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Research Article

Preparation and Bioavailability Analysis of Ferrous Bis Alanine Chelate as a New Micronutrient for Treatment of Iron Deficiency Anemia

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Abstract
Purpose: One of the most nutritional disorders around the world is iron deficiency. A novel iron compound was synthesized by chelating ferrous ions with alanine for prevention and treatment of iron deficiency anemia.
Methods: The newly synthesized compound was characterized both qualitatively and quantitatively by Fourier Transform Infrared (FT-IR) spectroscopy. The bioavailability of newly synthesized iron micronutrient was evaluated in four groups of Wistar rats. The group I was a negative control group and the other three groups received three different iron formulations. After 14 days, the blood samples were taken and analyzed accordingly.
Results: Calculations showed that more than 91.8% of iron was incorporated in the chelate formulation. In vivo studies showed that serum iron, total iron binding capacity and hemoglobin concentrations were significantly increased in group IV, which received ferrous bis alanine chelate compared with the negative control group ($p<0.05$) and also group II, which received ferrous sulfate.7H2O ($p<0.05$). It indicates that the new formulation considerably improves the blood iron status compared with the conventional iron compounds. There were no significant differences ($p<0.05$) in the serum iron between group IV and group III, which received ferrous bis glycine.
Conclusion: The results showed better bioavailability of ferrous bis alanine as a new micronutrient for treatment of iron deficiency anemia in comparison with ferrous sulfate. Ferrous bis alanine could be considered as a suitable supplement for prevention and treatment of iron deficiency anemia.

Introduction

Iron deficiency (ID) is one of the most nutritional disorders around the world. The people who suffer from ID are mostly infants, young children, women with heavy menstruations, and also pregnant and lactating women.[1] ID is a condition in which the iron storage of the body becomes depleted. In this situation available iron in the body becomes insufficient to produce normal red blood cells. Iron, as a micronutrient, plays a crucial role in cell function and also cell growth. Iron is an essential element for the distribution of oxygen to the cells.

In the developing countries the most common reason for ID is an inadequate dietary intake of iron.[2] However, decrease in iron consumption because of chronic diseases, gastritis or malabsorption disorders like celiac disease and also blood loss are the most prevalent causes of ID.[3] Blood loss can be a result of menstruation in premenopausal women or may be a chronic or acute condition like gastrointestinal bleeding in adult men and postmenopausal women.[4] ID's symptoms vary from one patient to another. ID is asymptomatic in many patients; however, in the severe cases, some general manifestations are prevalent such as weakness, fatigue, headache, exercise intolerance, and concentration problems.[5] Studies have suggested that iron fortification of food is one of the recommended plans for preventing iron deficiency.[6] When dietary is not adequate to replenish iron storages in the body, iron supplementation is needed. Nowadays the most preferred treatment of ID is oral iron therapy; because it is safe, cheap and effective.

There are several oral iron supplements with a variety of dosage forms which are different in some features such as salt and oxidation state of iron (ferrous and ferric form). In the past years, different ferrous iron salts such as ferrous sulfate, ferrous fumarate and ferrous gluconate were used more preferably for treatment of ID.[7] However, low bioavailability and undesirable side effects of these conventional products were some of the major challenges that physicians and patients were faced with.

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Patients who treated with these oral compounds often suffered from gastrointestinal disturbance including heartburn, abdominal pain, nausea, vomiting, diarrhea and constipation.[8] Moreover, the bioavailability of iron compound such as ferrous sulfate is influenced by the other compounds. For example, foods which contain poly phenol compounds or phytic acid decrease iron absorption, because they form complexes that are not absorbed from the gut.[9] Some of these foods are cereals such as wheat, rice, maize; vegetables such as spinach and spices; legumes such as soya beans; and beverages such as tea, coffee and cocoa.[10]

It is believed that some of the organic molecules form complexes with the iron ions and enhance the transportation and absorption through the gut lumen. It has been shown that amino acids such as glycine enhance the absorption of iron.[11,12] Therefore, it can be assumed that amino acids are one of the best choices to form iron chelate compound in order to enhance the bioavailability of this micronutrient. Amino acids are employed as bidendate ligands to chelate iron and form a metal complex.[13] Since amino acids are absorbed from the small intestine by the specific active transport mechanism, these complexes can be also absorbed from the gut lumen as a part of the chelate molecule.[14] In addition, studies have shown that there are some mechanisms which control the bioavailability and absorption of iron amino acid chelates.[11] This important fact reduces the concerns about the iron overload and toxic effect of these novel compounds.

The aim of the present study is to synthesize and characterize a novel iron chelate and also explore the therapeutic effect of the newly developed compound in vivo. Results showed that the newly synthesized iron chelate complex has better bioavailability in comparison with ferrous sulfate and also has a superior effect on the various blood indexes including serum iron level and hemoglobin concentration.

Materials and Methods

Materials

Alanine (ultra-pure), ascorbic acid and potassium bromide were purchased from Sigma-Aldrich Chemicals Co. (USA). Ferrous sulfate hepta hydrate (FeSO4.7H2O) obtained from Amin Pharmaceutical Company (Iran). All other chemicals and reagents were of analytical grade and used as received.

Synthesis of ferrous bis alanine chelate

Firstly, 20 ml of deionized water was boiled 5 minutes to remove the dissolved oxygen. Then the temperature of the water decreased to 70 °C and 3.47 grams of ferrous sulfate hepta hydrate (equivalent to 12.5 mmol) were added. The mixture was stirred constantly and the temperature was maintained at about 70 °C until the iron salt completely dissolved. By decreasing the temperature of the mixture to 45 °C, 4.27 grams of ascorbic acid (equivalent to 25 mmol) were added and stirred until the solution became clear. Then 2.25 grams of alanine (equivalent to 25 mmol) were added to the clear mixture with continuous mixing and the temperature was kept constant at 45 °C. The stirring followed for about further 4 hours. The prepared solution was dried using lyophilization process and kept for further analysis.

Determination of the chelation

Fourier Transform Infrared (FT-IR) spectroscopy is a method which by the formation of the chelate and also the percentage of chelated amino acid would be determined.[15,16] There were two significant peaks in the FT-IR spectrum of free alanine, which disappeared upon chelate formation. One of them is due to symmetric stretching of carboxyl group and appeared at 1409 cm^{-1}. The other one is at 1595 cm^{-1} and was assigned to asymmetric stretching of carboxyl group.[16]

In order to determine the percentage of iron chelate, the consecutive concentrations of ultra-pure alanine in potassium bromide were provided. Then all of the prepared samples were scanned by FT-IR spectrophotometer. By measuring the absorbance of the significant peak at 1409 cm^{-1} and comparing it with the absorbance of standard alanine at the same wave number, the percentage of the free alanine in the mixture is determined accordingly.

Sample preparation

5 milligrams of ultra-pure alanine were mixed completely with 995 milligrams of anhydrous KBr and grounded by mortar and pestle. After homogenization, 500 milligrams of the this powder were weighed and further mixed with the other 500 milligrams of KBr. Subsequent samples were provided from the stock mixture using successive dilutions. Seven samples were prepared ranging from 0.0078% to 0.5000% (W/W %) alanine in KBr.

FT-IR analysis

The infrared spectra of samples were recorded using Nicolet Magna FT-IR spectrophotometer. Samples which prepared previously were transferred to the chamber of FT-IR spectrophotometer and scanned from 400-4000 cm^{-1} with a resolution of 4 cm^{-1}. FT-IR analysis was performed by analyzing the absorbance of carboxyl group in various concentrations of alanine and fitting the best calibration curve.

Bioavailability studies

Animals and diets

Twenty eight male adult (at 8 weeks of age) Wistar rats weighing 201-300 grams were obtained from pharmacy school of Tehran University of Medical Science. This study was conducted in accordance with the ethics committee of Tehran University of Medical Sciences. The animals were kept in stainless steel cages and fed with water and a laboratory diet, freely. The temperature of the environment was maintained between 20 to 22 °C and the relative humidity was about 45%, constantly. A regulated 12 hours light and dark cycle was scheduled during this study. The animals were divided into four groups. The

group I was considered as a negative control group and the other three groups received an equivalent amount of elemental iron (1 mg/kg) in three different formulations by gastric intubation for 14 consecutive days. The group I was considered as a negative control group which received 1 ml of deionized water per day. Group II was the positive control group which received 1 mg/kg of the body weight, elemental iron as ferrous sulfate hepta hydrate per day. Group III received ferrous bis glycinate chelate (Ferrochel®) which was provided by Albion laboratories (USA) and considered as an alternative marketed product. Group IV received ferrous bis alanine chelate which was provided as the new formulation. Due to omit the effect of ascorbic acid in the formulation of ferrous bis alanine, equal amounts of ascorbic acid were added to the regimen of all the groups.

Blood sample preparation

After 14 days, all the rats were fasted for 12 hours and were anesthetized by 60 µl xylazine 1% and 100 µl ketamine. Blood samples were taken by heart puncture using sterile syringes. Each of the blood samples was transferred into two tubes. One tube was heparinized for determination of hemoglobin and CBC parameters meanwhile the other tube centrifuged to obtain the serum. In the separated serum samples, the concentration of iron and total iron binding capacity (TIBC) were measured.

Statistical analysis

Statistical differences between the groups were evaluated using One-Way ANOVA. A p value of 0.05 was considered to be statistically significant. Values were expressed as mean ± SD of seven samples. All the statistical analyses were evaluated using SPSS software (version 16.0).

Results

During the synthesis reaction, iron accepts the electron pairs from both carboxyl and ∝-amino group of the alanine. This electron exchange leads to form a complex in which the iron in a neutral state is bonded to the two molecules of alanine by coordinate covalent bonds (Figure 1).

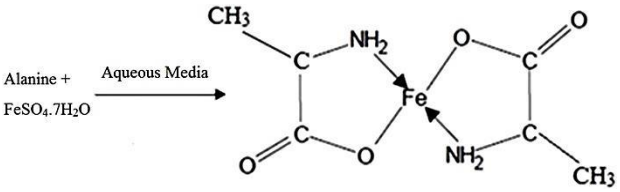

Figure 1. Scheme of the reaction between alanine and ferrous sulfate hepta hydrate in an aqueous media to produce ferrous bis alanine chelate.

FT-IR analysis detected the unbound alanine from chelated one. Free alanine exhibit a vibration peak at 2125 cm^{-1} that disappears upon chelate formation (Figure 2). Free alanine, which have zwitterions show an apparent peak at 2125 cm^{-1} due to the torsional vibration of $NH3^{+}$.[16] Disappearing of this peak indicates that a new coordinate bond is formed through the terminal amine.

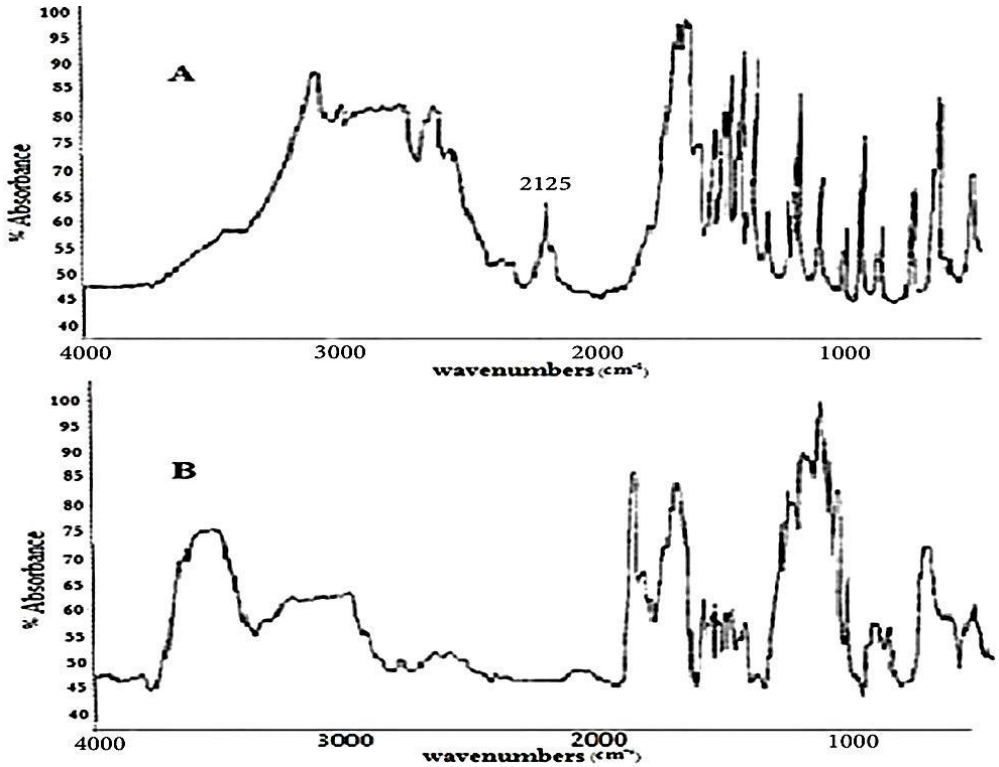

Figure 2. FT-IR spectrum of (A) alanine and (B) ferrous bis alanine chelate. The peak at 2125 cm^{-1} in alanine spectrum due to the torsional vibration of NH3+ disappears in the chelate form.

As previously mentioned, there are two significant peaks in the pure alanine crystal. Upon the chelation reaction the frequency of these two peaks was reduced accordingly. One of these peaks at 1409 cm^{-1} is assigned to the symmetric stretching of COO^- and the other one at 1595 cm^{-1} is assigned to the asymmetric stretching of COO^-. This fact indicates that as alanine molecules bond to the iron ions, the frequency of the COO^- band disappeared in a linear fashion (Figure 3).

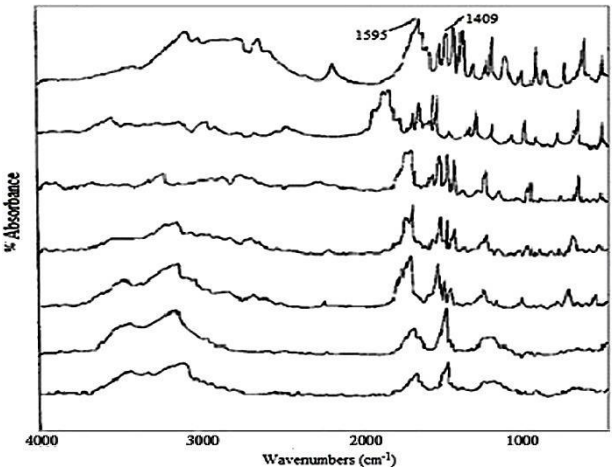

Figure 3. FT-IR spectra of successive concentrations of alanine in KBr.

The standard curve was prepared by plotting the absorbance of the symmetric stretching vibration of the alanine COO^- group versus alanine concentrations (Figure 4). The absorbance of the standard alanine concentrations are shown in Table 1. The equation of the standard curve was found to be Y=1.993X+0.114, where Y was the absorbance at 1409 cm^{-1} and X was the concentration of the free alanine in the mixture. The corresponding correlation coefficient of the obtained line was 0.998. After chelating reaction, the sample was transferred to the FT-IR chamber and scanned. By this method, the percentage of free alanine in the sample was determined. The total percentage of chelate was calculated by subtracting the free alanine form the feed alanine amount in the chelating reaction. Calculations showed that 91.8% of the total alanine were bonded.

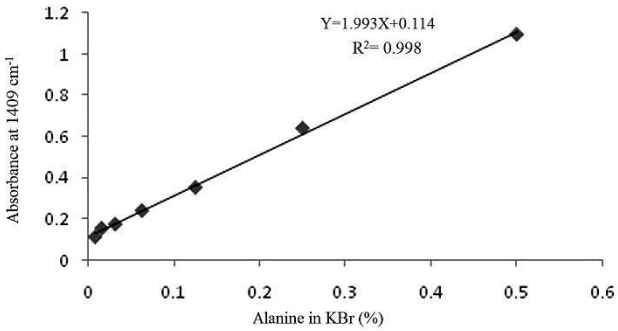

Figure 4. The percentage of standard alanine in KBr versus the absorbance at 1409 cm^{-1}.

Table 1. The concentrations of seven prepared samples and respective absorbances

Sample	Alanine[g]	KBr[g]	Total[g]	Alanine [%]	Absorbance[1409 cm^{-1}]
1	0.005000	0.995000	1.000000	0.5000	1.099
2	0.002500	0.997500	1.000000	0.2500	0.642
3	0.001250	0.998750	1.000000	0.1250	0.354
4	0.000625	0.999375	1.000000	0.0625	0.241
5	0.000313	0.999687	1.000000	0.0313	0.175
6	0.000158	0.999843	1.000001	0.0158	0.155
7	0.000078	0.999921	0.999999	0.0078	0.113

In order to investigate the therapeutic effect and the bioavailability of the newly developed ferrous bis alanine, in vivo study was performed in a rat model. One group was the negative control group and the other three groups were fed with a different iron compound for 14 consecutive days. After this period, blood samples were withdrawn from each rat and analyzed accordingly. The heparinized tubes which contained blood samples were used to analyze CBC parameters such as hemoglobin, hematocrit, mean corpuscular volume (MCV) and transferrin saturation which are all tabulated in Table 2. The non-heparinized tubes were centrifuged to separate blood serum. In the serum samples, total iron binding capacity (TIBC) and serum iron concentrations were measured. The results showed that serum iron concentrations (µg/dl) were significantly increased in group IV, which received ferrous bis alanine chelate in comparison with each of negative control group ($p<0.05$) and group II which received ferrous sulfate.7H2O ($p_1<0.05$). Hemoglobin concentrations (g/dl) were significantly increased in the ferrous bis alanine and ferrous bis glycine group in comparison with the negative control group ($p<0.05$) and also ferrous sulfate group ($p_1<0.05$). Total iron binding capacity (TIBC) concentrations (µg/dl) were significantly increased in the ferrous bis alanine group in comparison with the control group ($p<0.05$) and group II ($p_1<0.05$). In group III the concentration of TIBC (µg/dl) showed a significant increase compared with the control group ($p<0.05$) and ferrous sulfate group ($p_1<0.05$). There was no significant increase ($p_2<0.05$) in serum Fe, TIBC and also Hgb concentrations of group IV in comparison with group III.

The laboratory results showed a significant increase in the hematocrit percentage of group IV in comparison with control group ($p<0.05$), but there was not a significant increase in the percentage of hematocrit compared with each of group II ($p_1<0.05$) and III ($p_2<0.05$). Also, there was a significant rise in the MCV value of group IV, which received the newly developed formulation in comparison with group II ($p_1<0.05$) and also group III ($p_2<0.05$).

Table 2. Different blood indexes from in vivo studies. (Mean ± SD, n=7)

Blood parameters	Controls	Ferrous sulfate	Ferrous bis glycine	Ferrous bis Alanine
Serum Fe [µg/dl]	151.29±20.43	172.14±32.70 P	181.14±33.21 P P_1.N.S	187.86±31.29 P P_1 P_2.N.S
Serum TIBC [µg/dl]	426±26.10	437.57±27.30 P	474±20.42 P P_1	474±20.42 P P_1 P_2.N.S
Hgb [g/dl]	15±1.08	15.97±0.83 P	15.56±0.68 P P_1	15.51±0.74 P P_1 P_2.N.S
transferrin saturation[%]	35.59±4.82	39.54±8.79 P	38.26±7.08 P.N.S P_1.N.S	39.61±6.08 P P_1.N.S P_2.N.S
HCT [%]	45.29±3.70	47.59±3.32 P	47.17±2.43 P P_1.N.S	47.17±2.43 P P_1.N.S P_2.N.S
MCV [fL]	50.41±1.25	52.59±2.25 P	49.74±1.74 P P_1	50.77±0.98 P.N.S P_1 P_2

P: versus control group, P_1: versus ferrous sulfate group, P_2: versus ferrous bis glycine group, N.S: no significant. $P<0.05$, $P_1<0.05$, $P_2<0.05$

Discussion

New iron compounds such as iron chelates are being used as an alternative to the conventional products such as ferrous salts not only for prevention but also treatment of iron deficiency and iron deficiency anemia. Studies have demonstrated that there are significant differences between the bioavailability of the different sources of oral iron.[17] It has been reported that the intestinal uptake mechanism is one of the main factors which explains the differences between the bioavailability of the metal amino acid chelates and metal ions.[18] Iron is mostly absorbed by intestinal lumen through the passive absorbance and in small amounts uptake by an active transport mechanism involving a transporter called DMT-1. This transporter is not specific to iron, and also transfers the other divalent metal ions such as zinc and copper too.[19] Amino acids are known as the ligands that chelate the iron to form chemically inert structures which are absorbed actively through the mucosal membrane in an intact form.[17] It has been demonstrated that the metal amino acid chelates enter the intestinal lumen via the specific active transporters.[14] This fact indicates that chelate compounds are actively absorbed through the intestinal lumen in comparison with limited passive absorbance of iron ions. This active absorbance mechanism of iron chelates is resulted in higher bioavailability of this compound in comparison with iron ions as presented in this study. In addition, this new structure protects the iron from interaction with iron absorption inhibitors. Layrisse et al. reported in their study that these kinds of chelates protect the iron from undesirable interactions which inhibit its bioavailability.[20] Therefore, it can be concluded that chelation may improve the absorption and bioavailability of iron.

FT-IR is a reliable and accurate method that is used to assay the chelate composition in a dry mixture.[15] Using this method, the amount of iron amino acid chelate could be determined.

In this study alanine was used to form a new iron chelate formulation. Alanine as a ligand has two donor groups that bond to iron. During the reaction, iron ions bond to the carboxyl group and the α-amino group of the alanine via the coordinate covalent bonds. Ascorbic acid as a reducing agent is used in this formulation to protect the ferrous ion from undesirable oxidation reactions. In addition, studies have indicated that

ascorbic acid enhances the absorption of iron.[21,22] After the chelation reaction, iron chelate was freeze dried. The dried sample was transferred to the FT-IR chamber and analyzed. By comparing the FT-IR spectrum of iron chelated and free alanine, the formation of the chelate was proven. Moreover, using the calibration curve, the amount of chelated iron was calculated indirectly by determination of free alanine in the sample. According to the results, the considerable amounts of alanine (91.8%) were involved in the chelation reaction. After the formation of chelate, in vivo studies on rat model were performed to evaluate the bioavailability and also improvement in the blood indexes including Hgb, TIBC, MCV, and hematocrit. In vivo studies on rats demonstrated that ferrous bis alanine, as an oral iron preparation, significantly ($p<0.05$) increases serum iron level in comparison with the negative control group and group II. Some other blood parameters, such as Hgb, TIBC and MCV were also significantly ($p<0.05$) higher in group IV compared with group II. Studies have suggested that hemoglobin level is a valid parameter to evaluate whether the new iron formulations have improved absorption and bioavailability.[23] Higher level of mean hemoglobin concentration in group IV and group III demonstrates that iron chelate can be considered as a preferred formulation to increase the gastrointestinal absorption and bioavailability of iron. One aspect of this impressive absorption of iron is the neutralization of this ion during the chelating process which can prevent the iron interactions with phenolic compounds. Studies have reported that the amino acid moiety of the chelate has a protective effect on the sequestrated iron.[24] In addition, chelating iron keeps it soluble and facilitates iron absorbance into the cells. Researchers have represented that iron amino acid chelates cause less interaction between iron and the other minerals such as zinc.[25] The concentration of the serum iron and TIBC showed a significant ($p<0.05$) increase in group IV as compared to the negative control group and also compared to the group II. This finding indicates more bioavailability of iron amino acid chelate and increase in the serum iron level.

Conclusion

A novel iron chelate was synthesized with alanine for prevention and treatment of iron deficiency anemia. Formation of iron chelate was analyzed using FT-IR method. In vivo experiments in rat model demonstrated satisfactory results in iron bioavailability and blood indexes, including serum iron, Hgb and TIBC in comparison with ferrous sulfate. The higher bioavailability of ferrous bis alanine makes it an appropriate food fortifier because of the significant bioavailability. Altogether, it can be concluded that ferrous bis alanine could be considered as a suitable supplement for prevention and treatment of iron deficiency and iron deficiency anemia.

Acknowledgments

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Ethical Issues

This study was conducted in accordance with the ethics committee of Tehran University of Medical Sciences.

Conflict of Interest

The Authors report no declaration of interest.

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