Preliminary Studies for the Binding of ^{125}I –Anti CA 15-3 Antibody to the CA 15-3 in Human Breast Tumor Homogenate[+]

دراسات تمهيدية لارتباط الضاد المتخصص ^{125}I –Anti CA 15-3 Antibody مع CA 15-3 في مجانسات اورام الثدي في الانسان

Raad K. Muslih*[**] Sami A. AL-Mudhaffar[*] Salwa H.N.Al-Rubae'I

Abstract:

This study include the level of CA15-3 was determined in sera of (32) premenopausal malignant breast tumors patients, (15) postmenopausal malignant breast tumors patients, and (40) benign breast tumors patients matched with one group of (10) healthy women as control group by Immunoradiometric Assay (IRMA). The data obtained demonstrated highly significant increase (P<0.0001) in patients with malignant breast tumors, whereas slightly increase (P<0.05) in patients with benign breast tumors when matched with normal women. A modified Imunoradiometric Assay (IRMA) was used for determination of cytosolic carbohydrate antigen 15-3, using ^{125}I-anti CA15-3 antibody and found to be suitable for the assessment of those antigens in benign and malignant breast tumors. The data revealed an increment of CA15-3 in the cytosolic fraction in comparison to the nuclear fraction.

The binding of ^{125}I –anti CA 15-3 antibodies with CA 15-3 was studied in three groups: benign breast tumor (Fibroadenoma), pre-and post- menopausal malignant breast tumors (IDC). The optimum conditions measured for the binding of three groups were included :CA 15-3 concentration in tissue homogenate , ^{125}I -anti CA 15-3 antibody concentrations, temperature, time of the incubation, and optimum pH of the binding. The use of different halides and different divalent salts was shown to different effect on the binding between CA 15-3 and ^{125}I –anti CA 15-3 antibody .

المستخلص:

تضمنت هذه الدراسة تقدير مستوى الـــ CA15-3 في امصال مجموعتين من النساء المصابات باورام الثدي الخبيثة تتكون المجموعة الاولى من (٣٢) مصابة بأورام الثدي الخبيثة قبل انقطاع الطمث والمجموعة الثانية من (١٥) مصابة بأورام الثدي الخبيثة بعد انقطاع الطمث وضمت المجموعة الثالثة (٤٠) مصابة بأورام الثدي الحميدة بالاضافة الى مجموعة رابعة شملت (١٠) نساء طبيعيات كمجموعة سيطرة . تم اعتماد طريقة الاختبار الاشعاعي المناعي المتري (IRMA). لوحظ زيادة معنوية كبيرة (P<0.0001) في مستويات CA 15-3 لدى النساء المصابات باورام الثدي الخبيثة وزيادة معنوية نسبياً (P<0.050) لدى النساء المصابات باورام الثدي الحميدة مقارنة بالنساء الطبيعيات.

تم تطوير طريقة الاشعاع المناعي المتري (IRMA) بتقدير الـــCA15-3 السايتوسولي باستخدام االضاد المتخصص (^{125}I-anti CA15-3 antibody). وقد وجد ان هذه الطريقة مناسبة لتقدير هذه المستضدات في اورام الثدي الخبيثة والحميدة. اشارت النتائج الى زيادة انتشار هذه المستضدات في الجزء السايتوسولي عن النووي . تم دراسة

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[*]Assistant Professor /College of Science, Al-Mustansiriya University

[**]Professor/ College of Science, Baghdad University

تفاعلات الارتباط للـــCA15-3 السايتوسولي مع االضاد المتخصص (^{125}I-anti CA15-3 antibody) وتثبيت الظروف المثلى للتفاعل المتضمنة: تركيز CA15-3 في المجانس النسيجي وتركيز الضاد المتخصص ^{125}I-anti CA15-3 ودرجة الحرارة وزمن حضن التفاعل وpH المثلى للارتباط. وجد هناك تاثيرات مختلفة للهاليدات وبعض املاح العناصر ثنائية التكافوء على الارتباط بين الـــCA15-3 السايتوسولي مع االضاد المتخصص (^{125}I-anti CA15-3 antibody) باستخدام الظروف المثلى.

Introduction:

Tumor markers are one of the methods of investigation of cancer, the role of tumor markers in breast cancer is to enhance the clinicians, ability to provide more effective management of the disease [1]. Serum CA15-3 concentration was determined by using sandwich enzyme immunoassay of a double monoclonal antibody (ELISA)[2], automated chemiluminescent immunoanalyzer [3], immunoradiometric assay [4], and radioimmunoassay [5] in women with benign breast tumor and breast cancer.

CA 15-3 has been used in management of patients, with breast cancer. CA 15-3 has been evaluated for its ability to determine diagnosis, prognosis, monitor therapy and predict recurrence of breast cancer following curative surgery and radiation therapy [6]. Low incidences of CA 15-3 elevation in early stage cancer (stage I and stage II) have been observed [7].

Incidence of abnormal values of CA 15-3 in stage III and stage IV, and a very high CA 15-3 level have been correlated with metastases of breast cancer [8]. Therefore the development of immunoradiometric assay was planned to carry out the determination of the optimum conditions of ^{125}I-anti CA 15-3 antibody binding with CA 15-3 in breast tumor tissue homogenate, hence determination of CA 15-3.

Materials and Methods

Chemicals

All chemicals and reagents used in this study were of analytical grade, Immunoradiometric assay kit for CA 15-3 level from Diasorin Inc. (USA).Bovine serum albumin (BSA), urea, $ZnCl_2$, $CaCl_2$, NH_4Cl, NaBr, ethylendiamine-tetraaceticdisodium salt (EDTA) from Fluka:(Switzerland). $CuSO_4.H_2O$, NaK-tartarateglycine, NaOH,HCl, $NaCO_3$, NaF, NaCl, NaI, Na_2HPO_4, NaH_2PO_4 from BDH,limited,Poole (UK). Folin-Ciolteau from E. Merck AG. Dastmstapt, finally Blue dextran (2000), and sepharose CL-4B from Pharmacia fine chemicals (Sweden).

Instruments

Gamma counter type 1270-rack gamma II , Spectrophotometer ultra space type 4050 and Combicold rack were from LKB, UV-210 a double beam spectrophotometer from Shimadzu. pH-meter from Pye-Unicam. Cooling centrifuge; with a maximum speed 5000 r.p.m. from Hettich. Cooling centrifuge type 202-MK; with a maximum speed 13500 r.p.m. from Sigma. Memmert water bath, memmert incubator from West Germany. SM-shaker from England.

Patients

Three groups of breast tumors patients were included in this study.

Group I : Consisted of 40 patients with benign breast tumors
Group II : Consisted of 32 premenopausal patients with breast cancer.
Group III : Consisted of 15 postmenopausal patients with breast cancer.
Group IV : Consisted of 10 controls.

All patients were admitted for treatment to (Baghdad Teaching Hospital), (University Hospital, Al-Nahrain College of Medicine), (Nursing Home Private Hospital) and (Al-Arabi Private Hospital). Patients suffered from any disease that may interfere with this study were excluded. All surgical operation of breast tumors were carried out under the supervision of the following surgeons:

Dr. Saab Sedeq, Dr.Munthir Al-Aubaidi, Dr.Azam Qanbar Agha, Dr. Abd Al-Salam Al-Tai, Dr. Zuhair Abid Al-Hadi.

The host information of all patients and normal healthy subjects is summarized in table (1).

Table (1): The host information of breast tumors patients and healthy subjects studied.

Group	Patients	No.	Age	Type of tumor	Metastases
I	Benign breast tumor	40	18-42	23 fibroepithelial tumor (fibroadenoma) 17 fibrocystic changes (adenosis)	– –
II	Premenopausal malignant breast tumor	32	34-52	22 Infiltrative Ductal carcinoma 10 Ductal carcinoma	2 lymph nodes
III	Postmenopausal malignant breast tumor	15	55-73	Infiltrative Ductal carcinoma	4 lumph nodes
IV	Control	10	25-40		

Preparation of Blood Samples

Five milliliters of blood samples were obtained from patients by venipuncture just before surgery. Ten physically normal age volunteers were used as controls. Blood samples were left for 20 min. at room temperature. After coagulation, sera were separated centrifugation at 3000r.p.m for 10 min., and then sera were aspirated and stored at –20^{o}C until time analysis. The samples were not thawed and refrozen before testing.

Collections of Specimens

The tumors tissues were surgically removed from breast tumor patients by either mastectomy (cancer patients) or lumpectomy (benign tumor patients). The specimens were cut off and immediately rinsed with ice-cold isotonic saline solution. They were collected individually in plastic receptacle and stored at –20 ^{o}C until homogenization.

PBS–Buffer

Phosphate –buffered saline (PBS) 0.15 M, pH 7.2 was prepared as following:

A: Disodium basic phosphate (0.15M); 21.2940g Na_2HPO_4 and 9.0g of NaCl were dissolved in a final volume 1L deionized distilled water.

B:.Monobasic sodium phosphate (0.15M) 17.9970g of NaH_2PO_4 and 9.0g NaCl were dissolved in a final volume 1L deionized distilled water.

Phosphate buffer saline pH 7.2 was prepared by mixing a volume of solution A with appropriate amounts of solution B to obtain the required pH.

Preparation of Breast Tumors Tissue Homogenates

The frozen tissue were weighed, sliced finely and scalped in Petri dish standing on ice bath, and then homogenized with fivefold volumes of PBS buffer pH 7.2, using manual homogenizer [9]. The homogenate was filtered through four layers of nylon gauze in order to eliminate fibers connective tissues, and then centrifuged at 4000 r.p.m for 45 min. at 4 ^{o}C in order to precipitate the remaining intact cells and the intact nucleus. The supernatant fraction at this speed was separated, divided in aliquots and freeze -20 ^{o}C until use.

Statistical Analyses

Students' t-test was used to determine if the mean values of studied parameters were significant different in the individual groups included in this work. $P<0.05$ were considered significant[10].

Methods

A-Protein Determinations

Total homogenate protein content was determined by the method of Lowry [11], using bovine serum albumin (BSA) as the internal standard, fig. (1).

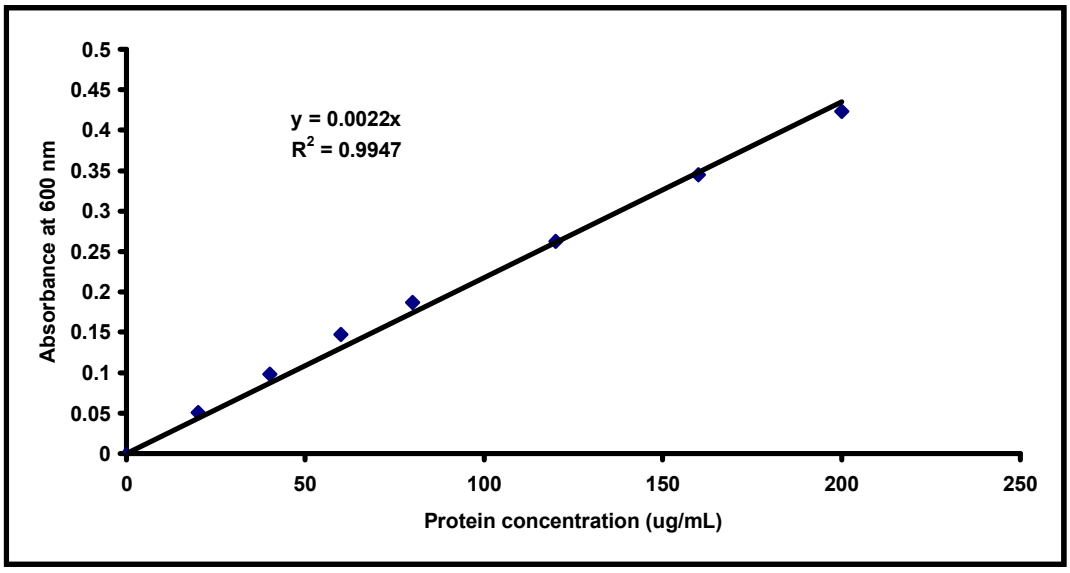

Figure (1): Standard curve of protein concentration.

B-Determination of CA 15-3 Levels in Sera of Breast Tumors Patients

Reagents:

The following reagents provided in the CA15-3 IRMA kit from Dia–Sorin-U.S.A. were used:

- Tracer: two vials each one contained 1.0 µ Ci/mL (37.1 KBa /mL). CA15-3 antibody labeled with ^{125}I in 10 mL / Tris buffer with protein stabilizer and preservative.
- CA15-3 standards: The vial contained 100 mL, which represented 0 U.mL^{-1}. There are four vials, 1.2 mL in each vial with different concentrations of human CA15-3 (25, 50, 100, 200) U.mL^{-1} in Tris. buffer with protein stabilizer and preservative.
- One bottle contained 100-coated beads, Anti-CA15-3-mouse, monoclonal.
- One vial contained 0.5mL CA15-3 control, CA15-3 in re-calcified human plasma with preservative.

Procedure:

The assay protocol is described in Table (2). The specimens and reagents must be brought to room temperature (20-30 ^{o}C) before opening. The reaction trays and data sheets were marked.

Table (2): IRMA protocol of serum CA 15-3 (U.mL^{-1})

	CA 15-3 standard (U.mL^{-1})					Control	Unknown samples	
	0	25	50	100	200		1	2-etc.
Reaction trays no.	1.2	3.4	5.6	7.8	9.10	11.12	13.14	15.16-etc.
Standard (µL)	200	200	200	200	200	_	_	_
Control serum or samples (µL)	_	_	_	_	_	200	200	200
^{125}I-anti CA15-3	200	200	200	200	200	200	200	200

First Incubation:

The specimens and the control were diluted to (1:15) prepared by adding 20 µL of specimen or control to 1000 µL CA15-3 standard, 0 U.mL^{-1} in a tube marked proper identification of specimen. Two hundred microliters of diluted specimen and control were pipette to their assigned wells. Two hundred microliters of each standard was pipette to its assigned well (standards are not to be diluted). One bead was dispensed into each well and the adhesive cover sealer was applied. After incubation for 2hrs at room temperature, the adhesive cover sealer was removed and the liquid was aspirated, then each bead was washed three times with 5 mL distilled water.

Second Incubation:

Two hundred microliters of ^{125}I-antiCA15-3 was pipetted on each bead. The adhesive cover sealer was applied again. After incubation time for 3hrs at room temperature, the cover was removed and the liquid was aspirated from wells, then the beads were washed as it is above. The beads were transferred to the counting tubes, and then the tubes were counted for 1 min.

Calculations:

The standard curve was constructed by plotting counts per min. (Y axis) versus concentration for CA15-3 standard (X axis), figure (2). Then the points were connected with straight-line segments.The CA15-3 concentration of specimens and control were determined directly from the standard curve.

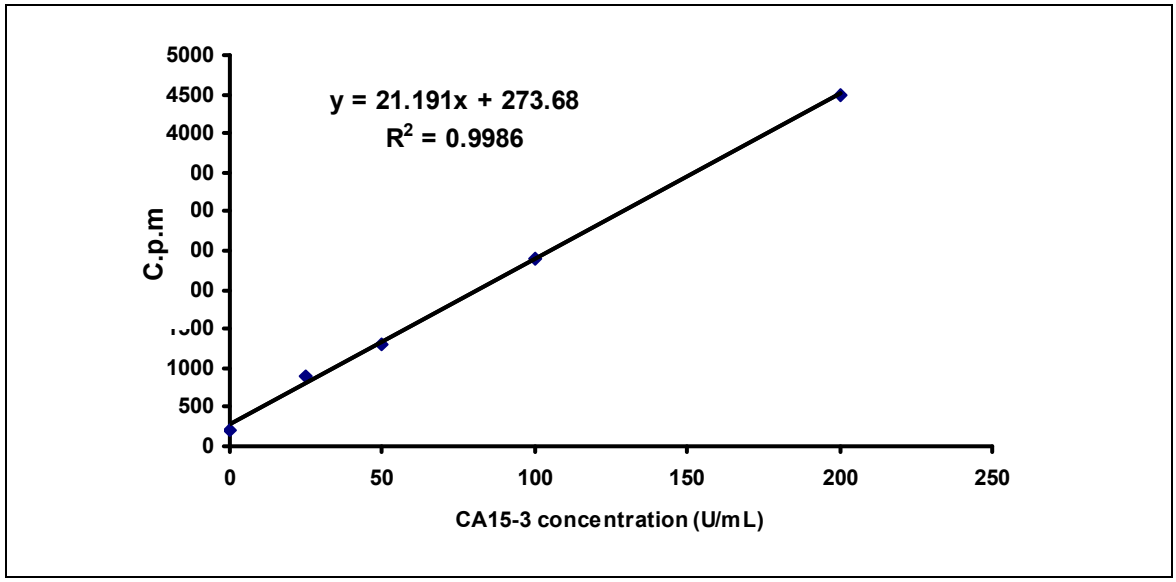

Figure (2):Standard curve of CA 15-3 determination in human sera by IRMA method.

C-Preliminary Test of CA 15-3 Binding to^{125}I –Anti CA15-3 Antibody in Breast Tumor Homogenate

Procedure

The supernatant and pellet were centrifuged and detected by using ordinary tubes. In order to detect CA 15-3, 100 µL of the supernatant breast homogenate having (900µg protein) were incubated with 50 µL (0.35 mg.mL^{-1}) of ^{125}I -anti CA15-3.The volume of reaction was completed to 500 µL with PBS buffer pH7.2, then incubated at 37 ^{o}C for 2hrs. The assay tubes were centrifuged at 4000 r.p.m. for 45 min. at 4 ^{o}C.

The supernatant was discarded, the rim at each tube was swabbed with cotton, and then gamma counter counted the complex formed for one minute. The pellet of CA 15-3 was estimated by dissolving the sediment in PBS-buffer pH 7.2 with the ratio 1:5 (weight: volume) shaking was then carried out. Hundred microliters of the supernatant fraction of the sediment having (540 µg.mL^{-1} protein) was added to 50 µL (0.35 mg.mL^{-1}) of ^{125}I -anti CA 15-3 antibody. The same steps mentioned in this experiment were followed to determine the radioactivity of the complex formed. For total count two additional tubes with 50µL of ^{125}I – anti CA 15-3 antibody were counted in gamma counter.

Calculations:

1. The counted radioactivity in each tube (expressed in c.p.m.) represents the bound fraction (B), (i.e., ^{125}I antiCA15-3 antibody/CA 15-3 complex).
2. The counted radioactivity in the tubes containing ^{125}I-anti CA15-3 antibody only represents the total count (T).
3. The (B/T) ratio for each tube counted as follows:

$$(B/T)\% = \frac{\text{Sample Counts } (B)}{\text{Total Counts } (T)} \times 100$$

Factors Effecting of ^{125}I-Anti CA-3 Antibody Binding to CA 15-3 in Breast Tumors Homogenates

D- The Effect of Different Amounts of Protein Concentration of the Tumor Homogenate on the Binding with ^{125}I-Anti CA 15-3 Antibody

Procedure

1. Fifty microliters (0.35 mg.mL^{-1}) of ^{125}I -anti CA 15-3 antibody were added to 100µL of the supernatant (benign Fibroadenoma, pre-and post-menopausal malignant breast tumors (IDC) respectively) containing increasing amounts of protein (50, 100, 150, 200, 250 µg.mL^{-1}) then completed to a final volume of reaction to 500 µL with 0.15 M PBS pH 7.2.
2. The assay tubes were then incubated for 2 hrs at 37^{o}C.
3. Two additional tubes, containing 50µL(0.35 mg.mL^{-1})of ^{125}I –anti CA15-3 antibody only, for total counts were set-aside until counting.
4. At the end of incubation, the assay tubes were centrifuged at 4000 r.p.m for 45 min at 4^{o}C.
5. The supernatant were decanted, the rims at the tube were swabbed with cotton piece.
6. The radioactivity of the complex were counted using gamma counter.

Calculations

1. The B/T percent were determined according to section (C).
2. The percent of binding values B/T were plotted versus the increasing amount of protein of the breast tissue homogenate**.**

E- The Effect of ^{125}I -Anti CA15-3 Antibody Concentration on the Binding

Procedure

1. Fifty microliters of increasing concentration (0.070,0.140,0.175,0.350, 0.701 mg.mL^{-1}) of ^{125}I -anti CA 15-3 antibody were added to 100µL of homogenate (benign breast tumor (Fibroadenoma), pre-and post-menopausal malignant breast tumors) (IDC) containing (100, 100, 200 µg.mL^{-1} protein) respectively.
2. The volume of reaction was made up to 500 µL with PBS pH 7.2.
3. Steps 2,3,4,5 and 6 of the experiment (D) were repeated.

Calculations

1. The same mathematical equation mentioned in section (D) was used to calculate (B/T)%.

2. Values of (B/T)% were plotted versus concentration of labeled antibody (^{125}I -anti CA15-3 antibody).

F- The Effect of pH on the Binding

Procedure

1. One hundred microliters of human homogenate (benign breast tumor (Fibroadenoma), pre-and post-menopausal malignant breast tumors (IDC)) containing (100,100,200, $\mu g.mL^{-1}$ protein) were added to 50µL (0.175, 0.175,0.140$mg.mL^{-1}$) of ^{125}I -anti CA15-3 antibody respectively.
2. Each mixture was completed to 500 µL with PBS of different pH ranging (6.8-8.0).
3. Step 2,3,4,5 and 6 of the experiment (D) were repeated.

Calculations

1.Values of (B/T) % were calculated as described in section (D).
2. (B/T)% were plotted against their corresponding pH.

G- The Effect of Different Halides on the Binding

Reagents

1. Phosphate buffer (PB) were prepared without the addition of NaCl .
2. Halid reagents were prepared in concentration of 0.01M PB at pH (7.0, 7.6 and 7.8) individually, by dissolving each of 0.021gm of NaF, 0.0292gm of NaCl, 0.0515 gm of NaBr, and 0.075gm of NaI in a final volume 50µL of PB and the pH was adjusted.
3. The breast tumors homogenates (benign breast tumor (Fibroadenoma)) were prepared using PB-buffer instead of PBS at the same pH and concentration carried out the homogenization.

Procedure

1. One hundered microliters of each group homogenate (benign breast tumors (Fibroadenoma) and pre-post menopausal malignant breast tumors (IDC) containing (100,100 and 200 $\mu g.mL^{-1}$ protein) were incubated with 50 µL of ^{125}I -anti CA 15-3 antibody concentration (0.175,0.175 and 0.140 $gm.mL^{-1}$). The volume was made up to 500 µL with PB pH (7.0, 7.6 and 7.8) containing 0.01 M of the following halides: NaF, NaCl, NaBr and NaI in each assay tube. (A sample without the addition of any salt was used as a control).
2. The assay tubes were then incubated for 90min. at 45, 15 and 45ºC for the three groups individually.
3. Steps 3, 4, 5 and 6 of the experiment (D) were repeated.

Calculations

1. The values of (B/T) % were calculated as described in section (D).
2. (B/T)% was plotted against halides concentrations.

H- The Effect of Monovalent and Divalent Cations on the Binding

Reagents

1. PB was prepared without addition of NaCl.
2. Monovalent and divalent cations salts were prepared in concentration of (0.025 M) PB at pH (7.0, 7.6 and 7.8) individually, by dissolving each of 0.0931gm of KCl, 0.0668gm of NH_4Cl, 0.2541 gm of $MgCl_2.6H_2O$, 0.1388 gm of $CaCl_2.2H_2O$, 0.2474gm of $MnCl_2.4H_2O$, 0.3150 gm of $CuSO_4.5H_2O$ and 0.1703gm of $ZnCl_2$, in a final volume 50 mL of PB and the pH was adjusted.

Procedure

1. The same steps mentioned in section (G) were followed to determinate the effect of monovalent and divalent of CA 15-3 in the tissues homogenates of (benign breast tumors (Fibroadenoma) and pre-and postmenopausal malignant breast tumors (IDC) with ^{125}I -anti CA 15-3 antibody, except the PB buffer containing (0.025M) of the following salts: KCl, $NH_4.Cl$, $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnCl_2$.
2. A sample without the addition of any salt was used as control.

Calculations

1. The values of (B/T)% were calculated as described in section (D)
2. (B/T)% was plotted against monovalent and divalent cations salts concentrations.

I-Recovery of CA 15-3

Reagents

1. All reagents are described previously.
2. Standard concentration of CA 15-3 200 $U.mL^{-1}$ was used.

Procedure

Known concentration of CA15-3 (200 $U.mL^{-1}$) was added to the three groups of tissues homogenates (benign breast tumors (Fibroadenoma), and pre-and post-menopausal malignant breast tumors (IDC). The experiment was carried out at optimum conditions that were obtained in the experements of (D,E,F,G). The CA15-3 was determined according to the experiment in section (C).

Calculations

1. The bound (c.p.m) of the reaction mixture (standard CA 15-3 was added to tissue homogenate) with ^{125}I -antiCA15-3 antibody, represent the measured value.

2. The bound (c.p.m.) of CA 15-3 in tissue homogenate with ^{125}I –antibody CA 15-3 antibody only , represent the expected value.
3. The recovery % (yield%) was calculated as follows:

$$\text{Recovery}\% = \frac{\text{Measured values}}{\text{Expected values}} \times 100$$

Results and Discussion

Determination of CA 15-3 Levels in Sera of Breast Tumors Patients

CA 15-3 levels in sera of patients with benign breast tumors (group I) and pre and post-menopausal malignant breast tumors (group II and group III) were measured by IRMA method. These three groups were matched with a group of control subjects. Table (3) summarizes the groups and the mean concentrations of CA15-3 for the control women and patients with benign breast tumors and pre-and post-menopausal malignant breast tumors.Table (3) showes that CA15-3 levels in three different groups (benign breast tumors and pre-and post-menopausal malignant breast tumors) were significantly elevated ($p<0.05$) for benign breast tumors and highly significantly elevation ($p<0.0001$) for pre-and post menopausal malignant breast tumors respectively, as compared with the control.The mean serum CA15-3 level of the control was found to be (17.26 ± 4.06 $U.mL^{-1}$) as shown in table (3), and the cutoff values was (25 $U.mL^{-1}$) that obtained from (mean +2 SD). This cutoff value is in agreement with Geraghty J.G [12], other study obtained that cutoff value of 40 $U.mL^{-1}$ [8], 30 $U.mL^{-1}$ [13]. It has shown that widely different cutoff value which was described ranging from 20-40 $U.mL^{-1}$ in different reference [14].

According to Bon et al [15] the upper limit of CA 15-3 of normal may be method-dependent. No association between the CA 15-3 and either age or menopausal status was found in the control group. Therefore , the cutoff values do not require adjustments related to these variables. These results were in agreements with Gion M.et.al. [16]. Figure (3) shows the distribution of the individual values of CA15-3 in sera of patients with benign breast tumors and pre-and post menopausal malignant breast tumors and control, were determined by using the standared curve in figure (2). It was found that the mean of serum CA 15-3 concentration in 20 patients with benign breast tumors was 21.9 ± 6.6 $U.mL^{-1}$ (mean ± SD). These results are in agreement with Hayes D.F. et.al [17]. The results show there was highly significant correlation between serum connections of CA15-3 in both groups pre-and post-menopausal status with control, while it was significantly lower in benign breast tumors status.

This is in agreement with Ichihara S. et.al [18] . Therefore all of the cases used in the binding studies were concentrated to this type of carcinoma (IDC) and this type is the common type of breast cancer. In Iraq very high levels of CA15-3 advanced disease and the value 5 to 10 times of normal suggest the presence of metastasis. Increasing numbers of metastatic sites correlate with increasing CA15-3 levels [19].

These findings suggest that higher levels of CA15-3 represent the breast cancer extent and reflect the cell differentiation and aggressiveness of the tumor. Therefore, it could be concluded that the determination of CA15-3 before surgical operative may be useful as a prognostic factor in breast cancer.

Table (3): Sera CA15-3 levels ($U.mL^{-1}$) in patients with benign and malignant breast tumors.

Group	Patients	No. of cases	Age range (Year)	Sera CA15-3 $U.mL^{-1}$ (mean ± SD)	P values
I	Benign breast tumors	20	18-42	21.9 ± 6.6	P<0.05
II	Premenopausal malignant breast tumors	16	34-52	37.3 ± 6.8	P<0.0001
III	Postmenopausal malignant breast tumors	12	55-73	60.3 ± 10.9	P<0.0001
IV	Control	10	25-40	17.3 ± 4.06	--

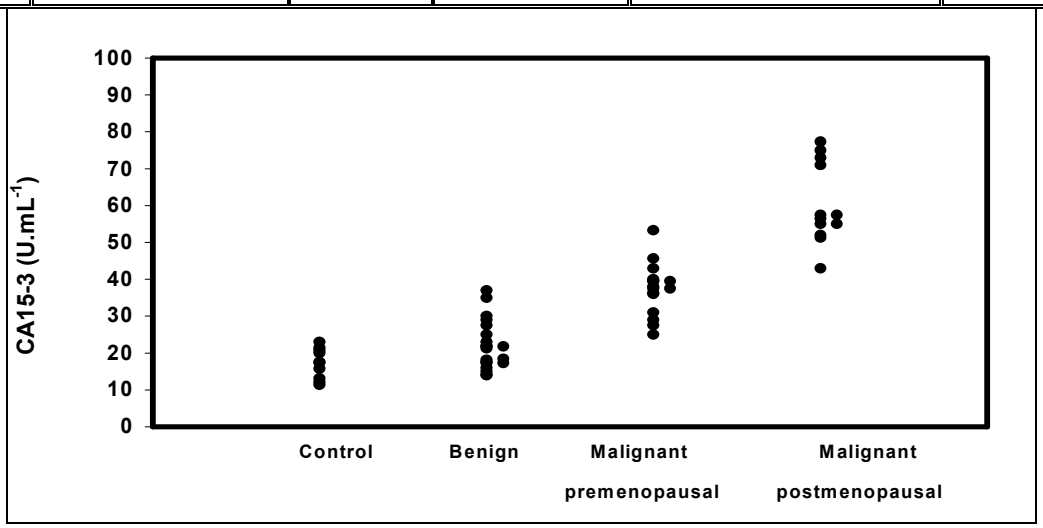

Figure (3): Distribution of the individual value of CA15-3 $U.mL^{-1}$ in the sera of benign and malignant breast tumors patients.

Binding Studies of ^{125}I-Anti CA15-3 Antibody with CA15-3 in Benign and Malignant Breast Tumors Homogenates

Preliminary Test of the Binding of ^{125}I-Anti CA15-3 Antibody with CA 15-3 in Breast tumor homogenate

Supernatant and pellet formed at speed (4000 r.p.m.) in three groups of human breast tumor homogenate (benign breast tumors, pre-and post-menopausal malignant breast tumors) were used in this experiment. In each fraction CA 15-3 was detected through the incubation of ^{125}I-anti CA15-3 antibody with supernatant fraction and pellet individually for 2hrs at 37ºC in PBS buffer as a medium to complete the reaction. The separation of the bound antibody from the unbound was carried out at 4000 r.p.m. for 45 min. to precipitate the (^{125}I-anti CA15-3 antibody/CA15-3) complex formed. Preliminary experimental conditions used in Table (4), which is show, the amount of binding B/T% in both fractions. The data revealed that CA15-3 was higher in incidence according to B/T%.

Table (4): Incidence of CA15-3 in supernatant and pellet fractions in three different breast homogenate.

Groups	(B/T)%		CA15-3 $U.mL^{-1}$ in supernatant fraction kit
	Supernatant Fraction	Pellet Fraction	

Benign	6.20	3.40	90
Premenopausal	8.04	5.53	356
Postmenopausal	6.31	4.64	144

B/T% in supernatant is more than in pellet fractions of this speed (4000 r.p.m.). According to these results supernatant fractions was collected and the pellet was then discarded. The CA15-3 levels in the supernatant of breast tumors homogenate were determined according to IRMA method.

In general, results show that CA15-3 concentration in pre-and post-menopausal malignant breast tumors homogenates is more than benign breast tumors homogenates. These results are in agreement with the result obtained from B/T% from IRMA developed method.

From these results, it can be said that developed method was useful for determination CA15-3 in breast tumors homogenate using ^{125}I-anti CA15-3 antibody.

Factors Effecting of ^{125}I-Anti CA15-3 Antibody Binding to CA15-3 in Breast Tumors Homogenates

The Effect of Different Amounts of Protein Concentration of the Tumor Homogenate on the Binding with ^{125}I-Anti CA 15-3 Antibody

To obtain the optimum protein of homogenate for the binding of CA15-3 with ^{125}I-anti CA15-3 antibody, the supernatant homogenate containing increasing amount of CA15-3 in the presence of fixed amount of ^{125}I-anti CA15-3 antibody as it was mentioned in practical section.

Figure (4) represents the quantitative precipitation curve in which the amount of (^{125}I-anti CA15-3 antibody/CA15-3) complex in three groups (benign breast tumors and pre-and-post menopausal malignant breast tumors) was plotted as a function of CA15-3 concentration.

As shown in this figure, in the first phase of the reaction no precipitate was formed.

The amount of precipitate increased until a point of maximum binding was reached. After this point as the amount of CA15-3 increased the amount of precipitate diminished; thus the increase in protein concentration which would increase the number of binding site and hence increase the percent of binding until the saturation state at (100, 100, and 200 $\mu g.mL^{-1}$) homogenate concentration for (benign breast tumors, pre-and post menopausal malignant breast tumors respectively).

The complex precipitate out of solution because of the multivalent nature of both molecules [20]. The radioactive antibody has two binding sites, it can cross-link antigenic sites of two different CA15-3 molecules and can produce maximum complex formation and therefore maximum precipitate will occur. When CA15-3 is in greater excess, large complex are again less probable.

In all subsequent experiments the amonts of (100, 100 and 200 $\mu g.mL^{-1}$ protein) of tissue homogenate in benign breast tumors and pre-and post menopausal malignant breast tumors were used according to the result obtained in this experiment.

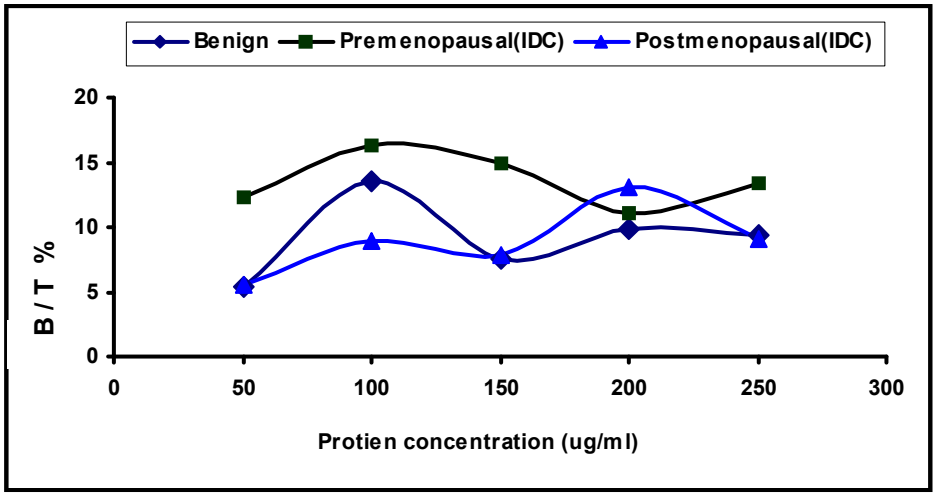

Figure (4): Influence of increasing protein concentration on the binding with ^{125}I-anti CA15-3 antibody.

The Effect of ^{125}I-Anti CA15-3 Antibody Concentration on the Binding:

The experiment was carried out in the presence of fixed amount of protein concentration of the homogenate and increasing concentration of ^{125}I-anti CA15-3 antibody. The results are illustrated in figure (5). Which represent ^{125}I-anti CA15-3 antibody binding curve with supernatant fraction of benign breast tumor, pre-and post-menopausal malignant breast tumors. As shown in figure (5) it is obvious that the amount of (^{125}I-anti CA15-3 antibody/CA15-3) complex rises gradually, and then the breast tumor protein was saturated with ^{125}I-anti CA15-3 antibody. When the amount of antibody is in moderate excess, the probability of cross-linking of Ag by Ab in the incubation mixture is more likely, and hence large complex formation is favored. Then the maximum B/T percent was detected. The presence of (0.175, 0.175, 0.14 mg.mL^{-1}) of ^{125}I-anti CA15-3 antibody in benign, pre-and post-menopausal breast tumors homogenates give the optimum concentration of ^{125}I-anti CA15-3 antibody in three groups. Then the binding percent decreased as the amount of ^{125}I-anti CA15-3 antibody increased. This is because all antigenic sites are covered with antibody and complex formation is inhibited [21]. These results indicate that the binding is principally dependent on the amount of the antibody in the reaction mixture [22]. According to the results of this experiment the above concentration of ^{125}I-anti CA15-3 antibody was used in the subsequent experiments.

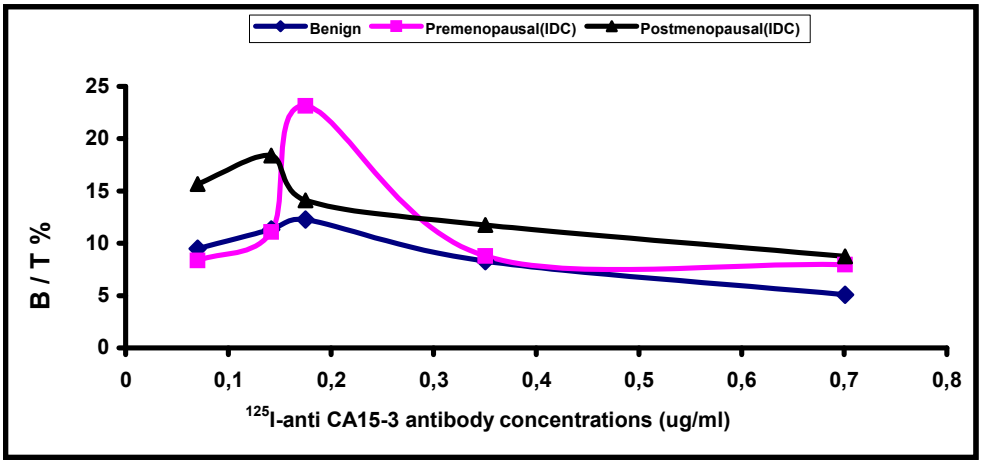

Figure (5): Effect of different concentrations of ^{125}I-anti CA15-3 antibody on the binding of with CA15-3.

The Effect of pH on the Binding

Figure (6) shows the values of the binding of ^{125}I-anti CA 15-3 antibody to CA 15-3 in benign breast tumor, pre- and post-menopausal malignant breast tumors, at different pH values. Maximum value of the binding occurs at (pH 7, pH 7.6, pH 7.8) for benign breast tumor, pre-and post-menopausal malignant breast tumors respectively.

The formation of (^{125}I-anti CA 15-3 antibody/CA 15-3) complex is usually performed at pH between 6.8-8.0; the results indicate that the shift in the pH of the environment may affect the properties of CA 15-13 molecules involved in the binding. This effect may include the protonation deprotonation processes occurring within the possible ionizable groups of the amino acids present in the binding domain of these molecules [23]

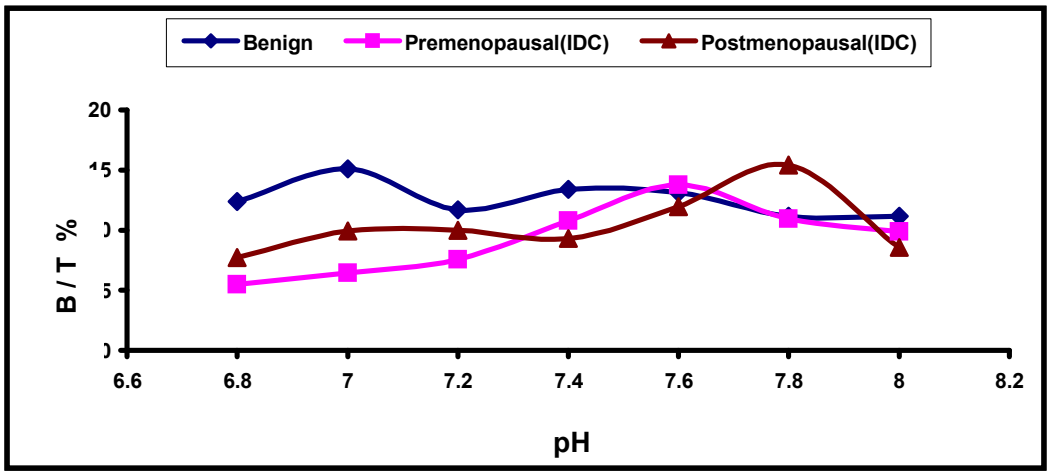

Figure (6): Effect of pH on the binding of ^{125}I-anti CA 15-3 antibody with CA 15-3 in breast tumors homogenates.

The Effect of Different Halides on the Binding

Different sodium halides at 0.01 M concentration were investigated to study their action on the binding ^{125}I-anti CA 15-3 antibody with CA 15-3 in the three groups (benign breast tumors, Pre-and postmenopausal malignant breast tumors), as shown in figure (7).

The presence of the sodium halides in the incubation medium tends to promote the binding of ^{125}I-anti CA 15-3 antibody to CA 15-3 in these groups, the following sequence of effects have occurred.

1.Benign breast tumor tissue homogenate

NaI > NaBr > NaCl > NaF

2.Premenopausal breast cancer tissue homogenate

NaCl > NaI > NaBr > NaF

3.Postostmenopausal breast cancer tissue homogenate

NaCl > NaBr > NaF > NaI

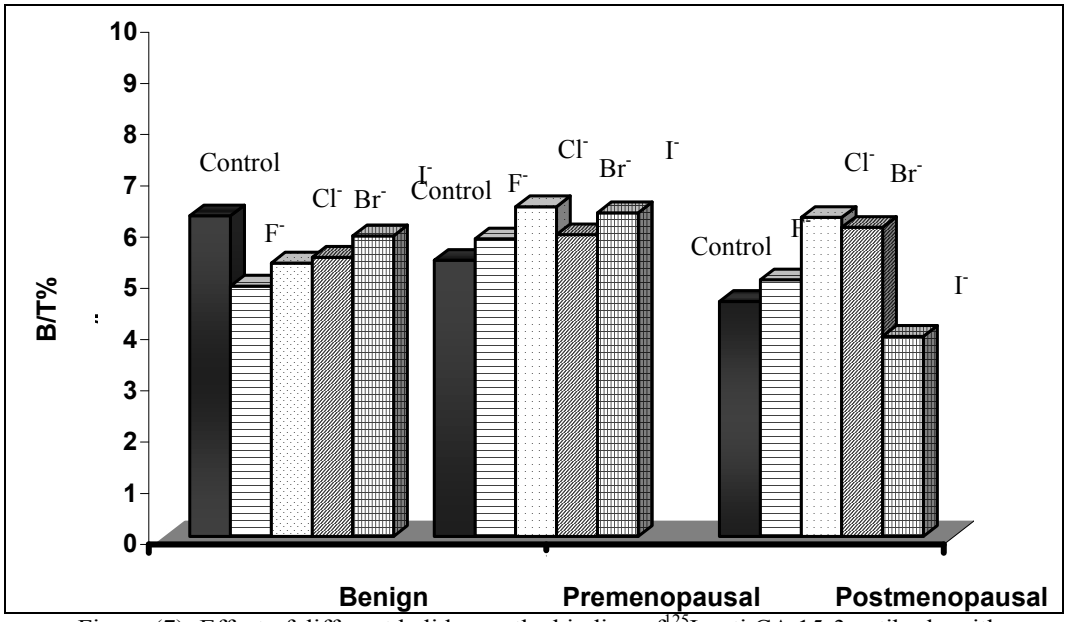

Figure (7): Effect of different halides on the binding of ^{125}I-anti CA 15-3 antibody with CA153.

As shown in figure (7), the sodium halides inhibited the binding in benign breast tumors, according to the decreasing ionic radius and increasing radius of hydration. It seemed that fluoride ion causes lower binding, this could be due to higher electro negativity of fluoride ion that tend to interact with the positive residue in the binding site of the antibody and/or the antigen which lead to decrease the interaction between CA 15-3 and its antibody [24].

Melander and Horvath (1977) reported that the effect of halide salt type on hydrophobic interactions is quantified by its molar surface tension increment (MSTI) that is a measure of the increase in surface tension by the salt [25]. On the other hand, figure (7) shows the effect of different halides salts at 0.01 M on the extent binding of ^{125}I-anti CA 15-3 antibody to pre-and postmenopausal malignant breast tumors homogenate. It seems that halides salts increased the binding, especially NaCl, this could be due to that NaCl in lower concentration (0.15M) or in physiological concentration, increased the binding between CA 15-3 and its antibody [26] .

The Effect of Monovalent and Divalent Cations on the Binding

The effect of different salts on the extent of binding of ^{125}I-anti CA 15-3 antibody to CA 15-3 in benign and malignant breast tumors are shown in figure (8). The results indicate

that the binding process is sensitive to the presence of cation metal ions. $CuSO_4.5H_2O$ at concentration (25mM) was shown to increase the binding more than other divalent cations, while $ZnCl_2$ increased the binding less than other divalent cations. One hypothesis assumes that salts may alter the nature of the hydrophobic forces controlling stabilization of the complex formed and these vary depending on the nature of the interacting groups [27]. From the results illustrated in figure (8), it is suggested that these salts maybe provide some conformational changes in the CA 15-3 and the charged groups of the binding domain of the antibody and antigen molecule [28] , that hinder maximal binding are shielded. If the interaction is dominated by ionic strength, high salt concentration lowers the affinity.

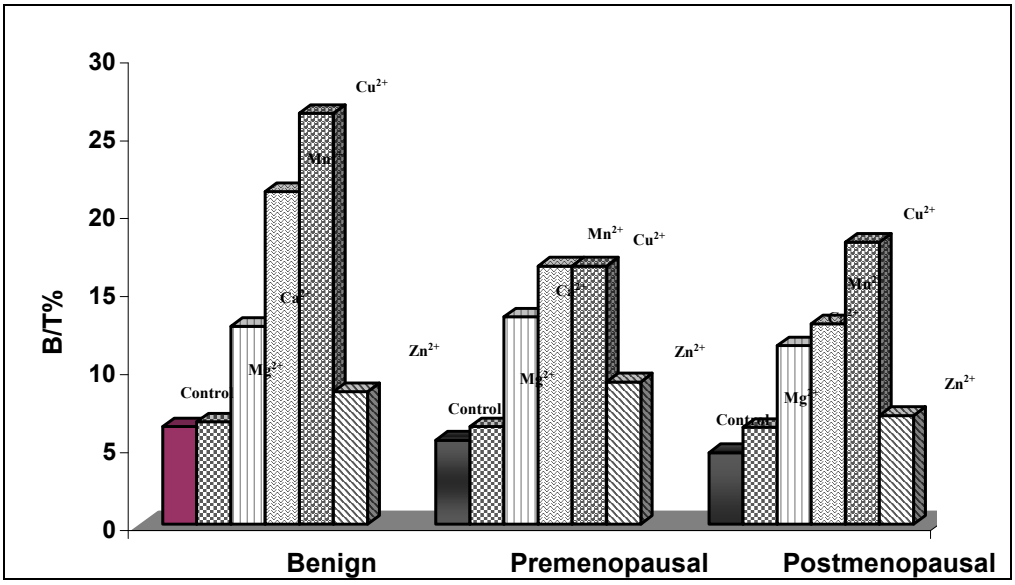

Figure (8): Effect of different divalent cations on the binding of ^{125}I-anti CA 15-3 antibody with CA 15-3.

Figure (9) shows the effect of monovalent cations (KCl and NH_4Cl) on the extent of the binding of CA 15-3 to its antibody ^{125}I-anti CA 15-3 in benign and malignant breast tumors. KCl at 25mM was shown to increase the binding in benign and premenopausal malignant breast tumors as compared with the control value, while KCl at the same concentration slightly inhibiting the binding in postmenopausal malignant breast tumors. These results may be due to conformational changes. NH_4Cl at 25 mM was shown to inhibit the binding but to a lesser extent. This result shows that NH_4Cl effect on the binding is nearly unremarkable. Presumably, the lesser degree of hydration permits greater interaction of the salt with an anionic group located in the antibody-combining site and then inhibits the complex formation.

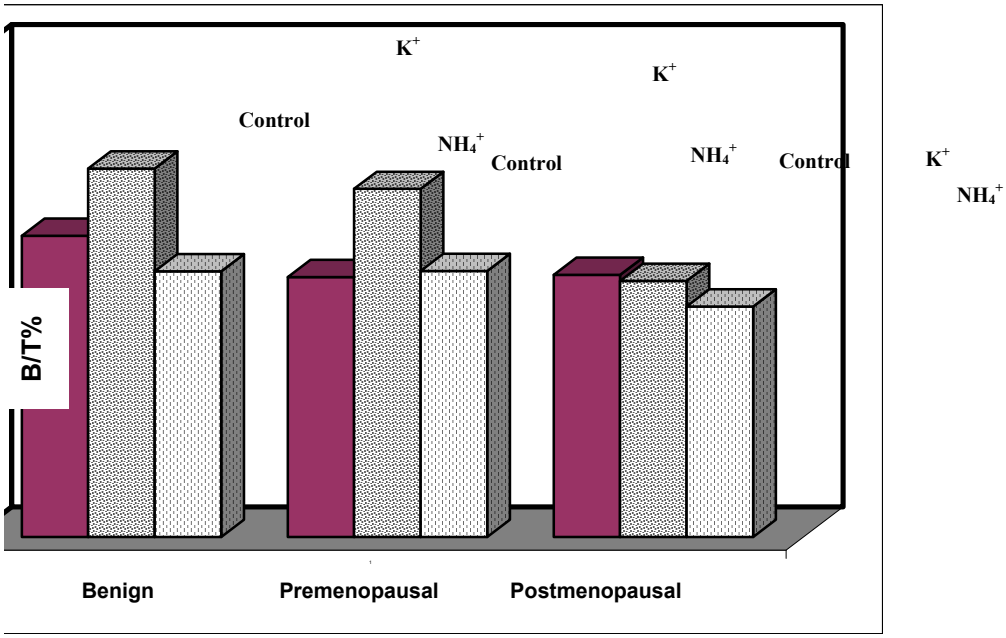

Figure (9) : Effect of different monovalent cations on the binding of ^{125}I-anti CA15-3 antibody with CA 15-3.

Recovery of CA 15-3:

This method was used to estimate the percent recovery of CA 15-3 in supernatant fractions of benign and malignant breast tumors homogenates. The results are summarized in table (5) and indicate that the CA 15-3 extracted from benign breast tumors were recovered less than malignant tissue homogenate. CA 15-3 extracted from postmenopausal malignant tissue homogenates were recovered more than CA 15-3 extracted from premenopausal malignant breast tumors homogenates. Also the results indicate that total CA 15-3 can be determined through the developed method of immunoradiometric assay, as well as the percent of recovery indicates the precision of the used method.

Table (5): Recovery of CA 15-3.

Type of CA 15-3	Measured B/T	Expected B/T	Recovery%
			Measured / Expected
Benign (Fibroadenoma)	16.23	24.84	65.34
Premenopausal (IDC)	20.04	27.64	72.50
Postmenopausal (IDC)	24.20	26.80	90.30

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