Application Note

Quantification of Hepcidin in Plasma Using the Immundiagnostik Hepcidin-25 LC-MS/MS Kit (Ruo) For Clinical Research

Davor Fielitz, Fabian Simon, Anne Arnold, Max Diesner, Stefanie Wernisch

Waters Corporation, Immundiagnostik AG

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Abstract

LC-MS/MS-based quantification of the small peptide hepcidin in plasma is challenging due to hepcidin´s multiple intramolecular disulfide bridges, its susceptibility to non-specific adsorption, and multiple charge states.

This application note presents a method for the measurement of low levels of hepcidin in citrate plasma for clinical research. The simple sample preparation technique utilizes reagents provided in Immundiagnostik´s Hepcidin-25 LC-MS/MS kit (RUO) in combination with Waters™ OASIS™ HLB technology in 96-well format for sample preparation. LC-MS/MS analysis is performed on an ACQUITY™ UPLC™ I-Class System with flow-through needle (FTN) connected to a Xevo™ TQ-S micro tandem-quadrupole Mass Spectrometer operated in ESI positive mode.

With this method, Hepcidin can be quantified in plasma with good linearity (R^2>0.99) and high precision (CV<10%). Accuracies of 95% and 98% were determined for the analysis of the 4+ and the 5+ charge states, respectively. Detection limits were determined to be below 2 ng/mL based on extrapolation from the signal-to-noise ratio of the lowest calibrator.

Benefits

- Immundiagnostik Hepcidin-25 kit (RUO) enables simple sample preparation and LC-MS/MS setup

- Waters OASIS HLB µElution plate and QuanRecovery™ collection plate minimize adsorptive losses and facilitate low-level quantification of hepcidin
- Waters ACQUITY I-Class UPLC (FTN) and TQ-S micro Mass Spectrometer enable quantification of hepcidin in plasma with excellent precision and accuracy

Introduction

Hepcidin is a small, cysteine-rich signal peptide comprised of 25 amino acids and containing four internal disulfide bonds. It was first isolated from human urine and named for the liver (hep-), its site of synthesis, and its in-vitro antimicrobial properties (-cidin).[1] Hepcidin is involved in mammalian iron metabolism.[2–4]

For measurement of hepcidin in plasma for clinical research, the disulfide bonds can be reduced. Due to its many chargeable sites, the intact peptide, is still prone to non-specific adsorption on charged surfaces, in particular at low levels. In this application note, we demonstrate the usefulness of consumables with low-bind surfaces for robust results. Solid-phase extraction removes potential matrix interferences from the sample and provides the basis for a robust chromatographic procedure. Optimization of the mass spectrometric parameters, among them the selection of the precursor ion charge state and fragments to monitor, allows for low-level quantification of hepcidin in citrate plasma in the low ng/mL range.

Experimental

The Immundiagnostik Hepcidin-25 LC-MS/MS kit (Immundiagnostik part no. KM4000 RUO) was used for sample preparation and LC separation setup according to its Instructions for Use (IFU). It contains the required reagents and solutions: mobile phases A and B, internal standard, activation solution, sample buffer, wash solutions 1 and 2, elution buffer, and dilution and reconstitution solutions.

Mobile phases, sample buffer, wash solution 2, and dilution and elution solutions were activated prior to analysis according to Immundiagnostik 25-Hepcidin LC-MS/MS kit manual. To avoid protein-surface interaction, samples were handled using Sørensen Low Binding Tips (Sigma-Aldrich, p/n: Z719579-1000EA) and Protein LoBind

Tubes, 15mL (Eppendorf, p/n: 0030122216)

Sample Description

Six levels of calibrators (CAL 1–6) and three levels of quality control samples (QC 1–3), were provided by Immundiagnostik within the Hepcidin-25 LC-MS/MS kit. The 19 samples of citrate plasma were also provided by Immundiagnostik.

Sample preparation was performed following a simple solid-phase extraction (SPE) protocol (see below) on an OASIS HLB µElution 96-well plate, using a positive pressure manifold and a QuanRecovery collection plate. Chromatographic separation of the analyte takes 5.5 minutes. The peptide was quantified in MRM (multiple reaction monitoring) mode via its four- or five-fold charged state and the corresponding, characteristic fragment ions.

Solid-phase extraction: Citrate plasma samples were centrifuged at 13,400 rpm for five minutes prior to SPE. The µElution plate was washed with 200 µL methanol and equilibrated with 200 µL water (Milli-Q Ultrapure). 200 µL of sample, CAL or CTRL were added to each well, together with 200 µL of internal standard. The µElution plate was washed with 200 µL each of washing solution 1, washing solution 2 and, again, washing solution 1. After installing the QuanRecovery collection plate, samples were eluted with 2 x 25 µL elution solution. The SPE eluate was diluted 1:2 with dilution solution. The UPLC injection volume was 20 µL.

LC Conditions

LC system:	ACQUITY UPLC I-Class (FTN) with column heater (CH)
Needle:	10 µL standard injection needle with 50 µL extension loop
Column:	XSelect™ CSH C_{18}, 3.5 µm, 2.1 x 100 mm
Pre-column:	XSelect CSH C_{18} VanGuard™ Cartridge, 130 Å, 3.5 µm, 2.1 mm x 5 mm

Column temperature:	50°C
Injection volume:	20 µL
Flow rate:	0.4 mL/min
Mobile phases	Immundiagnostik (proprietary)
Gradient:	15–98 % B

MS Conditions

MS system:	Xevo TQ-S micro
MS mode (Resolution):	MS1 and MS2 (0.75 FWHM)
Acquisition mode:	Multiple reaction monitoring (MRM)
Ionization mode:	ESI+
Source temperature:	150°C
Desolvation temperature:	650 °C
Desolvation gas flow:	1200 L/h
Cone gas flow:	0 L/h
Cone voltage:	See Table 1
Collision energy:	See Table 1

MS parameters	Hepcidin (4+)	Hepcidin (5+)	Hepcidin IS (4+)
Ionisation	ESI+	ESI+	ESI+
Parent *m/z*	698.2	558.8	703.5
Quantifier *m/z*	1040.1 (CE 26)	354.1 (CE 26)	354.1 (CE 35)
Qualifier *m/z*	354.1 (CE 36)	501.4 (CE 20)	–
Cone voltage (V)	20	24	20

Table 1: MRM parameters for hepcidin-25 and its stable-isotope labelled internal standard (IS). Dwell times were set automatically to 65 ms by dwell time calculator using 12 points per peak over a four-second peak width. CE, collision energy in eV.

Data Management

MS software: MassLynx™ 4.2 with TargetLynx™ XS

Results and Discussion

Optimization of MS precursor with IntelliStart™

The kit manual suggested to base the hepcidin analysis on the 698 > 354 (quantifier) and 698 > 1040 (qualifier) transitions, coming from the four times charged hepcidin parent $[M+4H]^{4+}$. However, the automated IntelliStart™ sample tuning function in MassLynx found higher ion intensities for the $[M+5H]^{5+}$ parent. IntelliStart™ readjusts mass position, cone voltage, and collision energy.

In this experiment, both parent signals and their corresponding MRM transitions were recorded and compared. For the 4+ species, the 698 > 1040 and 698 > 354 transitions gave almost equal signal intensity, but the former yielded slightly lower noise values. As lower noise levels can be critical in low-abundance situations, we deviated from the testing protocol suggested by Immundiagnostik and assigned the 698 > 1040 transition as quantifier and the 698 > 354 trace as qualifier.

Linearity

The calibration range was 3.5–132.7 ng/mL. Linearity of the method was determined by measuring two replicates of two independent sample preparations (four replicates total) for each calibration level. The coefficient of correlation (R^2) was above 0.993 for the $[M+4H]^{4+}$ species (Figure 1) and above 0.990 for the $[M+5H]^{5+}$ species (Figure 2). The variation of each individual measuring point was below 15% and thus within the acceptance criteria.

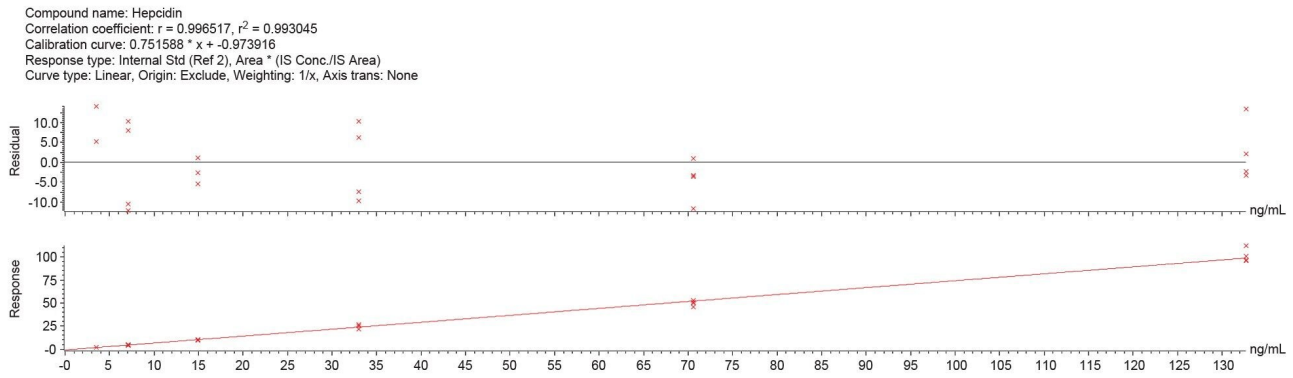

Figure 1: Calibration curve and residuals plot for hepcidin 4+ (698.2>1040.1).

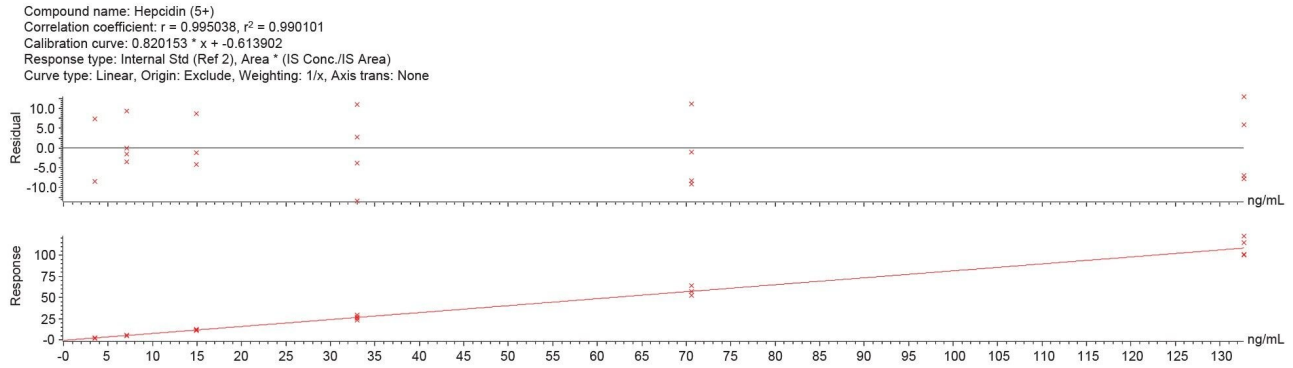

Figure 2: Calibration curve and residuals plot for hepcidin 5+ (558.8>354.1).

Precision

Intra-assay precision was determined based on eight measurements per QC level (low, medium, high). For all

three QC levels, two replicates each were prepared from two different QC samples (different sample container; same material batch). Each sample was injected twice, resulting in eight injections per level. Results are shown as coefficients of variation (CV) in Table 2.

	Low QC		Medium QC		High QC	
	Hepcidin 4+	Hepcidin 5+	Hepcidin 4+	Hepcidin 5+	Hepcidin 4+	Hepcidin 5+
CV%	8.6	6.9	6.7	7.7	5.7	5.6

Table 2: Intra-assay precision at the three levels of QC (low, medium, high). Data for N=8. CV, coefficient of variation.

Accuracy

Accuracy was determined by extracting and quantifying the hepcidin levels in all QC samples. The calculated sample concentrations were compared to the setpoint provided by Immundiagnostik in the kit´s IFU. All QC samples deviated from the expected values by less than 15% and are within the acceptance criteria (Table 3).

	Accuracy [%]			
	Low QC	Medium QC	High QC	Overall
Hepcidin 4+	98.8 ± 8.5	98.8 ± 8.5	98.8 ± 8.5	98.8 ± 8.5
Hepcidin 5+	98.1 ± 6.9	98.1 ± 6.9	98.1 ± 6.9	98.1 ± 6.9

Table 3: Accuracy of the three QC levels (estimated by the mean of eight injections per level) and over all accuracy.

Analytical sensitivity and selectivity

Chromatograms of a plasma sample with an estimated concentration of 2.9 ng/ml hepcidin are shown in Figures 3a and 3b. The quantification transitions 698 > 1040 (hepcidin 4+, 3a) and 559 > 354 (hepcidin 5+, 3b) enable reproducible peak integration and the quantification of Hepcidin in plasma even at low endogenous levels. With both methods, the qualification trace is detectable also at low levels and the ion ratio (*quantifier:qualifier*)

consistently meets acceptance criteria.

Quantification limits (LOQ) were determined by calculating the signal-to-noise (S/N) ratio for the lowest calibration point (CAL 1) and extrapolating to an S/N value of 10:1. LOQs for Hepcidin 4+ and Hepcidin 5+ are estimated at 1.4 ng/mL and 1.8 ng/mL, respectively. While it was not available at the time of this experiment, Immundiagnostik now offers a lower CAL 1 level (LOQ of 1.9 ng/mL) with the hepcidin kit.

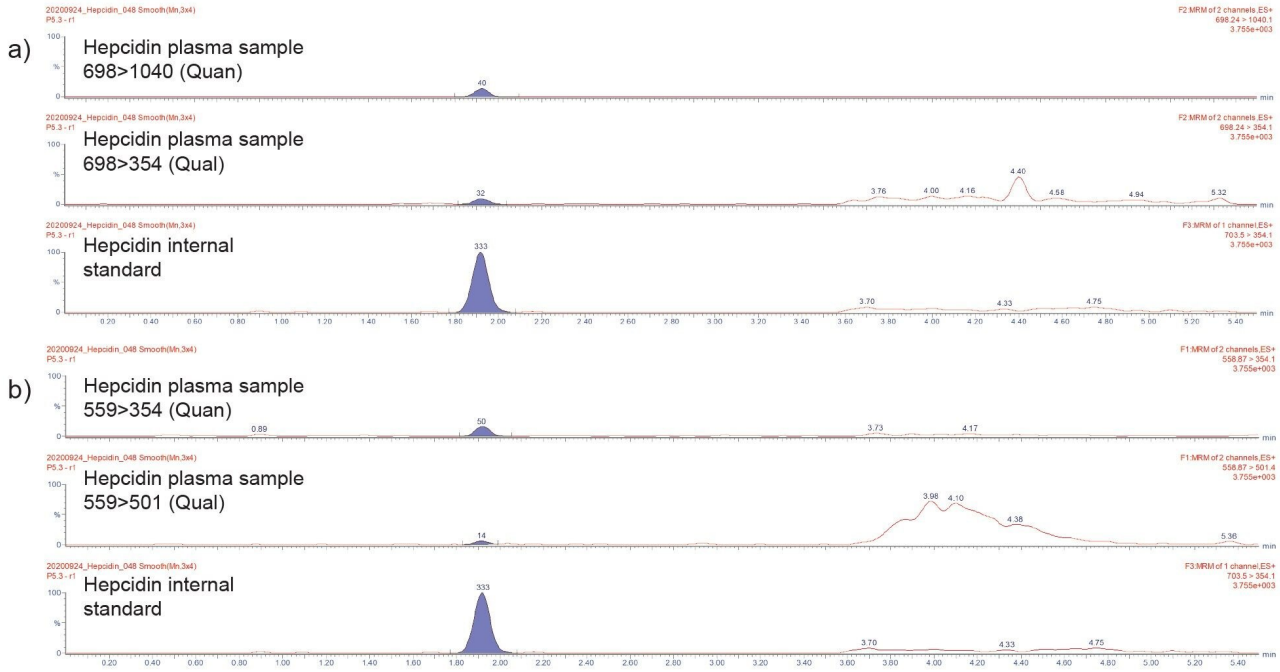

Figure 3: Chromatogram of a low level hepcidin plasma sample. A) MRM traces for Hepcidin 4+; b) MRM traces for Hepcidin 5+.

Comparison of the quantification transitions (hepcidin 4+ vs. hepcidin 5+)

Both transitions used for quantification of hepcidin in plasma are showing similar results regarding precision, accuracy, LOQ and linearity. Both charge states are present, giving same results within the error margins and both can be used without compromise.

Analysis of citrate-plasma samples / method comparison

Immundiagnostik AG provided 19 plasma samples (700 µL each) for quantification. Each sample was prepared

three times according to the testing procedure. Each individual sample preparation was injected once. The quantitative results for hepcidin 4+ and hepcidin 5+ were compared to the results obtained at Immundiagnostik AG, using the original method as described in kit IFUs (see Figure 4).

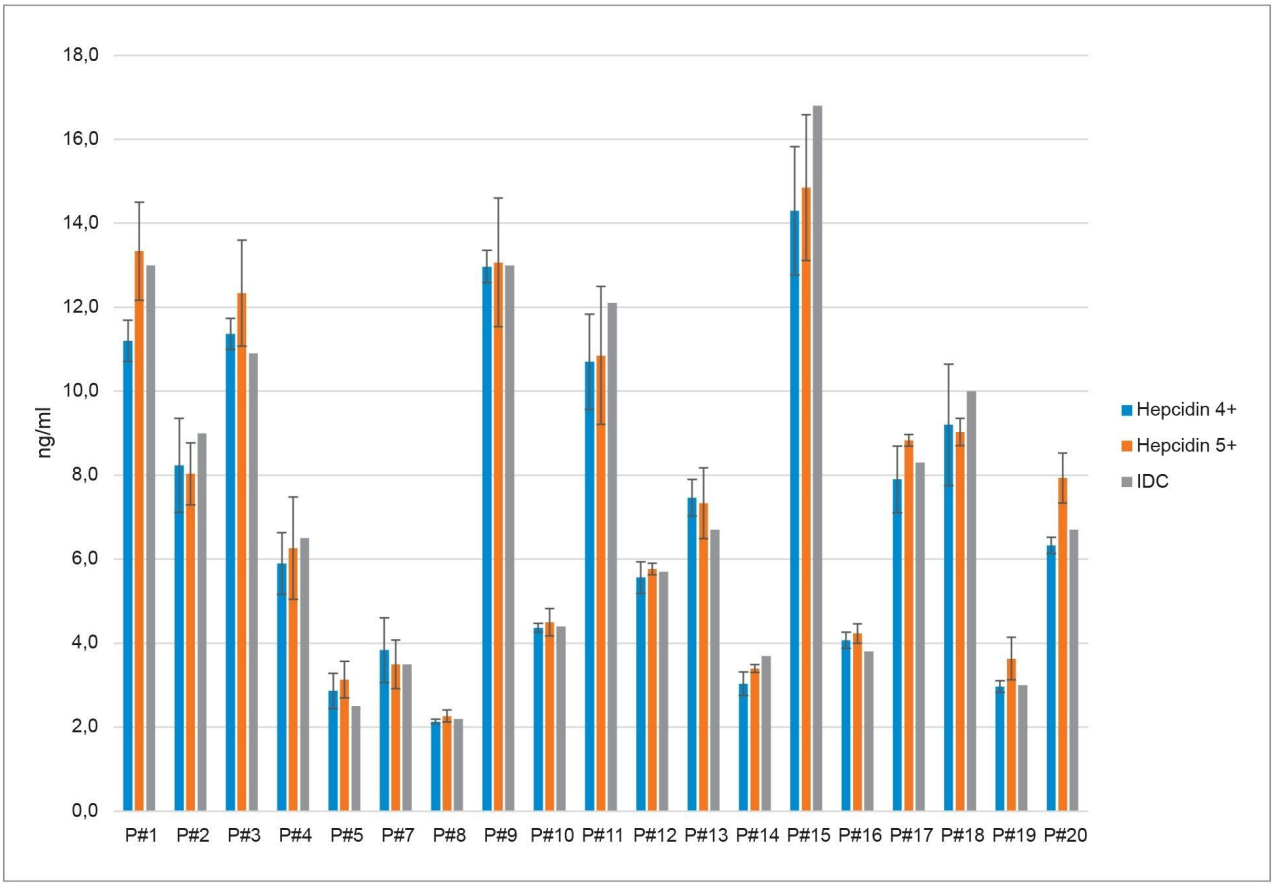

Figure 4: Calculated plasma concentrations and 95% confidence interval for hepcidin 4+ (blue), hepcidin 5+ (orange) in comparison to Immundiagnostik control value (IDC; grey) for 19 different samples.

Bland-Altman comparison of calculated concentrations for hepcidin 4+ and hepcidin 5+ to the original method provided by Immundiagnostik AG showed an excellent match of results. The mean bias for the calculated concentrations using hepcidin 4+ and 5+ compared to the results derived from original method was -0.4 and 0.03, respectively (Figure 5).

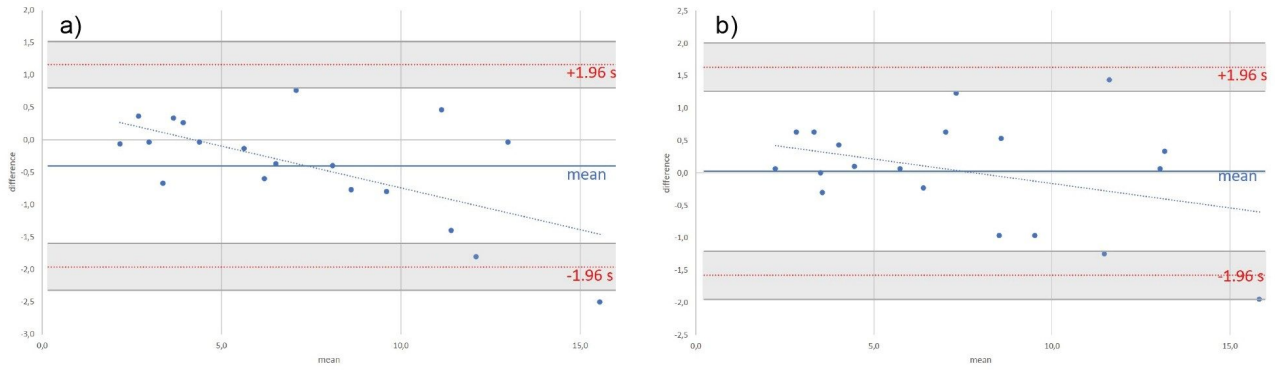

Figure 5: Bland-Altman comparison for the developed method using the hepcidin 4+ (5a) and 5+ (5b) transitions with the existing method described by Immundiagnostik.

The developed LC-MS/MS method for hepcidin analysis and quantification shows good linearity (r^2>0.99), precision (CV<10%) and accuracy (95±7% for hepcidin 4+; 98±7 for hepcidin 5+). Both charge states and their corresponding MRM transition can be used for accurate quantification. The quantitative results of the analyzed citrate-plasma samples were all within the limits of agreement compared to the original results.

Conclusion

The Waters ACQUITY UPLC I-Class / Xevo TQ-S micro System has demonstrated the capability to deliver analytically sensitive and selective performance with excellent precision and accuracy for the analysis of Hepcidin using the Immundiagnostik Hepcidin-25 LC-MS/MS RUO kit.

References

1. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1240030/.
2. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2855274/.

3. https://www.frontiersin.org/articles/10.3389/fphys.2019.01294/full.

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