Binding of CA125 to ^{125}I-antiCA125 Antibody in Breast Tumor Homogenates

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Abstract

The binding of CA125 to ^{125}I-antiCA125 antibody in the tissues of patients with benign and malignant breast tumors (malignant post-menopausal breast tumor patients, GI; the second contains the malignant pre-menopausal breast tumor patients, GII; and the third group comprise of the benign breast tumor patients, GIII) was studied using developed immunoradiometric assay kit equipped by Immunotech (France). The results showed that the supernatant fraction of the tissue homogenates contain higher CA125 level than the pellet fraction in all studied groups. Consequently, it was used in the further studies.

The effect of protein concentration, ^{125}I-antiCA125 antibody concentration, pH of the reaction medium, time of reaction and temperature was studied for the binding of CA125 to ^{125}I-antiCA125 antibody, in the tissue homogenates of benign and malignant breast tumors. The results of this study were considered the optimum conditions for binding in the studied groups. Optimum protein concentration was (0.81, 0.8 and 0.71 mg/ml) for (GI, GII and GIII) respectively, ^{125}I-antiCA125 antibody optimum concentration was (2.6, 2 and 1.6) mg/ml for (GI, GII and GIII) respectively. Optimum pH was (7.2, 7 and 7.4) for (GI, GII and GIII) respectively. All groups reacted at maximum at 5°C and for 2 hrs.

The effect of some factors like halides, medium polarity was studied. The results showed a decrease in binding with increasing atomic number of the halide and an increase with decreasing medium polarity.

Introduction

CA125 antigen is the tumor marker of choice in detection the ovarian cancer when measured in sera of those patients [1]. It was mentioned that CA125 antigen could be evaluated in sera of patients with other gynecological cancers like breast cancer [2]. Also, Jensen et al showed that serum level of CA125 antigen could utilizes the presence and monitoring the changes in breast cancer patients. Also, higher CA125 antigen levels were found in the sera of non-ovarian tumors such as those originating from the breast [3].

The problem of biological sample processing to deal is a complex one, because there is no simple system that will meet the requirements of all analytes [4]. This problem made the necessity of developing an assay for determination the CA125 in tissue homogenates of benign and malignant breast tumors.

Immunoradiometric assay technique has not been used, to the best of our knowledge, to study the binding of CA125 to the ^{125}I-antiCA125 antibody in breast tumor homogenates. Hence, the purpose of this study is to do so and the finding of the optimum reaction conditions, like antigen and antibody concentrations, pH, time and temperature and other factors affecting binding like halides and medium polarity.

Materials and Methods

Materials

Chemicals

All chemicals in this study were of analar grade. The specifications of these chemicals are tabulated in Table (1).

Table (1). Specifications of the used chemicals.

Chemicals	Company
Immunoradiometric Assay Kit for CA125 level	Immunotech (France)
Bovine serum albumin, Ethylendiamine tetraacetic disodium salt	Fluka (Switzerland)
$CuSO_4$, NaK tartrate, NaOH, HCl, Na_2CO_3, NaF, NaCl, NaI, Tris buffer, Polyethylene glycol 6000 (PEG 6000), Ethanol	BDH (UK)
Folin Ciltau	E. Merck AG, Darmstadt

Instruments

All instruments used in this study are listed in Table (2).

Table (2). Instruments and their manufacturer company.

Instruments	Company
Gamma counter type 1250-auto gamma II	LKB
Double Beam Spectrophotometer	Shimadzu
pH meter	Pye Unicam
Cooling Centrifuge, with a maximum speed 5000 r.p.m.	Germany
Memmert water bath, memmert incubator	Germany
Personal Computer PIII	-
Microsoft Excel software	USA

Patients

In the present work three groups of patients of breast cancers were selected according to the

investigation of a histopathologist. The first group includes the malignant post-menopausal breast tumor patients (GI), the second contains the malignant pre-menopausal breast tumor patients (GII) and the third group comprise of the benign breast tumor patients (GIII). The fourth group was considered as a control group clear from any source of CA125 antigen level elevation. Table (3) describes the host information for patients and control individuals in this study.

Table (3). The host information of patients and control individuals included in this study.

Group	Patients	No.	Age range	Type of tumor
GI	Post-menopausal breast tumor	23	55-86	Infiltrative ductal carcinoma
GII	Pre-menopausal breast tumor	17	23-48	Infiltrative ductal carcinoma
GIII	Benign breast tumor	25	19-52	Fibroadenoma
Control	Healthy individuals	16	23-36	-

All above selected patients were admitted for treatment at Al-Yarmook Teaching Hospital and Al-Kadhimya Teaching Hospital. All surgical operations of breast tumor were carried out under the supervision of surgeons.

Collection of Specimens

The tumor was surgically removed from breast tumor patients by either mastectomy (cancer patients) or lumpectomy (benign tumor patients). The specimens were immediately kept in a saline solution and stored at -20°C to the time of homogenizing.

Preparation of Tris-Buffer Solution

A total buffer concentration of 0.05 M of pH 7.2 of tris-buffer solution was prepared. Additives (sucrose and EDTA) used in preserving protein moiety were added to the buffer solution according to the following:

Tris-buffer (0.6075 g), sucrose (8.5575 g) and EDTA (0.1816 g) were dissolved in 100 ml deionized water and the pH was adjusted to 7.2.

Homogenization of Breast Tumor Tissues

The frozen tissue was sliced finely and scalped in Petri dish standing on ice, and then homogenized with three fold volumes of buffer pH 7.2 using manual homogenizer [5]. The homogenate was filtered through four layers of nylon gauze to eliminate fiber connective tissues. The filtrate was centrifuged at 4000 r.p.m. for 30 min at 4°C in order to precipitate the remaining intact cells and the intact nucleus. The supernatant and precipitate fractions were separated and frozed at -20°C until use.

Methods

Determination of Protein Concentration

Total protein was determined by Lowry method [6] using bovine serum albumin (BSA) as a standard. The standard curve of protein concentration was constructed by measuring the absorbance of the standards at 750 nm. The straight line equation for this standard curve was found and used for determination of homogenate protein content.

Preliminary Binding of CA125 to ^{125}I-Anti CA125 Antibody in Breast Tumor Homogenates

Reagents

1. Tris buffer of 0.05M for the binding experiments was prepared according to the following:
   Tris buffer (0.6075 g), bovine serum albumin (BSA) (1g), were dissolved in 100ml deionized water. The pH was adjusted to 7.2 and the buffer was freshly prepared at each experiment.
2. PEG 6000 was prepared by dissolving 1g in 10 ml of tris-buffer 7.2.

Procedure

- Three clean and dry tubes were counted for their background using Gamma counter.
- Fifty µl (2.9 mg/ml) of ^{125}I-CA125 Ab (tracer) was added to the first tube and denoted as total counts (T).
- Fifty µl of tracer was mixed in the second tube with 500µg of homogenate and denoted as "no precipitating reagent" (NP).
- Fifty µl of tracer was mixed with 500µg of filtrate of the homogenate in the third tube and denoted as "precipitating agent" (P).
- The volume of all tubes was completed to 500 µl using tris buffer of pH 7.2.
- The tubes were incubated at 25°C for 4 hrs.
- After 4hrs, 500µl 10% polyethylene glycol 6000 (PEG6000) was added to first (T) and third (P) tubes, and the incubation was continued for further 1hr.
- All tubes were centrifuged at 4000 r.p.m for 30 min at 4°C.
- The supernatant was decanted and the rim at each tube was swapped with cotton.
- The radioactivity of the formed antibody antigen complex was counted using gamma counter for 1 min.
- The pellet of the homogenate was dissolved in 1:3 tris-buffer at pH 7.2, and the protein concentration was measured by Lowry method. The steps mentioned above were repeated for the dissolved pellet to determine the radioactivity of the complex.

Calculations

1. The counted radioactivity in each tube (expressed in C.P.M) represents the bound (B).
2. The counts radioactivity in the tubes containing ^{125}I-Anti CA125Ab only represents the total counts (T).
3. The (B/T) ratio for each tube counted as follows:

$$\frac{B}{T} = \frac{\text{Sample counts}}{\text{Total counts}} \times 100$$

Factors Affecting of the Binding CA125 to ^{125}I-anti CA125 Antibody in Breast Tumor Homogenate

Effect of Different Amounts of Protein Concentration of the Tumor Homogenates on the Binding

Reagents

All reagents were prepared as described in the experiment of preliminary binding.

Procedure

1. Fifty micro liters (2.9 mg.ml^{-1}) of ^{125}I-AntiCA125Ab were used for the total radioactivity and an increasing amounts of protein (50, 100, 200, 300, 400, 500 and 600 µg.ml^{-1}) for the supernatant of (post-menopausal malignant breast tumors "GI", pre-menopausal malignant breast tumors "GII" and benign fibroadenoma "GIII") were added then completed to a final volume of reaction to 500µl with prepared buffer
2. The assay tubes were then incubated for 4hrs at 25°C.
3. PEG 6000 (500µl, 10%) was added to each tube and incubation continued for further 1hr.
4. At the end of incubation, the assay tubes were centrifuged at 4000 r.p.m for 30min at 4°C.
5. The supernatant was decanted and the rims of the tubes were swapped with cotton piece.
6. The radioactivity of the complex was counted using gamma counter.

Calculations

1. The B/T% was determined according to the experiment of preliminary binding.
2. The B/T% was plotted against the increasing amounts of protein of breast tumor homogenate.
3. The generated curves were treated with Microsoft Excel on the personal computer (PC).

Effect of ^{125}I-Anti CA125 Antibody Concentration on the Binding

Reagents

All reagents were prepared according to the experiment of preliminary binding.

Procedure

1. Increasing amounts (1, 2, 3, 4, 5 and 6 mg/ml) of ^{125}I-anti CA125 Ab were read for their radioactivity and then added to homogenate (GI, GII and GIII) containing (0.81, 0.8 and 0.71 mg/ml) of (GI, GII, and GIII) respectively, the reaction volume was completed to 500µl with tris buffer pH 7.2.
2. Steps 2, 3, 4, 5 and 6 mentioned in experiment of protein concentration were repeated.

Calculations

1. The B/T% was determined according to the experiment of preliminary binding.
2. The percent of binding was plotted against ^{125}I-CA125 Ab concentrations.
3. The generated curves were treated with Microsoft Excel using PC.

The Effect of pH on the Binding

Reagents

All reagents were prepared as mentioned in the experiment of preliminary binding except PEG6000. PEG6000 was prepared in a set of different pHs (6.8-8) by dissolving 1g of PEG6000 in 10ml of tris buffer of different pHs (6.8-8).

Procedure

1. Tracer (2.6, 2 and 1.6) mg/ml were added to (0.81, 0.8 and 0.71 mg/ml) of GI, GII and GIII respectively.
2. Each reaction mixture was completed to 500µl with tris-buffer at different pH (6.8-8).
3. Steps 2, 3, 4, 5 and 6 mentioned in experiment of protein concentration were repeated except the addition in PEG 6000 according to pH values of the reaction mixture.

Calculations

B/T% was plotted against their pH values

Time Course of the Binding

Reagents

All reagents were prepared according to the experiment of preliminary binding except 10% PEG6000 which was prepared according to the optimum pH of each group (i.e, GI, GII and GIII).

Procedure

1. (Tracer (2.6, 2 and 1.6) mg/ml were added to (0.81, 0.8 and 0.71 mg/ml) of (GI, GII and GIII) respectively.
2. Each mixture was completed to 500µl with tris-buffer at the optimum pH of each group. (pH 7.2, 7.4 and 7) for GI, GII and GIII respectively.
3. All tubes were incubated at 25°C at different time intervals (1, 2, 3, 4, 5 and 6) hrs.
4. Steps 3, 4, 5 and 6 mentioned in experiment of protein concentration were repeated.

5. To determine the time course of CA125 binding to ^{125}I-Anti CA125 Ab at different temperatures. Steps 1, 2, 3 and 4 in this experiment were repeated at different temperature (5, 37, 45)°C.

Calculations

The B/T % values were plotted against the time of incubation at different temperatures.

Effect of Different Halides on the Binding

Reagents

1. All reagents were prepared as according to the experiment of preliminary binding.
2. Halide reagent was prepared at concentration of 0.3M at the optimum pH of each group.

Procedure

1. Tracer (2.6, 2 and 1.6) mg/ml were added to (0.81, 0.8 and 0.71 mg/ml) of protein of (GI, GII and GIII) homogenate respectively.
2. Fifty µl of 0.3M NaF was added to the reaction mixture and then completed to 500µl with tris-buffer. The same step was repeated for the rest of halides.
3. Steps 3, 4, 5 and 6 mentioned in experiment of protein concentration were repeated.

Calculations

The B/T % was plotted against the halide type.

The Effect of Medium Polarity on the Binding

Reagents

1. All reagents were prepared according to the experiment of preliminary binding.
2. Absolute ethanol was used.

Procedure

1. Step (1) of effect of halide experiment was repeated.
2. Twenty five µl of absolute ethanol was added to the reaction mixture to prepare 5 percent of reaction mixture, then the volume was completed to 500 µl by tris-buffer at optimum pH. The same step was repeated to have (10, 15, 20 and 25%) of ethanol in reaction mixture.
3. Steps 3, 4, 5 and 6 mentioned in experiment of protein concentration were repeated.

Results and Discussion

Preliminary binding of the Binding of CA125 to ^{125}I-AntiCA125 Antibody in Breast Tumor Homogenates

Table (4) shows the results of the preliminary binding for the binding. The results reveal that the filtrate fraction contains higher CA125 content than the pellet fraction according to the (B/T%) values. On the other hand, the presence of precipitating agent increases the (B/T%) in relative to the (B/T%) in the absence of PEG6000, since the PEG6000 precipitate the (CA125/^{125}I-antiCA125 antibody) complex by salting out effect. According to these results, the cytosolic fraction of the homogenate was used in this study and the complex was precipitated using PEG 6000 to increase the detection of the complex. The pellet fraction was discarded.

Table (4). Results of preliminary binding for the binding of CA125 antigen to ^{125}I-anti CA125 antibody in cytosolic and pellet fractions of the tumor homogenates.

Group	B/T%					
	Filtrate			Pellet		
	T	P	NP	T	P	NP
GI	0.7	4.5	2.3	0.8	1.2	0.7
GII	0.9	5.6	2.9	0.8	0.9	0.7
GIII	0.6	4.3	3.1	0.5	1.3	0.9

T = ^{125}I-anti CA125 antibody alone (Tracer).
P = presence of precipitating agent (PEG6000).
NP = absence of precipitating agent.

Factors Affecting of the Binding of CA125 to ^{125}I-AntiCA125 Antibody in Breast Tumor Homogenates

Effect of Different Amounts of Protein Concentration of the Tumor Homogenates

This experiment was carried out at fixed ^{125}I-antiCA125 antibody and increasing amounts of protein concentration. The results of the three reactions for each group gave hyperbolic shapes when the B/T % was plotted against protein concentration.

The rate of reaction of ^{125}I-antiCA125 antibody with CA125 increases rapidly to a maximum point, then the rate begins to be almost constant that represents the saturation of the ^{125}I-antiCA125 antibody with CA125. This type of interaction was the same in all three groups.

The optimum protein concentration was determined by mathematical treatment for each curve. Each curve has two phases, rapid increase to a highest point and a plateau phase after the highest point. Two straight line equations were generated for each phase. The optimum protein concentration represents the intersection point between these two straight lines. This mathematical treatment was achieved by using Microsoft Excel Figure (1) shows the behavior of (B/T%) against protein concentration for the three studied groups.

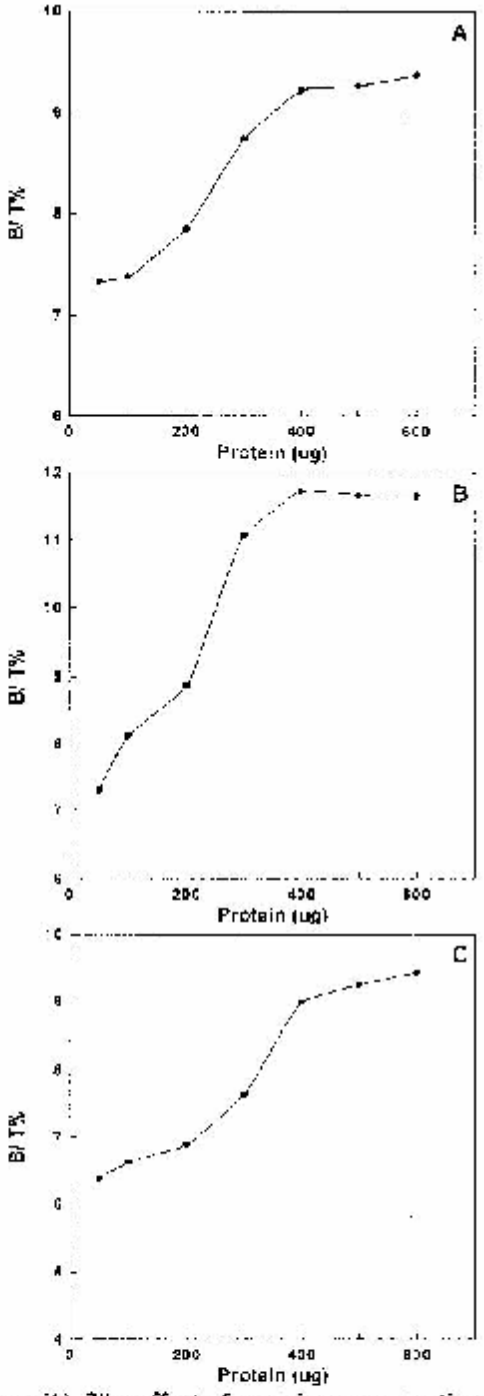


Figure (1). The effect of protein concentration on the binding of CA125 antigen to its ^{125}I-anti CA125 antibody in breast tumor homogenates, A) GI, B) GII, C) GIII.

Effect of ^{125}I-Anti CA125 Antibody Concentration on the Binding with CA125

Figure (2) shows the relation of (B/T%) against ^{125}I antiCA125 antibody concentration in the three studied group.

The optimum protein concentration was fixed in this experiment and the concentration of ^{125}I-antiCA125 antibody was increased within a range of (0.7-4.3) mg.ml^{-1}. The phases of the curve again gave hyperbolic shapes that agreed with that observed in the protein effect experiment. These two experiments suggest that the CA125 behaves in same manner whatever the source of its production, (i.e., GI, GII or GIII). The optimum ^{125}I-antiCA125 antibody concentration was determined in a similar mathematical treatment for previous experiment.

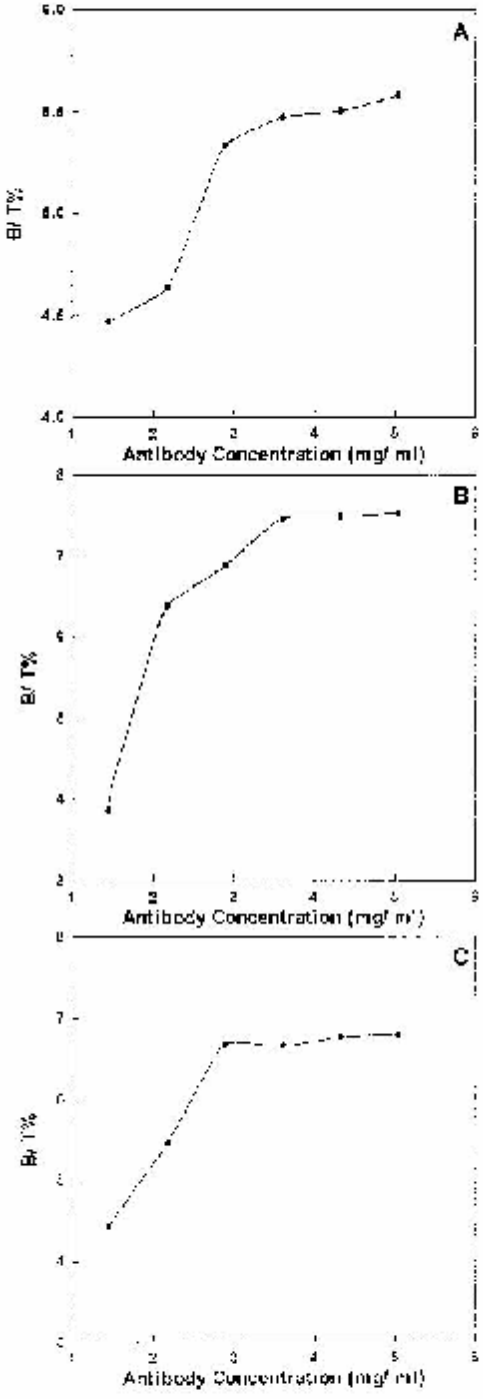


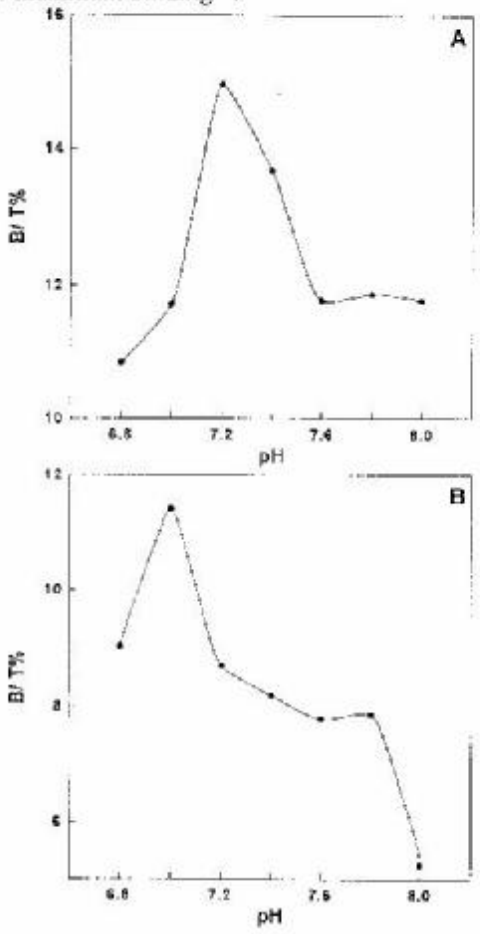
Figure (2). The effect of ^{125}I-anti CA125 antibody concentration on the binding with CA125 in breast tumor homogenates, A) GI, B) GII, C) GIII.

These hyperbolic shapes may indicate that the ^{125}I-antiCA125 antibody is monospecific antibody and directing toward a single and unique epitope which is not repeated within the structure of the

antigen that may prevent multivalent reactions. It should be noted that multivalent reactions between antibody and antigen might give biphasic response curve, which referred to "Hook" effect which has ascending and descending phases at low and high antigen concentration [7].

Effect of pH on the Binding

Figure (3) shows the effect of pH on (B/T%). All studied groups show the bell-shape curve. Although there is a small difference in optimum pH between these groups, it is considered to be significant and this difference may be due to presence of isomers of CA125 in the site of origin (i.e., GI, GII and GIII). On the other hand, this effect may include protonation-deprotonation processes occurring in the active site environment of this molecule. The optimum pH of binding is the pH at which the two proteins (CA125 and ^{125}I-ntiCA125 antibody) are in the right complementarity to each other. The change in pH will change the ionization state of the amino acid residues till reach to optimum ionization state of binding at which each negative charge on one of them will face a positive charge on the other to have maximum binding[8].



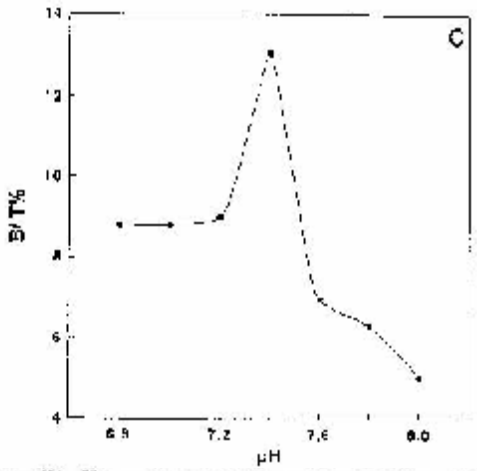


Figure (3). The effect of pH on the binding of CA125 to its ^{125}I-anti CA125 antibody in breast tumor homogenates, A) GI, B) GII, C) GIII. (All other details are explained in the text).

Time Course of the Binding of CA125 to ^{125}I-AntiCA125 Antibody in Breast Tumor Homogenates

Figure (4) shows the time course of binding of CA125 to ^{125}I-anti CA125 antibody. The process seems to be time and temperature dependent. In all the studied groups, the binding was increased when the time of reaction was lowered to 2 hrs. The binding continue to decrease when the time increase from 2 hrs to 6 hrs. The time of 2 hrs has been considered to be the optimum time for binding and it was agreed with that found by Masuho[9]. The optimum temperature was 5°C whereas the binding was decreased when the temperature increased to 45°C. The results reveal that the (CA125/^{125}I-antiCA125 antibody) complex formation is sensitive to temperature. The increase in temperature may affect the binding site in CA125 or ^{125}I-anti CA125 antibody or due to destruction of the binding forces of the complex. These results were agreed with that published previously [10]. The time course behavior was identical in all groups.

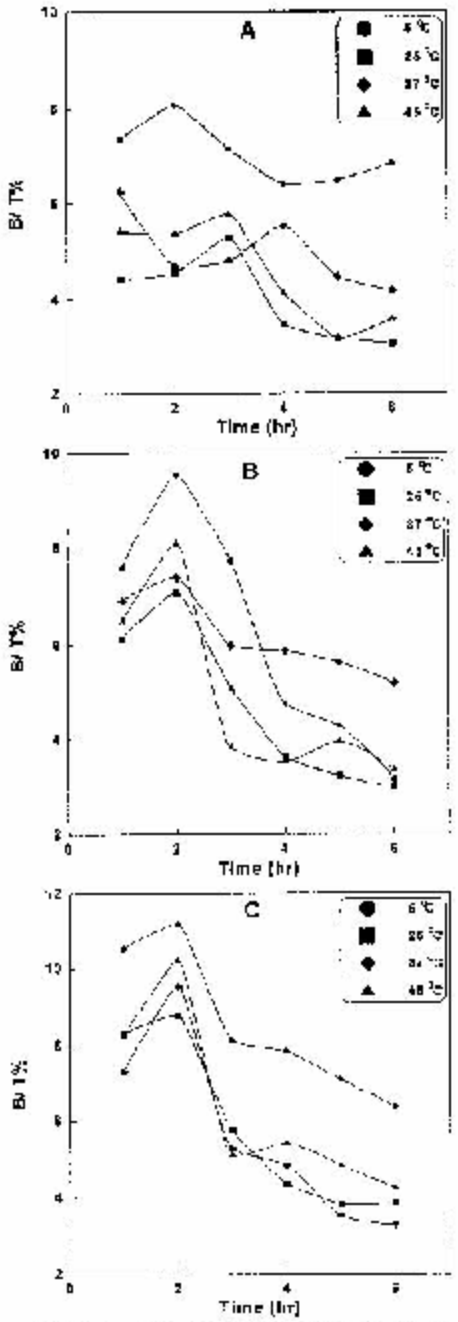


Figure (4). Time course of binding of CA125 to its ^{125}I-anti CA125 antibody in breast tumor homogenates, A) GI, B) GII, C) GIII.

Effect of Different Halides on the Binding of CA125 with ^{125}I-Anti CA Antibody

Edigington [17] found that chaotropic ions (SCN^-, I^-, Br^-, Cl^- and F^-) distort, particularly the three dimensional structure leading to the disruption of antibody-antigen interacting surfaces.

The effect of halides on binding of CA125 to its antibody in breast tumor homogenates was examined as shown in Figure (5). The findings is similar to Edigington study for our antibody-antigen interaction, which in turn substantiate the above explanation, where the B/T% decreases with the ion size in all studied groups.

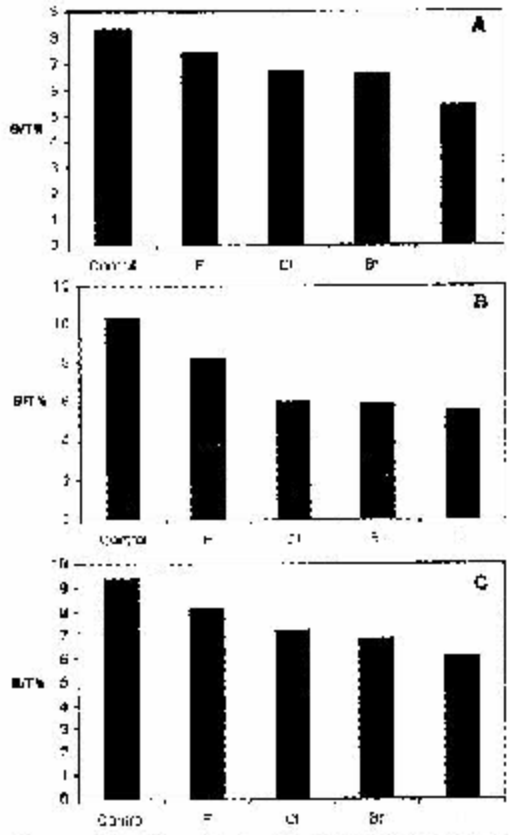


Figure (5). The effect of halides ions (0.3 M) on the binding of CA125 antigen to its ^{125}I-anti CA125 antibody in breast tumor homogenates, A) GI, B) GII, C) GIII.

Effect of Polarity on the Binding of CA125 with ^{125}I-AntiCA125 Antibody

This experiment was designed to study the polarity medium that may affect the active site of binding. The change of the environment around the amino acid residues in a protein may change the position of polar residues to be in surface or buried inside the protein according to polarity of the medium. Accordingly the change in the polarity of the medium may affect the percent of binding. The results show that the increase in ethanol % (i.e., decrease in polarity medium) led to an increase in binding percent (Figure 6). The formation (CA125 / ^{125}I-antiCA125 antibody) complex associated with the squeezing water molecules from binding sites that will lead to gaining entropy. The change of the microenvironment between the two surfaces (CA125 and ^{125}I-antiCA125 antibody) in close opposition decreases the local dielectric constant and enhancing the tightness of electrostatic or ionic binding [17].

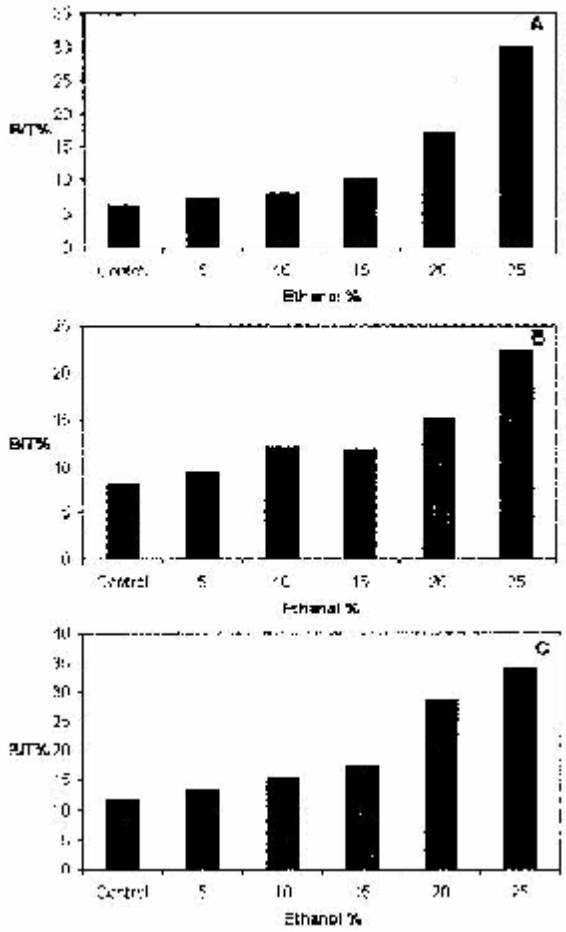


Figure (6). The effect of ethanol % on the binding of CA125 antigen to its ^{125}I-anti CA125 antibody in breast tumor homogenates, A) GI, B) GII, C) GIII.

References

1. Nusted, K., Lloyd, K. O., Nilsson, O. and O'Brien, T. J., Tumor Biol., 23, 303-314, (2002)
2. Jacobs, J. and Bast, R. C., Human Reprod., 4(1), 1-12, (1989).
3. David Wild, "The Immunoassay Handbook", Second Edition, Nature Publishing Group, U.K., (2001), p. 645.
4. Lawrance A. Kaplan, Amadeo J. Pesce, "Clinical Chemistry: theory, analysis and correlation", 2nd ed., Mosby Company, USA, (1989), p. 41.
5. Pal, S., Sanyal, V., Chattopadhyay, V., Int. J. Cancer, **60**, 759-765, (1995)
6. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randell, R.J., F. Biol. Chem., **193**, 265-275, (1951).
7. Ray Edwards, "Immunoassay An Introduction", William Heinemann Medical Books, London, (1985), pp. 52-53.
8. Clackson, T., Hoogenboom, H.R., Griffiths, A. D. and Winter, G., Nature, **352**, 624-628, (1991).
9. Davis, S., Davies, E., Tucknott, M., Jones, E. and van der Merwe, P., Proc. Natl. Acad. Sci., USA, **95**, 5490-5494, (1998).
10. Campbell, J. G., Nicolai, H. M., and Foulkes, W. D., Hum. Mol. Gene., **3(4)**, 598-594, (1994).
11. Edgington, T.S., J. Immunol., **106**, 673-689, (1971).
12. Hubsman, M., Yudkovsky, G. and Aronheim, A., Nucleic Acid Res., **294**, 18-26, (2001)