Binding of ^{99m}Tc to human serum albumin

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الخلاصة

تم اختبار الارتباط الانتقائي لمركب ^{99m}Tc-MDP المرتبط مع ألبومين مصل الدم البشري وأوضحت النتائج بان الارتباط غير الانتقائي كانت بحدود ٢١-٢٣ % من الارتباط الكلي . إن مستوى الأس الهيدروجيني الامثل لهذا الارتباط هو ٧,٤- ٧,٦ . وان التركيز الامثل لألبومين مصل الدم ليعطي أعلى ارتباط هو (٧٠) مكر وغرام . أوضحت النتائج إن الارتباط الامثل يتأثر بتركيز المعقد ^{99m}Tc-MDP و تركيز كل من أملاح الكلور للفلزات الأحادية والثنائية التكافؤ . إن قيمة ثابت الارتباط والارتباط الأعظم للمعقد ^{99m}Tc-MDP المرتبط في مواقع الارتباط الانتقائية لألبومين مصل الدم تم حسابه من علاقات سكاجارد scatchard and double reciprocal وأتضح من النتائج إن هذه القيم تتأثر بتغيرات درجة الحرارة .

ABSTRACT

The specificity of ^{99m} Tc-MDP binding to HSA was examined , the results showed that the non specific binding was 21-23% of the total binding . The range of optimum pH for the binding was 7.4-7.6 . The most suitable amounts for HSA to give maximum binding was (70µg)per incubation medium , binding seemed to be affected by ^{99m} Tc- MDP concentration and also mono and divalent metal chloride salts. The association constants (ka) and maximum binding capacity (B_{max}) of ^{99m} Tc-MDP binding to its binding sites of HSA was determined from scatchard and double reciprocal plots , the results revealed that (ka) and (B_{max}) values were temperature dependant.

INTRODUCTION :

Normally nearly many drugs, fatty acids and hormones ...etc are transport through the human body by serum albumin, plasma long-chain fatty acids, such as oleate, palmitate , linoleate , stearate and phospholipids are transported in association with serum albumin[(1)] . Albumin binds 1-2 fatty acid molecules to form a stable complex, this association stabilizes the fat and confers increased stability on the protein. About 99.9% bilirubin and long chain fatty acid are though to be albumin bound in normal plasma, this is important in neonates to whom entry into the brain tissue is a cause of bilirubin encephalopathy in neonatal jaundice and depends on the level of free plasma bilirubin[(2)] .

Many hormones are bound to plasma albumin and indicate percentage bound in normalplasma :-

Cortisol (30%) , corticosterone (30%) , thyroxin (10%) , aldosterone (60%) , testosterone (60%) .Other substances known to bind with albumin are bile pigments, prostaglandins ,some amino acids, triiodo thyronine,urate , salicylate , penicillin , ampicillin and other antibiotics, drugs barbiturates , sulphonamides , diphenylhydantion , warfarin...etc[(3)] transported as albumin bound compounds.

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Theoretical and practical aspects of the interaction of therapeutic drugs with transport proteins in human blood have been considered, during the last decade more attention was paid to diagnostic radio labeled compounds especially the technetium complexes which have become the most frequently used radiopharmaceuticals[4] .
Understanding the mechanism of their biolocalization in specific target organ tissues, as well as, their elimination (generally-their biokinetics) may depend on elucidation of their biochemical characteristics such as protein binding and specific protein binding (binding to individual circulatory proteins).

EXPERIMANTAL :

Preparation of ^{99m}Tc-MDP

a- Methylene diphosphonte (MDP)kit:-

The MDP kit was prepared according to the following procedure[5] :

1- 1.25g MDP was dissolved in 200ml nitrogen-purged distilled water in closed flask and stored under nitrogen pressure.

2- 0.6g $SnCl_2.2H_2O$ dissolved in slightly acidified distilled water with 0.1N-HCl , then after dissolving the solution was transferred to a closed vial and stored under N2-gas pressure to prevent the oxidation of $SnCl_2$ to $SnCl_4$.

3- 2.5ml of $SnCl_2$ solution was added drop wise with continuous stirring to the MDP solution, the solution should be clear, the pH of the solution must be adjusted to 6 using 1N-NaOH.

4- The final solution was filtered through Millipore filter and 1ml aliquots of the filtered solution was placed in vial under N_2-gas pressure and then lyophilized.

b- Technetium generator (300mci) was purchased from Amersham Bucks, England .

Labeling of HSA with ^{99m}Tc

1. MDP kit contains (5mg MDP and 0.5mg $SnCl_2$) was dissolved in 5ml phosphate buffer and left for 15 minutes before labeling MDP concentration was 2.8×10^{-3}M ,the other concentrations were prepared by serial dilution from this concentration using phosphate buffer pH 7.4 .

2. 10 μ ci ^{99m}Tc eluted with saline from the ^{99m}Tc-generator, was added to the MDP solution, incubated for 15 minutes then 100μl of HSA solution (750 μg/ml) was added and incubated to the required time(15,20,30,45&60 min.) or required temperature (20,30,37,50 and 60°c) (table-1) .

Table 1 . The specifically bound ^{99m}Tc-MDP to its binding protein (HSA) at optimum pH(7.4) and different temperatures.

Temp. °c	Specifically bound of 99mTc-MDP at equilibrium (×10-8M)
20	2.9
30	5.0
37	6.8
50	7.4

3. The samples was then applied to the surface of sephadex G.100 gel filtration column (1×30cm) elution was carried out using the same buffer to separate bound ^{99m}Tc-MDP-HSA complex from free ^{99m}Tc-MDP with flow rate of 12ml/h and fraction volume of 2ml. Accordingly three types of samples were applied to the surface of the same column by the following procedures.

(a) ^{99m}Tc:

10µl of ^{99m}Tc was diluted to 500µl with phosphate buffer(pH 7.4),then it was added to the surface of the same column used in the separation of ^{99m}Tc-MDP-HSA complex, elution was carried out using the same buffer, the radioactivity of each fraction was measured by gamma counter expressed in counts per minute (cpm).

The radioactivity of each fraction was plotted vs. corresponding fraction number.

(b) ^{99m}Tc-MDP :-

10µl ^{99m}Tc was added to 100µl MDP solution (2.8×10^{-6}M) then diluted to final volume of 500µl with phosphate buffer, incubated for 15 minute at 25°c, then this solution was added to the surface of the same column, elution was performed with phosphate buffer pH 7.4,the radioactivity of each fraction was measured expressed in (cpm).

The radioactivity (cpm) of each fraction was plotted against the corresponding fraction number .

(c) ^{99m}Tc-MDP-HSA:-

1- 10µl ^{99m}Tc was added to 100µl MDP (2.8×10^{-6}M),incubated for 15 minutes at 25°c .

2- 100µl of HSA(1mg/ml) was then added to ^{99m}Tc-MDP solution the funnel volume was completed to 500µl with phosphate buffer, incubated for 30 min at 25°c.

3- The solution was then added on the surface of the same column, and then eluted with same buffer.

4- The fraction volume 2ml were collected with flow rate of 12ml/h.

5- The activity of each fraction was measured with gamma counter.

6- The spectrum at wave length(200-300nm) of the same fractions collected in step (4) were measured.

The radioactivity of each fraction was plotted vs. corresponding fraction number.The absorbance of each fraction was plotted vs. its corresponding fraction number.

The effect of pH, monovelant and divalent metal salts on the binding of ^{99m} Tc to HAS was studied using phosphate buffer , KCl, NaCl ,$CaCl_2$, and $MgCl_2$ (50- 250) were used.

RESULTS AND DISCUSSION :

The bound 99mTc-MDP-HSA complex was separated by gel filtration chromatography[6] using sephadex G-100 column (1×30cm). The bound ^{99m}Tc-MDP complex was observed in fractions 4-9,whereas the non bound was observed in the fractions 11-16. as shown in Fig (1).

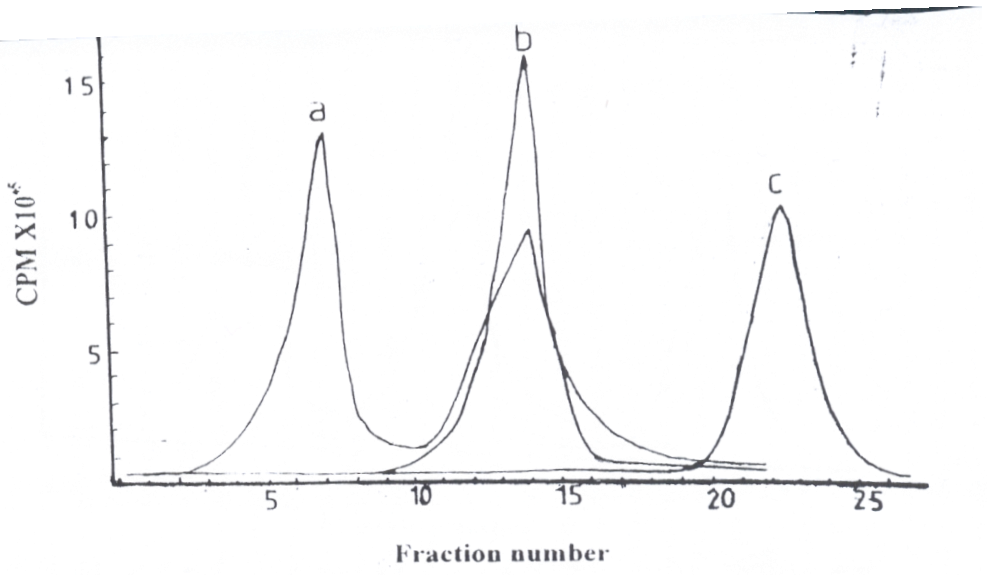

FIG 1 . SEPARATION OF TC, TC-MDP AND TC-MDP-HAS COMPLEX A-BOUND TC-MDP-HAS.(B) TC-MDP.(C) TC ONLY.

Fig (2) shows U.V spectra for four types of compounds MDP , ^{99m}Tc-MDP-HAS , ^{99m}Tc-MDP and HAS , ^{99m}Tc-MDP and MDP have the same spectra but differs from those of ^{99m}Tc-MDP-HSA and HSA , the last two compounds differ in the λ_{max} (278.8 , 253.5 , 219.4 nm for ^{99m}Tc-MDP-HSA and 278.1 , 253.1 , 215.1 nm for HSA). The results indicate that the bound ^{99m}Tc-MDP-HSA is new compound resulted from binding of ^{99m}Tc-MDP to HSA.

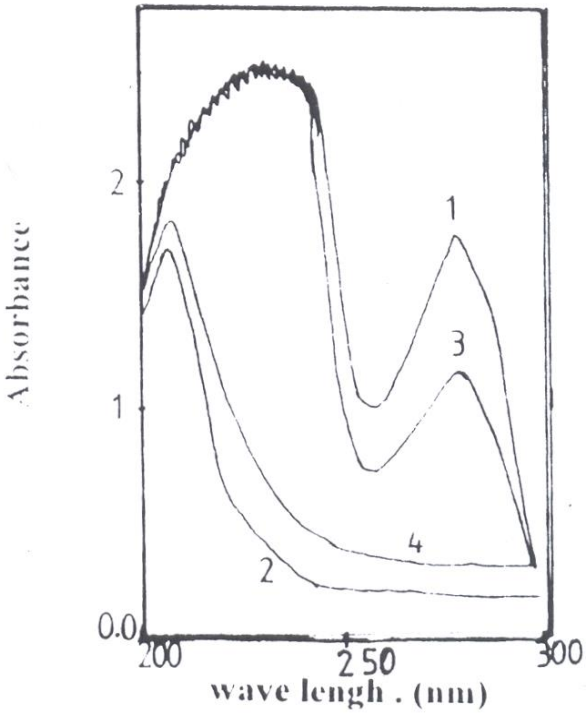

FIG 2 . UV-SPECTRA FOR : (1) HSA,(2)MDP,(3) TC-MDP-HAS (4) TC-MDP ONLY

These results have helped in the identification of fractions eluted from the column chromatography Fig (1) , the eluted bound ^{99m}Tc-MDP-HSA complex was observed in the earlier fractions (4 - 9) whereas non bound ^{99m}Tc-MDP appear in the fractions eluted later.
The most appropriate conditions of ^{99m}Tc-MDP binding to its binding HSA:-
The study of the binding of any ligand to its receptor or carrier necessitates the choice of the most appropriate conditions that lead to the maximum specific binding , hence the study of appropriate amount of HSA , the pH ,MDP concentration , salt concentrations , time and temperature on the extent of the binding of ^{99m}Tc-MDP to its binding site in HSA is quite necessary (table-2 & 3) .

Table 2 . Kd and B max values obtained from scatchard plot for the ^{99m}Tc-MDP binding with HSA at different temperature.

Temp. °c	K_d (nm)	B_{max}(pmol/mg protein)
20	15.5	3.81
30	15.9	4.8

37	17.0	5.89
50	17.5	6.88

Table 3 . Kd and B max values obtained from the double reciprocal plot for the ^{99m}Tc-MDP binding to purified HSA.

Temp. °c	K_d (×10-8M)	B_{max} (×10-8M)
20	5.14	1.50
30	6.72	1.59
37	8.84	1.76
50	11.16	1.92

The specific binding of ^{99m}Tc-MDP to HSA was enhanced with increasing amount of HSA , Fig (3). 100 μg -110 μg HSA gave the maximum value of the specific binding , this means that the depletion in the HSA concentration in the blood such as in the case of albuminanimia[7] effect the binding of ^{99m}Tc-MDP and lead to give various pattern of bone scanning depending on the concentration of HSA.

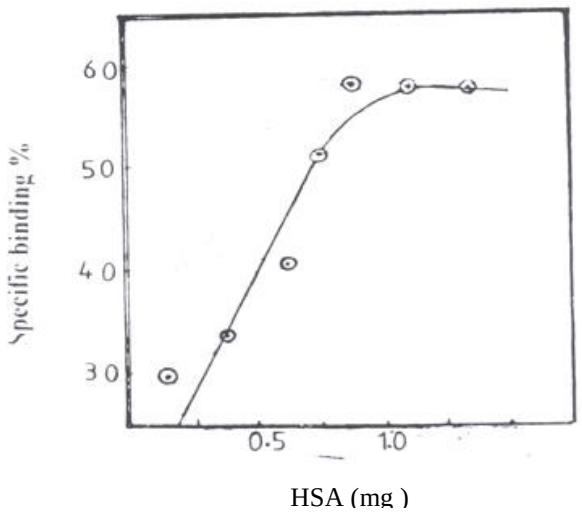

FIG 3 . THE EFFECT OF PROTEN CONTENT ON TC- MDP BIND TO HSA

Fig (4) show the effect of increasing pH from 7.0 to 8.0 on the binding of ^{99m}Tc-MDP to its binding sites of HSA , maximum value of the specific binding occurred at 7.4-7.6 (exhibits a narrow pH-optimum) , the results indicated that the binding was pH-dependent and the shift in the pH of environment may affect the properties of the HSA involved in binding , this effect includes the induction of protonation-deprotonation process occurring within the ionizable groups of the amino acids present in the binding domain of these macro molecules[8] , so protonation-deprotonation reaction are important in ^{99m}Tc-MDP interaction with its binding HSA and the ionic or electrostatic interactions may take part in stabilization of the complex[9]. Our results are similar in case of optimum pH to that obtained by Garnett et. al.[10] .

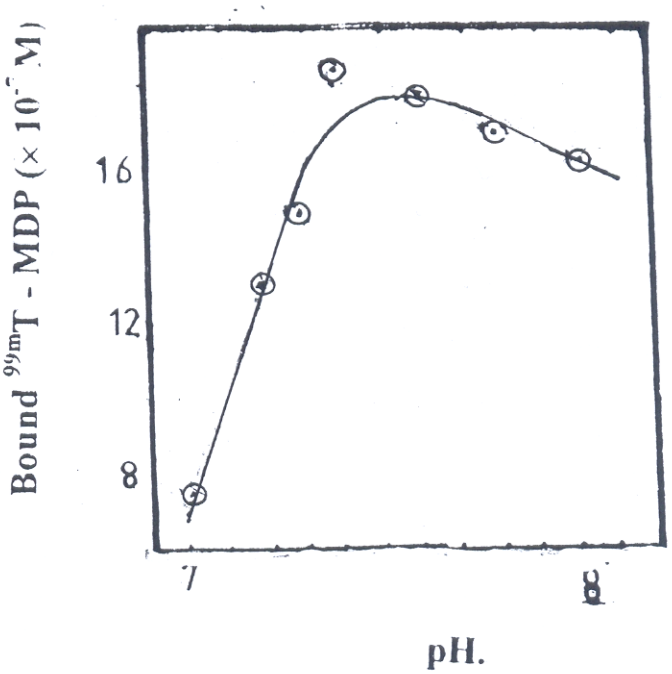

FIG 4 . PH DEPENDENCY OF TC- MDP BINDING TO PURIFIED HSA

Fig (5) show the effect of increasing amount of MDP on the binding of ^{99m}Tc-MDP to HSA , the total binding was increased to reach maximum bound at MDP concentration at 2.8×10^{-6} M-MDP and 2.2×10^{-7} M-$SnCl_2$ as reducing agent ; the increasing of MDP and $SnCl_2$ concentrations may leads to a decrease in the total binding , these may be due to the dissociation of ^{99m}Tc-MDP complex. Our results are nearly similar to that obtained by Poser et. al.[11].

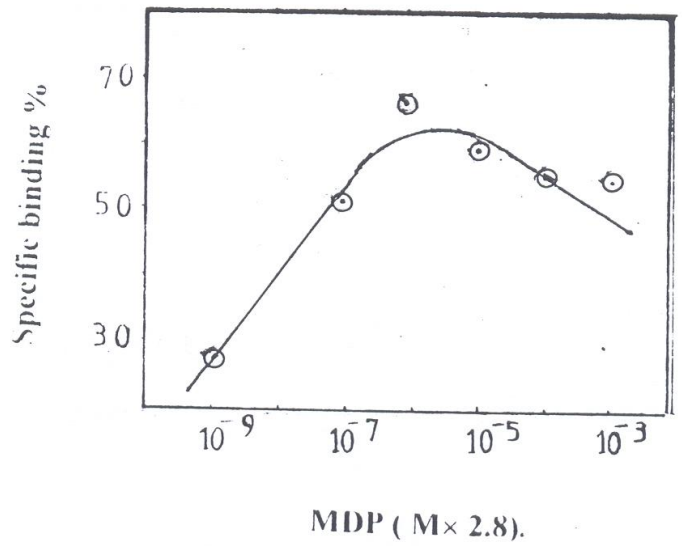

FIG 5 . BINDING OF INCREASING CONCENTRATION OF TC- MDP TO HSA

When monovalent salts (NaCl and KCl) and divalent salts ($CaCl_2$ and $MgCl_2$) were added with different concentration (50-250 mM) to the reaction mixture the results obtained indicated no significant effect on the ^{99m}Tc-MDP binding with HSA , as in Fig {6 (A and B)} show small decrease in the specific binding results from the effect of NaCl and KCl salts on the binding of ^{99m}Tc-MDP with HSA. Fig {6 (C and D)} show the effect of divalent salts ($MgCl_2$ and $CaCl_2$) on the specific binding , these results may be due to the interaction between ^{99m}Tc-MDP and the cations of the salt , then equilibrium was reached near 120 mM. The curves of Fig {6 (A,B,C and D)} indicate that a small amount of salt cause a high specific binding of ^{99m}Tc-MDP to HSA , any further increasing in salt concentration more than 50mM cause instability of the ^{99m}Tc-MDP-HSA complex , the reason may be due to the electrostatic interactions. Indeed if hydrophobic interactions were the force which stabilizes ^{99m}Tc-MDP-HSA , then increasing ionic strength would be expected to facilitate ^{99m}Tc-MDP-HSA interactions.

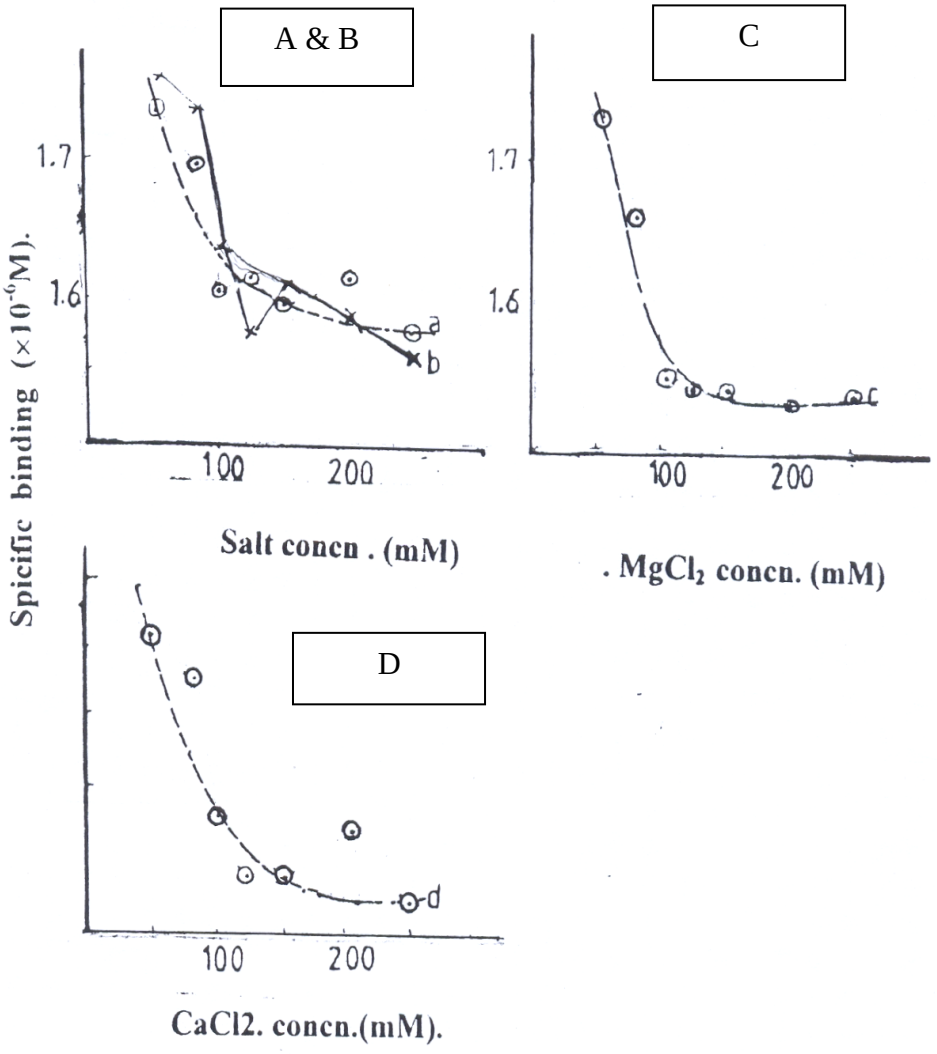

FIG 6 . THE EFFECT OF SALT CONCN. ON THE TC-MDP BINDING TO HAS USING : (A)NACL,(B)KCL,(C)MGCL2 ,(D)CACL2

In general , the mechanism by which these salts dissociate protein-protein complex is not completely clear , one hypothesis assumes that salts may alter the nature of the hydrophobic forces controlling the stabilization of protein-protein complex formed[12] . The high concentrations of the salts tend to destabilize the complexes as a result of their interaction with water molecules leading to diminution of protein-protein interaction and reversible denaturation of the protein[13,14] .

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