A new spectrophotometric method for direct determination of iron (III) in serum

*[1]Jafarian-Dehkordi A., [2]Saghaie L., [1]Movahedi N

[1]Isfahan Pharmaceutical Sciences Research Center, [2]Department of Medicinal Chemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Received 10 Jun 2007; Revised 29 Jan 2008 Accepted 5 Feb 2008

ABSTRACT

Bakground and purpose of the study: Hydroxypyridin-4-ones, a group of iron chelators have shown promise as potential compounds for the treatment of iron overload by the oral route. Their selectivity and high complex formation constant with iron makes them good candidates for iron determination. In this study the use of N-ethyl-2-methyl-3-hydroxy-pyridin-4-on (EMHP), a strong and selective ferric chelator, as a new ligand for measurement of μmolar concentrations of iron in aqueous solutions and biological fluids was investigated. This measurement is based on the color reaction of Fe^{3+} with EMHP.

Methods: After mixing serum sample and reagent, and incubating at room temperature, the absorbance of the resulting complex was measured at λ_{max}. The effect of analytical variables, such as the amount and the kind of the reagents, pH, ratio of EMHP/Fe (III) and presence of other ions in determination of iron were studied.

Results: The results showed that the optimum wavelength for the measurement was 456 nm. Formation of the complex was completed in less than 20 min and it was stable up to 24 hrs. Molar ratio of 6-10 EMHP/Fe (III) and pH = 5 were the optimum conditions for complex formation and determination of Fe (III). The detection limit was 2.5×10^{-6} M of Fe (III) in serum or plasma. Ions commonly associated with iron did not interfere in the present method.

Conclusion: This method which is simple and reproducible was found sensitive for determination of Fe (III) in several real samples at micromolar levels.

Keywords: Iron; Determination; Spectrophotometry; N-ethyl 2-methyl-3hydroxypyridin-4-on

INTRODUCTION

Iron plays important roles in both biological and environmental media (1-2). Due to its importance in the context of clinical diagnosis, intoxication, environmental pollution monitoring and … (3-5) many methods such as spectrophotometry (6-7), atomic absorption spectrometry (8-9), inductively coupled plasma-mass spectrometry (10), cathodic striping voltammetry (11), fluorimetry (12) and ion chromatography (13-14) have been proposed for determination of iron species in natural samples**.**

Among the most widely applied methods are those based on spectrophotometry, because of their experimental rapidity, simplicity and wide applications. Spectrophotometric techniques involvethe use of ligands that selectively bind to iron, or a particular redox state of iron, to produce a coloured complex with a high molar absorptivity. Iron selective ligands such as thiocyanate (15-16) or di(2-pyridyl)-N,N-di[(8-quinolyl)amino]methane (17-19) were among the first selective reagents to be used for the determination of iron. In most of these methods Fe (II) is involved in reaction with an appropriate ligand and color-generation (20), Fe (III) is then determined by subtraction the concentration of Fe (II) from total iron, which is determined either by reduction to Fe (III) or by conventional non-selective methods (21-22). The differential approach, however, often yields highly imprecise values for Fe (III) when the Fe (II) concentration is higher than that of Fe (III) (23). In addition, most above mentioned methods lack sufficient sensitivity for iron determination at μmolar or sub-μmolar levels. Therefore, ferrozine has been widely used for spectrophotometric determination of Fe (II), due to a sufficiently low detection limit and low blank values (4, 24). A potential problem with the classical ferrozine method is incomplete reduction of organic complex Fe (III) (25). This is probably why different reducing agents (mostly ascorbic acid and hydroxylamine hydrochloride) are used to optimize the reduction condition (7, 26). Several studies have also demonstrated that Fe (III) in solution can also react with ferrozine,

Correspondence: jafarian@pharm.mui.ac.ir

which interferes with the ferrous complex (7, 27). Increasing interest has therefore focused to develop new methods for determination of Fe (III).

Hydroxypyridin-4-one, a group of iron chelators have shown promising as potential compounds for the treatment of iron overload by the oral route. Their selectivity and high complex formation constant with iron makes them good candidates for iron determination (28-29). The aim of this study was to develop a fast, sensitive and selective spectrophotometric method for determination of Fe (III) ions in water and biological fluids using N-ethyl-2-methyl-3-hydroxypyridin-4-on (EMHP) as ligand.

MATERIAL AND METHODS

Chemicals and solutions

All reagents were of the highest available purity, at least analytical grade. Chemicals used were: iron (III) nitrate, acetic acid, sodium hydroxide, sodium chloride, sodium sulfate (Merck, Germany), N-ethyl-2-methyl-3-hydroxypyridin-4-on, potassium dihydrogen phosphate, sodium borate, potassium phosphate, potassium nitrate, sodium nitrate, calcium chloride, cupper sulphate, aluminium nitrate and sodium acetate (Aldrich). Serum samples were kept frozen at -20°C until analysis.

1. Standard Fe (III) solution (0.01 M) was prepared by dissolving 404 mg of ($Fe(NO_3)_3 \cdot 10H_2O$) in 100 ml of doubled distilled deionised water. The working solutions were prepared just before use by dilution of the standard solution with redistilled deionised water.
2. EMHP (0.01 M) stock solution: the aqueous stock solution of EMHP was prepared by dissolving 200 mg of EMHP in 100 ml of double distilled deionised water. The working solutions were prepared just before use by dilution of the standard solution with redistilled deionised water.
3. Acetate buffers of pH 4, 5 and 6 were prepared by mixing 0.05 M solutions of CH_3COONa and acetic acid.
4. Phosphate buffers of pH 7 and 8 were prepared by mixing 0.05 M solutions of KH_2PO_4 and 0.01 M NaOH.
5. Borate buffer of pH 9 was prepared by mixing 90 ml of 0.05 M solution of $Na_2B_4O_7$ and 10 ml of 0.5 M H_3BO_3 solution.
6. Standard solutions of K_3PO_4, KNO_3, NaH_2PO_4, $NaNO_2$, NaCl, $NaNO_3$, Na_2SO_4, $CaCl_2$, $CuSO_4$ and $Al(NO_3)_3$ were prepared by dissolving an appropriate amount of these salts in redistilled deionised water.

Instrumentation

The spectrophotometric analysis was performed on a double beam spectrophotometer Perkin Elmer 550 S (USA) using 1 cm quartz cells with a slit width of 1 nm.

Analytical procedure

Spectrophotometric condition

Into a 10-ml calibration flask an appropriate aliquot of 0.01 M Fe (III), 2 ml of buffer (pH = 5) and 1 ml of 0.01 M EMHP solution were placed. The mixture was filled to the mark with redistilled deionised water. Under the experimental conditions, the absorption spectra of EMHP and the Fe (III)–EMHP complex were scanned at 300-600 nm.

General procedure

A 100 µl aliquot of the working Fe (III) solution (0.01 M) was transferred into 10-ml volumetric flask and to it was added 1 ml of EMHP solution (0.01 M). The absorbance of the resulting solutions was measured after 20 min at 456 nm against blank. To find out the optimum conditions, the effects of pH (4, 5, 6, 7, 8, 9 and 10), time (0-60 min), EMHP concentration (EMHP/Fe molar ratio up to 20) and light were studied. Iron concentrations in the working standard solutions for the calibration curve were 2.5×10^{-6}, 5×10^{-6}, 7.5×10^{-6}, 1×10^{-5}, 5×10^{-5} and 1×10^{-4} M.

To prepare pooled serum or plasma for the use in sample blanks and the stock iron standard, ten random serum or plasma samples were ultrafiltered (using 0.25 µ filter) and tested for free iron by flame atomic absorption. Only "iron-free" serum or plasma ultrafiltrates were pooled, mixed and used to prepare a sample blank and iron standard solution (2.5×10^{-6}, 5×10^{-6}, 7.5×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4} M). EMHP (EMHP/Fe molar ratio = 10, same volume as serum or plasma) was added to samples and pH was adjusted to 5. After 20 min the absorbance was measured at 456 nm. Also, by using a standard kit containing ferrozine, standard solutions of iron in serum (2.5×10^{-6}, 5×10^{-6}, 7.5×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4} M) were measured spectrophotometrically at 562 nm.

Studies were conducted to determine whether other ions interfer with the spectrophotometric determination of iron. Different amounts of the potential interferences (K_3PO_4, KNO_3, NaH_2PO_4, $NaNO_2$, NaCl, $NaNO_3$, Na_2SO_4, $CaCl_2$, $CuSO_4$ and $Al(NO_3)_3$) were added to Fe (III) standard solutions, and the signals were compared.

To study the effect of light on the determination of Fe (III), three sets of experiment were carried out in the presence or absence (covering container by aluminium foil) of light.

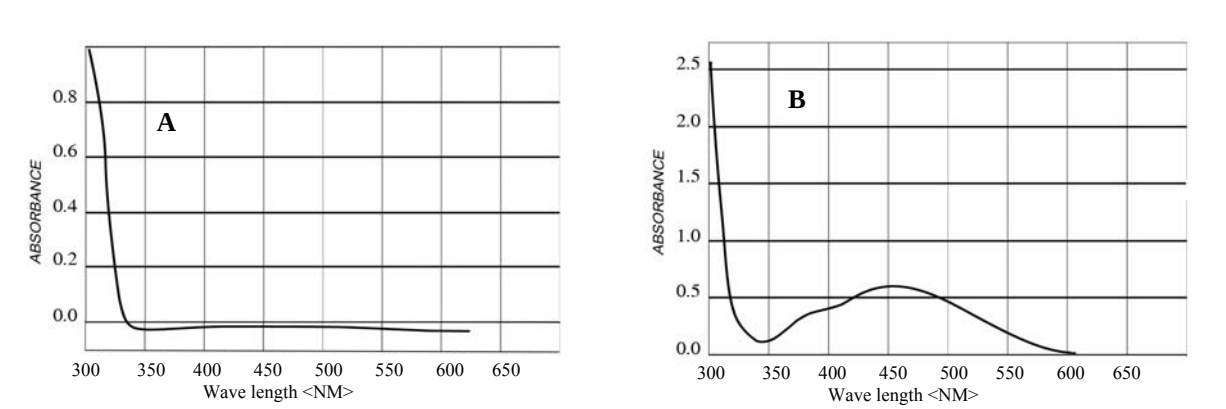

Figure 1. Absorption spectra: (A) Absorption spectra of the EMHP against water; (B) Absorption spectra of the EMHP-Fe (III) complex against reagent blank. pH=5, EMHP:Fe (III) molar ratio=10:1, (Fe (III)) =1×10^{-4} M, t=20 min.

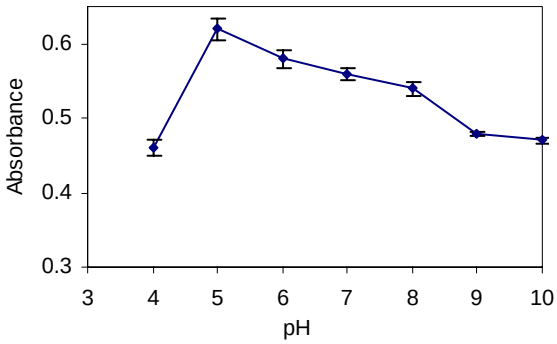

Figure 2. The effect of pH on the absorbance (means ±SD) of EMHP-Fe (III) complex. EMHP:Fe (III) molar ratio = 10:1, (Fe (III)) = 1×10^{-4} M, t = 20 min, λ = 456 nm, n = 9).

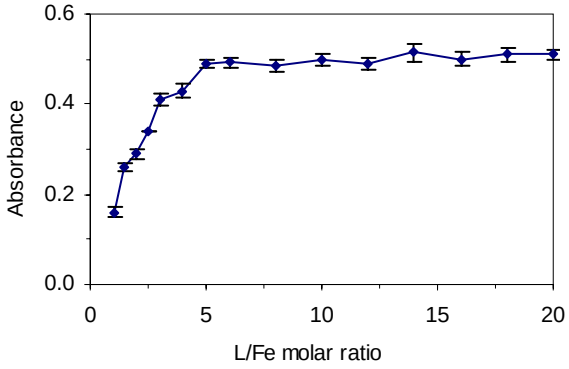

Figure 3. The effect of EMHP on EMHP-Fe (III) complex formation. pH=5, (Fe (III))=1×10^{-4} M, t =20min, λ=456nm. Each data point plotted represents the mean ± SD absorbance value for nine replicate absorbance readings.

To evaluate the validity of the method intraday and interday variation was studied. Briefly, for evaluation of intra-day variation, four sets of different solutions of Fe (III) in plasma or serum were prepared in one day and their iron concentrations were determined. For inter-day variation this procedure was repeated for four days.

RESULTS AND DISCUSSION

Spectrophotometric condition

The maximum absorption of Fe (III)–EMHP complex was at 456 nm, where EMHP by itself showed no absorption at this wavelength and therefore 456 nm was chosen as determination wavelength (Fig. 1).

Effect of pH

The effect of pH on the reaction of EMHP with Fe (III) is shown in Fig. 2. It is evident that pH 5 favors the complex formation; this value was selected as the working value.

Effect of EMHP concentration

Under the optimum pH value the effect of EMHP concentration on the absorbance profile is illustrated in Fig. 3. The molar ratio of 6-10 EMHP/Fe (III) was sufficient for complete complex formation. Considering the stoichiometry of the reaction between EMHP and Fe (III) (1:3) (30), since excess of EMHP reagent did not affect the absorbance of the complex, the molar ratio of 10:1 was used for experiments.

Table 1. Tolerance limit of electrolytes on the determination of iron

Electrolyte	Limiting concentration (molar ratio)
KNO_3	<1000
NaCl	<750
$NaNO_2$	<500
Na_2SO_4	<1000
$NaHCO_3$	<1000
$CaCl_2$	<1000
NaH_2PO_4	<1000
KH_2PO_4	<1000
K_3PO_4	<1000
$CuSO_4$	<1000
Al $(NO_3)_3$	<1000

pH = 5, EMHP:Fe (III) molar ratio = 10:1, (Fe (III)) = $1 * 10^{-4}$ M, t = 20 min, λ = 456 nm.

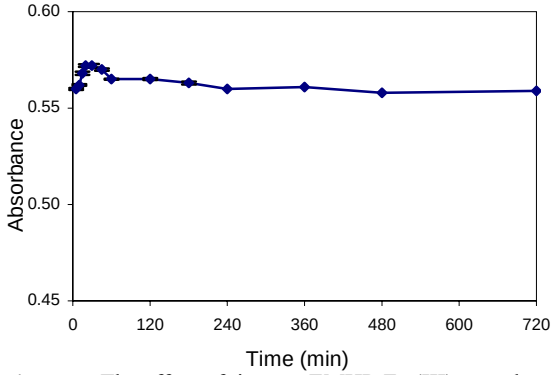

Figure 4. The effect of time on EMHP-Fe (III) complex formation. pH = 5, EMHP:Fe (III) molar ratio = 10:1, (Fe (III)) = 1×10^{-4} M, λ = 456 nm. Each data point represents the mean ± SD absorbance value for nine replicate absorbance readings.

Effects of time on complex formation and stability

The results from the optimization experiments showed that an incubation time of 20 min was adequate for quantitative complexation. The sample solution was examined with a Fe (III) standard solution (1×10^{-5} M) and no change in absorbance was observed up to 72 h (Fig. 4). The stability of these solutions provides an indication of the method robustness.

Other conditions

Although, temperature can affect complexation reactions, ambient temperature conditions were applied throughout the experiment, enabling the in situ application of the method. Our findings showed that light had no significant effect on absorbance up to 24 h.

Interference Studies

Studies were conducted to determine whether other cations or anions interfered with the spectrophotometric determination of iron. Different amounts of the potential interferences up to 1000 times molar ratio to that of Fe (III) were added to Fe (III) standard solutions (10^{-4} M), and the absorbance were compared. The results from these studies are shown in Table 1. Tolerance limits were determined for a maximum error of 5%. NaCl and NaNO2 interfered negatively with the spectrophotometric measurements when they were present at 1000 and 750 times of the iron concentration. The other tested metallic and anionic species had no adverse effect on the analytical signal(s) of Fe.

Analysis of real samples

The validation of the method was demonstrated by the analysis of Fe (III) at different concentrations in serum or plasma.

Linearity

Under the described spectrophotometric conditions, linear relationship was found between absorbance and Fe (III) concentration in serum (Fig 5), plasma and water. Standard deviation (SD) of intercept, slop and relative SD for slop are shown in Table 2.

Precision

The accuracy of the methods was validated by comparing the known amounts of Fe (III) which was added to serum and calculated concentration. The intra-day precision of the method was determined, under the optimal working conditions, by triplicate measurements of known Fe concentration. For determination of inter-day precision, the same procedure was repeated over a 4-day period. The findings for intra-day and the inter-day variation are illustrated in Table 3.

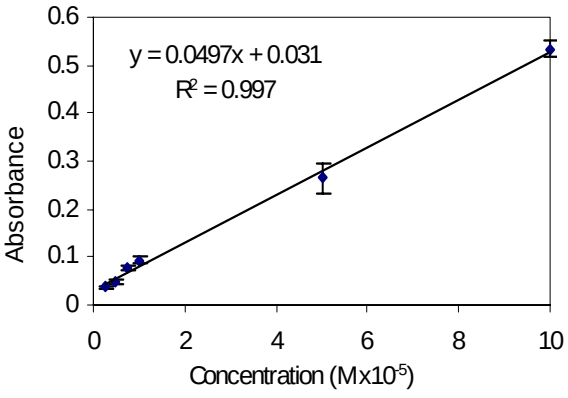

Figure 5. Standard curve for EMHP-Fe (III) complex in serum. pH = 5, EMHP:Fe (III) molar ratio = 10:1, t = 20 min, λ = 456 nm. Each data point plotted represents the mean ± SD absorbance value for nine replicate absorbance readings.

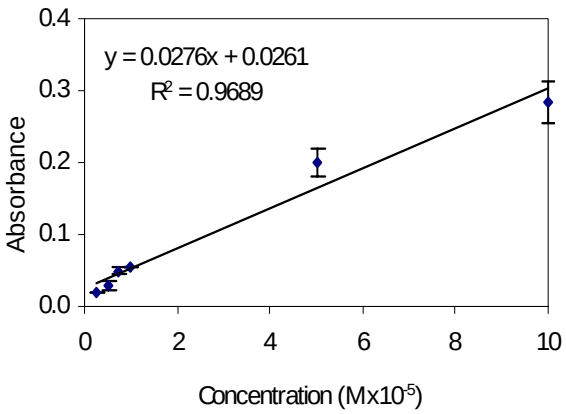

Figure 6. Standard curve for ferrozine-Fe complex in serum. pH = 4.5, ferrozine:Fe(III) molar ratio = 10:1, t = 20 min, λ = 562 nm. Each data point plotted represents the mean absorbance value for nine replicate absorbance readings. Error bars represent the standard deviation between consecutive measurements of each sample.

Table 2. Standard curves findings for iron determination in different samples

	Equation	R^2	Slope	RSD	Intercept
Serum	A=0.0497C+0.0310	0.9970	0.0497 ± 0.0014	2.81	0.031 ± 0.001
Plasma	A=0.0482C+0.0379	0.9874	0.0482 ± 0.0016	3.25	0.027 ± 0.001
Water	A=0.0515C+0.0182	0.9986	0.0515 ± 0.0011	2.74	0.034 ± 0.002
ferrozine-Fe complex in serum	A=0.0276C+0.0261	0.9689	0.0276 ± 0.0019	4.12	0.033 ± 0.003

A = absorbance, C = concentration in M, RSD = (SD/Mean)*100. All statistical calculations were based on nine replicates for each standard solution in the given range.

Table 3. Intra-day and Inter-day variation

Fe(III) Concentration (M) $\times 10^{-6}$	Calculated Concentration (M) $\times 10^{-6}$ (intra-day)				Calculated Concentration (M) $\times 10^{-6}$ (inter-day)			
	Mean	SD	%CV	%Error	Mean	SD	%CV	%Error
2.50	2.03	0.23	11.52	-18.80	2.18	0.35	16.01	-12.80
5.00	4.91	0.17	3.37	-1.80	4.66	0.35	7.68	-6.80
7.50	7.95	0.67	7.01	6.00	8.15	0.61	7.56	8.67
10.00	13.48	1.12	8.31	8.80	12.68	0.87	6.82	9.80
50.00	49.63	3.34	6.74	-0.74	46.52	2.68	5.69	-6.96
100.00	98.58	3.92	3.98	-1.42	101.29	3.29	3.26	1.29

To evaluate intra-day variation, four sets of different solutions of Fe (III) in serum were prepared in one day and their iron concentrations were determined. For inter-day variation this procedure was repeated for four days.

Limit of quantitation and detection

The detection limit indicates the smallest amount of analyte which can be detected with a reasonable degree of confidence under specified conditions. This is usually defined as the concentration or mass of analyte yielding a signal three times of the standard deviation of the blank signal (signal at zero analyte) (31). It was 2.5×10^{-6} M of Fe (III) in serum or plasma.

Comparison with other spectrophotometric methods

Results of this investigation showed that the proposed method was more sensitive than the method using a standard kit containing ferrozine (Fig. 6) and there was less variation in determination of iron compared to that of ferrozine kit. Several ligands have the ability to form a stable colored complex with Fe (III). The detection limit for Fe (III) when bitonol was used as a complex forming ligand was 1.7×10^{-5} (32). When thiocyanate, 4-capril-3-methyl-1-phenyl-5-pyrazolone, 2-hydroxy-3, 5-dimethl aceto-phenone, or 1,2 cyclohexane dioxamid are used as ligands, it is necessary to extract the colored complex with an appropriate solvent (15, 33-36). Also the sensitivity of some of those methods are less than that of the proposed method.

CONCLUSION

Compared to the reported spectrophotometric methods, the proposed method offers several noticeable advantages: 1. By this method, Fe (III) can be determined directly. 2. It is a fast method and the stability of colored complex is very good, The reaction of EMPH with Fe (III) was completed within 30 min, and the formed complex was stable up to 24 h. 3. EMHP, as a new reagent for spectrophotometric determination of Fe (III), is cheap and readily available, has very stable physicochemical properties and can be used to determine trace amount of Fe (III) conveniently. 4. The proposed method shows good selectivity. Since most anions and cations do not interfere with determination of Fe (III), this method may be applied in determination of Fe (III) in various complex samples such as water, serum or plasma without necessity of extraction by organic solvents.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from Research Council of Isfahan University of Medical Sciences.

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