PRELIMINARY STUDIES OF CARBOHYDRATE ANTIGEN 19-9 (CA19-9) ON HUMAN BREAST TUMORS

دراسات تمهيدية للمستضد الكاربوهيدراتي (CA 19-9) في مجانسات اورام الثدي في الانسان

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Abstract:

A solid-phase immunoradiomertric assay sandwich technique (IRMA) was used for the determination of the carbohydrate antigen 19-9 (CA19-9) defined by a monoclonal antibody ^{125}I-anti CA19-9 in sera of 10 premenopausal malignant breast tumors patients, 10 postmenopausal malignant breast tumors patients, and 10 benign breast tumors patients matched with one group of 10 healthy women as control. The data obtained demonstrated significant increase ($P<0.05$) in patients with benign and premenopausal malignant breast tumors, whereas highly significant increase ($P<0.005$) in patients with postmenopausal malignant breast tumors when matched with normal women. An Immunoradiometric Assay (IRMA) for the determination of cytosolic CA19-9 was developed, using ^{125}I-anti CA19-9 antibody and found to be suitable for assessment of those antigens in benign and malignant breast tumors. The data revealed an increment of CA19-9 in the cytosolic fraction in comparison to the nuclear fraction.

المستخلص:

استخدمت طريقة الاختبار المناعي المتري لتعيين المستضد الكاربوهيدراتي CA19-9 المعرف بواسطة الضاد للارتباط ^{125}I-anti CA19-9 antibody في امصال عشرة نساء مصابات باورام الثدي الخبيثة قبل انقطاع الطمث وعشرة مصابات باورام الثدي الخبيثة بعد انقطاع الطمث وعشرة مصابات باورام الثدي الحميدة وعشرة نساء سليمات كمجموعة سيطرة . لوحظ زيادة معنوية ($P<0.05$) في مستويات CA19-9 لدى النساء المصابات باورام الثدي الحميدة والخبيثة قبل انقطاع الطمث. في حين لوحظ زيادة معنوية كبيرة ($P<0.005$) لدى النساء المصابات باورام الثدي الخبيثة بعد انقطاع الطمث مقارنة بالنساء السليمات . تم تطوير طريقة الاختبار الاشعاعي المناعي المتري (IRMA) في تقدير الـــCA19-9 السايتوسولي باستخدام الضاد المتخصص (^{125}I-anti CA19-9 antibody) وقد وجد ان هذه الطريقة مناسبة لتقدير المستضدات في اورام الثدي الخبيثة والحميدة. اشارت النتائج الى زيادة انتشار هذه المستضدات في الجزء السايتوسولي عن النووي.

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Introduction:

The Carbohydrate antigen 19-9 (CA19-9) (Koprowski etal.,1979) [1], is specific carbohydrate fraction of a circulating antigen found in sera of normal adults [2], has sialyl Lewis^{a} structure and is present in individually expressing the Lewis^{a} and /or Lewis^{b} blood group antigen [3] . CA19-9 is identified as a glycolipid- that is , sialylated lacto-N-fucopentose II ganglioside [4]. In serum, it exists as a mucin , a high molecular mass (200-1000 KD) glycoprotein complex [5]. In Normal tissues, sialyl Lewis^{a} antigen is present in ductal epithelium of breast, kidney, salivary gland, and sweatglands [6]. CA19-9 is measured with a double monoclonal immuno-radiometric assay [7]. Another techniques used for the detection of CA19-9 in tissues and sera were performed by an immunoperoxidase assay [8] and by radioimmunoassay [9] of samples from patients, and enzyme immunoassay [10] for quantitative determination of CA19-9 in human serum. The upper limit of normal value 37.0 $U.mL^{-1}$ [11]. The abnormal expression of the sialyl Lewis ^{a} is closely correlated with various forms of cancer including pancreatic cancer [12], gall bladder [13] ,bile duct [14] cancer and cystic fibrosis [15].

A monoclonal antibody CA19-9 against sialyl Lewis ^{a} is a popular diagnostic agent for these tumors. The antibody is useless for cancer diagnosis when a patient is lacking the enzyme for the synthesis of sialyl Lewis ^{a}. In Japan, about 5-10% of the population lacks this enzyme leading to false negative results [16]. CA19-9 represents the most important and basic carbohydrate tumor marker. The immunohistologic distribution of CA19-9 in tissues is consistent with the quantitative determination of higher CA19-9 concentrations in cancer than in normal of tissues [17]. The reports indicates that serum CA19-9 level is frequently elevated in the serum subjects with pancreatic (80%), hepatobiliary (67%), gastric (40-50%), hepatocellular (30-50%), colorectal (30%) and breast (15%) cancer [18].

Research studies demonstrate that serum CA19-9 values may have utility in monitoring subjects with the above-mentioned diagnosed malignancies [19]. A declining CA19-9 value may be indicative of a favorable prognosis and good response to treatment [20]. Therefore, the development of immunoradiometric assay was planned to carry out the determination of the optimum conditions of ^{125}I-anti CA19-9 antibody.

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 and Blood Samples:

Thirty breast tumors patients and specimens, classified to three group of patients were include in this study, one group with benign and two groups with malignant breast tumors. The fourth group is a healthy women used as control.

- **Group I:** Consisted of 10 patients with benign (Fibroadenoma) breast tumors.
- **Group II:** Consisted of 10 premenopausal patients with breast cancer (Infiltrative Ductal carcinoma - IDC).
- **Group III:** Consisted of 10 postmenpausal patients with breast cancer (Infiltrative Ductal carcinoma - IDC).
- **Group IV:** Consisted of 10 normal healthy women.

All women patients were admitted for treatment to (Baghdad Teaching Hospital), (University Hospital, Al-Nahrain College of Medicine) and (Nursing Home Private Hospital) Patients suffered from any disease that may interfere with this study were excluded.

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 [21]. 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[22].

Methods

Determination of CA19-9 Levels in Sera of Patients with Benign and Malignant Breast Tumors

Reagents

The reagents IRMA-ELSA CA19-9 Kit was provided from CIS-bio international ORIS Group/France.

1. Anti CA19-9 monoclonal antibody coated on the ELSA fixed in the bottom of the tube.
2. Anti ^{125}I-CA19-9 monoclonal antibody, radioactivity content < 10 **µ**Ci (<370 KBq)
3. Six standard ready for use, Human serum, Human CA19-9 in sodium azide (0,14,30,66,130 and 255 U.mL^{-1}).
4. Diluent (0.0 U.mL^{-1}), human serum in sodium azide.
5. Control (35 U.mL^{-1}), human serum, human CA19-9 in sodium azide. Patients sera and control were used without dilution in this assay.

Procedure:

The assay protocol is described in table (1).

Table (1): IRMA protocol of serum CA19-9 (U.mL^{-1}).

	CA19-9 (U.mL^{-1})						Control		Unknown Samples	
	0	14	30	66	130	255	Level I	Level II	1	2 etc.
Coated tube no.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20
Standards (µL)	←						100 □L	→		
Control serum or samples (µL)	←						100 □L	→		
Buffer (µL)	←						200 □L	→		
	Incubation for 3 h. at 37 ^{o}C in water bath									
	The solution was aspirated, and washed the tubes 3 times with 3 mL distilled water									

^{125}I-anti CA19-9 (µL)	←	300 □L	→
All tubes were mixed gently with vortex-type mixer and			
Incubated for 3 hrs. at room temperature (18-25 oC)			
The solution was aspirated, the tubes were washed 3 times with 3 mL distilled water			
The remaining bound radioactivity was measured with gamma counter.			

Calculations:

1. The mean net count for each group of tubes was counted in gamma counter for 1 min, represents the bound c.p.m.
2. The standard curve was constructed by plotting counts per min. (Y-axis) versus concentration of CA19-9 standard (X-axis) figure (1). Then the points were connected with straight-line segments.

Preliminary Test of the Binding of CA19-9 in Breast Tumor Tissues with ^{125}I-Anti CA19-9 Antibody in Breast tumors Homogenates

Procedure:

The pellet and the cytosol fractions were obtained from the supernatant of breast homogenate were centrifuged at 4000 r.p.m. In order to detect CA19-9, 20 µL of crude cytosol fraction having 1100 µg protein were incubated with 60 µL (0.1356 mg.mL^{-1}) of ^{125}I-anti CA19-9 antibody. The volume of mixture was completed to 500 µL with PBS buffer pH 7.2, and then incubated at 37 ^{o}C for 3 hrs. The assay tubes were centrifuged at 4000 r.p.m. for 45 min. at 45 ^{o}C. The supernatant was discarded, the rims at tube were swabbed with cotton piece, then the complex formed was counted in gamma counter for 1 min. Pellet CA19-9 were determined by dissolving the sediment in PBS buffer pH 7.2 with ratio 1:5 (weight: volume), then 20 µL of supernatant fraction of pellet breast homogenate having 800 µg protein, was added to 60 µL (0.1356 mg.mL^{-1}) of ^{125}I-anti CA19-9 antibody. The same steps mentioned above were followed to determine the radioactivity of the complex formed. For total radioactivity two additional tubes with 60-µL of ^{125}I-anti CA19-9 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-anti CA19-9 antibody/CA19-9 complex).
2. The counted radioactivity in the tubes counting ^{125}I-anti CA19-9 antibody only represents the total radioactivity (T).
3. The (B/T) % ratio for each tube was calculated as follows:

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

Factors Effecting of ^{125}I-Anti CA19-9 Antibody Binding to CA19-9 in Breast Tumors Homogenates

Effect of Protein Concentration on the Binding

Procedure:

Sixty microliters (0.1356 mg.mL^{-1} protein) of ^{125}I-anti CA19-9 antibody were added to 20 µL of cytosolic fraction of benign (Fibroadenoma) and malignant (premenopausal IDC and postmenopausal IDC) breast tumors respectively, containing increasing amounts of protein (50, 75, 100, 150, 200 and 250 µg.mL^{-1}) and were completed to a final volume of 500 µL with 0.15 M PBS pH 7.2. The assay tubes were incubated for 3 hrs. at 37 ^{o}C. At the end of incubation, the assay tubes were centrifuged at 4000 r.p.m. for 45 min. at 4 ^{o}C. The supernatant was decanted; the rims at the tube were swabbed with cotton piece. The radioactivity of the complex formation was counted using gamma counter.

Calculations

1. The (B/T) % values were determined as described previously.
2. Values of (B/T) % were plotted against their corresponding amount of protein of the breast tumor homogenate.

Effect of ^{125}I-Anti CA19-9 Antibody Concentration on the Binding

Procedure :

Sixty microliters of increasing amounts (0.0226, 0.0452, 0.0565, 0.113, 0.1356, 0.226 mg.mL^{-1}) of ^{125}I-anti CA19-9 antibody were added to 20 µL of crude cytosolic fraction (100, 75 and 75 µg protein) for benign (fibroadenoma) and malignant (premenopausal IDC and postmenopausal IDC) respectively, completed to a final volume 500 µL with 0.15 M PBS pH 7.2. After incubation for 3 hrs at 37 ^{o}C the bound CA19-9 was determined as described previously.

Calculations:

1. The (B/T) % values were determined as described previously.
2. Values of (B/T) % were plotted versus the concentrations of ^{125}I-anti CA19-9 included.

Effect of pH on the Binding

Procedure :

Twenty microlites (100, 75 and 75 µg protein) of cytosolic fraction (fibroadenoma, premenopausal IDC and postmenopausal IDC respectively) were added to 25 µL (0.0565 mg.mL^{-1}) of ^{125}I-anti CA19-9 antibody respectively.The volume of the mixture was completed with PBS buffer of different pH (6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8 and 8.2) to a final volume 500 µL. After incubation for 3hrs at 37 ^{o}C, the bound CA19-9 was determined as previously described.

Calculations

1. The (B/T) % values were determined as described previously.

2. Values of (B/T) % were plotted versus the corresponding pH.

Effect of Temperature on the Binding

Procedure:

Twenty microliters (100, 75 and 75 µg protein) of cytosolic fraction (Fibroadenoma , premenopausal IDC and postmenopausal IDC) were added to 25 µL (0.0565 $mg.mL^{-1}$) of ^{125}I-anti CA19-9 antibody respectively. The volume of mixture was completed to a final volume 500 µL with PBS buffer at pH 7.8 for fibroadenoma , pH 8.0 for premenopausal (IDC) and pH 7.0 for postmenopausal (IDC). The experiment was carried out at (5, 15, 25, 37 and 45^{o}C) for 3hrs. After incubation the bound CA19-9 was determined as described previously.

Calculations:

1.The (B/T) % values were determined as described previously.

2.Values of (B/T) % were plotted versus the temperature.

Effect of Incubation Time on the Binding

Procedure :

Twenty microliters (100, 75 and 75 µg protein) of cytosolic fraction (fibroadenoma , premenopausal IDC and postmenopausal IDC) were added to 25 µL (0.0565 $mg.mL^{-1}$) of ^{125}I-anti CA19-9 antibody respectively. The reaction mixture was completed to a final volume 500 µL with PBS buffer pH (7.8 , 8.0 and 7.0) respectively. The experiment was carried out at 25 ^{o}C , 37 ^{o}C and 45 ^{o}C for fibroadenoma , premenopausal (IDC) and postmenopausal (IDC) respectively. The incubation was carried out at different time intervals (1, 2, 3, 4, 5 and 6 hrs). The bound CA19-9 was estimated as described previously.

Calculations:

1.The (B/T) % values were determined as described previously.

2.Values of (B/T) % were plotted versus incubation time.

Effects of Different Halides on the Binding

Reagents:

1. Phosphate buffer (PB) were prepared as described previously without addition of NaCl .
2. Halid reagents were prepared in concentration of 0.01M PB at pH (7.8, 8.0 and 7.0) individually, by dissolving each of 0.021gm of NaF, 0.0292gm of NaCl, 0.0515gm of NaBr, and 0.075gm of NaI in a final volume 50mL of PB and the pH was adjusted.
3. The breast tumors homogenates (fibroadenoma , premenopausal IDC and postmenopausal IDC) were prepared as described previously except using PB-buffer instead of PBS at the same pH and same concentration was carried out the homogenization.

Procedure:

The experiment was carried out at optimum conditions as mentioned previously, using three groups of human breast homogenate (i.e., fibroadenoma, premenopausal IDC and postmenopausal IDC), by incubating 20 µL of the homogenate from each group containing (100, 75 and 75 µg protein) respectively with 25 µL (0.0565 $mg.mL^{-1}$) of ^{125}I-anti CA19-9 antibody. The reaction mixture was completed to a final volume 500 µL with PBS buffer pH (7.8, 8.0 and 7.0) containing 0.01 M of each of the following salts: NaF, NaCl, NaBr and NaI in each assay tube (A sample without the addition of any salt was used as a control). The assay tubes were incubated for (4,1 and 6 h) at 25 ,37 and 45^{o}C for three group individually. The bound CA19-9 was estimated as described previously..

Calculations:

1.The (B/T) % values were determined as described previously..

2.Values of (B/T) % were plotted versus 0.01 M of NaX.

Effects of Monovalent and Divalent Cations on the Binding

Reagents:

1. Phosphate buffer (PB) were prepared as described previously. without addition of NaCl .
2. Monovalent and divalent cations (0.025 M) were prepared in PB buffer, and then the pH was adjusted to 7.8, 8.0 and 7.0 individually by dissolving each of 0.0931 gm of KCl, 0.0668 gm 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 $CuSO_4.5H_2O$, 0.1703 gm of $ZnCl_2$, in a final volume 50 ml of PB and the pH was adjusted.

Procedure:

The experiment was carried out at optimum conditions using three groups of human breast homogenate (i.e., fibroadenoma, premenopausal IDC and postmenopausal IDC) respectively. The same steps mentioned in experiment (Effects of Different Halides on the Binding) were followed to determine the effect of monovalent and divalent cations on the binding , except ; the buffer solution was PB (0.15 M) containing 0.025 M of the following salts: KCl , NH_4Cl , $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$ and $ZnCl_2$.

Calculations:

1.The (B/T) % values were determined as described previously.

2.Values of (B/T) % were plotted versus the 0.025 M of monovalent and divalent cations.

Recovery of CA19-9

Procedure:

The experiment was carried out at optimum conditions. Known concentration of CA19-9 (255 $U.mL^{-1}$) was added to the three group of benign (fibroadenoma) and malignant

(premenopausal IDC and postmenopausal IDC) breast tissues homogenates. The experiment was carried out at optimum conditions that was obtained from previously experements.

Calculations:

1. The bound (c.p.m.) of the reaction mixture added to tissue homogenate with ^{125}I-anti CA19-9 antibody, represent the measured value.
2. The bound (c.p.m.) of CA19-9 in tissue homogenate with ^{125}I-anti CA19-9 antibody only, represent the expected value.
3. The recovery % (yield) calculated as follows:

$$\text{Recovery} \quad \% = \frac{\text{Measured} \quad \text{values} \quad (\text{c.p.m})}{\text{Expected} \quad \text{values} \quad (\text{c.p.m})} x100$$

Results and Discussions:

Determination of CA19-9 levels in Sera of Patients with Benign and Malignant Breast Tumors

CA19-9 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 immunoradiometric assay. Three groups were matched with one group of control subjects. Table (2) shows the results obtained from this study. CA19-9 concentration of specimens and control were determined directly from standared curve in figure (1) .The level of serum CA19-9 in benign breast tumor patients was found to be 31.0 U.mL^{-1} ($p<0.05$), where that of (pre-and post-menopausal) malignant breast tumor patients were found to be 33.1 U.mL^{-1} ($p<0.05$) and 32.1 U.mL^{-1} ($p<0.0005$) respectively. While in control, the level was found to be 28.8 U.mL^{-1} .Matching case and control subject proved to be important for controlling undesired variability. The mean CA19-9 was significantly high in postmenopausal patients ($p<0.0005$) while in premenopausal and benign breast tumors the mean of CA19-9 was significantly low ($p < 0.05$ Student's t-test).

Table (2): Sera CA19-9 levels (U.mL^{-1}) in patients with benign and malignant breast tumors.

Group	Patients	No. of Cases	Age (year)	Serum CA19-9 U.mL^{-1} (mean ± SD)	P values
I	Benign breast tumors	10	18-35	31.0 ± 1.52	P<0.05
II	Premenopausal malignant breast tumors	10	35-43	33.1 ± 2.79	P<0.05
III	Postmenopausal malignant breast tumors	10	53-65	32.1 ± 0.13	P<0.0005
Control	Control	10	25-35	28.8 ± 0.631	

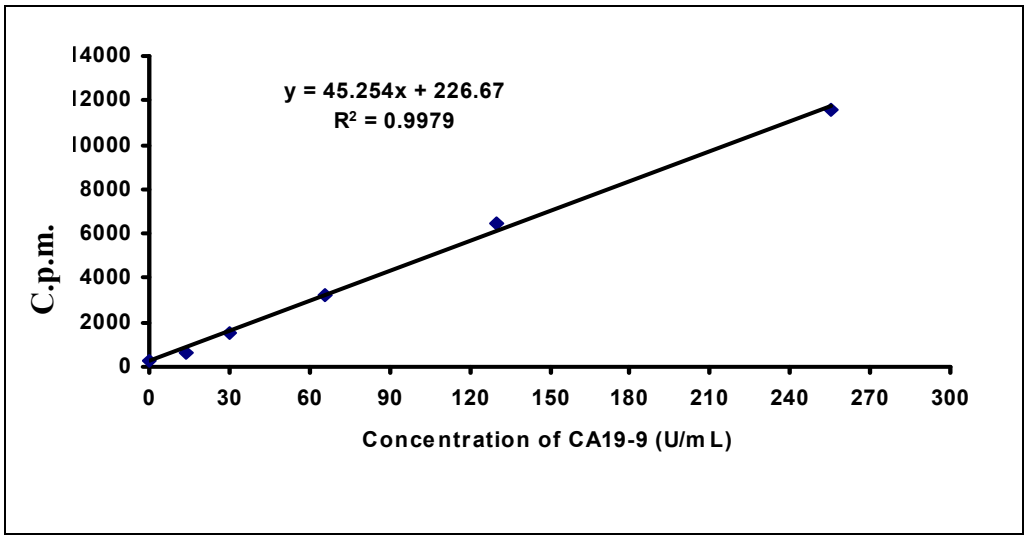

Figure (1): Standard curve of CA19-9.

CA19-9 was at low concentration in sera of healthy individuals, these results are in agreement with several authors previously [23]. There were few studies to evaluate CA19-9 in breast tumors patients. Several investigators [11] detected CA19-9 in bone metastasis in breast cancer patients and in patients without documented metastases and reported that CA19-9 level elevated in patients with metastases breast cancer. When patients were analyzed with respect to the menopausal status, significant differences between the metastases and non metastases cancer patients was detected [11]. Several studies proved the possibility of the role of carbohydrate antigen 19-9 as a tumor marker in colorectal cancer [24], pancreas [25], gastric [26], liver disease [27] and esophageal cancer[9]. Recently, European group proved that CA19-9 monitored in patients with tumors of gastrointestinal tract and endometrial cancer could be used as a tumor marker and can be helpful in monitoring patients with breast cancer. They observed significant increase of CA19-9 and CA15-3 levels in all patients [28].

Preliminary Test of the Binding of CA19-9 with ^{125}I-Anti CA19-9Antibody :

Supernatant and pellet obtained at speed (4000 r.p.m) were investigated in the three groups of human breast tumor homogenate (fibroadenoma, premenopausal IDC and postmenopausal IDC). In each fraction, CA19-9 was detected through the incubation of ^{125}I-anti CA19-9 antibody with crude fraction supernatant and pellet individually for 3 h at 37^{o}C in PBS buffer pH 7.2 as a medium to complete the reaction.

The separation of the bound antibody from unbound was carried out at 4000 r.p.m for 45 min. to precipitate the ^{125}I-anti CA19-9 antibody/CA19-9 complex formed.

Table (3): Incidence of CA19-9 in supernatant and pellet fractions in three different breast homogenate.

Groups	Age(year)	B/T %	
		Supernatant fraction	Pellet fraction
Benign	34	5.32	1.43
Premenopausal (IDC)	43	5.48	2.03
Postmenopausal (IDC)	63	5.86	2.47

Table (3) shows the amount of binding B/T % values of pellet and supernatant fractions. The data revealed that CA19-9 in cytosolic fraction obtained from supernatant was higher in incidence than in pellet fraction, according to these results cytosolic fraction was collected. CA19-9 collected and the pellet was then discarded.

Factors Effecting of ^{125}I-Anti CA19-9 Antibody Binding to CA19-9 in Breast Tumors Homogenates

Effect of Protein Concentration on the Binding

To obtain the optimum protein concentration of cytosolic fraction for the binding of CA19-9 with ^{125}I-anti CA19-9 antibody, cytosolic fraction containing increasing amount of soluble CA19-9 in the presence of fixed amount of ^{125}I-anti CA19-9 antibody was carried out. Figure (2) represent the formation of (^{125}I-anti CA19-9 antibody/CA19-9) complex in three cases (fibroadenoma, premenopausal IDC and postmenopausal IDC) and shows that (100, 75 and 75 µg protein) were the most appropriate concentration to give the maximum values of binding in crude fraction of three cases respectively. The decrease of the binding at high concentration of cytosolic fraction (in three cases) in the reaction mixture may be due to a conformational change in CA19-9 and ^{125}I-anti CA19-9 antibody rather than the formation of reversible inactive (^{125}I-anti CA19-9 antibody/CA19-9) complex [29] and may be due to splitting antigen into large fragments with proteolytic enzymes [30]. In all subsequent experiments an amount of (100, 75 and 75 µg protein in three cases respectively), were used in the incubation mixture.

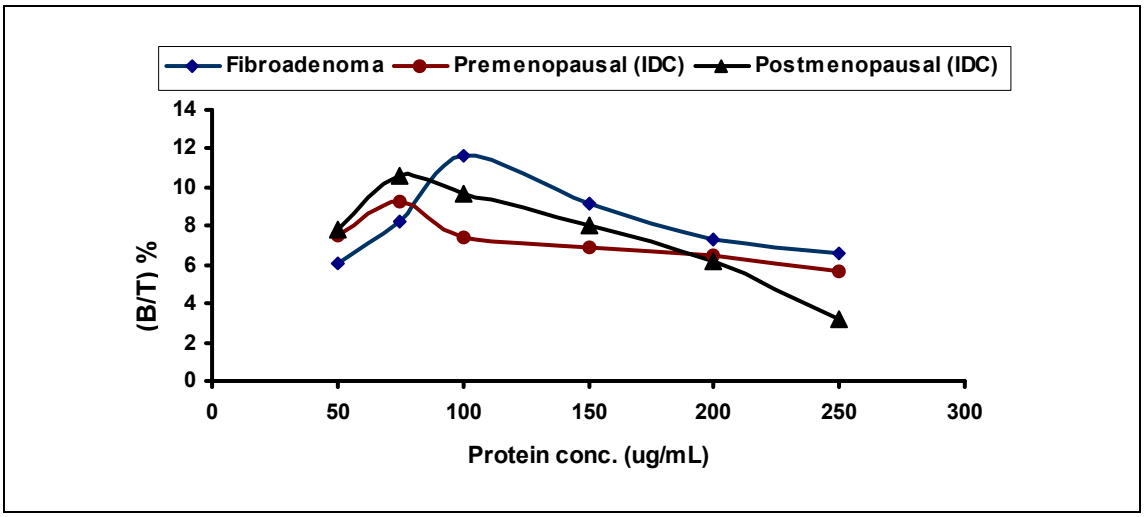

Figure (2): Influence of increasing protein concentrations on the binding of CA19-9 with ^{125}I-anti CA19-9 antibody.

Effect of ^{125}I-Anti CA19-9 Antibody concentration on the Binding

One of the most important factors that effect binding is the concentration of ^{125}I-anti CA19-9 antibody. To determine the suitable concentration of ^{125}I-anti CA19-9 antibody, cytosolic sample (100, 75 and 75 µg protein) in the three cases (fibroadenoma, premenopausal IDC and postmenopausal IDC) respectively were incubated with increasing concentration of ^{125}I-anti CA19-9 antibody, the incubation was carried out for 3 h at 37 ^{o}C. The results revealed that the optimum concentration of the ^{125}I-anti CA19-9 antibody to give the maximum binding in all three cases was (0.0565 mg.mL^{-1}). The results showed that an increase in the conc. of ^{125}I-anti CA19-9 antibody caused a decrease in the binding %. This is because the soluble complexes, and the excess of antibody cover all antigentic sites, which leads to complex formation inhibition. Accordingly in all subsequent experiments, 0.0565 mg.mL^{-1}of ^{125}I-antiCA19-9 was used as the optimum conc., which gives the highest binding %.

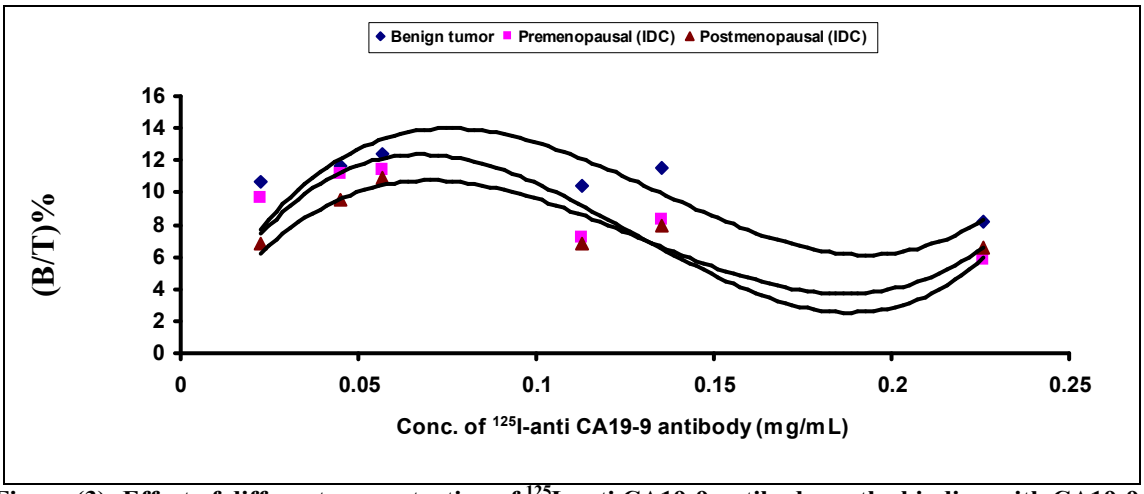

Figure (3): Effect of different concentration of ^{125}I-anti CA19-9 antibody on the binding with CA19-9.

Effect of pH on the Binding:

The effect of pH on the binding of radioactivity CA19-9 to its antigen CA19-9 was investigated. Figure (4) shows that the maximum binding of ^{125}I-anti CA19-9 antibody to its antigen CA19-9 was found to be (7.8, 8.0 and 7.0) in the three cases used (fibroadenoma , premenopausal IDC and postmenopausal IDC) respectively. The shift in pH of the environment may involve a protonation-deprotonation process occuring within the change of polar groups of the amino acids residues present in the binding domain [31] .According to these results, the pH of the buffer used in all subsequent experiments were (7.8, 8.0 and 7.0) for the three cases respectively.

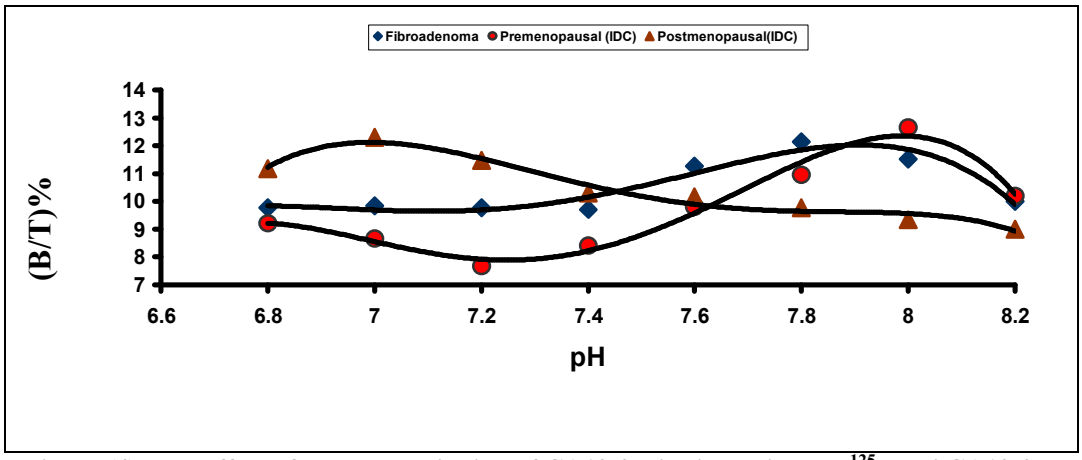

Figure (4): The effect of pH on the binding of CA19-9 with its antibody ^{125}I-anti CA19-9 antibody with CA19-9.

Effect of Temperature on the Binding:

Temperature dependency of the association of ^{125}I-anti CA19-9 antibody to its cytosolic fraction CA19-9 was investigated. Cytosol fraction of benign and malignant breast tumors was incubated for 3 hrs at different temperatures (5, 15, 25, 37 and 45 ^{o}C). Figure (5) reveals that the binding of ^{125}I-anti CA19-9 antibody to its cytosol fraction CA19-9 was increased when the temperature was raised from 5 to 25 ^{o}C in fibroadenoma and the maximal binding was obtained at 25 ^{o}C and from 5 to 37 ^{o}C in premenopausal (IDC) and the maximal binding was obtained at 37 ^{o}C. Finally from 5 to 45 ^{o}C in postmenopausal (IDC) and the maximal binding was obtained at 45 ^{o}C. The decrease in the binding at temperature higher than the optimum temperature is probably due to denaturation of CA19-9 molecules [32]or due to proteolytic degradation of enzyme [33]. According to these results (25 ^{o}C , 37 ^{o}C and 45 ^{o}C) respectively they will be used in all the subsequent experiments for the three cases used.

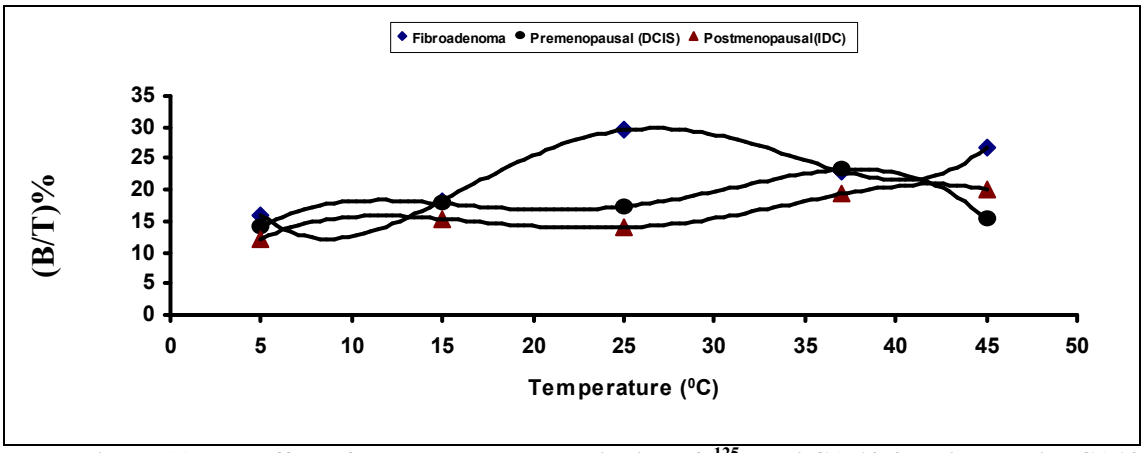

Figure (5): The effect of temperature on the binding of ^{125}I-anti CA 19-9 antibody with CA19-9.

The Effect of Incubation Time on the Binding:

To choose the most appropriate incubation time at (25, 37 and 45 ^{o}C) for the three cases used in this study (fibroadenoma, premenopausal IDC and postmenopausal IDC) respectively , the experiments were carried out at different time intervals. Figure (6) shows the results of this analysis. It seemed that the specific binding of ^{125}I-anti CA19-9 antibody to cytosolic fraction homogenate for the three cases were maximal at (4,1 and 6 hrs) respectively. In view of these results, the incubation time used in all subsequent experiments were (4,1 and 6 hrs) respectively.

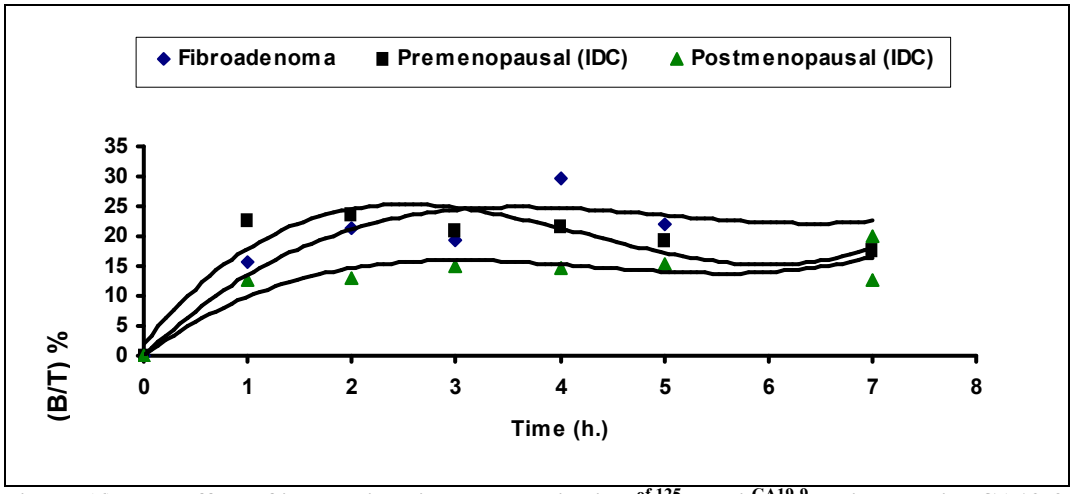

Figure (6): The effect of incubation time on the binding $^{of\ 125}$I-anti $^{CA19-9}$ antibody with CA19-9.

Effect of Different Halides on the Binding:

Figure (7) shows the effect of different halides salts (i.e., NaF, NaCl, NaBr and NaI) at 0.01 M concentration on the extent of ^{125}I-anti CA19-9 antibody binding to their cytosol fraction homogenate in benign and malignant breast tumors. The sodium halides (ion radius)

in the incubation mixture of benign and postmenopausal malignant breast tumors induced inhibition of the percent of binding according to the following sequence:

NaI>NaF>NaCl>NaBr

While the sodium halides in the incubation mixture of premenopausal malignant breast tumor (IDC) induced activation of the percent of the binding in the order:

NaF<NaCl<NaI<NaBr

Melander and Horvath (1977) reported that the effect of halide salt type on hydrophabic interactions is quantified by its molar surface tension increment (MSTI) which is a measure of the increasing in a surface tension by the salt [34], also they found that parameter increases as the following sequence:

NaF>NaCl>NaI

The same researches found that halides with higher MSTI values will strengthen the hydrophabic interactions while halides with lower MSTI values reverse this effect. Thus the dependence of the extent of the binding in benign and malignant (pre-and post-menopausal) breast tumors on MSTI values of the corresponding halide further implicates the low involvement of hydrphobic forces in maintaining the stability of (^{125}I-anti CA19-9 antibody /CA19-9) complex formed.

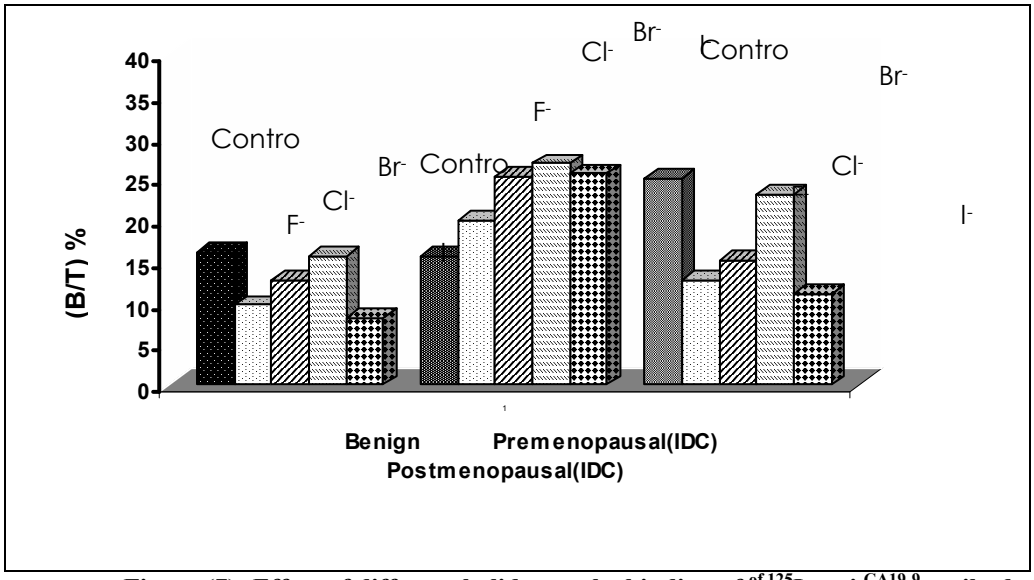

Figure (7): Effect of different halides on the binding of $^{of\ 125}$I-anti $^{CA19-9}$ antibody with CA19-9.

Effect of Monovalent and Divalent Cations on the binding:

Figure (8) and (9) show the effect of different divalent and monovalent cations respectively on the binding value in benign and malignant breast tumors. The results indicate that the binding process is sensitive to the presence of cation metal ions. $CuSO_4.5H_2O$ at concentration 25 mM was showed to increase the binding two folds than the control as compared with other divalent cations. $CaCl_2.2H_2O$ induced activation in the binding in benign (fibroadenoma) and malignant (premenopausal IDC), while induced inhibition in the

binding in malignant (postmenopausal IDC). $ZnCl_2$ decreased the binding in two groups (fibroadenoma and premenopausal IDC), while $ZnCl_2$ increased the binding in malignant (postmenopausal IDC). The frequency of the stimualtion of the binding of ^{125}I-anti CA19-9 antibody to its cytosolic fraction CA19-9 homogenate of the three groups by divalent cations is according to the following:

Postmenopausal breast cancer tissue homogenate (IDC)

$$Cu^{+2}>Zn^{+2}>Mn^{+2}>Mg^{+2}>Ca^{+2}$$

Premenopausal breast cancer tissue homogenate (IDC)

$$Cu^{+2}>Ca^{+2}>Mn^{+2}>Mg^{+2}>Zn^{+2}$$

Benign breast tumor tissue homogenate (Fibroadenoma)

$$Cu^{+2}>Ca^{+2}>Mg^{+2}>Mn^{+2}>Zn^{+2}$$

The binding of metal ions to proteins is a function of pH among the different classes of groups, such as carboxyl, amino, imidozol and tyrosyl (the unshared electron pairs for nitrogen , oxygen and sulfur atoms) [35]. The sites of binding of metal ions may range from elaborate chelate sites to simple complex formation which discrete single ligand groups in the protein. In short, chelation plays a dominant role in establishing the relative strengths of binding of a given metal ion by various sites in protein [36].Figure (9) shows that monovalent cations inhibite the binding in benign and malignant premenopausal (IDC), while the monovalent cations induce activation of the binding in-group of malignant postmenopausal (IDC). The alternation of increased and decreased binding percent between these cations may be ascribed to the differences in tissues studied. The variation of results obtained between these divalent cations may be ascribed to the difference in tissue studied.

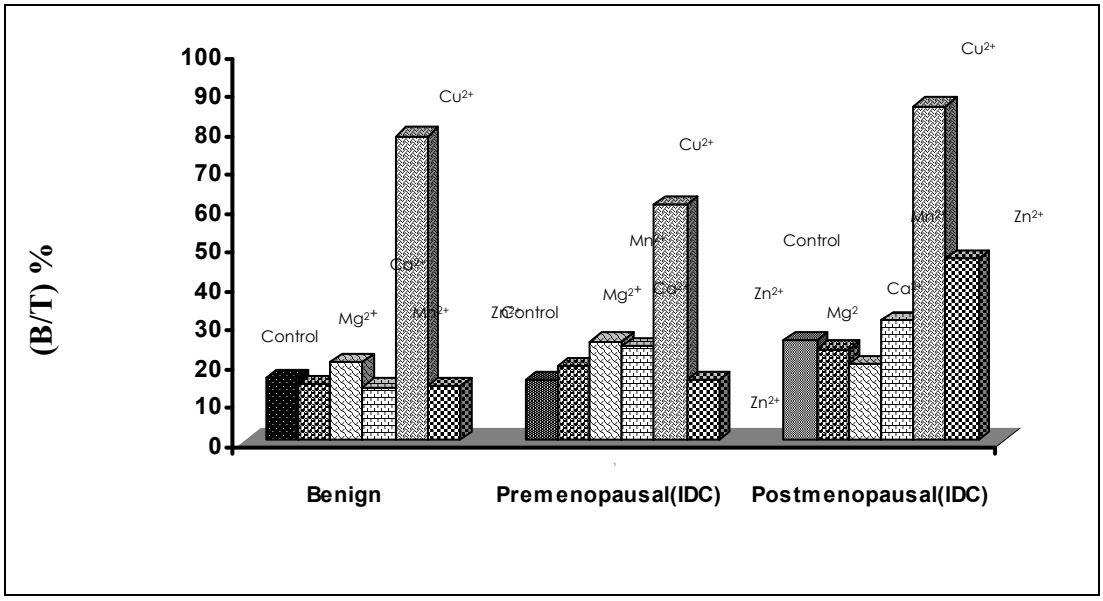

Figure (8): Effect of different cations on the binding of ^{125}I-anti $^{CA19-9}$ antibody with CA19-9 in different human breast tumor homogenate.

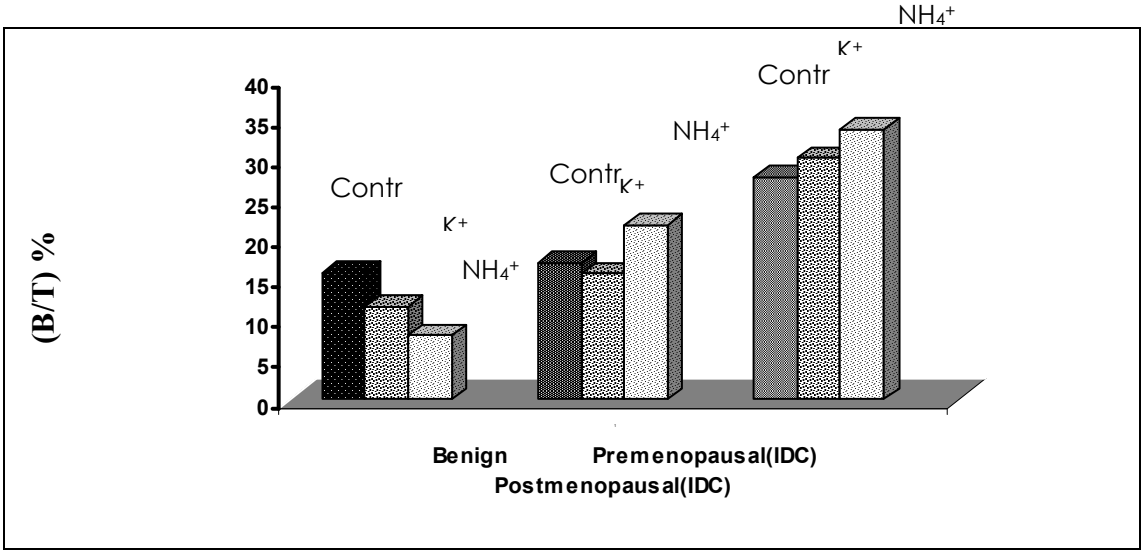

Figure (9): Effect of different monovaleat cations on the binding of ^{125}I-anti $^{CA19-9}$ antibody with CA19-9 in different human breast tumor homogenate.

Recovery of CA19-9**:**

The method used to estimate the percent recovery of cytosolic fractions of benign (Fibroadenoma) and malignant (pre-and post-menopausal IDC) breast tumors homogenates. The results summarized in table (4) indicate that CA19-9 extracted from malignant breast tumors homogenates was recovered less than CA19-9 extracted from benign breast tumor (fibroadenoma) and CA19-9 extracted from malignant premenopausal (IDC) malignant breast tumors homogenates was recovered more than CA19-9 extracted from postmenopausal (IDC) malignant breast tumors homogenates. Also the results indicate that total CA19-9 could determine through the developed method of immunoradiometric assay. The percent of recovery indicates the precision of the used method.

Table (4): Recovery of CA19-9 .

Type of CA19-9	Measured B/T %	Expected B/T %	Recovery % Measured/ Expected %
Benign	103	104	99
Premenopausal	192	195	98
Postmenopausal	166	175	95

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