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Original article

Development of a method for the sensitive and quantitative determination of hepcidin in human serum using LC-MS/MS

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ABSTRACT

Introduction: Hepcidin, a 25-amino acid peptide hormone, plays a crucial regulatory role in iron metabolism. Elevated hepcidin has been observed in response to inflammation and is speculated to be a causative factor in inflammatory anemia due to induction of functional iron deficiency. Hepcidin has been suggested as a biomarker of anemia of inflammation. An accurate assessment of human serum hepcidin is critical to understand its role in anemia. **Methods:** An LC-MS/MS method was developed to quantify hepcidin in human serum using chemically synthesized hepcidin as a standard and stable isotope labeled hepcidin as internal standard. Rabbit serum was used as a surrogate matrix for standards due to the presence of endogenous hepcidin in human serum. The method was validated to FDA criteria for bioanalytical assays. **Results:** The calibration curve was validated over the range of 2.5 to 500 ng/mL. Hepcidin was stable in serum for at least 16 h at room temperature, 90 days at −60 to −80 °C, and after three F/T cycles. Interday accuracy (% RE) and precision (%CV) were −11.2% and 5.6%, respectively at the LLOQ, and less than ±7.0% and 9.2%, respectively for higher concentrations. The mean accuracy of quality control samples (5.00, 15.0, 100 and 400 ng/mL) in 21 analytical batches was between −0.7 and +2.1%, with mean precision between 5.1% and 13.4%. Hepcidin was below 2.5 ng/mL in 31 of 60 healthy subjects, while the mean concentration was less than 10 ng/mL. Sepsis and chronic kidney disease patients had mean serum concentrations of 252 ng/mL ($n=16$, median 121 ng/mL) and 99 ng/mL ($n=50$, median 68 ng/mL), respectively. **Conclusions:** A fully validated LC-MS/MS method has been described for the determination of hepcidin in human serum. This method was applied to the determination of hepcidin in over 1200 human samples.

1. Introduction

Human hepcidin is a 25 amino acid peptide (DTHFP ICIFC CGCCH RSKCG MCCKT) with four disulfide bonds identified from both human urine (Park, Valore, Waring, & Ganz, 2001) and blood ultra filtrate (Krause et al., 2000) that controls normal iron homeostasis by inhibiting absorption of dietary iron and iron release from storage sites (Ganz & Nemeth, 2006). Exogenous expression of hepcidin has been established in mouse models to cause an iron-limited anemia (Roy, Mak, Akpan, Losyev, Zurakowski, & Andrews, 2007) and patients with elevated hepcidin due to production in liver adenomas have iron deficiency anemia (Weinstein et al., 2002). In addition to being produced in response to increased iron levels, hepcidin is also induced by inflammation (Anderson et al., 2002; Kemna, Pickkers, Nemeth, van der Hoeven, & Swinkels, 2005a; Nemeth et al., 2004; Nicolas et al., 2002; Roy et al., 2004). Hepcidin has been speculated to have a causal role in anemia of inflammation (AI) by creating a state of functional iron deficiency and thus depriving red cells of iron to drive hemoglobin production (Andrews, 2004; Ganz, 2003; Roy, Weinstein, & Andrews, 2003). Irrespective of its importance in the etiology of AI, its induction by inflammation has led to the suggestion that measuring hepcidin in anemia patients may allow prediction of AI (Sasu et al., submitted for publication). Anemia of inflammation is thought to impact many patient populations such as those with rheumatoid arthritis and to be a significant contributor to anemias associated with chronic kidney disease and cancer (Barany, vino Filho, & Bergstrom, 1997; Bertero & Caligaris-Cappio, 1997; Fine, 2002; Gunnell, Yeun, Depner, & Kaysen, 1999; Horl et al., 2000; Qureshi et al., 1998; Yeun, Levine, Mantadilok, & Kaysen, 2000; Zimmermann, Herrlinger, Pruy, Metzger, & Wanner, 1999). This is of clinical importance since in some of these patient populations data already exists showing that inflammation is correlated with impaired response to erythropoiesis stimulating agents (ESAs) (Barany et al., 1997; Bertero & Caligaris-Cappio, 1997; Fine, 2002; Gunnell et al., 1999; Horl et al., 2000; Qureshi et al., 1998; Yeun et al., 2000; Zimmermann et al., 1999). Hence, human serum hepcidin concentrations may give an indication of the potential for successful ESA therapy

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and so be used to determine optimal anemia treatment. Based on this, a robust method for accurate measurement of endogenous hepcidin in human serum was needed to profile hepcidin concentrations in a large number of samples from various patient populations expected to benefit from anemia therapy. LC-MS/MS was chosen as the analytical platform to support these experiments.

Immunoassay has been the traditional analytical technique used for the quantitative analysis of peptides and proteins and this has also been the case for human hepcidin. Currently-available immunoassays for hepcidin are limited. A semi-quantitative detection of hepcidin in urine by immunodot has been developed (Nemeth et al., 2003). Although this technique offers some benefit, it did not allow testing of hepcidin in serum. Other studies have utilized a prohepcidin ELISA (DRG Diagnostics) as a surrogate for hepcidin (Hsu, Chiang, Chien, & Hung, 2006; Taes, Wuyts, Boelaert, De Vriese, & Delanghe, 2004). One concern with that approach is that no studies have demonstrated a correlation between the prohepcidin determined using ELISA and hepcidin production. As an example of this, a study in humans injected with lipopolysaccharide (LPS) demonstrated a disconnection between the prohepcidin that was detected and hepcidin concentrations in the urine (Kemna, Pickkers, Nemeth, van der Hoeven, & Swinkels, 2005b). A competitive enzyme linked immunoassay (C-ELISA) for human hepcidin was recently reported (Ganz, Olbina, Girelli, Nemeth, & Westerman, 2008). This assay demonstrated good performance when used to measure total hepcidin concentrations in serum and urine. However, cross reactivity with prohepcidin and N-terminal truncated forms of hepcidin has yet to be determined.

The use of LC-MS/MS for the determination of peptide biomarkers has increased substantially over the last couple of years based both on the increased sensitivity of LC-MS/MS instruments and the growing appreciation that the multiple charge states of large peptides can generate a mass to charge ratio (m/z) which makes them compatible with detection using the mass analyzers in bench-top mass spectrometers. Examples of such methods include peptide biomarkers for Alzheimer's disease (Inoue, Garner, Ackermann, Oe, & Blair, 2005) and osteoarthritis (Berna et al., 2006; Li, Nemirovskiy, Fountain, Mathews, & Szekely-Klepser, 2007). Another approach involves the determination of signature peptides of proteolytic digestions performed *in vitro*, or so-called "tryptic peptides". Published reports have described the determination of peptide biomarkers for coronary heart disease (Kay, Gregory, Grace, & Pleasance, 2007), growth hormone (Kirsch, Widart, Louette, Focant, & De Pauw, 2007), prostate cancer (Bondar, Barnidge, Klee, Davis, & Klee, 2007; Wright, Han, & Aebersold, 2005) and the profiling of plasma proteins (Anderson & Hunter, 2006). In general, approaches making use of tryptic peptides appear to be less accurate and precise than the determination of native peptides, probably due to the variability inherent in the digestion process.

Mass spectrometry based approaches specifically for hepcidin have included the use of SELDI-TOF (Ganz et al., 2008; Tomosugi et al., 2006; Ward et al., 2008) to semi-quantitatively measure hepcidin in both urine and serum, and more recently, three quantitative methods using LC-MS/MS to measure hepcidin in serum (Kobold et al., 2008; Murao, Ishigai, Yasuno, Shimonaka, & Yoshinori, 2007; Murphy, Witcher, Luan, & Wroblewski, 2007). The LC-MS/MS based methods provided a more definitive measurement of hepcidin than methods published previously due to their selectivity, which allowed the precursors or degradation fragments of hepcidin to be differentiated from the target molecule.

This report describes the development and application of a method for the determination of hepcidin in human serum by LC-MS/MS. In contrast to previously published methods, this method is fully validated according to Food and Drug Administration (FDA) guidelines for bioanalytical methods validation (FDA, 2001; Viswanathan et al., 2007). It makes use of an efficient, single step solid phase extraction procedure and a stable isotope of hepcidin as an internal standard. Procedures are also described that are essential to prevent absorption losses of hepcidin during preparation of calibration standards and QC samples. This method has been used for the analysis of serum samples to determine serum hepcidin concentrations in healthy subjects as well as in various patient populations expected to have elevated hepcidin concentrations. The validated method was demonstrated to be robust and reliable.

2. Methods

2.1. Chemical and reagents

Methanol and water (HPLC grade) were obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid (reagent grade) was from Aldrich, Inc. (St. Louis, MO, USA). All other reagents were of analytical or HPLC grade. Oasis HLB μElution 96-well SPE plates were obtained from Waters Inc. (Milford, MA, USA). Control rabbit and human serum were supplied by Bioreclamation Inc. (East Meadow, NY, USA). Human serum used for evaluation of hepcidin in healthy subjects was obtained from Bioreclamation Inc. (East Meadow, NY, USA). Subjects were categorized as healthy based on their completing a health certification questionnaire and then a physical examination where various physical and vital signs were measured. Human serum used for evaluation of hepcidin concentrations in patients with either chronic kidney disease or sepsis was obtained from Lake Arrowhead Laboratory Consultants, Inc. (Lake Arrowhead, CA, USA). Chronic kidney disease patients were defined as donors demonstrating a glomerular filtration rate of <30 mL/min. Sepsis patients were defined as donors demonstrating a blood culture positive for bacterial infection. All patients gave consent for samples to be used for research purposes.

2.2. Peptide synthesis

Human hepcidin and the internal standard, human [$^{13}C_9$,$^{15}N_1$-Phe^4]-hepcidin, were chemically synthesized using an ABI433 synthesizer (Applied Biosystems, Foster City, CA) employing a N^{α}-Fmoc/side-chain ^{t}Bu orthogonal protection strategy with 1.0 M *N,N′*-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole hydrate (HOBT) (1:1) coupling chemistry in *N*-methyl-pyrrolidone (NMP) and 20% (v/v) piperidine/NMP deprotection chemistry. The synthesis was carried out on Fmoc-Thr(^{t}Bu)-Wang resin (0.12 mmol equiv scale, Novabiochem). Single amino acid coupling cycles at 1 mmol scale were used for the synthesis, and consisted of 58 min coupling times and 3 + 15 min Fmoc-deprotection times. The isotopically enriched hepcidin internal standard was prepared by the introduction of Fmoc-[$^{13}C_9$,$^{15}N_1$]-Phenylalanine-OH (98 atom% ^{13}C, 98 atom% ^{15}N, Sigma Aldrich) at residue 9 during solid-phase synthesis. Following side-chain deprotection and cleavage from the solid-support with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS)/3,6-dioxa-1,8-octane-dithiol (DODT) (92.5:2.5:2.5:2.5 v/v) for 2 h, the solution was filtered and then evaporated *in vacuo*. The residue was treated with ice-cold diethyl ether (250 mL) and the precipitated peptide collected by centrifugation (5 min at 3800 rpm), the ether solution was then decanted, and the peptide was dried *in vacuo*. The dried peptide was reconstituted in neat TFA (2 mL) and then diluted dropwise with stirring into a fresh buffered solution (100 mL) prepared by the 1:1 combination of 6 M guanidine pH 4.5 and 6 M guanidine/0.5 M Tris/20 mM EDTA pH 8.5. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 1 mmol) was added to the solution and stirred for 2 h. The reduced human hepcidin containing solution was then loaded onto a Phenomenex Jupiter 10 μm 300 Å C18 250 × 21.2 mm column for preparative purification and fractions containing the expected molecular mass of reduced human hepcidin were pooled. The pooled fractions were then diluted to 1 L with water and acetonitrile to give an approximate final acetonitrile composition of 25% (v/v). Disulfide bond formation was carried out for ~16 h in the presence of a glutathione/glutathione disulfide (GSH/GSSG) redox