Chapter 3
Results and discussion

Chapter 3

3.1.Radioiodination of AFP antigen:

Radioiodination of AFP antigen has been carried out by some variations of the currently used methods such as, chloramine-T, iodogen, N-bromosuccinimide and lactoperoxidase. With each oxidizing agent, optimization of the radioiodination conditions were studied in terms of oxidizing agent quantity, reaction time, pH of the reaction mixture and reaction volume. There were investigated interms of radiochemical yield percent, radiochemical purity percent, specific activity and immunoreactivity of the produced tracer.

3.1.1. Chloramine-T method:

Radioiodination of AFP was carried out according to Green Wood et al., (1963)[75].

3.1.1.1. Effect of reaction time:

The oxidation and iodination reaction proceed rapidly. Thus, the time allowed for iodination reaction must be strictly controlled in order to minimize the oxidation damage of the protein. So that different reaction times in the range of (0.5 to 7 min) were tested.

The influence of the reaction time on the radiochemical yield percent with chloramine-T as oxidizing agent was shown in table (1).

Table (1) Effect of reaction time on radiochemical yield percent of ^{125}I-AFP.

Reaction time (min)	^{125}I-AFP yield, %	Radiochemical purity, %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding (B_{500}%)	Non specific binding (NSB%)
0.5	29	80	12.4	13	10	3
1	58	97	25	45	11.5	3.1
3	56	92	24.2	42	11	3.2
5	50	90	22.5	40	9.5	3.1
7	46	84	20.2	23	9	3.2

B_o: Maximum binding at zero standard.

B_{500}: Binding at highest standard (500 IU/ml)

NSB: Non specific binding (without antibody).

Radioiodination conditions can be summarized as follows:

20 μg of AFP antigen, 50 μg of chloramine-T, 0.5 mCi of Na^{125}I, reaction volume was 60 μl and pH of the reaction mixture 7.4. These conditions were kept constant at all times under investigation.

Generally the radiochemical yield percent was increased with the increase of the reaction time up to one min , where a high radiochemical yield percent was achieved (58%) as shown in Fig(3). Further increase of the reaction time, the radiochemical yield percent start to decrease and reached to 46% after 7 min. Fig (4) show, some what sharp and big peak of a maximum radiochemical purity (97%) was obtained at optimum reaction time.

At reaction time one min, the highest binding percent was achieved (45%) in addition to the highest displacement percent between maximum binding percent and minimum binding percent was achieved (74.4%) where it is very important to differentiate between different standard points.

In conclusion, the reaction one min is just adequate to reach a complete reaction and the exposure time for the potential harmful chloramine-T was at lowest limits. For these reasons, reaction time one min is recommended for radioiodination AFP antigen.

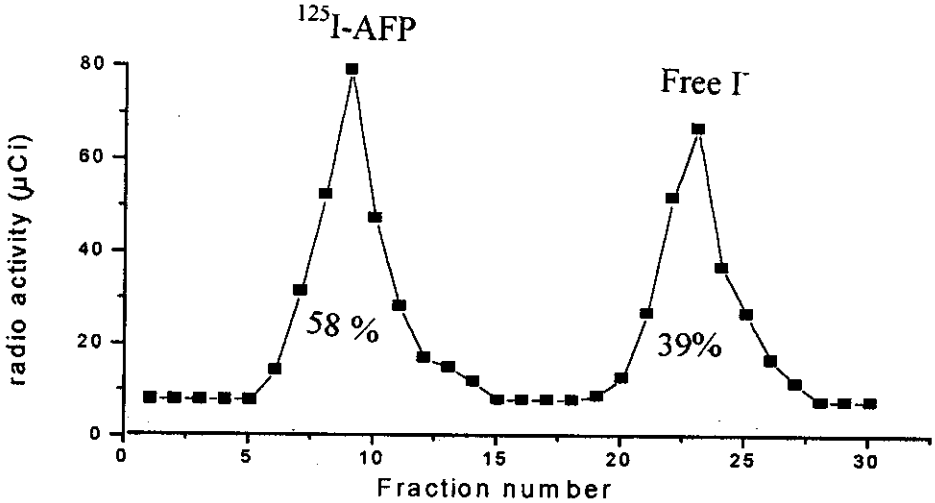


Fig (3): Purification profile of ^{125}I-AFP at optimum reaction time (1 min) using chloramine-T as oxidizing agent on PD-10 Sephadex column at a flow rate 0.8 ml/ 2min.

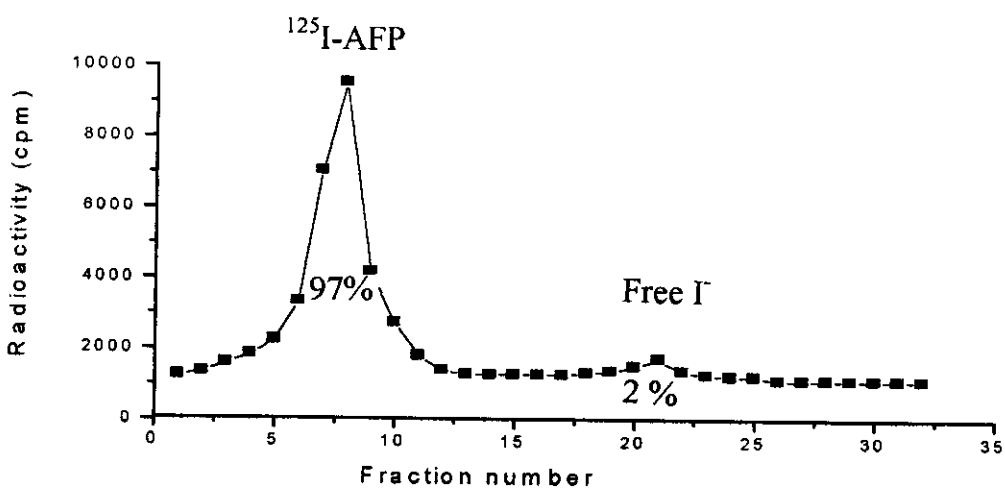


Fig (4): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction time (1min)

3.1.1.2. Effect of reaction volume :

The influence of reaction volume on the percent radiochemical yield was studied in the range of 40 to 115µl and all other parameters were kept constant. The results of this study were shown in Table (2).

Table (2): Effect of reaction volume on the percent radiochemical yield of ^{125}I-AFP:

Reaction volume (µl)	^{125}I-AFP yield,%	Radio-chemical yield %	Specific activity (µCi/µg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
40	48	90	18	45	11.2	3.1
50	55	91	19.2	48	11	3.2
60	58	95	20	51	10.8	3.5
70	42	88	16.3	44	12	2.5
115	18	79	12.2	40	11.2	3.3

Radioiodination conditions can be summarized as follows:

20 µg of AFP antigen- 50 µg chloramine-T- 0.5 mCi of Na^{125}I- one min reaction time and pH of the reaction mixture 7.4. These conditions were kept constant at all volumes under investigation.

The results in table (2) indicated that generally the radiochemical yield was increased with increasing the reaction volume up to 60 µl reaction volume. After that, the radiochemical yield was gradually decreased with increasing the reaction

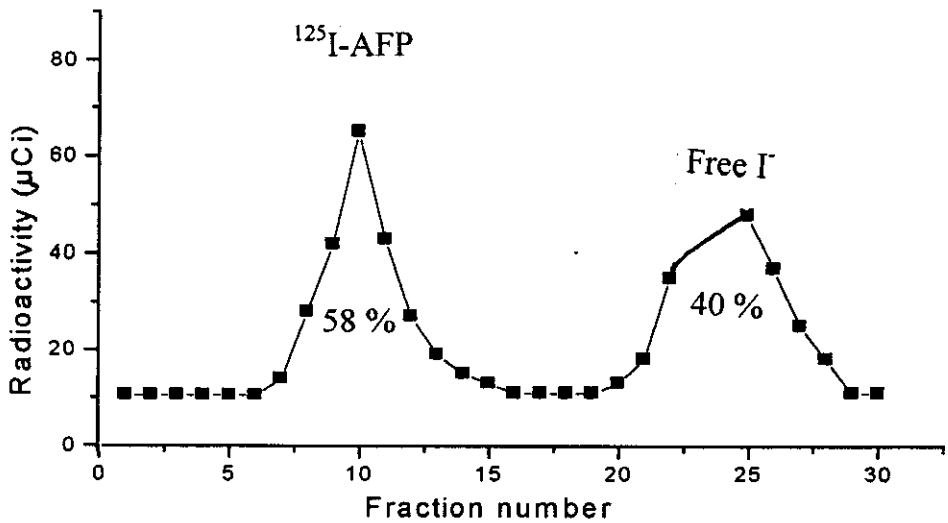
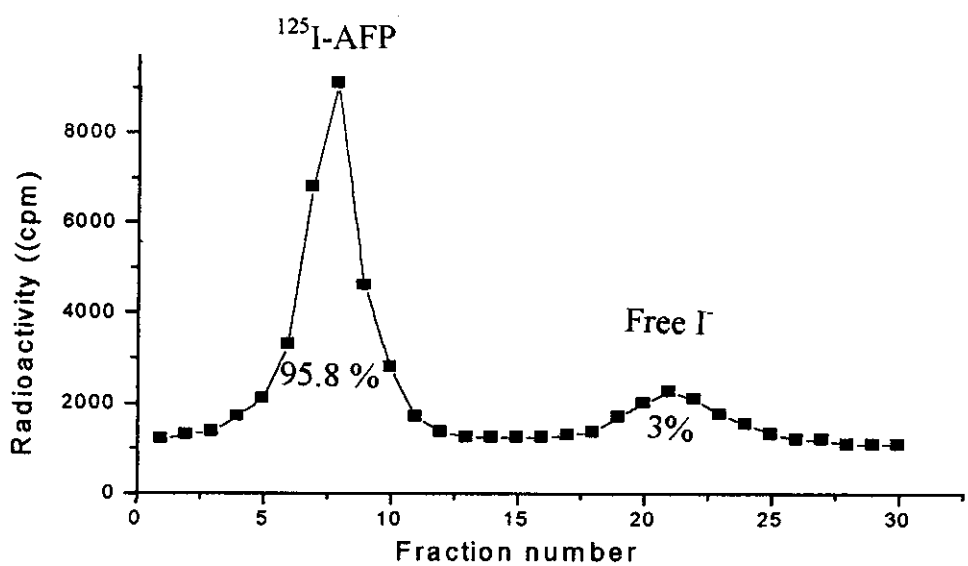


Fig (5): Purification profile of ^{125}I-AFP at optimum reaction volume 60 μl using PD-10 Sephadex column at flow rate 0.8 ml/ 2min fraction



Fig(6):Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction volume (60 μL)

volume more than 60 µl. The radioiodinated AFP was assessed for its immunoreactivity by using RIA technique. The results show, the highest binding percent (51%) and the highest displacement percent between a maximum binding percent (B_{50}%) was achieved (76%) at 60 µl reaction volume. Figs (5,6) show the highest radiochemical yield (58%) and highest radiochemical purity (95%) at optimum reaction volume (60 µl)

These results can be explained as follows:

With small reaction volume, good mixing for the reactants was difficult. On the other hand, with a big reaction volume, the reactants were separated and the chance for radioiodination reaction was decreased. Good chance for, radioiodination process was given at 60 µl reaction volume. So that, the volume ratio about 1:4 between substrate AFP antigen and all reagents is recommended.

3.1.1.3. Effect of the chloramine-T content:

To prevent excessive oxidation and protein damage, different AFP antigen and chloramine-T gram ratios were studied and the influence of the chloramine-T on the percent radiochemical yield was demonstrated in table (3).

Table(3) shows that the radiochemical yield was increased with increasing the amount of chloramine-T up to (59%)at ($20x10^{-6}$:$50x10^{-6}$) at gram ratio between

substrate AFP antigen and chloramine-T after that, the radio-chemical yield was decreased with increasing the chloramine-T content.

At gram ratio ($20x10^{-6}$: $50x10^{-6}$) of AFP antigen and chloramine-T, a high specific activity (25 μCi/μg) and a high percent binding (50%) were obtained. A highest displacement percent between a maximum binding (B_0%) and a minimum binding (B_{50}%) was achieved (77.6%). This due to the lowest excessive oxidation and protein damage was obtained as (1:2.5) gram ratio of AFP antigen and chloramine-T.

These results are in a good agreement with the results obtained by Hunter et al., [56,57] who suggested that the decrease of the percent radiochemical yield with a higher chloramine-T content at pH 7 can be attributed to destructive side reactions with respect to the extended reaction time. Under the above mentioned condition, the highest percent of radiochemical yield and the highest percent of radiochemical purity were achieved as shown in Fig (7,8).

Table (3): Effect of the gram ratio between substrate AFP antigen and chloramine-T on the percent radiochemical yield ^{125}I- AFP:

AFP (μg) 10^{-6}	Chloramine-T (μg) x 10^{-6}	^{125}I-AFP yield %	Radio-chemical yield %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
20	30	46.3	92.5	22	45	11.5	2
20	50	59	95.8	25	50	11.2	2.4
20	70	40	91.5	21.5	46	12	2.5
20	90	35	88.5	18	30	11.2	2.2
20	110	25	85.3	15	20	10.8	2.1

Radioiodination conditions can be summarized as follows:

20 μg of AFP antigen, 0.5 mCi Na^{125}I, reaction volume was 60 μl, one min reaction time and pH of the reaction mixture 7.4. These conditions were kept constant at all gram ratios under investigation.

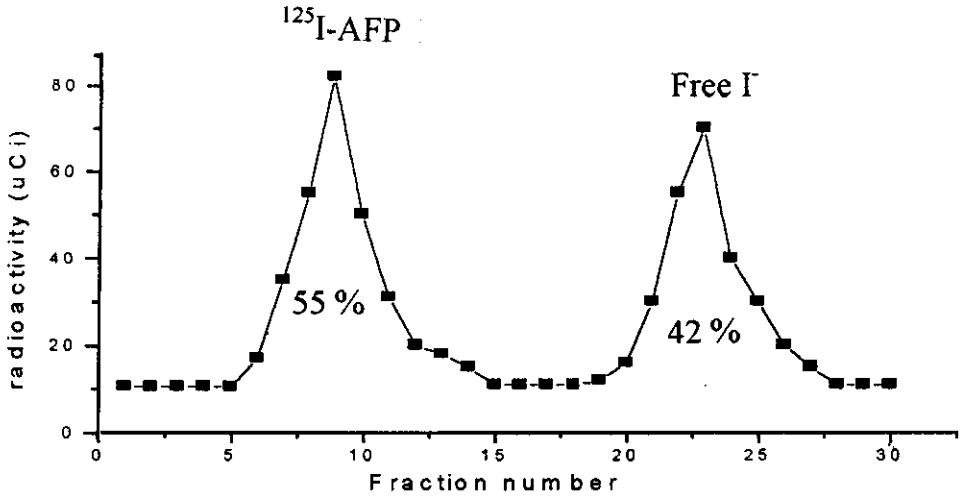


Fig (7): Purification profile of ^{125}I–AFP at optimum molar gram between substrate (AFP) antigen and chloramine-T as oxidizing agent on PD-10 Sephadex column.

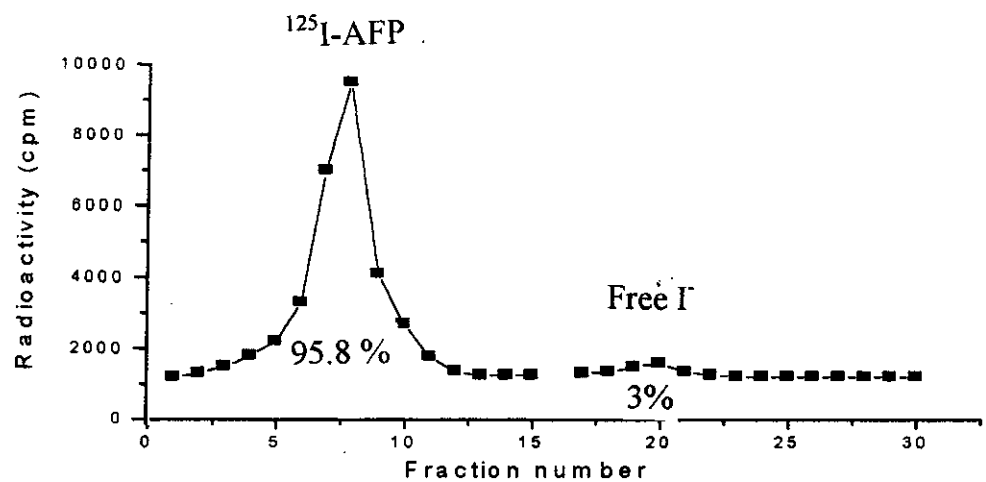


Fig (8): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum gram ratio between substrate (AFP) antigen and chloramine-T as oxidizing agent.

3.1.1.4. Effect of pH of reaction.

In order to compare the radiochemical products different radioiodination of antigen at variable pH of the reaction, it is necessary to have products with similar specific activities.

The influence of the pH of the reaction medium on the percent radiochemical yield of ^{125}I-AFP were presented in table (4), which clearly shows the highest radiochemical yeild (55%) and the highest radiochemical purity (96%) were obtained at pH 7.4 as shown in Figs (9,10).

The results show, the highest binding (48%) and the highest displacement percent (75.4%) between a maximum binding (B_0%) and a minimum binding (B_{50}%) were obtained at pH, 7.4. These results in a good agreement with El-Wetery et al [113] which they found an increase in the percent radiochemical yield with the increase pH of reaction and a sharp drop in percent yield was observed at higher pH value during their studies on the radioiodination of gammaglobulin using chloramine-T method.

The pH of reaction mixture seems to be critical as it was necessary to maintain the labelled solution at an alkaline pH (7.4). This is because the tyrosyl residues of proteins are, however, much more easily labelled under alkaline conditions. Also between pH 7 and 8 the ortho position in the aromatic

ring of tyrosine of peptides and proteins is activated for electrophilic attack, owing to the electron-donating effect of the neighboring hydroxyl group. For these reasons the optimum pH 7.4 is recommended in radioiodination of AFP antigen.

Table (4): Effect of pH of reaction mixture on the percent radiochemical yield of ^{125}I-AFP

PH of reaction	^{125}I-AFP yield, %	Radio-chemical purity, %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
3	30	85	13.8	33	11	2.6
5	41	91	23	38	12	2.1
7.4	55	96	27	48	11.8	2.2
9	40	90	23	35	11	2.3
11	33	86	13.5	28	11.5	2.4

Radioiodination conditions can be summarized as follows: 20μg of AFP antigen- 50μg chloramine-T- reaction volume 60 μl- one min reaction time and 0.5 mCi Na^{125}I.

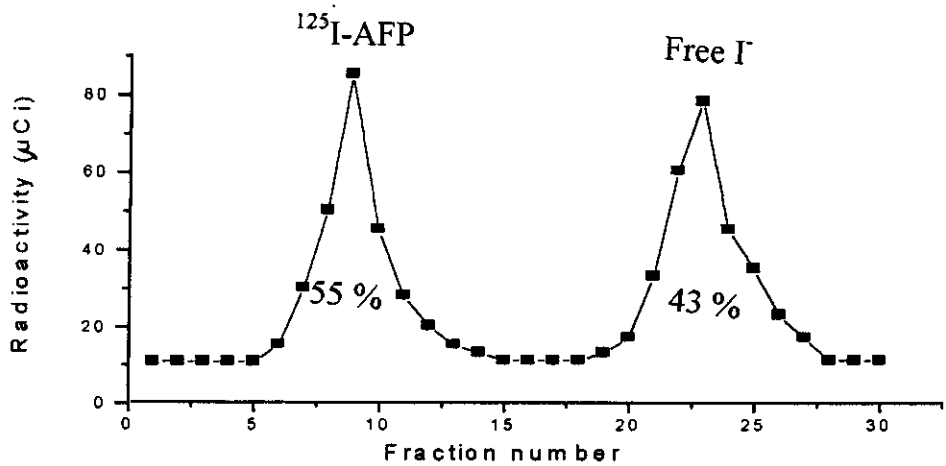


Fig (9): Purification profile of ^{125}I-AFP at optimum pH of the reaction (7.4) by using PD-10 Sephadex column at flow rate 0.8 ml/2min.

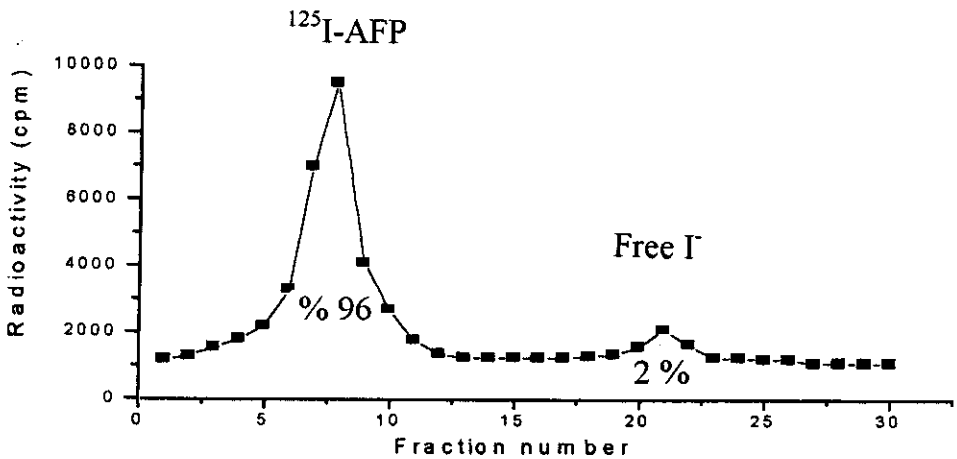


Fig (10): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum pH of the reaction (7.4) using chloramine-T method.

3.1.2. Iodogen method

Iodogen is a mild oxidizing agent and it can be used for oxidation radioiodination of proteins, ploypeptides, hormones and others. It is essentially insoluble in water. So that, it can be used as a thin film plated onto abottom of glass vials. The following experiments were performed to establish an ideal radioiodaintion of AFP procedure by iodogen method.

3.1.2.1. Effect of the reaction time:

Variable times in the range of 1.5 to 20 min were tested and the results obtained presented in the table (5). The big peak of high radiochemical yield percent (43%) obtained at the reaction time 10 min as shown in Fig (11) and the radiochemical yield percent start to decrease gradually with increase of the reaction time. At reaction time over 10 min, decrease of the radiochemical yield percent was observed, this indicated that reaction time 10 min, is adequate to complete the reactions. On the other hand, the reaction time less than 10 min is not adequate to complete the reaction. At the same time, the highest binding percent (44%) and a high specific activity (15.6 μCi/μg) were achieved. The highest displacement percent between a maximum binding percent (44%) and a minimum binding percent (11.8%) was achieved (73%). The highest radiochemical purity was obtained at 10 min reaction time and this due to , the reaction mixture was separated from iodogen at the end of the reaction time.

Fig (12) shows a high radiochemical purity percent (98.7%) which obtained at optimum reaction time. These results agree with El-Wetery et al[113] which suggested that the radiochemical yield increased rapidly between one and 10 min in the radioiodination of gamma-globulin. It is recommended to use a solid phase oxidizing agent (Iodogen) with reaction time 10 min.

Table (5) Effect of radioiodination reaction time at room temperature on the percent radiochemical yield of ^{125}I-AFP.

Reaction time (min)	^{125}I-AFP yield, %	Radio-chemical purity %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
1.5	30	93.5	13.6	30	13	3
5	38	95.8	14.9	39	12	2.9
10	43	98.7	16.6	44	11.8	3
15	30	92	12.1	31	11.5	2.9
20	23	90.8	10.2	26	12	3

The radioiodination conditions can be summarized as follows:

20 μg of AFP antigen-10μg of iodogen- reaction volume 60 μl- 0.5 mCi Na^{125}I and pH of reaction (7.4). These conditions were kept constant at all times under investigation.

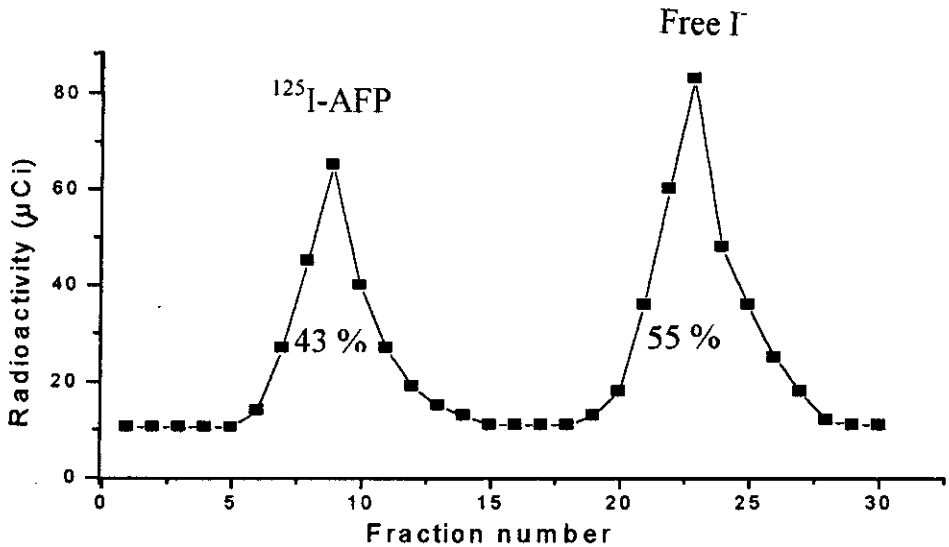
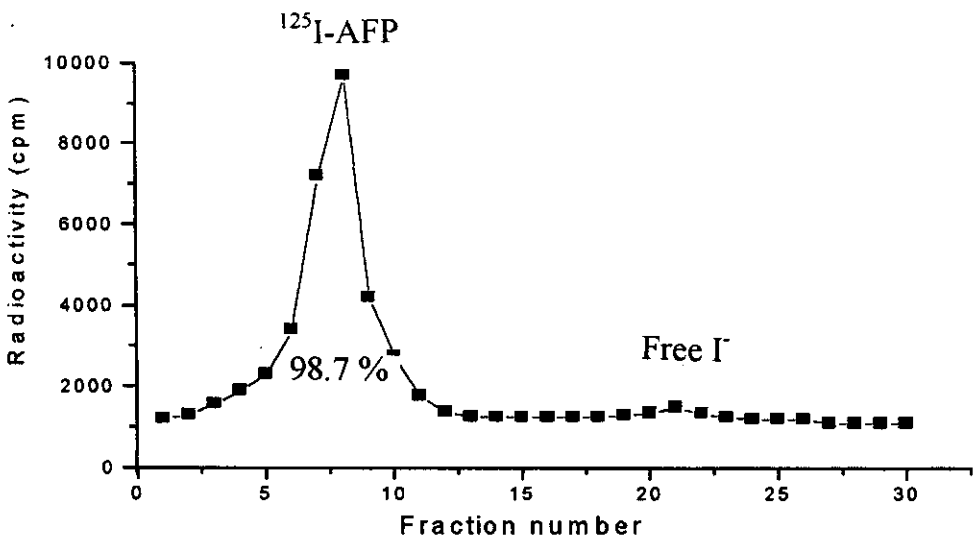


Fig (11): Purification profile of ^{125}I-AFP at optimum reaction time (10min) using iodogen as oxidizing agent on PD-10 Sephadex column at flow rate 0.8 ml/2 min.



Fig(12): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction time (10 min) using iodogen method.

3.1.2.2. Effect of reaction volume:

The influence of the reaction volume on the percent radiochemical yield was studied in the range of 30 to 165 µl and all other parameters were kept constant. The results of this study were shown in table (6). The results show a gradual increase in the radiochemical yield percent with increase of the reaction volume more than 50µl. With continuos increase of the reaction volume more than 50µl, the radiochemical yield started to decrease gradually. A radiochemical percent of 14% was obtained at a reaction volume equals 155 µl.

In addition to the highest radiochemical yield percent, a highest binding percent (40%) and a highest specific activity (14.6%) were achieved at reaction volume 50µl. At this reaction volume the displacement between a highest binding percent (40%) and a lowest binding percent (11.5%) was achieved (71%) this due to the reaction volume must be cover the surface of the thin layer of iodogen. So that, the small reaction volume leads to decrease in radiochemical yield percent and excess of reaction volume not give a good chance to complete the reaction where the reactant may not be incomplete the reaction where the reactant may not be in contact with the film of iodogen.

The reaction volume of 50 µl is the optimum reaction volume where the radiochemical yield percent and radiochemical purity percent obtained and clearly shown in Figs(13,14).

These results are in a good agreement with the data reported by Green Wood[75] who suggested that the volume ratio between reactant is critical and linear to an ideal reaction volume. So that , it is recommended that the reaction volume radioiodination reactants must be 1 :3 which is necessary for having a high radiochemical yield.

Table(6):Effect of radioiodination reaction volume at room temperature on the percent radiochemical yield of ^{125}I-AFP..

Reaction volume (µl)	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (µCi/µg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
30	28	90.2	10.2	30	11	3.1
50	45	94.6	14.6	40	11.5	3.2
70	38	92	9	30	11.2	3.1
105	26	88	8	22	11.3	3.2
155	14	79	7	16	11.5	3.2

The radioiodination conditions were 20 µg of AFP antigen- 10 µg of iodogen- 10 min reaction time- 0.5 mCi Na^{125}I and pH of the reaction (7.4).

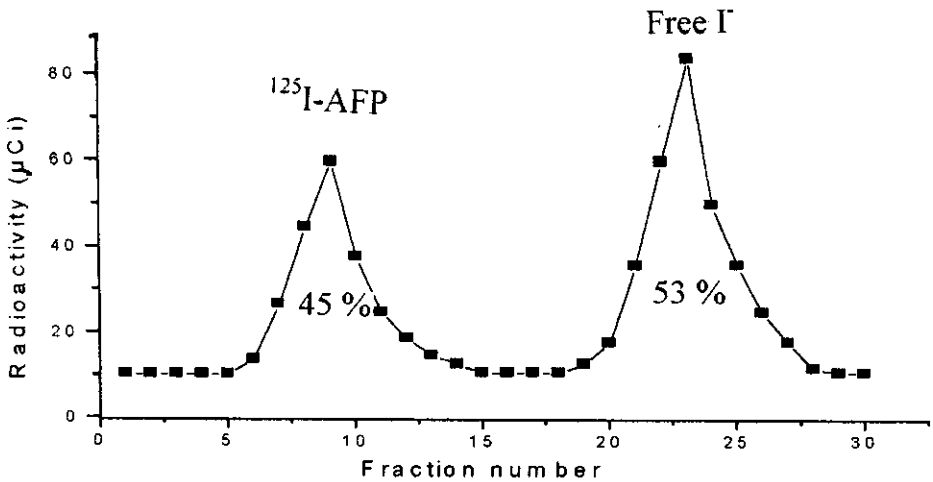
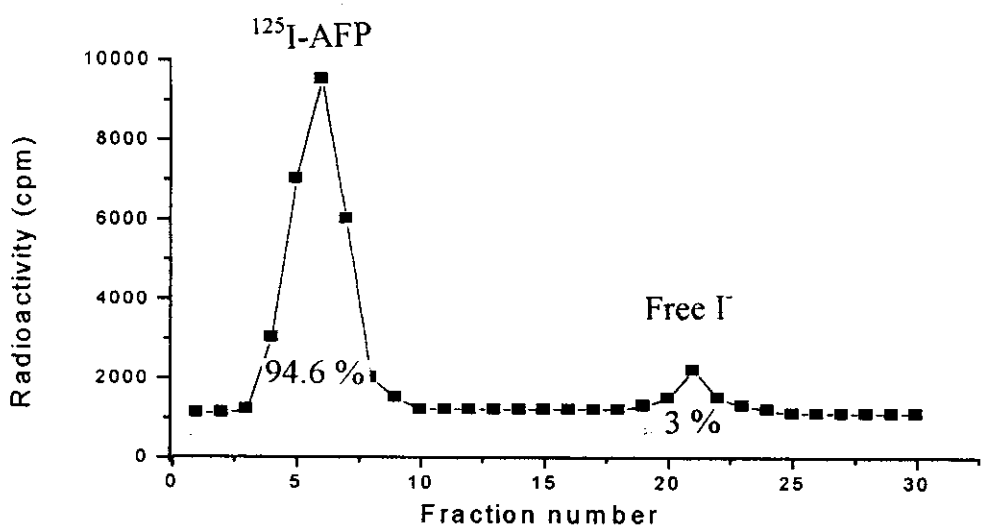


Fig (13): Purification profile of ^{125}I-AFP at optimum reaction volume (50μl) using iodogen as oxidizing agent on PD-10 Sephadex column at flow rate 0.8 ml/2 min.



Fig(14):Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction volume (50μl) using iodogen as oxidizing agent.

3.1.2.3. Effect of Iodogen content:

Variable gram ratio between substrate AFP and iodogen content were investigated. Increasing the amount of iodogen at fixed AFP content altered molar ratios. The results obtained presented in table (7) and it shows, the radiochemical yield percent increase gradually with increasing the gram ratio up to ($20x10^{-6}$: $15x10^{-6}$) and gradually decreasing observed in radiochemical yield percent with increase the gram ratio over ($20x10^{-6}$: $15 x10^{-6}$). A highest displacement percent between a highest binding percent (41%) and a lowest binding percent (10.8%) was achieved (73.6%) at the same time, at the gram ratio ($20x10^{-6}$: $15x10^{-6}$) a highest binding percent (41%) and a highest specific activity percent (18μCi/μg) were shown. This due to at the gram ratio 1:0.75 the oxidizing agent concentration is adequate to complete a radioiodination reaction but any other excess of oxidizing agent concentration lead to harmful oxidation damage of protein. On the other hand, iodogen content is not adequate to complete the radioiodination reaction at lowest molar ratio.

Figs (15,16) shows the highest percent radiochemical yield (49%) and the highest percent radiochemical purity (98%) which obtained at optimum molar ratio (1:1.0.75). This results agree with results of Hunter[57], who suggested that the decrease of labelling yield with a higher iodogen content and it can be attributed to destructure side reaction and oxidizing damage of protein.

Table(7): Effect of the gram ratio between substrate (AFP) and oxidizing agent (iodogen) concentration on the percent radiochemical yield of ^{125}I-AFP.

AFP (µg) x 10^{-6}	Iodogen (µg) x 10^{-6}	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (µCi/µg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
20	3	17	86	11	18	10	2.8
20	8	31	91	16	20	11	2.9
20	15	45	98	18	41	10.8	3.0
20	20	33	92	16	30	11	3.2
20	30	17	85	10.8	20	10.2	3.0

B_0 : maximum binding at zero standard.

B_{500} : binding at highest standard (500 IU/ml)

NSB : non specific binding (without antibody).

The radioiodination condition were 20 µg of AFP- 0.5 mCi Na^{125}I- 10 min reaction time- reaction volume 50 µl and pH of reaction (7.4).

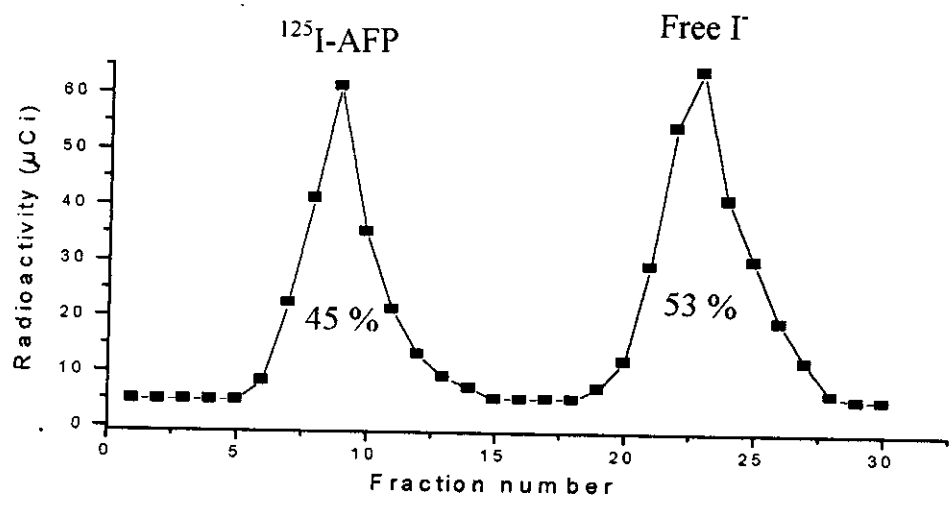


Fig (15): Purification profile of ^{125}I-AFP at optimum gram ratio between substrate (AFP) antigen and iodogen as oxidizing on PD-10 Sephadex column at flow rate 0.8 ml/ 2min.

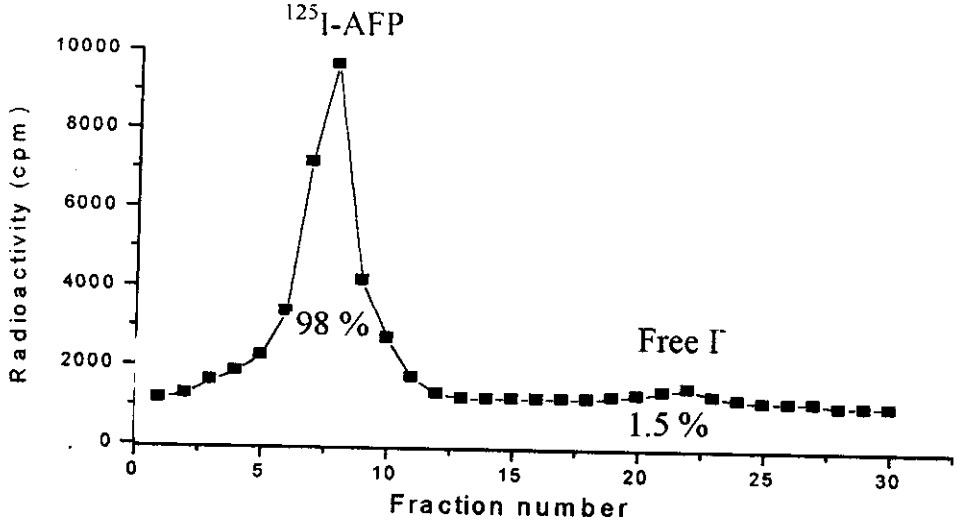


Fig (16): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum gram ratio between substrate (AFP) and iodogen concentration.

3.1.2.4. Effect of pH:

The influence of the pH of the reaction medium on percent radiochemical yield of ^{125}I-AFP are presented in table (8). It clearly shows a marked increase of the percent radiochemical yield with an increase pH of reaction. A maximum yield was achieved at pH 7.4, while a sharp drop in yield was observed at higher values.

At pH 7.4 the results were shown a sharp and big peak of maximum radiochemical yield (45%) and the highest percent binding (39%). The highest displacement percent between a maximum binding and a minimum binding (B_{50}%) was achieved (72%). The highest radiochemical yield percent (45%) and the highest radiochemical purity percent (98%) were shown in Figs(17,18). This due to, between pH 7 and 8 the ortho position in aromatic ring of tyrosine of peptides and proteins is activated for electrophilic attack, owing to the electron donating effect the neighboring hydroxyl group. So that, pH 7.4 is recommended.

Table (8) Effect of pH of radioiodination reaction at room temperature on the percent radiochemical yield of ^{125}I-AFP:

pH of reaction	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
3	20	86	16	20	10	2.8
5	30	92	17.8	33	11	3
7.4	45	98	21.8	39	10.9	2.9
9	27	85	17	25	11	3.0
11	19	81	16	20	11.2	3.1

Radioiodination conditions were 20 μg of AFP antigen- 0.5 mCi Na^{125}I- 10 min reaction time- 10 μg of iodogen- and 50 μl reaction volume.

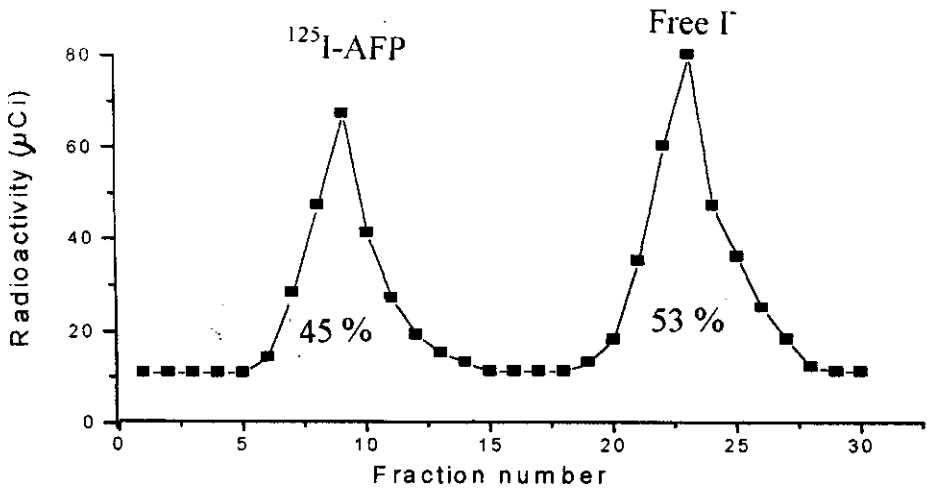
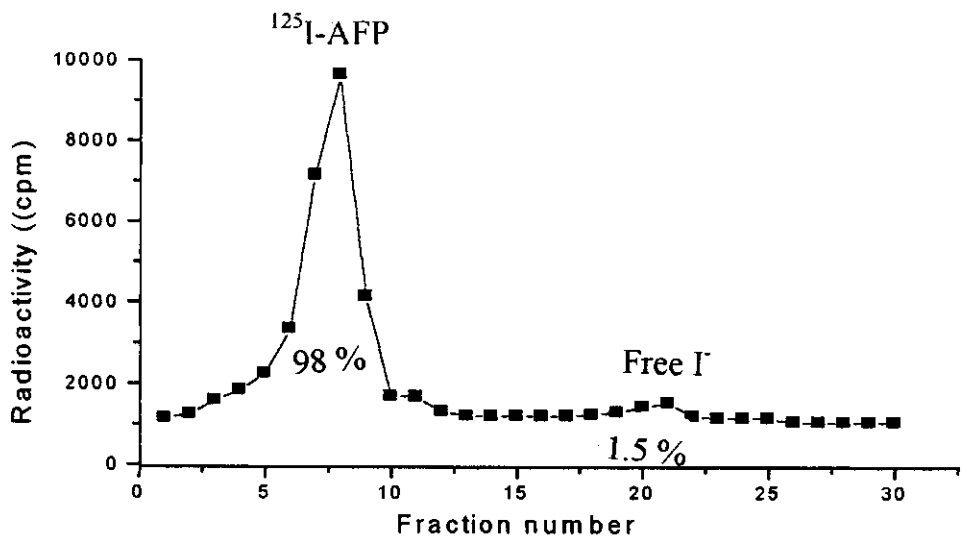


Fig (17): Purification profile of ^{125}I-AFP at optimum reaction pH 7.4 on PD-10 Sephadex column at flow rate 0.8 ml/ 2min.



Fig(18): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum pH of reaction (7.4).

3.1.3.N-bromosuccinimde method:

Exposure of antigen to oxidizing and reducing agents and lengthy exposure to radioiodine leading to loss of immunological activity. The N-bromosuccinimide method has been shows to involve minimal exposure to these hazards, while enabling satisfactory incorporation of iodine into protein.

3.1.3.1. Effect of reaction time:

Different reaction times in the range of one to ten min were tested and the results obtained presented in table (9). The highest radiochemical yield percent (48%) obtained at the optimum reaction time 5 min as shown in Fig (19). The results shows the highest binding percent (41%) and the highest specific activity (22 μCi/μg) obtained at reaction time 5 min. The displacement between a maximum binding percent (41%) and the lowest binding percent (11%) was achieved (73%).

Fig (19) shows the highest radiochemical purity percent (94%) to explain the results obtained which, the factor of time is critical and important. So that, to reduce chemical iodination damage, it is desirable to minims the exposure of the protein to oxidizing and strong reducing agents.

Table (9) shows the optimum reaction time which was 5 min and gradually decrease observed when the reaction time is increase more than 5 min or decrease

less than 5 min. this explain that, the reaction time 5 min is adequate to complete the reaction but when the time increase more than 5 min, the exposure of protein to oxidizing and reducing agents will increase and cause oxidation damage for the ^{125}I-AFP. On the other hand, when reaction time decreased less than 5 min, the time was not sufficient to reach the end of reaction. The results agree with Hunter and Green Wood[57] in preparation of iodine-131 labelled human growth hormone so that, the optimum reaction time 5 min is recommended with mild oxidizing agent N-bromosuccinimide.

Table (9): Effect of radioiodination reaction time on the percent radiochemical yield of ^{125}I-AFP:

Reaction time (min)	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
1	17	83	15	20	11	3
3	35	86	18	30	10.8	3.1
5	48	94	22	41	11	3.1
7	30	90	18.9	33	11	3.0
10	22	88	17	25	11.2	3.2

The Radioiodination conditions were 20 μg of AFP antigen, 50 μg N-bromosuccinimide- 0.5 mCi Na^{125}I- 50 μl reaction volume and pH 7.4 reaction mixture.

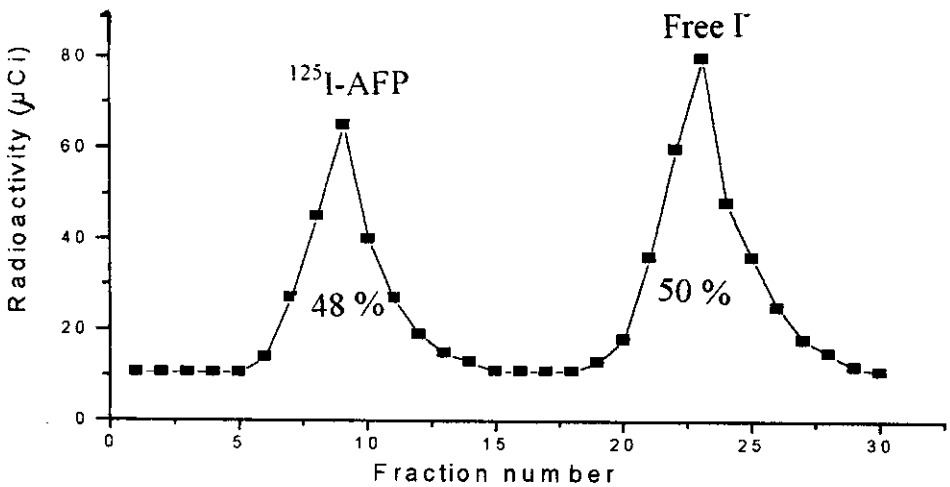


Fig (19): Purification profile of ^{125}I-AFP at optimum reaction time (5min) using N-bromosuccinimide on PD-10 Sephadex column at flow rate 0.8 ml/2min.

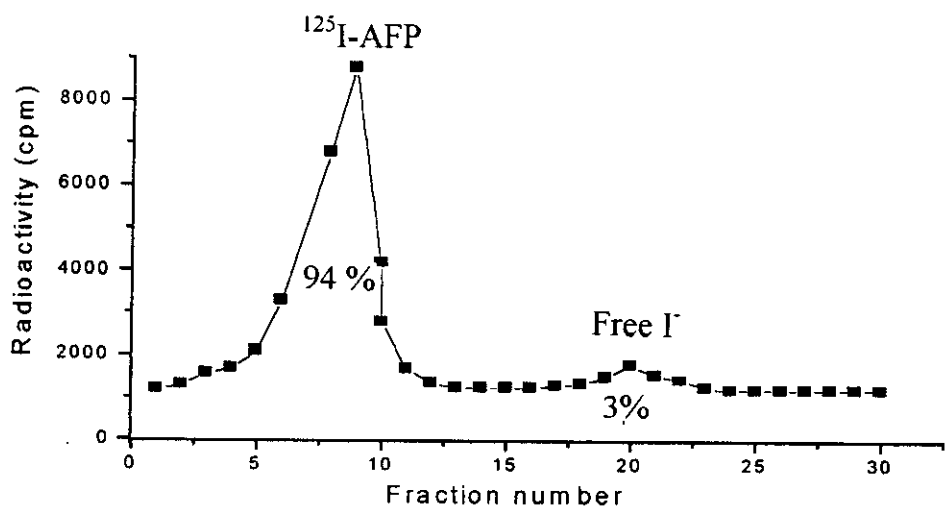


Fig (20): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction time (5 min) using N-bromosuccinimide.

3.1.3.2. Effect of reaction volume:

Different reaction volume in the range of 40 to 110 µl and all other parameters were kept constant. The results presented in table (10) and show gradually increase in radiochemical yield percent with decrease reaction volume up to 50 µl then the radiochemical yield decreased with decreasing reaction volume lower than 50 µl. A highest binding percent (43%) obtained at reaction volume 50 µl and a highest displacement percent was achieved (73.9%). This due to, that volume ratio between the reactants is important. At optimum reaction volume the was reached to the end and it was given the highest binding but any excesses in reaction volume over optimum reaction volume, lead to decrease in the radiochemical yield percent and this due to, the reactants may be not in good contact. Figs(21,22) show, the highest radiochemical yield percent (48%) and the highest radiochemical purity (96%) which obtained at optimum reaction volume 50 µl. It recommended, that, volume ratio between substrate AFP and reactant volume 1:3 may be suitable for radioiodination of AFP antigen.

Table (10): Effect of radioiodination reaction volume on the percent radio-chemical yield of ^{125}I-AFP.

Reaction volume (µl)	^{125}I-AFP yield, %	Radio-chemical purity %	Specific activity (µCi/µg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
40	37	88	11	22	11	3
50	48	96	22.8	43	11.2	2.8
60	30	93	18	39	11.1	2.9
70	20	90	15	25	11.2	3.1
110	17	88	11	20	11.5	3.2

Radioiodination conditions can be summarized as follows:

20 µg of AFP antigen - 50 µg of N-bromosuccinimide- 0.5mCi of Na^{125}I- 5 min reaction time and 7.4 pH of the reaction mixture.

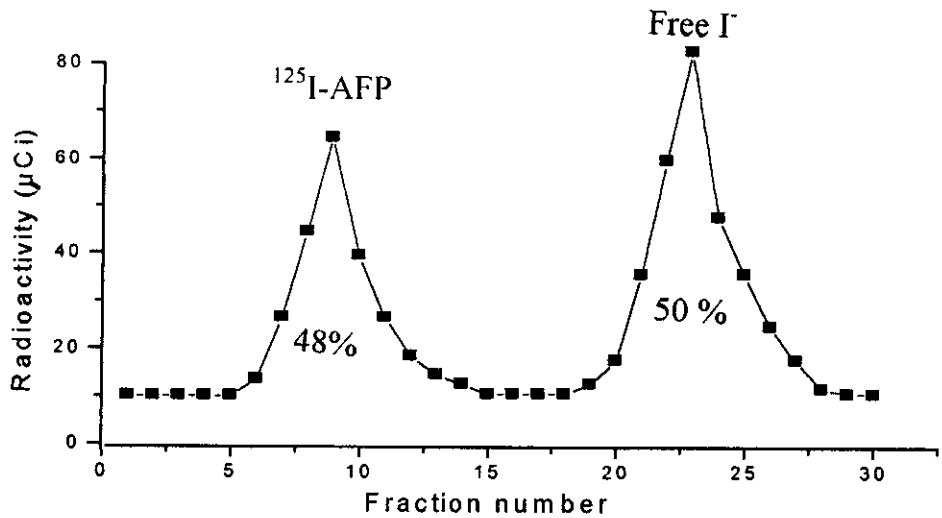


Fig (21): Purification profile of ^{125}I-AFP at optimum reaction volume (50 μl) using N-bromosuccinimide on pD-10 Sephadex column at flow rate 0.8 ml/2min.

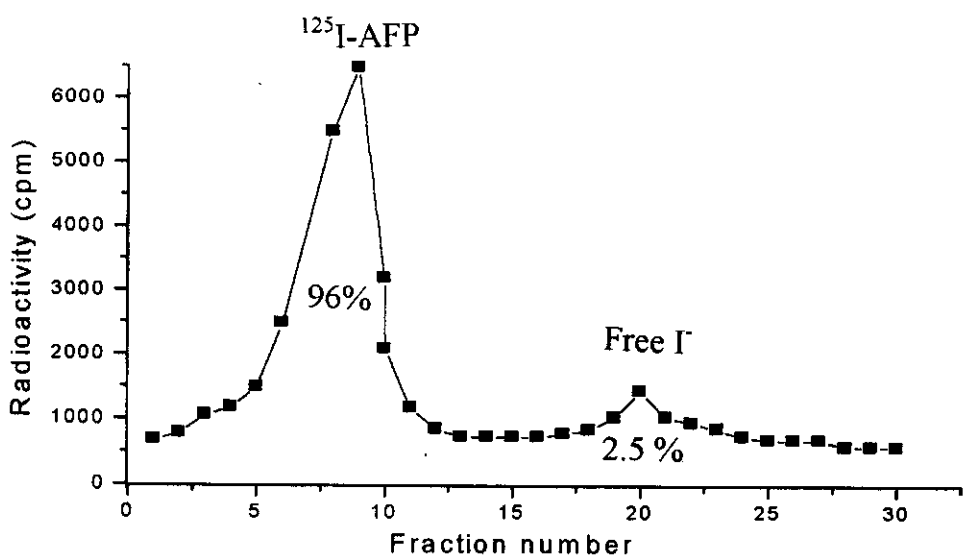


Fig (22): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction volume (50 μl).

3.1.3.3. Effect of N-bromosuccinimide content:

Different gram ratio between substrate AFP and N-bromosuccinimide content were tested. The results obtained presented in table (11) and it shows that, the percent radiochemical yield increased gradually with decrease the gram ratio up to (20×10^{-6}: 70×10^{-6}) gram ratio which give a highest specific radiochemical yield percent (45%) and the highest specific activity (21.8 μCi/μg) obtained.

The highest displacement percent between a maximum binding and a minimum binding was achieved (72.6%). This is due to, at the optimum gram ratio between substrate AFP and .N-bromosuccinimide, oxidizing agent,content is adequate to complete the radioiodination reaction but any excess of it may be lead to the chemical oxidation damage. On the other hand, the lowest gram ratio is not sufficient to complete the reaction.

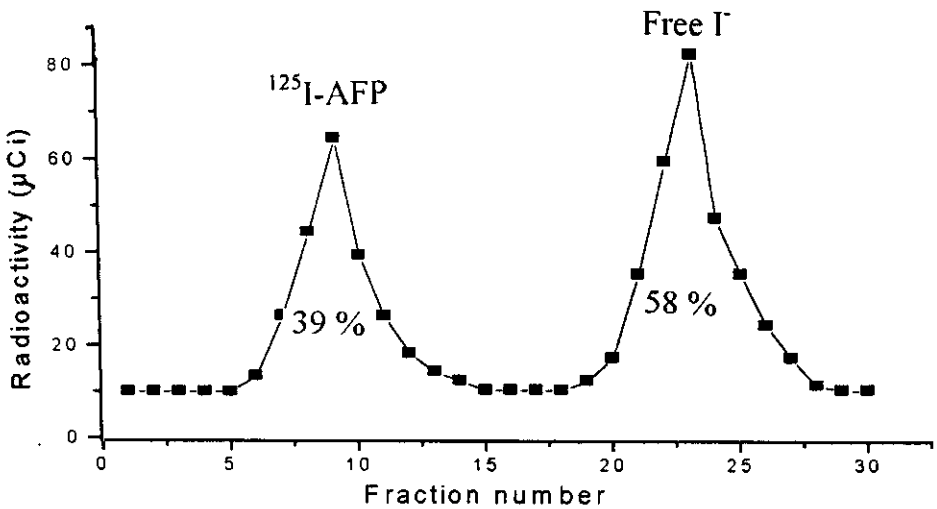
Figs(23,24) shows the highest percent radiochemical yield (45%) and the highest percent radiochemical purity (96%) obtained at optimum gram ratio (20×10^{-6} :70×10^{-6}). This results are in a good agreement with Paul Reay,[87] who use N-bromosuccinimide for iodination of proteins for RIA. So that (1:3.5) gram ratio between AFP antigen and N-bromosuccinimide is recommended.

Table(11): Effect of gram ratio between AFP antigen and N-bromo-succinimide content on the percent radiochemical yield of ^{125}I-AFP:

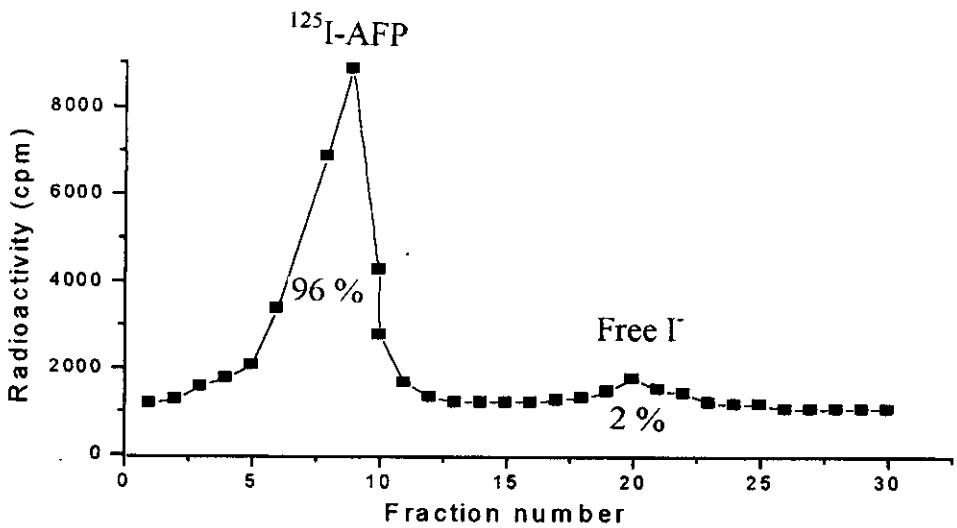
AFP (μg) 10^{-6}	N-bromosucc-inimide (μg) x10^{-6}	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
20	50	12	86	11.5	20	12	3.5
20	70	39	96	21.8	42	11.5	3.1
20	90	30	91	16	35	11.8	3.2
20	110	24	88	14	30	11.5	3.3
20	130	18	87	13	25	11.3	3.3

The radioiodination conditions can be summarized as follows:

20 μg of AFP, 0.5 mCi of Na^{125}I, 50 μl reaction volume, 5 min reaction time and 7.4 pH of the reaction mixture.



Fig(23): Purification profile of ^{125}I-AFP at optimum gram ratio between substrate AFP antigen and N-Bromosuccinimde as oxidizing agent on PD-10 Sephadex column at flow rate 0.8ml/ 2min.



Fig(24):Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum gram ratio between substrate AFP antigen and N-bromosuccinimide.

1.1.3.4. Effect of pH:

Different pH values in the range of 3 to 11 were investigated. The results obtained were presented in table (12) which show the highest percent radiochemical yield (48%) and the highest specific activity (20.8 μCi/μg) obtained at pH value (7.4). The highest binding percent (42%) and the highest displacement percent (74.3%) were achieved.

Table (12) show a sharp increase in radiochemical yield percent with increased pH value up to pH 7.4, then gradually decrease observed with continuos increase pH value over 7.4. This is due to between pH 7 and 8 the ortho position in aromatic ring of tyrosine of peptides or protein is activated as mentioned before. Figs (25, 26) show, the highest percent radiochemical yield (48%) and the highest percent radiochemical purity (96) obtained at the optimum pH (7.4).

These results show a good a greement with Poul Reay[87] who suggested that at pH 7.5, the highest percent radiochemical yield in radioiodination of BSA using N-bromosuccinimde was obtained. Finally, pH 7.4 is recommended in radioiodination of AFP antigen.

Table (12): Effect of radioiodination reaction pH on the percent radiochemical yield of ^{125}I-AFP:

Reaction pH	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
3	15	80	16	18	10	3.0
5	25	88	17	22	11	3.1
7.4	48	96	20.8	42	10.8	3.2
9	30	85	17	28	11	3.1
11	17	86	16	20	10.7	3.2

The radioiodination conditions can be summarized as follows:

20 μg of AFP antigen- 50 μg N-bromosuccinimide 0.5 mCi of Na^{125}I- 50 μl reaction volume and 5 min reaction time.

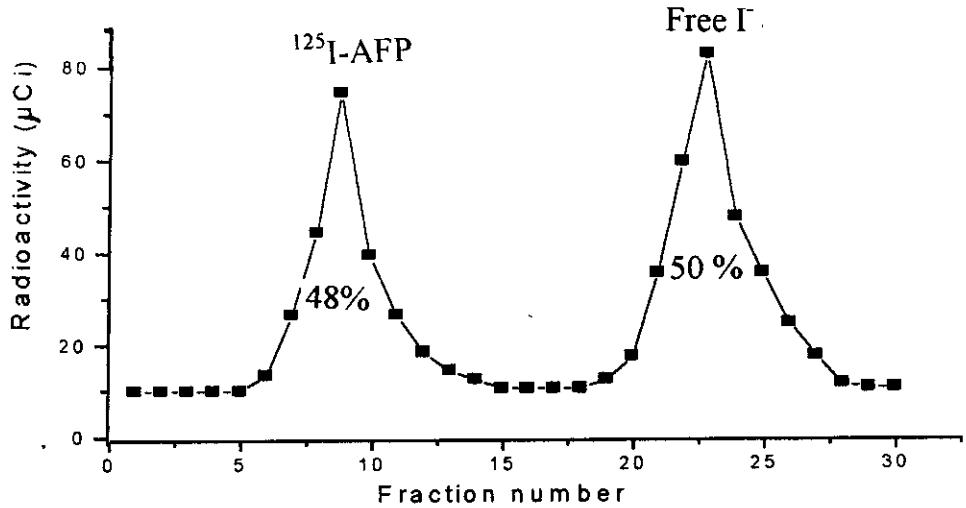


Fig (25): Purification profile of ^{125}I-AFP at optimum reaction pH value (7.4) on PD-10 Sephadex column at flow rate 0.8 ml/2 min using N-bromosuccinimde.

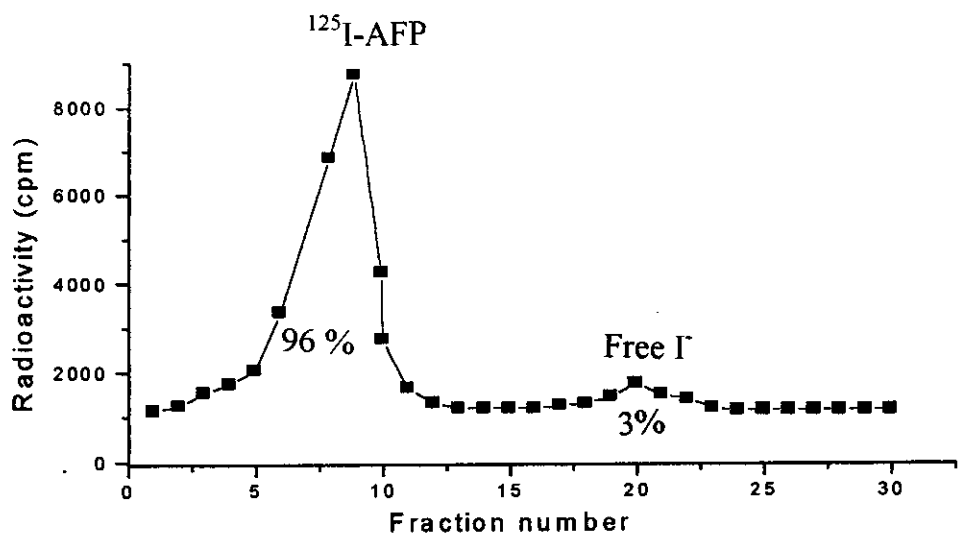


Fig (26): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction pH value (7.4)

3.1.4. Lactoperoxidase method:

Radiolabelling of AFP antigen with lactoperoxidase in liquid phase was performed essentially according to the methods of Marchalonis and Holohan et al[76] with some modifications.

3.1.4.2. Effect of the reaction time:

A comparison study of the different reaction time in the range of 10 to 50 min was carried out to reach the constant. The results obtained presented in table (13). Table (13) shows, linear relationship between reaction time and radiochemical yield percent i.e radiochemical yield percent was increased with increasing reaction time up to 40 min then the decrease in radiochemical yield percent with increase reaction time over 40 min was observed. The highest percent binding (45%) and the highest displacement percent (75.5%) were achieved. This due to, that lactoperoxidase is the enzyme and oxidizing agent so that, radioiodaintion reaction consume a long time in comparison with other oxidizing agents. Figs (27,28) show, the highest percent radiochemical yield and the highest percent radiochemical yield at optimum reaction time. These results agree with Karonen et al [114] who suggested the reaction time must be not decrease lower than 30 min in the radioiodination of BSA using lactoperoxidase method. Reaction time 40 min is recommended in radioiodination AFP antigen using liquid phase lactoperoxidase.

Reaction time 40 min is recommended in radioiodination AFP antigen using liquid phase lactoperoxidase.

Table(13): Effect of radioiodination reaction time on the percent radiochemical yield of ^{125}I-AFP:

Reaction time (m)	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
10	18	85	10	20	10.8	3
20	27	88	11	30	10.5	3.1
30	32	92	16	42	11	3
40	37	96	18.5	45	11	3
50	30	91	13.7	32	10.8	3.2

The radioiodination conditions can be summarized as follows:

20 μg of AFP antigen- 30 μg lactoperoxidase- 80 μl reaction volume - 0.5 mCi of $Na^{125}I$ and 7.4 pH of the reaction mixture.

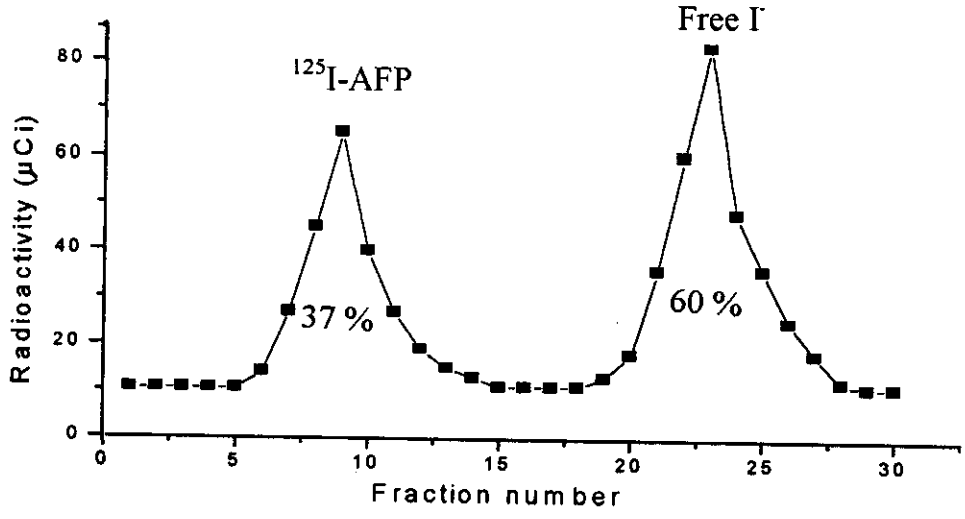


Fig (27): Purification profile of ^{125}I-AFP at optimum reaction time (40min) on PD-10Sephadex column at flow rate 0.8 ml / 2min,

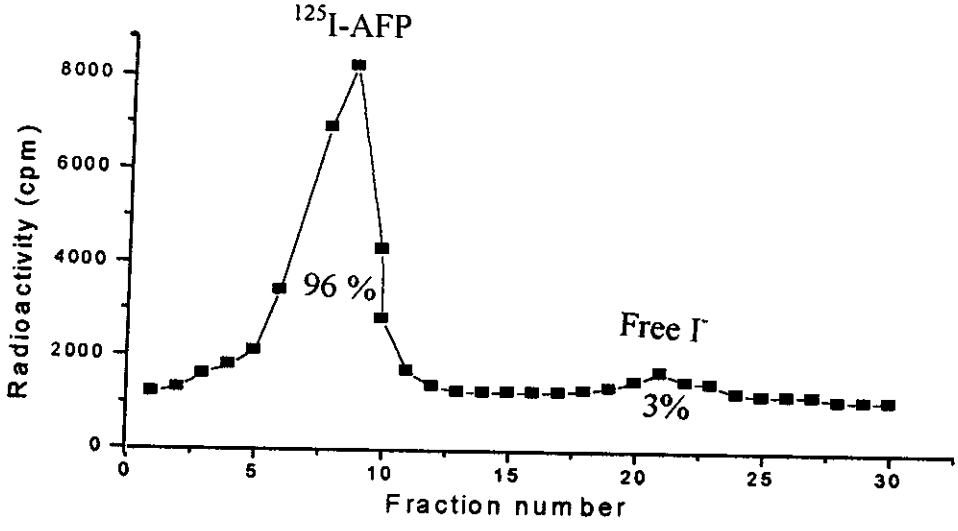


Fig (28):Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction time (40 min).

3.1.4.2. Effect of reaction volume:

A comparison study of different reaction volumes in the range of 40 to 120 µl was carried out and the results obtained presented in table (14). Table (14) shows the percent radiochemical yield which was increased with increase reaction volume up to 80µl then gradually decrease obtained with continuos increase in reaction volume. The highest percent binding (41%) and the highest displacement percent (73%) were achieved. The highest specific activity (22µCi/µg) and low nonspecific binding (2.7%) were achieved at reaction volume 80µl.

So that this study show that, the reaction volume 80 µl was the optimum reaction volume i.e. the volume ratio 1:5 where the volume AFP antigen was 16µl. At this volume ratio, the radioiodination reaction was complete and the excess of reaction volume over 80µl lead to decrease radiochemical yield percent because the reactants may be not in good contact with each other. Fig (29,30) show the highest percent radiochemical yield (43%) and a highest radiochemical purity percent (97%) obtained at optimum volume ratio (1:5). For these reasons, volume ratio (1:5) is a suitable for radioiodination of AFP antigen.

Table (14) Effect of radioiodination reaction volume on the percent radiochemical yield of ^{125}I-AFP.

Reaction volume (µl)	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (µCi/µg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
40	20	88	11.2	25	10.5	2.8
60	38	93	17.5	29	11	2.7
80	43	97	22.0	41	11	2.7
100	30	95	18.7	33	10.8	2.8
120	25	90	13.	26	10.8	2.7

The radioiodaintion conditions can be summarzied as follows:

20µg of AFP antigen, 20 µg of lactoperoxidase, 40 min reaction time, 0.5 mCi $Na^{125}I$ and 7.4 pH of the reaction mixture.

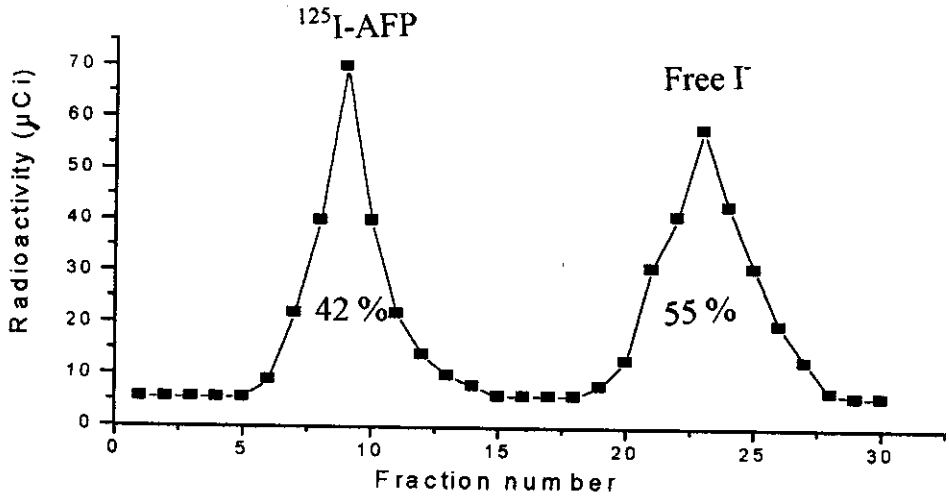


Fig (29): Purification profile ^{125}I-AFP at optimum reaction volume (80µl) on PD-10 Sephadex column at flow rate 0.8 ml/ 2min,

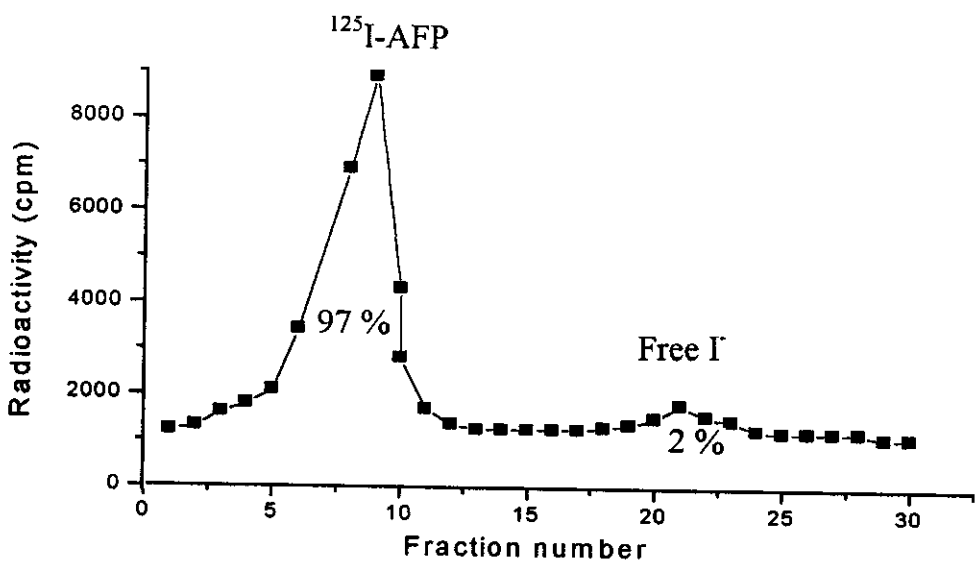


Fig (30): Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction volume (80µl).

3.1.4.3. Effect of lactoperoxidase content:

Different gram ratios between substrate AFP and lactoperoxidase content were studied and the results presented in Table (15). Table (15) shows, the highest percent radiochemical yield (38%) obtained at gram ratio (2:3) with a high specific activity (18μCi/μg). The highest displacement percent between a maximum binding percent (42%) and a minimum binding percent (11.8%) was achieved (71.9%). At lowest yield percent was obtained when the gram ratio increase more than 2:3 or decrease lower than 2:3. So that, gram ratio 2:3 is the optimum. , Figs (31, 32) show a highest radiochemical yield percent (38%) and a highest radiochemical purity percent (96%). This due to , at gram ratio 2:3, lactoperoxidase content is adequate to complete the reaction with lowest minimal immunological activity. These results agree with Karonen et al.[114] who radioiodinated BSA by liquid lactoperoxidase method. It is recommended to use gram ratio 2:3 in radioiodination of AFP antigen using liquid phase lactoperoxidase.

Table (15): Effect of oxidizing agent lactoperoxidase concentration on the percent radiochemical yield ^{125}I-AFP.

AFP (g) x 10^{-6}	Lactope-roxidase (g) x 10^{-6}	^{125}I-AFP yield %	Radio-chemical yield %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
20	5	16	80	11	22	12	3.3
20	10	27	90	16	30	11.5	3.1
20	30	38	96	18	42	11.8	3.2
20	50	24	87	13	32	11.8	3.2
20	100	19	85	11.5	25	12	3.1

The radioiodination conditions were 20 μg of antigen, 0.5 mCi of $Na^{125}I$, 40 min reaction time, reaction volume 80 μl and 7.4 pH of the reaction mixture.

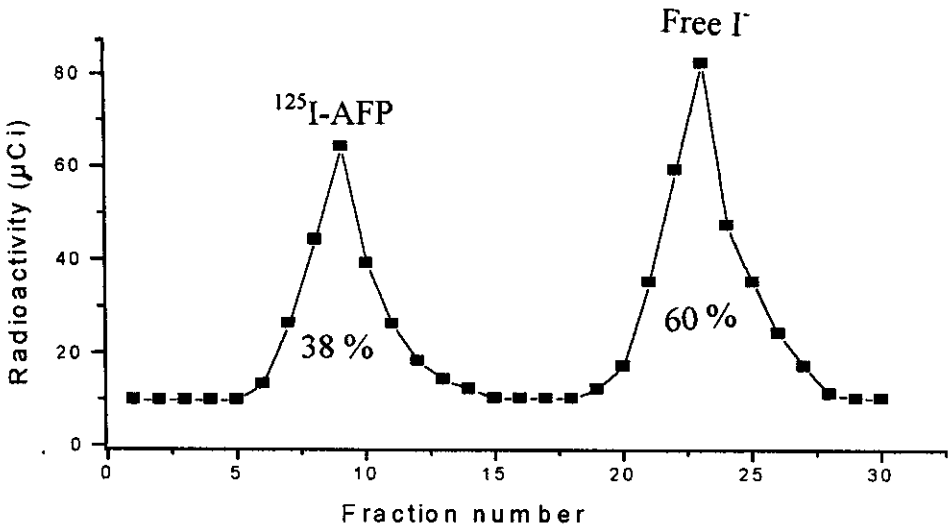


Fig (31):Purification profile of ^{125}I-AFP at optimum gram ratio between substrate AFP antigen and lactoperoxidase as oxidizing agent on PD-10 Sephadex column at flow rate 0.8 ml/2min.

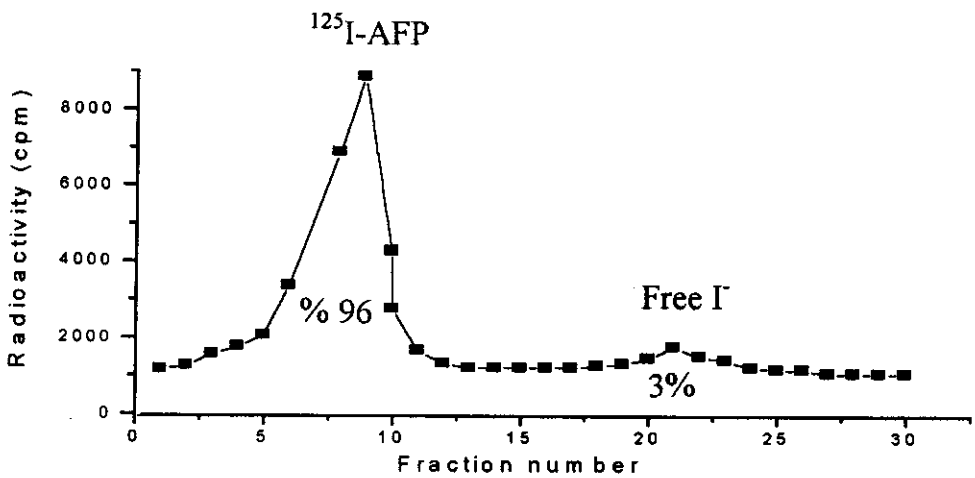


Fig (32):Electrophoretical pattern of radiochemical purity of produced ^{125}I-AFP at optimum gram ratio using lactoperoxidase as oxidizing agent.

3.1.4.4. Effect of pH :

Different pH values in the range of (3 to11) were tested and the results obtained presented in table (16). The results show, a sharp increase in radiochemical yield percent with increased pH value from 3 to 7.4. Then slightly decrease in radiochemical yield percent with continuos increasing pH value up to 11 was observed. The results are in good agreement with Thorell et al.,[115] where they found that it was better to choose a pH such as 7.5 using pH at optimal for tyrosine radioiodination, the highest percent of radiochemical yield was obtained . It is possible that not affect alteration in pH may affect the biological activity but not affect the immunoreactivity. Figs (33,34) show the highest percent radiochemical yield (39%) and the highest percent radiochemical purity (95%) which obtained at optimum pH value (7.4). This due to, between pH 7 and 8, the ortho position of tyrosine ring is active to electrophilic substitution as mentioned before. It is recommended pH value 7.4 as the optimum pH.

Table (16) Effect of pH value of the reaction mixture on the percent radiochemical yield of ^{125}I-AFP:

Reaction pH	^{125}I-AFP yield %	Radio-chemical purity %	Specific activity (μCi/μg)	Maximum binding (B_0%)	Minimum binding B_{500}%	Non specific binding (NSB%)
3	15	80	11	20	10	3.0
5	28	88	12	32	11	3.2
7.4	39	95	20	44	11	3.1
9	29	89	15	30	10.2	3.2
11	16	80.8	11.5	21	10.8	3.1

The radioiodination conditions can be summarized as follows:

20 μg of AFP antigen, 0.5 mCi of Na^{125}I, 40 min reaction time, 80μl; reaction volume and 30 μg of lactoperoxidase.

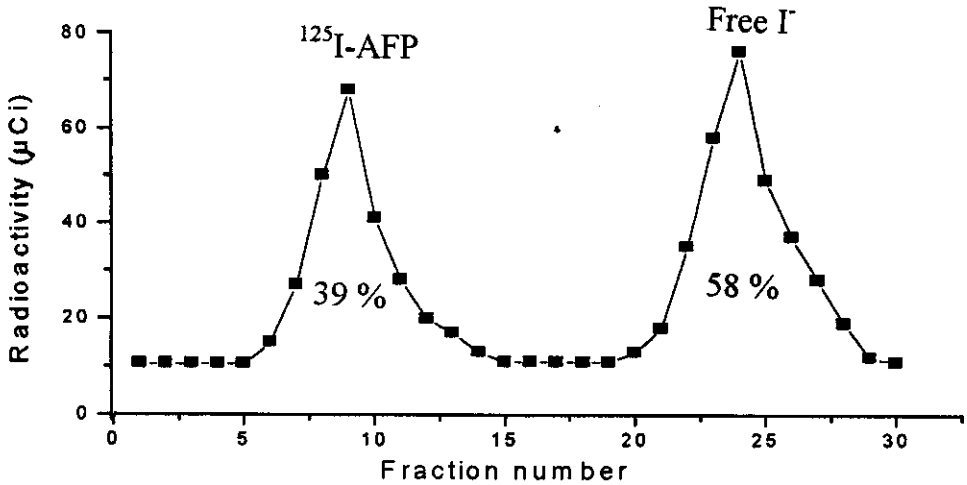


Fig (33): Purification profile of ^{125}I-AFP at optimum reaction pH (7.4) on PD-10 Sephadex column at flow rate 0.8 ml/ 2min.

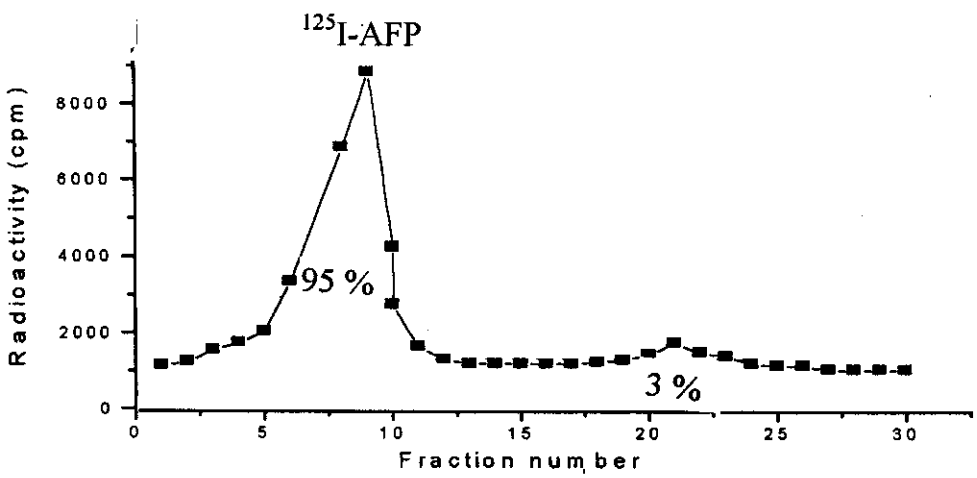


Fig (34): Electrophorical pattern of radiochemical purity of produced ^{125}I-AFP at optimum reaction pH (7.4). as oxidizing agent.

3.1.5 Effect of specific activity and storage conditions on immunoreactivity binding (Bo) and non specific binding (NSB):

Specific activity is the activity per unit mass of an element or compound containing the radioactive nuclide. It is usually expressed on molar basic (Ci/ mole or mCi/mmole). It can be also expressed on weight basis (mCi/mg or μCi/μg). because of self decomposition by self radiolysis, it is always advisable to use compounds at the lowest practical molar specific activity. General advice is not to prepare compounds of high specific activity, unless this is essential for the application and in this case the radiolabelled compound should be used within a few weeks of preparation depending on the rate of self-radiolysis under the storage conditions

Figs (35, 36, 37, 38, 39) indicated that in general immunoreactivity of produced ^{125}I-AFP tracer increased with the increase of the specific activity observed up to 10 weeks storage period then, gradually immunoreactivity decrease. After that immunoreactivity show unacceptable limits. In addition, these Figs show that specific activity of (20μCi/μg) was the most convenient. By applying it high specific activity was achieved with low rate of decay up to 10 weeks. After that the bind ability against the specific antibody was further lowered to a non acceptable levels. Also, it was observed from these Figs, that storage keeping of the proceed tracer frozen at – 20 °C or just cooled at 4°C is most proper to keep the decrease in immunoreactivity.

Furthermore Fig(36) shows that non specific binding increased, due to attachment of labelled degradation products to protein within incubation medium and that non specific binding will be a minimum at specific activity of 20 μCi/μg when the labelled product kept at –20 °C or 4 °C. Therefore, the present experiment shows that, the most convenient appropriate specific activity of the tracer product is that of 20 μCi/μg at which immunoreactivity persists in acceptable level ($\%B_0$=32 up to 10 weeks when stored frozen at –20 °C or just cooled at 4 °C).

Fig (35):Effect of specific activity and preservation method on immuno-reactivity stability of the prepared ^{125}I-AFP using chloramine –T method during 20 weeks period, a) specific activity 10 μCi/μg, b) specific activity 20 μCi/μg, c) specific activity 30μCi/μg, d) specific activity 40μCi/μg, e) specific activity 50μCi/μg.

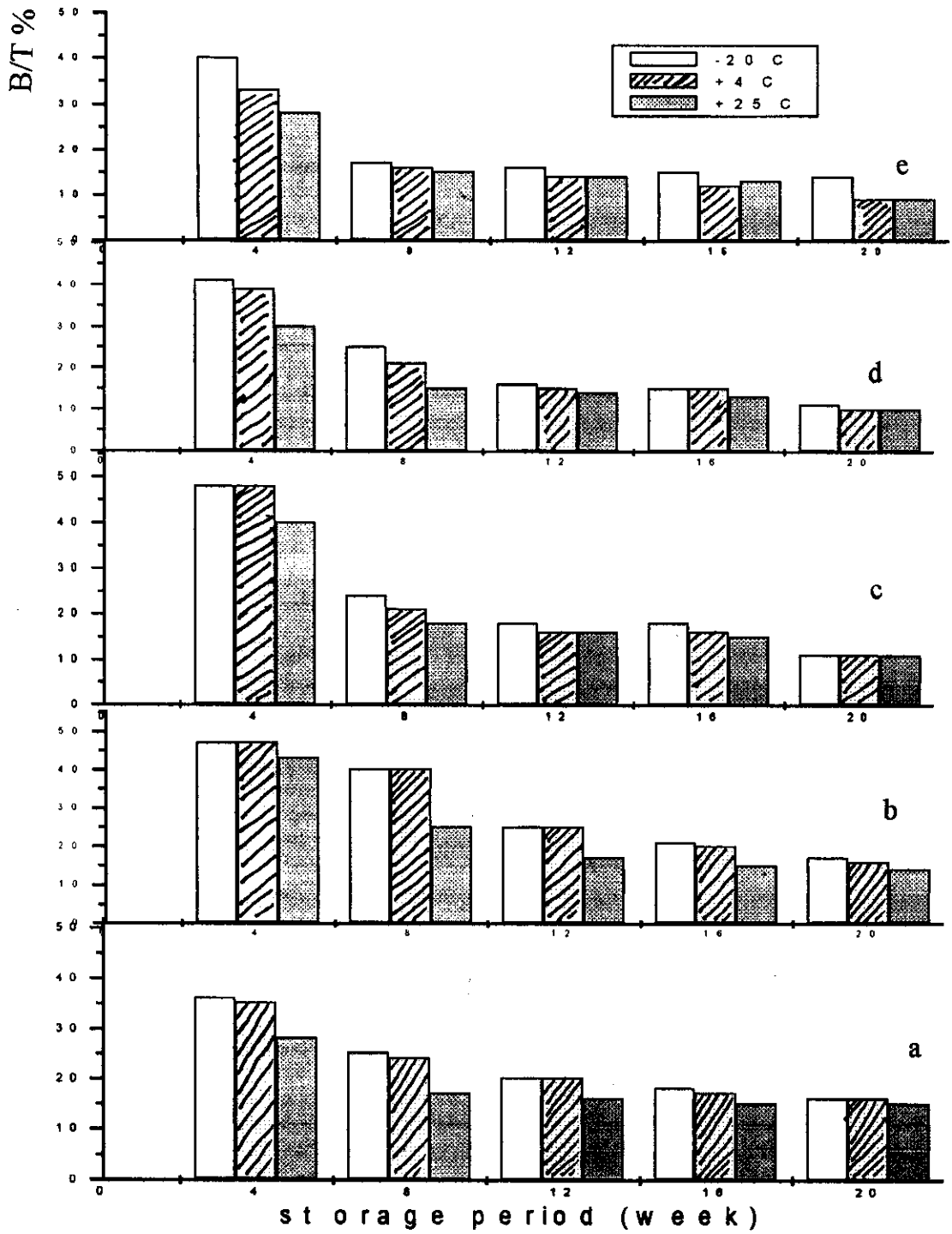


Fig (36):Effect of specific activity and preservation method on non-specific binding of the prepared ^{125}I-AFP using chloramine-T method during 20 weeks storage period, a) specific activity 10 μCi/μg, b) specific activity 20μCi/μg, c) specific activity 30 μCi/μg, d) specific activity 40 μCi/μg, e) specific activity 50 μCi/μg.

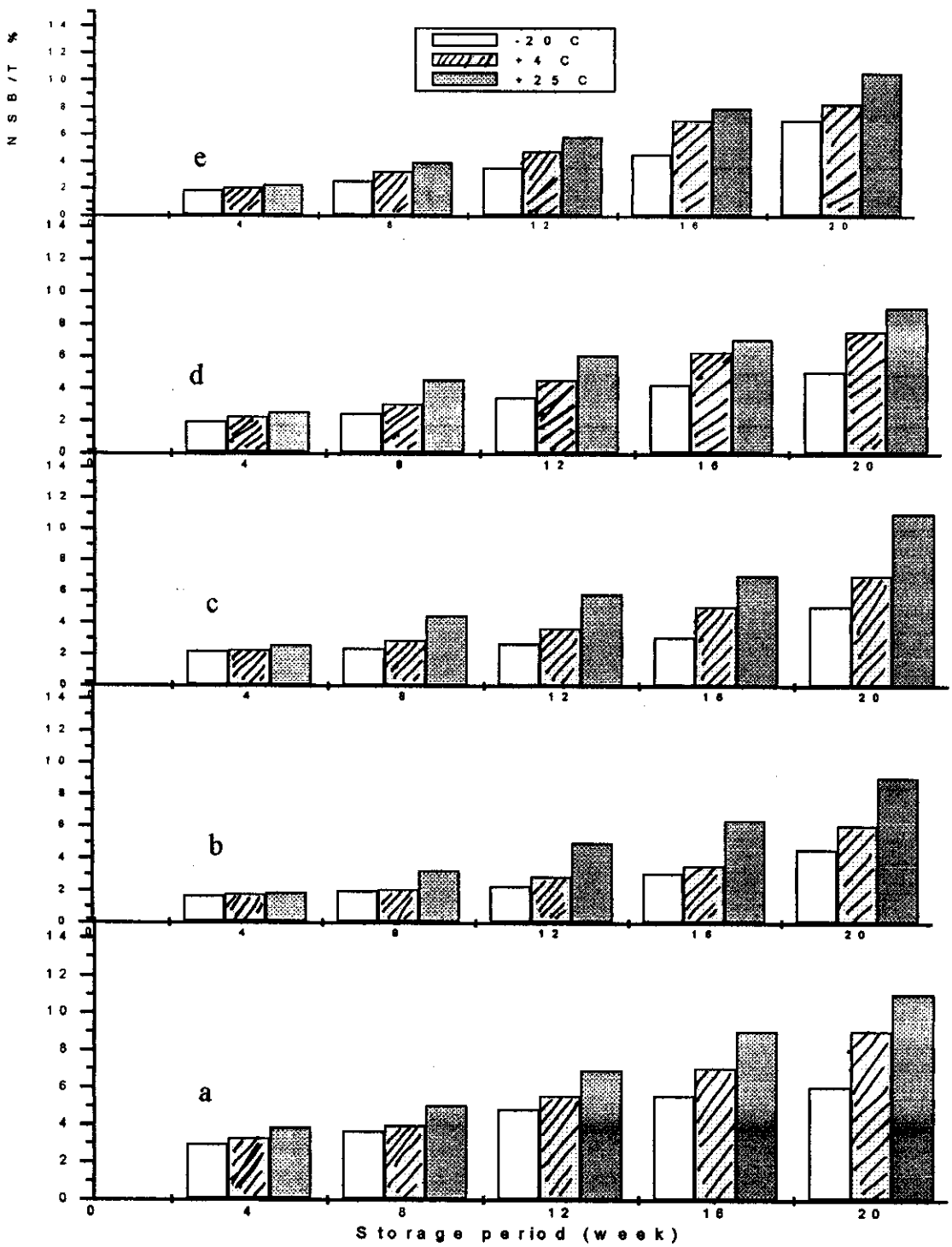


Fig (37):Effect of specific activity and preservation method on immuno-reactivity stability of the prepared ^{125}I-AFP using iodogen method during 20 weeks period, a) specific activity 10 μCi/μg, b) specific activity 20 μCi/μg, c) specific activity 30μCi/μg, d) specific activity , 40μCi/μg, e) specific activity 50μCi/μg.

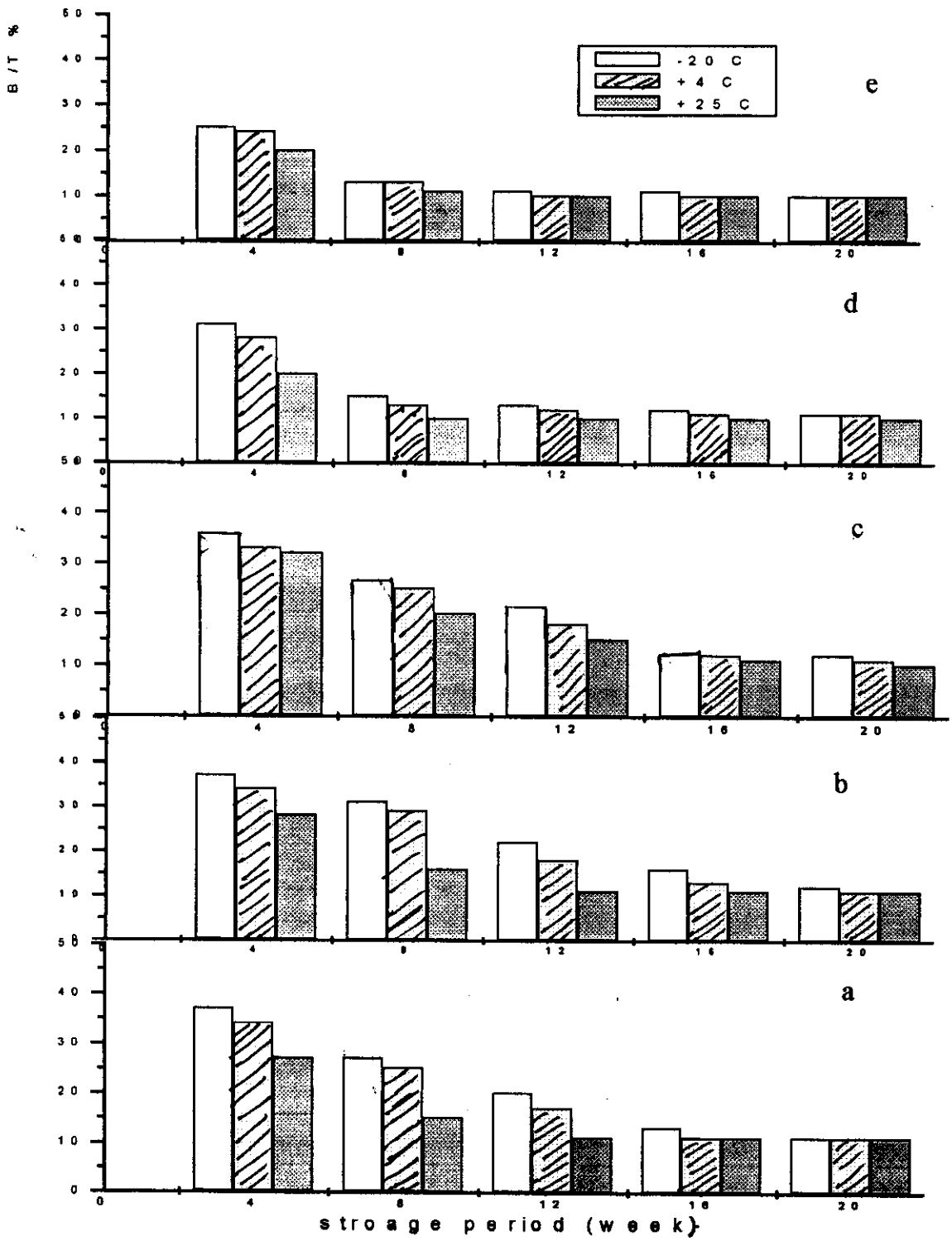


Fig (38):Effect of specific activity and preservation method on immuno-reactivity stability of the prepared ^{125}I-AFP using N-bromosuccinimide during 20 weeks period, a) specific activity 10 μCi/μg, b) specific activity 20 μCi/μg, c) specific activity 30μCi/μg, d) specific activity 40μCi/μg, e) specific activity 50μCi/μg.

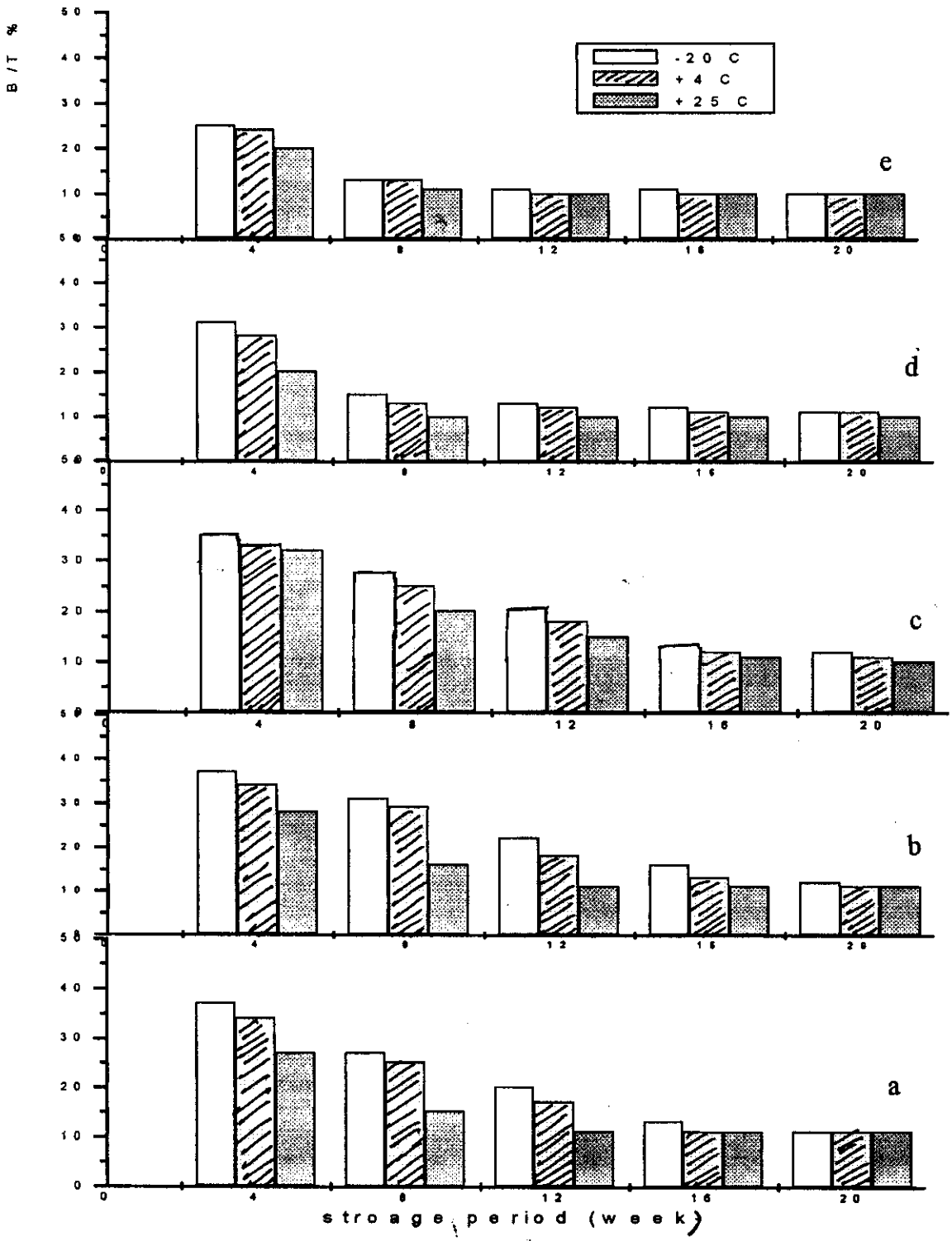
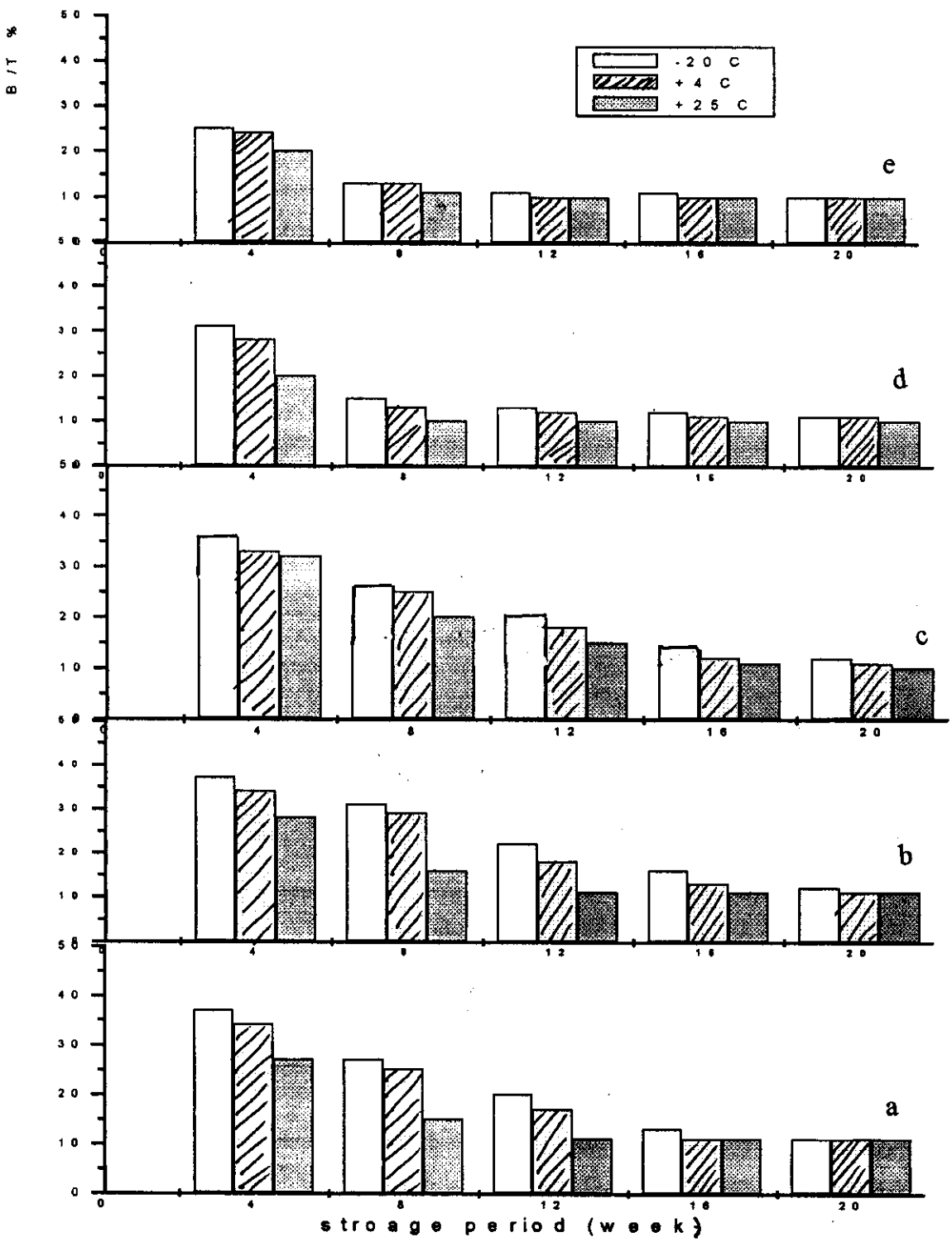


Fig (39):Effect of specific activity and preservation method on immuno-reactivity stability of the prepared ^{125}I-AFP using lactoperoxidase method during 20 weeks period, a) specific activity 10 μCi/μg, b) specific activity 20 μCi/μg, c) specific activity 30μCi/μg, d) specific activity , 40μCi/μg, e) specific activity 50μCi/μg.



3.1.6. Comparison study between different oxidizing agents

Comparison study between different oxidizing agents at optimum radioiodination conditions for radioiodination of AFP antigen . The results were presented in table (17) and show that, chloramine-T is prefer as oxidizing agent for several reasons: the first reason was, the short reaction time which was 1 min only. The second reason was, the highest of radiochemical yield percent, the highest of binding percent and the lowest non-specific binding percent.

The iodogen is the second one after chloramine-T because it is insoluble oxidizing agent in aqueous solution and it was given the highest radiochemical yield with high chemical purity.

Table (17): Comparison study between different oxidizing agents

Oxidizing agent	Chloramine-T	Iodogen	N-bromosuccin-imide	Lactoperoxidase
Reaction time (min)	1	10	5	40
gram ratio	1 : 2.5	1 : 0.75	1 : 3.5	1 : 1.5
Reaction volume ratio	1 : 4	1 : 3	1 : 3	1 : 5
pH of the reaction medium	7.4	7.4	7.4	7.4
Specific activity (μCi/μg)	21	20	19.5	18.4
Radiochemical yield percent	60	50	46	40
Radiochemical purity percent	95	98	96	97
Maximum binding percent	50	46	45	48
Non specific binding percent.	2.5	2.7	3	3.1

3.2. preparation of AFP standard and quality controls:

Cord blood are known to contain high level of AFP. The amount of AFP in the cord blood was estimated by IRMA technique using NETRIA-IRMA kits following NETRIA protocol. The results of IRMA-AFP assay of the cord blood samples are presented in Fig (40). Biorad quality controls were introduced in the IRMA assay and the observed values were in a good agreement with expected values of them as shown in table (18). Different dilution of cord blood 1:10, 1:100 and 1:1000) were introduced in parallel with Biorad quality controls. The results obtained show at a dilution of 1:100; the amount of AFP was 400 IU/ml and at a dilution of 1:1000, the a mount of AFP was 39 IU/ml as calculated from the standard curve. This indicate that, the amount of AFP antigen in cord blood pool was about 40,000 IU/ml.

Fig (40): standard curve for AFP using NETRIA-IRMA kit to estimate AFP antigen content in the cord blood pool.

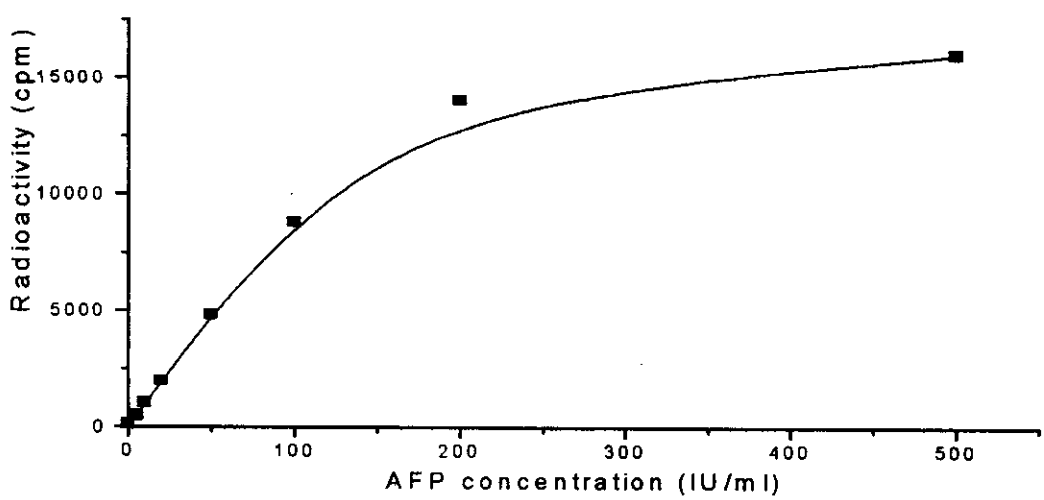


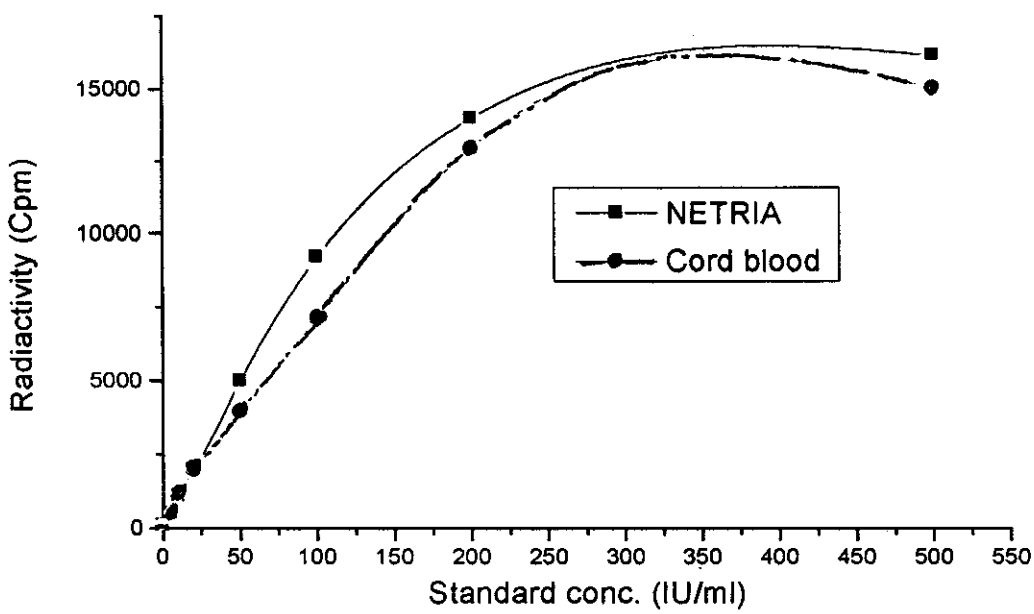
Table (18): Biorad quality controls and different dilution of cord blood were calculated from NETRIA-IRMA standard curve.

Biorad quality control	Radioactivity (cpm)	Observed value (IU/ml)	Expected value (IU/ml)
QC_1	1960	25	(20 – 30)
QC_2	9000	120	(105 – 150)
QC_3	14700	254	over than 200
Cord blood dilution			
1 : 10	17000	High	Over than 500
1 : 100	15100	400	40,000
1 : 1000	800	39	39,000

After that, the cord blood pool was purified following the ammonium sulfate precipitation method.[107] The UV spectrophotometer measurement was carried out. The obtained results show that the purified cord blood contain 39.8 KIU AFP/ml. Then, the estimated purified cord blood serum was diluted with IRMA assay buffer to make set of standard points as follows: 1:80, 1:200., 1:400, 1:800, 1:2000, 1:4000 and 1:8000 in order to give standard concentration points (500, 200, 100, 50, 20, 10 and 5 IU/ml).

At the end, the individual cord serum dilution were estimated in a comparison with NETRIA-IRMA system using series of IRP-AFP standard from Indonesia NETRIA-IRMA system.

Fig (41): Comparison between NETRIA standard curve and cord serum standard points curve.



Table(19): A comparison study between NETRIA-IRMA system and individual cord serum dilutions:

A) From NETRIA STD curve			B) From Cord blood curve		
IRP standard (IU/ml)	**Observed value**	**Obs./ exp%**	**IRP standard (IU/ml)**	**Observed value**	**Obs./ exp%**
0	0	100	0	0.01	99.9
5	4.9	98	5	4.8	96
25	25.7	102.8	25	24.8	99.2
50	50.5	101	50	49.8	99.4
100	99.5	99.2	100	98	98
400	399	99.7	400	396	99
Bio-rad QC(IU/ml)			**Bio-rad QC(IU/ml)**		
QC_1 (20-30)	24.5	98	QC1	23	92
QC_2 (105-150)	128	100.1	QC2	125	98
QC_3(over 200)	220	-	QC3	218	-

The values IRP standard and Biorad quality controls which obtained from NETRIA standard curve are compatible with that obtained from cord blood curve.

3.3. Anti-AFPantibody evaluation.

A- Titration curve of all bleeding and the dilution of antisera was carried out according to the method of Chapman (U.K) provided by IAEA cooperation project 4419/ RO.

Table (20): The production of polyclonal anti-AFP

Rabbit No.	Bleeding	Initial dilution / Final dilution	1:100 / 1:300	1:500 / 1:1500	1:1000 / 1:3000	1:5000 / 1:15k	1:10k / 1:30 k
R1	A		59.4	47.3	34.7	.....	.....
	B		65.4	61.9	59.4	43.1	34.5
	C		69.5	66.4	63.4	46.9	38.8
	D		70.9	67.8	63.8	48.1	39.0
	E		67.2	67.3	59.8	46.8	35.0
R2	A		62.9	56.0	48.1	.....	.....
	B		65.4	64.9	63.7	48.6	38.4
	C		68.8	66.3	65.6	53.7	42.9
	D		69.0	67.7	66.0	54.5	46.0
	E		67.7	67.2	63.2	49.1	37.0
R3	A		59.5	48.8	37.6	.....	.....
	B		62.8	59.6	55.1	33.9	23.5
	C		64.5	59.8	53.7	33.5	24.6
	D		.....	.....	.....	.....	.....
	E		.....	.....	.....	.....	.....
R4	A		56.4	45.2	37.6	.....	.....
	B		64.6	58.4	55.5	39.5	34.6
	C		64.0	61.5	56.8	40.9	33.8
	D		64.0	61.7	57.2	41.5	33.9
	E		59.8	58.6	55.1	38.6	29.5

-The data are presented as precent bound (B/TA)

A: 1 month after 1ry booster. B:3 wks after 1st booster. C: 3 wks after 2rd booster

D:3wks after 3rd booster; E:3wks after 4th booster.

The data presented in Table (20) revealed very interesting and consistent findings that can be summarized as follows:

- Rabbit No 3 was died before the 3rd booster. The same rabbit gave the lowest antibody titre (lowest Ab production).
- With respect to the other 3 rabbits, the date showed that the Ab production increased up to 3 weeks after 3rd booster followed by a slight decrease there after.

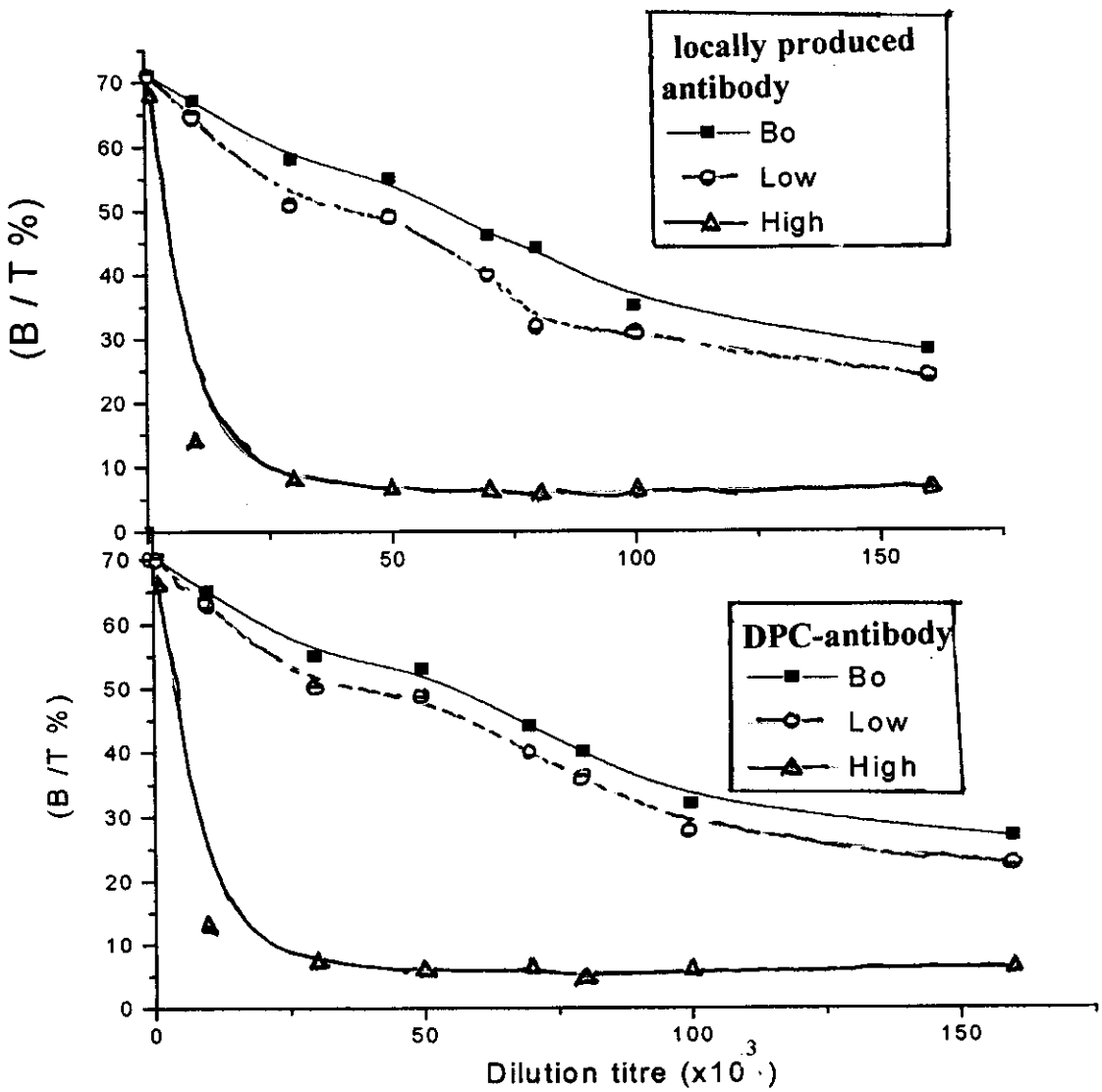
B- Estimation of AFP-antibody:

To obtain the best dilution for selected antibody (rabbit 3 Fr. D) an experiment was designed to get the highest displacement between zero, low and high standards. The anti-AFP antibody obtained was diluted with RIA assay buffer to give 1k, 10k, 30k, 50k, 70k, 80k, 100k,160k, (where $k=10^3$) and assayed with low and high standards in addition to zero standard. The DPC anti AFP antibody was used as a reference . The results obtained showed at Fig (40) and tabulated in Table (21). The results obtained show that, at dilution titer 50 K the highest displacement percent (87.7%) was obtained and the sensitivity was showed in the clear difference between zero and low standards (5%). These results compatible with the results, which obtained from DPC antibody.

Table (21): A comparison study between the displacement and sensitivity of locally produced antibody with DPC antibody as a reference.

Locally product antibody								
	1k	**10k**	**30k**	**50k**	**70k**	**80k**	**100k**	**160k**
B_0	71	67	58	55	46	44	35	28
Low	71	65	51	50	40	32	31	24
High	68	14	8.2	6.8	6.6	5.9	6.5	7.0
High-low	3	51	42.8	43.2	33.4	31.1	24.5	51.7
displacement	4.2	78	83.9	86.4	83.5	84	79.0	70
Reference DPC antibody								
B_0	70	65	55	53	44	40	32	27
Low	70	63	50	49	40	36	28	23
High	66	13	7.2	6	6.5	5	6	6.7
High-low	4	50	42.8	43	33.5	31	22	16.3
displacement	5.7	79	85.6	87.7	83	86	78	70

Fig(42): A comparison study between the displacement and sensitivity of locally produced antibody with DPC-antibody as a reference



3.4. Formulation of RIA double antibody of PEG liquid phase system for the estimation of AFP.

A-The reagent of radioimmunoassay required:

a- ^{125}I-AFP tracer: the tracer was prepared by optimized chloramine-T method.

b- Standards: The standards were prepared from cord blood pool as described before.

c- Antibody: the antibody obtained locally was used with dilution titre equals 1:60,000.

d- Separation method: PEG 8000 assisted second antibody precipitation technique consists of non immunorabbit serum and donkey anti rabbit IgG were used to separate the bound fraction from free fraction AFP-antigen. The precipitate was separated by centrifugation at low temperature.

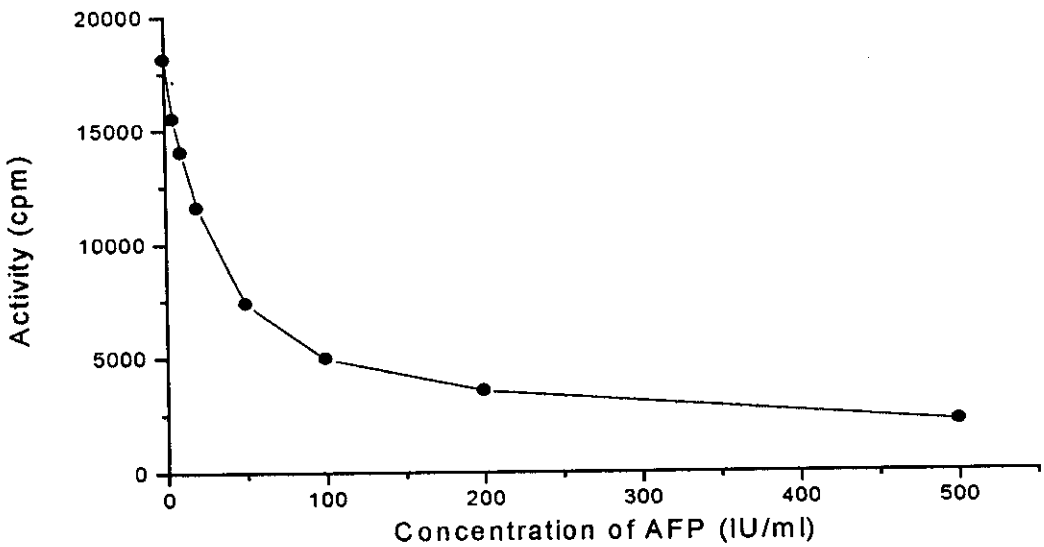
B- The protocal :

Into duplicate plane assay tubes 100 µl of STD, QC or samples, 100 µl of ^{125}I-AFP tracer and 100 µl anti AFP –antibody were added. Vortex mixed and incubated at 37 °C for 3 hrs. After that, 100 µl nonimmunorabbit serum , 100 µl donkey anti rabbit and 500 µl PEG (8%) were added . Then incubated for 30 min at room temperature. Finally, centrifugation was carried out at 4 °C and 4000 rpm for about 20 min. The tubes were aspirated or decanted carefully and counted in multicrystal gamma counter for 1 min. The results are shown in Fig (43) and presented in table (22).

Table (22): Typical AFP standard curve results with Bio-rad quality controls and three human serum samples:

Tube	Duplicate (cpm)	Average (cpm)	Percent bound (B/T)	Percent bound (B/B_0)	IU/ml
Total count	31824 32273	32047			
NSB	1010 1046	1028	3.2		
0 standard	19000 18720	18086	56	100	
Standards (IU/ml)					
5	15439 15573	15506	48.3	85.7	
10	13935 14123	14029	43.7	77.5	
20	11657 11525	11591	36	64	
50	7451 7248	7349	22.9	40.6	
100	4894 4992	4943	15.4	27.3	
200	3384 3713	3549	11	19.6	
500	2138 2124	2131	6.6	11.7	
Biorad quality controls (IU/ml)					
QC_1 (20-30)	10519 10724	10622	33	58.7	26
QC_2 (105-150)	4554 4934	4744	14.8	26.2	114.3
QC_3 (over 2000)	3540 3355	3447	10.7	19.5	221.5
Unknown samples					
Sample (1)	11519 11774	11647	36.7	64.3	19.7
Sample (2)	5373 5013	5193	16.2	28.7	94.8
Sample (3)	3259 3229	3244	10.2	17.9	264

Fig (43): Typical AFP standard curve using RIA double antibody PEG liquid phase technique.



3.5. Optimization of the RIA double antibody PEG liquid phase system:

3.5.1. Standard, sample or Q.C volume:

Three different standard points were assayed (5, 50 and 500 IU/ml) in addition to zero standard. Four different volumes (25, 50, 100 and 200 µl) of each standard were pipettes separately in plane tubes. The assay procedures were performed as mentioned earlier for each volume in separated assay. The data was presented in table (23) and shows that, the binding percent was increased with increasing the sample volume more than 100 µl. At standard, Q.C. or sample volume of 100µl, the assay can differentiate between zero standard, low, medium and high values of AFP. Therefore., 100 µl sample , standard or Q.C. volume was recommended.

Table (23): different standard, quality control or sample volume were studied with different standard points:

Sample volume / Standard used IU/ml	% radioactivity binding (%B)			
	25 µl	**50 µl**	**100 µl**	**200 µl**
0	28	34	48	50.6
5	19	33	46.2	49.2
50	16	20	28.5	30
500	8.9	10.8	11	10

3.5.2.Incubation time:

Different incubation times in the range of 1,2 and 3 hs at 37 °C were setup in parallel with 24 hs incubation time at +4 °C. Three different standard points were assayed (5, 50 and 500 IU/ml) in addition to zero standard. The data presented in table (24). As shown from the data presented in Table (24) 3 hs incubation time was optimum for the assay. In addition the values obtained at 3 hs incubation at 37 °C nearly equals to the values obtained after 24 hs incubation at 4 °C. This means that, the reaction proceeds slowly at low temperature and 3 hs was chosen for further assays to safe the time.

Table (24): Different incubation times studied with different standard points:

Incubation time / Standard used IU/ml	% radioactivity binding (%B)			
	1 h	2hs	3hrs	24 hs
0	35	40	46	48
5	33.5	38.8	44.5	46
50	21	25	28	29
500	9.6	10.2	10.8	10.7

3.5.3. Radioactivity counts:

Three different standard points were assayed (5, 50 and 500 IU/ml) in addition to zero standard using different ratio activities (10,000, 30000, 50000 and 70000 cpm) in 100 µl tracer were tested.

The data obtained were presented in table (25). The data shows that, the binding percent was increased with the increase of radioactivity. The variation of binding percent was little with increase of radioactivity recommended to use radioactivity about 30,000 cpm/100µl.

Table (25): Different radoactivities of tracer were studied with different standard points

Radioactivity (cpm) / Standard used IU/ml	% radioactivity binding (%B)			
	10,000	30,000	50,000	70,000
0	40	48	49.8	51
5	38.5	46.2	48	49
50	27	28.5	30.5	32
500	11.3	11	10.5	10

3.6. Solid phase coated tube RIA system:

The classical double antibody procedure offers a specific separation procedure but is often time-consuming and requires subststantial quantities of specific second antibody[7]. Recognizing that a fast, convenient simple method of separating antibody –bound from free tracers was needed. Catt and Tregear[116] developed the technique of using a plastic surface that had been coated with antiserum specific for the analyte of interest. The only reagent required other than the primary antiserum was the plastic tube. The advantages of the coated –tube immunoassay in general stem from the extreme simplicity of the method. Misclassification errors in separation of bound and free that could arise from tracer breakdown. Only tracer material that is recognizable by the antibody is counted in the bound fraction. Non specific binding is another problem common to double antibody. Adsorption of tracer to plastic surfaces can occur, but washing the antibody- coated tube prior to use with a buffer containing a protein such as gelatin or bovine serum albumin (BSA) will fill up uncoated plastic sites and render hydrophobic tracer less likely to stick. The elimination of centrifugation is one of the foremast advantages of using antibody –coated tube. Variable decanting techniques and carry-over of precipitates are also done a way with. A coated tube separation of bound and free is accomplished by simple aspiration or decantation. Washing of the tubes with buffer or distilled water has been used to improve tube replication. For these advantage of coated tube, the coated tube was carried out by two techniques. The first, one was the phosphate

method and the second one was the borate method. The assay carried out as the protocol mentioned before in chapter 2. From table (26) the results show the high binding observed with the tubes which coated using phosphate method.

Table (26): Comparison study between phosphate and borate method.

Coated method / Binding %	Borate method	Phosphate method
B_0	36	45
B_5	30	38
B_{500}	12	11
NSB	2	1.5

The complete standard curve with coated tubes was carried out. After that, to optimize the assay conditions such as reaction time, sample volume radioactivity of the tracer and washing manners, different assay were carried out.

Table (27): Typical AFP coated tube standard curve results with Biorad quality controls and three different human serum samples:

Tube	Duplicate cpm	Average cpm	Corrected cpm	Percent bound (B/T)	Percent bound (B/B_0)	IU/ml
Total count	34290 34095	34193				
NSB	513 545	529		1.5		
Zero standard	17392 17365	17379	16850	49.2 %	100	
Standards, Points (IU/ml)						
5	16329 16477	16403	15874	46.4	94.2	
10	15842 15991	15917	15388	45	91.3	
20	15299 15105	15202	14673	42.9	87	
50	12886 12830	12858	12329	36	73	
100	9766 9830	9798	9269	27	55	
200	6100 5900	6000	5470	16	32.4	
500	4600 4664	4632	4103	12.0	24.3	
Bio-rad quality controls (IU/ml)						
QC_1 (20-30)	14000 14100	14050	13521	39.5	80	21.7
QC_2 (105-150)	9100 9000	9050	8521	24.9	50.5	108.9
QC_3 (over 200)	5700 5800	5750	5221	15.2	30.9	209.7
Unknown samples						
1	16015 16071	16043	15531	45.4	92	5.2
2	9641 9994	9817	9288	27.1	55	100
3	5500 5400	5450	4921	14.4	29.2	221.9

The standard curve constructed between concentration of AFP (IU/ml) on x-axes and the activity (cpm) of bound fraction on y-axis as shown in Fig (44)

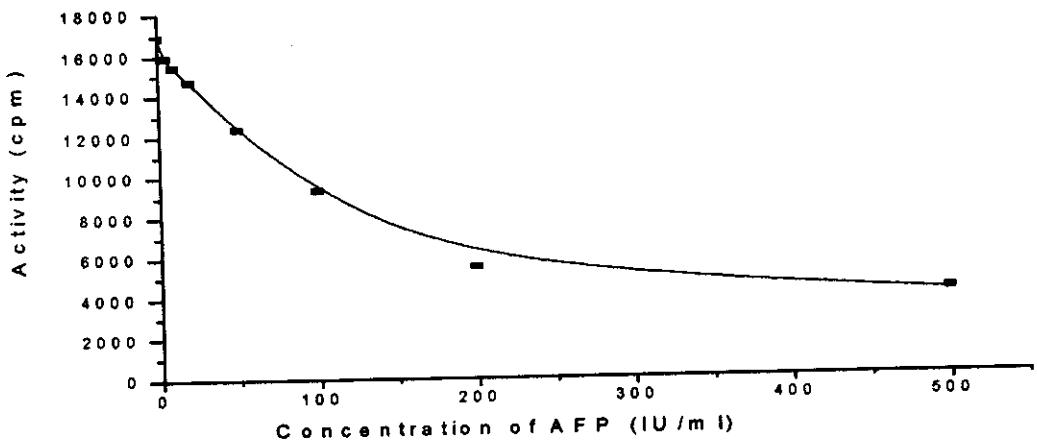


Fig (44):Typical AFP coated tube standard curve with Biorad quality controls and different three human serum samples.

3.6.1. Optimization of solid phase RIA –coated tube system:

3.6.1.1. Standard, sample or QC volume:

Table(28): Different standard, quality control or sample volume were studied with different standard points:

Sample volumes / Standard used IU/ml	% radioactivity binding (%B)			
	25 µl	50 µl	100 µl	200 µl
0	40	48	49	49.1
5	39	46.2	47.3	47
50	25	29	30	30.5
500	10.5	10.3	10.4	10.8

Three different standard points were assayed (5, 50 and 500 IU/ml) in addition to zero. Four different volumes (25, 50, 100 and 200 µl) of each standard were pipetted separately in coated tubes. The assay procedures were performed as mentioned earlier for each volume in separate assay. The results was presented in table (28) and shown generally that the binding present was increased with increase of the sample volume. 50 µl is recommended because after that, the binding increase slowly.

3.6.1.2. Incubation time :

Different incubation times for AFP assay were setup in parallel using incubation of (1, 2 and 3hs) at 37 °C and 24 hs at + 4 °C. Three different standard points were assayed (5, 50 and 500 IU/ml).

Table(29): Different incubation times were studied with different standard points:

Incubation time / Standard used IU/ml	% radioactivity binding (%B)			
	1h	2hs	3hs	24 hs
0	36	42	48	49
5	35	40.8	46.3	47.2
50	21	27	29.9	30
500	9	10	10.4	10.5

The results show, the binding percent was increased with the increase time and the displacement percent between different standards were acceptable. From the results, incubation time 3hs at 37 °C was recommended because the binding percent nearly equals to the value obtained after 24hs incubation at 4 °C.

3.6.1.3. Radioactivity counts:

Three different standard points were assayed (5, 50 and 500 IU/ml). Different activities (10000, 30000, 50000 and 70000 cpm) in 100 µl were used. The results presented in Table (30). The results show, the binding percent was increased with the increase radioactivity. From the results obtained radioactivity of 50,000 cpm was recommended because the binding percent at 50,000 cpm nearly equals that one at 70,000 cpm and low radioactivity is prefer to decrease the radiation weste.

Table (30): Different radioactivities of tracer were studied with different standard points.

Radioactivity (cpm) / Standard used IU/ml	% radioactivity binding (%B)			
	10,000	**30,000**	**50,000**	**70,000**
0	40	48	50	51.5
5	38.8	46.3	48	50
50	25	28.2	30	33
500	11.2	10.8	10.7	11

3.6.1.4. Washing method:

PBS buffer and 0.05 M phosphate buffer were used as washing buffers in addition to distilled water. The results obtained tabulated in table (31). Different volume from (PBS) were used and the results tabulated in table (32):-

Table (31): Study of different washing buffer in comparison with water:

Washing buffer / Standard used (1U/ml)	Dist. water	0.05 M Phosphate buffer	PBS
0	50	49.8	47.5
5	47	46.8	46
50	28	27.5	26.7
500	11	10.5	10

The results shown that phosphate buffer saline was the best washing solution which yield very low error. Variable volume of the chosen washing buffer were tested and results were presented in table (32).

Table (32): Comparison study between different volumes of PBS which used as a washing buffer.

% Radioactivity binding (%B)					
PBS buffer / Standard used IU/ml	1 ml once	1 ml twice	2 ml once	2 ml twice	Without Washing
0	50	49	49.3	49	52
5	47	46	46.5	46	48
50	29	28	28	27.8	30
500	11.5	11	11.3	11	12.2

3.6.2. Performance characteristics of the coated tube AFP-RIA assay:

A developed assay protocol required validation to established suitability before proceeding to practical application. In broad terms validation should test the assay in the following ways:

Determination the sensitivity of the locally prepared RIA-AFP system, determination the precision, effect of dilution, effect of recovery and comparison with other established methods.

3.6.2.1. Determination the sensitivity of the locally prepared RIA-AFP system:

The sensitivity of an assay (the lowest concentration of analyte which can be distinguished from zero) is a function of the slope of the calibration curve and the precision at each dose. Twenty zero standard point coated tubes were processed in a single RIA assay with a set of other standard points and quality controls for AFP. The results are presented in table (34) and shown in Fig(45). The mean zero response was calculated 95.4% and related to 1.5 IU/ml

Fig.(45): Standard curve to determine the sensitivity of the local coated tube of AFP RIA system.

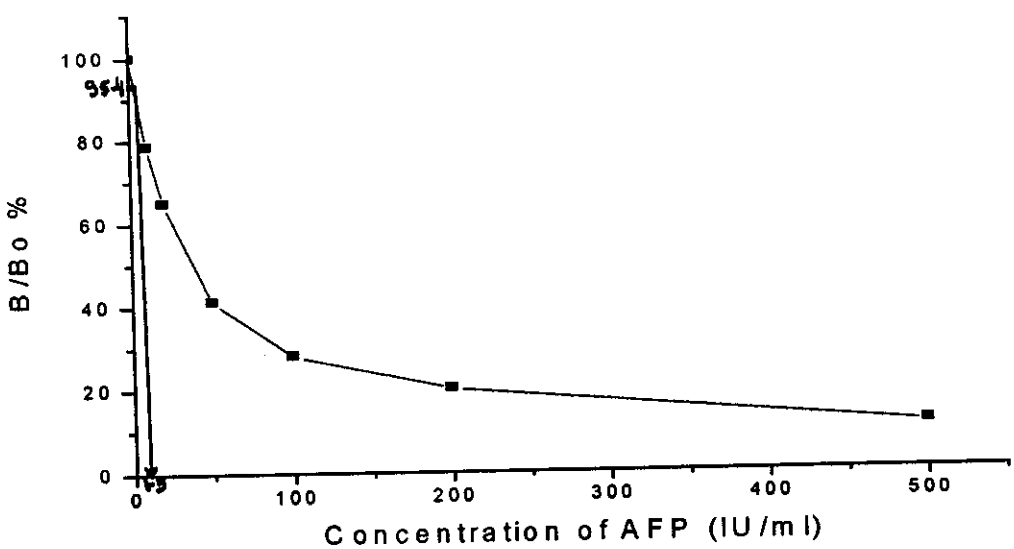


Table (33) :Bio-rad quality controls calculated from standard curve.

Biorad QCs	cpm	Mean (x)	Obs. Value (IU/ml)
QC_1	14300 -14250	14275	260
QC_2	9100 - 9050	9075	122
QC_3	2100 - 2090	2095	24

Table (34): Determination of the sensitivity of the locally prepared RIA-AFP system.

$\bar{X}$	SD	SEM	2SD	$\bar{X}$ – 2SD	minimal detectable dose	Minimal detectable dose (IU/ml)
15495 (cpm)	± 357.8	80	715.8	14779.2	95.4%	1.5

3.6.2.2. Determination of the precision profile:

Precision in RIA refers to reproducibility of the results obtained when the test is carried out several times on the same sample under the same conditions (Inter assay). It also refers to the consistency of the results when many replicates of the same sample are included in one assay (Intra-assay). In this case the agreement between the replicates is an indication of the precision of the assay. The precision of the technique can be estimated by means of coefficient of variation, C.V(%).

$$C.V\% = \frac{SD}{Mean} \times 100$$

3.6.2.2.1. Intra-assay (within-run):

Twenty replicates of the same three pooled human serum samples low medium and high are included in one RIA assay for estimated of AFP. The meen ($\bar{x}$) and standard deviation were calculated. Then the coefficient of variation (CV%) was calculated and the results are presented in table (35). The results shown, the values of CV% were 5.17% at pool 1, 1.8% at pool 2 and 9.8% at pool 3. These results were in a good agreement with allowed limits which they lower than 10%.

Table (35):Twenty replicates of the same three pooled serum are included in one RIA assay (Intra-assay) for estimation of the precision of the local system.

Pool 1 (IU/ml)	Pool 2 (IU/ml)	Pool 3 (IU/ml)	Pool 1 (IU/ml)	Pool 2 (IU/ml)	Pool 3 (IU/ml)
29	105	201	30	103	205
28	101	210	28	104	206
26	109	215	29	100	207
26	110	217	26	109	208
28	108	215	28	108	209
30	107	210	29	105	212
27	109	222	29	107	218
26	105	224	26	106	220
30	102	213	25	102	217
28	100	212	27	103	202

Table (36)Estimation of coefficient of variation from intra assay:

Human serum (IU/ml)	$\bar{X}$	SD	CV%
Pool 1	26.45	1.34	5.17
Pool 2	105.15	3.8	1.8
Pool 3	212.15	15	9.8

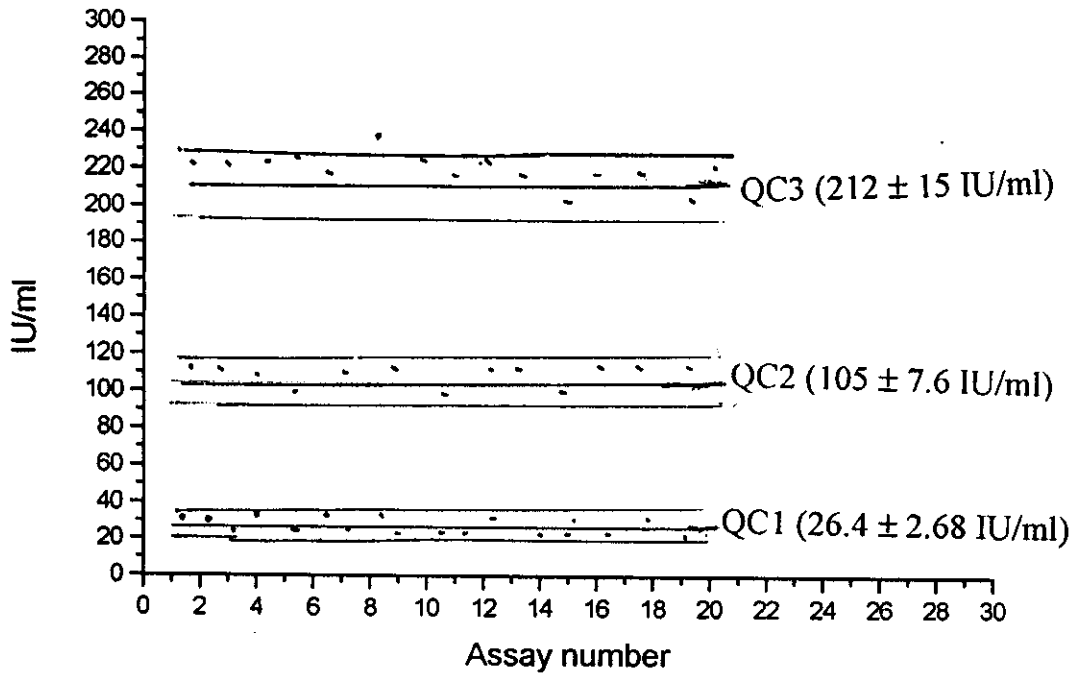
3.6.2.2.2. Inter-assay: (run to run):

Twenty replicates of the same three pooled human serum samples (low, medium and high) are included in twenty RIA assay for established AFP under the same conditions. The statistics were calculated and the results obtained were tabulated in table (37).

Table(37):Twenty replicates of the same three pooled human serum samples are included in twenty RIA assays (Inter assay) for estimation of the precision of the local system:

Pool 1	Pool 2	Pool 3	Pool 1	Pool 2	Pool 3
27	110	220	28	109	224
26	111	222	28	110	226
28	109	225	27	111	228
26	108	227	28	110	221
28	109	220	26	109	223
27	112	225	25	109	227
25	111	222	26	110	228
26	110	230	28	111	223
27	111	226	25	110	228
29	112	225	26	111	223

Fig. (46) The range of internal quality controls from intra assay and the values of inter assay were applied with the values of Inter assay.



3.6.2.3. Effect of dilution:

It means measure concentration of specimens at various dilutions in matrix and assays linearity. So that, three serum samples were assayed out undiluted and diluted with zero standard point for AFP. Each sample was diluted (1:1, 1:5 and 1:7) separately. The expected value for each dilution was calculated. The observed results were obtained from the standard curve. The observed data was divided over the expected ones and the results were presented in table (30). The results shown the observed value divided over expected value was in the range of ±10

observed value was estimated from the standard curve as mentioned earlier the observed values were divided by the expected values and the results presented in table (40).

Table(40): Effect of recovery:

Standards (IU/ml)	Sample + StD (1:1)	Obs.	Recovery %
0	25	24.5	98
5	27.5	28	101.8
10	30	31	103
20	35	35.5	101.4
50	50	52.5	105
100	75	73.5	98
200	125	125.7	100
500	275	281	102

The results shown, the recovery percent was obtained in the range of 100 ± 3% and this agreement with allowed limits reach to 100 ± 10%.

3.6.2.5. Method comparison:

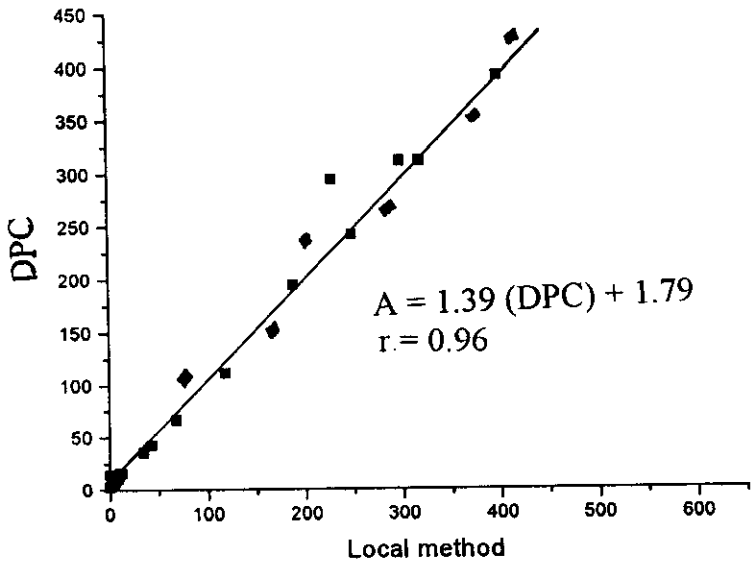
The statistical analysis were under taken to compare AFP results of 30 different human serum samples obtained by commercially available kits (DPC, double antibody) and NETRIA-IRMA coated beads to those obtained by the present system. It was found that the present method had produced clinically

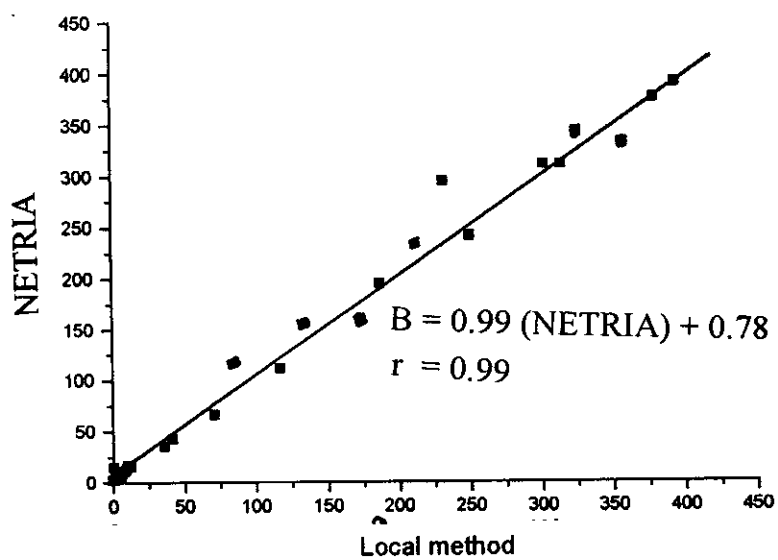
acceptable results. Normal and abnormal subjects are reasonably and accurately distinguished using the present method. The statistical analysis showed good correlation between the results obtained from the follows:

Linear regression analysis between local system and DPC system yield the following relationship A = 1.39(DPC) + 1.79 and correlation coefficient (r) equal to 0.96. Then the linear regression analysis between local system and NETRIA system yield the following relationship B = 0.99 (NETRIA) + 0.78 and correlation coefficient (r) equal to 0.99.

The equation Y = a + bX show that, the good linearity regression analysis can obtain when the intercept (a) equal to zero and the slope (b) equal to one. At these values, The correlation coefficient equal to one.

Fig(47): Estimation of correlation coefficient between local system and NETRIA-IRMA coated beed method in parallel with DPC kit.





DPC/Local (r) = 0.96818

NETRIA/Local (r) = 0.995754