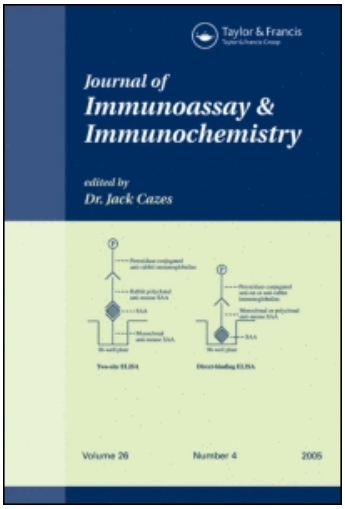

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Physico-Chemical Studies on the Thyroid Stimulating Hormone Immunoradiometric Assay

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Abstract: Thyroid stimulating hormone (TSH), which is known as thyrotropin, is an anterior pituitary hormone and a glycoprotein with a molecular weight of about 28,000 Daltons. This hormone increases the secretion of thyroxine and triiodothyronine from the thyroid gland. In the present study, the immunoradiometric (IRMA) technique was used for the estimation of TSH, and also the various thermo-kinetic parameters of the assay were determined including temperature, antibody volume, tracer volume, and incubation time. Many thermo-kinetic parameters were calculated, such as the free energy change of (ΔG°), the enthalpy change (ΔH°), and the entropy change (ΔS°) of the reaction. The results obtained were used to determine the order of the reaction and the optimum conditions to perform the assay. The optimum conditions that were obtained from the present studies were used to perform the assay for patient's samples and the results obtained were compared with that of a commercially used kit; the results indicated the high correlation between the traditional (commercial) method and the studied one.

Keywords: Hormone, IRMA, Kinetics, Stimulating, Thermodynamics, Thyroid

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INTRODUCTION

Thyroid stimulating hormone is a glycoprotein with a molecular weight of about 28,000 Daltons. This hormone is secreted from the anterior pituitary gland; it stimulates the thyroid gland to secret the thyroid hormones.[1] Thyroxine withdrawal or recombinant TSH is used for the stimulation of thyroglobulin (Tg) and whole body scanning iodine-131 treatment in patients with thyroid carcinoma.[2] In recent years, serum thyroglobulin measurement during thyroxine treatment and/or after stimulation by endogenous TSH or recombinant human (TSH) has eclipsed other diagnostic procedures in managing patients with differentiated thyroid cancer.[3]

Measurements of TSH were originally based on bioassays such as the stimulation of colloid droplet formation in the guinea pig thyroid gland and the release of labeled thyroidal iodide into mouse blood.[4] These early *in-vivo* bioassays, however, were of limited sensitivity and precision and were not applicable to the measurement of TSH in unfractionated serum. Sensitive detection of TSH in unfractionated serum is possible by using a cytochemical bioassay,[5] but this procedure is technically difficult and time consuming. At present, immunoassay is the standard procedure for the measurement of serum TSH in the clinical laboratory. The first immunological assay for TSH was based on the cross reaction of human and bovine TSH in a hemagglutination inhibition test;[6] however, this method was too insensitive for clinical purposes. Immunoassay measurement of serum TSH level did not become a routine test until the purified pituitary hormone became available for immunization and iodination. The traditional radioimmunoassay (RIA) for TSH was based on competition between endogenous and radio-labeled hormone for binding sites on a limited amount of antibody. Separation of antibody-bound and free radio-legends was conveniently performed by double antibody precipitation (enhanced by the addition of polyethylene glycol) or by using a solid phase second antibody procedure. The amount of labeled TSH bound to the antibody was inversely related to the amount of unlabeled TSH present in the serum specimen. The determination of serum TSH by RIA proved to be very valuable in assessing the elevated TSH values in primary hypothyroidism. However, because they could only detect 1 mIU/L, most conventional RIAs could not distinguish normal values from abnormal values associated with hyperthyroidism. New immunometric techniques are now capable for measuring TSH at levels that routine clinical laboratories cannot detect them. These new sensitive methods resulted from the application of the immunometric sandwich configuration, in which a serum TSH molecule forms a bridge between two or more distinct anti-TSH antibodies. The first antibody (of monoclonal origin) is often directed at the specific β-subunit and is

anchored to a solid phase separation system. This antibody is present in excess; it selectively immunoextracts the majority of TSH molecules from the serum specimen. Bound hormone is then quantitated using a second TSH antibody, of either monoclonal or polyclonal origin, which is directed against a distinctly different antigenic site on the TSH molecule (for example, the α-subunit). This detected antibody is labeled with a signal molecule, such as a radioisotope, enzyme, fluorophore, or luminescent molecule. The separation of the free and the bound portion is accomplished in one or two steps. The two step procedure has less interference, but it is harder to achieve. The double binding results in a labeled insoluble (sandwich) concentration which is directly proportional to the amount of TSH in the specimen.[7,8] In immunoradiometric assay, TSH is captured between monoclonal anti-TSH antibodies immobilized on the surface of the spherical beads and the radiolabeled polyclonal anti-TSH tracer. Unbound ^{125}I-labeled anti-TSH antibody is removed by aspiration and followed by a washing step. The TSH concentration is directly proportional to the radioactivity present on the surface of bead after washing step.[9] In some immunometric assays, the separated antibody is attached to plastic beads, test tube walls, microtiter plates, magnetizable particles or glass fiber paper.[10,11]

EXPERIMENTAL

Materials and Methods

In this study, many reagents were used for the establishment of an IRMA assay for TSH, such as ^{125}I-TSH-Ab, iodinated anti-TSH goat polyclonal antibody supplied in liquid form and eight vials of TSH standards; labeled from A to H and of lyophilized TSH standards from Diagnostic Products Corporation (DPC, USA). Mouse monoclonal anti-human TSH was supplied from Scottish Antibody Production Unit (SAPU), Scotland, and beads were supplied from Nitria Co., London. Bovine serum albumin, D-mannitol anhydrous and hydrolyzed gelatin were purchased from Sigma Chemical Co., USA. All other chemical reagents were analytical (AR) grade obtained from reputed manufacturers. The present research plan was achieved through the local preparation of the following:

Preparation of Coated Beads

The coated beads were prepared by adding coating buffer (0.1% bovine serum albumin (BSA) and 0.1 NaN_3), to 1,000 beads in a beaker to cover

all beads, followed by a degassing step; then, the beads were incubated over 3 nights at 298 K. A blocking step was carried out using 6 g of hydrolyzed gelatin in 600 mL coating buffer (blocking buffer). After 3 hours, the beads were washed three times using wash buffer (one liter containing 25 mL 1 M PO_4, 50 mL 3 M NaCl, 1 mL 10% Triton and 1 g NaN_3) followed by addition of a second antibody diluted in priming buffer (600 mL containing 15 mL 1 M PO_4^{-3}, 30 mL 3 M NaCl, 1 mL 0.6 g BSA and 1 g NaN_3) then, it was left at 298 K overnight. After aspirating the solution, the wash step was repeated three times followed by addition of monoclonal antibody which was diluted in priming solution to cover all beads. After the degassing step, the beads were incubated for 24 hrs at 298 K. Glazing solution, 600 mL containing 15 mL 1 M PO_4, 30 mL 3 M NaCl, 12 g mannitol and 6 g hydrolyzed gelatin was added instead of priming buffer after the washing step and the beads were left for about 30 minutes at 298 K. The solution was aspirated and the beads were left to air dry overnight. Dried beads were kept in labeled metallized plastic film bags.

Preparation of TSH Standards

Lyophilized TSH standards were reconstituted with 3 mL distilled water except standard zero with 6 mL distilled water.

Assay Design

Into each tube, one of the previously prepared coated beads was added, followed by 100 μL of high standard (60 μIU/mL) and 150 μL of assay buffer. All tubes were incubated in a rotator for one hr at 298 K (first incubation). After the first incubation, the solution was aspirated followed by addition of 3 mL wash buffer to each tube, then aspirated. The washing step was repeated three times; then, 100 μL tracers and 150 μL assay buffer were added to each tube, then incubated for one hour in the rotator at 298 K (second incubation). After the second incubation, the aspiration step was followed by a washing step. All tubes were counted using a gamma counter in counts per minute (cpm). The binding percent from total activity (B/T%) with time (in hours) for the reaction of TSH with Ab and Ab* at different concentrations and temperatures (277, 298, and 310 ± 1 K).[12] All these steps were performed at variable concentrations of TSH antigen (Ag), tracer (^{125}I-TSH Ab), and anti-TSH antibody (MO Ab) at three different temperatures (277 K, 298 K, and 310 K) to calculate the order

of the reaction, the reaction rate law, and the activation energy (E_{act}) of the reaction, as well as the thermodynamic parameters (ΔG°), (ΔH°), and (ΔS°) accompanying this reaction.

Theory and Calculation

The initial rate of reaction (V_o) was obtained from the Equation (13):

$$\text{Rate}\,(V_o) = k[Ag]^x[Ab^*]^y[Ab]^z\,M/h \tag{1}$$

where, x, y, and z are the orders of reaction with respect to Ag, Ab^*, and Ab concentrations, respectively.

$$\text{Ln}(k_1/k_2) = -[E_{act}/R]\ [T_2 - T_1/T_1T_2] \tag{2}$$

where k_1 and k_2 are the specific rate constants of the reaction at the absolute temperatures T_1 and T_2. R is the universal gas constant ($8.3145\,J \cdot mol^{-1} \cdot K^{-1}$).[13,14]

The distribution coefficient K_d was obtained from Equation (15).

$$K_d = [(A_o - A)/A] \cdot (V/m)\,ml/g \tag{3}$$

(A_o) is the initial activity, (A) is the activity of solution after equilibrium, (A_o – A) is the activity on solid sorbent in units of counts per minute (cpm), i.e., =B, (V) is the volume of solution in mL and (m) is the mass of solid sorbent in grams.

The standard changes in free energy, enthalpy, and entropy were obtained from the following Equations:[13,15]

$$\Delta G^\circ = -RT \ln K_d\,kJ/mol \tag{4}$$

$$\text{Log}\,K_d = (-\Delta H^\circ/2.303\,RT) + \text{constant} \tag{5}$$

$$\Delta H^\circ = -2.303\,R \times \text{slope}\,kJ/mol \tag{6}$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T\,J \cdot mol^{-1} \cdot K^{-1} \tag{7}$$

Both values, E_{af} and E_{ab}, are related to the enthalpy change of the reaction by Equation (8)[13]

$$\Delta H = E_{af} - E_{ab}\,kJ/mol \tag{8}$$

RESULTS AND DISCUSSION

Kinetic Studies

The main aim of the chemical kinetic studies is to deduce the reaction rate law and the order of the reaction under the experimental conditions. The method for determining the initial rate, V_o (i.e., rate at $t = 0$), is a principal in this respect in which it is the largest rate and the most readily measured. The initial rate (V_o) in units M/unit time of reaction is the slope of the line tangent to the curve at zero time, i.e., at the start of experiment ($t = 0$).[13]

Thermodynamic calculations enable us to predict whether or not a reaction will proceed to the right, as written, but there is a very important matter that thermodynamics cannot tell us about the rate at which a reaction will occur. Time is not a thermodynamic variable.[13]

Figures 1–9, show the variation of the binding percent from total activity (B/T%) with time (in hours) for the reaction of TSH with Ab and Ab* at different concentrations and temperatures (277, 298, and 310 ± 1 K). From the slopes of tangents of the obtained curves at $t = 0$, the initial rate V_o of the reaction in M/h was obtained at different reactions and different experimental temperatures. The obtained initial rates V_o from Figures 1–9 were drawn against reactant concentrations and represented in Figures 10–12. Straight lines were obtained in each figure. The order of reaction with respect to each reactant was obtained from the slope of respective lines and these obtained orders are tabulated

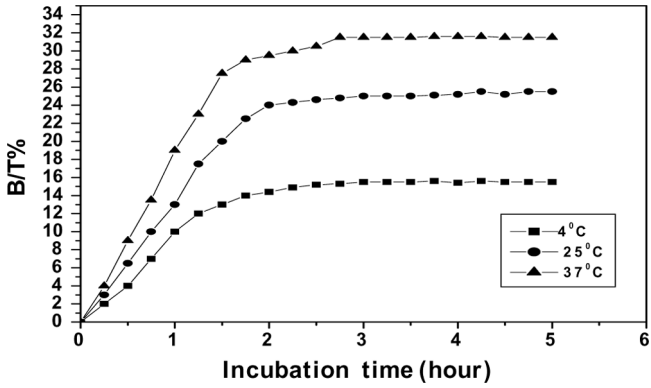

Figure 1. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at: $[Ag] = 1.37 \times 10^{-8}$ M, [Ab*] = (0.5X) M and [Ab] = 1:1000 dilution, (0.1Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

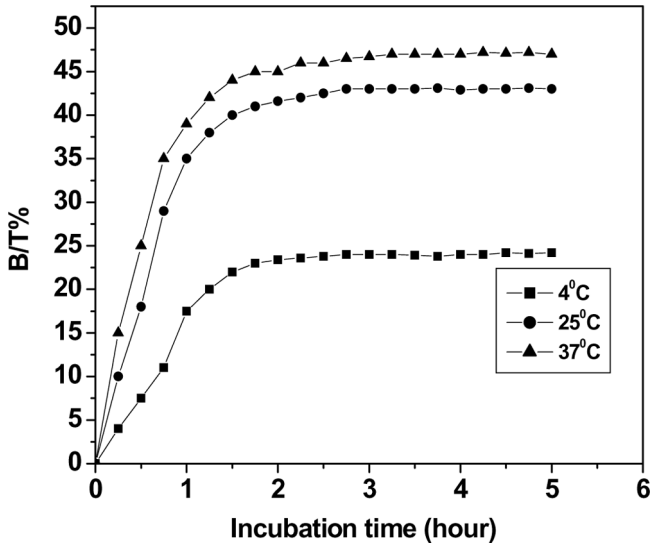

Figure 2. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at: $[Ag] = 6.84 \times 10^{-8}$ M, [Ab*] = (0.5X) M and [Ab] = 1:1000 dilution, (0.1Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

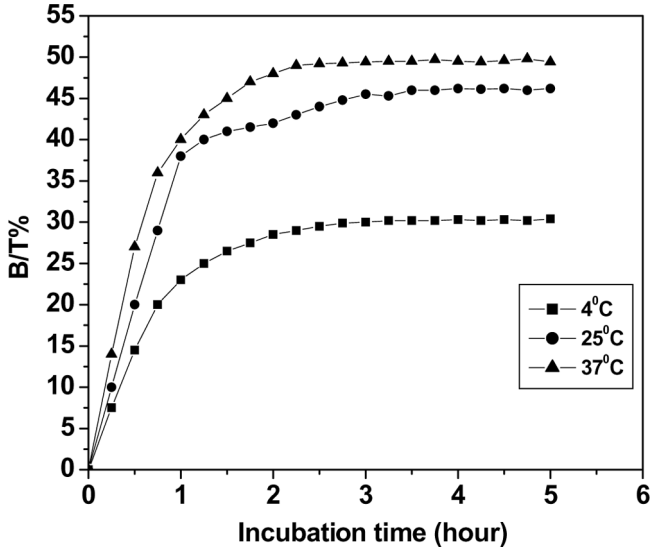

Figure 3. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at: $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.5X) M and [Ab] = 1:1000 dilution, (0.1Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

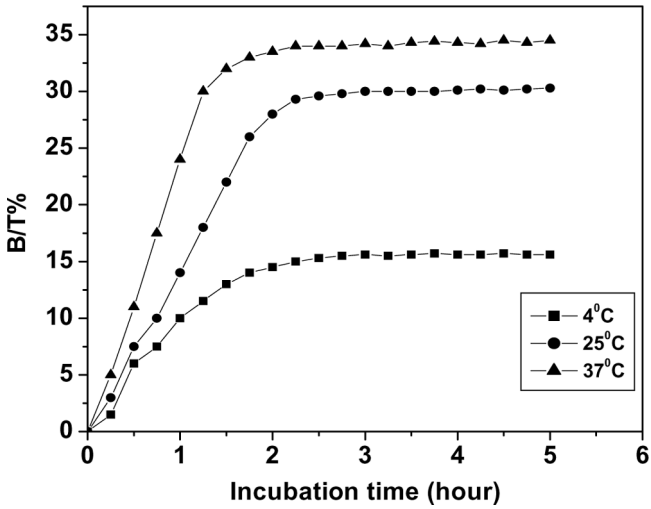

Figure 4. Variation of B/T % against incubation time at in reaction of TSH with both Ab and Ab* at: $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.25X) M and [Ab] = 1:1000 dilution, (0.1Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

in Table 1. From these data, the reaction rate law can be expressed as follows:

$$\text{Rate}\,(V_o) = k[Ag]^{0.486}[Ab^*]^{1.62}[Ab]^{0.023} \quad (1)$$

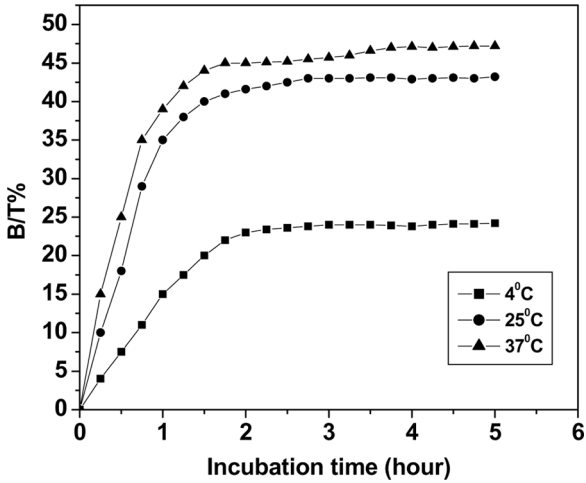

Figure 5. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at: $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.5X) M and [Ab] = 1:1000 dilution, (0.1Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

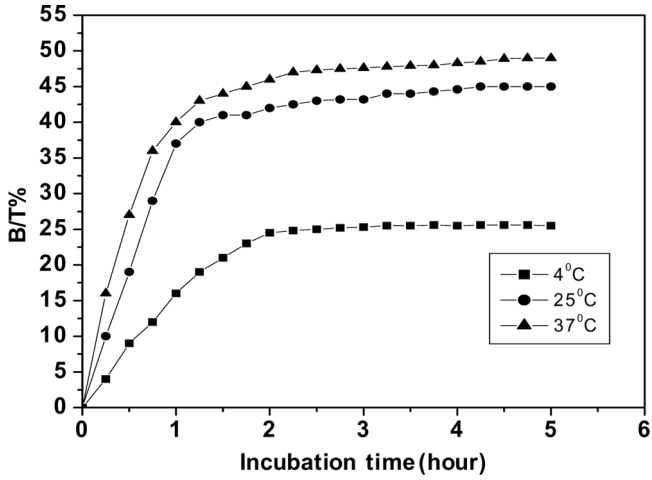

Figure 6. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at: $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (1.0X) M and [Ab] = 1:1000 dilution, (0.1Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

where k is the specific rate constant of the reaction and it varies with temperature like the rate (V_o). The overall order of the reaction was the summation of the respective orders of individual reactants and was found to be 2.13. According to this temperature, like rate (V_o) and k,

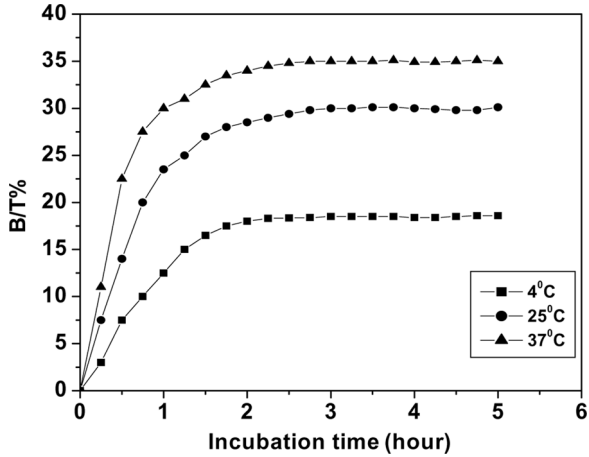

Figure 7. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.5X) M and [Ab] = 1:5000 dilution, (0.02Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

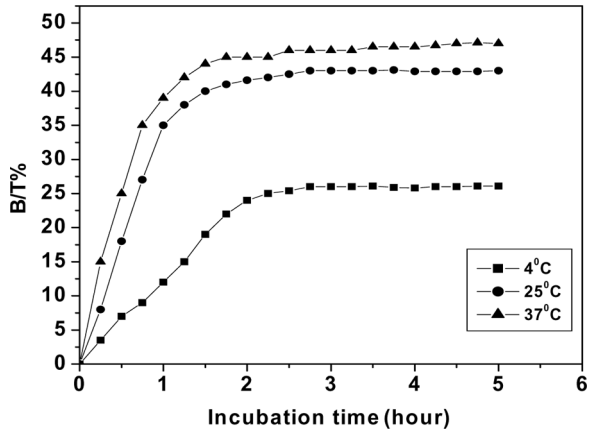

Figure 8. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at: $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.5X) M and [Ab] = 1:1000 dilution, (0.1Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

the activation energy of the reaction (E_{act}) was obtained according to the logarithmic form of the Arrhenius equation:[13]

$$\mathrm{Ln}(k_1/k_2) = -[E_{act}/R]\ [T_2 - T_1/T_1T_2] \quad (2)$$

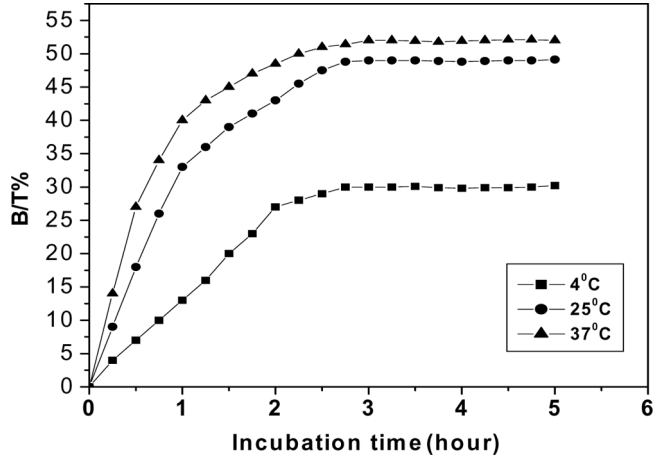

Figure 9. Variation of B/T% against incubation time in reaction of TSH with both Ab and Ab* at: $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.5X) M and [Ab] = 1:100 dilution, (1.0Y) M, at T = ■ 277 K; ● 298 K; ▲310 K.

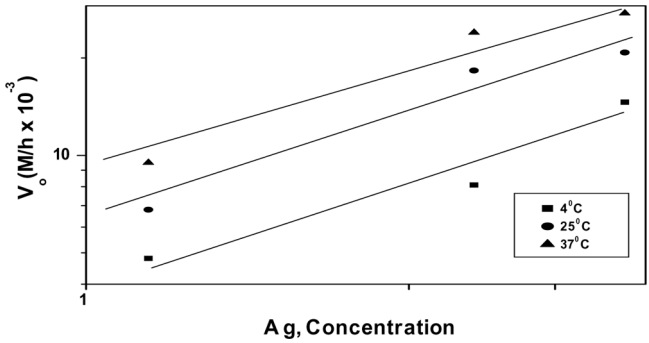

Figure 10. Effect of [Ag] on V_o at T = ■ 277 K; ● 298 K; ▲310 K, in reaction of TSH with both Ab and Ab*. [Ab] = 1:1000 dilution, (0.1Y) M and [Ab*] = (0.5X) M.

By applying Equation (1) at two different temperatures, V_{o1} and k_1 at T_1 and V_{o2} and k_2 at T_2 were obtained. By dividing the equation of V_{o1} by the equation of V_{o2} at the same Ag, Ab, and Ab* concentrations, k_1/k_2 was calculated and is expressed in Equation (2), from which the activation energy (E_{act}) of the reaction was calculated under the experimental conditions and found to be 31 ± 2.43 kJ/mol. This energy is necessary for reactants to reach the transition state or the activated complex to give the final product. It was found, from Figures 1–9, that B/T % was increased with increasing temperature, i.e., the binding percent B/T % at 310 K > B/T% at 298 K > B/T% at 277 K. The V_o values increased as temperature increased in all cases, as illustrated in Figures 10–12. It is well known that the order of a chemical reaction can be zero, an integer, or fractional, although it is

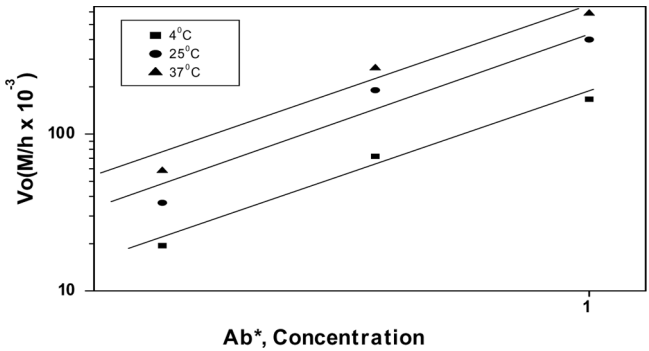

Figure 11. Effect of [Ab*] on V_o at T = ■ 277 K; ● 298 K; ▲310 K, in reaction of TSH with both Ab and Ab*. [Ag] = 1.37×10^{-7} and [Ab] = 1:1000 dilution, (0.1Y) M.

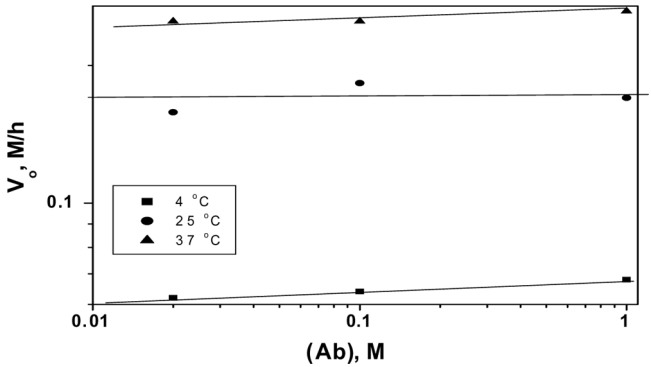

Figure 12. Effect of [Ab] on V_o at T = ■ 277 K; ● 298 K; ▲310 K, in reaction of TSH with both Ab and Ab*. [Ab*] = (0.5X) M and [Ag] = 1.37×10^{-7} M.

most frequently a small integer.[13,14] The results of Figures 3, 5, and 8 were obtained from the same assay performed using the same conditions but at different times, where the assay was performed at variable (Ag), (Ab*), and (Ab) concentration, these results indicated the better reproducibility (precision) of the assay, where the results of the three curves were closely correlated.

Thermodynamic Studies

The thermodynamic studies on this reaction were based on the obtained K_d values due to the reaction between the reactants and the coated beads. K_d (distribution coefficient) was calculated from Equation (3):

$$K_d = [(A_o - A)/A] \cdot (V/m)\ \text{ml/g} \tag{3}$$

$$\Delta G^\circ = -RT\ \ln K_d\ \text{kJ/mol} \tag{4}$$

Table 1. Experimentally obtained reaction orders (n) of Ag, Ab* and Ab at 277, 298 and 310 K

	n			
System	277 K	298 K	310 K	Average
Ag	0.495	0.50	0.462	0.486
Ab*	1.56	1.65	1.66	1.62
Ab	0.025	0.025	0.0184	0.023

Table 2. Thermodynamic data for the reaction of TSH (Ag) with Ab and Ab*

System	K_d (298)	Log K_d (298)	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° ($J \cdot mol^{-1} \cdot K^{-1}$)	$T\,\Delta S^\circ$ (kJ/mol)
(1) [Ag] = 1.37×10^{-7} M [Ab*] = 0.5X M(100 µl) [Ab] = 0.1Y M 1:1000 dilution	1.511 ± 0.049	0.1793 ± 0.011	−1.022 ± 0.065	+19.71 ± 0.106	+69.57 ± 0.467	+20.73 ± 0.00467
(2) [Ag] = 1.37×10^{-7} M [Ab*] = 0.5xM (100 µl) [Ab] = (1Y) M 1:100 dilution	1.72 ± 0.031	0.236 ± 0.007	−1.347 ± 0.37	+20.82 ± 0.079	+74.39 ± 0.3184	+22.17 ± 0.003184

ΔH° (the enthalpy change or the heat content change) was obtained from the logarithmic form of Van't Hoff Equation (10):[13]

$$\text{Log}\,K = (-\Delta H^\circ/2.303RT) + \text{constant} \tag{5}$$

Considering two investigated systems, the conditions of system (1) were of conditions $[Ag] = 1.37 \times 10^{-7}$ M, $[Ab^*] = 0.5X$ M for (100 µl) and $[Ab] = 0.1Y$ M for (1:1,000) and for system (2) were $[Ag] = 1.37 \times 10^{-7}$ M, $[Ab^*] = (0.5X)$ M for (100 µl) and $[Ab] = 1.0Y$ M for (1:100).

The obtained values of $\log K_d$ at different absolute temperatures, are given in Table 2. By plotting the obtained $\log K_d$ values together with their standard deviations (S.D.) versus the reciprocal of absolute temperatures, Figures 13 and 14 were obtained for systems (1) and (2), respectively. ΔH° values for both systems were calculated from the slopes of the obtained straight lines according to the following Equation (6):[13]

$$\Delta H^\circ = -2.303\,R \times \text{slope}\,\text{kJ/mol} \tag{6}$$

The entropy change of the reaction (ΔS°) was obtained from Equation (7):

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T\,J \cdot mol^{-1} \cdot K^{-1} \tag{7}$$

The obtained values ΔG°, ΔH°, ΔS° and $T\,\Delta S^\circ$ for the two experimented systems were also tabulated in Table 2. It is obvious from the data that the reaction of Ag with Ab and Ab* proceeded spontaneously

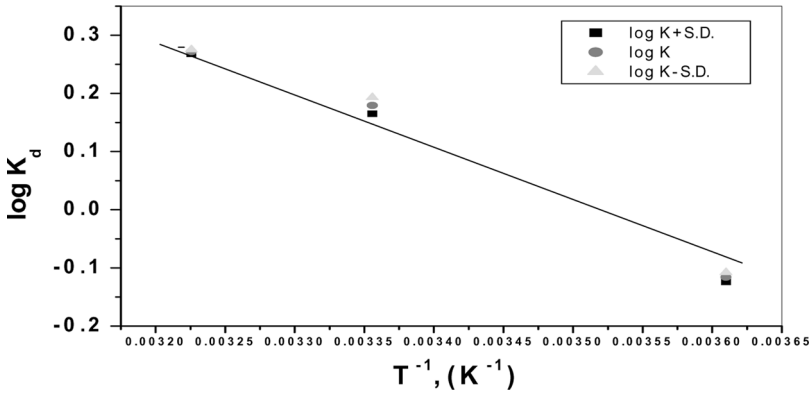

Figure 13. Log K_d against 1/T for reaction of Ag with Ab and Ab* at $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.5X) M and [Ab] = 1:1000 dilution, (0.1Y) M, System (1).

(negative ΔG° value), and the reaction was endothermic where B/T% increased with temperature, and this is thermodynamically obvious from the obtained positive ΔH° values of the two systems. Also, the reaction proceeded in the two systems with entropy gain (i.e., it is entropy favored or entropy driven). By comparing the data in the two systems, as illustrated in Table 2, it can be concluded that the system (2) of 1:100 dilution of antibodies on the coated beads (which has highest Ab concentration) was more optimum for this reaction, which gave the highest

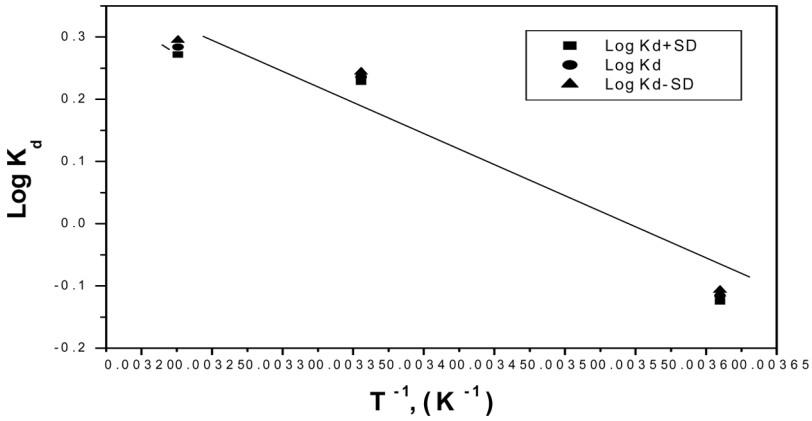

Figure 14. Log K_d against 1/T for reaction of Ag with Ab and Ab* at $[Ag] = 1.37 \times 10^{-7}$ M, [Ab*] = (0.5X) M and [Ab] = 1:100 dilution, (1.0Y) M, System (2).

binding percent at 310 K, and this was concluded from the thermodynamic data in Table 2, where higher K_d and $\log K_d$ values were obtained with more negative ΔG° values and more positive ΔS° values with comparable ΔH° values in between. The obtained ΔH° values were consistent with the obtained E_{act} values for these endothermic reactions where raising the temperatures facilitated the binding between Ag with Ab and Ab*.

The positive ΔH° value indicated an endothermic reaction and the obtained positive ΔH° and ΔS° values were the characteristics of inner-sphere complexes.[16]

The energy that the reactants must acquire to reach the activated state is the activation energy of the forward reaction, (denoted E_{af}). For the backward reaction to take place, the same activated state must be attained. The activation energy of the backward reaction is denoted E_{ab}.

$$\Delta H = E_{af} - E_{ab}\,\text{kJ/mol} \tag{8}$$

For an endothermic reaction, as in the present case, the reactants were at a lower potential energy than the products. The activated state was at a higher energy than either the reactants or the products. The relatively slower rates obtained with the investigated systems, due to the obtained fractional orders, can be understood in terms of the relatively higher activation energy which, consequently, has a higher potential energy barrier. By applying Equation (8) on the investigated systems and considering $E_{af} = 31 \pm 2.43$ kJ/mol and knowing ΔH° values, E_{ab} can be obtained for both systems as follows:

For system (1), $\Delta H^\circ = +19.71 \pm 0.106$ kJ/mol and
$E_{ab} = +11.29 \pm 1.9$ kJ/mol;
For system (2), $\Delta H^\circ = +20.82 \pm 0.079$ kJ/mol and
$E_{ab} = +10.18 \pm 1.92$ kJ/mol

Generally, The positive ΔH° values obtained for both systems are consistent with the obtained E_{act} values for these endothermic reactions, where raising the temperature facilitates the binding between Ag with Ab and Ab*, and requires higher activation energy to overcome the potential energy barrier in order to attain the transition state and to form the activated complex which instantaneously transformed to the products.[10]

Standard Curve

Complete standard curve under obtained thermodynamics and kinetics optimum conditions was obtained using set of standards (0.15, 0.5, 1.5,

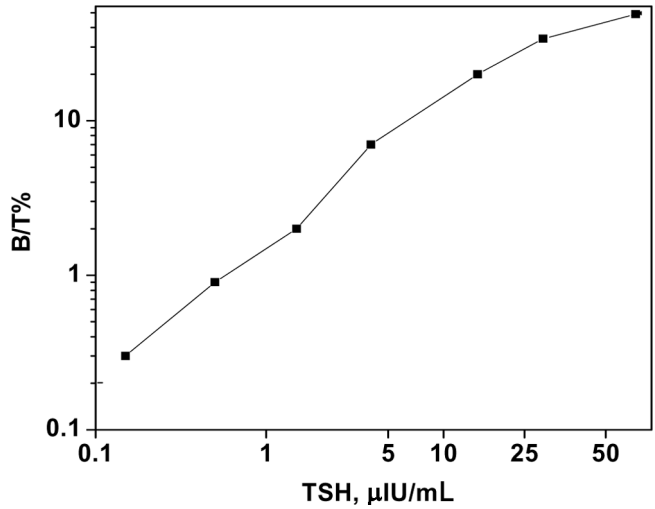

Figure 15. A standard curve constructed using the optimum conditions, Incubation temperature 310 K, incubation time 3 hrs, $[Ag] = 1.37 \times 10^{-7}$ M (100 µl of 60 µIU/mL), [Ab*] = (0.5X) M (100 µl of 31,600 cpm) and [Ab] = 1:100 dilution titer (1.0Y) M.

4.0, 15, 30, and 60 µIU/mL). Forty TSH human serum samples were assayed by the optimum conditions obtained from the present study and calculated from this standard curve, (Figure 15).

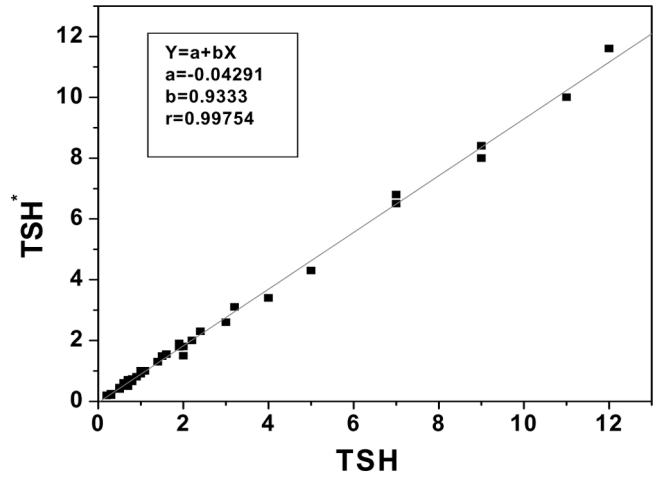

Figure 16. Regression line equation and correlation coefficient (r) between levels of TSH obtained by DPC method and the method which used the calculated parameters (TSH*).

Method Comparison

The results of patient samples of locally prepared system were compared to that of DPC IRMA coated tube system. Forty TSH human serum samples were assayed by the two systems and the TSH values (ranged from 0.2 to 12 μIU/mL) were calculated. From the obtained results, the linear regression (correlation coefficient) was calculated; (r) is equal to 0.99754, as indicated in Figure 16, and this result indicated high correlation between the two methods and the validity of the studied new method.

CONCLUSION

From the previously mentioned results, the following orders of the reaction were obtained, 0.486th order with respect to [Ag], 1.62th order with respect to Ab*, and 0.023th order with respect to Ab, the overall order of this reaction was $0.486 + 1.62 + 0.023 = 2.129$. At the incubation temperature 37°C, the optimum condition for the reaction between Ag, Ab, and Ab* were: $[Ag] = 1.37 \times 10^{-7}$ M (100 μl of 60 μIU/ml), [Ab*] = (0.5X) M (100 μl of 31,600 cpm), and [Ab] = 1:100 dilution titer (1.0Y M). The reaction proceeded spontaneously at the experimental temperature ($\Delta G^\circ = -1.347$ kJ/mol) and was entropy favored ($\Delta S^\circ = +$ 74.4 J/mol/K). The positive value of ΔH° (+20.82 kJ/mol) was matched with the required activation energy ($E_{act} = 31 \pm 2.43$ kJ · mol^{-1}). The positive value of ΔH° indicated an endothermic reaction and the obtained positive ΔH° and ΔS° values are the characteristics of inner-sphere complex compounds. The optimal conditions that were obtained from the previously mentioned study were used to perform the assay of TSH and the standard curve was constructed. Patient samples were measured using the prepared system and the results were compared to those of the commercially used one, where the results indicated the accuracy and sensitivity of the obtained parameters of the prepared system. Also, the results indicated the high correlation between the traditional (commercial) method and the studied one.

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