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RESEARCH ARTICLE

A new method for the determination of dissociation constant (kd) on the binding of CA19-9 to its antibody in type 2diabetic patients by enzyme linked immunosorbent assay (ELISA) with some modifications.

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Manuscript Info	***Abstract***

Background:-In order to quantify the interactions between molecules of biological interest, the determination of dissociation constants (Kd) is essential. Several methods are known for calculating these constants, most of which are based on linearization procedures, such as the Scatchard (1949) plot, Lineweaver and Burk (1934) plot, etc.
This linearization does not require direct ligand labelling or the absolute concentration of the complex and is, therefore, especially suitable for Kd determination from an enzyme-linked immunosorbent assay (ELISA).
Objectives: - Present a new method for the determination of a dissociation constant (Kd) of the binding of CA19-9 to its antibody in type 2 diabetic patients using Scatchard plot through development of ELISA.
Methods:- This study included eighty individuals with mean age (46.5 ± 1.14 years) were divided into two groups. Group Ⅰ ,forty patients with type Ⅱ diabetes mellitus have a mean duration 6.6± 0.94.Group Ⅱ consisted of forty healthy individuals were classified as control group. CA19-9 level were measured in serum by ELISA technique.Select the highest value from the first group and other value within the normal range chosen from the second group in addition to the CA19-9 standard (100U/ml) to determine the dissociation constant (kd) using external CA19-9 monoclonal antibody and calculated by Scatchard plot.
Results:- The dissociation constant of the interaction between antibody and antigen from the data of direct, non-competitive enzyme linked immunosorbent assays (ELISA) by Scatchard plot is (15.6006, 12.5313 and 4.1271 U/ml) for Standard (100 U/ml), Patient (99.568 U/ml) and Control (23.494 U/ml) respectively.
Conclusion:-A simple linearization procedure developed to determine the dissociation constant of the interaction between antibody and antigen from the data of direct, non-competitive enzyme linked immunosorbent assays (ELISA) by Scatchard plot.

List of abbreviation :-
CA19-9: Carbohydrate antigene CA 19-9, T2DM: Type Ⅱ diabetes mellitus, kd: Dissociation constant.

Introduction:-
In 1976, Koprowski et al.[1] determined that carbohydrate antigen 19-9 (CA19-9) is a tumor-associated antigen. CA19-9 was originally defined by a monoclonal antibody produced by a hybridoma prepared from murine spleen cells immunized with a human colorectal cancer cell line.

In humans, CA19-9 is expressed by the exocrine pancreas in vivo, and is elevated in the blood of many patients with pancreatic cancers, cancers of the upper gastrointestinal tract, ovarian cancer, hepatocellular cancer and colorectal

cancer [2]. Furthermore, this antigen has a high value during the diagnosis of pancreatic cancer, because it has a sensitivity of 70–90% and a specificity of 68–91% [3].

CA19-9 levels are higher in patients with diabetes than in non-diabetic controls, as well as in patients with poor glycemic control, relative to those with good glycemic control [4–9].

Diabetes has been claimed to be a risk factor for pancreatic cancer, which is increasing its incidence and has one of the lowest survival rates of all cancers[10].

In order to quantify the interactions between molecules of biological interest, the determination of dissociation constants (Kd) is essential. Several methods are known for calculating these constants, most of which are based on linearization procedures, such as the Scatchard (1949) plot [11], Lineweaver and Burk (1934) plot [12], etc. The main advantage of linearization methods is their simplicity. Previously, a simple linearization procedure was developed to determine the Kd of antigen –antibody (Ag – Ab) interactions [13,14]. This linearization does not require direct ligand labelling or the absolute concentration of the complex and is, therefore, especially suitable for Kd determination from an enzyme-linked immunosorbent assay (ELISA).

Sang-Han Lee et al., 1996 obtained the dissociation constant (Kd) of antigen-antibody complex using Scatchard equation through direct ELISA [15].

In this study we present a new method for the determination of a dissociation constant (Kd) of the binding of CA19-9 to its antibody in type 2 diabetic patients using Scatchard plot through development of directnon-competitive enzyme linked immunosorbent assays (ELISA).

Methods:-
The study was executed during the term from February 2015 to May 2015. The study included eighty individuals (35 male and 45 female) have a standard error of mean age (mean ± SE) 46.5 ± 1.14 years, were divided into two groups. Group Ⅰ ,forty patients with type Ⅱ diabetes mellitus have a mean duration 6.6± 0.94. Group Ⅱ consisted of forty healthy individuals were classified as control group.

Blood sample was collected from all 80 subjects from Al-Imamain Al-Kadhimain Medical City, Baghdad, Iraq. The approval of the Al-Nahrain University/ college of Medicine Research Ethics Committee.

Patients with any malignancies or suffered from pancreatic, thyroid, liver, and renal diseases in their medical history, pregnant women and smokers were excluded.
Blood samples were centrifuged at 3000 rpm and serum was stored at -20°C. Serum CA 19-9 level was measured by monoclonal antibody Enzyme Linked Immuno Sorbent Assay (ELISA) technique. Select the highest value from the first group and other value within the normal range chosen from the second group in addition to the CA19-9 standard (100U/ml) to determine the dissociation constant (kd) using external CA19-9 monoclonal antibody and calculated by Scatchard plot. The data obtained was analyzed by Microsoft excel 2013.

Results:-

Standard solution (0, 10, 50, 100, 250, 500U/ml) of CA 19-9 ELISA kit used to determine the standard curve as figure (1):
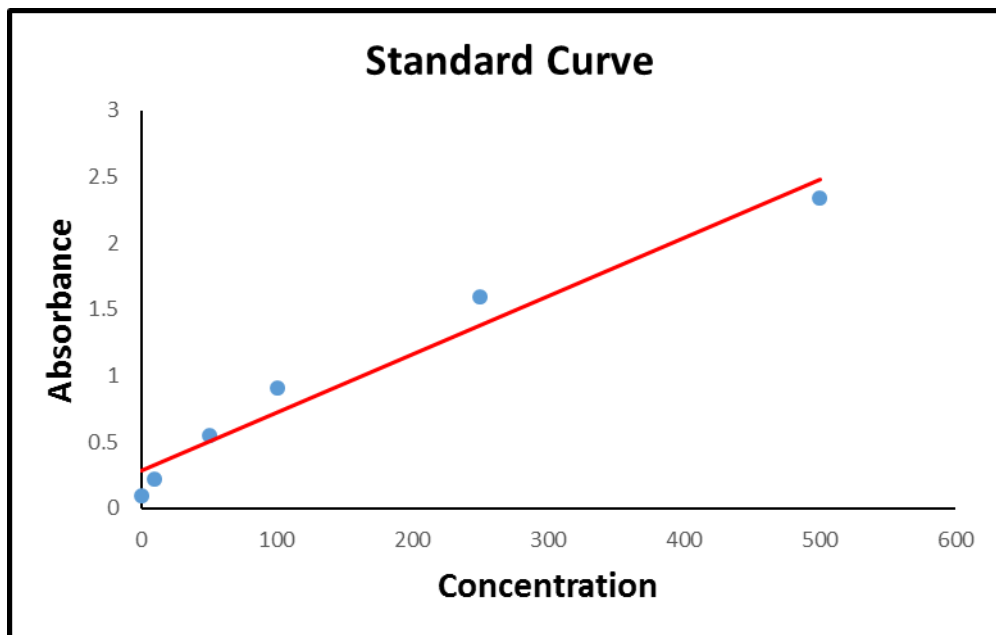

Figure (1):Standard Curve

The original concentration of Monoclonal antibody solution was 100 μg /100 μl, as a result when made up the volume with 0.01M Phosphate Buffer Saline to 1 ml the concentration become 10 μg/ml = 0.01 mg/ml and used the last as a stock solution.

Table (1):- Monoclonal Antibody dilution

No.	Dilution	Absorbance	Concentration (U/ml)
1	25 μl of Ab	0.135	0.020
2	20 μl of Ab + 5 μl of PBS	0.142	0.033
3	15 μl of Ab + 10 μl of PBS	0.169	0.232
4	10 μl of Ab + 15 μl of PBS	0.218	8.240
5	5 μl of Ab + 20 μl of PBS	0.379	21.634
6	25 μl of PBS	0.905	100.002

To find the concentration of Ab in each well used the following equation:-

$$\text{Conc. Of Ab in the well} = \frac{\text{Volume of Ab in the well}}{\text{Total incubation volume}} \times \text{Conc. Of Ab (stock solution)}$$

Table (2):- The concentration of Monoclonal Antibody in each well

No.	vol. of Ab (μl)	Total incubation volume (μl)	Conc. Of Ab (stock solution) (mg/ml)	Conc. Of Ab in the well (mg/ml)
1	25	50	0.01	0.005
2	20	50	0.01	0.004
3	15	50	0.01	0.003
4	10	50	0.01	0.002
5	5	50	0.01	0.001

- To find the concentration of Ag equivalent to the Ab concentration in each well applied the following equation:-

$$\text{The equivalent conc. of Ag} = \frac{\text{Conc. of Ab in this well}}{\text{Conc. of Ab in stock solution}} \times \text{Conc. of Ag}$$

Table (3):- The equivalent concentration of Ag for CA19-9 standard (100U/ml)

No.	Conc. Of Ab in this well (mg/ml)	Conc. Of Ab in stock solution (mg/ml)	Conc. Of Ag (U/ml)	Equivalent conc. of Ag (U/ml)
1	0.005	0.01	100.002	50.001
2	0.004	0.01	100.002	40.0008
3	0.003	0.01	100.002	30.0006
4	0.002	0.01	100.002	20.0004
5	0.001	0.01	100.002	10.0002

- To calculate the x-axis values (Bound) applied the equation below:-

$$\text{Bound} = \frac{\text{Absorbance of Ab}}{\text{Absorbance of PBS}} \times \text{The equivalent conc. of Ag}$$

Table (4):- *Bound value (x-axis in scatchard plot) for CA19-9 standard (100U/ml).*

No.	Absorbance of Ab	Absorbance of PBS	Equivalent conc. of Ag (U/ml)	Bound (x-axis value) (U/ml)
1	0.135	0.905	50.001	7. 4587
2	0.142	0.905	40.0008	6. 2763
3	0.169	0.905	30.0006	5.6023
4	0.218	0.905	20.0004	4.8177
5	0.379	0.905	10.0002	4.1879

- To calculate the free values used the following equation:-

> Free value = (Total Concentration) – Conc. Of Ab (Bound value)

Table (5):- Free and (Bound/free) values for CA19-9 standard (100U/ml).

No.	Conc. Of PBS (Total Concentration) (U/ml)	Conc. Of Ab (Bound value) (U/ml)	Free value (U/ml)	Bound/Free (B/F)(y-axis value)
1	100.002	0.020	99.982	0.0002
2	100.002	0.033	99.969	0.0003
3	100.002	0.232	99.77	0.0023
4	100.002	8.240	91.762	0.0897
5	100.002	21.634	78.368	0.276

Scatchard plot:-
Applied the following equation to find dissociation constant Kd:-

$$\frac{B}{F} = \frac{1}{K_d} \times (B_{max} - B)$$

Where:

B= The bound of CA19-9 to its immobilized antibody (B) that can be obtaining from ELISA curve.
F= the free concentration of CA19-9 which represent the first incubation in solution that can be deduced from the total binding (TB) of CA19-9 obtained from the standard curve of sandwich ELISA and B.
Where
F= TB-B
Kd = The dissociation constant

Bmax= The maximal binding capacity.

The plot of B/F ratios vs. the B values gives a linear relationship. The value of the dissociation constant of the binding Kd can be calculated from the slope of the straight line.
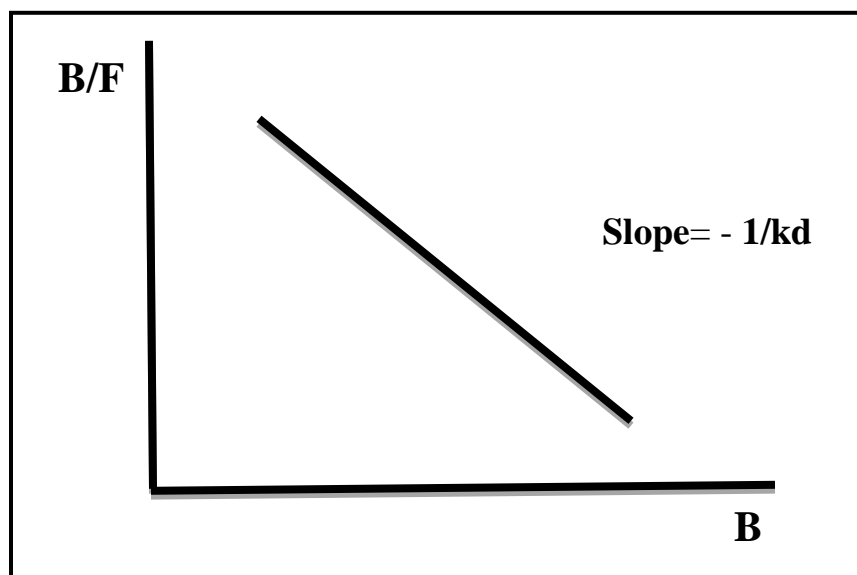

Figure (2): Scatchard plot
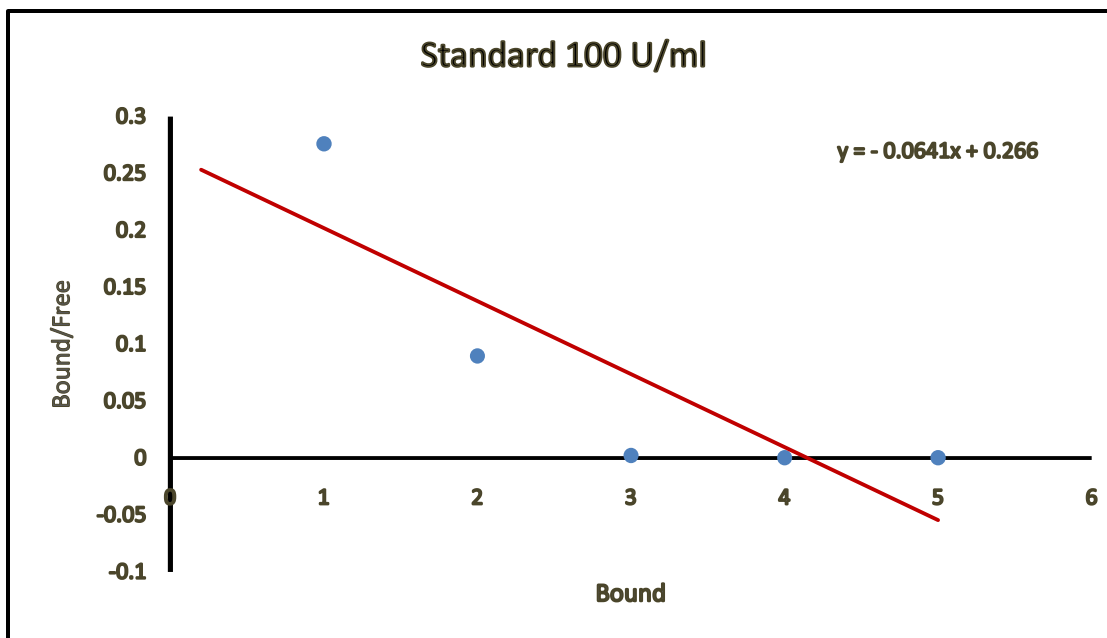
Standard 100 U/ml

$y = -0.0641x + 0.266$

Figure (1):Scatchard plots of standard CA19-9 (100U/ml)
Then Kd value equal 15.6006 U/ml.

The same previous equations were applied for patient has a CA19-9 level (99.568U/ml) and for healthy individual has a CA19-9 level (23U/ml), which were gave the following results:-
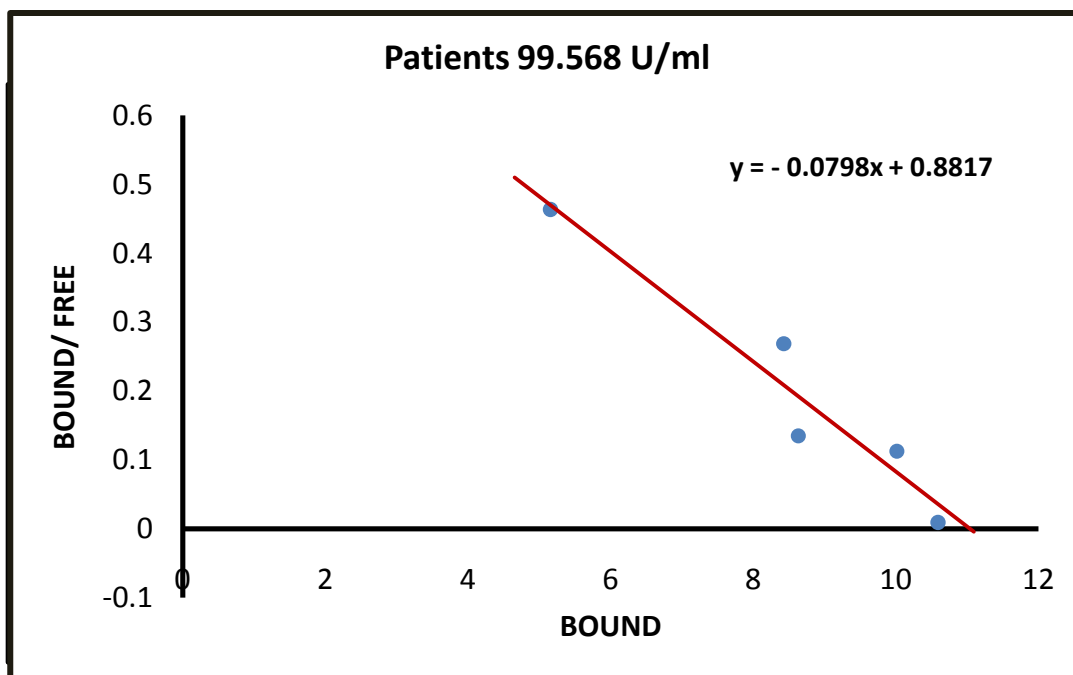
Patients 99.568 U/ml

$y = -0.0798x + 0.8817$

Figure (2): Scatchard plot of patient has a CA19-9 level (99.568U/ml)
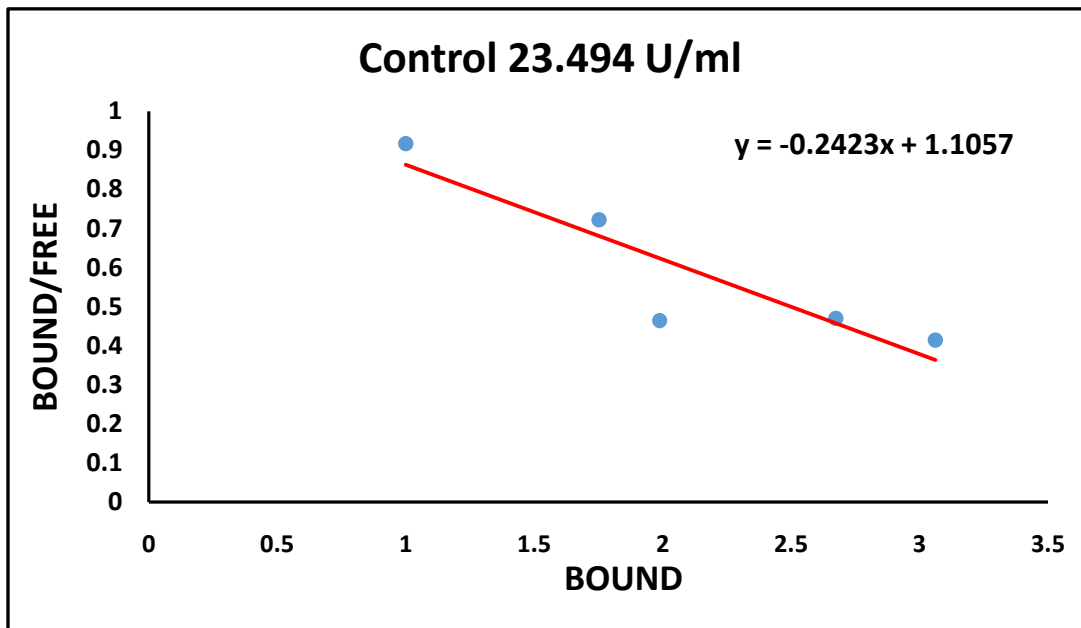
Then Kd value equal 12.5313 U/ml

Figure (**Error! No text of specified style in document.**): Scatchard plot for Healthy individual has a CA19-9 level (23.494 U/ml).

Then Kd value equal 4.1271 U/ml

Table (6):- Summary of Scatchard plot

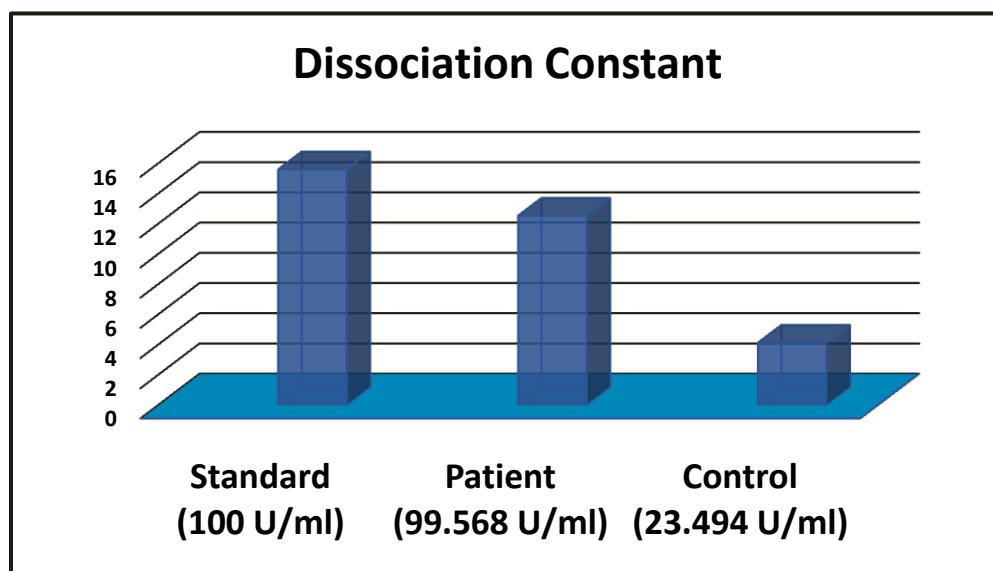| Cases	Slope	Kd (U/ml)	B max
Standard (100 U/ml)	- 0.0641	15.6006	0.266
Patient (99.568 U/ml)	- 0.0798	12.5313	0.8817
Control (23.494 U/ml)	- 0.2423	4.1271	1.1057

Figure (3):- Summary of Scatchard plot

Bradford method is used to determine the concentration of dissociation constant in µg/ml instead of U/ml, the unknown concentration was extracted from its absorbance using standard curveas showed in figure (4)
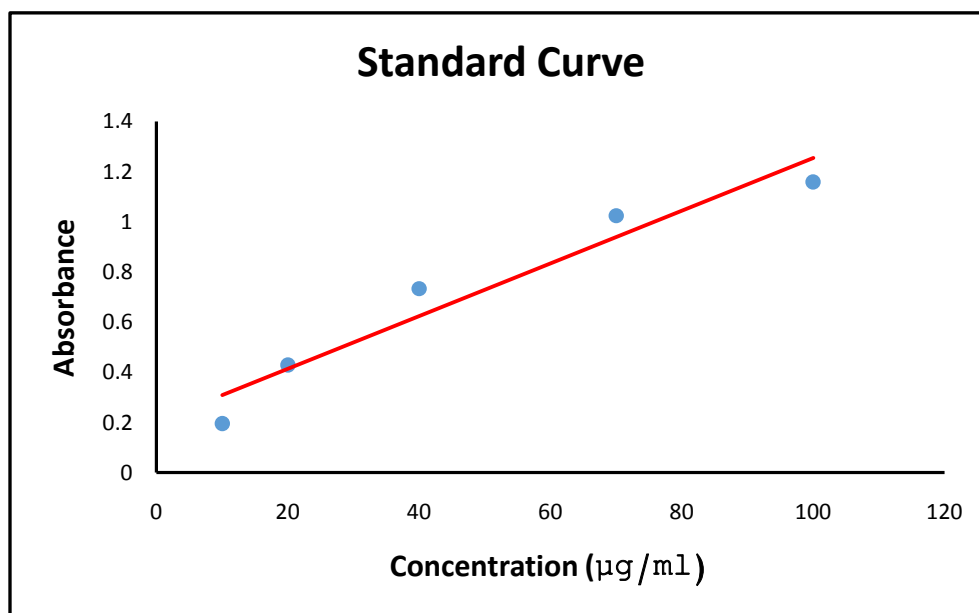

Figure (4): Standard Curve of Bradford method

Then the converter factor can be used to convert the concentration of carbohydrate antigen CA19-9 from (U/ml) to (µg/ml) is **1.010388**

Table (6):-Dissociation constant (Kd) with different units

Dissociation constant Kd (U/ml)	Dissociation constant Kd (μg/ml)	Dissociation constant Kd (mg/ml)
15.6006	15.76266	0.01576266
12.5313	12.6615	0.0126615
4.1271	4.16997	0.00416997

Discussion:-

Enzyme-linked immunosorbent assay (ELISA) is one of immunoassay methods using antibody to capture an antigen (target antigen) then using an enzyme labeled antibody for estimate the antigen amount. [16]

It is widely used as a clinical diagnostic tool to detect a vast range of diseases from infection diseases to cancer biomarkers. ELISA instrument is described as a versatile, precise, quantifiable and sensitive diagnostic method. [17, 18]

Several methods are known for calculating dissociation constant, most of which are based on linearization procedures, such as the Scatchardplot (1949) [11], Lineweaver and Burk (1934) plot[12], etc.

The present study suggest a simple linearization procedure developed to determine the obvious dissociation constant of the interaction between antibody and antigen from the data of direct, non-competitive enzyme linked immunosorbent assays (ELISA) by Scatchard plot.
FerencOrosz and JuditOva´di concluded no linearization procedure has been described for determination of dissociation constant (Kd) from displacement ELISA [14].

Liliom, K et al. described a linearization procedure for determination of dissociation constants (kd) of antigen–antibody interaction using data from the enzyme-linked immunosorbent assays (ELISA). [13]

Katsumi discussed the impact of dissociation constant on the standard curve from the below equation of antigen-antibody interaction,

$$Kd = (H - b)x(R - b)/b$$
$$b2 - (Kd + H + R)b + RH = 0$$
$$b = \frac{Kd + H + R - \sqrt{(Kd + H + R)2 - 4HR}}{2}$$

Where antibody concentration: R, bound antigen concentration: b, initial antigen concentration: H, dissociation constant: Kd (unit:M)
And conclude that the standard curve shift to right depending on the value of dissociation constant (Kd), which indicates the sensitivity of assay is related to Kd. Therefore if Kd is small (the affinity constant is large), the sensitivity becomes excellent.[16]

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Author contribution:-
The authors are grateful to the staff of Chemistry and Biochemistry Department and Center of Diabetes and
Dr. Hassan H. AL-Saeed suggests the study; Dr.MahmoodShakirKhudhair select the suitable patients and both of them co-writes the manuscript for study and Miss Russul R. AL-Hamaoy collected the blood samples, conducted the necessary analysis of the study, writes the paper and analyzed the results statistically.

Conflict of interest:-

There was no conflict of interest.

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