Measurement of Hepcidin-25 in Serum Using the Agilent 6490 Triple Quadrupole LC/MS with iFunnel Technology

Application Note

Clinical Research

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Abstract

A novel nano HPLC/MS method has been developed for the rapid, robust, reliable, and accurate determination of hepcidin-25 levels in human serum. It relies on straightforward pre-analytical steps that are cost effective, avoiding the need for solid phase extraction. This Application Note demonstrates how nano HPLC/MS can be used to deliver better reproducibility and a limit of detection (LOD) for hepcidin less than half that of current C-ELISA assays.

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Introduction

Both intestinal iron absorption and macrophage iron recycling are regulated by hepcidin, a 25 amino-acid (aa) cysteine-rich peptide first characterized in 2001[1–3]. Isoforms of the peptide (20, 22, and 24-amino acids) also exist[4], but their clinical relevance is unknown.

A number of methods have been developed for this purpose using multiple technologies, including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and more recently, mass spectrometry analysis (SELDI-TOF and LC/MS/MS)[7–9]. The immunoassays employ antihepcidin antibody, with the potential to recognize the different isoforms. The methods based on mass spectrometry can specifically detect all the hepcidin isoforms, and they can provide absolute quantitation through the use of a spiked internal standard. All of these methods have demonstrated limited specificity and reproducibility as well as lack of concurrence in quantified levels of hepcidin, across the assay technologies.

In an effort to resolve these issues, the different methods have been recently compared through "round robin" studies[7,8]. The results demonstrated that development of a reliable assay remains quite challenging. The use of an internal standard was recommended for mass spectrometry based methods. These studies also called for development of a calibrator mimicking human serum and development of a consensus on calibrator levels.

This Application Note describes a recently published liquid chromatography tandem-mass spectrometry (LC/MS/MS) analytical method using a nano HPLC-chip on the Agilent 6490 Triple Quadrupole LC/MS for the reliable quantification of hepcidin-25 in human serum[10]. The method was evaluated for specificity, reproducibility, repeatability, and determination of LODs, limits of quantitation (LOQs), and linearity. These results were compared to the well-characterized C-ELISA method used to quantify serum hepcidin-25 in human samples[11]. Although discrepancies were observed in the absolute values of hepcidin measured, there was good correlation between the two approaches ($R^2 = 0.96$).

Experimental

Reagents and standards

Reagents were obtained as described[10]. The human endogenous hepcidin standard (DTHFPICIFCCGCCHRSKCGMCCKT) and the internal hepcidin standard (DTHFPICIFCCGCCHRSKCGMCCKT) [$^{13}C_6$, $^{15}N_4$] Arg[16] were purchased from Eurogentec (Seraing, Belgium) with a purity > 97 % assessed by reverse phase HPLC and mass spectrometry.

Samples

The serum samples included in this study were part of a biobank (officially registered # DC-2008-417) and all participants gave their informed consent to use their sample for research conducted in accordance with the local ethics committee.

Instruments

This method was developed using an Agilent 1290 Infinity HPLC-Chip/MS System coupled to an Agilent 6490 Triple Quadrupole LC/MS equipped with a nano ESI source. The instrument conditions are listed in Table 1.

Sample preparation

Serum samples (50 µL) were extracted using trichloroacetic acid as described[10].

Data analysis

Agilent MassHunter Quantitative Analysis Software was used for data treatment. For the calibration curve, all replicates results were loaded into the software database, and the automatic quantification method was then used to obtain the appropriate calibration curve type with the best fit to the experimental data. Statistical analyses were performed using MedCalc (7.3) software. Bland and Altman[12] and Deming adjusted regression curves[13] were used to test the commutability of the methods.

Table 1. HPLC-Chip and MS instrument conditions for the hepcidin quantification method.

LC conditions	
HPLC-Chip	ProtID-Chip-43 (II) 43 mm 300 Å C18 chip with 40 nL trap column (p/n G4240-62005)
Injection volume	4 µL in 15 % B, followed by 7 µL of flush volume
Mobile phase	A = 97:3:1 v/v/v (water/acetonitrile/formic acid) B = 10:90:1 v/v/v (water/acetonitrile/formic acid)
Run time	9 minutes
Flow rate	2.5 µL/min for sample loading, 0.6 µL/min for separation
Gradient program	7 minutes linear gradient from 3 % to 100 % B, then 2 minutes wash (100 % B) and 1 minute equilibration (97 % A)
MS conditions	
Mode	ESI mode, positive ionization; MRM
Capillary tension	1,700–2,100 V
Nebulizer gas flow	11 L/min
Nebulizer gas temperature	150 °C
Cell accelerator voltage	4 V
Delta EMV	200 V
Fragmentor voltage	380 V
Dwell time	40 msec
MS1	Wide
MS2	Wide
Ion funnel voltage pressure	Low: 80 V; High: 180 V

Acquisition parameters

The acquisition parameters used in the multiple reaction monitoring (MRM) method for both endogenous hepcidin and the isotopically-labeled hepcidin internal standard are shown in Table 2.

Table 2. Acquisition parameters for the hepcidin MRM method.

	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Endogenous hepcidin	559.4	694.8†	11
		1042	19
		983	19
		764.6	11
		645	11
Isotopically-iabeled hepcidin internal standard	560.6	696.8†	11
		1,045.5	19
		985	19
		766.7	11
		646,2	11

†Quantifier ion; all others are qualifier ions.

Results and Discussion

Analytical validation

A typical total ion chromatogram and extracted ion chromatograms using MRM are shown in Figure 1 for serum spiked with endogenous hepcidin standard and isotopically-labeled hepcidin standard. Calibration curves were constructed in MassHunter software over the range 0–200 ng/mL, based on hepcidin quantification values determined using orthogonal analysis methods. The calibration curve equation was linear ($y = 1.045346x + 0.030337$) when the origin was ignored and the equation was weighted 1/y. The calibration coefficient (R^2) was 0.9566 (Figure 2).

The specificity of the method was confirmed by the absence of a hepcidin-25 peak at the appropriate retention time in blank samples (normal goat serum containing 0 ng/mL of hepcidin-25).

Analysis of four replicates of one calibration curve point on the same day was used to evaluate intra-assay precision and accuracy. The intra-assay precision was 7 % at 50 ng/mL and 28 % at 5 ng/mL. The intra-assay accuracy was approximately 100 % at 20, 50, 100, and 200 ng/mL and almost 80 % at 5 and 10 ng/mL (Table 3). Inter-assay precision and accuracy were evaluated by quantifying the 50 and 200 ng/mL calibration standards on four separate days. Inter-assay precision was around 9–10 %, and the accuracy was close to 80% at both concentrations. The LOD and LOQ were 2 ng/mL and 6 ng/mL, respectively.

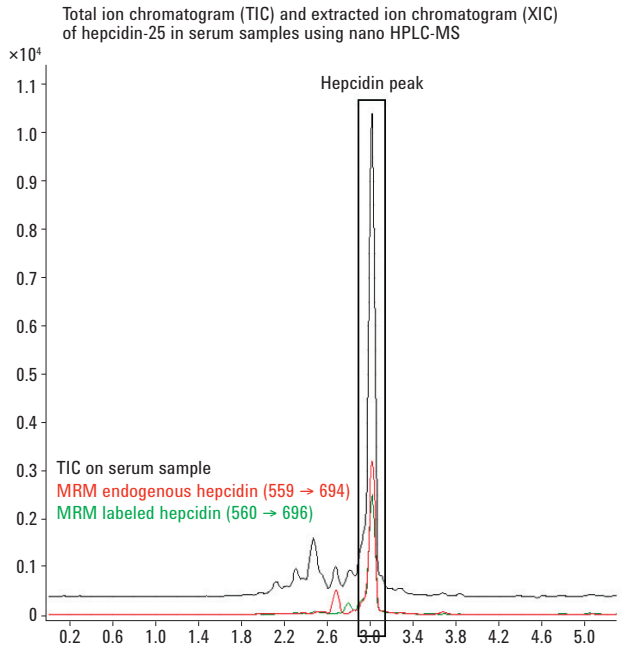

Figure 1. Typical TIC and extracted ion chromatograms (EICs) for endogenous hepcidin standard and isotopically-labeled hepcidin spiked into human serum.

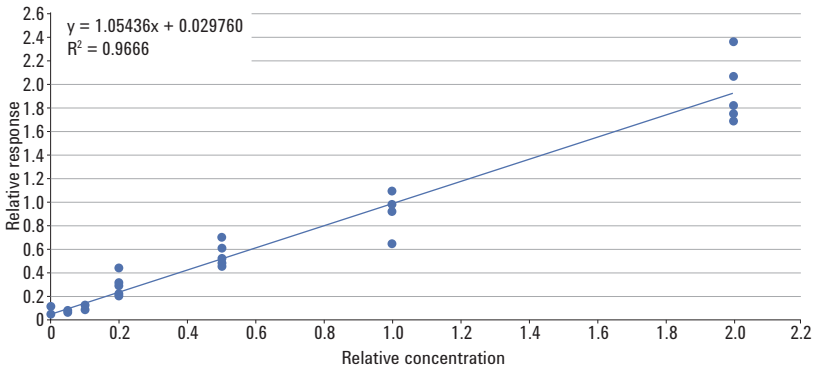

Figure 2. Calibration curves of hepcidin-25 using model matrix (normal goat serum) in the concentration range of 0 to 500 ng/mL (0, 5, 10, 20, 50, 100, 200, ng/mL). The calibration equation was linear when ignoring origin and weighted 1/y. $R^2 = 0.9665$.

Table 3. Intra-assay coefficient of variation and accuracy (%).

Hepcidin-25 concentration (ng/mL)	Intra-assay coeffcient of variation (% CV)	Intra-assay accuracy (%)
5	28	76
10	21	82
20	8	106
50	7	103
100	17	100
200	13	109

Comparison to competitive ELISA

Serum samples previously quantified using the C-ELISA method[11] were analyzed using this nano HPLC/MS MRM method. A positive significant correlation with $R^2 = 0.89$ and Pearson coefficient $P < 0.001$ (Figure 3) However, the slope of the linear correlation (5,0255x) indicates that the MRM assay provided values five times lower than those obtained with the C-ELISA. This result is likely related to the fact that the MS methods quantify only the 25 aa isoform and, therefore, are fully specific. The difference may also be due in part to the differences in the origin and purity of the material used for the standard curves in each of the two methods.

This nano HPLC/MS method generated a coefficient of variation (CV) of only 9 % for 30 participants, while the CV for the C-ELISA study described by Ganz *et al*. ranged from 5–19 %[11]. The LOD for the nano HPLC/MS method is also less than half of the LOD determined for the C-ELISA method, at 2 ng/mL, versus 5.5 ng/mL. We have, therefore, developed a robust and reliable analytical method for hepcidin-25 suitable for quantification of hepcidin-25 in human serum.

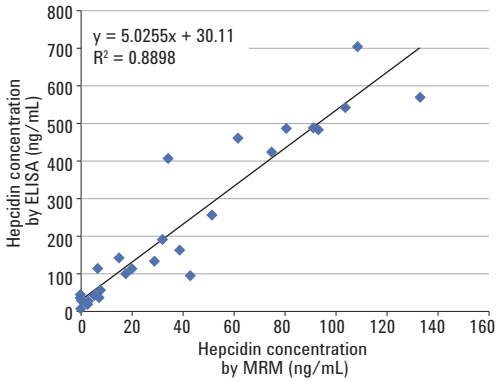

Figure 3. Comparison of hepcidin-25 concentrations measured in serum samples using nano HPLC/MS (MRM) and ELISA methods. A positive significant correlation has been obtained with an R^2 value of 0.89 and a Pearson coefficient $P < 0.001$.

Conclusions

This nano HPLC/MS method developed on the Agilent 6490 Triple Quadrupole LC/MS is suitable for the accurate, robust, and reproducible measurement of hepcidin in human serum samples. It relies on straightforward pre-analytical steps that are cost effective and avoid the need for solid phase extraction. While the obtained hepcidin levels differed from those determined using the reference C-ELISA assay, this nano HPLC/MS method has the advantage of being able to detect and quantify the truncated 20, 22, and 24-amino acid isoforms of hepcidin, whose clinical relevance is not yet known.

References

1. C.H. Park, *et al.* "Hepcidin, a urinary antimicrobial peptide synthesized in the liver." *J. Biol. Chem*. 276, 7806-7810 (**2001**).

2. C. Pigeon, *et al.* " A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload" *J. Biol. Chem*. 276,7811-7819 (**2001**).

3. G. Nicolas, *et al.* "Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice" *Proc. Natl. Acad. Sci. USA* 98, 8780-8785 (**2001**).

4. B. Rochat, *et al.* "Validation of hepcidin quantification in plasma using LC-HRMS and discovery of a new hepcidin isoform" *Bioanalysis 5*, 2509-2520 (**2013**).

5. J.J. Kroot, *et al.* "Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders" *Clin. Chem*. 56, 1570-1579 (**2010**).

6. J.J. Kroot, *et al.* "Hepcidin in human iron disorders: diagnostic implications" *Clin. Chem*. 57,1650-69 (**2011**).

7. J.J. Kroot, *et al.* "Results of the first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization" *Haematologica* 94,1748-1752 (**2009**).

8. J.J. Kroot, *et al.* "Second round robin for plasma hepcidin methods: first steps toward harmonization" *Am. J. Hematol.* 87, 977-983 (**2012**).

9. C. Delaby, C. Beaumont, "Serum hepcidin assay in 2011: where do we stand?" *Ann. Biol. Clin.* (Paris) 70, 377-386 (**2012**).

10. C. Delaby, *et al.* EuPA Open Proteomics, Article in revision.

11. T. Ganz, *et al.* "Immunoassay for human serum hepcidin" *Blood* 112, 4292-4297 (**2008**).

12. J.M. Bland, D.G. Altman "Statistical methods for assessing agreement between two methods of clinical measurement" *Lancet* 1 (8476), 307-310 (**1986**).

13. P.J. Cornbleet, N. Gochman "Incorrect least-squares regression coefficients in method-comparison analysis" *Clin. Chem*. 25, 432-438 (**1979**).

14. B.J. Sasu, *et al.* "Serum hepcidin but not prohepcidin may be an effective marker for anemia of inflammation (AI)" *Blood Cells Mol. Dis*. 45, 238-245 (**2010**).

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