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*Original Article*

# Manganese and Iron Binding to Human Transferrin

Ali Asghar Moshtaghie[a*], Akbar Badii[a], Mohammad Fazilati[b], Taghi Hassanzadeh[a] and Hadi Kharazi[a]

[a]*Department of Clinical Biochemistry, School of Medicine, Razi University, Kermanshah, Iran.* [b]*Department of Food Sciences, School of Agricalture, Isfahan University of Technology, Isfahan, Iran.*

**Abstract**

The characteristics of manganese and iron binding to human apotransferrin (apo-tf) have been investigated and compared in this study. Both metal ions were taken up by human apo-tf and formed complexes, with the maximum absorbances observed at 410 and 340 nm for manganese-transferrin (Mn-tf) and 465 nm for iron-transferrin (Fe-tf). Addition of manganese (1.5 µg/ml) to the reaction mixture containing iron and apo-tf, reduced Fe binding to apo-tf by 20 percent, in comparison to the control sample. The binding of both metals to apo-tf appears to be time and pH dependent processes.

Using the equilibrium dialysis technique, the binding constant of manganese to apo-tf was also determined. The binding constatnt of Mn to apo-tf was calculated, using the Scatchard plot analysis. The calculated Ka was $3.1 \times 10^9$ $M^{-1}$.

The binding of manganese and iron to human apo-tf has been discussed and compared in this work, using different biochemical techniques.

**Keywords:** Manganese; Iron; Transferrin; Binding activity.

## Introduction

The trace element manganese (Mn) is essential for normal development and body functions of animal life (1). Mn is a required co-factor for many enzymes throughout the body. Mn is a required co-factor for arginase (2), which is responsible for urea production in the liver, superoxide desmutase, which is critical in the prevention of cellular oxidative stress, and pyruvate carboxylase, an essential enzyme in gluconeogenesis (3).

Thermodynamic modeling of Mn (II) in serum suggests that Mn exists in several forms, including an albumin-bound form, as a hydrated ion, and in complex with bicarbonate, citrate and other small molecular weight ligands (4). Similar modeling of Mn (III) in serum suggests that it is almost 100% bound to transferrein (5, 6). Mn (II) may be oxidized to Mn (III), which is rather reactive and more toxic than Mn (II). Mn (III) rapidly associates with transferrin to from a stable complex (7, 8).

Manganese toxicities have been reported through occupational exposure, which could lead to the occurrence of pathophysiological disorders. Neurotoxicity due to the inhalation of airborne Mn, has been reported in miners working in manganese oxide mines, workers in dry cell battery factories and welders (9).

The exact mechanism by which manganese produces neurotoxicity is still a matter of speculation. It has been reported that manganese

* Corresponding author:
E-mail: Moshtaghie@pharm.Mul.ac.ir

neurotoxicity may be associated with its interaction with other essential trace elements including iron (10), zinc and copper (11). Studies have indicated that chronic exposure to manganese appears to be associated with an altered blood iron (Fe) concentration, due to Mn-Fe interaction at certain iron containing proteins (12).

As manganese binds to plasma transferrin (tf), transport of manganese-transferrin (Mn-tf) complex into brain has been suggested to rely on a transferrin receptor (TFR), which competes with iron-transferrin (13) to enter the cells. Transferrin, the metal-combining globulin of blood plasma, possesses two metal binding sites (14) and binds to a variety of metals including chromium (15), cobalt (16), cadmium (17), zinc (18), indium (19, 20) etc.

With regards to the interference of manganese with iron binding to trasnferrin in those with manganese overload, the present study was established in order to conduct a series of methods, to investigate and compare binding characterstics of manganese and iron to transferrin.

## Experimental

### Materials

Human apo-tf iron free was purchased from Sigma Chemical Company, and further purified. The purification process was as follow (21): human apo-tf (5 mg/ml) was dissolved in Earle's medium, placed in prewashed visking sacs, and dialyzed twice against 100 volume of 50 mM acetate buffer, pH 5.2, first for 6 h and then for a further16 h. Protein solutions were then successively dialyzed against 100 volume of 0.15 mM NaCl, 0.02 M $NaHCO_3$ in 0.15 M NaCl, and finally Earle's medium (pH 7.4). The homogeneity of the prepared apo-tf was checked by SDS-polyacrylamide gel electrophoresis, as reported elsewhere (21). No aggregation was noted in the solution, as checked at 280 nm.

### Methods

*Preparation of iron and manganese citrate complex*

Separate stock standard solutions of $FeCl_3$. $6H_2O$ (3.0 mM) and/or $MnCl_2$. $4H_2O$ (3.0 mM), were prepared in deionized water and mixed with an equal volume of 60.0 mM citric acid. The solutions were adjusted to pH 7.4 with 1 M NaOH and made up to a final concentration of 1.5 mM iron and manganese.

Freshly, 50 ml of each stock standard solution was prepared in the beginning of each series of experiments.

*Spectrophotometeric titration technique of metal binding to apo-tf*

The binding of iron and/or manganese to apo-tf was carried out using a Perkin-Elmer UV/visible spectrophotometer (Model 5515), at room temperature ($22\pm1$ °C).

Approximately, 1.0 ml (5 mg) of the prepared tf in Earle's medium was added to a standard 1-Cm Pre-acid washed glass cuvet. Aliquots (1-100 µl) of 1.5 mM metal ion as the citrate complex, were added to separate cuvets. The cuvets were covered with parafilm, mixed thoroughly by vortexing, and left for 2 h at room temperature.

The absorbance of samples prepared were individually measured at wavelengths of 465 nm for Fe-tf and 410 nm for Mn-tf, respectively. The same conentration of apo-tf was used as a blank. Throughout this study, all the glasswares were soaked overnight in 10% nitric acid and then thoroughly rinsed with distilled and deionized water, in order to minimize iron and manganese contamination. Plasticwares were individually perwashed with 10 mM EDTA, followed by three washes of distilled and deionized water.

*The equilibrium dialysis technique*

The binding of manganese and iron to human serum apo-tf was also investigated, using the equilibrium dialysis technique (22) at room temperature (20-22 °C) in a chamber gassed with a 95% $O_2$ / 5% $CO_2$ mixture. A solution of apo-tf (5 mg/ml) was placed in a dialysis sac, open to the atmosphere, which was immersed in a plastic vessel containing 1600 ml of a pH 7.4 Earle's medium. 300 µl aliquots of (1.5 mM) iron as ferric-citrate or (1.5 mM) manganese as manganese chloride solutions were added at set time intervals to the buffer solution (1600 ml) surrounding the dialysis sac, with the aid of a magnetic stirrer and kept at the required pH by

bubbling through the $O_2/CO_2$ mixture. After 24 h, a 100 µl of sample was taken from the inside and another 100 µl of sample from the outside of the dialysis sac and analysed for iron and manganese concentration. Iron concentration was determined using phenanthrolin as chromogen (23).

Manganese level was determined, using a flameless Perkin-Elmer model 3030 atomic absorption spectrophotometer. The binding constant of manganese to apo-tf was calculated, using the Scatchard plot analysis (24).

## Results

*Spectrophotomeric titration studies*

Binding of iron and manganese to the prepared purified apo-tf was studied by spectrophotometric and equilibrium dialysis techniques.

The first experiment carried out was the measurement of the absorption spectrum of iron-trasnferrin complex. This was performed at room temperature, as mentioned in the experimental section. The absorption spectrum of the iron-transferrin complex shows a broad peak in the 465 nm region. The same experiment was conducted for the determination of the absorption spectrum of manganese-transferrin complex. The absorption spectrum of the manganese-transferrin complex shows two peaks at 410 and 340 nm (Figure 1 and 2).

The next series of experiments were undertaken to study the binding of manganese and/or iron to apo-tf. Initially, increasing amounts of iron (0.84-8.4 µg/ml) were added to apo-tf solutions (5 mg/ml), present with the Earle's medium (pH 7.4) and incubated at room temperature for 2 h. The absorbance at 465 nm was indicating the binding of iron to apo-tf (Figure 3).

When the apo-tf was titrated with iron in the presence of 1.5 µg/ml of manganese as the complex with citric acid, Fe binding to apo-tf was reduced by 20 percent (Figure 3).

In order to investigate the effect of pH on iron and/or manganese binding activities of ap-trasnferrin, to a series of plastic tubes containing buffer (2 ml), at different pH values within the range of 3-10 and apo-tf (5 mg/ml), aliquots of Fe-citrate and Mn-citrate were added. The tubes were treated as mentioned above. The maxiumum binding of Fe to apo-tf was observed at pH 7.0, whereas the binding of Mn to apo-tf took place at pH 7.5 (Figure 4).

*Equilibrium-dialysis studies*

In order to confirm the binding of iron and/or Mn to apo-tf, equilibrium dialysis techniques were used next.

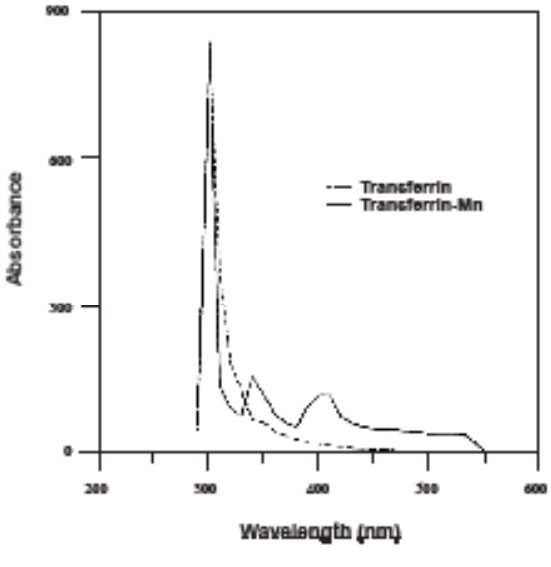


**Figure 1.** Absorption spectrum of Mn-transferrin (Apo-tf, Mn-tf). APO-tf was titrated with manganese as a complax with citric acid. The maximum wavelength was obtained as mentioned in the experimental section.

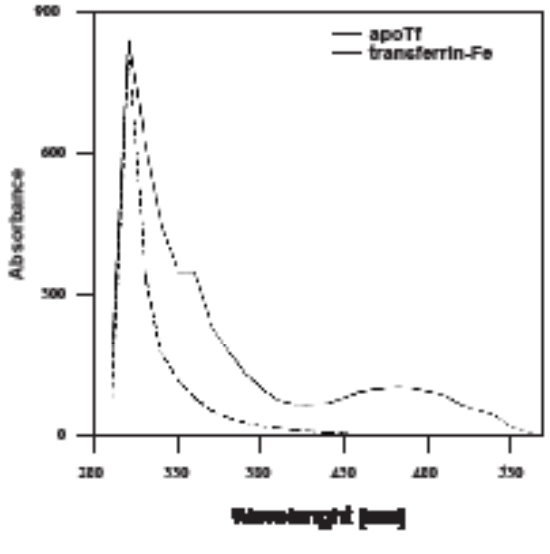


**Figure 2.** Absorption spectrum of Fe-transferrin (Apo-tf, Fe-tf). APO-tf was titrated with iron as a complex with citric acid. The maximum wavelength was obtained as mentioned in the experimental section.

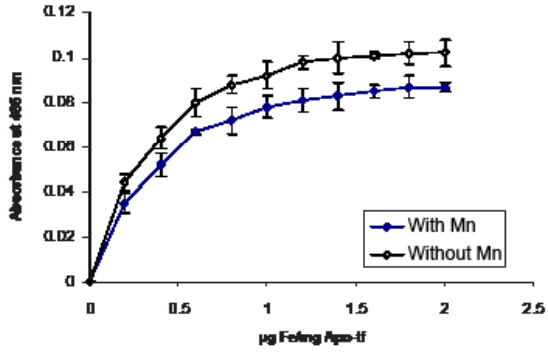


**Figure 3.** Spectrophotometric titration of human apo-tf with iron in Earle's medium at pH 7.4 and the effect of Manganese. Each point is the mean±SD of three separate experiments.

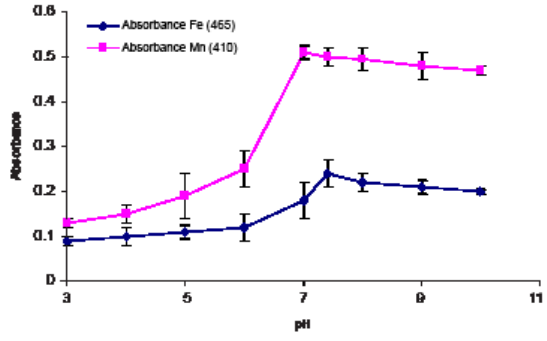


**Figure 4.** Effect of pH on iron and manganese binding to apo-tf. Each point is the mean±SD of three separate experiments.

To achieve this goal, a dialysis sac containing 20 ml of 5 mg/ml apo-trasnferrin was placed in the dialysis chamber, as described in the experimental section. Aliquots of ferric cirate and/or manganes-cirate were added and the binding of iron and/or manganese to apo-tf studied as desricbed earlier. Results obtained are presented in Figures 5 and 6. Figure 5 shows the binding of iron to apo-tf and the effect of 200 µg/l of manganese on the binding activity, whereas Figure 6 shows the binding of manganese to apo-tf.

Reduction in iron uptake by apo-tf was seen when manganese was added to the outside of dialysis sac.

*Calculation of binding constant*

In order to find out the association constant of manganese to apo-tf, the Scatchard plot analysis technique was used. For this purpose, this data shown in figure 6 was used and the binding constant for manganese binding to apo-tf calculated as follows: free manganese ion concentration from outside the sac was subtracted from the total Mn ion concentration within the sac. This was considered as the bound fraction. The approximate calculated binding constant for manganese apo-tf complex was $3.1\times10^{9}$ $M^{-1}$ (Figure 7). The calculated binding constant values of Mn to apo-tf are presented in Table 1 and the Scatchard plot was constructed using the bound/free values versus the bound values.

Finally, the effect of varying concentrations of citric acid on manganese binding to apo-tf was investigated and the results obtained are presented in Figure 8.

It was shown that the binding of manganese

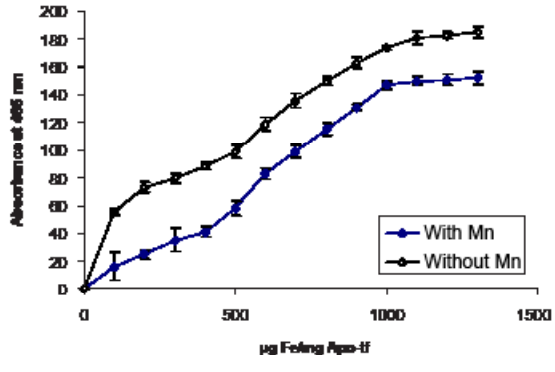


**Figure 5.** The equilibrium dialysis study, showing the binding of iron to human apo-tf in Earle's medium at pH 7.4 and the effect of manganese. Each point is the mean±SD of three separate experiments.

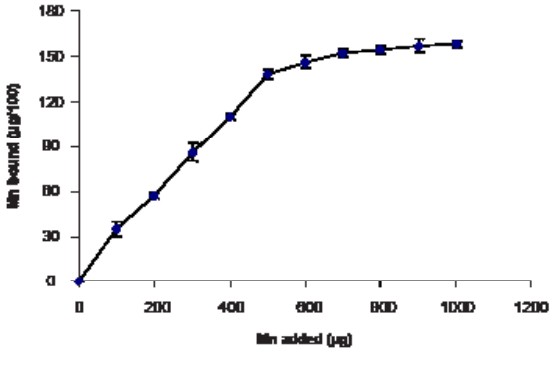

**Figure 6.** The equilibrium dialysis study, showing the binding of manganese to human apo-tf in Earle's medium at pH 7.4. Each point is the mean±SD of three separate experiments.

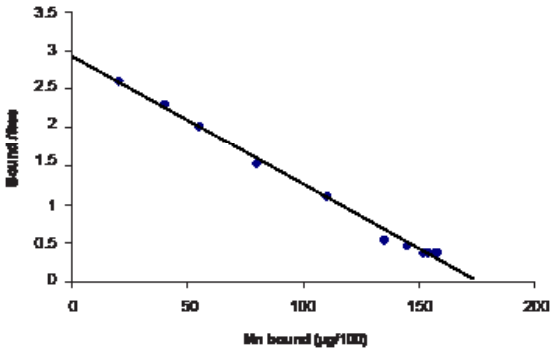

**Figure 7.** A typical Scatchard plot for the binding of manganese to apo-tf.

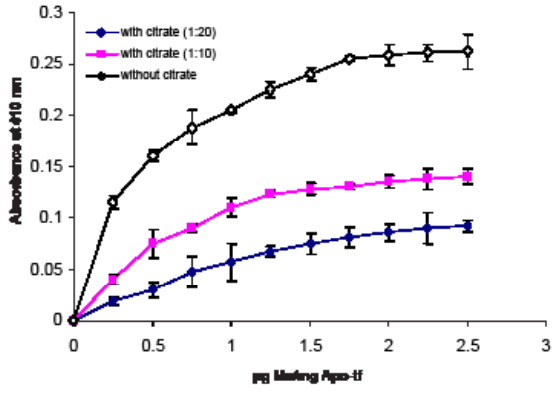


**Figure 8.** The effect of citrate on the manganese binding to apo-tf.

to apo-tf decreased with increasing the concentration of citric acid. This confirmed that citric acid is not necessary for binding of manganese to apo-tf.

## Discussion

Transferrin has been proposed as the major manganese binding ligand in the plasma. Earlier reports from this laboratory showed that cerroluplasmine (Ferroxidase) may play an important role in the binding of manganese to apo-tf (25).

Absorption spectra obtained in this study, indicated the presence of two peaks at 410 and 340 nm for the iron-transferrin complex (Figures 1 and 2), whereas earlier reports from other laboratories suggested two peaks at 430 and 330 nm for the manganese-trasferrin complex and the same spectrum for the iron-transferrin complex (25). The discrepancies in these findings could be due to the buffer composition and other experimental conditions.

**Table 1.** The calculated values for the binding constant of manganese to apo-tf

| Bound/free | Mn bound µg/100 |
|---|---|
| 2.68 | 20 |
| 2.38 | 40 |
| 2.01 | 55 |
| 1.53 | 80 |
| 1.18 | 110 |
| 0.95 | 133 |
| 0.47 | 143 |
| 0.36 | 152 |
| 0.36 | 154 |
| 0.37 | 157 |
| 0.37 | 158 |

Mn (III) can effectively compete with ferric ion for binding to transferrin in the blood circulation, and thus it is generally accepted that Mn (III) is transported extracellulary as a transferrin complex. Fe-transferrin complex binds to transferrin-receptors at the cell membranes and iron releases into the cells for further biochemical pathways (27). It has been reported that manganese-transferrin complex may also follow the same procedure, undergoing internalization within endosomal vesicles (28). The endosomal vesicle undergoes acidification, presumably releasing Mn (III) from the transferrin-complex (13), resulting in the reduction in iron uptake by the cell.

Although citric acid was necessary for iron binding to apo-tf, the binding of manganese to apo-tf dose not require citric acid. These discrepancies might be due to the chemical structure of manganese. The effect of different pH values on the binding activity of manganese to apo-tf is in agreement with this suggestion. Data presented here show that the maximum binding occurs at an alkaline pH of 7.5 (Figure 4), rather than an acidic pH. In fact, this binding activity of manganese seems to be dependent on the pH of the solutions. Data obtained from both the spectrophotomeric titration and equilibrium dialysis studies conducted confirm the binding of manganese to human apo-tf, with a subsequent reduction in iron uptake. This might

lead to the accumulation of manganese in those exposed to this metal and could be linked to the development of severe neurological disorders or the appearance of anemia.

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