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ELISA METHOD FOR SERUM HEPCIDIN QUANTIFICATION IN BULGARIAN POPULATION

V. Manolov, B. Atanasova, V. Vasilev, K. Tzatchev and M. Velizarova

Department of Clinical Laboratory and Clinical Immunology, Medical Faculty, Medical University, Sofia, Bulgaria

Summary. Hepcidin is a 25-aminoacid cysteine-rich iron regulating peptide. Hepcidin quantification in human blood may provide further insights for the pathogenesis of disorders of iron homeostasis and might prove a valuable tool for clinicians for the differential diagnosis of anaemia. This study describes ELISA immunoassay for hepcidin quantification in human serum. We used a sandwich ELISA method from USCN Life Science inc., that consists of ready to use, pre-coated 96-well strip plate with 2 antihepcidin-25 monoclonal antibodies. A recombinant hepcidin in 16 µg/l concentration is used as a standard; it reconstitutes with 1.0 ml standard diluent to prepare a stock solution. We correlated ELISA results of hepcidin-25 measurements in healthy population with hemodialysis patients. The sandwich ELISA was highly specific for hepcidin-25, having a low limit of quantification of 0.020 µg/l. Hepcidin-25 concentrations were increased in hemodialysis patients (median 33.05 µg/l, range 22.31-60.98 µg/l, n = 10) compared with healthy individuals (median 12.41 µg/l, range 6.05-18.53 µg/l, n = 40). The use of 2 monoclonal antibodies in a sandwich ELISA format provides a robust, convenient and not very expensive method for measuring concentrations of the active form of hepcidin. It should help to improve our understanding of the role of hepcidin in regulating iron metabolism.

Key words: *hepcidin, quantification, ELISA, hemodialysis, iron metabolism*

INTRODUCTION

Hepcidin is a 25-aminoacid cysteine-rich peptide, present in human serum and urine [11, 17]. It is synthesized predominantly by hepatocytes as an 84-aminoacid precursor protein and its mature form is released in circulation [23]. Hepcidin acts by binding to ferroportin (FPN1), the only known cell iron exporter [7], inducing its internalization and subsequent degradation in the

cytoplasm [16]. In systemic level, hepcidin upregulation results in inhibition of iron absorption from intestinal enterocytes and iron recycling from macrophages [9, 16]. Hepcidin expression is up-regulated by iron and inflammation and down-regulated by anaemia and hypoxia [2].

Evidence has emerged that in certain types of hereditary hemochromatosis, the cause of the disease is a deficiency of hepcidin, resulting in unregulated uptake of iron and subsequent iron overload [2, 20]. In contrast, in anemia of chronic disease and anemia of cancer, data suggest that hepcidin concentrations are increased, causing decreased absorption of iron and increased sequestration of iron in the reticuloendothelial system, which together account for the observed anemia [1, 2, 14, 19, 22]. It is also suggested that patients with chronic kidney disease may have increased hepcidin, which could contribute to their observed renal anemia that is treated with erythropoietin and oral iron [4, 24].

Several methods for hepcidin quantification in human serum and urine have been reported. There are two antibody-based techniques, a dotblot assay, which has been used to measure hepcidin in urine and is considered semi-quantitative [15, 25], and a commercially available ELISA assay measuring serum prohepcidin. The diagnostic utility of the latter is controversial, since prohepcidin represents a processing intermediate rather than a biologically significant form [12, 21].

AIM

Our study describes a specific and non-operator demanding immunoassay for hepcidin quantification in human sera. It was designed to introduce the method into Bulgarian clinical laboratory practice as a routine method for quantification of hepcidin serum levels in the Bulgarian population, as well as to establish the reference ranges of serum hepcidin in our population.

MATERIALS AND METHODS

Subjects

This study included 40 healthy controls and 10 hemodialysis patients. The study was approved by the ethics committees of the participating institutions. Informed consent was obtained from all patients and controls and study procedures were conducted in accordance with to the Declaration of Helsinki.

40 serum samples from healthy volunteers (ages 22-60 years, mean age 33.4 years) were collected. We also obtained 10 serum samples from hemodialysis patients. After obtaining protocol approval from an institutional review board and proper informed consent, all samples were collected, stored, and deidentified to protect patient privacy. Samples were stored at −70°C before analysis of hepcidin levels. Ferritin analysis was performed by using a Roche Diagnostics turbidimetric immunoassay.

ELISA procedure

96-well microtiter plates (USCN Life Science Inc.) pre-coated with specific antibody were used. As calibrator we used hepcidin25-His diluted in standard diluent (8, 4, 2, 1, 0.500, 0.250, 0.125, 0.065 µg/l). Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for hepcidin. Next, avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contain hepcidin, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change of color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of serum hepcidin in the samples was then determined by comparing the O.D. of the samples to the standard curve.

DATA ANALYSIS

MSD software and SigmaPlot version 8.0 were used for fitting ELISA calibration curves. Data were plotted by using version 2.98 of the program FigP (Biosoft). For each group of individuals or patients studied, the median, 25th percentile, 75th percentile, and interquartile range were determined. Comparisons of hepcidin-25 concentrations between respective groups were performed by using the Wilcoxon nonparametric rank sum test. In each case, a P value of <0.05 was considered to indicate statistical significance.

RESULTS

We compared sandwich ELISA hepcidin-25 results in hemodialysis patients (n = 10) to results of healthy individuals (n = 40). These comparisons (Table 1) demonstrated that hepcidin-25 concentrations were increased in hemodialysis patients (median 33.05 µg/l, range 22.31 – 60.98 µg/l) compared with healthy individuals (median 12.41 µg/l, range 6.05 – 18.53 µg/l).

The sandwich ELISA method produces a typical calibration curve for the recombinant hepcidin25-His (Fig. 1). The analytical limit of detection of the ELISA assay, defined as the concentration corresponding to the mean signal +3 SD of 10 replicates of the zero calibrator was 0.020 µg/l. The measurement range was 0.0625 – 8 µg/l. For the statistical analysis of the reproducibility, linearity and recovery of the hepcidin ELISA assay, we used 3 serum samples ranging from low (21.15 µg/l) to high (214.84 µg/l) concentrations chosen from 40 normal sera tested. The intra-assay coefficients of variance (CVs) were 1.69-4% as evaluated by assaying 12 replicates of each sample in a single assay (Table 2). The inter-assay CVs were 0.89-4.54% as evaluated by 5 subsequent measurements of the test samples (Table 3). Intra- and inter-assay CV for the standard curve were 0.65% and 7.82%, respectively. Analytical recovery was studied by adding the calibrator at 8 and 2 µg/l in

each serum sample and was found to range from 96% – 109% with a mean recovery index of 103% (Table 4).

Table 1. Comparison of serum hepcidin-25, serum iron, TSAT, hemoglobin and RBC in healthy individuals with the concentrations in hemodialysis patients

		Control	HdP
serum Hepcidin	min	6.05	22.31
	max	18.53	60.98
	aver	12.41	33.05
serum Iron	min	10.60	5.60
	max	39.00	34.10
	aver	19.14	13.42
TSAT	min	17.19	10.41
	max	63.93	61.13
	aver	30.90	30.42
Hemoglobin	min	117.00	84.00
	max	172.00	115.00
	aver	143.70	99.20
RBC	min	3.80	2.58
	max	6.14	3.79
	aver	4.96	3.28

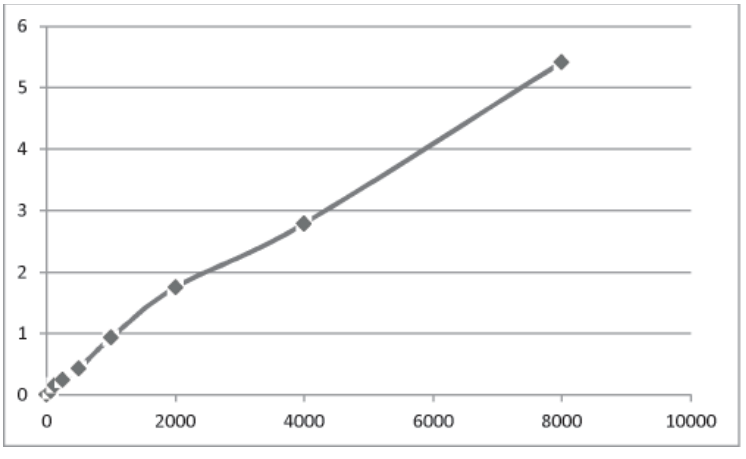

Fig. 1. Representative calibration curve for recombinant hepcidin25-His. The range of the assay is 0.0625 – 8 μg/l

Table 2. Intra-assay variation

real	Smpl 1	Smpl 2	Smpl 3
	21.15	108.92	214.84
intra-assay variation	20.45	108.84	210.14
	21.15	104.55	209.87
	20.98	106.98	215.47
	21.01	109.84	216.28
	22.98	110.01	211.44
	21.29	115.52	204.55
	20.01	107.51	213.85
	20.87	108.14	214.88
	21.11	109.51	217.85
	19.47	110.84	215.01
	21.17	111.1	212.88
	21.22	111.14	211.84
aver	20.98	109.50	212.84
SD	0.84	2.71	3.59
CV	4.00	2.47	1.69

Table 3. Inter-assay variation

real	Smpl 1	Smpl 2	Smpl 3
	21.15	108.92	214.84
inter-assay variation	21.15	108.92	214.84
	20.11	107.01	215.99
	19.98	106.99	211.74
	22.14	110.52	214.99
	21.7	107.85	216.78
aver	21.02	108.26	214.87
SD	0.95	1.49	1.92
CV	4.54	1.38	0.89

Table 4. Recovery of calibrator added to human serum samples

real	21.15		108.92		214.84	
analytical recovery with 8 ng/ml		%		%		%
	21.96	103.83	111.47	102.34	212.84	99.07
	20.95	99.05	116.88	107.31	209.89	97.70
	20.41	96.50	118.74	109.02	224.98	104.72
	21.65	102.36	118.77	109.04	226.14	105.26
	21.5	101.65	119.78	109.97	221.85	103.26

real	21.15		108.92		214.84	
analytical recovery with 4 ng/ml		%		%		%
	22.14	104.68	111.14	102.04	217.11	101.06
	21.99	103.97	109.89	100.89	214.55	99.87
	20.19	95.46	114.14	104.79	219.54	102.19
	21.45	101.42	107.89	99.05	212.87	99.08
	22.08	104.40	112.45	103.24	222.54	103.58

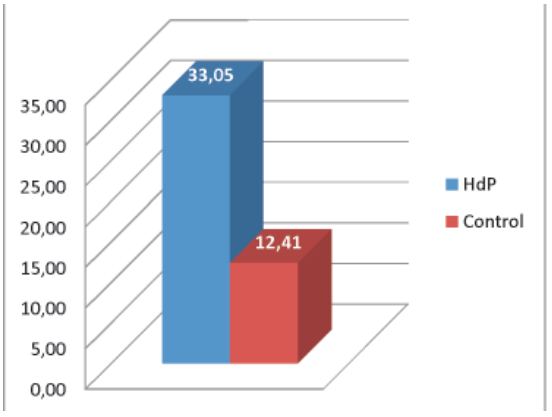

Fig. 2. Hepcidin serum concentration in healthy controls (control) and hemodialysis patients (HdP)

Significant difference compared to control is indicated by asterisk ($p < 0.05$).

Serum hepcidin levels in 40 healthy controls ranged from 6.05 to 18.53 μg/l (Fig. 2), and the levels correlated with serum iron levels (Pearson correlation: 0.133, $p < 0.001$) and with TSAT (calculation based on serum iron levels and total iron binding capacity) (Pearson correlation: 0.206, $p < 0.001$) (Fig. 3).

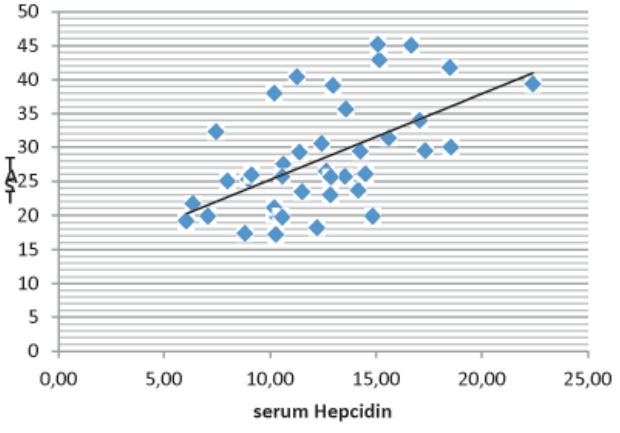

Fig. 3. Correlation between serum hepcidin and TSAT in healthy controls (r = 0.32)

DISCUSSION

The present study describes an immunological assay for hepcidin quantification in human serum, based on the use of a recombinant hepcidin peptide and a polyclonal antibody.

After examining the analytical characteristics of this assay, which were found satisfactory, we proceeded in determining the clinical value of it by measuring hepcidin levels in patients with iron disorders in chronic kidney diseases (in particular hemodialysis). We found significantly higher hepcidin levels in hemodialysis patients, as expected. These results allow us to conclude that this ELISA system can effectively quantify hepcidin in human serum.

Our results demonstrate that the sandwich ELISA is capable of measuring hepcidin-25 concentrations in human serum.

Compared with existing assays that use a competitive format (either ELISA or RIA), this method has advantages inherent in the sandwich assay format. In particular, responses are directly correlated with increasing hepcidin concentrations. In contrast, in competitive ELISA methods, absorbance values are inversely correlated with hepcidin concentrations [2, 3, 5, 10, 13]. Another advantage of this assay is its improved limit of quantification of 0.020 μg/l compared with existing assays.

In patients with anemia of chronic disease that is unresponsive to erythropoietin, this hepcidin ELISA may be used to verify that increased serum hepcidin is at least partly responsible for the erythropoietin-resistant anemia. Likewise, in the setting of chronic renal disease, this ELISA could be used to determine serum hepcidin concentrations to better direct therapy.

This sandwich ELISA could also be used to help diagnose iron deficiency anemia in difficult cases in which it may coexist with anemia of chronic disease. In uncomplicated iron deficiency, hepcidin concentrations would be expected to be quite low, whereas in anemia of chronic disease without coexisting iron deficiency, hepcidin concentrations would be expected to be increased [2, 8, 18, 26]. Thus, in patients with anemia of chronic disease, a relatively low serum hepcidin concentration might also indicate the presence of coexisting iron deficiency.

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✉ *Corresponding author:*
V. Manolov
Department of Clinical Laboratory and Clinical Immunology
Medical Faculty, Medical University
1 Sv. Georgy Sofiyski st.
Bg – 1431 Sofia