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Investigation and Biological Activity of Some Nonpolar Iron Amino Acid Chelates

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Abstract: The nonpolar Fe (III) phenylalanine and glycine chelates have been synthesized and characterized by using different tools, in particular; elemental analysis, molar conductance, infrared and electron paramagnetic resonance spectra. The elemental analysis showed the formation of 1:3 [M: L] ratio. The molar conductance measurements revealed a non-electrolytic nature. The infrared spectral data displayed the complexation behavior which takes place through the nitrogen and oxygen atoms of the present amino acids. The electron paramagnetic resonance data showed the existence of an octahedral geometry structure. Thirty-eight adult rats divided into four groups; group I: included 10 rats received 0.5 mL physiological saline, group II: included 9 rats received 0.5 mg ferrous fumarate/kg body weight, group III: included 9 rats received 0.5 mg Iron phenylalanine chelate/kg body weight/day, group IV: included 10 rats received 0.5 mg Iron glycine chelate/kg body weight/day. All rats were killed then blood, serum samples and liver homogenate were obtained, in blood were measured Hb and HCT.

Key words: Nonpolar amino acids, $FeCl_3.6H_2O$, hemoglobin, kidney and liver functions

Introduction

Iron deficiency continues to be one of the most prevalent nutritional deficiencies in the world. For physiological reasons, the most commonly affected groups are infants, children, adolescents and women of childbearing age (DeMayer and Adiels, 1985). A simplest way to improve Iron status of individual suffering from Iron deficiency occurs by supplementation with Iron tablets. Pharmaceutical Iron supplementation is generally indicated in adults when hemoglobin concentration fall below 12g/dl (Schumann, 1998). Fortification of food with Iron is the best way of preventing Iron deficiency, most difficult technical hurdle is finding the adequate combination of Iron fortification compound and food vehicle and the selection of an appropriate Iron compound (Olivares et al., 1997). Harvey et al. (1998) were reported that oral Iron supplements in the form of ferrous salt (ferrous sulfate), are more likely to cause gastrointestinal irritation. In order to enhance Iron bioavailability and avoid its side effects, chelating Iron with amino acid have been employed, since amino acids are absorbed well from intestinal lumen by specific active transport mechanisms. The amino acid compounds are bidentate ligands and used as chelating ligands to form many transition metal complexes with the most periodic table elements (Hawary et al., 1975). Three chelates of Fe (III) ion with alanine, histidine and aspartic acid were prepared and investigated by using different tools; in terms of elemental analysis, infrared and electronic spectra. An octahedral geometry was proposed for these chelate (Abdel-Gayoum et al., 2001).

The present paper aims to synthesis two chelates of Fe (III) with nonpolar amino acids (phenylalanine and glycine) and to characterize their geometrical structures and to elucidate whether Iron supplements in the form of ferric amino acid chelates would increase the bioavailability of the Iron. Meanwhile, delivered ferric Iron would be as effective as the ferrous Iron in improving the hematology and serum Iron status.

Materials and Methods

Solvents and reagents: All chemicals used in this study were laboratory pure, including Phenylalanine, glycine, Iron (III) chloride hex hydrate ($FeCl_3.6H_2O$), dimethylsulphoxide (DMSO) and alkaline medium and double distilled water, ferrous fumarate tablets were purchased from drug store (limassol-Cyprus-Europe).

Synthesis of chelates

Synthesis of Iron (III) phenylalanine chelate: The chelate under investigation was synthesized by mixing $25cm^3$ of an aqueous solution of the phenylalanine (0.01mole; 1.65g) with (0.01mo1; 2.71g), Iron (III) salt in same solvent and the obtained mixture was reflexed for 2hrs. Few drops of alkaline solution were added to adjust the pH at which the solid chelate was separated. The formed chelate was filtrated, collected and washed several times with hot double distilled water until the filtrate becomes colorless. The chelate was dried in a desiccator over anhydrous $CaCl_2$ under vacuum. The yields were estimated to be about 70% and the purity of the complex was confirmed by the elemental analysis and TLC technique.

Phenylalanine +FeCl₃. 6H₂O → Alkaline Media
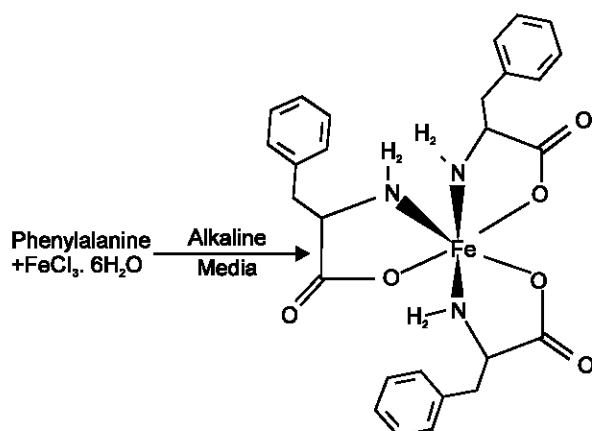
Synthesis of Fe (III) glycine chelate: The synthesis of this chelate was performed by mixing an aqueous solution (25 mL distilled H_2O) of glycine (0.01mol; 0.75g) with Fe (III) salt (0.01mo1; 2.71g) in same amount of solvent, then adjust the pH to desired ratio. The obtained mixture was reflexed for 2hrs until the chelate isolated. The precipitate was separated by filtration and washed several times with hot double distilled water. The resulted chelate was dried in a desiccator over anhydrous $CaCl_2$ and the analyses were confirmed by the elemental analysis and TLC technique.

Glycine+ FeCl₃. 6H₂O → Media Alkaline
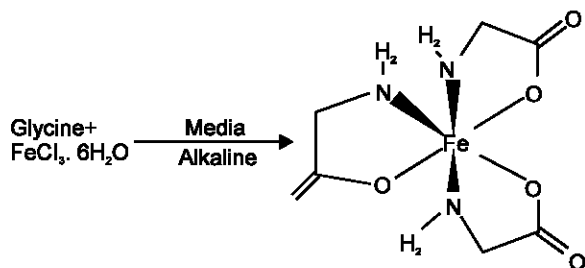
Physical measurements: The elemental analyses (C, H, N) of the chelate was carried out by 2400 elemental analyzer. The molar conductance was carried out in DMSO solvent using conductivity meter model CMD 650 digital. The infrared spectra were obtained by KBr disc on using IFS-25DPUS/IR spectrometer (Bruker) 1998Y. The electron paramagnetic resonance spectra of the chelates were carried out using EMX ESR spectrometer (Bruker) 1998Y. All the mentioned analyses were done at Micro-Analytical Center, Giza, Egypt.

Animals: Thirty eight female adult Sprague Dawley rats weighing 158-261g obtained from the Central Animal House of Garyounis University, Benghazi, Libya, were used in this study. The animals were housed in stainless steel cages with a free supply of diet and water and maintained under constant 12hrs light and dark cycle in an environmental temperature of 20-30°C, they were divided into 4 groups:

Group I: Included 10 rats received 0.5 mL physiological saline.
Group II: Included 9 rats received 0.5mg ferrous fumarate/kg body weight.
Group III: Included 9 rats received 0.5mg Fe (III) phenylalanine chelate/kg body weight/day.
Group IV: Included 10 rats received 0.5 mg Fe (III) glycine chelate/kg body weight/day.

*All Iron forms were dissolved in 0.5 mL of normal saline and given by gastric intubations for 10 consecutive day.

Sampling: All rats were killed by decapitation and fasting blood sample was taken using sterile syringe and transferred into two tubes: one tube contains heparinzen for Hb, pack cell volume and other tube was for separation of serum. In serum samples, Iron concentration, TIBC concentration, % transferrin saturation, liver function tests (ALT, AST, Total protein, albumin and total bilirubin), kidney function tests (Urea and Creatinine) we measured and liver homogenate were done for liver ferritin.

Statistical analysis: The statistical analysis was done by using student's t-test. A $p < 0.05$ value was considered statistically significant. All values are presented as Mean±SD.

Results

The condensation of ferric chloride hexahydrate with amino acids was done in double distilled water. All the results are shown in Table (1-6). In serum we measured Iron concentration, total Iron binding capacity, % transferrin saturation, total bilirubin, total protein, creatinine, urea, albumin, alanine transaminase (ALT) activity, aspartate transaminase (AST) activity, in liver homogenate were measured liver ferritin. Serum Iron (µg/dl) concentration was a significantly increased in ferric phenylalanine chelate compared with each of control group ($p < 0.001$), ferric glycine chelate ($p_1 < 0.002$) and ferric glycine chelate ($p_2 < 0.001$). Hemoglobin concentration (g/dl) significant increases ($p < 0.010$) in ferrous fumarate and ferric phenylalanine chelate ($p < 0.001$) compared with control group and also in ferric phenylalanine ($p < 0.025$) compared with ferric glycine chelate. Serum TIBC (µg/dl) concentration significantly increased ($p < 0.014$) in ferric glycine chelate group, but decreased ($p < 0.001$) in ferric phenylalanine chelate compared with controls. Liver ferritin (ng/gm tissue) concentration was significantly increased in ferrous fumarate ($p < 0.001$), ferric phenylalanine chelate ($p < 0.001$) and ferric glycine ($p < 0.002$) compared with controls, but there were significant decrease in ferric glycine chelate ($p_1 < 0.002$) compared with ferrous fumarate and there were significant increase ($p_2 < 0.001$) in ferric phenylalanine chelate compared with ferric

Table 1: Elemental analysis and molar conductance of chelates

| Chelates | Molecular weight | % C | | % H | | % N | | Λ_m |
		Calculate	Found	Calculate	Found	Calculate	Found	
[Fe (Phe)$_3$]	548.04	58.63	59.17	4.31	5.52	7.07	7.67	2.16
[Fe (Gly)$_3$]	277.63	25.57	25.96	4.82	4.36	14.22	15.14	3.00

Λ_m = molar conductivity (ohm^{-1} cm^2 mol^{-1})

Table 2: Infrared and electron paramagnetic resonance of chelates

| Ligand/chelates | IR (cm^{-1}) | | | | | | g_{eff} |
	υ C = O	υ COO$^-$	υ NH$_3^+$	υ NH$_2$	υM-N	υ M-O	
Phenylalanine (Phe)	1628	1562	3150	-	-	-	-
[Fe (Phe)$_3$]	1605	1496	-	3300	586	463	2.007
Glycine (Gly)	1504	1585	3166	-	-	-	-
[Fe (Gly)$_3$]	1612	1612	-	3400	594	421	2.004

IR = Infrared. g_{eff} = E.P.R.

Table 3: Mean values±significant of initial and final body weight of control and studied groups

Group Parameter	Controls No. (10)	Ferrous fumarate (9)	Ferric glycine chelate (10)	Ferric phenylalanine chelate (9)
Initial body weight (g)	237±18.18	184±2.86	157.30±20.97	217.22±1.92
Final body	214±19.6	203.56±12.62	185.00±26.93	238.78±9.24
weight (g)	p<N.S	p<0.001	p<0.019	p<0.000

P: Initial body weight versus final body weight, Signifiant p>0.05., NS. No Significant

Table 4: Mean values±S.D of serum urea (mg/dl), serum creatinine mg/dl) in controls and studied groups

Group Parameter	Controls No. (10)	Ferrous fumarate (9)	Ferric glycine chelate (10)	Ferric phenylalanine chelate (9)
serum	18.20±6.11	35.11±8.92	22.90±6.82	30.44±3.84
Urea		p<0.001	p. N.S	p<0.001
(mg/dl)			p1<0.004	p1. N.S
				p2<0.010
serum	0.31±0.088	0.24±0.073	0.30±0.08	0.40±0.18
Creatinine		p. N.S	p. N.S	p. N.S
(mg/dl)			p1. N.S	p1<0.029
				p2. N.S

P. Versus controls. P1. Versus ferrous fumarate, P2. Versus glycine, NS. No Significant

glycine chelate. % transferrin saturation there were significant increases in ferric phenylalanine chelate compared with controls (p<0.001) ferrous fumarate (p_1<0.001) and ferric glycine chelate (p_2<0.001). The results of the present study also demonstrated that the Iron amino acid chelate have no harmful effects on the liver or kidney as indicated by liver and kidney function tests, there were no serious side effects from the supplementation of the Iron amino acid chelates. We concluded that, in the inorganic form, which has been the form of choice for supplementation and fortification ferrous salt demonstrated restricted bioavailability with some side effects on liver. Modification on the inorganic forms of Iron gains some improvement in absorption. Iron amino acid chelates may be the best form for supplement and fortification of Iron into human diets.

Discussion

Microanalysis and molar conductance: The elemental analysis results of the prepared chelates (Table 1) revealed the stoichiometric of measurements 1:3 [M:L] ratio. The isolated chelates are air stable and insoluble in most common organic solvents, but partially soluble in dimethylsulphoxide (DMSO). The molar conductance measurements of the chelates indicate a non-electrolyte nature (Raman *et al.*, 2001).

Infrared spectra: The infrared spectral data of the two chelates under investigation displayed two bands at 1605 and 1612 cm^{-1} due to C = O of COOH group and these bands changed on comparing with their original position in the free ligands (1628 and 1504 cm^{-1}). That indicating the involvement of C = O group in chelation with Fe (III) ion (Condrate and Nakamoto, 1965). Where the NH$_2$ group of the amino acids was effected by the chelation with the same metal ion (Table 2) and the appearance of new bands which are not seen in the spectra of the free ligands attributed to υ (M-N) and υ (M-O) vibrations respectively supporting the participation of nitrogen and oxygen atoms in chelation with Fe (III) ion (Faniran *et al.*, 1976).

Electron paramagnetic resonance spectra: The electron paramagnetic resonance spectral data of these chelates exhibited g_{eff} values (Table 2) corresponding to the presence of an octahedral structures (Aml Jarbou, 2006).

Table 5: Mean values±S.D of Hb (g/dl), RBC/106/µl), HCT%, serum iron (µs/dl), serum total iron binding capacity (TIBC) (µs/dl), liver ferritin µ tissues) and % transferring saturation in controls and studied groups

Group Parameters	Controls (10)	Ferrous fumarate (9)	Ferric glycine chelate (10)	Ferric phenylalanine chelate (9)
Hb	11.76±0.70	12.94±1.05	12.64±1.19	13.74±0.66
(g/dl)	0	p<0.010	p. N.S	p<0.001
			p1. N.S	p1. N.S
				p2<0.025
RBC	6.99±0.49	7.08±0.44	6.65±0.58	7.37±0.48
(10⁶/µl)		p. N.S	p. N.S	p : N.S, p1 : N.S
			p1.N.S	p2<0.010
HCT	40.27±2.29	40.40±2.51	39.45±4.21	42.03±2.02
(%)		p. N.S	p. N.S	p. N.S, p1. N.S
			p1. N.S	p2. N.S
serum	196.93±25.13	214.78±39.03	203.00±52.55	323.11±81.75
Iron		p. N.S	p. N.S	p<0.001
(µg/dl)			p1. N.S	p1<0.002
				p2<0.001
serum	368.20±57.61	378.78±62.74	455.40±82.95	103.78±27.88
TIBC		p. N.S	p<0.014	p<0.001
(µg/dl)			p1<0.038	p1<0.001
				p2<0.001
liver	2.70±0.55	4.35±0.64	3.46±0.38	7.39±0.64
ferritin		p<0.001	p< 0.002	p<0.001
(ng/gm tissue)			p1<0.002	p1<0.001
				p2<0.001
% transferrin	54.90±12.01	57.78±14.08	45.70±13.47	332.78±125.21
saturation		p. N.S	p. N.S	p<0.001
			p1. N.S	p1<0.001
				p2<0.001

P. Versus controls., P1. Versus ferrous fumarate, P2. Versus glycine, NS No Significant

Table 6: Mean values±S.D values of serum got (U/L), G, PT (U/L), ALP (U/L), total protein g/dl, albumin (g/dl) and total bilirubin (mg/dl) in controls and studied groups

Group Parameters	Controls (10)	Ferrous fumarate (9)	Ferric glycine chelate (10)	Ferric phenylalanine chelate (9)
Serum	80.40±36.28	169.44±26.01	116.40±29.05	101.56±15.75
AST		p<0.001	p<0.025	p. N.S
(U/L)			p1<0.001	p1<0.001
				P2. N.S
Serum	25.90±12.00	72.89±18.43	14.60±5.32	18.89±6.15
ALT		p<0.001	p<0.014	p. N.S
(U/L)			p1<0.001	p1<0.001
				p2. N.S
Serum	113.50±50.36	191.22±64.04	132.70±36.45	109.33±38.53
ALP		p<0.009	p. N.S	p. N.S
(U/L)			p1<0.024	p1<0.005
				p2. N.S
Serum	7.060±0.4835	6.59±0.831	6.40±0.73	6.98±1.161
Total protein		P. N.S	p<0.029	p. N.S, p1. N.S
(g/dl)			p1. N.S	p2. N.S
Serum	2.660±0.51	2.36±0.347	2.21±0.42	2.97±0.68
albumin		p. N.S	p<0.045	p. N.S
(g/dl)			p1. N.S	p1<0.028
				p2<0.009
Serum	0.15±0.05	0.19±0.13	0.21±0.13	0.34±0.19
Total Bilirubin		p. N.S	p. N.S	p<0.006
(mg/dl)			p1. N.S	p1. N.S, p2. N.S

P. Versus controls., P1. Versus ferrous fumarate, P2. Versus glycine, NS No Significant

Biochemical data: In the present study, Iron supplementation (0.5 mg kg⁻¹ body weight) in the form of ferrous fumarate, ferric phenylalanine and ferric glycine chelates was done. Serum Iron was increased significantly (p<0.001) in ferric phenylalanine chelate group (64.07%) in comparison with control group, but increased non significantly in ferrous fumarate (9.06%) and ferric glycine chelate (3.08%). Puurcell and Kotz (1980) explained why more Iron can be absorbed from the gastro-intestinal tract into the mucosal cell as a

chelate than as a salt, first is that in the amino acid chelated form, the sequestered atom of Iron has no polarity (neutral). The amino acid chelated Iron is not as reactive with food a ingredient which leaves more of the Iron available for absorption. So that, there is a protective effect of the amino acid moiety both chelating and protecting Iron from inhibitors of the diet and keeping it soluble and available for incorporation into the cell (Mazariegos *et al.*, 2004). Second, Iron amino acid chelate enters into fewer absorptive inhibiting reactions in the gut. Third, absorption of the Iron amino acid chelate did not inhibit by the presence of other minerals in the diet. There was a significant increase in the mean hemoglobin level in ferrous fumarate (10.03%) and ferric phenylalanine chelate (16.84%) groups compared to controls but non significant increase (p>0.05) was found in glycine chelate group. The mean of hemoglobin level in ferric phenylalanine increased but insignificantly as compared to ferrous fumarate. These results demonstrated that, the mean of hemoglobin concentration was significantly higher in fed Iron amino acid chelates especially ferric phenylalanine chelate but there was no statistically difference in the packed cell volume. These results concluded that most of the absorbed Iron from the chelate incorporated into (16.84%) increase in hemoglobin level as regard phenylalanine chelate rather than being stored in the liver. Fomon *et al.* (1988) reported that (90%) of the absorbed Iron is used for hemoglobin and is a valid method for comparing the absorption and bioavailability of different chemical forms of Iron within the same individual. Faiweather-Tait *et al.* (1992) noted that the amount of Iron absorbed from the chelate source must have been far greater than was reflected in the increased hemoglobin in order to meet the anabolic needs of the rapidly growing animal. This was confirmed in our study that showed significant increases in ferrous fumarate (p<0.001) group, ferric glycine chelate (p<0.019) group, ferric phenylalanine chelate (p<0.001) group in comparison of final body weights with initial body weights and non significantly changed (p>0.05) in controls. The greater bioavailability of the Iron amino acid chelate allows for the rapid incorporation of Iron into hemoglobin first, followed by a quicker repletion of tissue Iron stores. This was seen in our study in which there was a significant increases (p<0.001) in mean liver ferritin in all rats supplemented with ferrous salt compared to controls and also in rats supplemented with both Iron amino acid chelates compared to ferrous fumarate group (Faiweather-Tait *et al.*, 1992). Our results demonstrated that, the greater bioavailability of the Iron amino acid chelate allows for satisfying hemoglobin requirements and a more rapid repletion of tissue Iron stores, so more Iron was stored from ingesting Iron amino acid chelate than equivalent amounts of Iron from salts. The increasing in mean liver

ferritin could be attributed to the daily Iron supplement in the present study causing surplus of Iron in the blood and uptake by the liver. This was confirmed by the % transferrin saturation which was significantly increased in ferric phenylalanine chelate (p<0.001) as compared to control. Increased % saturation of transferrin indicates the presence of an adaptive mechanism of the body to absorb Iron when it is greatly needed as indicated by the significantly increase of Iron as result of absorption after oral doses. Our findings confirmed that the Iron phenylalanine chelate is indeed a highly bioavailability form of Iron amino acid chelate because it was found to be statistically effective in raising hemoglobin level, serum Iron and % transferrin saturation as compared to ferrous fumarate. This research study showed that Iron from Iron phenylalanine chelate is preferentially absorbed over Iron from ferrous fumarate. Pizarro *et al.* (2002) suggested that these differences could be explained on the basis of physical and chemical characteristics that determine different Iron bioavailabilities and so we suggested that the increase in absorption of Iron amino acid chelate was probably due to the chemical structure of this compound, which partially prevents Iron inhibitors interaction. The results of the present study also demonstrated that the Iron amino acid chelate preferentially phenylalanine had no harmful effects on the liver or kidney as indicated by liver and kidney function tests, there were no serious side effects from the supplementation of the Iron amino acid chelates. This suggested that in people with normal Iron levels, there is a little potential danger of overloading or subsequent toxicity when consuming foods fortified with nutritionally appropriate amounts Iron amino acid chelate. This result was in agreement with the study of Jeppsen (2001).

Conclusion: We concluded that, in the inorganic form, the choice for supplementation and fortification ferrous salt demonstrated restricted bioavailability with some side effects on liver. Modifications on the inorganic forms of Iron gain some improvement in absorption. Iron amino acid chelate may be the best form for supplement and fortification of Iron into human diets. Increasing in absorption of Iron shown by increasing of the amount of hemoglobin, so the amino acid chelated with Iron is suitable for hemoglobin repletion in Iron deficiency anemia and allowed daily lower doses of Iron than would be expected.

Recommendations: We recommended that the use of Iron amino acid chelate as phenylalanine source of Iron in food intended for the general of population, food supplements and food for particular nutritional uses including foods intended for infants and young children.

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