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## Clinical measurement of Hepcidin-25 in human serum: Is quantitative mass spectrometry up to the job?

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### ABSTRACT

From its discovery, hepcidin has generated many hopes in terms of diagnosis and management of a wide variety of iron-related diseases. However, in clinical use its accurate quantification remains a challenge due to the limited sensitivity, specificity or reproducibility of the techniques described. In this work, we adapted a highly specific and quantitative mass spectrometry method based on selected reaction monitoring (SRM) to measure hepcidin. Our objective was to adapt the feasibility and reproducibility of the workflow to a clinical environment. Analytical validation was performed according to ISO 15189 norms for determining the limit of detection (LOD, 2 ng/mL), limit of quantification (LOQ, 6 ng/mL), repeatability, reproducibility and linearity (up to 200 ng/mL). Using the serum of patients with various iron-related diseases we compared our SRM detection method to the well-characterized competitive ELISA (cELISA) test. The two methods were commutable (Bland–Altman plot) and we found a positive and significant correlation ( $r^2 = 0.96$ , Pearson correlation coefficient  $p < 0.001$ ) between both methods, although the absolute concentration determined is different from factor 5. The validation of our SRM method encourages us to propose it as an alternative approach for accurate determination of hepcidin in human samples for clinical diagnosis, follow-up and management of iron-related diseases.

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**Abbreviations:** SRM, selected reaction monitoring; ELISA, enzyme-linked immunosorbent assay; aa, aminoacid; TCA, trichloroacetic acid.

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## 1. Introduction

Hepcidin is a 25 amino-acid (aa) cysteine-rich peptide involved in iron metabolism: it regulates both intestinal iron absorption and macrophage iron recycling. There are several clinical applications for hepcidin measurements such as iron overload disorders and anemia, chronic inflammation, kidney disease and cancer [1,2]. This peptide can also be found in the 20, 22 and 24-aa isoforms [3]; however the histopathological relevance of these isoforms still needs to be determined and they are most likely the result of the degradation of the bioactive 25-aa isoform. Since 2001 [4–6], various methods have been published on accurate quantification of hepcidin [7–9]. Among them, we can mention radio-immuno assay (RIA), Enzyme-linked immunosorbent assay (ELISA) and more recently, mass spectrometry analysis (SELDI-TOF and LC-MS/MS). Immunoassays are based on the use of anti-hepcidin antibodies (therefore potentially identifying the different isoforms) and mass spectrometric approaches to specifically detect the different types of hepcidin. Furthermore, absolute quantitation is possible through normalization via a spiked internal standard of hepcidin. These methods have their limits, especially in regards to specificity (for the 25 aa-isoform) and assay reproducibility for repetitive quantification of a same sample. In addition, the different approaches differ greatly in terms of absolute values of hepcidin. These discrepancies are specifically related to the inherent properties of the peptide's structure, its low immunogenicity, its propensity to aggregate as well as the existence of smaller isoforms, which could affect the immunochemical assay results. However, the various studies comparing these approaches have reported a good correlation between the different methods [7,8].

Recently and in order to standardize the quantitation of hepcidin, different methods have been compared via inter-laboratory studies [7,8]. These studies have underlined the challenges in developing a reliable assay and recommended the use of internal standards for MS-based methods, using a calibrator mimicking human serum and setting a consensus on calibrator levels.

We have developed a nano-liquid chromatography tandem-mass spectrometry (LC-MS/MS) to reliably quantify hepcidin-25 in human serum. Nano-LC system are generally not recommended in clinical applications since they are known for not being as robust as micro-LC. However in our workflow we used a nano-Chip/MS system which is known to be more versatile and reliable than regular nano-LC system. Firstly, we performed an analytical validation to test the specificity, reproducibility and repeatability of LC-MS/MS as well as determining its LOD, LOQ and linearity. We then compared our results to the well characterized C-ELISA method to quantify serum hepcidin-25 in human samples [10]. Despite discrepancies in the absolute values of hepcidin measured, we showed a good correlation between these two methods ( $r^2 = 0.96$ ), thus validating our LC-MS/MS assay for accurate measurement of hepcidin in human serum samples. Furthermore, we underlined the relevance of this measurement in iron-deficiency anemia, anemia of inflammation and other iron-related diseases.

## 2. Materials and methods

### 2.1. Chemicals used

Trichloroacetic acid (TCA), ref. T9159-250G (Sigma-Aldrich); Water ULC-MS, ref. 23214102, formic acid ULC-MS (FA) ref. 069141A8, acetonitrile ULC-MS (ACN) ref. 01204101 all from Biosolve (Dieuze, France); Normal Goat serum, ref. S-1000 Clinisciences (Nanterre, France); Protein LoBind tube 1.5 mL, ref. 022431081 Eppendorf (Le Pecq, France); glass vial insert ref. 5181-1270 and ProtID-Chip-43 II ref. G4240-62005 both from Agilent Technologies (Santa Clara, CA, USA). Human hepcidin standard (DTHFPICIFCCGCCHRSKCGMCKKT) and internal hepcidin standard (DTHFPICIFCCGCCHRSKCGMCKKT) [ $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ ] Arg $^{16}$  were purchased from Eurogentec (Seraing, Belgium) with a purity >97% assessed by RP-HPLC and mass spectrometry. Standard peptides were synthesized in lyophilized form with the same amino acid sequence (25 AA) and folding (4 disulfite bridges Cys 7–23; 10–13; 11–19; 14–22) as the endogenous human hepcidin-25.

### 2.2. Human samples and ELISA determination of hepcidin

In France, since hepcidin is already an accepted serum analyte used in clinical settings, which is determined using mass spectrometry or validated competitive C-ELISA [10], there was no need for a specific authorization from an Ethics committee for this work. However, serum samples included in this study were part of a biobank (official registration # DC-2008-417) and all patients signed an informed consent form to authorize the use of their samples for research conducted in accordance with the local Ethics committee.

### 2.3. Liquid chromatography (LC) separation

Nano-LC separation was carried out on a 1290 nano-LC system (Agilent technologies). Peptides were loaded on a ProtID-Chip-43 (Agilent technologies) containing a 43 mm  $\times$  75  $\mu\text{m}$  analytical column and a 40 nL trap-column packed with Zorbax 300SB-C $_{18}$  5  $\mu\text{m}$ . The mobile phase was composed of H $_2$ O/ACN/FA (phase A 97:3:0.1, v/v/v and phase B 10:90:0.1, v/v/v). The sample was loaded on the trapping column with a flow rate of 2.5  $\mu\text{l}/\text{min}$  using the capillary pump to deliver an isocratic enrichment phase composed of 15% B. Furthermore, 7  $\mu\text{l}$  of flush volume was used for cleaning the trapping column from un-retained compound. The, trapped peptides were separated from the analytical column using the nanopump. A 10-minute gradient was performed, starting with 3% of solvent B and linearly ramped to 100% in 7 min. The column was then washed for 2 min and re-equilibrated during 1 min with 97% of solvent A.

### 2.4. Multiple reaction monitoring analysis

Mass spectrometric detection was performed using a 6490 triple quadrupole with a nano-ESI source operating in positive mode and in SRM mode (Agilent technologies, Waldbronn, Germany). The control of the LC-MS/MS was done

with MassHunter Software (Agilent technologies, Waldbronn, Germany). The ESI nano spray was set-up according to the following specificities: capillary tension 1700–2100 V, nebulization gas flow 11l/min and temperature at 150 °C. We selected the most abundant ion detected after standard peptide injection. Precursor ions followed were 559.4  $m/z$  ( $z = 5$ ) for the endogenous Hepcidin and 560.6  $m/z$  ( $z = 5$ ) for the heavy standard. Precursors ions were transferred inside the first quadrupole with an accelerator voltage of 4 V while ion funnel RF high pressure was set to 180 V and low pressure to 80 V. Precursor ions were fragmented in “Product Ion Scan” mode and the 5 most abundant generated fragments were selected to constitute the SRM method (560.6 → 1045.5, 985, 766.7, 696.8, 646.2; 559.4 → 1045, 983, 764.6, 694.8, 645). Collision energies (CE) were optimized as described in Table 1.

## 2.5. Preparation of Hepcidin-25 standards

Lyophilized standards were resuspended at 200 ng/μL with H<sub>2</sub>O/ACN/FA (66.2:33.8:0.1, v/v/v). The solution was then separated into aliquots of 50 μL in LoBind tubes and stored at –80 °C. The internal standard was prepared at 5 ng/μL using the same protocol. Isotopically-labeled hepcidin was chosen as the internal standard because of its physicochemical properties which are similar to hepcidin. Both compounds exhibited the same behavior during the preparation and chromatographic process while the mass difference of 10 amu, provided by the heavy aa of the internal standard, permitted the independent detection of each compound.

Appropriate dilutions of 200 ng/μL hepcidin stock solutions (0.125, 1.25, 25 μg/mL) were made with H<sub>2</sub>O/ACN/FA (79:20:1, v/v/v) to prepare the matrix-based calibration curve (normal goat serum) at the concentration range of 0–200 ng/mL (0, 5, 10, 20, 50, 100, 200 ng/mL). The internal standard was spiked in the model matrix and biological sample at a final concentration of 100 ng/mL (Table 2).

## 2.6. Hepcidin-25 pre-fractionation protocol

All experiments were performed at 4 °C. In a 1.5 mL LoBind tube, 50 μL of serum sample was mixed with 1 μL of internal hepcidin standard solution (5 ng/μL), vortexed 10 s and then 1:1 ratio of 4% trichloroacetic acid (TCA) solution was added. Samples were then vortexed a few seconds and centrifuged at 17,000 ×  $g$  during 5 minutes to obtain a clear supernatant, followed by a new centrifugation step at 17,000 ×  $g$  during 5 min after thawing on ice. The supernatant was transferred into a new LoBind tube and dried in a vacuum concentrator (Lab-conco, Kansas city, USA). The samples were resuspended with 10 μL of H<sub>2</sub>O/ACN/FA (20:1:79, v/v/v) and vortexed at 1000 rpm for 10 min. The LC vial was then centrifuged 3 min at 17,000 ×  $g$  at room temperature before transferring the sample and making sample preparations in duplicates.

## 2.7. Method validation

To validate this method we evaluated specificity, linearity, LOD, LOQ, precision and accuracy. Specificity was assessed by confirming that there were no interference peaks at the same retention time than those of the analytes in the blank sample.

**Table 1 – Optimized LC-SRM method and selected compound targets.**

| Compound name           | ISTD  | Precursor ion | MS1 res | Product ion | MS2 res | Dwell time | Fragmentor | Collision energy | Cell accelerator voltage | Polarity | Fragment                             |
|-------------------------|-------|---------------|---------|-------------|---------|------------|------------|------------------|--------------------------|----------|--------------------------------------|
| DTHFPICFCGGCCHRSKCGMCKT | True  | 560.6         | Wide    | 1045.5      | Wide    | 40         | 380        | 19               | 4                        | Positive | [y19] <sup>2+</sup>                  |
| DTHFPICFCGGCCHRSKCGMCKT | True  | 560.6         | Wide    | 985         | Wide    | 40         | 380        | 19               | 4                        | Positive | [y18-H <sub>2</sub> O] <sup>2+</sup> |
| DTHFPICFCGGCCHRSKCGMCKT | True  | 560.6         | Wide    | 766.7       | Wide    | 40         | 380        | 11               | 4                        | Positive | [y21] <sup>3+</sup>                  |
| DTHFPICFCGGCCHRSKCGMCKT | True  | 560.6         | Wide    | 696.8       | Wide    | 40         | 380        | 11               | 4                        | Positive | [y19] <sup>3+</sup>                  |
| DTHFPICFCGGCCHRSKCGMCKT | True  | 560.6         | Wide    | 646.2       | Wide    | 40         | 380        | 11               | 4                        | Positive | [y23] <sup>4+</sup>                  |
| DTHFPICFCGGCCHRSKCGMCKT | False | 559.4         | Wide    | 1042        | Wide    | 40         | 380        | 19               | 4                        | Positive | [y19] <sup>2+</sup>                  |
| DTHFPICFCGGCCHRSKCGMCKT | False | 559.4         | Wide    | 983         | Wide    | 40         | 380        | 19               | 4                        | Positive | [y18-H <sub>2</sub> O] <sup>2+</sup> |
| DTHFPICFCGGCCHRSKCGMCKT | False | 559.4         | Wide    | 764.6       | Wide    | 40         | 380        | 11               | 4                        | Positive | [y21] <sup>3+</sup>                  |
| DTHFPICFCGGCCHRSKCGMCKT | False | 559.4         | Wide    | 694.8       | Wide    | 40         | 380        | 11               | 4                        | Positive | [y19] <sup>3+</sup>                  |
| DTHFPICFCGGCCHRSKCGMCKT | False | 559.4         | Wide    | 645         | Wide    | 40         | 380        | 11               | 4                        | Positive | [y23] <sup>4+</sup>                  |

**Table 2 – Appropriate dilutions of light and heavy hepcidin stock solutions to prepare the calibration curve using model matrix (Normal Goat serum) in the concentration range of 0 to 200 ng/mL (0, 5, 10, 20, 50, 100, 200 ng/mL). Internal standard (Heavy hepcidin) was spiked in the model matrix and in the biological sample at a final concentration of 100 ng/mL.**

| Normal goat serum<br>Volume ( $\mu\text{L}$ ) | Heavy hepcidin           |   |                                | Light hepcidin           |   |                                |
|---|--------------------------|---|--------------------------------|--------------------------|---|--------------------------------|
|   | Volume ( $\mu\text{L}$ ) | Initial concentration<br>(ng/ $\mu\text{L}$ ) | Final concentration<br>(ng/mL) | Volume ( $\mu\text{L}$ ) | Initial concentration<br>(ng/ $\mu\text{L}$ ) | Final concentration<br>(ng/mL) |
| 50  | 1                        |   | 100                            | 0                        |   | 0                              |
|   | 1                        |   | 100                            | 2                        | 0.125   | 5                              |
|   | 1                        |   | 100                            | 4                        |   | 10                             |
|   | 1                        | 5   | 100                            | 0.8                      |   | 20                             |
|   | 1                        |   | 100                            | 2                        |   | 50                             |
|   | 1                        |   | 100                            | 4                        | 1.25  | 100                            |
|   | 1                        |   | 100                            | 8                        |   | 200                            |
|   | 1                        |   | 100                            |                          |   |                                |

In order to do this, a 0 ng/mL point was added to the calibration curve and the software took it into account for computing it. Calibration curves were constructed over the 0–200 ng/mL range. Intra-assay precision and accuracy were evaluated by analyzing the replicates of the calibration curve on the same day. Inter-assay precision and accuracy were assessed by analyzing the same quality control (QC) samples on series of analyses performed on different days. The LOD has been determined using the signal obtained with the replicates at 100 ng/mL. S/N average obtained was of 148. LOD is calculated based on  $S/N = 3$ . LOD of 2 ng/mL was obtained. LOQ was determined in the same way but using  $S/N = 10$ . LOQ of 6 ng/mL was obtained. Accuracy was calculated using the Agilent software (MassHunter Quantitative analysis) based on the calibration curve equation. Formula used was “Accuracy = [(calculated concentration)/(expected concentration)]  $\times$  100”.

### 2.8. Data treatment and statistics

We used the MassHunter Quantitative Analysis software (Agilent technologies) to conduct bioinformatics data treatment. All replicate results for the calibration curve were loaded into the software database. An automatic quantification method was used to treat all data in order to obtain the equation curve with the best fit for the experimental points. The two transitions 559.4  $\rightarrow$  694.8 (light hepcidin) and 560.6  $\rightarrow$  696.8 (heavy hepcidin) were used as quantifiers and were automatically detected on specific retention time windows. Other transitions were used as qualifiers. Statistical analyses were performed with the MedCalc software (7.3). Unless indicated, we used non-parametric tests (Kruskal–Wallis) to compute all available data. We also used the Bland and Altman plot [11] and Deming adjusted regression curves [12] to test the commutability of the methods.

## 3. Results and discussion

### 3.1. Pre-fractionation optimization

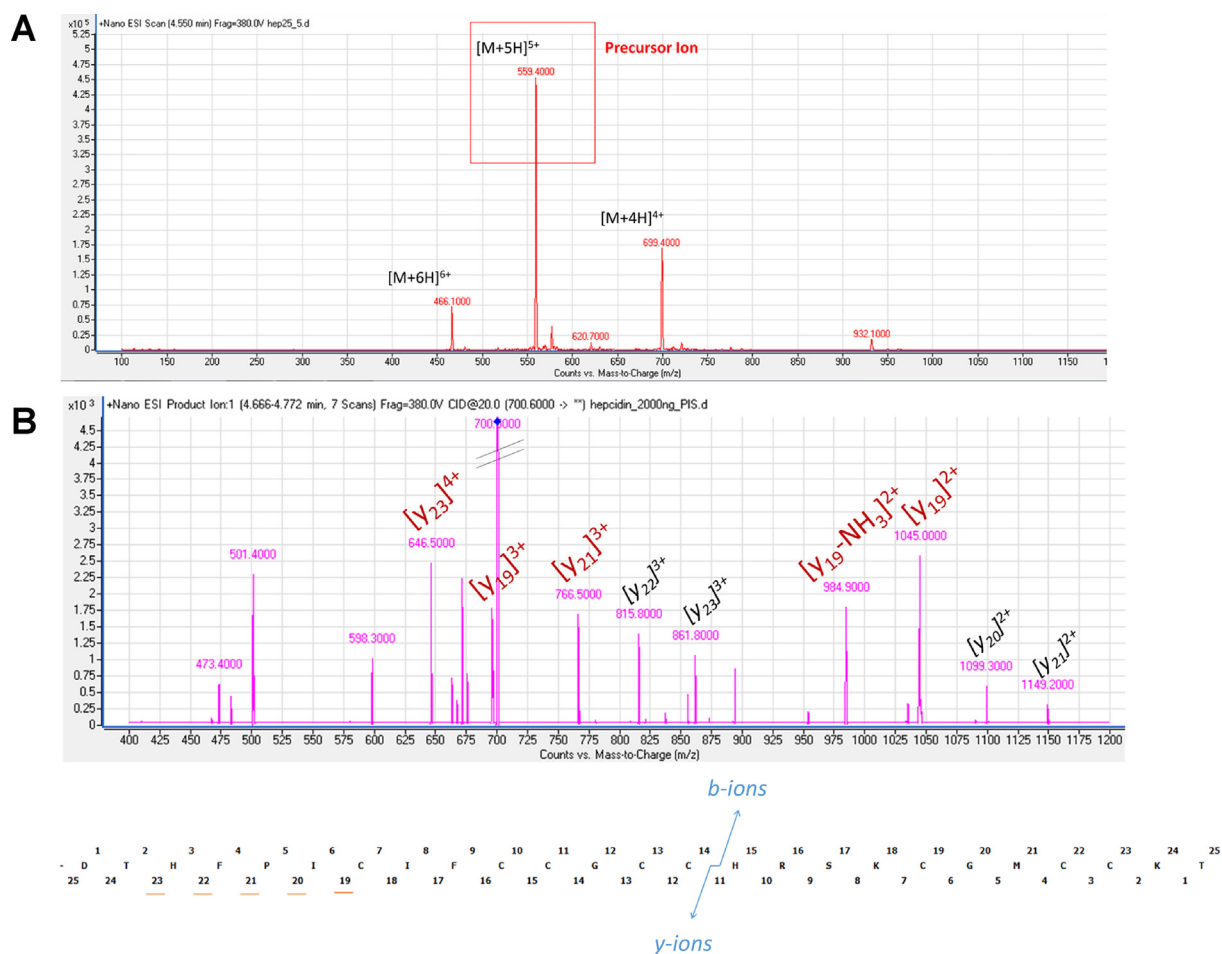
In order to measure the hepcidin-25 level using LC-SRM method, we adapted the pre-fractionation protocol from Murao et al. [13]. Based on protein precipitation, this method is fast, simple, reproducible, cost-effective and overall very

adapted to a routine clinical environment. TCA precipitation showed a sufficient recovery for peptides with a molecular weight under 3 kDa, and allowed a direct injection of the sample without evaporation or dilution. With this sample preparation, we were able to detect 5 times more hepcidin-25 than with direct analysis (not shown), probably due to sample simplification and consequently matrix effect reduction. On a serum sample, the reproducibility test was calculated using an area ratio endogeneous hepcidin/heavy hepcidin and it showed a coefficient of variation of 1.5% (see below).

### 3.2. LC-SRM method optimization

Firstly, transmission of ion precursor through the triple quadrupole was optimized. For this purpose, cell accelerator voltage (CAV) and RF of ion funnel were optimized to increase ion transmission inside the Q1. Optimum CAV and ion funnel RF enabled a gain of signal with respectively 49% and 30% compared to reference values. The CAV optimum value was found at 4 V (reference 7 V) and the ion funnel RF at 180–80 V (reference 210–110 V). Then 1  $\mu\text{L}$  of 10  $\mu\text{g}/\text{mL}$  solution hepcidin-25 standards (light and heavy) were injected to determine the most abundant ion detected in “full-scan mode” for the light and heavy standards (Fig. 1A). The mass spectra showed a similar series of ions, mainly the quadruply, quintuply and sextuply charged molecular ions for both standards. A highest S/N were found at  $m/z$  559.4 ( $M + 5H$ )<sup>5+</sup> for the light standard ions and at 560.6  $m/z$  ( $M + 5H$ )<sup>5+</sup> for the heavy standard ions. Precursor ions were fragmented in “product ion scan” mode to observe the subsequent generated fragments. Five transitions per peptide were followed and two transitions 559.4  $\rightarrow$  694.8 (light hepcidin) and 560.6  $\rightarrow$  696.8 (heavy hepcidin) were used as quantifiers (higher S/N ratio). The sequence of the peptides chosen was located in the unfolded N termini area of the protein sequence (y19 to y23 ions with a mix of charge states) (Fig. 1B). Accordingly, the SRM method was optimized for the collision energy (CE) transition by transition. This step was performed automatically with “Peptide Optimizer” software using a ramping CE in order to find the optimum. CE was a predominant parameter for the optimization, with an SRM Signal enhancement of up to 6-fold compared to the method of reference.

Optimum conditions were achieved by re-suspending samples in 20% acetonitrile/1% formic acid/79% water and storing



**Fig. 1 – (A) Light hepcidin-25 standard chromatograms obtained by nano-HPLC-MS in “full-scan mode” showing quadruply, quintuply and sextuply charged molecular ions. (B) MS/MS spectra of the  $[M + 5H]^{5+}$  precursor of light hepcidin-25 standard. In red, the peptide fragment chosen located in the unfolded N termini area of the protein sequence (y19 to y23 ions).**

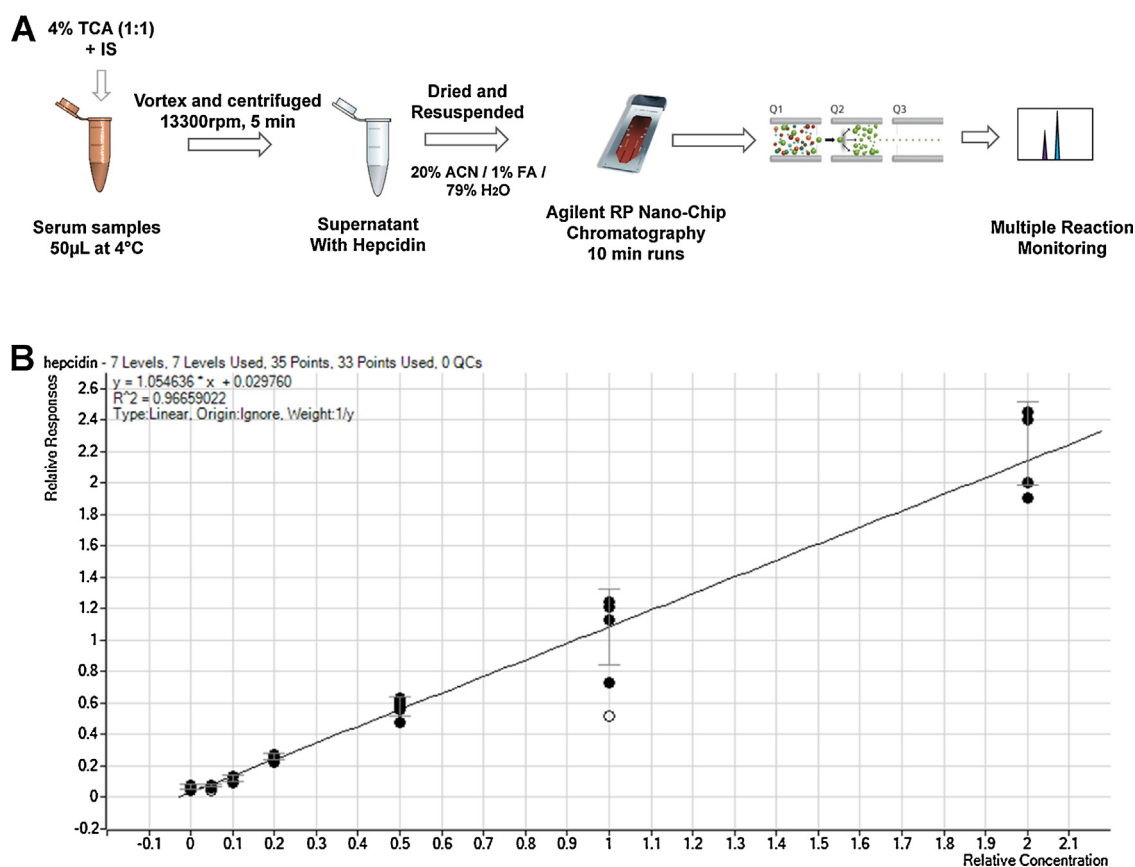
the sample in a LC glass vial. The samples were then loaded with 15% of B phase (90% CAN, 0.1% FA) at 2.5  $\mu\text{L}/\text{min}$  flow rate using the capillary pump. This increased the signal/noise ratio about 30-fold probably due to a reduction of nonspecific interactions between proteins and surfaces, and a reduction in sample complexity linked to the fact that in this situation a number of hydrophilic peptides were eluted before the analysis. To clean the trapping column from un-retained compound we used 7  $\mu\text{L}$  of flush volume as well as a 10-min chromatographic separation gradient starting with 3% of solvent B and linearly ramped to 100% in 7 min. The column was then washed for 2 min and re-equilibrated for 1 min with 97% of solvent A. Under these conditions, elution time of the hepcidin-25 peptide was 3 min (65% phase B).

### 3.3. Analytical validation and quantitation

The specificity of the method was evaluated by validating the absence of interference peaks in blank sample at the retention time of the analytes (0 ng/mL of hepcidin-25 in normal goat serum). This blank sample was integrated by the Mass Hunter software into the calibration curve performed using a model matrix (normal goat serum) free of hepcidin-25. Calibration curves were constructed over the

0–200 ng/mL range based on hepcidin values found with the orthogonal analysis method. The equation was linear when ignoring origin and weighted  $1/y$ .  $r^2$  was obtained with the following equation  $y = 1.54636 + 0.02970x$  and was equal to 0.9665 (Fig. 2B). Finally, the quantitation of hepcidin-25 in cohort samples did not exceed 140 ng/mL therefore the calibration curve (0–200 ng/mL) is perfectly suitable for dosage.

Each LC-SRM dosage was performed in duplicate in order to calculate a coefficient of variation. Intra-assay precision and accuracy were assessed by analyzing four replicates of the calibration curve point on the same day. Intra-assay precision was around 7% for the 50 ng/mL point and 28% for the lowest concentration point (5 ng/mL). Intra-assay accuracy was close to 100% for the 20, 50, 100 and 200 ng/mL points and around 80% for the lowest concentration points (5 and 10 ng/mL) (Table 3). In order to evaluate inter-assay precision and accuracy, we chose 2 points on the calibration curve (50 and 200 ng/mL) as quality control (QC) samples which were analyzed on four separate days. Inter-assay precision was around 9–10%, accuracy was close to 80% for the two QCs and LOD was determined using the signal obtained with the replicates at 100 ng/mL. S/N average obtained was 148. LOD is calculated based on  $S/N = 3$ . LOD of 2 ng/mL was obtained. LOQ was determined in the same way but using  $S/N = 10$ . LOQ of 6 ng/mL was obtained.



**Fig. 2 – (A) Workflow for nano-HPLC–MS quantification of hepcidin-25 in human serum samples. (B) Calibration curves of hepcidin-25 using model matrix (normal goat serum) in the concentration range of 0–200 ng/mL (0, 5, 10, 20, 50, 100, 200 ng/mL). The equation was linear when ignoring origin and weighted 1/y.  $r^2$ , obtained with the following equation  $y = 1.054636 + 0.029760$ , was equal to 0.9665.**

However, one cannot exclude that, in some human samples, interfering compounds could affect the precision of the assay, and additional validation to comply with the ISO 15189 norm might be needed.

### 3.4. Comparison of LC-SRM and C-ELISA approaches for hepcidin quantitation

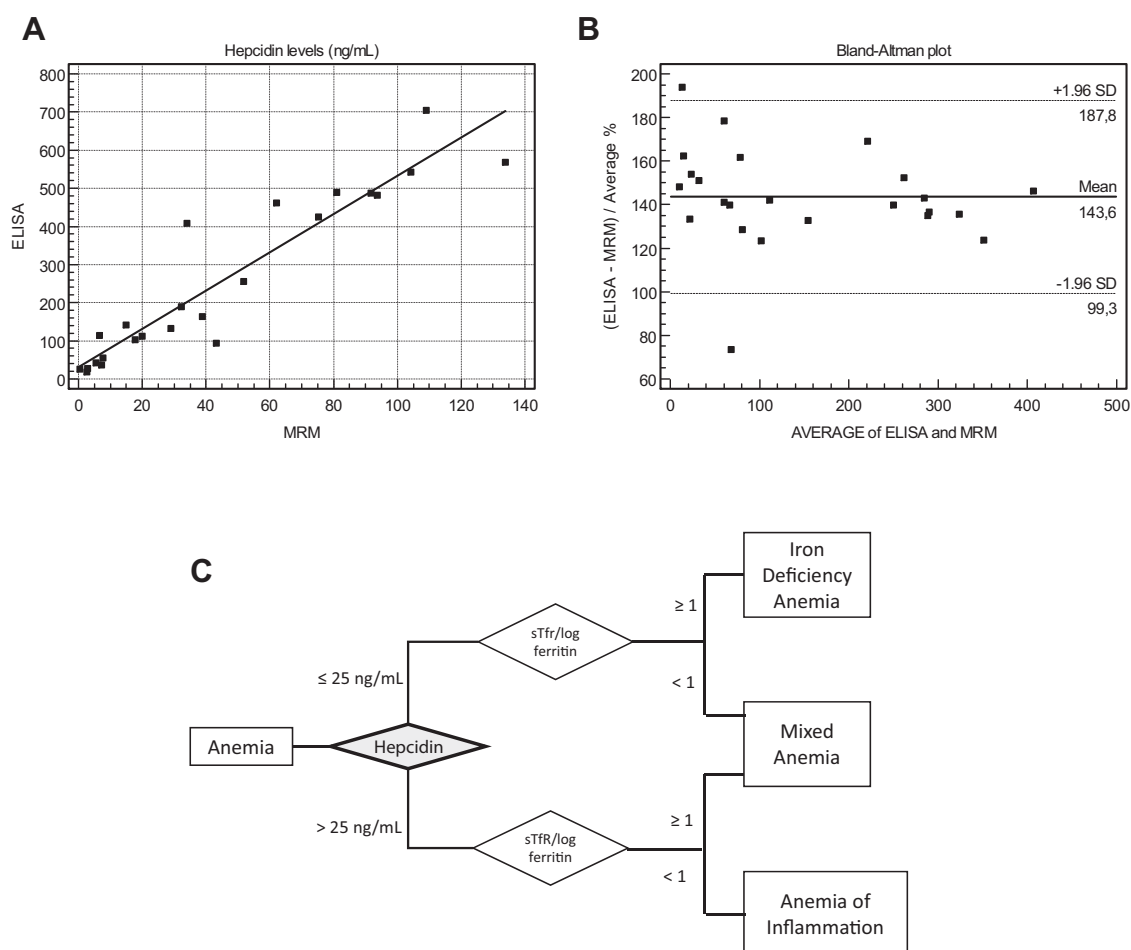
Among the different methods used to quantify bioactive hepcidin-25 in serum, the competitive C-ELISA developed by Ganz et al. in 2008 [10] is a very valuable method that correlates well with a specific weak cation exchange (WCX) time-of-flight MS assay [1]. We further analyzed serum samples previously

measured with this C-ELISA method using our MS protocol (Fig. 3A). We found a significant positive correlation ( $r^2 = 0.96$ , Pearson's  $r P < 0.001$ ) and the Bland–Altman plot (Fig. 3B) validated the commutability of these methods. However, the slope of the linear correlation (5.0255x) indicated that our assay provided values, which were five times lower than those obtained with the C-ELISA. This result is likely related to the fact that MS methods are fully specific (i.e. only the 25aa isoform is quantified) and to the difference in origin and purity of the material used for the standard curves. Furthermore, the LC-SRM CV for the 30 patients in our study was 9% compared to 5–19% in the study previously described by Ganz et al. In addition, our LOD is 2 ng/mL compared with a LOD at 5.5 ng/mL for the reference C-ELISA method. Finally, our method shows reproducibility under 10%, compared to the reproducibility of C-ELISA which rises to 12%. Thus, our experimental results underline the robustness and reliability of our quantitative assay for hepcidin-25, as well as its suitability for the relevant evaluation of hepcidin-25 in human serum within a clinical environment.

As expected, hepcidin-25 levels differed for patients suffering from iron deficiency (mean value of 29.87 ng/mL or 49.97 ng/mL for patients with ferritin <300 ng/mL and >300 ng/mL, respectively) and our results show an appropriate correlation between hepcidin and ferritin ( $r^2 = 0.38$  or by splitting into 2 groups,  $r^2 = 0.42$  and 0.74 for patients with

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

| Light concentration (pg/µL) | Intra-assay CV (%) | Intra-assay precision accuracy (%) |
|-----------------------------|--------------------|------------------------------------|
| 5                           | 28                 | 24                                 |
| 10                          | 21                 | 18                                 |
| 20                          | 8                  | 6                                  |
| 50                          | 7                  | 3                                  |
| 100                         | 17                 | 0                                  |
| 200                         | 13                 | 9                                  |



**Fig. 3 – (A) Comparison of hepcidin-25 concentrations measured in serum samples from patient using nano HPLC-MS and ELISA. A significant positive correlation was obtained ( $r^2 = 0.96$ , Pearson product-moment correlation coefficient (Pearson's  $r$ )  $P < 0.001$ ). (B) Commutability of the methods (ELISA and SRM) using a Bland-Altman plot. The Deming adjusted regression curve equation is:  $y = 11,7691 + 4,9683x$ . (C) Proposed patient stratification scheme for biochemical discrimination of iron deficiency versus inflammatory status as set up in this assay (note: the origin and purity of the standard material may modify this value).**

ferritin  $< 300$  ng/mL and  $> 300$  ng/mL, respectively), thus reflecting the regulation of both proteins by iron stores. Moreover, hepcidin values also differed in patients with an inflammatory status (mean value of 4.64 ng/mL for patients with CRP  $< 10$  mg/dL, vs. mean value of 55.85 ng/mL for patients with CRP  $> 10$  mg/dL). The values of hepcidin in our cohort were distributed between 10 and 100 ng/mL. Though, this assay allows biochemical discrimination of iron deficiency status and inflammatory status of patients as proposed in the decision tree illustrated in Fig. 3C based on the article by Sasu et al. [14].

#### 4. Conclusion

These past 10 years, numerous assays relying on immunochemical and mass spectrometry methods have been developed and described to measure hepcidin in human samples of blood and urine. The experimental method described in the present work matches the appropriate standard for clinical measurement of hepcidin-25 in human serum. Hepcidin

levels obtained differed from a reference C-ELISA assay which validated the need for a calibrator mimicking human serum and establishing a consensus on calibrator levels. This method also has the ability to detect the truncated 20, 22 and 24-aa isoforms of hepcidin (not shown) even though the relevance of these isoforms for clinical applications still needs to be established [15]. More importantly, our method relies on simple, robust, reproducible and straightforward cost-effective pre-analytical steps that are specifically adapted to a clinical environment, thus avoiding the need of solid phase extraction for example. In conclusion, our approach is compatible with routine hepcidin measurements in daily clinical practice and for discriminating between iron-deficiency anemia and anemia of chronic disease.

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