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Validation of ELISA method for serum erythroferrone evaluation

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PURPOSE / OBJECTIVES

Haematologists often meet patients with iron deposition in their organism, including iron-deficiency anaemia or insufficient iron for bone marrow function (anaemia in chronic diseases or malignant processes). Other patients are subjected to phlebotomy in order to remove iron from organism and/or suppression of iron delivery to bone marrow in policytemia vera, iron deposition from food intake or hemochromatosis, hemosiderosis, blood transfusions, and porphyria cutanea tarda. Some patients needs blood transfusion despite iron deposition in organism. Investigations on erythroferrone might bring to new therapeutic approach, through influence on hepcidin secretion and absorption, deposition and mobilisation of iron, which may lead to more specific and quick results compared to methods used in our days, such as iron supplementation, chelation and phlebotomy.

Hepcidin regulation on ferroportin function is mostly described in macrophages, which assimilate iron through phagocytosis from sequestrated erythrocytes, in hepatocytes, where iron deposits in form of ferritin, and in duodenal enterocytes, where actual iron absorption from food presents. An animal model shows that erythropoietic stress occurs after blood lost or haemolysis, induction of erythroferrone brings iron to erythroblasts, and recovery of circulating erythrocyte number is presented very quick. Deficiency of erythroferrone leads to increasment of serum hepcidin, worsening iron restriction and anaemia, slowing down recovery of inflammatory induced anaemia.

Potential role of erythroferrone comes down to modulation of hepcidin synthesis in cases of iron accumulation in hemochromatosis and restriction of trace element in anaemia of chronic inflammation through changes of signalling pathways and inflammatory cytokines. One of the approaches might be suppression of erythroferrone in patients with beta-thalassemia or conditions with iron accumulation during non-effective erythropoiesis. Other therapy can be expressed with increasment of erythroferrone which may influence on anaemia in chronic inflammation, especially in patients with unaffected by therapy inflammatory process.

MATERIALS & METHODS

Principle of ELISA method is based on reaction antigen-antibody through labeled antibodies. Usually the labeling involves not the antibody that reacts with antigen (named first antibody), but anti-immunoglobulin antibody, that responds to first antibody (named second antibody). Finally the method includes at least three phases: a) reaction between first antibody and antigen; b) reaction between second and first antibodies; and c) measurement of labeled second antibody. This reaction is very appropriate for routine laboratory analyses and can be processed as both quantitative and qualitative methods.

ELISA usually runs in 96-strip plates made by absorbing surface for proteins. Originally in the strip is added antigen solution, left to clue on the bottom and walls of wells. The excess of antigen is then removed and wells are filled with indifferent protein, most often bovine serum albumin. The wells are once again washed and first antibody solution is added. After specific incubation the solution is removed and second antibody is added. Finally a substrate for color reaction is placed in the wells which catalyzes enzyme that marked second antibody. ELISA belongs to solid phase immunological methods. The common thing is that in the beginning antigen or antibody is adsorbed on a surface.

For serum erythroferrone quantification we used ELISA method based on sandwich principle. Microplates are loaded with specific antibodies against human erythroferrone. Standards and samples are added in amount of 100 µl, mixed with biotin-conjugated antibodies, specific to erythroferrone. Avidin conjugated horseradish peroxidase is then added. A substrate which leads to change of colour is then dripped in microplates. Enzyme-substrate reaction is then stopped by addition of sulphuric acid solution. Colour of reactionary solution is photometrically evaluated on ELISA reader on 450 nm. Erythroferrone concentration in samples is reported based on calibration curve from 8 standards (20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml, and 0.156 ng/ml). Standard diluent is used as blank (0 ng/ml).

RESULTS

Validation of ELISA serum erythroferrone quantification follows the next steps:

a)determination of analytical range through calibration curve;

b)determination on limit of detection;

c)verification of accuracy of the curve by low level of quantification (LLOQ), middle point of quantification (MPQ) and upper level of quantification (ULOQ);

d)determination of reliability by recovery method (added/found);

e)determination of intra-assay and inter-assay precision.

Calibration curve

The first step of serum erythroferrone ELISA method validation is construction of calibration curve. For this purpose we used standard, which is recombinant human erythroferrone with value 20 ng/ml. From this standard by dilution we prepared eight clinically significant values of erythroferrone (10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml, 0.156 ng/ml). Each standard was measured twice. The received values were corrected by result of blank (standard diluent). Calibrations curve is four parametric, X – logarithmic, and Y – linear was duilded.

The blank was ten times measured for establishment of limit of detection. The result of 0.056 ng/ml permits very high diagnostic sensitivity of this method.

Validation of ELISA serum erythroferrone quantification follows the next steps: determination of analytical range through calibration curve (four parametric, X – logarithmic, and Y – linear); determination on limit of detection (0.056 ng/ml); verification of accuracy of the curve by low level of quantification (LLOQ), middle point of quantification (MPQ) and upper level of quantification (ULOQ); determination of reliability by recovery method (added/found); and determination of intra-assay and inter-assay precision (average 2.73% and 2.63%, resp.).

During the steps of validation of ELISA method for serum erythroferrone quantification we found: wide range of quantification; very low limit of detection; very high diagnostic sensitivity; and low analytical variation. All these analytical characteristics showed that selected ELISA method for serum quantification is reliable for routine laboratory application in diagnosis and monitoring of therapy in different diseases, that involves dysregulation of iron homeostasis.

RESULTS

Using three of standards (0.156 ng/ml, 1.25 ng/ml, and 10 ng/ml) and measured each five times LLOQ, MPQ and ULOQ were established.

(n=5)	LLOQ	MPQ	ULOQ
CV %	6,145	5,057	2,979
bias %	8,754	3,47	0,77

Reliability of used ELISA method for serum erythroferrone quantification was established by procedure recovery (added/found). The area of recovery from 96.7% to 97.9% was proved.

Parameter	Sample 1	Sample 2
Base level	9.10 ng/ml	4.19 ng/ml
Added	10.00 ng/ml	10.00 ng/ml
Expected value	19.10 ng/ml	14.19 ng/ml
Found value	19.51 ng/ml	14.68 ng/ml
Recovery	97.9 %	96.7 %

Intra-assay precision was established by five times measurement of three random chosen patient samples with already knows erythroferrone levels.

X_0	sample 1 [0,301 ng/ml]	sample 2 [5,457 ng/m]	sample 3 [12,425 ng/m]
X_i	0,30	5,46	12,43
SD	0,01	0,02	0,01
CV %	4,17	2,11	1,91

Inter-assay precision was established by five times measurement of three random chosen patient samples with already knows erythroferrone levels;

X_0	sample 1 [0,557 ng/ml]	sample 2 [5,178 ng/ml]	sample 3 [12,505 ng/ml]
X_i	0,56	5,18	12,51
SD	0,02	0,01	0,03
CV %	3,74	1,98	2,17

SUMMARY/CONCLUSION

During the steps of validation of ELISA method for serum erythroferrone quantification we found:

a)wide range of quantification;

b)very low limit of detection;

c)very high diagnostic sensitivity;

d)low analytical variation.

e)we established low CV during evaluation of low limit of quantification (6.15%), middle point of quantification (5.06%), and upper limit of quantification (2.98%);

f)high CV in recovery of this method was established – 96.7% and 97.9%;

g)very low intra-assay (average 2.73%) and inter-assay precision (average 2.63%) were found.

All these analytical characteristics showed that selected ELISA method for serum quantification is reliable for routine laboratory application in diagnosis and monitoring of therapy in different diseases, that involves dysregulation of iron homeostasis.

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