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Effects of a Single Dose of Oral Iron on Hepcidin Concentrations in Human Urine and Serum Analyzed by a Robust LC-MS/MS Method

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Abstract

BACKGROUND—The measurement of serum hepcidin, a peptide hormone that regulates iron metabolism, is clinically important to the understanding of iron homeostasis in health and disease. To date, the quantification of serum hepcidin levels by conventional immunological detection methods has proven problematic due to challenges in obtaining high quality antibodies which demonstrate good reproducibility. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) has been employed recently for more sensitive quantification of hepcidin; however, this method has high background levels and therefore less than optimal specificity.

METHODS—In order to increase the specificity of the mass spectrometry based assay, we developed a robust, ultra-performance liquid-chromatography-tandem mass spectrometry (UPLC-MS/MS) protocol using multiple selected reaction monitoring (mSRM) for quantification of hepcidin levels in urine and serum of human subjects. With this assay, we assessed levels of hepcidin before and for up to 8 h after oral ingestion of ferrous sulfate in ten adult human subjects without known disease.

RESULTS—The linear response of hepcidin quantitation on each instrument was measured, and the correlation coefficients of these calibrations were $r^2 = 0.9512 \pm 0.0202$ (n=5) for urine and $r^2 = 0.9709 \pm 0.0291$ (n=5) for serum [$r^2 = \text{mean} \pm \text{SD}$]. Compared to baseline, the levels of urinary hepcidin between 2–4 h and 4–8 h of both women and men showed significant increases with $p < 0.05$ and $p < 0.001$, respectively. The levels of serum hepcidin between 4 h and 8 h in both women and men showed significant increases, compared with baseline values, with both $p < 0.01$. Interestingly, we also observed some degree of oscillation of levels, occurring at later time points.

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CONCLUSIONS—We have developed and validated a new method for measuring hepcidin concentrations in human serum and urine and used it to demonstrate early increases with iron supplement in both urinary and serum levels of hepcidin, which return to baseline levels, except in urine samples from men.

Keywords

Hepcidin; Oral Iron; Mass Spectrometry; LC-MS/MS; Selected Reaction Monitoring

1. Introduction

Hepcidin is a peptide hormone that plays a key role in regulation of iron metabolism and innate immunity [1-6]. Hepcidin is synthesized principally in hepatocytes as an 84-amino acid (aa) pro-hormone and cleaved by proteolysis to generate the biologically active 25-aa peptide that is secreted into serum and urine. In contrast to the serum, where the 25-aa hepcidin (hepcidin-25) is most abundant, urine primarily contains N-terminally truncated peptides of 22-aa and 20-aa [4, 7].

In recent years, hepcidin has emerged as the key regulator of iron release both from enterocytes of the proximal small intestine and from macrophages and monocytes, where much iron, derived from the heme of senescent erythrocytes, is salvaged and recycled. Hepcidin performs these functions chiefly by altering the expression of ferroportin (Fpn) on the plasma membranes of these cells. Specifically, when circulating levels of hepcidin are high, hepcidin binds to and leads to internalization of Fpn, thus decreasing iron transport out of enterocytes and macrophages. In contrast, when levels of hepcidin are low, there is increased Fpn expression and function, leading to increased release of iron from enterocytes and macrophages [8-13].

Numerous factors have been found to modulate formation and release of hepcidin by hepatocytes, including HFE, HJV, TFR2, BMPs, pro-inflammatory interleukins (especially IL-6), hypoxia and signals from the bone marrow [8, 14]. Thus, many (if not most) proteins involved in iron metabolism and homeostasis influence or are influenced by hepcidin. Most forms of pathologic iron overload (hemochromatosis) have been associated with inappropriately low levels of hepcidin in serum, including the most common form of hereditary hemochromatosis, due to defective expression of HFE, resulting from a single homozygous mutation of the *HFE* gene (C282Y+/+) [15-19]. To date, the quantification of serum and urine hepcidin levels using commercially available immunological assays has proven unreliable due to the difficulty of procuring high quality antibodies that demonstrate good reproducibility [20]. Recently, assays using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry have been developed for sensitive quantification of hepcidin [7, 21-24]. To increase specificity of the assay, liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple selected reaction monitoring (mSRM) mode has been developed [25-29]. However, sample preparation strategies for hepcidin have remained challenging and difficult because components of serum and urine samples interfere with hepcidin enrichment and reverse-phase LC-MS/MS. In this paper, we describe a more robust hepcidin assay using WCX magnetic nanoparticles and multiple selected reaction monitoring (mSRM) with LC-MS/MS, and we use this method to quantify hepcidin in urine and serum of ten normal human subjects before and for 8 hours after a single oral dose of ferrous sulfate.

2. Materials and Methods

2.1. Human subjects and iron administration protocol

Five male and five women volunteers of widely varying age (19 – 68 y) were recruited to participate in this study. All subjects considered themselves healthy and with no known acute or chronic medical disorders. However, as noted below, after sample collection and testing, one woman was found to be iron deficient. Subjects were asked to fast for at least 8 hours, prior to collection of baseline samples of blood and urine, which were collected from all participants at 8 am. Immediately following initial sample collection, participants orally ingested 650 mg ferrous sulfate, taken with orange juice (8 oz) containing ~81.6 mg ascorbic acid, to encourage iron absorption. Blood samples were collected at 1, 2, 4, and 8 hour time points, while urine was collected in time intervals from 0-2 hours, 2-4 hours, and 4-8 hours. Participants continued fasting until after the 4 hour time point, after which they were free to eat. Blood samples were obtained through venipuncture, and voided urine was collected in sterile urine cups. After collection, blood samples were allowed to clot and then centrifuged for 10 minutes at 1500 rpm and the sera were separated and stored (-80°C) as 0.5 mL aliquots. The urine was centrifuged and separated into 1mL aliquots. All samples were kept frozen at -80°C until assays were performed (within 74 days of collection). This protocol was approved by the Carolinas Medical Center Institutional Review Board.

2.2. Clinical laboratory assays

Measurements for hemoglobin (Hgb), serum iron, serum transferrin saturation and serum ferritin were performed by the clinical laboratory at Carolinas Medical Center. Hematological parameters (e.g. Hgb) were measured using a Coulter LH 750 Series Analyzer. Serum iron (timed-endpoint method) and serum transferrin (turbidimetric method) concentrations were quantitated using a Beckman Synchron LX/DX System. Serum ferritin was determined by Access Ferritin assay (two-site immunoenzymatic, assay) using a Beckman DXI.

2.3. Chemicals, supplies, and reagents

Water, acetonitrile of LC-MS grade and formic acid were purchased from EMD Chemicals (Darmstadt, Germany). Sodium phosphates monobasic and dibasic were obtained from Calbiochem, citric acid from J. T Baker, and sodium citrate from EM science. PALL Acrodisc 0.2 μm syringe filter for filtering urine samples was purchased from Pall Corporation (Ann Arbor, MI, USA) Heavy isotopic hepcidin-25 at 24-aa, with [$^{13}\text{C}_6$, $^{15}\text{N}_2$] L-lysine (Hepcidin-25H) which contains four disulfide bonds, was synthesized by Anaspec (Fremont, CA, USA). Magnetic nanoparticle weak cation exchange (WCX) beads, Dyna-WCXTM, were obtained from Invitrogen (Carlsbad, CA, USA). LC-MS/MS was performed on custom nanoflow-split Waters ACQUITY Ultra-Performance Liquid Chromatography (UPLC) coupled to a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer and/or a Thermo LTQ linear ion trap mass spectrometer equipped with a New-Objective electrospray ionization source.

2.4. Urine hepcidin enrichment

All sample preparation was performed at room temperature. Urine samples were thawed on ice and vortexed briefly. Samples were filtered with PALL Acrodisc 0.2 μm syringe filter. Subsequently, 10 pmol 8Da heavy hepcidin internal standard was added to 1.5 mL urine sample and vortexed briefly. 20 μL Dyna-WCX magnetic nanoparticles were activated by washing twice with 50 mM sodium phosphate pH 7.0/ 1 M sodium chloride (Buffer A), then equilibrated by washing twice with 50 mM sodium citrate pH 5.0/ 50 mM sodium chloride (Buffer B). The urine samples were mixed with 0.5 mL 200 mM sodium citrate pH 5.0/ 200

mM sodium chloride then added to 0.25mg of the activated and equilibrated beads. The beads were rotated for 1 hour at room temperature and pelleted on a DynaMag-2 (Invitrogen) magnets [25]. The supernatant was discarded and the beads were washed three times with Buffer B and twice with HPLC grade deionized water. The bound hepcidin was eluted with 100 μ L 0.5% trifluoroacetic acid three times, dried, and resuspended in 25 μ L 20% acetonitrile, 5% formic acid for LC-MS/MS analysis. 5 μ L of enriched sample, which is equivalent to 300 μ L of starting urine, was analyzed three times.

2.5. Serum hepcidin enrichment

All sample preparation was performed at room temperature. Serum samples were thawed on ice and filtered with 0.45 μ m cellulose acetate spin-filter (Thermo) at 3000 X g for 5 min in a Microfuge 22 R centrifuge (Beckman Coulter). 5 pmol/ μ L Hepcidin-25H was added to 200 μ L serum and vortexed briefly. Followed by the addition of 95 μ L of acetonitrile, samples were mixed at 1500 rpm for 10 min in a Mixmate (Eppendorf). The samples were then centrifuged at 12000 X g for 30 min at 4 °C, after which 210 μ L of supernatant was transferred to a clean tube. Dynabeads™ (Invitrogen) weak cation exchange beads (Dyna-WCX) were used for peptide enrichment from samples. The magnetic bead separations were performed according to the manufacturer's protocol. Briefly, the magnetic beads in 35 μ L of a well mixed suspension were activated with Buffer A and equilibrated with Buffer B. The beads were resuspended with 40 μ L Buffer B and added to the sample. The total volume was brought to 2 mL with Buffer B. The bead-sample mixture was rotated at room temperature for one hour. The bead-sample complex was then washed with 50 mM sodium citrate buffer (pH 5.0)/ 0.5 M NaCl solution (Buffer C) then water, 3 times each. The peptide fraction was eluted with 100 μ L 0.5 % trifluoroacetic acid three times. The eluate was collected into a clean tube and evaporated using a centrifugal vacuum concentrator (Labconco) and resuspended in 20% acetonitrile, 5% formic acid. After UPLC-MS/MS analysis, raw data were analyzed for determination of the endogenous hepcidin level, which was calculated by the ratio of area under the curve (AUC) between endogenous hepcidin and Hepcidin-25H using the following equation:

$$\text{Plasma hepcidin} = \text{Injected Hepcidin-25H (fmol)} \times \frac{\text{AUC of endogenous hepcidin}}{\text{AUC of Hepcidin-25H}}$$

2.6. UPLC and tandem mass spectrometry analysis

The relative quantification of urine and serum hepcidin was carried out by liquid chromatograph-tandem mass spectrometry (LC-MS/MS) analysis as described previously with modifications [25, 26]. Briefly, the LC-MS/MS system consisted of a Waters ACQUITY UPLC (Milford, MA) with custom C18 trap (100Å Magic C18 5 μ m, 0.15 mm $\times$ 50 mm) and analytical columns (100 Å Magic C18, 5 μ m, 0.1 mm $\times$ 100 mm) and a ThermoFisher TSQ Quantum Ultra triple quadrupole MS and LTQ-XL linear ion trap mass spectrometers with electrospray ionization. Hepcidin was separated by a 10 minute linear gradient from 90% (v/v) Solvent I (0.1% formic acid by volume in water)/ 10% Solvent II (0.1% formic acid in acetonitrile) to 60% Solvent I/ 40% II at a flow rate of ~500 nL/min with custom double-split UPLC. The quantification of endogenous and 8Da heavy isotope hepcidins was performed by using multiple selected reaction monitoring (mSRM) of the transition of parent ions at 698.18 and 700.16 m/z (z=4), both of which generate the same two product ions, 354.1 m/z (b3) and 501.2 m/z (b4) (Fig. 1A, B). These n-terminal ions showed the most reproducible fragmentation pattern during the transition.

The measured urinary hepcidin level was expressed as “nmol hepcidin/ mmol urinary creatinine concentration” in urine samples. The serum hepcidin concentration was expressed as pmol/mL.

2.7. Statistical analysis

Changes in urine and serum hepcidin concentrations were further analyzed by the statistical program SAS version 9.2 (SAS Institute Inc., Cary, NC) using a paired t-test, linear regression and correlation. For outcome variables that were measured at several time-points for each subject, repeated measures ANOVA using generalized estimating equations (GEE) was employed. GEE was needed to account for correlations in the data that arise from multiple measurements on the same individual. Two-tailed p -values < 0.05 were considered statistically significant.

3. Results

3. 1. Demographic features and response to iron loading

We measured changes in hepcidin levels in response to iron loading in 10 adult volunteers (5 women and 5 men) who considered themselves to be normal and free of disease. The age range of the five women was 19-47 with a median of 28.2 y. The age range of the five men was 20-68 with a median of 34.4 y. Hematological and iron parameters were determined for all study subjects (Table 1). Blood hemoglobin (Hgb) concentrations were within the normal range. Serum iron levels increased over time (baseline to 4h) after iron supplementation. Subject FE was judged to be iron deficient, based upon the results shown in Table 1. Worthy of note is that those with lower levels of serum iron and transferrin saturation at baseline [FE, MF] experienced the largest relative and absolute increases after iron ingestion, indicative of their greater iron absorption of the gut and lack of a pronounced first-pass effect in the porto-hepatic circulation.

3.2. Quantification, recovery rate, and linearity

Endogenous hepcidin from 1.5 mL urine and 200 μ L serum was enriched using Dyna-WCX magnetic beads and spiked with 10 pmol and 5 pmol heavy isotope labeled internal standard (Hepcidin-25H), respectively. The quantification of urine and serum hepcidin was performed by multiple selected reaction monitoring (mSRM) using TSQ-Quantum Ultra and LTQ-XL instruments as described in methods and their representative mSRMs are shown in Fig. 1C.

The recovery rates of urine and serum samples using heavy isotope hepcidin were 21.6 ± 2.8 % (mean $\pm$ SE, $n=50$) and 47.5 ± 4.7 % (mean $\pm$ SE, $n=40$), respectively. The linear response of hepcidin quantitation on each instrument was measured before or after sample analysis, and the representative standard curves from TSQ-Quantum Ultra and LTQ-XL are shown in Fig. 2. The correlation coefficients of these lines were $r^2 = 0.9512 \pm 0.0202$ ($n=5$) for urine and $r^2 = 0.9709 \pm 0.0291$ ($n=5$) for serum [$r^2 = \text{mean} \pm \text{SD}$]. The quality control run in the linear standard curve showed $1.54 \text{ pmol} \pm 0.11$ [mean $\pm$ SE, $n=13$] accuracy for 1.6 pmol internal standard.

3. 3. Hepcidin concentration of urine and serum

The mean hepcidin concentrations of the subjects' urine and serum at each time point after 650 mg ferrous sulfate treatment are shown in Fig. 3. The ratio of area under the curve (AUC) between endogenous hepcidin and Hepcidin-25H was multiplied by the spiked Hepcidin-25H concentration to calculate the relative quantity of the hepcidin levels of urine and serum. Note that there were increases in both urinary and serum levels of hepcidin at 1 h after oral iron, but that levels fell back to near baseline at 2 h (serum) or 2-4 h (urine). The

urine samples from men did not demonstrate this trend, however. Further oscillatory behavior was observed at later time points, with increases occurring at 4 and 8 h (serum) and during the 4–8 h interval (urine). The significance of the differences in hepcidin levels among time points after ferrous sulfate treatment is shown in Fig. 3. The levels of urinary hepcidin between 2–4 h and 4–8 h of both women and men showed significant increases with $p < 0.05$ and $p < 0.001$, respectively (Fig. 3A and B). The levels of serum hepcidin between 4 h and 8 h of both women and men showed significant increases with both $p < 0.01$ (Fig. 3C and D).

The relationship of hepcidin in urine and serum at baseline to baseline serum ferritin of women showed no significant correlation (Fig. 4). However, comparison of the hepcidin levels to the serum ferritins of men showed a strong trend of significance, both for urine ($r^2 = 0.7613$, $p = 0.054$) and serum ($r^2 = 0.7641$, $p = 0.053$).

The effects of a single oral dose of ferrous sulfate (650 mg, equivalent to 130 mg iron) on serum iron and serum transferrin is shown in Fig. 5. As expected, there were significant increases in these levels with time, both in men and women with monotonically increasing levels ($p \leq 0.038$, for trends). Results for each subject are shown in Supplemental Fig. 2 (urine) and Fig. 3 (serum), respectively. With but one exception [MJ], all subjects showed increases in urinary hepcidin levels at one or more time intervals after iron ingestion. Similarly, except for the woman who was iron-deficient [FE], all subjects showed increases in serum hepcidin levels after iron ingestion. Furthermore, several showed evidence of oscillations in levels, suggesting a rapidly responsive self-regulating negative feed-back loop governing hepcidin production and/or release.

4. Discussion

The measurement of hepcidin levels in serum, urine or tissues is still in its infancy. However, “inappropriately” low levels of hepcidin relative to the degree of hepatic or total body iron burden) are a hallmark of certain diseases, such as hereditary hemochromatosis [30, 31] and liver diseases often associated with iron overload, such as chronic hepatitis C [32–34] or alcoholic liver disease [35]. In addition, the increasingly frequent non-alcoholic fatty liver disease (NAFLD) is often associated with elevated levels of serum ferritin and sometimes with increased stainable iron in the liver [35], suggesting that levels of hepcidin may be inappropriately low. However, because serum ferritin is an acute phase reactant, as well as a measure of body iron status, and because NAFLD may be associated with increased inflammatory cytokine levels, [IL-6, TNF α , and others], interpretation of elevated levels of serum ferritin in NAFLD is not straight-forward. In addition, hepcidin itself is also an acute phase reactant. We analyzed the relationship between age and urinary or serum hepcidin levels, baseline iron, and ferritin using Pearson’s correlation coefficients. We found that there was no correlation of age with any of these variables. It has been reported previously that there is no correlation between serum iron levels and age in adults [36]. Similarly, another more recent paper reported no association of serum hepcidin and age in healthy adults [37]. However, interpretation of levels of hepcidin in serum or urine needs to take into account numerous factors, including body iron stores, presence and types of liver disease, and presence of inflammatory conditions.

The unstable and inefficient fragmentation of the four disulfide bonds in hepcidin is the major challenge in performing reproducible and sensitive quantification using LC-MS/MS. To overcome this intrinsic problem for high cysteine containing peptides, some researchers utilize the immonium ion for SRM transitions with high cleavage energy, which results in reduced sensitivity [27]. In this study, we found that the N-terminal residue of hepcidin, namely, H₂N-Asp-Thr-His-Phe-Pro-Ile, which is free of cysteines, demonstrated a linear dissociation efficiency by collision-induced dissociation and SRM transitions. Therefore, we

selected b3 and b4 ions generated by dissociation of this peptide for use in the quantification of hepcidin.

Unlike HPLC analysis with UV detection, mass spectrometry analysis requires high temperature ionization which is not compatible with high concentrations of analytes and/or contaminants from biofluids. Therefore, sample clean-up is crucial in LC mass spectrometry analysis. We tested various solid-phase extraction methods, including C18, SCX, MCX, and WCX. We found that the C18 material caused clogging of nano-spray columns and ion carrier tubes more frequently than SCX material. In our hands, the optimal condition for hepcidin enrichment from urine and serum samples was achieved with use of magnetic based WCX material. We compared the results of experiments conducted using WCX beads from multiple vendors and found Dyna-WCX to be the best. Unfortunately, this product has been discontinued. Bruker manufactures the closest/ best alternative to the Dyna-WCX product. Further evaluation of beads from other vendors is ongoing. We successfully quantified hepcidin from urine and serum of human subjects using mSRM methodology on LC-MS/MS ranging from 100 femtomoles to 6 picomoles per 200 μ L. From our optimization experiments, we determined that a filtering procedure prior to snap freezing is necessary to achieving acceptable urine hepcidin recovery rate [89.6 ± 5.3 %, mean $\pm$ SE, n=8] (Supplemental Fig. 1). We underestimated the importance of this step of clinical sample processing and observed >4 fold lower recovery of the hepcidin from clinical urine samples. Therefore, in the future we plan and recommend filtration of urine samples prior to the snap freezing.

Interestingly, even in this relatively small data set we observed a weak trend for oscillation of hepcidin levels in both urine and serum after iron administration (Supplemental Figs. 2 and 3). We think it likely that this oscillatory behavior is due to a rapidly responsive negative feedback loop whereby rapid iron uptake by the proximal duodenum leads to influx of iron into portal blood and uptake by hepatocytes, leading, in turn to rapid, but transient increases in hepcidin production and/or release. These, in turn, produce rapid decreases in further iron absorption and hence oscillatory decreases in hepcidin levels. It will be interesting in larger, longer term studies to see how long such oscillations may continue after a single oral dose of iron. We acknowledge that interpretation of our results is rendered more difficult by the fact that our subjects were allowed to eat various foods (according to their preferences) after 4 h of continued fasting following iron ingestion. Thus, it is likely that their intakes of iron after noon on the days of study were variable. However, we did not feel that we could justify, nor could we afford, more prolonged strict control of oral iron intakes. We may be able to study these oscillations and related phenomena more precisely in cell cultures of hepatocytes and/or in mice, both wild type and with inherited abnormalities in *HFE*, *HJV*, *TFR2*, *FPN* or other relevant genes.

In summary, we have developed an accurate and precise method for measuring levels of hepcidin in human serum and urine. With it, we show that levels are directly correlated with body iron stores in normals [as reflected in serum ferritin levels] and respond quickly to the ingestion of a single dose of oral ferrous sulfate. Furthermore, our data suggest subsequent oscillation in levels, consistent with a self-damping negative feedback loop.

We plan further studies of hepcidin levels in NAFLD, which is the hepatic manifestation of the metabolic syndrome, and which is rapidly increasing in prevalence in the USA and, indeed, throughout the world. Levels of hepcidin are also likely to be helpful for differential diagnosis of anemias and to unravel other disorders of iron homeostasis [31].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We report a robust quantification of hepcidin in urine and serum using UPLC-MS/MS.
- Serum iron, TfS, and serum and urinary hepcidin levels increase after FeSO₄[p<0.05].
- Oscillation of hepcidin levels in study subjects suggests a negative feed-back loop.

Fig. 1.

Optimization of mSRM transitions of endogenous hepcidin-25 and isotopic hepcidin-25. (A) MS spectra of endogenous and heavy isotopic hepcidin-25. The most intense parent ions (charge: 4+), 698.18 (endogenous hepcidin-25) and 700.16 (Hepcidin-25H) m/z , were chosen fragmentation to product ions, (B) Representative MS/MS spectra. The product ions, 354.1 (b_3) and 501.2 (b_4) m/z were chosen for mSRM transition. (C) Extracted ion chromatogram (upper) and selected reaction monitoring of product ions (lower).

Fig. 2. Representative standard curves of the Hepcidin-25H calibration. (A) A linear response of Hepcidin-25H quantitation between 0.5 to 6 picomoles was demonstrated in TSQ Quantum Ultra, (B) The linear response of the Hepcidin-25H quantification between 100 to 500 femtomoles was demonstrated in LTQ-XL linear ion trap instrument.

Fig. 3.

Mean hepcidin concentrations in urine and serum of normal subjects. (A, B) Urine hepcidin concentration: Samples were obtained from 10 subjects (women, A, n=5; men, B, n = 5) at 4 time points (0, 2, 4, and 8 h) after 650mg ferrous sulfate treatment at t=0, (C, D) Serum hepcidin concentration: Samples were obtained from 10 subjects (women, C, n=5; men, D, n = 5) at 5 time points (0, 1, 2, 4, and 8 h) after 650mg ferrous sulfate treatment at t=0 (The bars show mean $\pm$ SE), n=5 for each gender. * p < 0.05, ** p < 0.01, and *** p < 0.001.

Fig. 4. Relationship of (A) urine and (B) serum hepcidin to serum ferritin at baseline. The best fit lines for men are defined by the following equations: A. $y=0.084x-0.059$, $r^2=0.7613$, $p=0.0536$, B. $y=0.106x+1.372$, $r^2=0.7641$, $p=0.0526$.

Fig. 5.

Effects of a single dose of oral iron on (A) serum iron and (B) serum transferrin saturation in subjects studied (mean $\pm$ SE). The increases are highly significant [$p \leq 0.0378$] for both men and women. As expected, levels are lower in women than men at all time points, except for serum iron concentrations at 4 h after oral iron.

Table 1

Characteristics and Selected Lab Results of Study Subjects.

Subject*	Gender	Blood Hgb (g/dL)	Serum Iron (µg/dL)			Serum Transferrin Saturation (%)			Baseline Serum Ferritin (ng/mL)
			Baseline	t=2h	t=4h	Baseline	t=2h	t=4h	
FA	F	12.9	83	147	193	18	30	39	17
FB	F	13.4	74	96	126	21	25	34	32
FC	F	13.3	109	142	130	22	26	25	17
FD	F	14.5	62	143	133	13	29	28	30
FE	F	12.2	24	253	442	5	47	89	4
MF	M	14.9	56	231	321	16	61	88	12
MG	M	15.5	140	198	238	45	63	81	222
MH	M	16.1	141	217	214	37	58	57	20
MI	M	15.2	97	142	148	24	33	37	64
MJ	M	15.4	99	112	156	32	37	51	73

* The subject FE is adjudged to be iron deficient based upon baseline serum ferritin =4ng/ml and transferrin saturation = 5%. It is worthy of note that those with lower levels of serum iron and transferrin saturation at baseline [FE, MF] had the largest relative and absolute increases in these variables following iron ingestion, indicative of their greater absorption of iron from the gut.

**The "t" represents time after iron ingestion.