Brief report

Novel urine hepcidin assay by mass spectrometry

Erwin Kemna, Harold Tjalsma, Coby Laarakkers, Elizabeta Nemeth, Hans Willems, and Dorine Swinkels

The hepatic peptide hormone hepcidin is the central regulator of iron metabolism and mediator of anemia of inflammation. To date, only one specific immuno-dot assay to measure hepcidin in urine had been documented. Here we report an alternative approach for quantification of hepcidin in urine by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). Peptide peaks were detected corresponding to the 3 forms of hepcidin normally found in urine. The identity of the peptide peak equivalent to hepcidin-25 was confirmed using synthetic human hepcidin-25. Validation of our MS data on samples with various hepcidin levels showed a strong correlation with previous immuno-dot assay results (Spearman R = 0.9275, $P < .001$). Most importantly, this hepcidin assay clearly discriminates between relevant clinical iron disorders. In conclusion, this novel MS urine hepcidin assay is easy to perform and available to a wide audience. This enables the implementation of hepcidin measurements in large clinical studies. (Blood. 2005;106:3268-3270)

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Introduction

Hepcidin is a small, cysteine-rich cationic peptide produced by hepatocytes,[1-3] secreted into plasma, and excreted in urine. Hepcidin expression is induced by iron stores and inflammation[3] and suppressed by hypoxia and anemia.[4] Hepcidin is proposed to be the key regulator of iron metabolism and its discovery has changed our understanding of the pathophysiology of iron disorders. It now appears that hepcidin deficiency is the cause of most types of hereditary hemochromatosis and that hepcidin excess mediates anemia of inflammation.[5] Measurements of hepcidin concentrations could therefore be useful in diagnosis of iron disorders and would provide further insight into hepcidin regulation in vivo. However, assays for hepcidin detection and quantification in plasma or urine have not been generally available, and the development of reagents has been hampered by technical difficulties.[6,7] The development of immunochemical methods based on the production of specific antihepcidin antibodies is difficult due to the small size of hepcidin (25 amino acids), conservation between animal species,[8] and the limited availability of the antigen, as production of synthetic hepcidin in its native conformation[9] or the isolation of hepcidin from urine[2] involves complex, time-consuming procedures. To date, only one immunochemical assay was successfully used to quantify urinary hepcidin in clinical studies.[10]

We sought to develop a more widely available, high-throughput assay. Here we report a new quantification method for hepcidin in urine by the use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). This provides a fast assay with increased simplicity and accessibility that circumvents the difficulties encountered with antibody and antigen production. Moreover, this assay has also the potential to provide insight in the proportional contribution of the 3 known hepcidin isoforms that can be found in urine (hepcidin-20, -22, and -25).

Study design

Urine collection and storage

Approval was obtained from the Radboud University Nijmegen Medical Centre institutional review board for these studies. Informed consent was provided in accordance with the Declaration of Helsinki. From healthy volunteers, second-morning urine was collected. Clinical urine samples from patients in the intensive care unit with severe sepsis (selection by protocol and APACHE [Acute Physiology and Chronic Health Evaluation] score) were collected by urine catheter. Samples from patients with (1) secondary iron overload (myelodysplasia [MDS]; World Health Organization [WHO] classification of refractory anemia [RA] or RA with ring sideroblasts [RARS] with blood-transfusion dependency), (2) iron-deficient anemia (hemoglobin level < 7.5 mM and mean corpuscular volume [MCV] < 85 fL), and (3) hereditary hemochromatosis (homozygous C282Y, in various stages of phlebotomy treatment, urine samples at least 1 week after the last phlebotomy) were collected during visits at the outpatient department of the Radboud University Nijmegen Medical Center, The Netherlands. Healthy volunteers and outpatients lacked clinical signs of inflammation.

Freshly collected urine was centrifuged for 10 minutes at 2600*g*, and the supernatant was divided in aliquots and stored at −20°C. Patient laboratory characteristics are shown in Table 1.

Protein chip preparation and SELDI-TOF-MS measurements

The preparation procedure was based on protocols from Ciphergen and previous reports.[11,12] 8-spot hydrophilic Normal Phase chips (ProteinChip

Table 1. Laboratory characteristics of patients and healthy volunteers who provided urine samples for the clinical validation

	Hemoglobin, mM	MCV, fL	Serum iron (Fe), μM	Fe/TIBC (TS), %	Ferritin, μg/L	CRP, mg/L
Normal, n = 7	8.1 (7.8–9.1)	NA	20 (14–29)	32.8 (24.6–52.7)	80 (32–190)	< 5 (< 5–8)
Secondary iron overload, n = 8	5.6 (3.4–6.2)	NA	39 (29–63)	96.0 (77.1–100.0)	3088 (568–14 496)	< 5 (< 5–< 5)
Hereditary hemochromatosis, n = 7	8.7 (7.2–9.6)	NA	27 (9–39)	50.9 (16.1–93.5)	54 (12–2118)	<5 (< 5–< 5)
Iron deficiency anemia, n = 6	7.1 (3.3–7.4)	78 (69–84)	10 (2–11)	16.0 (2.4–19.6)	8 (2–11)	< 5 (< 5–6)
Endotoxemia model, n = 6	8.1 (7.2–9.1)	NA	16 (4–22)	25.6 (8.0–38.6)	65 (15–219)	8 (5–33)
Sepsis, n = 4	5.4 (4.9–6.2)	NA	1 (1–4)	7.1 (3.8–17.4)	370 (280–505)	192 (124–227)

Data are expressed as the median with the range in parentheses.
NA indicates not analyzed; CRP, C-reactive protein; and TIBC, total iron binding capacity.

NP20; Ciphergen Biosystems, Fremont, CA), mimicking normal-phase chromatography with silicate functionality were used for their binding characteristics of proteins through hydrophilic and charged residues.

Urine samples were thawed, vortexed, and centrifuged for 10 minutes at 2600*g*. Urine supernatant (7 μL) was applied to the chip and incubated for 30 minutes in a humidity chamber. When protein overload was expected, a reduced sample volume was applied (minimum 1 μL). Spots were washed 3 times with 10 μL ultraPURE distilled water (Invitrogen, Breda, The Netherlands) and air-dried for 10 minutes. Finally, 0.8 μL of a saturated solution of sinapinic acid in 0.5% (vol/vol) trifluoroacetic acid and 50% (vol/vol) acetonitrile, used as energy-absorbing matrix (EAM), was applied to each spot surface, allowed to air-dry, and reapplied. Mass spectrometry was performed with a PBS IIc mass spectrometer (Ciphergen Biosystems). Data were collected using the following settings: 2 warming shots at laser intensity 185 (not collected); collection of 50 shots at laser intensity 180 every 5 positions between 29 and 89; high mass 50 000 Da; detector voltage 2900 V; detector sensitivity 9. The acquired mass range was from a mass overcharge (m/z) ratio of 1500:10 000. External mass calibration was performed with synthetic human hepcidin (Peptides International, Louisville, KY). The bioactivity of this synthetic peptide was shown to be compable to the synthetic hepcidin-25 used in the immuno-dot assay.[13] The mass of the synthetic hepcidin-25 from Peptides International was verified by matrix-assisted laser desorption/ionization (MALDI)–TOF-MS (2787.80 m/z) and corresponded well with data from the supplier (2789 m/z) and previously reported values (2789 m/z) for hepcidin-25.[2] Peak annotation was performed with Ciphergen ProteinChip Software (version 3.2.0), after baseline subtraction and adjustment (fitting 8 times expected peak width). Peak intensity levels were normalized to urinary creatinine values and reported as Mega intensity/mmol creatinine. We found that changes in solvent and matrix (eg, sample dilution) can influence the flight behavior of peptides during SELDI-TOF-MS analysis.[11] Therefore, we aimed at a semiquantitative method for hepcidin quantification in urine.

Statistic analysis

Analysis were performed with GraphPad Prism software (version 4.0; GraphPad Software, San Diego, CA). Correlation was tested by Spearman rank test. Group differences were tested for statistical significance by paired *t* test and Mann-Whitney *U* test.

Results and discussion

Hepcidin tracing by mass spectrometry

To investigate the feasibility of a mass spectrometry–based assay for the quantification of urine hepcidin, a pilot SELDI-TOF-MS was conducted where the spectra of a patient with septicemia and a healthy volunteer were generated. Figure 1Ai-ii shows in both spectra a clear peak at 2788 m/z that corresponds with the peak mass of 2789 m/z from the synthetic human hepcidin-25 peptide (Figure 1Aiii). Besides hepcidin-25, the urine spectra also show peaks that correspond with reported masses of the N-terminally truncated hepcidin-20 and -22 (respectively 2192 m/z and 2436 m/z, as measured by MALDI-TOF-MS).[2] As expected, the intensi-

ties of the hepcidin peaks are strongly increased (about 3-fold) in the case of septicemia (Figure 1Ai-ii). The results indicated hepcidin was detectable and quantifiable in urine samples by SELDI-TOF-MS. As the lack of commercially available peptides hampers the mass confirmation of the 20– and 22–amino-acid hepcidin forms, measurements will be based on the hepcidin-25 peptide until new insights on the 20– and 22–amino-acid peptides will approve a change in the data analysis protocol.

Validation of SELDI-TOF-MS measurements

To validate SELDI-TOF-MS measurements, we performed SELDI-TOF-MS on urine samples from our previous study,[14] in which
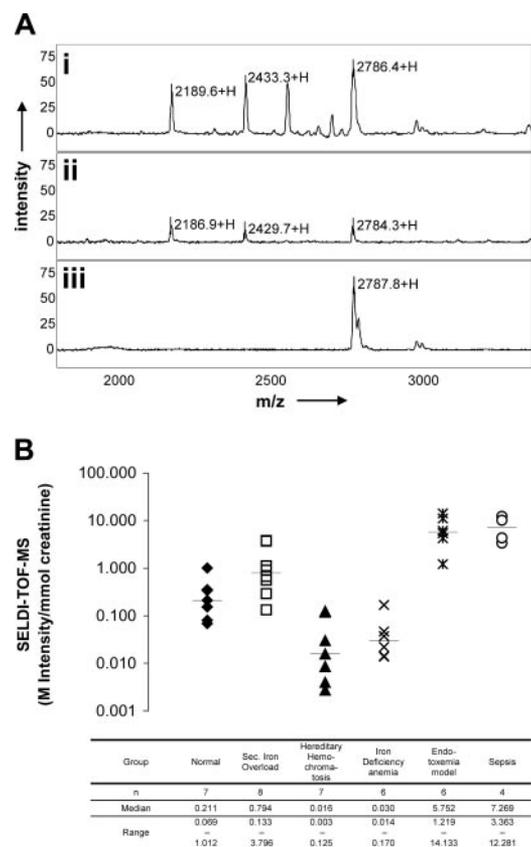

Figure 1. Urine hepcidin profiles by SELDI-TOF-MS, and urine hepcidin levels in different iron metabolism disorders. (A) SELDI-TOF-MS spectra from urine samples of a septicemia patient (i), healthy subject (ii), and human synthetic hepcidin-25 (iii). Urine hepcidin-25 peaks from spectra shown in panel i and panel ii correlate with the reference human synthetic peptide (spectrum shown in panel iii). The annotated peak masses correspond with hepcidin-20, -22, and -25.[2] (B) Urinary hepcidin excretion in patients with transfusion-induced iron overload (□), treated hereditary hemochromatosis (▲), iron deficiency anemia (×), endotoxemia after LPS injection (✳), and sepsis (○) compared with healthy subjects (♦). In each group the median is indicated.

hepcidin concentration was determined by the immuno-dot assay. The samples were from 10 volunteers injected with lipopolysaccharide (LPS) from whom we collected urine at 4 time points within a 22-hour time frame.[14] Statistic analysis showed a strong significant correlation between the 2 methods (Spearman R = 0.9275, $P < .001$) and no significant differences between methods for each volunteer at each time point (Paired t test $P > .05$). These results prove that the SELDI-TOF-MS approach for urinary hepcidin measurements is comparable to the published immunoassay method. In addition to providing accurate results, the assay is fast, simple, and high-throughput, and therefore suitable for large experimental clinical studies.

Implementation in clinical practice

To investigate whether hepcidin quantification by mass spectrometry can distinguish between different clinical iron metabolism disorders, urine from patients with several iron-related diseases were used for SELDI-TOF-MS measurements. Figure 1B shows that patients suffering from septicemia as well as those injected with LPS had significant elevated urinary hepcidin excretion compared with healthy subjects (Mann-Whitney U test, $P < .05$). Patients with iron deficiency anemia and (partly) compensated hereditary hemochromatosis showed significant reduced hepcidin excretion compared with healthy subjects ($P < .05$). Patients with MDS with transfusion-induced iron overload, serum transferrin saturation values higher than 77%, and ferritin levels over 500 µg/L showed relatively increased but greatly varying hepcidin levels. This variety precludes differentiation of patients with secondary iron overload from healthy individuals ($P = .054$), while median difference with acute infection patients is still significant ($P < .05$). These results are consistent with previous reports on hepcidin levels in physiologic and pathophysiologic states.[10,15-17] In addition, the SELDI-TOF-MS method would be suitable for differentiation between (hepcidin-induced) anemia of inflammation, and iron deficiency anemia where hepcidin excretion is physiologically reduced.

In conclusion, we present a novel mass spectrometry–based assay for the high-throughput measurement of hepcidin levels in urine. We anticipate that this will become an important tool to increase our insight in the role of hepcidin in iron metabolism–related disorders.

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