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Evaluation of Chitosan As a Potential Medical Iron (III) Ion Adsorbent

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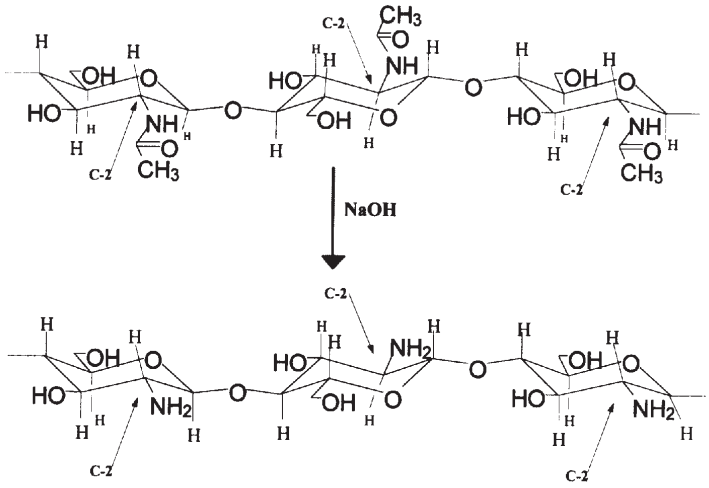
Abstract: Chitosan, a derivative of the natural polysaccharide chitin, is known for its biocompatibility and metal-binding capacity. The aim of this study was to combine these two properties of chitosan and investigate its potential as a new iron (III) ion (ferric ion) adsorbing agent. The development of a safe and orally active iron chelating agent is especially important for the treatment of thalassemics. In this study, the physicochemical parameters affecting the ability of chitosan flakes to adsorb iron (III) ions were studied by complexometric titration. The results showed that the iron (III) adsorption capacity of chitosan increases with the amount of chitosan, degree of deacetylation of chitosan, concentration of ferric ions in solution and with the pH of the medium. The amount of ferric ions that adsorb on the polymer increases with time until an equilibrium is reached between adsorbed iron (III) ions and those in solution. Preliminary *in vitro* results obtained in human blood serum indicate that chitosan is capable of adsorbing iron (III) ions in the body fluid medium and may be a suitable iron-adsorbing agent in biological systems.

Key Words: chitin, chitosan, iron-chelation, iron-adsorption, thalassemia.

Introduction

Chitin is a natural polysaccharide obtained from crab and shrimp shells or from similar sources. Chitosan is the deacetylated form of chitin:

The main advantage of chitosan over chitin is that it is soluble in dilute acid solutions through protonation of amine groups. Chitin and chitosan have a very diverse range of applications such as in the food, textile and



cosmetics industries in addition to environmental and biomedical applications (1,2). Chitin and especially chitosan have been used as bone and tooth filling material, drug conjugates, microparticulate controlled drug delivery systems and in enzyme immobilization (2,3). The ability of chitosan to chelate metal ions both in solution and in the solid state have been studied, suggesting the possibility of using this polymer in waste-water treatment for environmental purposes (2,3). However, no reports in the literature could be found on any biomedical application of the ion-chelation capacity of chitin or chitosan.

Iron overload in the human body may result in damage in the heart, liver and other organs. Iron overload is an especially important problem with thalassemia patients who have to undergo blood transfusions frequently. Beta-thalassemia, also called Cooley's anaemia, thalassemia major, Mediterranean Anemia or Hereditary Leptocytosis, is a type of blood disorder characterized by a deficiency of hemoglobin, the blood protein that transports oxygen to the tissues. Thalassemia is caused by genetically determined abnormalities in the synthesis of one or more the of polypeptide chains that make up the globin part of hemoglobin. Combined red blood cell transfusion and iron-chelation therapy remains the primary treatment of Cooley's anaemia. To prevent disability and death from transfusional iron overload, daily prolonged intravenous or subcutaneous infusions of the iron-chelating agent deferoxamine are needed. Deferoxamine B mesylate (Desferal), which is a very widely used iron-chelator was first introduced in 1962. Although chelation therapy with deferoxamine increased the life expectancy of thalassemics to more than forty years, this therapy is very demanding physically, psychologically and financially. Recently, an orally active iron-chelating agent, deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one, also known as L1, CP20 and DMHP), has been introduced. Clinical evaluation of this agent has provided evidence that deferiprone is effective in decreasing body iron to a tolerable level. However, some patients have developed a life-threatening drop in their white cell counts (neutropenia or agranulocytosis) during deferiprone treatment (4).

The development of a safe and orally active iron-chelator still remains a major problem and is given number one priority in Recommendations for Research by the National Institutes of Health – National Heart, Lung, and Blood Institute (4). Our *in vitro* study on chitosan has given promising results, suggesting that the polymer is worth studying as an oral, parentral or subcutaneuos iron-chelating agent if prepared in the form of microspheres of dimensions of the order of 30 – 100 nm (5). There are applications and *in vivo* studies on oral applications of chitosan as a dietary food (6), and as an oral adsorbent for urea and ammonia (7,8,9). It may prove to be an alternative to deferoxamine or deferiprone when its chelation capacity, biocompatibility, biodegradability and cost are considered. The toxicity of chitosan and chitosan derivatives in living systems is still being investigated (10, 11).

Material and Methods

Chitin was purchased from Sigma. Chitin flakes were cleansed of any proteins and lipids by treatment with 1.0 M NaOH solution for 3h at about 80°C and washing in water. They were then digested for 12h in 1.0 M HCl solution at room temperature. The alkali and acid treatments were repeated twice and the chitin was decolorized by refluxing in acetone (12). *Sodium hydroxide* was from Merck, *hydrochloric acid, iron (III) nitrate* and *EDTA* were from BDH, and *hydrobromic acid* was from Analar. All these reagents were used without any further purification.

Preparation of Chitosan: Chitin flakes were treated for 1,2 and 3 hours with 47% NaOH solution at 110°C under nitrogen atmosphere, and washed with water at about 80°C to neutrality (12). Three chitosan samples (C1, C2 and C3) with different fractions of deacetylated units were obtained. The samples obtained had only limited solubility in dilute acetic acid. Only about 40% of sample C3 was soluble in dilute acetic acid.

Determination of Degree of Deacetylation: Chitosan hydrobromide salts from the soluble fraction of chitosan samples were prepared and the free amino group contents were determined by direct titration with standard 0.1 M NaOH solution (13). The deacetylation percentages are given in Table 1. Sample C3 was found to have a deacetylation value of 57% by this method. However, in this study chitosan was used as a solid sample which was composed of soluble and insoluble fractions. Therefore, a solid state ^{13}C CP/MAS NMR spectrum of sample C3 was taken, which gave us a more realistic deacetylation value equal to 73%. CP/MS NMR spectrum of the sample was taken at a spinning rate of 8 kHz, with a repetition time of 3s and contact time of 1ms. The number of scans was 15 000. Line integrals were normalized at the C-1 signal of chitosan at 105 ppm. The spectrum is shown in Figure 1. Carbonyl carbon

Table 1. Iron (III) Adsorption Capacities of Chitosan Samples (mmol Fe^{+3}/g chitosan) in 50 mL,10 mM Fe^{+3} Solution at a pH of 1.8 and at 20°C at Different Contact Hours.

Sample	DD (%)	3 hours	6 hours	72 hours
C1	40	0.276	0.537	0.673
C2	50	0.534	1.24	1.53
C3	57	0.552	1.27	1.54

and methyl carbons of the N-acetyl group appear at 174 ppm and 24 ppm respectively. The peaks at 106, 84, 76, 60 and 56 ppm belong to C-1, C-4, C-5 and C-3, C-6 and C-2 carbons of the pyranose ring of chitosan respectively.

Determination of Molar Mass of Chitosan: Dilute solution viscometry with a home- made Ubbelohde-type viscometer was used to determine the viscosity average molar mass of the soluble fraction of chitosan, sample C3, according to the method of Terbojevich and Cosani (14), who reported K and a values at 25°C as 3.2*10-4 and 0.76 in 0.5M acetic acid/ 0.2M sodium acetate solution. The M_v of the soluble fraction was calculated as 1.24*104 g/mol.

Determination of Adsorbed Iron: The effect of the degree of deacetylation of chitosan flakes on the amount of adsorbed iron was determined by adding 285 mg of the chitosan samples of different degrees of deacetylation (C1, C2 and C3) and 100 ml samples of aqueous 10 mM $Fe(NO_3)_3.9H_2O$ solution of pH 1.8 to the reaction flasks thermostatically maintained at 20 ±0.5°C. After 3, 6 and 72 hours, the mixtures were filtered, treated with 10% sodium acetate solution to attain a pH of 2-3 and 2% sulfosalicylic acid was added as indicator to form the violet-red complex. The solution was then heated to 40-50°C and the amount of Fe^{+3} ions remaining in solution was determined by titration with 0.1 M EDTA solution (15). Then the amount of adsorbed ions was calculated by subtracting the amount of Fe^{+3} ions from the initial amount.

In a second set of experiments, the effects of the concentration of Fe^{+3} ions in solution and pH on the amount of adsorbed ions onto chitosan were investigated. The same procedure described above was repeated using 50 mg of samples of C3 and 100 mL, 1mM and 0.5 mM solutions of Fe^{+3} with pH values of 3, 5 and 7 adjusted with sodium acetate solution. EDTA solution at a concentration of 0.01 M was used as the titrant. No precipitation of Fe^{+3} ions in any form was observed under the conditions mentioned above within the time interval used for the adsorption profiles. Each experiment was repeated twice and the results for each sample agreed with each other within +/- 5% error.

Determination of Adsorbed Iron in Human Blood: Preliminary *in vitro* studies of normal human blood and

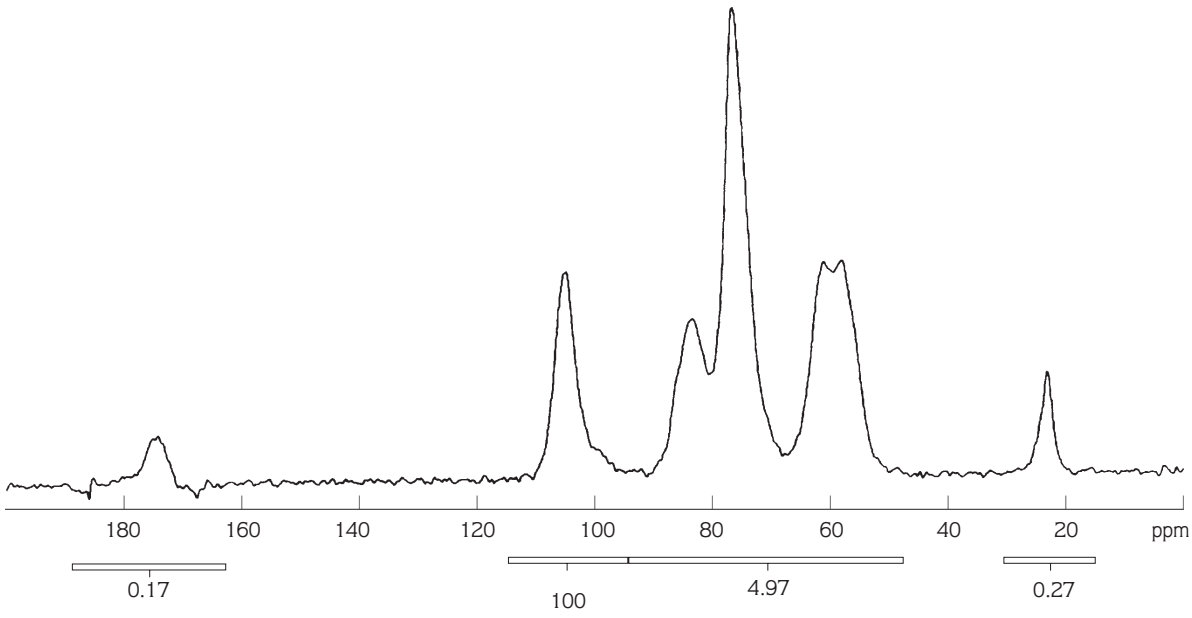


Figure 1. ^{13}C CP/MAS spectrum of chitosan (C3).

of thalassemia patients' blood were carried out. Sample C3, in the form of a flake, was brought into contact with the above mentioned blood samples for 3 hours at 37°C. The blood samples were analyzed for their iron content by using an Irma-mat Ferritin test kit. 300 μl of Iodine-125 –anti-ferritin agent was added to 25μl of sample from the patient pipetted into a tube, and incubated for 2 hours at room temperature on a horizontal shaker. The liquid was aspirated and the tubes washed with 2 ml of 0.9% NaCl solution. The radioactivity was measured (CPM) using a Cap-Ria 16 multi channel gamma counter by Capintec Inc. The validity and precision of the results were checked with the control sample included with the kit.

Results and Discussion

Analysis of Iron-Adsorption Capacity of Chitosan

The adsorption of Fe^{+3} ions on the surface of insoluble chitosan particles takes place as the formation of a surface complex which involves nitrogen and oxygen atoms in the repeat unit of chitosan:

$$CHI\text{-}NH_2 + Fe^{+3} \Leftrightarrow [CHI\text{-}NH_2\text{---}Fe\text{---}]^{+3} \quad (1)$$

The effects of time and degree of deacetylation on iron-adsorption capacities of chitosan samples C1, C2 and C3 in mmol Fe^{+3}/g chitosan are compared in the Table 1.

The adsorption capacity at a pH of 1.8 at 20°C in a ferric ion solution of concentration 10mM increases with the degree of deacetylation of chitosan, indicating that free amino groups take part in binding the Fe^{+3} ions onto the chitosan surface. Hydroxyl groups and carbonyl groups may also take part in the process in cooperation with amino groups. Different mechanisms for the interaction of metal ions with chitosan solutions involving amine and hydroxyl groups have been proposed (16,17). It can be seen that the amount of iron adsorbed increases in time for all the samples studied. Since sample C3 produced the best result, further studies were carried out with this sample only. The behavior of sample C3 under the conditions above is shown in Figure 2. The amount of adsorbed iron(III) ions increases with time, almost reaching equilibrium within 6 hours.

The effects of time, pH and concentration of ferric ions in the medium on the amount of iron adsorbed were studied. The results obtained at pH=3, pH=5 and pH=7 at 20°C, in 10mM, 1mM and 0.5 mM ferric ion solutions using 50.0 mg C3 sample in each experiment are summarized in Figures 3, 4 and 5. The amount of iron adsorbed on chitosan increases with time reaching equilibrium at around 5 hours. At all the ferric ion concentrations studied, increasing pH resulted in an increase in the amount of adsorbed ferric ions.

Figure 6 shows that there is a linear relationship between pH of the medium and the amount of the adsorbed iron (III) ions. The increasing pH of the medium decreases the probability of having any soluble fraction of chitosan in the medium with protonated amino groups. As the fraction of free, unprotonated amino groups increases, the number of nitrogen atoms with free electrons increases in the medium. The sites available for complexation with ferric ions increase in the medium and

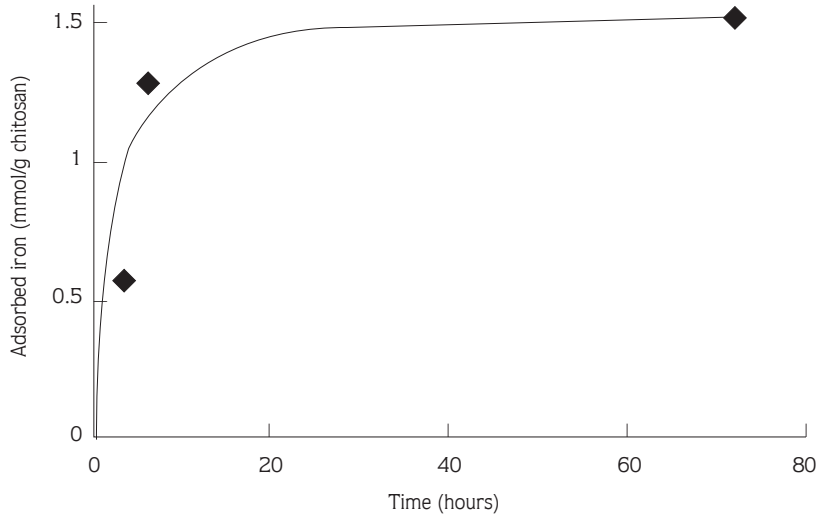


Figure 2. Adsorption equilibrium of iron (III) ions by Chitosan in 10 mM Fe^{+3} solution at 20 °C and pH=1.8.

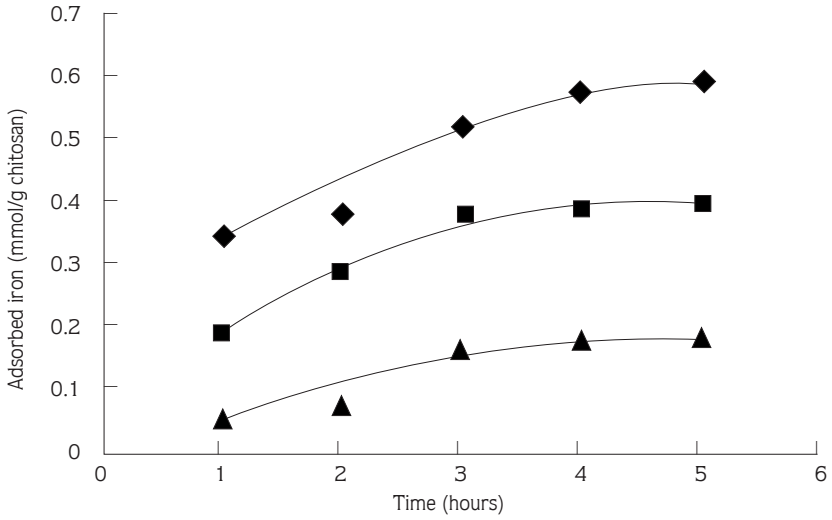


Figure 3. Adsorption equilibria of iron (III) ions by chitosan in 10 mM Fe^{+3} solution at 20°C at pH = (◆: 7, ■: 5, ▲: 3).

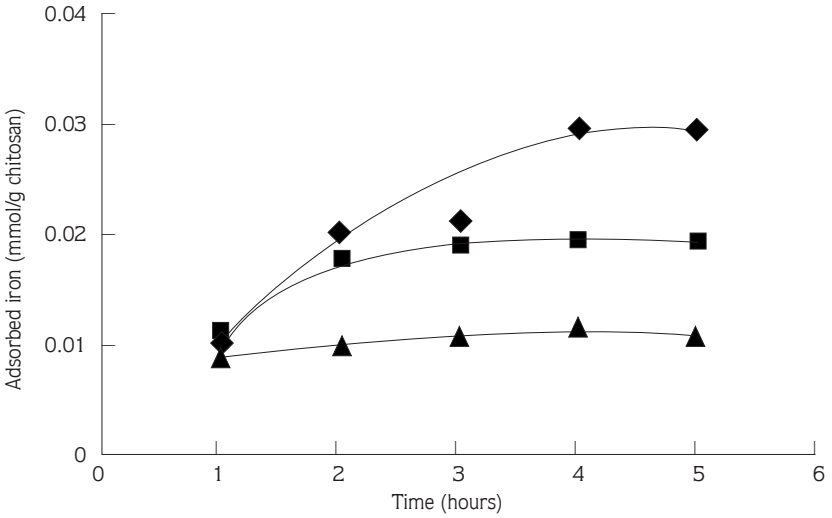


Figure 4. Adsorption equilibria of iron (III) ions by chitosan in 1 mM Fe^{+3} solution at 20°C at pH = (◆: 7, ■: 5, ▲: 3).

this is reflected in an enhanced adsorption of ferric ions onto chitosan. Another factor that may have an effect on the increase in ferric ion adsorption with pH may be the increasing tendency of iron (III) ions to separate from the solution.

Figure 7 shows that the amount of adsorbed iron (III) increases as the equilibrium concentration of iron (III) ions in solution increases. This behavior can be explained with equation (1), which represents the adsorption of the ions on the polymer surface as an equilibrium reaction. Equation (1) also explains why adsorption is parallel with the degree of deacetylation of chitosan.

It is also worth noting that the adsorption capacity of chitosan increases when greater amounts of chitosan are present in a given volume of the adsorption medium. This is also due to the increase in the amount of free amino groups in the adsorption medium. When 50 mg of chitosan is placed in a 10 mM Fe^{+3} solution at a pH of 1.8, after 5 hours the maximum adsorption capacity is 0.075 mmol Fe^{+3}/g chitosan (Figure 6). When 285 mg chitosan is used under the same conditions as shown in Figure 1, the adsorption capacity of the polymer increases to about 0.75 mmol Fe^{+3}/g chitosan. The ferric ion adsorption capacity of the currently used chelating agent Desferal is

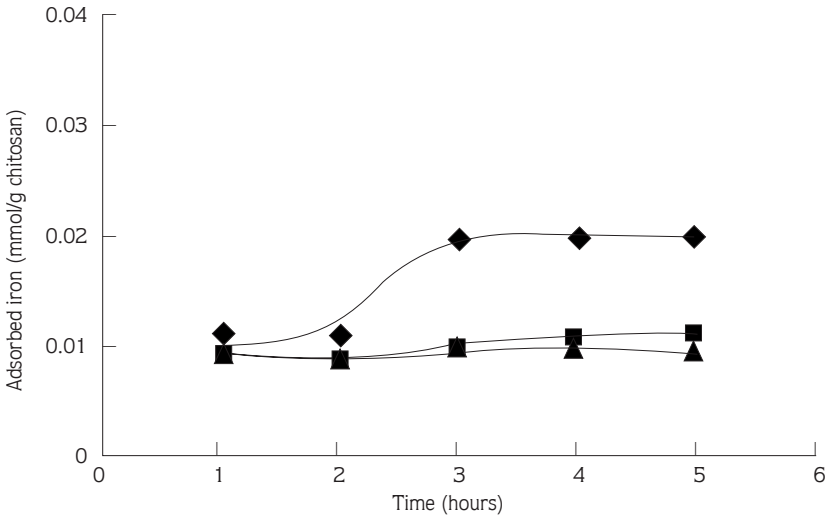


Figure 5. Adsorption equilibria of iron (III) ions by chitosan in 0.5 mM Fe^{+3} solution at 20°C at pH = (◆: 7, ■: 5, ▲: 3).

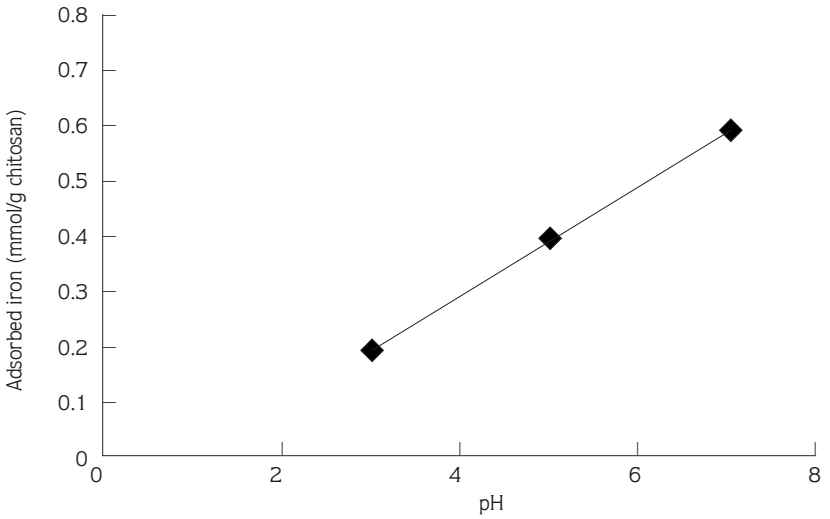


Figure 6. Adsorption behavior of iron (III) ions onto chitosan with pH at 20°C in 5 hours in 10 mM Fe^{+3} solution.

reported as 8.5 mg iron/100 mg Desferal theoretically. This is 1.5 mmol iron/g Desferal. Then, it is obvious that chitosan is comparable to Desferal in terms of its iron chelation capability.

Analysis of Iron-Adsorption Capacity of Chitosan in Human Blood

In light of the analysis given above, preliminary *in vitro* studies were carried out using human blood. In 3.0mL normal human blood of iron content, 40 μg/dL chitosan flakes, (20.0 mg) adsorbed 3 μg/dL of Fe^{+3}. The new iron content of the blood serum was determined as 37 μg/dL after being in contact with chitosan (C3) for 3 hours. However, when 20.0 mg C3 samples were brought into contact with the blood serum of a thalassemia patient with iron contents of 5795 μg/dL, which may be taken to be equivalent to a 1 mM iron (III) solution, it resulted in a decrease in the iron content of the blood serum equivalent to 620 μg/dL. If interpreted in terms of mmol iron (III) adsorbed/g chitosan, these results correspond to 0.55 mmol Fe^{+3}/g chitosan. This value is promising when compared to the iron binding capacity of Desferal, which is 1.5 mmol Fe^{+3}/g Desferal. The results obtained in human blood are in agreement with the results obtained in the chemistry laboratory. In a medium of high Fe^{+3} content, i.e. in the blood of

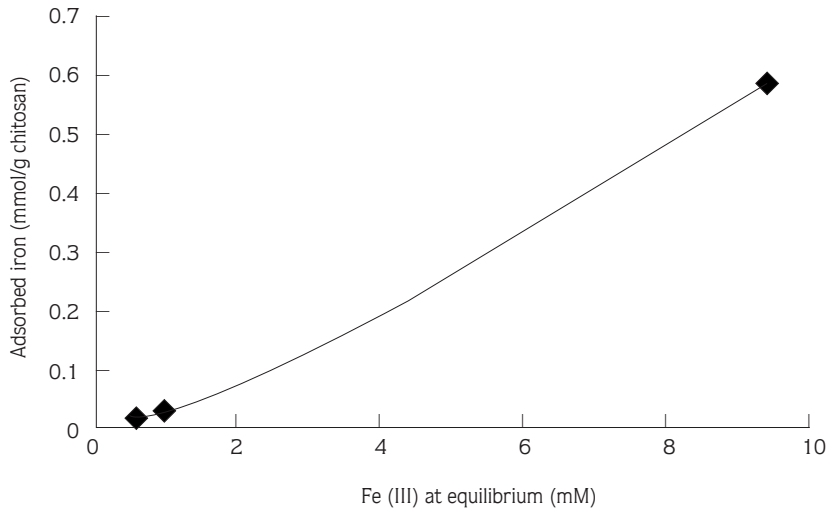


Figure 7. Iron (III) adsorption isotherm for iron (III)/chitosan system at pH=7, at 20°C in 5 hours.

thalassemia patients, the adsorption capacity of chitosan is much higher than in a medium of low Fe^{+3} content, in normal blood.

Since chitosan has an iron binding capacity that is comparable with that of Desferal, it is worthwhile investigating the potential of chitosan as an oral, parentral or subcutaneous iron adsorbing agent. Factors that might affect the iron adsorption capacity of chitosan such as the particle size, molecular weight and physical form are still under investigation. Microspheres of chitosan will be tested for their iron adsorption capacities in the near future. In addition, studies of human blood will be carried out to see if chitosan has any adverse effects on the composition of blood when its cholesterol, metal ion and protein binding capacities are known.

Acknowlegement

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