The Application of Ion Chromatographic Method for Bioavailability and Stability Test of Iron Preparations

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Postabsorptive serum iron level was determined after oral administration of the compounds to human. In serum and whole blood, Fe^{3+} was measured by ion chromatography (IC) using a pyridine-2,6-dicarboxylic acid (PDCA) as an eluent. The serum sample solutions were pretreated with I N HCl and 50% TCA. The whole blood sample solutions were treated with 3 N HCl for 30 min at 125°C. The limit of detection (LOD) of the IC technique is 0.2 μM for Fe^{2+} and 0.1 μM for Fe^{3+} The area under concentration (AUC) can be obtained by the above analytical condition. In addition, to compare the stability of Fe^{2+} to that of Fe^{3+} in pharmaceutical preparations, accelerated stability test was carried out. After storing the samples under 40°C, 75%RH in light-resistant container for various time intervals, the contents of iron of different valencies were determined separately by the IC technique and the change and/or the interchange of among those iron species in preparations was investigated. Iron raw materials are stable, but Fe^{2+} in Fe^{3+} source materials was slightly converted to Fe^{3+} by oxidation. Fe^{2+} in Fe^{3+} source raw materials and Fe^{3+} in Fe^{2+} raw materials are determined as impurities. Therefore, IC technique is found to be an appropriate method for comparative evaluation of dissimilar bioavailability of Fe^{2+} and Fe^{3+}, stability of Fe^{2+} and Fe^{3+} raw materials and preparations.

Key words : Ion chromatography (IC), Bioavailability, Stability, AUC, LOD, Fe^{2+}, Fe^{3+}

INTRODUCTION

Absorption and utilization of Fe^{2+} and Fe^{3+} compounds used as oral ferrotherapy of iron deficiency have been studied by many investigators in past (Choi *et al.*, 1998; Kaltwasser *et al.*, 1987; Bothwell *et al.*, 1979; Brise *et al.*, 1962). However, their evaluations of therapeutic efficacy of iron from those two different sources lead to contradictory results and these long-standing discrepancies about optional ion therapy still remain unresolved (Danielson *et al.*, 1996; Muller and Geisser 1979; Dietzfelbinger *et al.*, 1977; Dietzfelbinger *et al.*, 1979; Heinrich *et al.*, 1979; Heinrich *et al.*, 1983). In this study, to compare the bioavailability of iron of different valency state, we examined post absorptive serum iron level as an indicator of their bioavailability. Ferrous sulfate, ferrous succinate and ferrous fumarate as ferrous iron source and ferric hydroxide polymaltose complex, iron proteinsuccinylate and ferritin extractive as ferric iron source were investigated in present experiment and serum iron levels after oral administration of the preparations were determined by IC. Although there are many different methods of determination for serum iron concentration, wide variation exists in estimated iron concentrations (Tietz *et al.*, 1994). The conventional spectrophotometric assay has problems such as coprecipitation and occlusion of iron during sample pretreatment (Ryall *et al.*, 1970) and twin isotope technique, a commonly used technique in the elucidation of bioavailability of ferrous and ferric iron, requires the labeling of commercial product in manufacturing procedure (Geisser *et al.*, 1987). A capillary electrophoresis has been applied in the determination of the iron level in human serum (Che *et al.*, 1995), but this method has the problem that phenanthroline complex should be formed to have a strong absorbance at 270 nm. Iron in chicken conalbumin was separated by capillary electrophoresis (Huang *et al.*, 1977). The metals (Zn, Cu, Fe) in healthy school-children were determined by flame atomic absorption spectrometry (Alarcon *et al.*, 1997). In this study, the method originally developed by Abdulla W. Al-Shawi *et al.* (1996) was used with a slight modification to apply for bioavailability and stability studies for iron preparations. Procedure of serum, blood sample pretreatment was developed to use on bioavailability test of iron preparations.

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MATERIALS AND METHODS

Materials

Pyridine-2,6-dicarboxilic acid (PDCA), 4-[2-pyri-dylazo] resorcinol (PAR), ferrous chloride and ferric fluoride were obtained from Dionex (Sunnyvale, CA, USA). Acetic acid, sodium acetate, trichloroacetic acid, sodium hydroxide and ammonium hydroxide were purchased from Wako Co. (Osaka, Japan). Deionized water was prepared with a Milli-Q system (Millipore, Bendford, MA, USA). Human serum and whole blood samples were collected from volunteers.

Equipments

A model DX-300 Ion chromatograph equipped with a pump, a UV/Visible absorbance detector, reaction coil and postcolumn reagent delivery module was purchased from Dionex. The analytical column, containing the substrate surface grafted with a quaternary ammonium group, coated with sulfonated cation exchange latex and with aminated anion exchange latex (25 cm×4 mm I.D, 13 μm, particle size), was purchased from Dionex. A model SpectraSystem UV1000 UV/Visible absorbance detector (Thermo Separation Products, mountain view, CA, USA) was set at 520 nm. An AI-450 software (Dionex, sunnyvale, CA, USA) was used to collect the data. An incubator (Vision scientific, Korea) was used to store the iron raw materials for stability test. The eluent was prepared with 6 mM PDCA, 50 mM acetic acid and 50 mM sodium acetate, and postcolumn reagent was prepared with 0.3 mM PAR, 1M acetic acid and 3M ammonium hydroxide. All solutions were prepared using deionized (DI) water. The flow rate of postcolumn reagent was 0.5 ml/min.

Preparation of standard solutions

Ferrous and ferric iron standard solutions were prepared by dissolving ferrous chloride ($FeCl_2 4H_2O$) and ferric fluoride (FeF_3) with DI water. If necessay, and these solutions were diluted to appropriate concentrations.

Preparation of sample solution for bioavailability

The procedures for serum sample treatment have been significantly modified from those described in the literature (Bjerve, *et al.*, 1976). To 100 μl serum, 200 μl 1 N HCl and 50 μl 50% trichloroacetic acid (TCA) was added. The mixture was sonicated for 30 s to assure that lumps of precipitated protein were disintegrated into small pieces. This was allowed to stand for 10 min, then centrifuged at 5700×g for 10 min. The supernatant was injected to IC System. Iron level in serum was determined 4 hours after volunteers took the six iron preparations respectively, since iron is absorbed in the stomach for 3~4 hours. In case where preparation of sample solution from whole blood is necessary, five hundred microliters of 3 N HCl was added to 500 μl human whole blood. The mixture was stored in the autoclave (at 125°C) for 30 min, centrifuged at 5,700×g for 10 min, the supernatant injected on to IC system.

Preparation of sample solution for stability test

The iron raw materials in the light resistant container were stored in an incubator at 40°C, 75 % RH for six months. Each raw material was accurately weighed to contain 10 mg as iron at one month intervals. The weighed sample was dissolved with 10 ml 1 N HCl, sonicated for 10 min and diluted to 100 ml with deionized water. The sample solution was obtained by a further dilution of above solution. Iron proteinsuccinylate was dissolved with 0.1 N NaOH instead of 1 N HCl. The pHs of the solution was adjusted to 2.5, 7.0 and 12.5 respectively. The commercial products were accurately weighed to contain 10 mg iron as iron elements, and sample solution was obtained by a procedure similar to the above.

Relative recovery

Hundred microliter of iron standard solution was added to 100 μl serum. Sample solution was prepared by the same method of sample solution for bioavailability test.

RESULTS AND DISCUSSION

Optimal condition of IC for separation

Fig. 1 shows IC chromatogram of non-pretreated serum. Representative IC chromatogram of a serum sample is shown in Fig. 2, which demonstrates that the interferences with Cu, Zn and Mn are completely eliminated. The iden-tification of peaks was carried out by injection of sample solution spiked with standard solution (Fe^{2+}; 10 μg/ml, Fe^{3+}; 10 μg/ml). The determination of iron in the human serum is performed with the optimal condition described in equipments. This procedure was found to have no interference by other components. The IC chromatogram of human whole blood is shown in Fig. 3. This result shows that determination of iron in the human whole blood is not influenced by components in the sample. Determination of iron in serum is not influenced by iron (impurity) in HCl because iron level contained in HCl added to serum sample solution is lower than iron level in serum and can be ignored. Fig. 4 is an IC chromatogram of iron preparations, indicating that Fe^{2+} and Fe^{3+} can be determined simultaneously without any interference of components in the preparations and reagents.

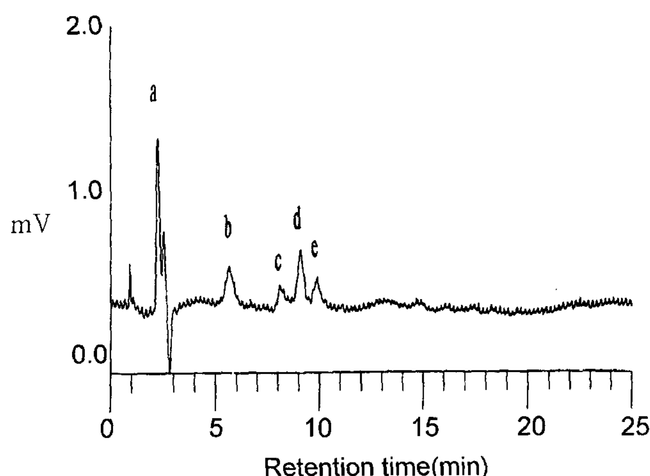

Fig. 1. IC chromatogram of untreated serum. Peak a, c: unknown, b: iron (Fe^{3+}), d: copper, e: zinc. The analytical conditions are as follows; analytical column containing the substrate surface grafted with a quaternary ammonium group, coated with sulfonated cation exchange latex and with aminated anion exchange latex (25 cm×4 mm I.D, 13 μm, particle size), detector: UV/Visible absorbance detector (set at 520 nm), eluent: prepared with 6 mM PDCA, 50 mM acetic acid and 50 mM sodium acetate, flow rate: 1.0ml/min, postcolumn reagent: prepared with 0.3 mM PAR, 1M acetic acid and 3M ammonium hydroxide (flow rate: 0.5 ml/min), injection volume: 100 μl.

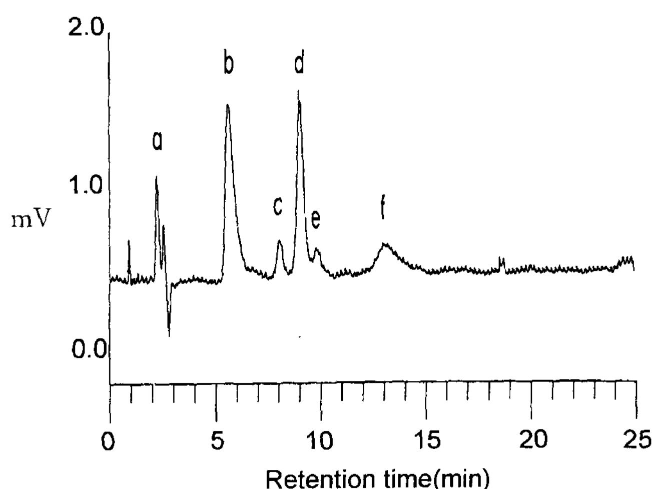

Fig. 2. Representative chromatogram of serum sample treated with HCl/TCA, Peak identification: a, c: unknown, b: Fe^{3+}, d: copper, e: zinc, f: manganese, The analytical conditions are identical to those in Fig. 1.

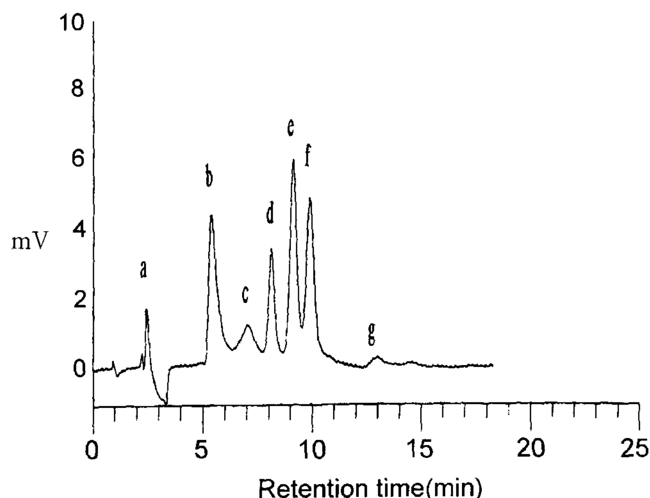

Fig. 3. IC chromatogram of whole blood, Peak identification: peak a,c,d: unknown, b: Fe^{3+}, e: zinc, f: zinc, g: manganese, The analytical conditions are identical to those in Fig. 1.

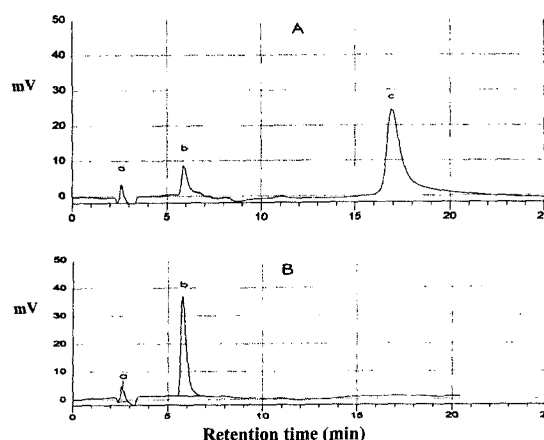

Fig. 4. IC chromatogram of commercial products containing ferrous sulfate (A) and ferritin extractive (B). peak a: unknown, b: Fe^{3+}, c: Fe^{2+}, The analytical conditions are identical to those in Fig. 1.

Sensitivity and linearity

The limit of detection (LOD) of IC technique is as low as 0.2 μM for Fe^{2+} and 0.1 μM for Fe^{3+} with signal-to-noise ratio of 5. This LOD was about 4 times less sensitive than that obtained with high performance capillary electrophoresis method (LOD of Fe^{2+}: 0.05 μM) at 270 nm (Che *et al.*, 1995). The assay for iron (Fe^{3+}) in serum is linear from 1 to 30 μM. Iron concentration in human serum is ranged from 10 to 35 μM as determined in 4-time diluted samples. Therefore, linearity between 2.5 and 8.7 μM is quite relevant in our studies. The assay for iron (Fe^{3+}) in whole blood is linear from 1.8 mM to 9 mM. In case of stability test, the detector response is linear from 18 μM to 360 μM (for both Fe^{2+} and Fe^{3+}).

Relative recovery

The results of recovery test are shown in Table I. The recovery of Fe^{2+} and Fe^{3+} is ranged from 97.1% to 99.0%.

Effect of different serum treatment methods on the determination of iron

The method used for the pretreatment of serum

Table I. The percent recovery of I.C method on the determination of iron in serum

C_{Fe} in serum (μM)		Stanandard C_{Fe3+} Added (μM)	Measured (μM)	Recovery (%±S.D.)
Fe^{2+}	31.7	10	41.8±0.2	98.3±1.2
	32.8	20	52.5±0.4	97.9±1.6
	32.1	30	60.1±0.2	96.1±2.1
Fe^{3+}	31.2	10	40.5±0.1	97.3±0.9
	31.8	20	52.3±0.5	99.0±1.1
	33.5	30	62.5±0.4	98.4±1.5

n: 5, S.D.: standard deviation. The analytical conditions are the same as those in Fig. 1.

Table II. The effect of pretreatment

Sample treatment			Iron level in serum [μM Fe^{3+}]
Sonication	IN HCl	50% TCA	
Yes	Yes	Yes	36.5
Yes	Yes	No	33.4
Yes	No	Yes	5.1
No	no	No	0.0

n=3. The analytical conditions are the same as those in Fig. 1.

samples significantly affects the accuracy of the determination of iron level. In order to study the accuracy of our technique, we compared our method with HPCE method at 270 nm. The data listed in Table II shows that the addition of 1 N HCl plays an important role in the accuracy. Table II shows that the the addition of 1 N HCl and 50% TCA resulted in a better accuracy than the addition of 50% TCA alone. Therefore, Fig. 1 and 2 shows that iron concentrations in serum are different according to pretreatment procedure. Also table II shows that an accuracy in case of sonication is better than those without.

Determination of iron in human serum and whole blood after the oral administration of iron preparations

Table III shows that only Fe^{3+} can be determined in serum and whole blood after raw materials (Fe^{2+}, Fe^{3+}) are administered. Iron concentration is different among postabsorptive serum and whole blood, and those before administration. AUC can be determined readily, indicating that this method can be applied in the determination of iron in serum and whole blood after the administration of various iron preparations.

Stability of iron raw materials

Iron raw materials were comparatively stable but Fe^{2+} may change to Fe^{3+} by oxidation. Iron raw materials are chemically converted by oxygen, heat, humidity and other factors to free iron, Fe^{2+} and Fe^{3+}. Table IV shows that iron raw materials are stable but Fe^{2+} in Fe^{2+} source raw materials slightly changes to Fe^{3+} by oxidation. Korean Food and Drug Administration (KFDA) has required the specification of iron raw materials, ruled limit of Fe^{2+} and Fe^{3+} as impurities. Therefore, simultaneous determination iron content of raw materials and impurities, such as this method, would be very useful. In case of iron proteinsuccinylate sample solution (pH 2.5), only about 50% of iron content was determined. Iron content in other samples (pH 7.0, 12.5) were not detectable. Therefore, iron proteinsuccinylate can not be determined reliably with this method.

Assay of the commercial products

Iron (Fe^{2+}, Fe^{3+}) in the pharmaceutical preparations, marketed in Korea, was determined by the IC technique. Table IV lists that Fe^{3+} contained in raw materials. Fe^{2+} contained in Fe^{3+} raw materials was found to be converted to Fe^{3+} by oxidation. Ferric raw materials (ferritin extractive, iron hydroxide polymaltose complex) was more stable than ferrous raw materials (ferrous sulfate, ferrous fumarate, ferrous succinate).

CONCLUSION

The IC technique and procedure of pretreatment for the quantitative determination of serum iron has been modified and developed. The method is sensitive, rapid, and accurate and requires only 100 μl serum sample and 500 μl whole blood. The separation could be achieved within 20 min and the components such as

Table III. Determination of iron in human serum and whole blood after administration of iron preparations

Iron preparations	Assay (Fe^{3+}, μM)			
	SB	SA (4 hr)	WB	WA (1 day)
A (Fe^{2+}, 34 mg)	32.3±1.6	34.1±2.0	310.7±25.6	318.9±25.1
B (Fe^{2+}, 10 mg)	29.4±1.2	29.7±2.2	295.4±20.7	298.2±22.1
C (Fe^{2+}, 35 mg)	25.1±1.1	26.3±1.5	279.9±18.5	289.5±18.9
D (Fe^{3+}, 80 mg)	33.6±2.5	35.1±2.7	350.2±22.1	379.1±15.4
E (Fe^{3+}, 20 mg)	30.7±2.2	31.1±1.7	305.3±15.4	310.5±20.6

SB: Serum iron level before oral administration, SA: Serum iron level after oral administration, WB: Whole blood iron level before oral administration, WA: Whole blood iron level after oral administration.

Table IV. Accelerated stability test of iron rae materials

Samples	Assay [Fe^{2+}/Fe^{3+}] (%)					
	1	2	3	4	5	6(month)
Ferrous sulfate	98.5/0.0	98.3/0.1	98.1/0.5	95.3/2.1	94.3/3.2	94.1/4.2
Ferrous Succinate	98.3/0.0	98.1/0.2	97.2/0.4	97.1/1.1	96.7/2.5	96.3/2.8
Ferrous fumarate	99.2/0.0	99.0/0.1	98.2/1.0	97.2/2.0	96.9/2.3	96.7/2.4
FHPC	0.0/98.8	0.0/98.7	0.1/98.5	0.5/98.3	1.0/97.2	1.5/97.1
Ferritin extractive	0.0/98.	0.0/98.4	0.2/98.3	0.3/98.1	0.7/97.1	1.0/97.3

402°C, 75%RH. FHPC: Ferric hydroxide polymaltose complex, %: Percent for label claim.

Table V. Determination of iron in the commercial pharmaceutical preparations

Samples	Dosage form	Component	Assay (%)	Impurity (%) (Fe^{2+} or Fe^{3+})
A	Soft cap.	Ferrous sulfate	95.6	5.2
B	Soft cap.	Ferrous Succinate	97.5	3.5
C	Film coated Tab.	Ferrous fumarate	98.7	4.7
D	Sugar coated Tab.	FHPC	99.7	–
E	Hard cap.	Ferritin extractive	99.5	–

FHPC: Ferric hydroxide polymaltose complex

copper, zinc and manganese etc. in blood do not interfere with the separation. LODs for Fe^{3+} and Fe^{2+} are 0.1 μM and 0.2 μM respectively. Percent recovery of Fe^{2+} and Fe^{3+} is ranged from 97.1 to 99.0%. Iron in raw materials and impurities are readily determined by this method simultaneously. Therefore, these results indicate that this method and serum pre-treatment procedure is applicable in the bioavailability test and stability test of the iron pharmaceutical preparations.

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