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Time-of-flight mass spectrometry analysis of the ferroportin–hepcidin binding domain complex for accurate mass confirmation of bioactive hepcidin 25

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Dear Editor

Because of its central role in the regulation of blood iron levels, hepcidin has emerged as a leading analytical target related to multiple disorders including the anemias of chronic inflammation, aging, cancer and autoimmune disorders as well as iron loading in hereditary hemochromatosis and transfusion-dependent anemias [1–6]. Mutations in the hepcidin gene (HAMP) cause hemochromatosis type 2B, also known as juvenile hemochromatosis, a disease characterized by severe iron overload that results in cardiomyopathy, cirrhosis, and endocrine failure [7].

Although triple quadrupole multiple reaction monitoring (MRM) assays are widely used due to their sensitivity and specificity, caution is needed when dealing with quality control and specificity of analysis [8]. Highlighting this need for caution in MS based testing are examples including the 2004 metanephrine quantitation discrepancies [9], recent Vitamin D lab results from a major testing laboratory [10], and more specifically, the 3rd World Congress of the International BioIron Society round robin for hepcidin quantification [11].

Recent reports suggest that hepcidin 25 may differ between serum and urine in both confirmation (folding) and molecular weight [12–14]. Prior to implementing new biomarkers for iron homeostasis into clinical practice, *verification* of a target compound identity and specific bioactivity is vital. This process involves measuring the exact same analyte across many individuals and many testing sites — preferably on different instrument platforms and/or differing analytical principles [15].

In 2008, De Domenico et al. reported details of a binding assay to measure hepcidin bioactivity in serum [16]. By design, this functional binding assay measures only bioactive (defined here as ferroportin interacting) hepcidin 25 present in human serum. It was noted that the binding assay does not detect hepcidin in urine, which may suggest some difference in mass or structure of hepcidin 25 as it passes through the kidney and is eliminated from the body. Although several methods have been reported to measure hepcidin, important questions remain such as; what form of hepcidin is bioactive? What are the specific differences between serum and urine hepcidin? What are the primary causes of the loss of biological activity? To date, accurate mass confirmation of the ferroportin bound form of hepcidin has not been reported.

In brief, ferroportin hepcidin binding domain (HBD) peptides (RRFDCITGTGYAYTQGLSGSILSRR — MW 2720.38) were immobilized covalently to agarose beads using the AminoLink Plus Immobilization Kit (Pierce, Rockford IL). An aliquot (100 μ l) of human serum or urine was added to HBD-conjugated beads. The samples ($n = 4$) were incubated at 37 °C for 12–18 h, the beads were washed, then bound and unbound fractions collected. The use of human serum and urine samples was approved by the University of Utah Institutional Review Board. After the affinity capture, bound and unbound fractions were prepared for analyses as reported by Merrell et al. [17]. Briefly, 100 μ l of each sample was mixed with 200 μ l acetonitrile for 30 s, incubated at room temperature for 30 min, and centrifuged for 10 min at 15,000 $\times$ g. Supernatant was removed, lyophilized and reconstituted in 10 μ l water containing 0.1% formic acid. All samples remained above 25 °C until analysis to avoid premature dissociation of the HBD/hepcidin complex [16].

A 1 μ l aliquot of each sample was analyzed by nanoflow reversephase LC/MS using the Agilent 6510 Q-TOF, Chip Cube (G2240A), and 1200 nano-HPLC system (Agilent Technologies, Santa Clara, CA). Peptide separation was performed on a C-18SB-ZX Chip (40 nl trap, 75 μ m $\times$ 43 mm, and 5 μ m particles). Mobile phase consists of 5% acetonitrile and 0.1% formic acid in water, and 5% water and 0.1% formic acid in acetonitrile. Total gradient time was 12 min. Time-of-flight data acquisition (2 GHz) was performed with MassHunter QTOF Acquisition software (ver. B.02) with an acquisition rate of 2 scans per sec. The TOF analyzer was tuned to resolution >12,000 and calibrated prior to the experiments for a mass accuracy of <2 ppm. Autosampler temperature was also set to 25 °C to minimize any premature dissociation of the HBD/hepcidin complex. TOF profile scans were processed using 2 different deconvolution algorithms in MassHunter Qualitative Analysis software (ver. B.02). Accurate mass was also confirmed by manual calculation using multiple charge states (+3, +4, and +5) for hepcidin 25 and the HBD peptide.

Hepcidin 25 was captured from human serum or urine during incubation with ferroportin peptide (HBD) covalently linked to agarose beads. Bound and unbound fractions were then analyzed by TOF mass spectrometry (MS). Fig. 1 shows the TOF mass spectrum of bound fractions (in black) and unbound fractions (in red) demonstrating efficient capture of hepcidin 25 isolated from human serum. The accurate mass of bioactive hepcidin 25 from human serum was calculated using charge state deconvolution to be 2787.035 Da, which agrees with the theoretical value of 2787.0248 Da. Hepcidin 25 accurate mass was also manually verified by isotope patterns of TOF profile scans at three different peptide charge states (+3, +4, and +5).

As shown in bold in Table 1, the HBD bead capture of bioactive serum hepcidin 25 was very efficient (>99%). In contrast, isolation of ferroportin binding hepcidin 25 was much less efficient in urine (<3%). The lower binding affinity of hepcidin 25 in urine is likely due to modifications (such as oxidation) of native hepcidin 25 that would normally bind

ferroportin (HBD). Reported isoforms of hepcidin 20 and hepcidin 22 were not found in the bound fraction. Of interest, TOF acquisition of the unbound fraction revealed urine hepcidin 25 in at least 3 different isoforms: 1) minor amounts (<5%) of bioactive hepcidin, 2) oxidized hepcidin (modified by the addition +15.99 Da) eluting at the same retention time as bioactive hepcidin and 3) an isomer of bioactive hepcidin eluting at ~0.35 min longer retention time, suggesting a possible structural change.

Jordan et al. reported new structural information for hepcidin confirming that methionine 21 is readily accessible [14]. In urine, we propose the oxidation (+15.99 Da) of methionine 21 to form a sulphoxide. MS/MS fragmentation at varied collision energy confirmed the presence of this modification in urine and serum, and argues against the formation of naturally occurring sulfinothioic acid ester from a disulfide bridge (data not shown).

Variability in the analytical specificity of measuring hepcidin 25 is related to the presence of 8 cysteine residues and differing oxidation states. Furthermore, isoforms of molecular weight and folding may affect not only the bioactivity of the peptide, but analytical sensitivity of different measurement methodologies. This highlights the need for adequate chromatographic separation and accurate mass measurement.

One advantage of measuring hepcidin using MRM acquisition on a triple quadrupole MS is the ability to simultaneously analyze serum and urine samples. However, the assay must be carefully designed to properly measure only the desired isoforms. Prior to validation of a new clinical assay, source material used for calibration and controls should not only be carefully evaluated, but also readily available in a pure physiological relevant form. The existence of multiple isoforms of hepcidin in serum and urine may be a potential source of variability among methods, including poor agreement with immuno-based measurement and site to site variation. Availability of high quality reference material is necessary prior to implementing any clinical diagnostic test for hepcidin. Improperly folded or partially oxidized standards would result in overestimation of concentrations in patient samples. Accurate and reliable quantitation of hepcidin in biological samples will allow clinical investigators to study the role of hepcidin in various disease states and pathologic conditions.

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Fig. 1.

A) TOF full scan acquisition of bound (in black) and unbound (in red) fractions from the ferroportin hepcidin binding domain/hepcidin complex isolated from human serum. B) Accurate mass measurement of ferroportin binding (bioactive) hepcidin 25 in human serum using resolved isotope deconvolution was 2787.035 Da.

Table 1Summary of ferroportin hepcidin binding domain (HBD) bead capture of hepcidin 25 in serum and urine.^a

<i>Serum</i>			
Retention time (min)	7.12	7.11	7.46
Molecular weight (theoretical)	2787.025	2803.018	2787.028
Molecular weight (measured)	2787.035	2803.022	2787.034
Percent bound ^b	99.8	0.1	0.1
Percent unbound ^b	0.2	7.4	2.9
<i>Urine</i>			
Retention time (min)	7.15	7.14	7.49
Molecular weight (theoretical)	2787.025	2803.018	2787.025
Molecular weight (measured)	2787.035	2803.023	2787.034
Percent bound ^b	2.9	0.7	2.1
Percent unbound ^b	74.3	21.6	4.1

^aMethod details reported in De Domenico et al. Cell Metab 2008, 8, 146–156.^bCalculated from relative quantitation of total peak area.