# Comparative Binding Studies of Titanium and Iron to Human Serum Transferrin

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## ABSTRACT

It is believed that titanium may interfere with iron metabolism in terms of absorption, transportation, utilization and storage in the cells. The present investigation was designed to study and compare the binding of iron and titanium to human serum Apo transferrin (apo-htf). Present results show that Ti(III) ions bind to transferrin and form a new complex and the calculated apparent association constant is $1.03 \times 10^7$ $M^{-1}$ based on the Equilibrium dialysis technique. The binding of both metals to apo-htf appears to be pH dependent, changing with both increase and decrease pH. Titration studies demonstrate that transferrin specifically binds two moles Ti(IV) as complex with citrate per mol protein. spectrophotometery technique indicated that Ti(IV) ions cause a 13% reduction in binding of Fe(III) to transferrin. These results show that titanium competes with iron in binding to apo-htf. Although, the binding sites for these two ions seem to be similar, the binding of iron to apo-htf seems tighter.

**Key words:** Titanium, transferrin, iron, binding constant

## INTRODUCTION

Serum transferrin is a single-chain glycoprotein (MW 80 kDa) which transports Fe (III) ions to cells where it is taken up via receptors-mediated endocytosis (Zhong *et al.*, 2002; Sun *et al.*, 1999; Byrne *et al.*, 2010). The polypeptide chain is folded into two globular lobes, each lobe contains a distorted octahedral Fe(III) binding site consisting of two Tyrosine, one Histidine and one Aspartic acid and a carbonate anion (the synergistic anion in N-lob and C-lobe derived from buffer) (Sun *et al.*, 1999; Guo *et al.*, 2000a; Harris and Messori, 2002; Gomme *et al.*, 2005). Transferrin concentrations is high (2-4 mg $mL^{-1}$) in vertebrate sera (Jabeen and Afzal, 2010) but it is only about 30% saturated with iron in normal condition. Therefore, it has substantial binding capacity for the other tripositve, tetrapositive and bipositive metal ions that enter the blood (Zhong *et al.*, 2002; Sun *et al.*, 1999; Harris and Messori, 2002). Thus, transferrin is an important serum transport agent for metal ions therapeutic, diagnostic or toxic including $Al^{3+}$ (Moshtaghie and Skillen, 1986; Moshtaghie *et al.*, 1992; Fatemi *et al.*, 1991), $Ga^{3+}$ (Harris and Pecararo, 1983), $In^{3+}$ (Moshtaghi and Ghaffari, 2003; Biver *et al.*, 2008) and $Bi^{3+}$ (Ge and Sun, 2007).

Titanium is the ninth most abundant element on Earth and the second most abundant transition metal, yet is often thought to be inert in biology and environment (Messori *et al.*, 1999).

Consumption of this metal and number of biochemical applications of Ti has recently increased. Some titanium compounds have been shown to exhibit high antitumor activates against a wide range of murine and human tumors with less toxic side effects than cis platin (Guo *et al.*, 2000a; Melendez, 2002; Desoize, 2004). Titanium (IV) citrate has been tested against tumors in rats (Collins *et al.*, 2005). In a research, rats with Jensen`s sarcoma were treated with injections of Ti (IV) citrate. The survival rates for titanium-treated group were more than for control group (Collins *et al.*, 2005; Schwietert and McCue, 1999). In addition, some Ti (IV) complexes show anti bacterial activity and $^{45}$Ti is potentially useful as a pharmaceutical (Guo *et al.*, 2000a).

The ability of transferrin to interact with several metal ions and the chemical similarities between titanium and iron, prompted us to study the interaction of human serum Apo-transferrin with titanium (IV) complex by the electronic absorption and equilibrium dialysis technique. The interaction of iron (III) and titanium (IV) ions with each other on the binding to hTf was also investigated.

## MATERIALS AND METHODS

**Materials and sample preparation:** Apo-htf was purchased from Sigma Chemical Co. (catalog No. T0519). The concentration of apo-htf was determined by using its molecular weight 80000 g $M^{-1}$ and also spetrophotometery from the absorbance at 278 nm using an extinction coefficient of 93000 $M^{-1}$ $cm^{-1}$ (Du *et al.*, 2008). All other chemicals used in this project were analytical grade and purchased from Sigma Chemical Co and Merck.

All glassware including cuvettes were routinely soaked in a solution of concentrated nitric acid (1 N) and then rinsed with distilled and deionized water before use to minimize metal ions contamination. For preparation of Titanium (IV) citrate, titanium (III) chloride was mixed with a 1.2 fold excess of sodium citrate at pH 3. The prepared solution was then exposure to air caused quantitative oxidation of titanium (III) citrate to colorless titanium (IV) citrate (Messori *et al.*, 1999).

Uv-Visible spectroscopy: A computer controlled Shimadzu multispec-150 spectrometer was used together with 1 cm path length cuvettes maintained at room temperature.

Apo htf solutions were prepared by diluting aliquots of stock apo-htf solutions to ca. $1 \times 10^{-5}$ M with 50 mM Tris buffer pH 7.4. Immediately before the titration, sufficient $NaHCO_3$ was added to produce a 20 mM $HCO_3^-$ concentration (Harris and Pecararo, 1983). For spectrophotometeric experiments, 200 µL of an apo-htf solution (100 µM) was added to the sample and reference tube. Aliquots of 1.2 mM Ti (IV) citrate or Fe (III) citrate complex was then added. To achieve titration, aliquots of Ti or iron ions (5-50 µL) were added to sample tube, while equal volumes of distilled, deionized water were added to the reference tube. The solutions were then mixed; the tubes were capped and left for up to 120 min at room temperature.

**Equilibrium dialysis technique for metal binding to Apo-htf:** The binding of titanium and iron to human apo-htf was also investigated using equilibrium dialysis at 2-4°C. A solution of apo-htf (20 µM) was placed in a dialysis sac open to the atmosphere, which was immersed in a glass vessel containing 1000 mL, Tris-HCl buffer 50 mM buffer, pH 7.4, 0.02 M $NaHCO_3$ (Moshtaghie and Badii, 1996), 100 µL of iron (60 mM) as ferric-citrate or titanium (60 mM) as titanium-citrate. Solutions were prepared and aliquots added at time intervals to the buffer surrounding the dialysis sac with the aid of a magnetic stirrer. Samples (100 µL) were taken from inside and outside of the dialysis sac and analyzed for titanium iron. Iron and titanium concentrations were determined using atomic absorption spectrometery. The binding constant of iron or titanium to apo-htf was calculated using Scatchard equation (i.e., $[B]/[F] = -1/K[B] +$

n[E]$_T$/K$_S$) and plot of [B]/[F] against [B]. Where, [B] is the concentration of bound ion or titanium to protein, [F] is the concentration of free ions, n is the number of binding site, [E]$_T$ is the concentration of total protein and K$_S$ is the dissociation constant (Scatchard, 1984).

**The effect of pH on the binding of iron and titanium to apo-hTf:** To a series of pre-acid washed test tubes containing 200 µL apo-htf (100 µM), 40 µL of iron solution (1.2 mM) or titanium (1.2 mM) as complex with citric acid were added and the volumes made up to 2 mL by Tris-HCl buffer within the pH range 2 to 12. The solutions were mixed and incubated for 120 min at room temperature. The absorbance of the test tubes was measured by spectrophotometer.

**Metal ions interactions with each other for binding to apo-htf:** The binding of iron to apo-htf and the effect of titanium on iron binding activity and vice versa, were also investigated by spectrophotometric titration. Firstly, a solution of apo-htf (10 µM) in 50 mM Tris-HCl containing 20 mM NaHCO$_3$ at pH 7.4 was equilibrated with 20 µM of Ti(IV) citrate and then Fe$^{3+}$ solution was added in 5-60 µL aliquots to the sample tube and equal volumes of distilled, deionized water added to the reference tube. Spectra were taken 2 h after each addition.

Secondly, a similar titration was carried out for Fe-transferrin (2:1 molar ratio) using Ti(IV) citrate solution as a titrant. This study was carried out in laboratory of Biochemistry, School of Pharmacy, Isfahan University of Medical Sciences at 2005.

## RESULTS

**UV difference spectra:** Reaction of human serum transferrin with Ti(IV)-citrate or Fe(III)-citrate and displacement of Ti(IV) were studied by UV-Vis spectroscopy under physiologically important conditions. The spectra characterization of Ti(IV)-transferrin and Fe(III) transferrin complexes was carried out by scanning within the range of 200-600 nm. The binding of Ti(V) to human serum transferrin leads to the production of two new absorbance bands at 242 and 295 nm which is similar to reactions of other metal ions with apo-htf and a new broad band at 320 nm in the difference spectrum (Fig. 1) (Zhong *et al.*, 2002; Sun *et al.*, 1999). The broad band near 321 nm
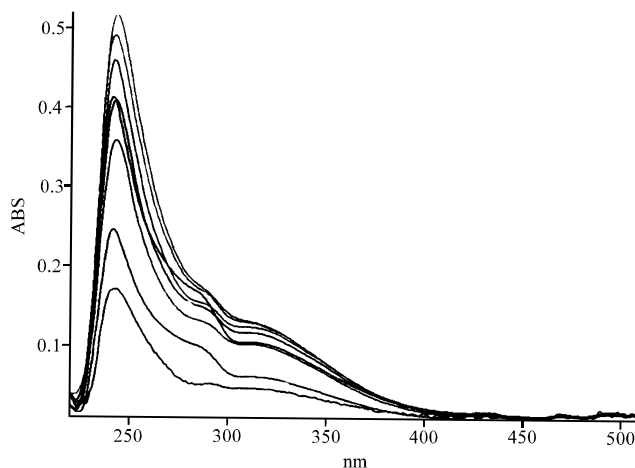


Fig. 1: Different spectra for titration of apotrasferrin (1×10$^5$ M) with Ti(IV)-citrate in 50 mM Tris-HCl buffer, 20 mM NaHCO$_3$, pH 7.4 at 298 K. The molar ratios of Ti to transferrin from bottom to top are: 0.5, 0.75, 1.25, 1.75, 2, 2.5 and 3
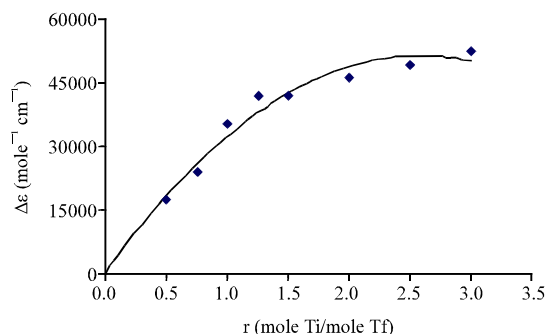
Fig. 2: Titration curve for additions of Ti(IV)-citrate to apotrnsferrin; $\Delta\varepsilon$ equals the absorbance at 240 nm dividing by the apotransferrin concentration, other conditions as in Fig. 1
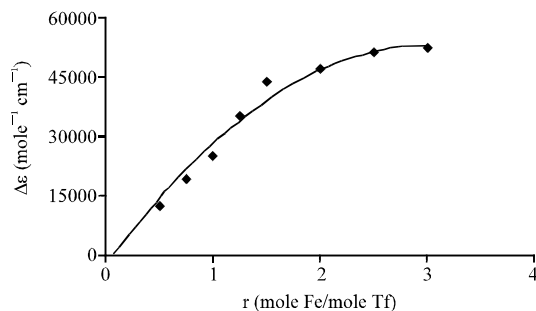


Fig. 3: Titration curve for additions of Fe(III)-citrate to apotrnsferrin; $\Delta\varepsilon$ equals the absorbance at 240 nm dividing by the apotransferrin concentration, other conditions as in Fig. 1

exhibits a similar pattern as those bands due to Ligand to Metal Charge Transfer (LMCT) transitions of metal ions with phenolate ligands, thus this should be attributed to the LMCT transition of Ti(IV) with phenolate ligands (Sun *et al.*, 1999).

A titration to investigate the stoichiometry of Ti (IV) and Fe (III) was performed at pH 7.4 in 50 mM Tris-HCl buffers, 20 mM $NaHCO_3$, 298 K. Apo human serum transferrin was titrated with a solution of Ti (IV)-citrate and different uv spectra recorded after the addition of each aliquots of titanium as shown in Fig. 1. The absorbencies data have been converted to absorptivities, $\Delta\varepsilon$, by dividing the maximum absorbance at the 240 nm by the total transferrin concentration. Titration curve is prepared by plotting $\Delta\varepsilon$ vs. r, the ratio of the Ti(IV) concentration to the total transferrin concentration as shown in Fig. 2. The shape of the curve indicates that transferrin binds two moles Titanium (IV) per mol protein, in good agreement with the hypothesis that titanium (IV) ions occupy the empty metal binding sites of transferrin.

A similar titration was performed for Fe (III) citrate. Values of $\Delta\varepsilon$ were calculated from the absorbance maximum at 240 nm and plotted as a function of r the ratio of total iron concentration to transferrin concentration as shown in Fig. 3. Using the Fig. 3, it was found that each mole of apo-htf was saturated with 2 mole of Fe (III).

**pH dependent properties:** The effect of different pH within the range of 2-12 on the stability of iron and titanium apo-htf was investigated (Fig. 4a and b). The results indicate that $Ti_2Tf$ is stable within the pH range 6-9 whereas $Fe_2Tf$ is more stable at high pH (6-11) which is in line with previous studies (Guo *et al.*, 2000a).
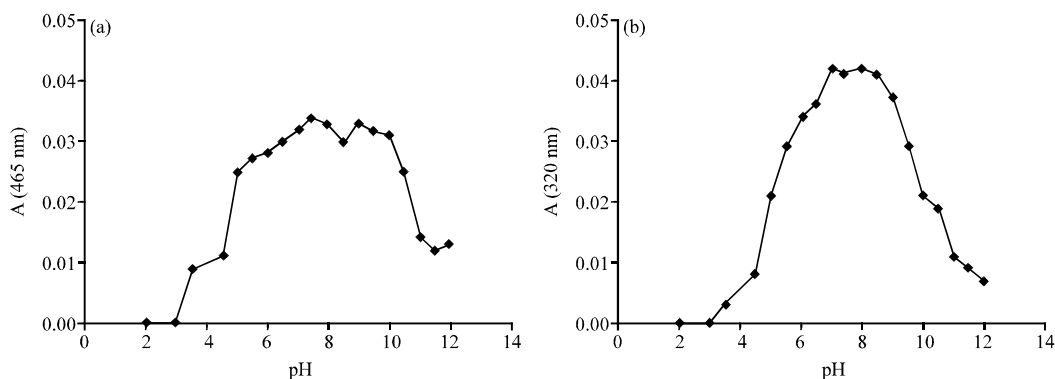
Fig. 4: Comparative study of Effect of different pH (2 to 12) on (a) iron binding and (b) titanium binding to apo-tf. Each point is the mean of three separate experiments. ([Apo-htf] = $1 \times 10^5$ M, [Fe$^{+8}$] = [In$^{+8}$] = 24 μM, at 298 K
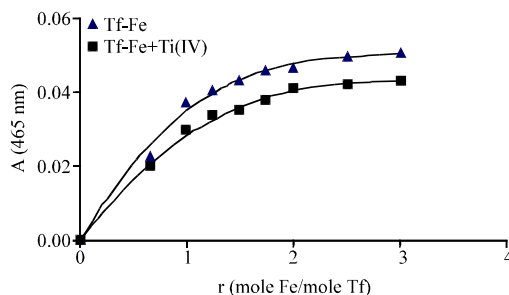


Fig. 5: Titration curve for addition of different concentrations of Fe(III)-citrate(0-30 μm) to apotransferrin in the absence (▲) and presence (■) of titanium (30 μm). [Apo-htf] = 10 μm, in 50 mM Tris-HCl buffer, 20 mM NaHCO$_3$, pH 7.4 at 298 K

**Metal interaction:** The Tf-Fe (III) absorption band in the visible region at 465 nm was used to monitor the competitive binding of Fe(III) and Ti(IV) to apo-htf. Based on the previously reported studies, the uv region was not used because the phenolic groups of tyrosine side chains and therefore similar absorbance changes occur in this region for both ions (Fatemi *et al.*, 1991).

First, Ti$_2$-hTf was titrated with 0-30 μM Fe-citrate (1:1.2) in 50 mM Tris-HCl, 20 mM NaHCO$_3$ buffer solution at pH 7.4. The absorbencies of test tubes were measured at 465 nm. The same experiment was also without titanium (Fig. 5). The results obtained suggest that Fe (III) may displace Ti (IV) from the apo-transferrin because the absorbencies of Tf-Fe(III) at 465 nm was reduced by approximately 13%.

In another experiment, Fe$_2$-htf (30 μM) was titrated with Ti (IV)-citrate in, the same buffer solution and the same temperature. The LMCT band for Fe$_2$-hTf at ca. 465 nm (Baker and Lindley, 1992) showed little decrease in intensity. The results obtained are presented in Fig. 6, Suggesting that iron binds stronger than titanium to apo transferrin. Therefore, both sets of results indicate that Ti (IV) can not displace Fe (III) completely but Fe (III) can easily replace Ti (IV).

**Equilibrium dialysis:** In order to obtain the binding constant of Ti (IV)-transferrin complex and also to compare the binding constant of titanium and iron to apo-htf, equilibrium dialysis technique
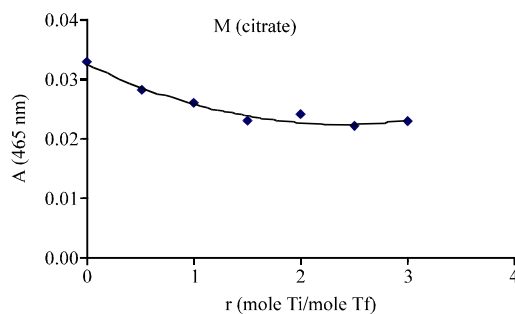
Fig. 6: Titration of apotransferrin with addition of Ti(IV)-citrate in the presence of Fe(III), conditions as in Fig. 1
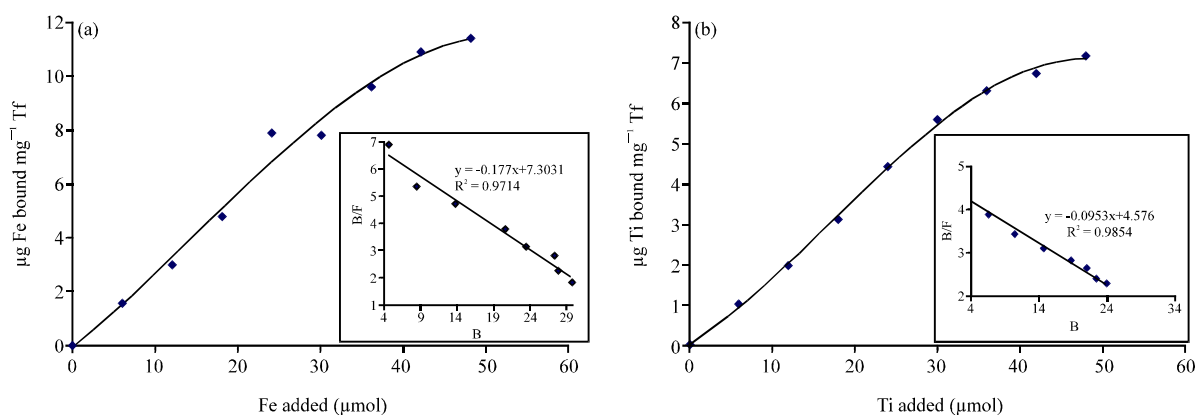


Fig. 7: The study binding of titanium and iron to apo-htf by equilibrium dialysis and Scatchard plot for the binding constant of (a) iron and (b) titanium to apo-htf. ([Apo-htf] = 20 μM pH 7.4, t = 4°C)

was used. Figure 7 shows the results of titration of titanium and iron by this technique. Using Scatchard plot analysis and the data in Fig. 7, the binding constant for titanium or iron binding to apo-htf was calculated. The approximate binding constant for titanium-transferrin complex, was $1.03 \times 10^7$ $M^{-1}$ and for iron-transferrin, it was $1.7 \times 10^7$ $M^{-1}$ (Fig. 7a, b).

## DISCUSSION

Human serum transferrin is known to act as a general ligand for many metal ions of different charges and sizes besides the physiological iron (III) ions (Messori *et al.*, 1999). Since, *in vivo* transferrin is largely present as the apo form, this protein is often involved in the distribution and metabolism of metal ions that are known to be either trace elements or pollutants (Messori *et al.*, 1999) or may be responsible for the transport of anti tumor complexes to tumor cells (Guo *et al.*, 2000a; Richardson *et al.*, 2009).

On the other hand, previous reports suggest that due to biochemical similarity of few cations with iron, they may interfere with iron metabolism and cause some disorders (Moshtaghie and Skillen, 1986; Punnonen *et al.*, 1994; Vidaud *et al.*, 2007).

In the present study, we first studied the binding of Ti (IV) to human apo transferrin which appeared uv difference spectrum (Fig. 1).

196

The changes in the uv spectrum of hTf reaction with Ti(IV) are very similar to those observed previously for the binding of other metal ions to the specific Fe(III) sites (Sun *et al.*, 1999; Guo *et al.*, 2000a). The two sharp new bands near 240 and 295 nm are typical of phenolat groups (π-π*transitions) generated by binding of metal ions to tyrosine residues in the specific iron binding sites. The third broad band centered near 320 nm, lies in the range typical of LMCT transitions of Ti(IV) with phenolat ligands (Sun *et al.*, 1999; Guo *et al.*, 2000b). The titration curve obtained by monitoring the absorbance change at 320nm gave a break near the ratio of Ti/hTf 2:1, suggesting that Ti(IV) binds to the specific metal binding sites in both the N lobe and C lobe of the protein and that two tyrosine were involved in the binding Ti(IV) in both lobes as is the case for Fe(III) (Sun *et al.*, 1999).

Our UV-Vis experiment showed that Ti2-hTf is stable over the pH range 6-9 whereas Fe2-Tf is more stable at high pH 6-11.$Ti_2Tf$ exhibits an unusual pH dependence profile; in fact, while this derivative is stable in the pH range 6-9, it breaks down above pH 9. Thus, while being considerably stable at low pH, it is relatively unstable at high pH. The lower stability of $Ti_2Tf$ at high pH compared to other metalotransferrins probably reflects the fact that transferrin is no longer able to prevent titanium(IV) hydroxide formation, as previously observed in the case of thallium and titanium transferrin (Messori *et al.*, 1999).

When, pH was decreased to acidic pH, release of these two ions from transferrin occurred. The release of titanium was more than iron. This might be due to the affinity of titanium to apo-htf which is less tight than iron.

The reduction in iron up take by titanium and vice versa and the competition of iron with titanium on the other hand suggest that these two metal ions may compete for the same binding site on the apo-htf. The same results were obtained when other trivalent elements were added to the apo-htf (Aisen, 1966; Aisen *et al.*, 1969; Moshtaghie *et al.*, 1992; Du *et al.*, 2008).

Using equilibrium dialysis technique and Scatchard plot, it was found that the binding constant for iron and titanium to apo-htf were 1.7×107 M$^{-1}$ and 1.03×107 M$^{-1}$, receptively. This study appears to be the first report of binding constant of titanium (IV) citrate to transferrin by equilibrium dialysis technique. Thus we can conclude that iron ions binds to apo-htf tightly more than 1.5 fold in compare to titanium ions. The difference between obtained binding constant for iron to apo-htf (Aisen, 1966) could be due to the discrepancies in the methods of binding and the factors such as composition of buffer. Overall, it seems that both iron and titanium bind to the same sites of transferrin. Thus titanium ions may interact with iron metabolism. More investigations should be carried out to elucidate the exact mechanism of these competitions particularly at the cellular levels.

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