IMMUNOCHEMICAL AND MASS-SPECTROMETRY-BASED SERUM HEPcidin assays for iron metabolism disorders

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Background

Hepcidin:

- is iron-regulatory peptide hormone
- consists of 3 isoforms:
  - bioactive hepcidin-25
  - inactive hepcidin-22
  - inactive hepcidin-20

- Instrumental in the diagnosis
- Monitoring of iron metabolism disorders

- Reliable methods for its quantification in serum are sparse
- isn’t knowledge of their relative analytical strengths and clinical utility
Main objective of this study

• Determined:
  • the differences in absolute concentrations
  • The degree to which the presence of hepcidin isoforms influenced the concentrations

• Ability to differentiate between samples from patients with IDA and those with IDA and anemia of chronic disease (ACD)
Methods

Developed methods:

• A competitive (c) – ELISA

• An immunocapture TOF mass-spectrometry (IC-TOF-MS) assay

• Weak cation exchange (WCX)-TOF-MS

Measured serum hepcidin concentrations in 186 patients and 23 healthy controls
Competitive ELISA

- 96-Well plates were coated overnight with goat-antirabbit IgG (Fc) Ab
- Blocked with BSA 2h
- Incubated with rabbit-antihuman hepcidin antibody for 2h
- Study samples and biotinylated hepcidin-25 calibrator were added to the wells and incubated overnight at 4°C
- Plates were incubated for 1h with conjugate a substrate was added for 15 min

The color reaction was stopped and absorbance measured at 492 nm.
***Immunocapture TOF-MS***

- Rabbit-antihuman hepcidin antibody was coupled to protein A sepharose beads
- Serum and the internal standard (hepcidin-24) were incubated for 1h with the beads-antibody complex
- Hepcidin was eluted from the beads (50% ACN and 0.5% TFA)
- 1 ul was applied to a MicroScout Plate 96 polished steel plate and m/z spectra were generated by using TOF-MS
WCX-TOF-MS

- This method is a combination of WCX bead-based hepcidin enrichment followed by TOF-MS
- As internal standard (hepcidin-24) was used for quantification
- m/z spectra were generated by using MALDI-TOF-MS

Total hepcidin concentration was defined as the sum of hepcidin-25, -22 and -20 concentration
Sample selection for the overall comparison of the 3 hepcidin assays

<table>
<thead>
<tr>
<th>Samples selection</th>
<th>number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>23</td>
</tr>
<tr>
<td>IDA patients</td>
<td>10</td>
</tr>
<tr>
<td>ACD patients</td>
<td>10</td>
</tr>
<tr>
<td>multiple myeloma patients</td>
<td>6</td>
</tr>
<tr>
<td>HFE-hemochromatosis (HH) patients at presentation</td>
<td>9</td>
</tr>
<tr>
<td>iron-depleted HFE-HH patients</td>
<td>8</td>
</tr>
<tr>
<td>C282Y/H63D HFE compound-heterozygous HH patients at presentation</td>
<td>5</td>
</tr>
<tr>
<td>Iron-depleted hemojuvelin-mutated HH patients</td>
<td>3</td>
</tr>
<tr>
<td>chronic kidney disease (CKD) patients</td>
<td>84</td>
</tr>
<tr>
<td>coronary artery bypass graft surgery patients</td>
<td>22</td>
</tr>
<tr>
<td>septic shock (sepsis) patients</td>
<td>19</td>
</tr>
<tr>
<td>healthy volunteers who were injected with lipopolysaccharide</td>
<td>5</td>
</tr>
<tr>
<td>metabolic syndrome patients</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total of samples</strong></td>
<td><strong>209</strong></td>
</tr>
</tbody>
</table>
# Analytical characteristics of hepcidin assays

<table>
<thead>
<tr>
<th></th>
<th>WCX-TOF-MS</th>
<th>c-ELISA</th>
<th>IC-TOF-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analytical LLOD, pmol/L</strong></td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.8</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Functional LLOD, pmol/L</strong></td>
<td>500</td>
<td>26.5</td>
<td>100</td>
</tr>
<tr>
<td><strong>Intraassay CV, range, %</strong></td>
<td>2.2–3.7</td>
<td>4.8</td>
<td>3.9–13.1</td>
</tr>
<tr>
<td><strong>Interassay CV, range, %</strong></td>
<td>3.9–9.1</td>
<td>11.2</td>
<td>—</td>
</tr>
<tr>
<td><strong>Recovery, range; mean, %</strong></td>
<td>96–102; 99</td>
<td>86–114; 98</td>
<td>95–105; 100</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Parallelism</strong></td>
<td>—</td>
<td>Yes</td>
<td>—</td>
</tr>
<tr>
<td><strong>Cross-reactivity hepcidin-20, %</strong></td>
<td>0</td>
<td>68</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Cross-reactivity hepcidin-22, %</strong></td>
<td>0</td>
<td>47</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> NA, not applicable; —, not determined; analytical LLOD, lowest level that can be detected based on a protein standard; functional LLOD, lowest level that can be detected based on human serum samples.

<sup>b</sup> No cross-reactivity was indicated because MS results are specific for hepcidin-25.
Relation between the serum hepcidin methods

A

\[ y = 0.79x - 0.81 \]

\[ R = 0.912^* \]

B

\[ y = 0.85x - 0.58 \]

\[ R = 0.920^* \]

C

\[ y = 0.26x + 0.05 \]

\[ R = 0.743^* \]

D

E

F

% Difference ELISA and WCX-TOF-MS

Mean ELISA and WCX-TOF-MS (hepcidin-25) (nmol/L)

Mean ELISA and WCX-TOF-MS (total hepcidin) (nmol/L)

Mean ELISA and IC-TOF-MS (hepcidin-25) (nmol/L)
Fig. 2. Bland–Altman plots for the comparison between c-ELISA and WCX-TOF-MS hepcidin-25 and total hepcidin concentrations for (A,B) 84 CKD patients with (n = 67) and without (n = 17) hepcidin isoforms (bias = 32.6%, SD = 40.5%; bias = 8.1%, SD = 32.8%, respectively) and (C,D) in all patients without isoforms, CKD patients excluded (n = 104 of 209) (bias = 33.7%, SD = 60.3%, for both).
Hepcidin concentrations in the various iron disorders as measured by the WCX-TOF-MS (hepcidin-25, closed bars, total hepcidin, striped bars) and c-ELISA (total hepcidin, open bars) hepcidin assays.
Clinical utility of low hepcidin concentrations as assessed by the different assays
Results

• They found:
  • The relative differences in median hepcidin concentrations in various diseases to be similar
  • The absolute concentrations measured with c-ELISA and WCX-TOF-MS differed
  • Hepcidin isoforms contributed to differences in hepcidin concentrations between methods
    Chronic kidney disease
  • Hepcidin concentrations measured by c-ELISA and IC-TOF-MS correlated with ferritin concentrations <60 μg/L and were suitable for distinguishing between iron deficiency anemia (IDA) and the combination of IDA and anemia of chronic disease.
Conclusions

• **c-ELISA**
  + Method of choice for the large-scale quantification of serum hepcidin concentrations
  + Low limit of detection
  + Low cost
  + High-throughput
  - Not specificity for bioactive hepcidin-25

• **WCX-TOF-MS**
  + this method can be used to distinguish variable concentrations of hepcidin isoforms – chronic kidney disease
  - Relatively expensive equipment
All data, tables and graphs were used from this article:


Immunochemical and Mass-Spectrometry–Based Serum Hepcidin Assays for Iron Metabolism Disorders

Thank you for attention.
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