A semi-automated method for the measurement of hepcidin in plasma by on-line extraction coupled to liquid chromatography-tandem mass spectrometry

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ABSTRACT

Introduction: The iron regulatory peptide hormone, hepcidin, is a promising biomarker for the diagnosis and monitoring of iron metabolism disorders. The mainstay of hepcidin analysis has been through the availability of relative analytical methods. The aim of the investigation was to develop a liquid chromatography-mass spectrometric method for the measurement of hepcidin that could be used in future human biomarker investigations of this hormone. Method: Samples (100 µL) were processed using an ion-exchange coupled to LC-MS/MS. The on-line extraction involved the loading of sample (100 µL) onto pre-conditioned Hysphere C8 extraction cartridges (10 µm, Spark Holland). The cartridges were sequentially washed with water (100 µL), 25% ammonium hydroxide (300 µL), 25% ammonium hydroxide in 0.1% formic acid (300 µL), and 0.1% formic acid (300 µL). The peptides were eluted using 2% ammonium hydroxide in 0.1% formic acid (300 µL) and lyophilized using a vacuum pump. The eluate was resuspended in 0.1% formic acid (100 µL) prior to LC-MS/MS analysis. Results: Samples were run using a reversed phase C18 column (100 x 2.1 mm, 5 µm, Waters) under gradient conditions at a flow rate of 0.3 mL/min. The peptides were eluted using 2% ammonium hydroxide in 0.1% formic acid (0.5 mL), then 2% formic acid (2 mL), followed by a 2-minute re-equilibration. Mass spectrometric detection was by selected reaction monitoring (hepcidin, labeled internal standard). Accuracy and precision were determined using quality controls across the analytical range (5, 15, 40 and 80 ng/mL) and above the upper limit of quantification (250 ng/mL). A pilot study was undertaken to compare hepcidin and ferritin concentrations using patient samples from routine iron investigations (n = 34). Results: The measured hepcidin concentrations for 79 patients with data stratified according to high and low C-reactive protein groups were 12.6 mg/L (n = 55) and 33.4 mg/L (n = 31), respectively. A statistically significant relationship between hepcidin and ferritin was observed (Figure 2, P = 0.0001). CONCLUSION: The HPLC-MS/MS method described can facilitate the validation of this new biomarker, hepcidin, for iron studies in various disease states. The method described offers the benefits of semi-automated sample preparation and relatively high throughput of approximately 7 minutes/sample, making it applicable to the clinical setting.

INTRODUCTION

Hepcidin is the master regulator of iron homostasis in humans. It is a peptide hormone that is produced primarily by the liver and excreted via the kidneys. Hepcidin expression is regulated by iron requirements, erythropoietic activity, inflammation and renal disease. Hepcidin is the master regulator of iron homostasis in humans. It is a peptide hormone that is produced primarily by the liver and excreted via the kidneys. Hepcidin expression is regulated by iron requirements, erythropoietic activity, inflammation and renal disease. Hepcidin is involved in the loading of sample (100 µL) onto pre-conditioned Hysphere C8 extraction cartridges (10 µm, Spark Holland). The cartridges were sequentially washed with water (100 µL), 25% ammonium hydroxide (300 µL), 25% ammonium hydroxide in 0.1% formic acid (300 µL), and 0.1% formic acid (300 µL). The peptides were eluted using 2% ammonium hydroxide in 0.1% formic acid (300 µL) and lyophilized using a vacuum pump. The eluate was resuspended in 0.1% formic acid (100 µL) prior to LC-MS/MS analysis. Results: Samples were run using a reversed phase C18 column (100 x 2.1 mm, 5 µm, Waters) under gradient conditions at a flow rate of 0.3 mL/min. The peptides were eluted using 2% ammonium hydroxide in 0.1% formic acid (0.5 mL), then 2% formic acid (2 mL), followed by a 2-minute re-equilibration. Mass spectrometric detection was by selected reaction monitoring (hepcidin, labeled internal standard). Accuracy and precision were determined using quality controls across the analytical range (5, 15, 40 and 80 ng/mL) and above the upper limit of quantification (250 ng/mL). A pilot study was undertaken to compare hepcidin and ferritin concentrations using patient samples from routine iron investigations (n = 34). Results: The measured hepcidin concentrations for 79 patients with data stratified according to high and low C-reactive protein groups were 12.6 mg/L (n = 55) and 33.4 mg/L (n = 31), respectively. A statistically significant relationship between hepcidin and ferritin was observed (Figure 2, P = 0.0001).

RESULTS AND DISCUSSION

Under the chromatographic conditions, the predominant precursor ion for hepcidin was the triply charged species, (M+3H)+. To minimize the reported [4] and observed (data not shown) issues with hepcidin stability and absorption effects on LC-MS/MS analysis, the sample preparation was performed only with water dilution as pre-treatment. Figure 1 shows a representative chromatogram of a patient sample with a measured hepcidin concentration of 24.7 ng/mL. The method was found to be linear over the analytical range of 5 to 100 ng/mL (r² = 0.999, n = 12). Inter- and intra-day accuracy and precision of the method, based on quality controls across the analytical range, was 95.4 – 106% and < 10%, respectively (Table 2). The inter- and intra-day accuracy and precision of the method, based on a quality control (250 ng/mL) outside the analytical range, was 96.2 – 108% and < 7%, respectively (Table 2). The measured hepcidin concentrations for 79 patients with data stratified according to high and low C-reactive protein groups were 12.6 mg/L (n = 55) and 33.4 mg/L (n = 31), respectively. A statistically significant relationship between hepcidin and ferritin was observed (Figure 2, P = 0.0001).

The measured hepcidin concentration, for the low and high ferritin groups were 19.0 mg/L (n = 48) and 33.4 mg/L (n = 31), respectively. A statistically significant relationship between hepcidin and ferritin was observed (Figure 2, P = 0.0001). The measured hepcidin concentration, for the low and high C-reactive protein groups were 12.6 mg/L (n = 55) and 33.4 mg/L (n = 31), respectively. A statistically significant relationship between hepcidin and C-reactive protein was observed (Figure 2, P = 0.0007). The measured hepcidin concentrations for 79 patients with data stratified according to high and low ferritin and C-reactive protein results. The solid dot represents the median hepcidin concentration and the error bars are the 95% confidence intervals.

CONCLUSION

The HPLC-MS/MS method described can facilitate the validation of this new biomarker, hepcidin, for iron studies in various disease states. The method described offers the benefits of semi-automated sample preparation and relatively high throughput of approximately 7 minutes/sample, making it applicable to the clinical setting.

ACKNOWLEDGEMENTS

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REFERENCES


Table 3. Stability of quality control samples at room temperature and frozen at -20°C and -80°C (n = 3)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Duration</th>
<th>Measured hepcidin concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room Temperature</td>
<td>16 hours</td>
<td>15</td>
</tr>
<tr>
<td>Frozen -20°C</td>
<td>131 days</td>
<td>14.1</td>
</tr>
<tr>
<td>Frozen -80°C</td>
<td><strong>P = 0.0007</strong></td>
<td><strong>P = 0.0001</strong></td>
</tr>
</tbody>
</table>

Table 1. Some clinical scenarios in which hepcidin measurement may have diagnostic and therapeutic applications

<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>Possible utility of hepcidin determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemosiderosis due to deficiency anemia (IDA) with underlying conditions such as: Chronic inflammatory disease Chronic renal disease Iron overload disease</td>
<td>Hemochromatosis Hemoglobinopathy Stomatitis Gastropathies Skeletal abnormalities</td>
</tr>
</tbody>
</table>