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Proteomics and
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Mass Spectrometry–Based Hepcidin Measurements in Serum and Urine: Analytical Aspects and Clinical Implications

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Background: **Discovery of the central role of hepcidin in body iron regulation has shed new light on the pathophysiology of iron disorders. Information is lacking on newer analytical approaches to measure hepcidin in serum and urine. Recent reports on the measurement of urine and serum hepcidin by surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) necessitate analytical and clinical evaluation of MS-based methodologies.**

Methods: **We used SELDI-TOF MS, immunocapture, and tandem MS to identify and characterize hepcidin in serum and urine. In addition to diagnostic application, we investigated analytical reproducibility and biological and preanalytical variation for both serum and urine on Normal Phase 20 and Immobilized Metal Affinity Capture 30 ProteinChip arrays. We obtained samples from healthy controls and patients with documented iron-deficiency anemia, inflammation-induced anemia, thalassemia major, and hereditary hemochromatosis.**

Results: **Proteomic techniques showed that hepcidin-20, -22, and -25 isoforms are present in urine. Hepcidin-25 in serum had the same amino acid sequence as hepcidin-25 in urine, whereas hepcidin-22 was not detected in serum. The interarray CV was 15% to 27%, and interspot CV was 11% to 13%. Preliminary studies showed that hepcidin-25 differentiated disorders of iron metabolism. Urine hepcidin is more affected by multiple freeze-thaw cycles and storage conditions, but less influenced by diurnal variation, than is serum hepcidin.**

Conclusion: **SELDI-TOF MS can be used to measure hepcidin in both serum and urine, but serum requires a standardized sampling protocol.**

The iron-regulating peptide hepcidin is produced by hepatocytes and secreted into plasma *(1–3)*. Increased iron stores and inflammation induce hepcidin expression *(3)*, whereas suppression occurs during hypoxia and anemia *(4)*. Although recognition of the central role of hepcidin in body iron regulation has increased our understanding of the pathophysiology of iron disorders *(5, 6)*, few investigative tools are available; these include an immunodot method for measuring urinary hepcidin *(7)* and a urinary hepcidin assay based on surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)[3] for differentiating various disorders of iron metabolism *(8)*. A SELDI-TOF MS–based serum assay is under development for measuring hepcidin in serum *(9)*, but this method requires optimization and evaluation to facilitate clinical investigation of hepcidin measurement in serum compared with urine and the biological variation of hepcidin in body fluids. In this study, we used SELDI-TOF MS, immunocapture, and tandem MS to identify and characterize hepcidin in serum and urine.

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[3] Nonstandard abbreviations: SELDI-TOF, surface-enhanced laser desorption/ionization time-of-flight; MS, mass spectrometry; OMIM, Online Mendelian Inheritance in Man; NP20, normal phase 20 ProteinChip array; IMAC30, Immobilized Metal Affinity Capture 30 ProteinChip array; EAM, energy-absorbing matrix; ACN, acetonitrile; TFA, trifluoroacetic acid; TIC, total ion current; MS/MS, tandem mass spectrometry; DTT, dithiothreitol; PBS, phosphate-buffered saline; PBS-Tx, phosphate-buffered saline with Triton X-100.

Materials and Methods

STUDY PARTICIPANTS

Study participants included a control population of healthy volunteers (laboratory personnel), hereditary hemochromatosis (*HFE*[4] C282Y homozygous) patients cross-sectionally selected from a family screening program (various stages of phlebotomy treatment), iron deficiency anemia patients (Hb ≤8.3 mmol/L men, ≤7.3 mmol/L women; mean corpuscular volume ≤80 fL; ferritin ≤10 μg/L), and thalassemia major patients treated with chelation therapy. The patients were recruited by their physicians during outpatient clinic visits (all in Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, except for the thalassemia major patients, who were in Ospedale Sant'Eugenio, Rome, Italy). Endotoxemia samples from volunteers injected with lipopolysaccharide were selected from a human endotoxemia project *(10)*. Written informed consent was obtained from all study participants, according to the Declaration of Helsinki. Characteristics of the study participants are shown in Table 1. In addition, collaborators from several medical centers in The Netherlands provided urine and serum samples from 3 patients with distinct forms of hereditary hemochromatosis not associated with the hemochromatosis (*HFE*) gene [Online Mendelian Inheritance in Man (OMIM) type 2a, homozygous G320V HJV variation *(11)*; OMIM type 4, N144H-caused ferroportin disease *(12)*]. Characteristics of these patients are shown in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue4.

[4] Human gene: *HFE*, hemochromatosis.

We collected samples randomly between December 2005 and June 2006, at no specified time of day, except from the endotoxemia patients, from whom samples were collected according to a tight time schedule *(10, 13)*. Urine and blood samples were centrifuged immediately after collection, divided into aliquots to avoid multiple freeze-thaw cycles, and stored at −80 °C. We performed the hepcidin assay within 2 months after collection.

LABORATORY MEASUREMENTS

Using an Abbott Aeroset analyzer, we measured total serum iron and latent iron binding capacity by the ascorbate/FerroZine colorimetric method, urine creatinine by enzymatic/colorimetric detection (Roche Diagnostics), and C-reactive protein by immunologic agglutination detection with latex-coupled polyclonal anti–C-reactive protein antibodies (Abbott Laboratories). We quantified serum ferritin by a solid-phase, 2-site chemiluminescent immunometric assay (Immulite 2000 and 2500, Diagnostic Products Corp.) and routine hematology characteristics by use of a Sysmex XE-2100 analyzer.

SELDI-TOF MS

We performed nonblinded hepcidin measurements by use of SELDI-TOF MS as previously reported *(8)*. In brief, a 5-μL sample was applied to Immobilized Metal Affinity Capture 30 (IMAC30), Normal Phase 20 (NP20), or cation exchange ProteinChip arrays (CM10), all equilibrated with appropriate buffers according to the manufacturer's instructions (Ciphergen Biosystems). Loaded arrays were incubated in a humidity chamber for 30 min and then washed according to the manufacturer's instructions and

Table 1. Characteristics of participants who provided sample material for assay validation.[a]

	Control	Endotoxemia (model)	Iron deficiency anemia	Thalassemia major[b]	Hereditary hemochromatosis[c]
n	20	28	6	5	14
Sex (male:female)	9:11	NA[d]	3:3	1:4	10:4
Age, years	44 (24–62)	NA	47 (14–61)	35 (25–44)	56 (23–82)
Hemoglobin, mmol/L	8.7 (7.8–11.0)	NA	6.7 (5.0–8.3)	6.3 (6.1–7.3)	9.3 (7.5–12.0)
MCV, fL	88 (84–93)	NA	77 (76–79)	86 (78–88)	90 (83–95)
Serum iron (Fe), μmol/L	19 (9–38)	18 (7–38)	6 (4–7)	52 (47–62)	22 (10–48)
Fe/TIBC, TS, %	35 (14–73)	40 (23–79)	8 (5–10)	98 (93–100)	56 (21–100)
Ferritin, μg/L	60 (11–191)	120 (31–233)	6 (6–10)	784 (272–1710)	64 (28–1361)
C-reactive protein, mg/L	<5	9 (<5–33)	<5	<5	<5
Urinary hepcidin-25 (NP20), M Int/mmol Cr	0.52 (0.09–2.97)	6.16 (1.27–19.78)	0.01 (0.01–0.02)	0.21 (0.04–0.98)	0.15 (0.02–1.25)
Urinary hepcidin-25 (IMAC30), M Int/mmol Cr	1.45 (0.52–7.83)	12.97 (1.95–30.81)	0.10 (0.01–0.19)	0.59 (0.24–2.96)	0.66 (0.08–4.50)
Serujm hepcidin-25 (IMAC30), M Int/L	4.38 (0.58–9.95)	12.69 (4.11–27.33)	0.41 (0.35–0.60)	0.28 (0.18–2.42)	1.60 (0.19–9.39)

[a] Data are median (range).

[b] Cross-sectional selection of thalassemia major patients with variable transfusion history and treated with different iron chelators (desferrioxamine and/or deferiprone).

[c] Cross-sectional selection of homozygous C282Y patients from family study; 11 patients are under phlebotomy treatment.

[d] NA, not available; TIBC, total iron binding capacity; M Int/mmol Cr, megaintensity/mmol creatinine.

air-dried for 15 min. Finally, 1 μL energy-absorbing matrix (EAM), made up of a 12.5 g/L solution of sinapinic acid in 500 mL/L acetonitrile (ACN) containing 5 mL/L trifluoroacetic acid (TFA), was applied twice onto each spot surface with the use of polymer-free polypropylene pipette tips and allowed to air-dry for 5 min. Mass-to-charge (m/z) spectra were generated using a Ciphergen Protein Biology System IIc TOF mass spectrometer at laser intensity 180 (NP20 and CM10) or 175 (IMAC30); detector sensitivity 9; high mass to acquire 50 kDa; optimization interval 1500 to 10 000 Da. External mass calibration was performed with a mixture of synthetic human hepcidin-25 peptide (2789.4 Da, Peptides International), hepcidin-22, and hepcidin-20 (2436.1 and 2191.8 Da, respectively; kindly provided by E. Nemeth, University of California, Los Angeles). Peak annotation was performed with Ciphergen ProteinChip Software version 3.2.0. From unpublished experiments we found that for serum and urine, normalization to total ion current (TIC) did not improve the hepcidin measurements and was therefore not applied in this study. Urine hepcidin measurements showed a relationship with TIC and total peptide content, which was predominated by hepcidin under the applied experimental conditions and instrumental settings (data not shown). Consequently, normalization of urine hepcidin values to TIC leads to loss of differentiation between samples. In contrast, due to the relatively stable protein content of serum samples, the serum hepcidin concentrations did not significantly change upon normalization to TIC (unpublished observations). Although the reabsorption and excretion characteristics of hepcidin are unclear, we used urinary creatinine to normalize all peak intensities for hepcidin-25 in urine. Normalization on creatinine is a prerequisite for comparison of urine hepcidin measurements because it is the best method to correct for the highly fluctuating concentration differences between urine samples. Relative concentrations were expressed as mega-intensity units per millimole of creatinine. Relative concentrations of serum hepcidin-25 were expressed as mega-intensity units per liter. Information on the assay can be found on: www.UMCN.NL/Hepcidin.

PURIFICATION OF PEPTIDES FROM SERUM AND URINE

We first fractionated serum samples by use of spin columns containing Q HyperD F resin (Pall Corp.), eluted the flow-through fraction by centrifugation, and eluted bound proteins with buffers of pH 9, 7, 5, 4, and 3. We analyzed fractions by use of IMAC30 arrays. Every serum fraction or urine sample was adjusted to final concentrations of 5% ACN and 0.5% TFA and bound to PLRP-S beads (Polymer Laboratories, Varian). Bound proteins were eluted with 5%, 10%, 20%, 30%, 40%, 50%, and 60% ACN solutions containing 0.1% TFA. We detected proteins in eluted fractions by profiling 1 μL of each fraction on an NP20 array using Protein Biology System IIc MS.

IDENTIFICATION OF PEPTIDES BY TANDEM MASS SPECTROMETRY

We analyzed peptides of interest for the presence of disulfide bonds. Aliquots of the 30% ACN fractions were air-dried on an NP20 array. A solution containing 10 mmol/L dithiothreitol (DTT) in 50 mmol/L ammonium bicarbonate, and the sample loaded arrays, was preheated to 70 °C. Then we loaded 10-μL aliquots of the DTT solution onto the spots and air-dried them at 70 °C. After complete evaporation of solutions and cooling to room temperature, we applied EAM. We acquired single MS spectra for unreduced and DTT-reduced samples by use of a Q-STARXL MS/MS (Applied Biosystems) equipped with a Ciphergen PCI-1000 ProteinChip Interface. We used the reduced samples to acquire tandem mass spectrometry (MS/MS) spectra. We subjected the ions of interest (m/z values of 2198 for hepcidin-20, 2442 for hepcidin-22, and 2796 for hepcidin-25) to collision-induced dissociation and submitted the results to the database-mining tool Mascot (Matrix Science) for peptide identification.

IMMUNOCAPTURE

We captured hepcidin from urinary samples by use of Protein G coupled to IDM beads (Ciphergen Biosystems) and polyclonal rabbit antihepcidin serum (generous gift from E. Nemeth and T. Ganz, University of California, Los Angeles). We performed all incubation steps at room temperature. Protein G beads were first incubated with rabbit antiserum diluted 20 times in 0.01 mol/L phosphate-buffered saline (PBS; Sigma-Aldrich Chemie BV) supplemented with 0.1% Triton X-100 (PBS-Tx). We washed the beads 3 times with PBS-Tx to remove unbound serum proteins and resuspended the beads in urine, diluted 20 times in PBS containing a final concentration of 0.25 mol/L NaCl and 0.1% Triton X-100 ($PBS_{0.25}$-Tx). We washed the beads 6 times with $PBS_{0.25}$-Tx to remove unbound and nonspecifically bound proteins. Finally, we eluted bound proteins with 500 mL/L ACN containing 3 mL/L TFA. To obtain profiles of Protein G–captured proteins, we applied eluates to a CM10 array equilibrated with 0.1 mol/L ammonium acetate (pH 3) and incubated them for 30 min in a humidity chamber. To obtain reference spectra, we diluted untreated urine samples once in equilibration buffer before on-spot incubation. Spots were washed, allowed to air-dry, and followed by sinapinic acid application.

ANALYTICAL ASSAY CHARACTERISTICS

We performed spot-to-spot precision for hepcidin-25 on NP20 and IMAC30 ProteinChip array with 2 human urine samples with medium and high hepcidin concentrations. Both samples were applied onto the first 6 positions of the 8-spot array, followed by addition of the EAM. The last 2 spots were used for control samples. For serum, we followed the same procedure for a single sample.

We used the same urine and serum samples for a chip-to-chip reproducibility study. We collected single measurements for serum and urine on NP20 and IMAC30 array types from randomized chip positions for 10 days (8 days for serum application on NP20 arrays). Every day a new sample aliquot was thawed, and freshly prepared EAM was applied. From these data, we calculated means, SDs, and CVs.

We measured linearity of peak intensities by dilution of the urine and serum samples used for the reproducibility tests with human urine or serum (dilution samples) from a patient with hepcidin concentrations below those detectable by SELDI-TOF MS. Immediately before sample application, we diluted 1 to 18 μL sample in a polypropylene microcentrifuge container to a 20 μL end volume with the dilution sample. After pipette mixing, 5 μL of the diluted sample was spotted on the array.

We created standard curves of synthetic hepcidin-25 for both serum and urine applications. After dissolving the lyophilized peptide in distilled water (100 μmol/L), the peptide was diluted 4500-fold with dilution sample for serum or urine (22.2 nmol/L). Dilutions of 18, 16, 12, 8, 4, 2, and 1 μL of the 22.2 nmol/L solution with the dilution sample to the final volume of 20 μL yielded a standard curve ranging from 22.2 to 1.11 nmol/L.

PREANALYTICAL AND BIOLOGICAL INTERFERENCES

We evaluated the influence of multiple freeze-thaw cycles for both serum and urine using IMAC30 arrays. Five sera and 4 urine samples from different participants and different intensity concentrations underwent 4 freeze-thaw cycles. After every cycle, we analyzed hepcidin batchwise by single measurement.

We studied the existence of a circadian rhythm for hepcidin in serum and urine by performing a 24-h time course in 3 healthy volunteers (1 man, 2 women). Blood and urine were collected every 3 h for analysis of routine iron measurements and serum and urine hepcidin (IMAC30 array), starting at 6:00 AM with fasting blood and urine samples, after which the fasting state was ended. All samples were processed as described above and stored at −80 °C before analysis (batchwise by single measurement).

STATISTIC ANALYSIS

We performed statistical analyses with GraphPad Prism software, version 4.0. Pearson correlation was used for comparison studies. P values <0.05 were considered significant.

Results

HEPCIDIN IDENTIFICATION AND CHARACTERIZATION

The presence of hepcidin-25 in human urine *(8)* and serum *(9)* has been demonstrated. Our current investigation revealed that, in addition to hepcidin-25, hepcidin-20 can be verified in both urine and serum with the use of a purified synthetic reference human hepcidin peptide, whereas hepcidin-22 can be identified only in urine (Fig. 1A). This supports the hypothesis that both the 20- and 25–amino acid peptides are secreted in the circulation after production in hepatocytes, whereas the 22–amino acid peptide merely is a urinary degradation product of hepcidin-25 *(14)*.

In addition to mass tracing, we investigated immunocapture of all 3 known forms of hepcidin from urine. Immunoaffinity assays using polyclonal rabbit antihepcidin antibodies [the same as those used in hepcidin dotblot assay *(7)*] showed that the 3 peaks annotated as hepcidin-25, -22, and -20 can be specifically captured from a human urine sample of a healthy individual (Fig. 1B). MS/MS analysis of peptides in a urine sample from an endotoxemia patient confirmed that the peaks with m/z of 2198, 2442, and 2796 corresponded to hepcidin-20, -22, and -25, respectively (see Fig. 1A in the online Data Supplement). Similarly, we identified the m/z 2796 peak in serum as hepcidin-25. Notably, single MS spectra of urine and serum samples displayed a mass shift of +8 Da for all hepcidin forms after reduction with DTT, as exemplified for urine hepcidin-25 (see Fig. 1B in the online Data Supplement). This observation confirms the presence of 4 disulfide bonds in these peptides, which is a typical characteristic of hepcidin *(15)*. Finally, the MS/MS spectra for serum and urine peptides with m/z of 2796 displayed mostly the same fragment ions, strongly suggesting that both parent ions corresponded to the same peptide hepcidin-25 (see Fig. 1C in the online Data Supplement).

ANALYSIS OF SERUM AND URINE HEPCIDIN-25 ON NP20 AND IMAC30 PROTEINCHIP ARRAYS

Because the roles of hepcidin-20 and -22 in iron metabolism are unclear *(2, 14)*, hepcidin-25 is used for optimization of serum and urine hepcidin measurements. To evaluate the effect of ProteinChip array type on the performance of the hepcidin-25 assay, we analyzed 73 urine and serum sample pairs from controls and patients with various iron metabolism disorders using the previously reported NP20 and IMAC30 arrays *(8, 9)*. Although hepcidin concentrations measured using NP20 arrays are lower than those measured with IMAC30 arrays, both arrays correlate for the urinary application ($R = 0.928$, $P < 0.0001$; Pearson correlation; Fig. 2A). Sensitivity was higher with the use of IMAC30 compared with the NP20 arrays, likely because of highly specific binding of hepcidin to the IMAC-Cu surface. In contrast to the IMAC30 arrays, NP20 arrays did not bind detectable concentrations of serum hepcidin-25; therefore, we could not perform correlation analyses for serum and urine on NP20 arrays. In contrast, IMAC30 arrays showed a significant correlation ($R = 0.822$, $P < 0.0001$; Pearson correlation; Fig. 2B).

Chip-to-chip variation of the urine application for IMAC30 ranged from 22% at a high intensity of 48 to

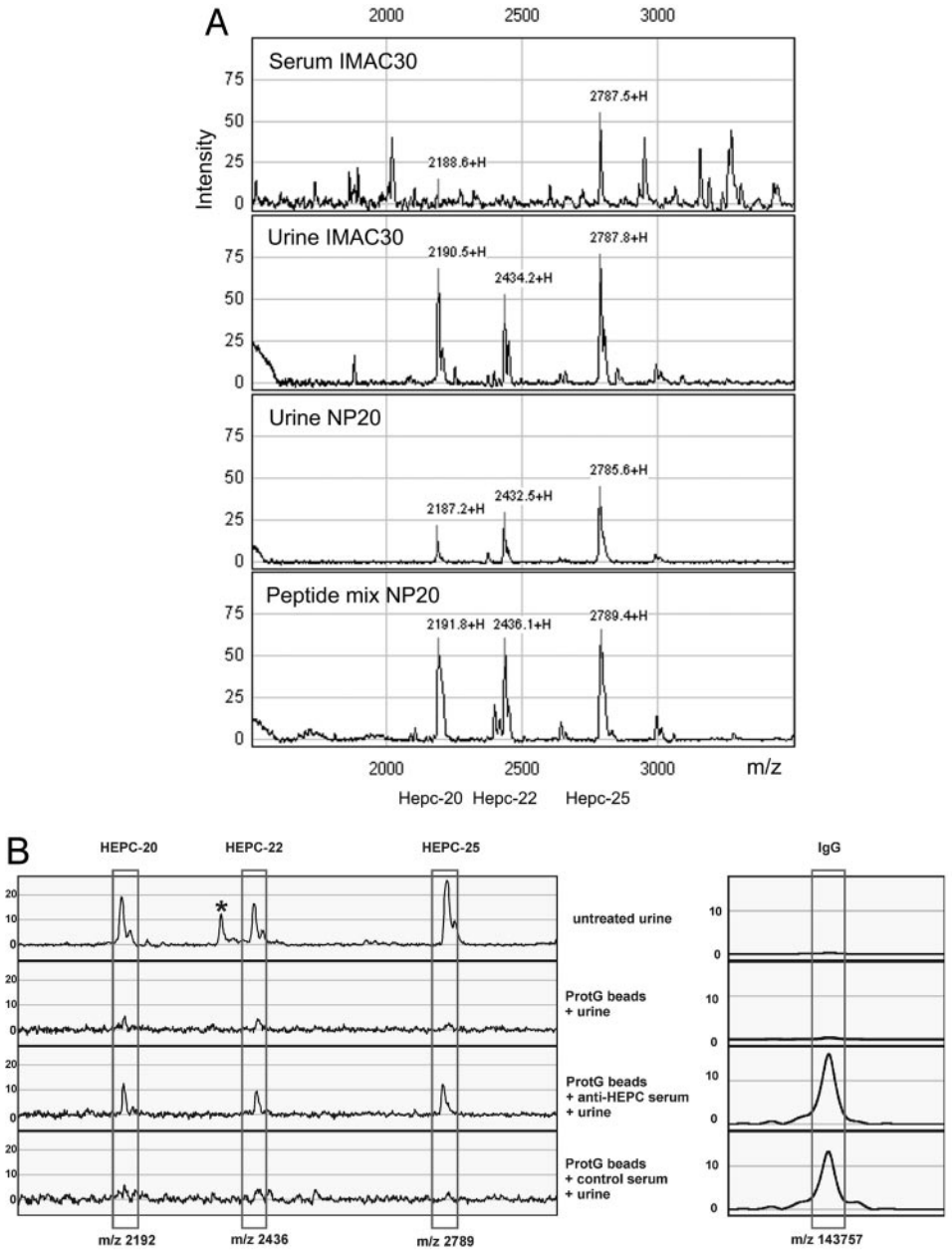

Fig. 1. Hepcidin identification and characterization.

(*A*), SELDI-TOF MS profiles in serum and corresponding urine samples from a participant with an inflammation response after lipopolysaccharide injection. The annotated peak masses matched to the masses in the reference peptide mix that contained purified synthetic human hepcidin-20, -22 (E. Nemeth, University of California, Los Angeles), and -25 (Peptides International). (*B*), *i*mmunocapture of urine hepcidin. Urine samples from a healthy individual were incubated with Protein G beads loaded with either a polyclonal antihepcidin rabbit antibody or control rabbit IgG. Immunocaptured proteins were analyzed by SELDI-TOF MS, and protein profiles were compared with the protein profile of an untreated urine sample (*upper panel*). Peaks corresponding to 3 forms of urine hepcidin *(8)* and those corresponding to rabbit IgGs are indicated. A peak with *m/z* 2436 that is present in untreated urine, but not present in the captured fractions, is marked (*), as it shows that the capture of the 3 hepcidin forms was specific. Notably, on-spot incubation of the same urine sample with antihepcidin antibodies bound to Protein G-loaded arrays did not yield detectable hepcidin concentrations (our unpublished observations).

27.5% at a lower intensity of 20 (Table 2), whereas NP20 variation reached 34% for the same samples. Precision was also better for urine hepcidin measurements using the IMAC30 chip (CVs 11% and 13%) compared with the NP20 chip (CVs of 16% and 23%; Table 2).

For serum, the reproducibility was strongly affected by differences in protein binding capacity of both arrays. Intensities measured from the same sample differed, from an intensity of 5 with relatively high CVs on NP20 array to an intensity of 50 with low CVs on IMAC30 array. We checked the linearity of the urinary and serum hepcidin application on IMAC30 array by measuring a dilution series of urine and serum samples from an inflammation patient (single measurements). Fig. 2, A and B, in the online Data Supplement shows a high degree of linearity for both urine and serum ($R = 0.993$ and 0.971, respectively). Intensity values >55 seem to deviate from linearity, perhaps because of MS detector saturation. Therefore, an intensity value of 55 was considered to be the upper level of detection.

The construction of a calibration curve with synthetic human hepcidin-25 showed that both NP20 and IMAC30 arrays are capable of producing a 6-point calibration curve based on a urine matrix (see Fig 2, C and D, in the online Data Supplement). Again, intensity values >55 deviated from linearity and therefore were considered as exceeding the upper level of detection. Construction of

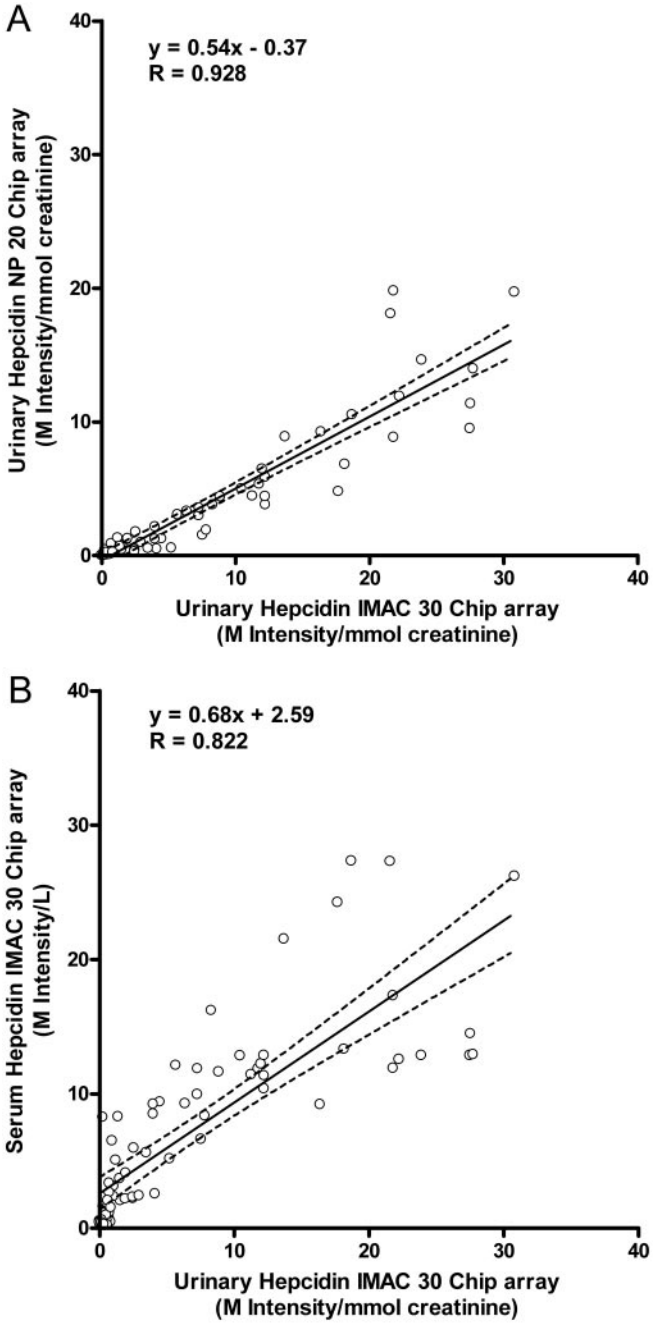

Fig. 2. Detection of serum and urine hepcidin-25 on NP20 and IMAC30 arrays.

Correlation of urinary hepcidin-25 values as measured by NP20 and IMAC30 ProteinChip arrays (*A*) and coupled serum and urine hepcidin-25 concentrations measured on IMAC30 array (*B*). The *solid line* represents the regression line, and the *dotted line* indicates the 95% CI of the regression line. It should be noted that the apparent increased scatter in absolute variation on high hepcidin values compared with low values, as presented in panel *A* of this figure, is because absolute concentrations are used in this plot. The relative average for the high values is not more scattered than the low-end values (data not shown), which is in concordance with the CV values shown in Table 2. As it is currently unknown whether and how much hepcidin is reabsorbed in the tubulus, we cannot indicate whether this aspect contributes to the scatter in panel *B* of this figure.

the same concentration interval on IMAC30 array in a serum matrix showed hepcidin-25 only for a concentration of 22.2 nmol/L and above (results not shown).

Table 2. Reproducibility of hepcidin-25 analysis by SELDI-TOF MS in NP20 and IMAC30 chip arrays in both serum and urine.

	NP20		IMAC30	
	m/z	Intensity	*m/z*	Intensity
Urine application				
Chip-to-chip[a]				
Level I				
Mean	2788.48	13.10	2788.65	19.84
SD	0.94	4.37	1.96	5.46
CV, %	0.03	33.4	0.07	27.5
Level II				
Mean	2788.37	41.55	2788.45	47.84
SD	0.89	10.00	1.64	10.50
CV, %	0.03	24.1	0.06	22.0
Spot-to-spot[b]				
Level I				
Mean	2787.13	15.23	2788.53	17.83
SD	1.00	2.62	1.55	1.97
CV, %	0.04	16.5	0.06	11.0
Level II				
Mean	2787.88	40.61	2788.24	40.89
SD	0.53	9.47	1.00	5.25
CV, %	0.02	23.3	0.04	12.8
Serum application				
Chip-to-chip[c]				
Level I				
Mean	2788.40	5.26	2788.02	51.96
SD	1.41	1.73	1.56	7.76
CV, %	0.05	32.9	0.06	14.9
Spot-to-spot[b]				
Level I				
Mean	2787.54	4.27	2788.33	43.89
SD	0.60	1.14	0.59	5.06
CV, %	0.02	26.6	0.02	11.5

[a] Ten-day single measurement with randomized chip position.
[b] One chip (6 replicates).
[c] Eight-day single measurement (NP20 array) and 10-day single measurement (IMAC30 array) with randomized chip position.

DIAGNOSTIC APPLICATION

We categorized urine and serum sample pairs into 5 groups after clinical diagnosis. Fig. 3 shows that, despite the variation within each group and a slight overlap, differentiation between inflammation-induced and iron deficiency anemia is clear for both serum and urine hepcidin concentrations measured on IMAC30 array. The results are comparable with the results obtained with NP20 and consistent with previous reports *(7, 8)*. The broad ranges of the thalassemia group and the hemochromatosis group are in accordance with the individual heterogeneity in genetic makeup, extent of anemia, and treatment *(16–18)*.

In this study, we found serum and urine hepcidin concentrations close to zero in 2 brothers treated for juvenile hemochromatosis (OMIM type 2a) and values in

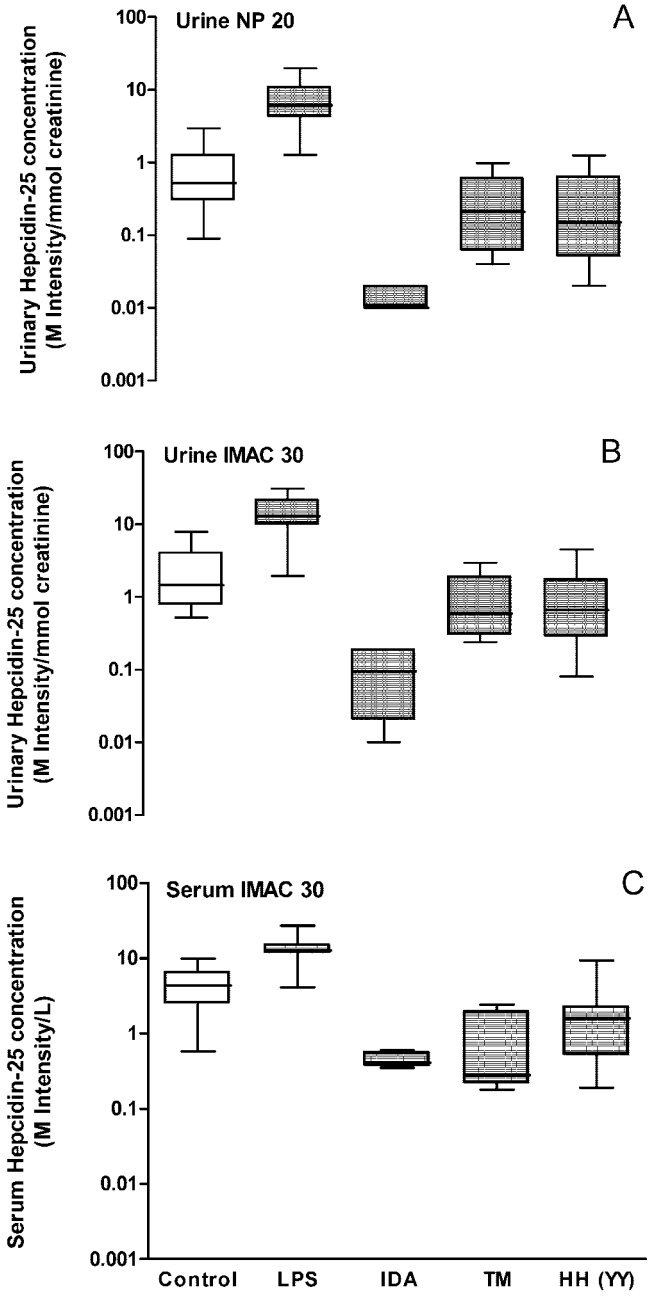

Fig. 3. Urinary and serum hepcidin-25 concentrations in selected clinical populations.

Urinary hepcidin-25 concentrations measured on NP20 array (*A*) and IMAC30 array (*B*) and serum hepcidin-25 measured on IMAC30 array (*C*). *LPS*, volunteers injected with lipopolysaccharide (6 h after injection); *IDA*, iron deficiency anemia patients; *TM*, thalassemia major patients; *HH(YY)*, C282Y homozygous hemochromatosis patients. *Box plots* show 25th and 75th percentile with median for every group. *Error bars* represent minimum and maximum values. Characteristics of each population are shown in Table 1.

the upper level of the reference range for a patient treated for ferroportin disease (OMIM type 4; see Table 1 in the online Data Supplement). These data confirm the potential of hepcidin analysis in prescreening for the presence of non-*HFE* hemochromatosis.

PREANALYTICAL AND BIOLOGICAL DETERMINANTS THAT INFLUENCE HEPCIDIN-25 MEASUREMENTS IN SERUM AND URINE

Decreases in hepcidin concentrations in serum samples due to multiple freeze-thaw cycles is of minor importance compared with those in urine (see Fig. 3 in the online Data Supplement). Preliminary results from urine samples stored at −80 °C and −20 °C during a 6-month course showed that (*a*) hepcidin was stable only at −80 °C and (*b*) addition of protease inhibitor phenylmethylsulfonyl fluoride *(19)* had no beneficial effect (unpublished observations).

Hepcidin concentrations in serum follow a clear circadian rhythm, such that the concentrations were 2- to 6-fold higher at 1500 than at 0600. Urinary concentrations, however, show a more blunted response, and thus less

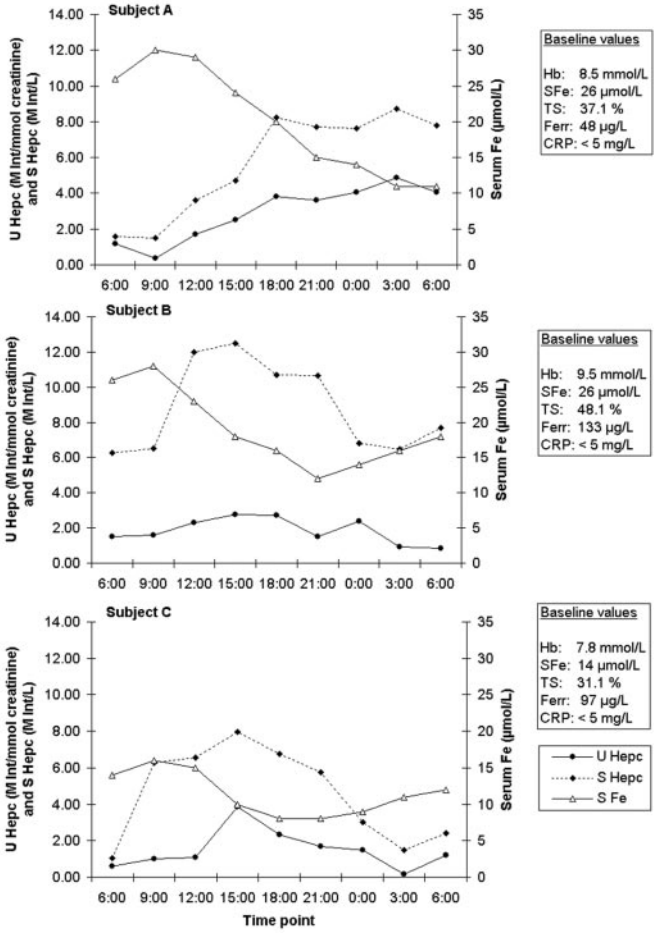

Fig. 4. Circadian influence on urinary and serum hepcidin-25 concentrations.

Urinary and serum hepcidin concentrations and serum iron concentration in 3 healthy volunteers during a 24-h time course (participants A and C, premenopausal women; participant B, man). Circadian influence is seen on all 3 variables. Individual differences are visible on amplitude and frequency pattern and relative concentration difference between urinary and serum hepcidin. Note that in participant A, C-reactive protein concentrations increased to 10 mg/L in the last 6 h of the time course, which suggests that an inflammatory process may have contributed to the persistent increase in hepcidin and the decrease in serum iron during this period.

diurnal variation. The course of serum iron shows an inverse association with serum and urine hepcidin values (Fig. 4).

Discussion

The IMAC30 array–based urinary hepcidin assay correlated significantly with the previously described urinary hepcidin MS application using NP20 arrays *(8)* and showed greater sensitivity and reproducibility, resulting in improved analytical performance. The increase in protein-binding capacity of the IMAC30 array makes it particularly suitable for serum analysis and measurement of low hepcidin concentrations in urine.

The total assay variation includes analytical variation, biological or diurnal variation, and preanalytical variation, all contributing to the widening of ranges of the clinical utility clusters. In the future, we plan to routinely perform duplicate measurements and investigate isotope dilution *(20)* or hepcidin derivatives as internal standards to reduce analytical variability.

Biological variation in hepcidin concentrations due to a circadian rhythm correlated inversely with daily variations in serum iron concentrations *(21)*, in accordance with previous reports that transferrin receptors 1 and 2 on the outer hepatocyte membrane act as sensors of circulating iron and TS, thereby linking low serum iron with increased hepcidin synthesis *(22, 23)*. Circadian rhythm may also lead to important variation in outcome if sampling time is not standardized, thereby contributing to the wide variation in hepcidin concentrations of the control population (Fig. 3C). Sampling according to protocol led to decreased variation in hepcidin concentrations within the lipopolysaccharide group. The low variation within the iron deficiency anemia group is likely to be due to the dominant down-regulating influence of an iron-deficient erythropoiesis on hepcidin that prevents circadian increases.

Variation in hepcidin results is also attributable to preanalytical conditions. According to our preliminary results, urine samples are more susceptible than serum to variation from multiple freeze-thaw cycles, which should be avoided, and urine samples should be stored at −80 °C as soon as possible *(24, 25)*.

Measurement of serum and urine hepcidin by the same technique, and under the same circumstances, enabled comparison of serum with urinary values. Although their association was strong, confirming the previously reported correlation between hepcidin mRNA expression in liver cells and urinary hepcidin excretion *(26)*, differences in serum and urine composition prohibit absolute comparison of analytical characteristics such as binding competition on the array surface and flight behavior during SELDI-TOF MS analysis *(27)*. Aspects such as ionization efficiency or ion suppression also can play a role and should be investigated in future studies. Meanwhile, differences in specimen behavior preclude calculations of the kinetics of hepcidin release by hepatocytes and its excretion from the blood into the urine. Construction of a calibration curve by addition of the same amount of hepcidin-25 to serum or diluted urine confirms this effect, leading to speculation that a binding protein in serum prevents the binding of free hepcidin on the chip surface at low concentrations *(27)*. To circumvent differences in binding characteristics or possible matrix interferences, methodologic approaches such as plasma fractionation *(27)* or the use of magnetic reversed-phase beads *(28)* have to be investigated for utility on hepcidin measurements.

Our reported SELDI-TOF MS method detected all 3 isoforms of hepcidin, improved urinary hepcidin analysis, and enabled measurement of serum hepcidin by IMAC30 array. We show for the first time a high correlation of concentrations measured in corresponding serum and urine samples. Selection of the optimal body fluid for analysis, however, is influenced by preanalytical, analytical, and biological variations that effect serum and urine differently. Urine is more vulnerable to multiple freeze-thaw cycles and storage temperatures other than −80 °C but less influenced by diurnal variation. Serum is more susceptible to circadian variation, and therefore standardization of sampling time is needed in clinical studies with serum. Serum is likely to be more sensitive than urine, however, for monitoring short-term kinetics of body hepcidin concentrations. Therefore, the specimen of first choice depends on study design and practical aspects such as availability of materials and equipment. The ability to measure hepcidin in both serum and urine confirms that the learning process on hepcidin characteristics, kinetics, and clinical utility has only just begun.

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