

Hepcidin Assays: Ironing Out Some Details

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Iron is the fourth most common element in the earth's crust and the most abundant transition metal in the human body. It is an essential element required for growth and survival. Maintaining the correct iron balance is crucial for health. All living organisms evolved sophisticated mechanisms to maintain appropriate iron levels in their cells and within their body. It was shown recently that the kidney is also involved in iron metabolism. Our understanding of the molecular control of iron metabolism has increased dramatically in the past 5 yr as a result of the discovery of hepcidin. This is a circulating antimicrobial peptide that is synthesized mainly in the liver, which was recently proposed as a factor that regulates the uptake of dietary iron and its mobilization from macrophages and hepatic stores.

The major circulating bioactive forms of hepcidin consist only of the carboxy-terminal portion (peptides of 25, 22, and 20 amino acids) (1). The exact location of the final prohormone processing is unknown. Propeptide convertases could be located in the blood or in the cell membrane of capillaries. Still no reliable information is available on normal serum levels of mature hepcidin (20, 22, and 25 amino acids); however, 60–amino acid prohepcidin is easily detectable in serum. Hepcidin-25, the major form of mature hepcidin, is cleaved from prohepcidin by convertases. Hepcidin-20 and hepcidin-22 may be directly generated from prohepcidin by convertases or indirectly by degradation of hepcidin-25. Hepcidin-25 and hepcidin-20 are a major form of hepcidin in urine, whereas hepcidin-22 is a minor one (2). The same may be true for serum. The nuclear magnetic resonance structure studies indicated that hepcidin-20 exists as a monomer, whereas hepcidin-25 readily aggregates (1).

Hepcidin-25 was initially identified in human blood using a mass spectrometric assay (3). Then Park *et al.* (2) isolated urinary hepcidin-25 and hepcidin-20 using cation exchange chromatography and reverse-phase HPLC. There is no consensus on the best assay method for hepcidin, and assays for hepcidin detection and quantification in serum or urine have not been generally available. The detection and quantification of hepcidin in plasma and serum have been hampered by technical difficulties (small size of hepcidin; limited availability of the antigen; isolation of hepcidin from urine involves complex, time-consuming procedures). Moreover, its conservation among animal species complicates the elicitation of an immune response in host species. Urinary hepcidin assays seem to be

preferable for studies on iron metabolism because serum hepcidin levels are below the detection limit of the currently used methods. This might be due to a rapid clearance of free serum hepcidin by its binding to ferroportin and its subsequent cellular internalization (4); however, reliable information still is not available on normal serum levels of mature hepcidin (20, 22, and 25 amino acids). The urinary hepcidin excretion could be due either to physiologic incomplete reabsorption (overflow) or to impaired tubular reabsorption during chronic inflammation. Further research has now complicated the scenario. Ganz *et al.* (5) successfully developed an assay for detection and quantification of urinary hepcidin, but this assay seems to be available only in their laboratory. A method for assay of urinary hepcidin-25 by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry was reported (6); however, measurement of urinary hepcidin is impossible in patients who have anuria and undergo dialysis. To tackle and overcome the technical problems of hepcidin determination in patients who are on dialysis, Tomosugi *et al.* (7) used a technology, the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), to detect serum hepcidin in renal failure. Other serum hepcidin assays that exploited liquid chromatography tandem mass spectrometry (LC-MS/MS [8]) or isotope dilution micro-HPLC–tandem MS (9) were reported; however, the use of non-hepcidin-related peptides as internal standard may affect the accuracy and reproducibility of the hepcidin concentration measurements. Li *et al.* (10) developed an LC-MS/MS method to quantify hepcidin in human serum using chemically synthesized hepcidin as a standard and stable isotope-labeled hepcidin as internal standard. Rabbit serum was used as a surrogate matrix for standards because of the presence of endogenous hepcidin in human serum. The method was validated to Food and Drug Administration criteria for bioanalytical assays. As shown by Swinkels *et al.* (11), there was almost 10-fold difference between lower limit of detection in SELDI (in study by Swinkels *et al.* [11]) and immunodot (study by Nemeth *et al.* [4]) in urinary hepcidin. The same difference was found between the assay by Ganz *et al.* (12) and the assay by Ashby *et al.* (13). In the studies by Ganz *et al.* (12) and Zaritsky *et al.* (14) reported in this issue of *CJASN* (using the same ELISA), the difference between detection limit was 10-fold, with 0.5 ng/ml in the study by Ashby *et al.* (13) and 5 ng/ml in the assay developed by Ganz *et al.* (12). Values of hepcidin in both studies varied the normal range for healthy volunteers was 2 to 55 ng/ml (fifth through 95th percentiles) versus 29 to 254 ng/ml for male patients and 18 to 288 ng/ml for female patients. In patients with CKD, hepcidin

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levels ranged from 3.1 to 153.0 with a median of 26.5 ng/ml; in patients on dialysis, hepcidin levels ranged from 27.6 to 158.0 ng/ml with median of 58.5 ng/ml; whereas in the study by Zaritsky *et al.* (14), in adults with CKD, median hepcidin was 269.9 ng/ml, in 26 pediatric patients who were on dialysis, it was 652.4 ng/ml, and in healthy volunteers, it was 72.9 ng/ml. Both assays are immunoassays. Ganz *et al.* (12) performed the assay by using a functional biotinylated synthetic peptide (hepcidin analog) as a tracer (Intrinsic LifeSciences, La Jolla, CA) and another one for the construction of the standard curve (Bachem, King of Prussia, PA), whereas Ashby *et al.* (13) used synthetic hepcidin-25 (Bachem, UK) conjugated to Keyhole limpet hemocyanin. Binding was assessed by competition with I^{125} -labeled hepcidin-25, separated by secondary antibody. This assay is linear up to 200 ng/ml, whereas Ganz *et al.* (12) claimed that their assay can detect hepcidin within a range of 5 to 4000 ng/ml. It would be nice to know the hepcidin levels for samples obtained from Girelli (who contributed to both studies of Ganz *et al.* and Swinkels *et al.*, which yielded different magnitude of results) using both methods ELISA and TOF MS. Of interest is that correlation between ELISA values and MS in the recently published study by Koliaraki *et al.* (15) was only modest ($r = 0.863$, $P = 0.027$) in samples obtained from six patients. They described also an immunoassay for serum hepcidin quantification, this time based on a recombinant hepcidin peptide (hepcidin25-His) and a polyclonal antibody (confirmed by Western blot). According to Koliaraki *et al.* (15), the analytical detection limit of their ELISA assay defined as the concentration corresponding to the mean signal ± 3 SD of 10 replicates of the zero calibrator was $5.4 \mu\text{g/L}$, similar to the assay of Ganz *et al.* (12). This assay is capable of measuring serum hepcidin within the range of 10 to $1500 \mu\text{g/L}$, which is between both assays of Ganz *et al.* (12) and Ashby *et al.* (13).

Enormous progress has been achieved in the field of hepcidin measurement; however, as suggested by Pietrangelo and Trautwein (16), “the reliability of methods must be proved (or disproved) by studies designed specifically for this purpose, and that validation requires that the assay (whether it uses blood or urine) can be transferred to and reproduced in different laboratories.”

Disclosures

None.

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See related article, “Hepcidin—A Potential Novel Biomarker of Iron Status in Chronic Kidney Disease,” on pages 1051–1056.