Pharmacokinetics study of hemin in rats by applying $^{58}$Fe-extrinsically labeling techniques in combination with ICP-MS method

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**A B S T R A C T**

Iron is a challenging element due to its high background in various matrixes including blood, tissues even in the air and it is urgent to develop a method for the accurate determination of iron in bio-samples. After optimization of mass spectrometric conditions using collision cell technology and compensating for interference using a mathematical correction equation, an inductively coupled plasma mass spectrometry (ICP-MS) method for the quantitative determination of $^{58}$Fe originating from hemin extrinsically labeled avoiding endogenous interference was developed. After a single step of dilution, analysis of each sample was completed within 1.5 min. The assay was linear in the concentration range from 0.005 to 1.0 μg/ml. The precisions and accuracies determined within three consecutive days were in acceptable limits and there was no significant matrix effect. The optimized method was successfully applied to a pharmacokinetic study of $^{58}$Fe originating from hemin extrinsically labeled and iron absorption measured in rats was 1.07%. Those indicated that extrinsically label techniques in combination with ICP-MS will become a new tool for the analysis of iron preparations and other endogenous substances.

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1. Introduction

Iron is one of the important essential trace elements in the human body. Iron deficiency is currently the most common micronutrient deficiency worldwide affecting more than 40% of women at child-bearing age in developing countries [1]. To reduce the prevalence and severity of iron deficiency, many countries such as Mexico [2], Sri Lankan [3], and Peruvian [4] require iron supplements into their daily diet. There are various iron preparations on the market and they can be either heme-iron or nonheme-iron and heme-iron absorption was considered to be greater than nonheme-iron absorption [5]. The iron in heme is in its oxidized state, while it is in reduced state in hemin, iron (III) protoporphyrin chloride (IX) (chemical structure shown in Fig. 1) [6]. That means the use of hemin in absorption study of iron instead of heme is very meaningful considering the instability of heme molecule, and this view is supported in Villarroel and Jahn’s in vitro study [6,7]. One of the first papers to describe intrinsic labeling using stable isotope for iron absorption measurement. However, it is difficult to evaluate the absorption of iron from heme in vivo because of its high background in blood. Thus the determination of iron in bio-samples is considered to be of great importance.

Presently the most widely used method is probably based on the quantitation of elemental iron by atomic absorption spectroscopy [8–10]. However, one of the major limitations of these methods is the lack of adequate sensitivity required for pharmacokinetic studies. For example, the limit of quantitation for iron was only 1.0 μg/ml and the analytical range was just from 1.0 to 4.0 μg/ml in Shang’s method [8]. Due to the poor sensitivity, it is very difficult to extend these methods to bio-sample analysis. Also, these methods were developed for the quantitative determination of iron in food or pharmaceutical preparations while iron is a challenging element due to the substantially high background in various matrices including blood, tissues even in the air and water and it is very difficult to distinguish between endogenous and exogenous iron. Therefore, a method using labeled iron as a tracer is required for the accurate determination of iron in bio-samples.

Radioisotopes of iron including $^{57}$Fe and $^{59}$Fe were usually used as tracers in the research of uptake and metabolism of iron.
In Swati’s method, $^{59}\text{Fe}$ was successfully used to evaluate the effects of dietary factors on iron uptake from ferritin in Caco-2 cells [13]. However, due to the risk associated with exposure to radiation, stable isotopes may be a better choice. Iron has 4 naturally occurring isotopes, including $^{54}\text{Fe}$ (5.85%), $^{56}\text{Fe}$ (91.75%), $^{57}\text{Fe}$ (2.12%) and $^{58}\text{Fe}$ (0.28%). The existence of polyatomic ion interferences particularly for $^{54}\text{Fe}$, $^{58}\text{Fe}$ [14] and the lowest abundance for $^{58}\text{Fe}$ in blood make it a good opportunity for pharmacokinetic research [15]. While to our knowledge, no successful attempts to extrinsically label hemin with $^{58}\text{Fe}$ have been published.

Since 1983, when inductively coupled plasma mass spectrometry (ICP-MS) became commercially available firstly [16], it has been demonstrated to be a valuable tool for the determination of drugs containing metals, due to its excellent sensitivity and selectivity [17]. What’s more, compared to negative thermal ionization mass spectrometry (NTIMS), there are many advantages for ICP-MS, such as multi-element capabilities, much easier sample preparation and the speed of isotopic analysis [18,19]. Recently a large number of papers reporting the use of ICP-MS for iron isotope ratio determinations have been published [20–22]. However, in Whittaker’s method, the sample analysis was too time consuming and the isotope ratio, not the isotope concentration was treated as the information for absorption study. More importantly, this method has not been validated systematically [14,23]. In fact, the similar phenomenon was existed in Ronny Schoenberg’s research, the sample analysis was too time consuming and working solutions were stored at 4°C.

2. Materials and methods

2.1. Chemicals and reagents

Stable isotope, iron-58, as metal powder (>99.86%, enrichment), was obtained from ISOFLEX USA (San Francisco, CA, USA). Hemin extrinsically labeled with $^{58}\text{Fe}$ was supplied by State Key Laboratory of Natural Medicines, School of Basic Science, China pharmaceutical university. Germanium Plasma Emission Standard (>99.999%, purity) was supplied by Shanghai ANPEL Scientific Instrument Co., Ltd. (Shanghai, China). Triton X-100 of chemical purity and Nitric acid of MOS grade were from Sinopharm Chemical Reagent Company (Shanghai, China). N-butyl alcohol of analytical grade was purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Standard stock solution of $^{58}\text{Fe}$ was prepared in nitric acid (2%, v/v) at 2 mg/ml and germanium (Ge) was in a solution (nitric acid 2%, v/v) at 1 mg/ml, then were serially diluted to working solution with nitric acid (2%, v/v). All the stock and working solutions were stored at 4°C.

2.2. Apparatus

All analytical experiments were carried out on an ICP-MS (XSERIES 2, Thermo Scientific Waltham, MA, USA), which was operated with the Plasma Screen Plus sensitivity enhancement option fitted, Xt interface cones and with peltier cooling of the spray chamber. A standard quartz nebulizer was used, together with a standard quartz impact bead spray chamber, standard single piece, and 1.5 mm i.d. injector quartz torch. The instrument was operated using collision cell technology (CCT) mode (using 7% (v/v) H$_2$ in He as the collision gas). Plasma Lab software was applied for instrument control, data acquisition and analysis.

2.3. Determination of $^{58}\text{Fe}$ by ICP-MS

The instrumental and operating condition were optimized with the multi-element tune solution. Typically, this solution gave readings of $^{114}\text{In}$: $>4 \times 10^4$ c/s; $^{59}\text{Co}$: $>1.5 \times 10^4$ c/s; $^{238}\text{U}$: $>8.0 \times 10^4$ c/s. Performance was checked daily. Ge (50 ng/ml) was added online using of three-way valve as internal standard.

The operating parameters of ICP-MS instrument at CCT mode were as follows: RF power 1500 W, coolant gas flow 14.3 L/min, CCT gas flow (7% H$_2$/He): 3 ml/min; auxiliary gas flow 0.55 L/min; nebulizer gas flow 0.87 L/min; pump rate 1.0 ml/min; peak jumping data acquisition mode: dwell time 10 ms; uptake timings 40 s and three replicates per sample.

For the detection of iron, the signals of $^{54}\text{Fe}$, $^{58}\text{Fe}$, $^{60}\text{Ni}$, $^{53}\text{Cr}$ and $^{72}\text{Ge}$ (IS) were monitored but only $^{58}\text{Fe}$ were used for calculations. Other elements were used to correct interference, correction formula was as follows:

\[
^{58}\text{Fe}_{\text{act}} = \text{Total counts at mass 58} - 0.05593 \times (^{54}\text{Fe} - 0.24921 \times ^{53}\text{Cr}) - 2.59021 \times ^{60}\text{Ni}
\]

2.4. Sample preparation

An aliquot of 0.05 ml rat plasma was diluted to a final volume of 1.0 ml using the dilution solution containing Triton X-100 (0.01%, v/v), nitric acid (0.05%, v/v) and N-butyl alcohol (2%, v/v). After vortex-mixing, the aliquot was injected for analysis by ICP-MS. All the samples were prepared using plastic pipes.
2.5. Method validation procedures

Full validation according to the FDA guidelines was performed for the method.

The selectivity of the assay for $^{58}$Fe versus endogenous substances in the matrix was assessed by comparing the scans from six different blank rat plasma, blank rat plasma spiked with analyte and the rat plasma sample. These samples were treated according to the sample preparation procedure described above.

The calibration curves were prepared at different days. The plasma samples with concentrations of 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.8, 1.0 µg/ml for $^{58}$Fe were freshly prepared with blank plasma and working solution under the same conditions as the test samples. Modified CPS (counts per second) was used for calculations. Quality control samples containing three different concentrations (0.01, 0.1, 0.8 µg/ml) were prepared in the same way for calibration curve samples. The low limit of quantitation (LLOQ) was calculated as the concentration at a signal-to-noise ratio of 10:1.

For precision validation, five QC samples at three different concentrations were evaluated for three successive days. The accuracy was determined by calculating the percentage of deviation observed in the analysis of QC samples and expressed as the relative error (RE%). The precision was evaluated by the relative standard deviation (RSD%). Both RE and RSD should generally be less than 15%, and less than 20% in the vicinity of lower limit of quantification. The absolute recoveries and matrix effects for $^{58}$Fe from plasma were calculated at three QC levels according to the modified equation in Erin Chambers’s research [24].

Stability experiments were performed to evaluate the stability for $^{58}$Fe using five QC samples at three different levels in plasma samples under different temperatures and timing conditions: short-term stability storage at room temperature for 4 h, post-preparative stability at 4 °C for 24 h, three cycles of freeze-thaw (room temperature) stability, and long-term stability storage at −20 °C for 15 successive days. Samples were considered to be stable if 85–115% of the initial concentration was recovered.

2.6. Application in pharmacokinetic studies

Male and female Sprague-Dawley rats ($n = 24, 200 \pm 20$ g) were provided by the Shanghai Sino-British Sippr/BK LAB Animal Co. Ltd (Shanghai, China) and certificate number was scxk (hu) 2008-0016. Animals were allowed to adapt to the housing environment (20 ± 2 °C, 50 ± 20%, with natural light–dark cycle) for 1 week prior to study. The animals were fasted overnight (12 h) before the experiment and had free access to water throughout the experimental period. All animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

Rats were randomly divided into two groups: i.v. and i.g., with three sub-divisions for different dosages in i.g. group and six rats in each group. The dosages were 2 mg/kg (equivalent to $^{58}$Fe at 0.18 mg/kg) with the volume of 4 ml/kg for i.v. and 40, 80 and 120 mg/kg (equivalent to $^{58}$Fe at 3.55 mg/kg) with the volume of 10 ml/kg for i.g. Blood-sampling times were arranged as 0, 0.5, 1, 1.5, 2, 4, 8, 15, 24, 36 h and 0, 0.17, 0.5, 1, 2, 4, 8, 15, 24, 36 h post-dose for i.g. and i.v. groups, respectively. When each time point reached, blood samples (about 200 µl) were collected from the fossa orbitalis and centrifuged immediately at 12,000 g for 3 min. Plasma of 50 µl volume was finally harvested. All samples were stored at −20 °C until analysis.

2.7. Data analysis

The pharmacokinetic parameters were calculated using the pharmacokinetic software DAS 2.1.1 (China) by non-compartmental method.
interference on the determination of $^{58}\text{Fe}$ and the intercept has dropped to 1.597 from 770.8 while the coefficient has been promoted to 0.999 from 0.990.

As shown in Table 1, there is still much spectral interference such as $^{34}\text{Ar}^{+}/^{54}\text{Fe}^{+}$, $^{34}\text{Ar}^{+}+^{13}\text{C}(1.1\%),^{17}\text{OH}^{+}+^{36}\text{Ar}(0.3\%),^{14}\text{N}^{+}+^{37}\text{Cl}(24.5\%),^{12}\text{C}^{+}+^{41}\text{K}(6.9\%)$ in the analysis of $^{58}\text{Fe}$ by ICP-MS. Compared with standard mode, CCT mode using 8% (v/v) $\text{H}_2$ in He as the collision gas could greatly improve the polyatomic ion interferences. The spectrum in the absence or presence of $^{58}\text{Fe}$, and the peak at $^{58}\text{Fe}$ was almost at very low background level.

3.1.2. Selection of internal standard

In this method Ge was chosen as IS for its similar mass with iron and the abundance is low enough to ignore in the sample solution as one of rare element.

3.1.3. Carry-over effect

To evaluate and minimize the carry-over effect, the signals of water following the ULOQ (upper limit of quantitation) calibration sample were detected. A 40 s rinse time with 2% HNO$_3$ was required to avoid a memory effect from the preceding high concentration sample.

3.2. Method validation

3.2.1. Selectivity and linearity

The selectivity was checked with six different blank plasma samples. No significant interferences from the endogenous plasma components were observed at the same mass of the analyte.

Standard curves for $^{58}\text{Fe}$ in spiked rat plasma exhibited good linearity over the concentration ranges 0.005–1.0 $\mu$g/ml with the correlation coefficients ($R^2$) > 0.99.

The LLOQ for $^{58}\text{Fe}$ in spiked rat plasma was proved to be 0.005 $\mu$g/ml.

3.2.2. Assay precision and accuracy

Assay precision and accuracy were calculated after analysis in three different analytical runs. The data indicated good accuracy and precision of the method. As shown in Table 2, the accuracy values for intra- and inter-day were all within 100 ± 10% of the actual values at each QC level; the intra- and inter-day precisions of the assay were below 10.86% for $^{58}\text{Fe}$. The results indicated that the method was reproducible. In general the accuracy (10.8%) achieved analytically in this paper has shown to be much better than regular researches [2] and conformed to the requirements of FDA.

3.2.3. Absolute recovery and matrix effect

The mean plasma absolute recoveries for $^{58}\text{Fe}$ at different levels were from 87.3 to 111.3%. The overall yields were shown in Table 3 and the results showed that data were consistent and precise.

After optimization of mass spectrometric conditions and compensates for interference, no apparent ionization interference was found in the analysis for $^{58}\text{Fe}$. Results of matrix effect were no less than 88.8%, showing that there was no significant difference between the CPS of samples spiked into matrix after the pretreatment procedure and from the dilution solution. It indicated that the interferences from matrix have been compensated well.

3.2.4. Stability

No significant reduction of $^{58}\text{Fe}$ was detected for the plasma samples under different conditions by simulating the same conditions during sample analysis. The results were summarized in Table 4.

3.3. Application in the pharmacokinetic study

The method was successfully applied to the pharmacokinetic study of $^{58}\text{Fe}$ originating from hemin extrinsically labeled. Non-compartmental pharmacokinetic parameters of $^{58}\text{Fe}$ originating from hemin extrinsically labeled in plasma after intravenous and oral administration for rats are shown in Table 5 and concentration-time profiles of $^{58}\text{Fe}$ in rat plasma following administrations are presented in Fig. 3. After intragastric administration to rats, the peak plasma concentrations were reached at about 2 h after dosing and the mean terminal half-life was 6.62 ± 1.55, 7.92 ± 2.49, 8.10 ± 2.37 h at the doses of 40, 80 and 120 mg/kg (equivalent to $^{58}\text{Fe}$ at 3.55 mg/kg, respectively). Compared with exposure of intravenous dosing group in rats, the absolute bioavailability of $^{58}\text{Fe}$ originating from hemin extrinsically labeled was 1.07 ± 0.21% at 40 mg/kg.

Systemic exposures (AUC) for intragastric administration at different dose levels (while equivalent to $^{58}\text{Fe}$ for the same dose at 3.55 mg/kg) were compared and as shown in Table 5, there were significant statistically differences between the low dose

### Table 1

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Abundance (atom %)</th>
<th>Interfering species</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{58}\text{Fe}$</td>
<td>0.28</td>
<td>$^{58}\text{Ni}(67.8%),^{40}\text{Ar}^{+}+^{18}\text{O}(0.2%),^{32}\text{C}^{+}+^{40}\text{Ti}(7.9%),^{17}\text{OH}^{+}+^{37}\text{Fe}(6.9%),^{11}\text{H}^{+}+^{57}\text{Fe}(2.2%),^{14}\text{N}^{+}+^{44}\text{Ca}(2.1%)$</td>
</tr>
<tr>
<td>$^{54}\text{Fe}$</td>
<td>5.80</td>
<td>$^{54}\text{Cr}(2.4%),^{40}\text{Ar}^{+}+^{13}\text{C}(99.2%),^{34}\text{Ar}^{+}+^{20}\text{Ne}(90.6%),^{12}\text{C}^{+}+^{46}\text{Ti}(72.6%),^{14}\text{N}^{+}+^{39}\text{K}(92.7%),^{17}\text{OH}^{+}+^{37}\text{Cl}(24.5%),^{12}\text{C}^{+}+^{41}\text{K}(9.5%)$</td>
</tr>
<tr>
<td>$^{53}\text{Cr}$</td>
<td>0.095</td>
<td>$^{53}\text{Cr}(9.5%)$</td>
</tr>
<tr>
<td>$^{58}\text{Ni}$</td>
<td>26.23</td>
<td>$^{58}\text{Ni}(100%),^{40}\text{Ar}^{+}+^{28}\text{Ne}(90.6%),^{12}\text{C}^{+}+^{46}\text{Ti}(72.6%),^{14}\text{N}^{+}+^{39}\text{K}(92.7%),^{17}\text{OH}^{+}+^{37}\text{Cl}(24.5%),^{12}\text{C}^{+}+^{41}\text{K}(9.5%)$</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>Intra-day (n = 5)</th>
<th>Inter-day (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Concentration</td>
<td>Accuracy</td>
</tr>
<tr>
<td>$^{58}\text{Fe}$</td>
<td>Added (ng/mL)</td>
<td>Measured (ng/mL)</td>
<td>RE (%)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.5 ± 0.7</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>102.5 ± 0.8</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>785.6 ± 29.8</td>
<td>1.8</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng/mL)</th>
<th>Recovery (%)</th>
<th>Matrix effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{58}\text{Fe}$</td>
<td>Mean ± SD</td>
<td>RSD (%)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>10</td>
<td>111.3 ± 9.3</td>
<td>8.4</td>
<td>88.8 ± 4.8</td>
</tr>
<tr>
<td>100</td>
<td>873.3 ± 3.5</td>
<td>4.0</td>
<td>99.0 ± 1.7</td>
</tr>
<tr>
<td>800</td>
<td>968.6 ± 1.8</td>
<td>1.7</td>
<td>98.0 ± 3.8</td>
</tr>
</tbody>
</table>
group and other groups. This disproportional increase at the high-dose level suggested that uptake process, rather than excretion process, are saturated. Due to the lack of observed sex-related differences in pharmacokinetics, male and female data were combined.

Currently, there are three methods for estimation of iron absorption. One method is to study the incorporation of stable iron isotopes into erythrocytes after isotope administration. Those indicated variation can be found using a developed ICP-MS method after pharmacokinetic study and this view was supported in Michael B's study [29]. However, a recent paper investigated the availability of hemin in humans and found a bioavailability of 5% [30]. Due to analytical problems it is very hard to find other references about availability of hemin in human or rat, species difference should be investigated in the future study.

In our study, isotope concentration, not ratio was treated as an acceptable reference for further study. In the future, the method established in this paper could be used in absorption study for similar metallotherapeutic drugs including endogenous isotope such as calcium.

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng/mL)</th>
<th>Room temperature stability for 4 h</th>
<th>Post-preparative stability at 4 °C for 24 h</th>
<th>Freeze-thaw stability for three cycles</th>
<th>Frozen stability for 15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>58Fe</td>
<td>10</td>
<td>102.8 ± 12.1</td>
<td>89.4 ± 11.0</td>
<td>99.9 ± 15.3</td>
<td>93.7 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>102.7 ± 1.6</td>
<td>100.1 ± 1.9</td>
<td>105.4 ± 1.6</td>
<td>102.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>101.8 ± 0.8</td>
<td>101.4 ± 1.1</td>
<td>101.3 ± 1.4</td>
<td>102.1 ± 0.8</td>
</tr>
</tbody>
</table>

### Table 5

Mean pharmacokinetic parameters of 58Fe in plasma after administration of hemin at different dose levels (while equivalent to 56Fe for the same dose at 3.55 mg/kg) to rats, respectively (mean ± S.D, n = 6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>i.g.</th>
<th>i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>2.3 ± 0.8</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>Cmax (ng/L)</td>
<td>197.5 ± 50.0</td>
<td>141.4 ± 33.8</td>
</tr>
<tr>
<td>AUC0−1 (µg.h/L)</td>
<td>1837.1 ± 365.4</td>
<td>1032.8 ± 355.6*</td>
</tr>
<tr>
<td>AUC0−∞ (µg.h/L)</td>
<td>1916.4 ± 373.2</td>
<td>1086.2 ± 368.5*</td>
</tr>
<tr>
<td>Vf (L/kg)</td>
<td>14.7 ± 3.6</td>
<td>25.9 ± 14.5</td>
</tr>
<tr>
<td>CL (L/kg.h)</td>
<td>1.6 ± 0.3</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>6.6 ± 1.6</td>
<td>6.4 ± 3.3</td>
</tr>
<tr>
<td>MRT0−1 (h)</td>
<td>8.4 ± 1.7</td>
<td>7.9 ± 2.5</td>
</tr>
<tr>
<td>Bioavailability (F, %)</td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>

i.v.: intravenous administration; i.g.: intragastric administration. *p < 0.05 vs low dose group.

**Fig. 3.** Concentration–time profiles of 58Fe in rat plasma following administrations of hemin extrinsically labeled to rats. i.g., 40 mg/kg (equivalent to 56Fe at 3.55 mg/kg); i.v., 2 mg/kg (equivalent to 56Fe at 0.18 mg/kg) (mean ± S.D, n = 6).

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