pool 3	45.0	3.1	6.9	44.5	3.87	8.4

Accuracy:

Accuracy is defined as the degree of agreement between the measured value and the true value. The assay accuracy was checked in this study by recovery and dilution tests.

a. Recovery test:

Recovery test measures the concentration in human samples before and after adding known amounts of pure analyte (TSH). The recovery data of the present study (Table 5) are in a good agreement with that of Pillai &

Bhandarkar (1998) and El-Bayoumy (2012) who stated that the recovery of the assay should be 100±15%. This indicates that standards and samples were identical.

Table (5): Recovery for TSH-IRMA.

Sample No.	Endogenous TSH- μIU/ml	Standard μIU/ml	Expected (E)	Observed (O)	O/E%
1	0.4	0.5 15	0.9 15.4	0.84 15.0	93.3 97.4
2	10.0	0.5 15	10.5 25.0	10.0 25.5	95.2 102.0
3	30.0	0.5 15	30.5 45.0	30.8 46.0	100.9 102.0

b. Dilution:

The results in Table (6) reveal the concentrations of three human serum samples, undiluted and at various dilutions in the matrix of the assay (zero standard) to assess the linearity of the assay. Edwards (1996) reported that non-linearity indicated inaccurate calibration or an inappropriate matrix of both. The results obtained in this study showed that the procedure of the present study of TSH-IRMA maintained good linearity under dilution.

Table (6): Dilution for TSH-IRMA.

Sample No.	TSH- μIU/ml	Dilution Factor	Expected (E)	Observed (O)	O/E%
1	4	1:2 1:4 1:8	2 1 0.5	2.1 0.95 0.52	105 95 104
2	30	1:2 1:4 1:8	15 7.5 3.75	15.3 7.2 4.0	102 96 106

Method comparison:

The statistical analyses were undertaken to compare TSH results of 20 different human serum samples obtained (Siemens Medical Solution Diagnosis, USA) to those obtained by the present system. It was found that the present technique gave clinically acceptable results. The statistical analysis showed highly positive correlation (r= 0.999) between the results obtained from the present system using TSH-IRMA system and the commercially available kits (Siemens) as illustrated in Fig. (3).

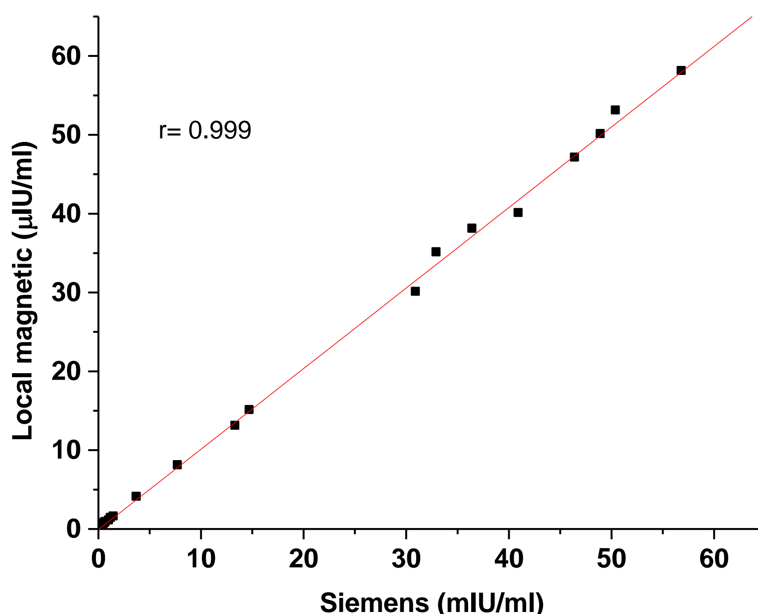

Fig. (3): Correlation coefficient (r) between TSH values obtained by Siemens method and the present technique.

CONCLUSIONS

In conclusion, the technical simplicity of this sensitive, specific, precise and accurate method may suggest that the low cost TSH-IRMA technique of the present study should be suited for routine laboratory use. It has been used effectively as a decisive diagnostic kit in the etiology of thyroid disorders.

The study presented here may be of interest particularly to laboratories in developing countries, who may prefer our procedure to the more expensive diagnostic kits available from commercial sources.

REFERENCES

1. Chan D.W. and Sell S. "Tumor Markers" In Tietz Fundamentals of clinical chemistry. C.A. Burtis and E.R. Ashood, 5th ed., W.B. Sanders Co., Phiadephia, 390-413 (2001).
2. Mandel S.J., Brent G.A. and Larsen P.R., Ann. Intern. Med., 119: 491 (1993).
3. Klee C.G. and Hay I.D., Clin. Endocrinol. Metab., 64(3): 461 (1987).
4. Martin D.W., Mayes P.A. and Rodwell V.W., Harpers Review of Biochemistry 18th ed., Middle East Edition (1981).
5. Ekins R.P., In: "Monoclonal antibodies and development in immunoassay, A. Albertini, P.R. Ekins (Eds.), Elsevier-North Holland Amsterdam, p. 3 (1981).
6. Hunter and Budd P.S., J. Immunol. Meth., 45: 255 (1981).
7. Mehany N.L., El-Kolaly M.T., Meky N.H. and Ebeid N.H., Isotope & Rad. Res., 414(2): 1403-1419 (2009).
8. Shafik H.M., J. Radioanal. Nucl. Chem., DOI 10. 1007/S 10967-009-0043-5 (2009).
9. Persoon T.J.: In: Berner D.R., Langan J.K., Wells L.D. (eds.), Nuclear medicine technology and technique. The C.V. Mosby Company, Sr. Louis, Toronto, London, pp. 433 (1981).
10. Steinbuch M. and Audran R., Arch Biochem. Biophys., 134, 2-79 (1969).
11. Fraker P.J., Speck J.C., Biochem. Biophys/ Res. Common, 80: 849 (1978).
12. Mougy E.H. and Mason I.W., Anal. Biochem., 6: 233 (1963).
13. Mehany N.L., El-Kolaly M.T., Ayoub S.M. and Hassan S.E.M., Radioanal. Nucl. Chem., 265(1): 63 (2005).
14. Forrest, G.C. and Rattle, S.J., In: Immunoassay for Clinical Chemistry, W.M. Hunter and J.E.T. Corrie (eds.), 2nd Churchill Livingstone, Edinburgh, p. 147 (1983).
15. Bauer J.D., In: Clinical Laboratory Methods, 9th ed., I.D. Bauer, The C.V. Mosby Company London, 411-415 (1982).

16. Pillai M.R.A. and Bhandarkar S.D., In: Radioimmunoassay: Principle and Practice, 2nd (ed.), Hear Isotope Division, Bhabha Atomic Energy Center, India, p. 89 (1998).
17. Goding J.W., In: Monoclonal Antibodies, H.B. Jovanovicn Publisher 2nd (ed.), Academic Press Ltd. London.
18. Jyotsna T., Pillai M.R.A., Pal M., Gupta J.H., Desai C.N. and Mani R.S., J. Radioanal. Nucl. Chem., 97, 45 (1986).
19. Pillai M.R.A., Jyosna T., Gupta J.H. Desai C.N., Mani R.S., in: Proc. Intern. Symp. on African Radioactivity, RUNE, PAR, p. 41 (1985).
20. El-Kolaly M.T., Mehany N.L., Hassan S.E.M. and Ayoub S.M., Arab J. Nucl. Sci. Appl., 39(2): 320 (2006).
21. El-Bayoumy A.S.A., J. Rad. Res. Appl. Sci., 5(2), 353 (2012).