Original article

Verification of the Automated ELISA Assay for Hepcidin-25 in Human Serum

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Abstract

Introduction: Hepcidin-25, the bioactive form of hepcidin, is the master protein in regulating iron homeostasis. Serum concentrations, measured by different methods, are often incomparable and complicate results interpretation.

Materials and Methods: The aim was to verify the first fully automated enzyme-linked immunosorbent assay (ELISA) method, using the DRG Hybrid XL analyzer (DRG Instruments, Marburg, Germany) standardized against the mass spectrometry method. Intra- (CVi) and inter-assay (CVg) precision and bias were performed using commercially available controls with low (C1) and high (C2) concentrations. The reference interval was verified by analyzing serum samples of 20 healthy males.

Results: CVi = 9.1% (C1), 4.5% (C2); CVg = 8.9% (C1), 5.6% (C2); calculated bias was 33% for C1 and 20% for C2, respectively.

Conclusion: Verification of the fully automated ELISA method for hepcidin-25 in serum on the DRG Hybrid XL analyzer met the analytical acceptance criteria.

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Introduction

Hepcidin-25, the bioactive 25 amino acid peptide is the master iron-regulatory hormone and is predominantly generated in the liver by proteolytic cleavage of prohepcidin at the C-terminus (1). N-terminal removal of hepcidin-25 can result in short peptides of 20-24 amino acids which have reduced activity but can interfere with some immunoassays (2). Hepcidin-25 is the only active form of hepcidin in serum and has the main role in iron status regulation by down-regulating the expression of ferroportin. Hepcidin binds to the cell surface iron transporter – ferroportin – inducing its internalization and degradation (3). The result is increased intracellular iron storage, decreased dietary iron absorption and decreased serum iron concentration (1). In conditions of increased demand for iron, (iron deficiency, hypoxia, anemia and erythropoiesis) hepatocellular hepcidin synthesis decreases (4, 5). Hepcidin deficiency causes hereditary hemochromatosis, characterized by body iron overload that may progress into liver cirrhosis (6). In addition, low hepcidin-25 concentration can be induced in iron-loading anemias and chronic hepatitis C (7, 8). In contrast, hepcidin synthesis is induced by inflammation and infection (9). High hepcidin-25 concentration has been found in iron-refractory iron-deficiency anemia, during infection, chronic kidney disease as well as in cancer (9–11). Therefore, hepcidin-25 serum levels are valuable for identifying and differentiating specific diseases or conditions related to iron homeostasis (7, 10, 12).

So far, two main techniques have been used for serum hepcidin determination: mass spectrometry (MS) and immunochemistry – solid phase enzyme-linked immunosorbent assay (ELISA) (13–15). Immunochemistry assays are commonly used to carry out routine tests in laboratories. However, the ELISA method is not suitable for individual and random-access testing because results provided by different methodologies show high discrepancies.

Analytical method verification is required for providing objective evidence that an analytical procedure meets the requirements suitable for scientific research and routine application in the laboratory (16). In 2016, the first fully automated immunoassay, an important step forward, was introduced to the market. Until then, only manual immunoassays were available.

The aim of this study was to verify in a clinical laboratory the fully automated ELISA method validated by DRG company by MS (results not published) for measuring concentrations of hepcidin-25 in human serum.

Materials and methods

Subjects and blood sampling

Commercially available hepcidin samples were obtained from DRG company (DRG Instruments, LLC (GmbH), Marburg, Germany): C1-normal level (4.6 µg/L) and C2-high level (44.3 µg/L), were used as standards for the verification procedure. Additionally, blood samples from healthy subjects (regular male blood donors aged 18 years or older), were collected in August 2020 at the Department of Transfusion Medicine, Osijek University Hospital, following the guidelines on venipuncture for blood donation. All subjects were previously screened for anemia at the same Department according to the guidelines for blood donors. The exclusion criteria were hemoglobin concentration of less than 135 g/L. Inclusion criteria were iron, ferritin and transferrin levels within the reference range. Indicators of iron status were conducted at the Institute of Clinical Laboratory Diagnostics. Informed consent was signed by all participants. Venipuncture was performed, and blood was collected using tubes (5 mL) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) without anticoagulants. The tubes were centrifuged for 10 minutes at 2,000 g and the serum was used for measuring the concentration of hepcidin-25.

Analytical methods

Analytical verification of the method was performed according to the Clinical and Laboratory Standards Institute (CLSI) guideline EP15-A2 on Method Verification (16). Method verification procedure included the assessment of the accuracy of series (repeatability) and day-to-day (intermediate precision) accuracy, measurement uncertainty and reference interval verification. After reconstituting the lyophilized control samples following the manufacturer's instructions, verification steps were performed. Control standard samples were analyzed in triplicate for five consecutive days and the data were used to calculate both accuracy and variability. The normal reference range was checked from 20 serum samples from healthy blood donors.

Hepcidin-25 concentrations were measured by the automated ELISA method using the DRG Hybrid XL (DRG Instruments, LLC (GmbH), Marburg, Germany) analyzer. The manufacturer declared that the hepcidin-25 ELISA method was standardized against the mass spectrometry method.

All data were calculated using the Excel program: mean value, standard deviation, coefficient of variation and bias according to the formulas provided in Supplement Table 1. Reference interval verification was performed using MedCalc for Windows, version 12.4.0.0 (MedCalc Software, Mariakerke, Belgium). The reference interval provided by the manufacturer was confirmed in serum samples. The distribution of the results was normal, calculated data obtained in Excel were used for the reference interval verification – the interval in which the central 95% values of a healthy subject lie. The used limits of normality: mean ± 1.96 x SD.

Results

In this research, we performed method verification in terms of intra- and inter-assay precision and bias assessment. Method verification was performed by analyzing two concentration levels of commercial controls. Results are summarized in Table 1. We confirmed the declared inter-assay precision for both concentration levels considering the concentration values. Namely, < 10.7% and < 10.0% for normal (C1) and high (C2) control levels, respectively. Calculated bias was higher in C1 (> 30%) than in C2 (20%).

Table 1. Coefficients of variation for control samples

	CVi	CVg	Total CV	Bias
C1	9.0%	8.9%	11.6%	33%
C2	4.5%	5.6%	6.7%	20%

C1=control sample level 1; C2=control sample level 2; CVi=intraindividual coefficient of variation; CVg=interindividual coefficient of variation; CV=coefficient of variation

The mean hepcidin-25 level in serum samples was 10.2 µg/L (SD 9.9) which is within the normal reference range stated by the manufacturer (DRG Instruments, LLC (GmbH), Marburg, Germany) declared for the 2.5th and 97.5th percentile as of 0.2–34.4 µg/L obtained on 20 healthy male blood donors in the current study.

Supplemental Table 1: Measured values of control samples during five consecutive days in triplicate

1. Intra- and interassay precision control sample level 1					
Day:	1	2	3	4	5
1	5,25	5,95	5,87	5,70	6,92
2	6,25	5,90	6,13	5,93	7,08
3	5,38	5,20	7,54	5,47	6,29
Mean	5,63	5,68	6,51	5,70	6,76
Sd	0,54	0,42	0,90	0,23	0,42
Sr	0,55				
CV%	9,06				
grand mean	6,06				
Xd - grand mean	0,19	0,14	0,21	0,13	0,50
Sb	0,54				
CV%	8,90				
Sl	0,70				
CV%	11,56				
2. Intra- and interassay precision control sample level 2					
	1	2	3	4	5
1	54,30	49,70	59,70	54,10	53,70
2	55,30	56,00	60,80	56,20	53,50
3	52,30	51,00	59,10	61,90	54,80
Mean	53,97	52,23	59,87	57,40	54,00
Sd	1,53	3,33	0,86	4,04	0,70
Sr	2,49				
CV%	4,48				
grand mean	55,49				
Xd - grand mean	2,33	10,63	19,13	3,64	2,23
Sb	3,08				
CV%	5,55				
Sl	3,69				
CV%	6,65				

SD= standard deviation; CV= coefficient of variation; Sb= standard grand mean deviation; Sr= mean standard deviation; SI= total standard deviation

Discussion

A study designed by Aune et al. described a framework for optimizing hepcidin measurement and improvements in method standardization (17). Prior to the Aune et al. study, Diepeveen et al. standardized hepcidin methods (18). Both studies proposed an international standard for methods calibration. Aune et al. compared 16 different methods for hepcidin and obtained a mean accuracy of 145% (CI 76–540%), for all methods (17). Interestingly, among 9 MS and 7 ELISA methods, and described here, the ELISA DRG Hybrid XL bioactive hepcidin-25 method, the declared accuracy was 125% (CI 109–147%). Other ELISA methods in the Aune et al. study had similar accuracies with only one method having a higher accuracy (340% (CI 274–540%)). The lack of standardization is reflected by the large variation in results. It is important to note that only hepcidin-25 is the biologically active isoform to be measured, but with ELISA methods there is a certain rate of cross-reactivity

with other hepcidin isoforms, usually declared by the manufacturer.

In the current study, the optimal minimum CV for precision was 2.27% and 1.12%, calculated as CVi x 0.25 pointing out that the ELISA DRG Hybrid XL method has optimal CV when compared to the literature data (< 12.2%) (19).

We must point out that the available data about hepcidin-25 biological variation were obtained on mice and a few human samples, in a study by Murphy et al. (19). The obtained intraindividual biological variation of hepcidin-25 was 48.8% while the inter-individual coefficient of variation was 154.1%. Here described study obtained CVi and CVg around 10%.

The main advantages of the automated ELISA method are straightforward procedure, high sensitivity, ready-to-use reagents, short analysis time (2 hours), small sample volume (160 µL), and the possibility of individual sample analysis compared to MS analysis which is multiplex and delays in results. A significant advantage of this method is its standardization against the MS.

There are limitations to this current study. The DRG company reagent manufacturer did not report the desirable bias or inaccuracy for the method. Also, the DRG company used patient' samples for the initial method validation, while our study verification was performed with commercial control samples so there is a difference in the matrix. In addition, we did not compare the DRG method with other hepcidin-25 methods. For the verification, we used control samples provided by the manufacturer. Therefore, we could not compare our data with the manufacturer's validation data which were conducted on the patient samples. During the verification study, we were not able to perform commercial controls of a third-party manufacturer. Finally, the reference interval verification was performed using only serum samples of healthy males.

Conclusion

The verification of the fully automated ELISA method for determining hepcidin-25 levels in serum samples performed on the DRG Hybrid XL analyzer showed desirable analytical reproducibility and met all acceptance criteria.

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Disclosure

Funding. No specific funding was received for this study.
Competing interests. None to declare.

List of abbreviations:

MS = mass spectrometry
ELISA = solid phase enzyme-linked immunosorbent assay
C1 = control sample level 1 (normal value)
C2 = control sample level 2 (high value)
CLSI = Clinical and Laboratory Standards Institute
CVi = intraindividual coefficient of variation
CVg = interindividual coefficient of variation
CV = coefficient of variation
SD = standard deviation
CI = confidence intervals

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