The Kinetic studies of LH Binding in Benign and Malignant Uterine Tumors Homogenate with ^{125}I-Anti LH Antibody.

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Summary

Kinetic studies were carried out on the uterine tumor homogenate. Time-Course of the association of ^{125}I-anti LH antibody with LH in benign and malignant uterine tumors at four different temperatures revealed the time and temperature dependency. Association kinetics indicated pseudo first order kinetics for the binding.

Introduction

The uterus is a muscular organ lying in the true pelvis between the bladder and the rectum [1] it can be divided in to three parts, the fundus, the cervix and the body of the uterus. The uterus supported on both sides by four sets of ligaments [2]. The uterus wall is composed of three layers Perimetrium, myometrium and endometrium [3]. The myometrium is the thickest of the three layers of the uterine wall and forms the major portion of the wall [4, 5]. The benign tumors of the uterus include endometriosis, adenomyosis, leiomyomas and endometrial polyps [6]. Although the tow main malignant tumors of the uterus cancers of the cervix and that of endometrium, this is the second most common cancer in the female genital organs while the cervix being the first [7,8].

Luteinizing hormone is a glucoprotiein hormone of an approximate molecular weight of 28 kDa, it is produced in the gonadotrophic cells of the pituitary. this hormone is responsible for the gametogenesis and steroidogensis in the gonads [9,10]. Luteinizing hormone place a key ruls in the control of reproductive function and act a specific in the cell membrance of target cells in the gonads[11].

Experimental

Chemicals

All laboratory chemicals and reagents were of analar grade. Tris buffer, were obtained from Fluka Company, Switzer land. Hydrochloric acid, were obtained from BDH limited pool, U.K. kit of radio active (^{125}I- anti LH antibody) was manufactured by ***Immunotech***, ***Abeckman coulter company***. The radioactivity of (^{125}I-anti LH antibody) was approximately 370 KBq.

Instruments

The instruments used in this work were, LKB gamma counter type 1270-Rack gamma II, centrifuge type Hitachi, Pye-Unicam pH meter.

Patients

Two groups of postmenopausal patients where include in this study, group I consisted of 10 postmenopausal patients with benign uterine, (Age = 51- 60 years), group II consisted of 10 postmenopausal patients with malignant uterine tumor, (Age = 54- 62 years).

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All patients were admitted for treatment to **(Medical City, Baghdad Teaching Hospital), Al-Yarmuk Teaching Hospital, Al Habibia Hospital,** under the supervision of specialists, **Dr. Nada Salih Ameen, Dr. Akram Al-Shareef, Dr. Wafaa Al- Omary.**

They were histologically proven from the supervision of specialists **Dr. Raja Al-Hadethy, Dr. Eman Alash**. The patients were newly diagnosed and not underwent of any type of therapy. Patients did not suffer from any disease that may interfere with our study were excluded.

Collections of Specimens

The tumor tissues were surgically removed from uterine patients by hysterectomy. The specimens were cut off and immediately rinsed with ice-cold isotonic saline solution. They were collected individually in plastic receptacles and stored at –20 °C until homogenization.

Preparations and Uterine Tissue Homogenate.

The frozen tissue was thawed, sliced finely with a scalped in petridish standing on ice bath. The slices were further minced with scissors, then homogenized at 4 °C in (Triss) buffer solution (0.01M) with ratio of 1:5 (weight:volume), using manual homogenizer. The homogenate was filtered through several layers of nylon gauze, and then centrifuged at 1500xg for 15 min in a cooling centrifuge at 4 °C. The supernatants were used through out our study.

Solutions

The Triss buffer solution (0.01M, pH = 7.6) was prepared by dissolving (0.303 gm) of Triss buffer in 200ml distilled water. The required pH was adjusted by adding HCl solution (0.2M), and then the volume was completed to 250ml by distilled water.

Methods

The kinetic Studies of LH Binding in Benign and Malignant Uterine Tumors Homogenate with ^{125}I-Anti LH Antibody.

The Time Course of The Binding of LH in Benign and Malignant Uterine Tumors Homogenate to ^{125}I-Anti LH Antibody

1- Twenty-five microliters (50µg protein) of postmenopausal benign tumor homogenate was added to 25µl (93.67µg protein) of ^{125}I-anti LH antibody in duplicate tubes. The volume was made up to 400µl with triss buffer pH 7.6
2- The reaction mixture was incubated at 25°C for several times intervals (10, 20, 30, 60, 90, 120 and 150 min).
3- After incubation, the tubes were centrifuged for 15min in order to separate the complex formed.
4- Supernatant was discarded by decanting the assay tubes. Then, the tubes were inverted on a filter paper for 10min.
5- Rims of the tubes were swabbed with cotton piece.
6- Amount of bound radioactivity (C.P.M) was counted in a gamma counter for one min.
7- Experiment above was repeated at different temperatures (4, 10 and 45 °C) for several time intervals (10, 20, 30, 60, 90, 120 and 150 min).
8- To determine the time course for other groups, postmenopausal malignant tumor homogenate (50µg protein) 20µl (74.93µg protein) of ^{125}I-anti LH antibody

and triss buffer, pH 7.4, and different time intervals (10, 20, 30, 60, 90, 120 and 150min).

Calculations

1- The counted radioactivity in each tube (expressed in C.P.M) represents the bound fraction (B), (^{125}I- anti LH antibody/LH) complex.

2- The counted radioactivity in the tubes containing ^{125}I – anti LH antibody only represents the total activity (T).

3- The B/T ratio for each tube was counted as follows :

$$(B/T)\% = \frac{\text{Sample mean counts (B)}}{\text{Total activity mean count(T)}} * 100$$

4- The percent of binding values were plotted against the different times of incubation at each temperature.

Determination of The Affinity Constant (K_d) and The Maximal Binding Capacity (B_{max}) of LH in Benign and Malignant Uterine Tumors Homogenate Associated with ^{125}I –Anti LH Antibody

1- Twenty-five microliters (50µg protein) of postmenopausal benign tumor homogenate was incubated with increasing volumes (10, 20, 25, and 30µl) of ^{125}I-anti LH antibody (37.47, 74.93, 93.67 and 112.39 µg protein). The final volume was made up to 400µl with triss buffer pH (7.6). The incubation was carried out at 25 °C for 120min.

2- After incubation, the tubes were centrifuged for 15min in order to separate the complex formed.

3- Supernatant was discarded by decanting the assay tubes. Then, the tubes were inverted on a filter paper for 10min.

4- Rims of the tubes were swabbed with cotton piece.

5- Amount of bound radioactivity (C.P.M) was counted in a gamma counter for one min.

6- Previous steps were performed at different temperatures (4, 10 and 45 °C). The times of incubation needed to get to the equilibrium state were as the following :

Temp(°C)	Time(min)
4	120
10	90
45	90

7- Steps 1 to 5 of this experiment were repeated by using postmenopausal patients with malignant tumor homogenate (50µg protein), triss buffer pH (7.4). The times of incubation needed to get the equilibrium state were 90 min at (4 °C), 60 min at (10 and 25 °C), and 120 min at (45 °C).

Calculations

1- The B/T % ratio was computed for each tube, where:

B: is the bound radioactivity mean counts (c.p.m), which represents the (^{125}I-anti LH antibody/LH) complex.

F: is the free radioactivity mean counts (c.p.m), which represents the non-bound ^{125}I-anti LH antibody.

T: is the total radioactivity mean of the counts.

F = Total counts (T) – Bound radioactivity (B)

2- The concentration of the (^{125}I-anti LH antibody/LH) complex in mg/ml that formed after time (t) was calculated from the following equation:

$$B(mg/ml) = \frac{B(c.p.m)}{T(c.p.m)} *$$

Concentration of ^{125}I- anti LH antibody in the incubation medium (mg/ml).

3- The affinity constant and maximal binding capacity were determined according to scatchard equation[12].

$$B/F = 1/K_d (B_{max} - B)$$

$$K_a = 1/K_d$$

Where:

K_a =Affinity constant.

K_d = Dissociation constant.

B_{max} = Maximal binding capacity.

4- The values of the ratio B/F were plotted against the values of the (B) in (mg/ml), give a linear relationship. The values of the affinity constant of the binding (K_a) at each temperature can be calculated from the slope of the straight line, while the value of the total concentration of LH (B_{max}) in Benign and malignant uterine tumor tissue was calculated from the intercept with the x-axis.

Results and Discussion

The Kinetic Studies of LH Binding in Benign and Malignant Uterine Tumors Homogenate with ^{125}I-Anti LH Antibody.

The Kinetics of Interaction of ^{125}I-Anti LH Antibody with Benign and Malignant Uterine Tumors.

To examine the characteristics of the binding of ^{125}I-anti LH antibody with LH in benign and malignant uterine tissue homogenate, the experiment was carried out at four temperatures and for different incubation time.

Figure (1 A and B) shows the time course of the formation of (^{125}I-anti LH antibody/LH) complex at four different temperatures (4, 10, 25 and 45°C). The concentration of the ^{125}I-anti LH anti body /LH complex that formed after time (t) was calculated from the following equation:

B(mg/ml) = [B(c.p.m)/T(c.p.m)] * Concentration of ^{125}I- anti LH antibody in the incubation medium in (mg/ml)

The results of the time-course patterns at different temperatures revealed that the binding of ^{125}I-anti LH antibody to LH in benign and malignant uterine tissue homogenate is a temperature and time dependent process with a maximum binding occurs at 25°C and the incubation time 120min for the postmenopausal patients with benign uterine tumor, and 25°C with 60min incubation for the postmenopausal patients with malignant uterine tumor.

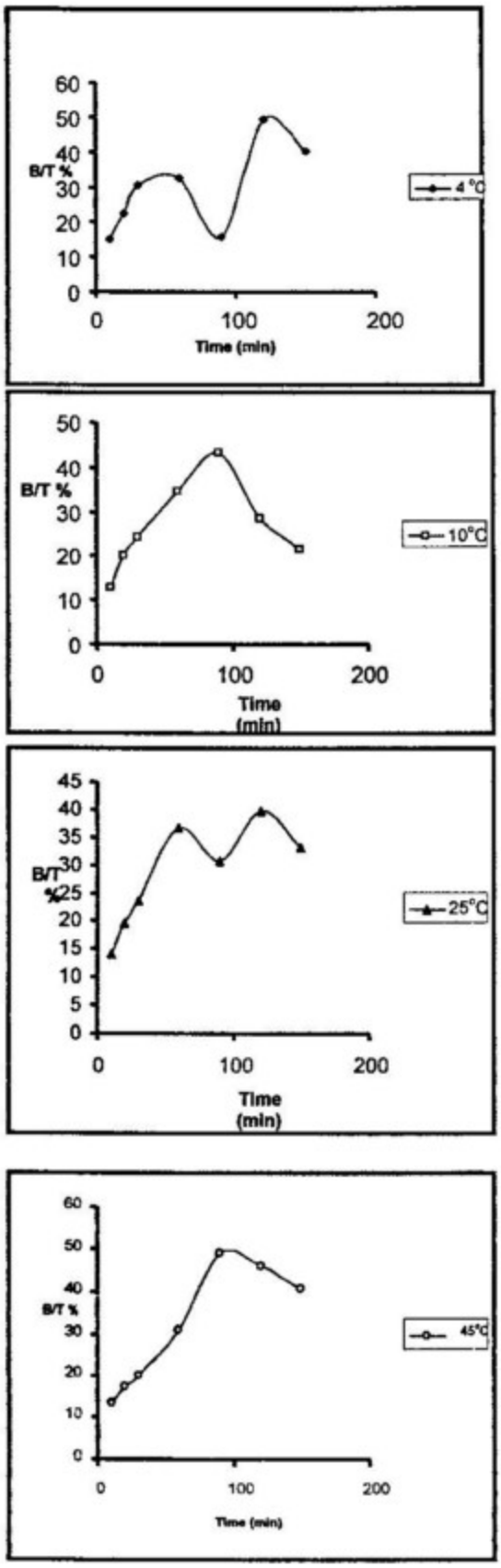


Figure (1): Time-course of ^{125}I-anti LH antibody binding with LH in:

A: Postmenopausal patients with benign uterine tumor

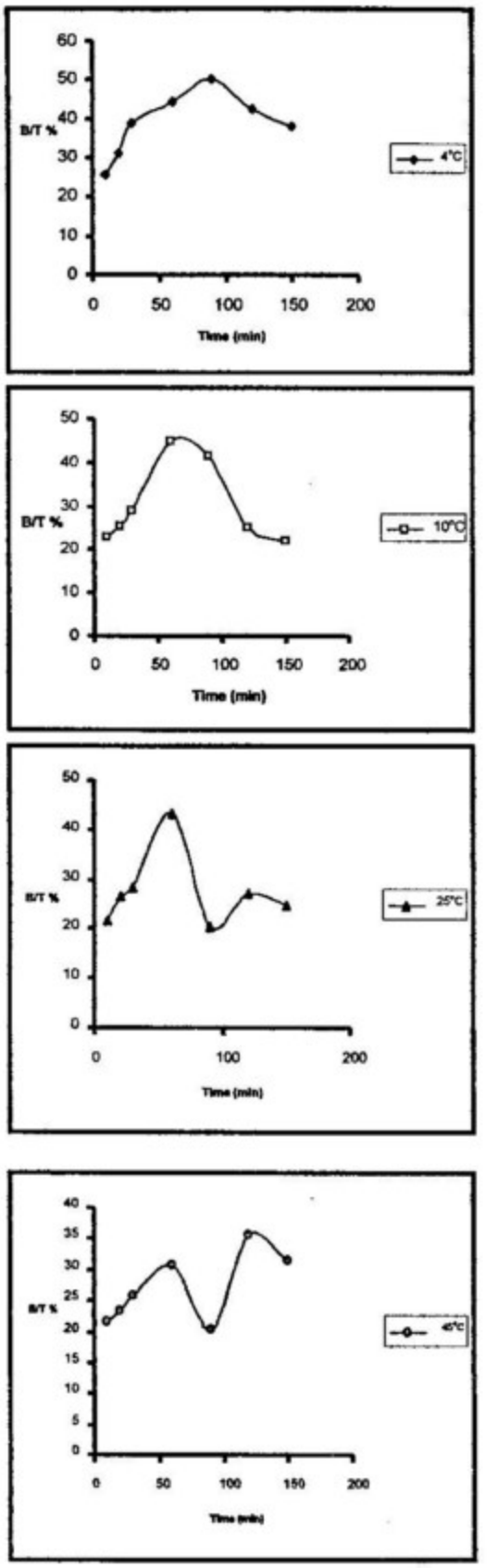


Figure (1): Time-course of ^{125}I-anti LH antibody binding with LH in:

B: Postmenopausal patients with malignant uterine tumor

Determination of Kinetic Parameters of ^{125}I-Anti LH Antibody Binding with LH in Benign and Malignant Uterine Tumors Homogenate

The time course of ^{125}I-anti LH antibody binding to LH in benign and malignant uterine tumor was carry out to describe the kinetic parameters of the binding.

The simplest proposed model representing the interaction of ^{125}I-anti LH antibody with LH could be expressed by the following equation:

$$^{125}\text{I-anti LH antibody} + \text{LH} \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} {}^{125}\text{I-anti LH antibody/LH}$$

(^{125}I-Ab) (Ag) (^{125}I-AbAg)

Where:-

K_{+1}: is the rate of the association of ^{125}I-anti LH antibody with LH.

K_{-1} : is the rate of the reverse reaction of the dissociation of the complex formed under the same condition.

At equilibrium: -

$$K_a = \frac{[\,^{125}\text{I- AbAg}\,]}{[^{125}\text{I- Ab}][\text{Ag}]} \quad \ldots\ldots\ldots\ldots\ldots(1)$$

$$K_d = \frac{[^{125}\text{I-Ab}]\,[\text{Ag}]}{[^{125}\text{I-AbAg}]} \quad \ldots\ldots\ldots\ldots\ldots(2)$$

Thus,

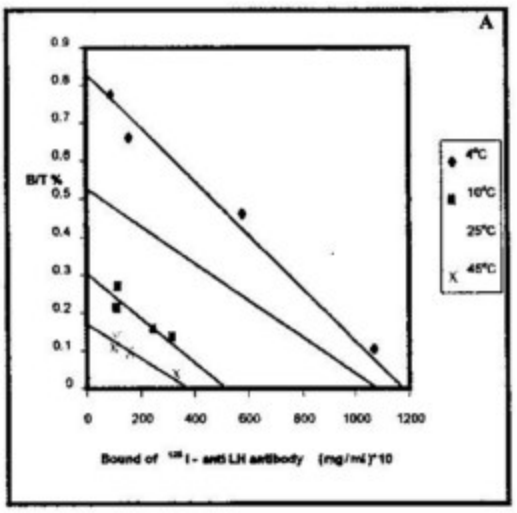
$$K_a = \frac{1}{K_d} = \frac{K_{+1}}{K_{-1}} \quad \ldots\ldots\ldots\ldots (3)$$

Where:-

K_a: is the equilibrium constant of the association (affinity constant).

K_d: is the equilibrium constant of the dissociation of (^{125}I-AbAg) complex.

The values of K_a and maximal binding capacity (B_{max}) were calculated form scatchard plot at four temperatures, as shown in Figure (2 A & B) and Table (1).



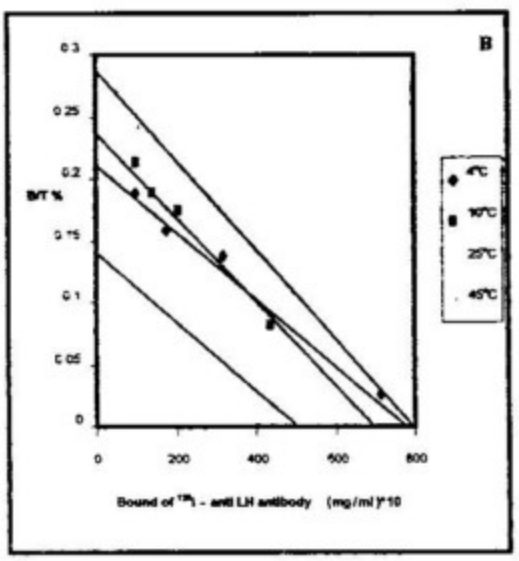


Figure (2): Scatchard plot of the ^{125}I-anti LH antibody binding with LH in:
A: Posmenopausal patients with benign uterine tumor.
B: Postmenopausal patients with malignant uterine tumor.

Table (1): The Kinetic parameters of ^{125}I-anti LH antibody binding to LH in benign and malignant uterine tumors.

Groups	Kinetic Parameters	Temperature (°C)			
		4	10	25	45
Postmeno pausal patients with benign uterine tumors.	Binding Capacity (mg/ml)	1.15	0.5	1.07	0.35
	$K_a = K_{+1}/K_{-1}$ (mg^{-1}.ml)	0.722	0.6	0.48	0.51
	$K_d = K_{-1}/K_{+1}$ (mg/ml)	1.14	0.67	2.06	1.95
Postmeno pausal patients with malignant uterine tumors.	Binding Capacity (mg/ml)	0.79	0.7	0.5	0.8
	$K_a = K_{+1}/K_{-1}$ (mg^{-1}.ml)	0.26	0.34	0.28	0.36
	$K_d = K_{-1}/K_{+1}$ (mg/ml)	3.76	2.92	3.57	2.75

Results in Table (1) show that K_a value at 4°C of postmenopausal patients with benign uterine tumors is higher than (10, 25 & 45°C) and the K_a value at 45°C of postmenopausal patients with malignant tumors is higher than (4, 10 & 25 °C). These results indicate that when the K_a value is high, then the binding affinity between LH and ^{125}I-anti LH antibody in benign and malignant uterine tumors is increased.

The value of K_d calculated by using equation (3) shows that the lowest K_d values of (^{125}I-anti LH antibody/LH) complex occurs at (10 °C) in benign postmenopausal uterine tumor homogenate and 45 °C for malignant postmenopausal uterine tumor homogenate.

Different equations are used for the determination of the association rate constant (K_{+1}) of ^{125}I-anti LH antibody with LH at four temperatures. These include the following:

$$\text{Ln}(\text{AbAg})e\left[\frac{(Ab)_T - (AbAg)_t(AbAg)_e / (Ag)_T}{(Ab)_T[(AbAg)_e - (AbAg)_t]}\right] = K_{-1}\, t\left[\frac{(Ab)_T(Ag)_T - (AbAg)_e}{(AbAg)_e}\right] \quad \text{-------(4)}$$

This equation could be simplified to equation (5) in order to fit the data of the first order kinetics [13].

$$\text{Ln}\frac{(AbAg)_e}{(AbAg)_e - (AbAg)_t} = K_{+1}\, t[(Ab)_T(Ag)_T / (AbAg)_e] \quad \text{----(5)}$$

Where: -

K_{+1}: is the kinetic association constant.

$(Ab)_T$: is the total concentration of ^{125}I-anti LH antibody.

$(Ag)_T$: is the total concentration of LH in uterine tissue homogenate.

$(AbAg)_e$: is the concentration of (^{125}I-anti LH antibody /LH) complex formed at equilibrium.

$(AbAg)_t$: is the concentration of (^{125}I-anti LH antibody /LH) complex formed after time (t).

Since in some cases of our work the percent of binding was small. And most of the ^{125}I-anti LH antibody remained free and only a small fraction of ^{125}I-anti LH antibody is bounded even at equilibrium (pseudo-first order conditions). So that the following equation could be used in order to fit the data of first order kinetic:

$$\text{Ln}\frac{(AbAg)_e}{(AbAg)_e - (AbAg)_t} = t\,K_{obs} \quad \text{------(6)}$$

Figure (3 A & B) shows that the plotting of

$$\textbf{Ln}\frac{\textbf{(AbAg)e}}{\textbf{(AbAg)e} - \textbf{(AbAg)t}}$$

against time (t) gives a straight line with a slope equal to the observed value of first order rate constant (Kobs) in min^{-1}. The association rate

constant (K_{+1}) was calculated from the following formula:

$$K_{obs}=K_{+1}\frac{(Ab)_T(Ag)_T}{(AbAg)_e} \quad \text{--------(7)}$$

The half-life time of association ($t\ ½$)ass., which represents the time needed for the formation of half amounts of the complex at equilibrium, was determined from the concentration of the complex at equilibrium and the time course curve, while the half-life time of dissociation $(t\ ½)_{diss.}$ was determined from:

$$(t\ ½)_{diss}=\frac{Ln\ 2}{K_{-1}}=\frac{0.693}{K_{-1}} \quad \text{---------(8)}$$

The values of Kobs, K_{+1}, K_{-1}, $(t\ ½)_{ass.}$ and $(t\ ½)_{diss.}$ at different temperatures are summarized in Tables (2).

Table (2):The effect of different temperatures on the kinetic parameters of ^{125}I-anti LH antibody binding with postmenopausal benign and malignant uterine tumors

Groups	Kinetic Parameters	Temperatures (°C)			
		4	10	25	45
Postmenopausal patients with benign uterine tumors	K_{obs} (min^{-1})	0.03	0.029	0. 037	0. 02
	K_{+1}(mg^{-1}.ml.min^{-1})	1.304	2.636	1.423	2.857
	K_{-1} (min^{-1})	1.806	4.393	2.928	5.558
	$(t½)_{diss.}\cdot10^{-2}$ (min)	38.4	15.8	23.7	12
	$(t\ ½)_{ass.}$ (min)	23	25	20	50
Postmenopausal patients with malignant uterine tumors	K_{obs} (min^{-1})	0.045	0.04	0.048	0.05
	K_{+1}(mg^{-1}.ml.min^{-1})	2.81	2.67	4.1	2.27
	K_{-1} (min^{-1})	10.56	7.77	14.28	6.25
	$(t½)_{diss.}\cdot10^{-2}$ (min)	6.5	8.9	4.8	11
	$(t½)_{ass.}$ (min)	10	11	12	9

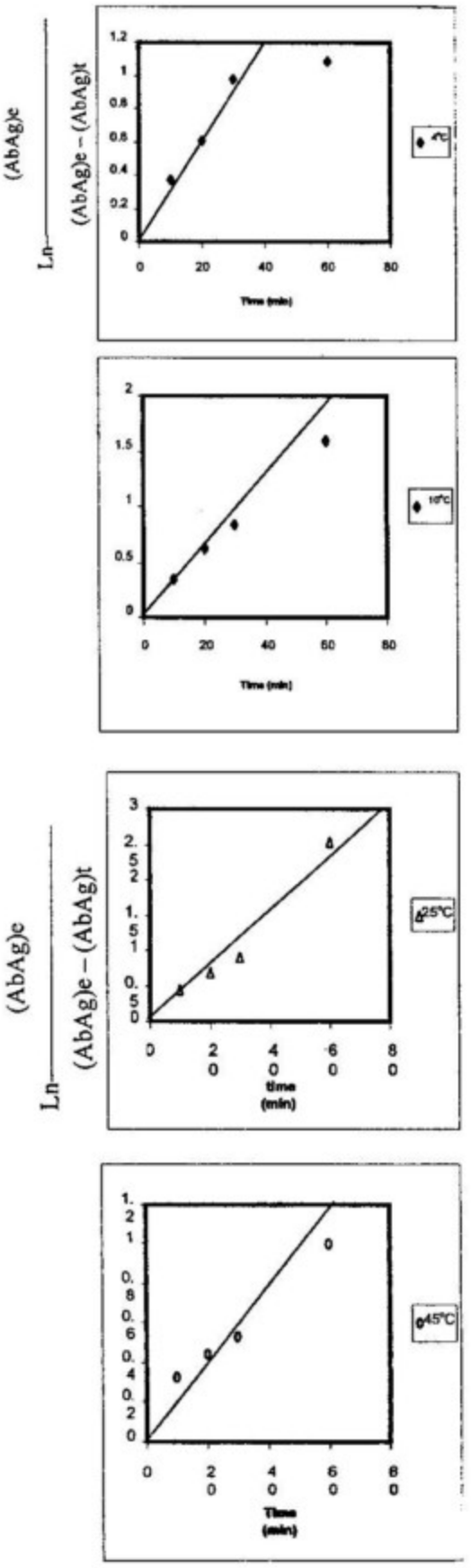


Figure (3): Kinetics of 125I-anti LH antibody binding with LH in: A: Postmenopausal patients with benign uterine tumor.

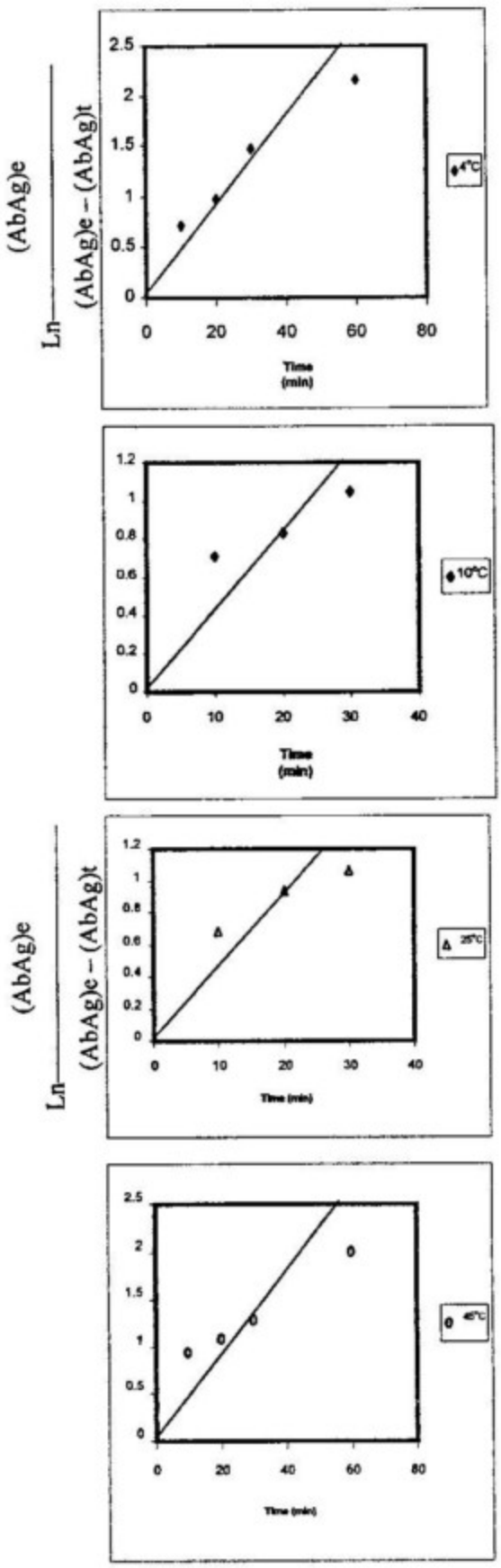


Figure (3): Kinetics of ^{125}I-anti LH antibody binding with LH in: B: Postmenopausal patients with malignant uterine tumor.

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الدراسات الحركية لارتباط الهرمون الليوتيني باورام الرحم الحميدة و الخبيثة مع الجسم المضاد (^{125}I-Anti LH)

هبة اعتماد نهاب *

***جامعة بغداد – كلية العلوم – قسم الكيمياء**

الخلاصة

أنجزت الدراسات الحركية على الرحم . المدى الزمني لارتباط LH بضده المعلم بنظير اليود المشع ذو العدد الكتلي 125 في الانسجة الورمية الحميدة و الخبيثه في اربع درجات حرارية مختلفة يعتمد على الحرارة و الزمن ، الرابطة الحركية تشير الى أن تفاعل الارتباط هو من المرتبة الاولى الكاذبة.