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Construction of a QCM Biosensor for free Hemoglobin Assay

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Protein responsible for oxygen transfer, hemoglobin, is an important marker in biochemistry that can be measured in blood and blood plasma. Meaning of the assays are different in the two fluids. While blood hemoglobin serving as a marker of anemia and some oncotic processes is simply measurable by spectroscopy, hemoglobin content in plasma, a marker of hemolytic processes, is significantly lower and the assay should be done by more elaborative techniques. In this paper, piezoelectric biosensor containing QCM platform with basic frequency of oscillations 10 MHz and gold circle electrodes on the opposite sites, antibody against hemoglobin and iron oxide nanoparticles as blocker and antibody layer stabilizer was prepared. The assay exerted limit of detection equal to 0.063 mg/ml and the calibration well correlated ($r^2 = 0.995$) with standard ELISA. No interference by immunoglobulins and albumin was proved. The assay appears to applicable for clinical praxis and outside of central hospital laboratories. Reasonably limit of detection, low price of the biosensor and device, simple procedure of sample use, no necessity of specific reagents and label free type of assay are the major advantages of the biosensor.

Keywords: affinity; antibody; biosensor; biorecognition; blood; erythrocyte; hemoglobin; immunochemistry; label free assay; piezoelectric; quartz crystal microbalance; QCM

1. INTRODUCTION

Hemoglobin is a protein responsible for oxygen transfer in the blood system where it is located in erythrocytes. The irreplaceable role of oxygen in the processes is well known and it is an abundant blood protein studied in relations to human physiological functions and various pathologies like diabetes where hemoglobin become glycated and serve for diagnostic purposes [1-5]. Content of hemoglobin in blood samples is typically measured by a hemoglobinometer that can be a portable device working on spectral analysis and disposable cuvettes [6]. The blood hemoglobin is measured for recognition of anemia which is confirmed when hemoglobin level in blood is under 135 g/l in a man or 120 g/l in a woman. Under some conditions like living in high altitudes or due to taking erythropoietin, hemoglobin

level can be increased. High increase can be due to some types of cancer and hemoglobin can exceed upper physiological level 175 g/l for man and 155 g/l for woman. Because of high concentration of hemoglobin in blood and its ability to absorb light with maximum at approximately 560 nm respective 540 nm and 590 nm for oxyhemoglobin, direct spectroscopy is quite easy.

Measurement of hemoglobin in plasma is more complicated compared to the hemoglobin in blood. The plasma hemoglobin is entitled free hemoglobin and it comes from broken erythrocytes and the measurement helps reveal hemolytic anemia. Extrinsic hemolytic anemia caused by autoimmune disorders or infections and intrinsic one caused by thalassemia, congenital spherocytic anemia and sickle cell anemia can be mentioned as examples. Free hemoglobin in plasma is thousand times lover than the one in the whole blood as approximate plasma level is equal to 0.12 mg/ml [7]. Concentration of free hemoglobin up to 0.3 mg/ml is not alarming, moderately elevated level is up to 0.7 mg/ml and pathological processes probably appears when free hemoglobin exceeds 0.7 mg/ml. The free hemoglobin can be also measured in serum but the results can be influenced by loosing of hemoglobin during blood clot formation hence plasma should be preferred because results from serum measurement can suffer from a false negative error.

Because of low physiological level of hemoglobin in blood plasma, it is not so easy to make an assay in field or home care conditions. In this paper, a biosensor assay for the determination of hemoglobin sensitive enough to be applicable for plasma samples is invented on the piezoelectric bases using Quartz Crystal Microbalance (QCM) sensors. The assay is organized as a label free which means that it is organized to be suitable for hemoglobin measurement without adding any specific reagent or sample processing. The study expects that the biosensor could replace standard assays when equipped biochemical laboratories are not available. Principle of the assay is depicted as a graphical abstract in figure 1.

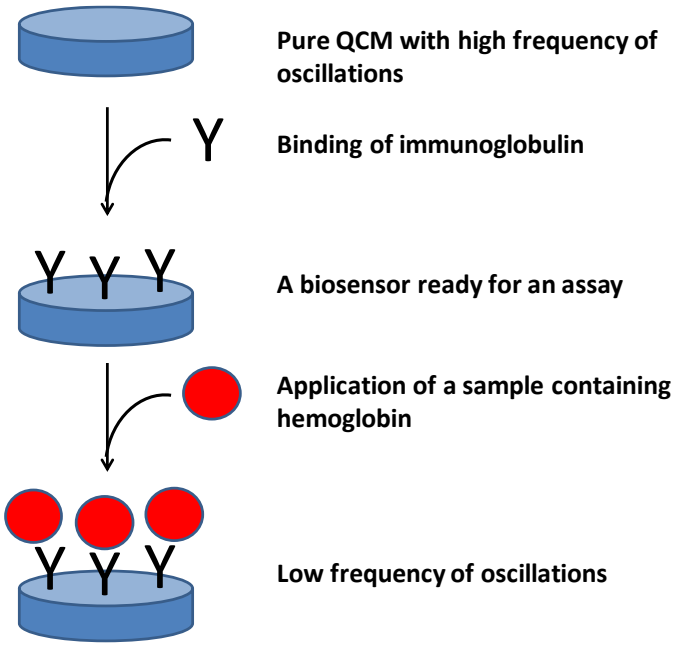


Figure 1. Principle of biosensor construction and hemoglobin assay.

2. MATERIALS AND METHODS

2.1. Chemicals

Following chemicals and reagents were used in the experiments. Ethanol 96 % v/v, pure gelatin, sulfuric acid and hydrogen peroxide were purchased from Penta (Prague, Czech Republic). Cysteamine, glutaraldehyde, phosphate buffered saline (PBS) pH 7.4, iron oxide (II,III) magnetic nanoparticles 30 nm containing carboxylic acid on their surface, human hemoglobin in form of pure lyophilized powder, human immunoglobulin G (IgG), human serum albumin, goat produced anti-rabbit polyclonal antibody labelled with peroxidase and anti-human hemoglobin polyclonal antibody produced in rabbit were bought from Sigma-Aldrich (St. Louis, MO, USA). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, o-phenylenediamine and Tween 20 were purchased from Litolab; Chudobin; Czech Republic. Deionized water was prepared by Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) device.

2.2. Biosensor construction

For purpose of this study, QCM sensors with basic frequency of oscillations 10 MHz were bought from Krystaly (Hradec Kralove, Czech Republic; http://www.krystaly.cz/en/). The QCMs had external diameter 20 mm and contained gold electrodes in the middle of quartz discs. The electrode was placed on chromium interlayer and was circle shaped with diameter 7 mm. Thickness of the quartz disc was equal to 0.166 mm. The QCM sensors were modified by immobilization of an antibody against human hemoglobin. The immobilization procedure was done under standard ambient temperature and pressure (temperature 25 ºC; atmospheric pressure 101 kPa) and it consisted from following steps:

- New QCMs were washed by dipping into 96 % v/v ethanol for 30 minutes and then let to dry.
- 50 µl of cysteamine solution 50 mg/ml in deionized water was spread per one electrode and let to activate the gold surface in a wet box for 5 hours.
- The electrodes were rinsed with deionized water and dried under laboratory conditions.
- The previously activated gold electrodes were modified by 50 µl/electrode of glutaraldehyde 5 % w/w in water, the electrodes were again let to interact with the solution in wet box for another 5 hours.
- The electrodes were again washed by deionized water and dried.
- Anti-human hemoglobin antibody dissolved in PBS 1:8,000 was applied per one electrode in wet box for 5 hours. In a total 50 µl of solution was given per one electrode, let to incubate in wet box for 12 hours.
- The electrodes were rinsed by PBS and dried.
- A batch of magnetic nanoparticles with volume 5 ml and solid content 5 % was washed with PBS for three times and the particles were separated by an external magnet between each step. After the third washing, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride 20 µg/µl solved in PBS was added to magnetic particles previously isolated by magnet and the suspension was let to incubate for half of an hour. Finally, the activated nanoparticles were washed again with PBS.

- The magnetic particles were shaken and then applied in an amount 50 µl per one electrode of QCM and let to incubate in wet box for 12 hours.
- The prepared biosensors were rinsed with PBS and then dried under laboratory conditions.

2.3. Assay by biosensor

Biosensor was connected by an elastic cord with ICM Level Oscillator 10.000 MHz (ICM, Oklahoma City, OK, USA) and frequency counter UZ 2400 (Grundig, Nuremberg, Germany) and osculation frequency was measured. After that, a sample (solution of hemoglobin in PBS, pure PBS or tested interferents) sized 50 µl was spread over electrode and let to incubate for 10 minutes. After that, the biosensor was rinsed with by PBS with 0.1 % (w/w) Tween 20 followed by pure PBS and dried under laboratory conditions. Oscillation frequency was measured again and difference of oscillations Δf was calculated.

2.4. Validation assay

The biosensors were validated to standard Enzyme-Linked Immunosorbent Assay (ELISA). The assay was performed on standard 96-well microplates with flat bottom (MaxiSorp, Nunc, Roskilde, Denmark). In the first step, tested sample (the same as used for biosensor performance) in an amount 50 µl was applied per one well and let to incubate in a wet box for 12 hours. After the interval, the solution was sucked out and 100 µl of 0.1 % (w/v) gelatin was injected per one well and left for 2 hours incubation. The solution was sucked out and anti-human hemoglobin polyclonal antibody produced in rabbit and then goat produced anti-rabbit polyclonal antibody labelled with peroxidase were applied. The antibodies were mixed with PBS in a ratio: 8,000 as the manufacturer recommended, given in an amount 100 µl and left to incubate for 4 hours. The second antibody was applied after sucking out the first one. Finally, the antibodies were sucked out the wells were washed by PBS with 0.1 % (w/w) Tween 20 followed by pure PBS and o-phenylenediamine 0.5 mg/ml with hydrogen peroxide 5 mmol/l in total amount 100 µl was given per one well and let to incubate for five minutes. Sulfuric acid 2 mol/l in a volume 100 µl was given per one well and optical density at 450 nm was detected.

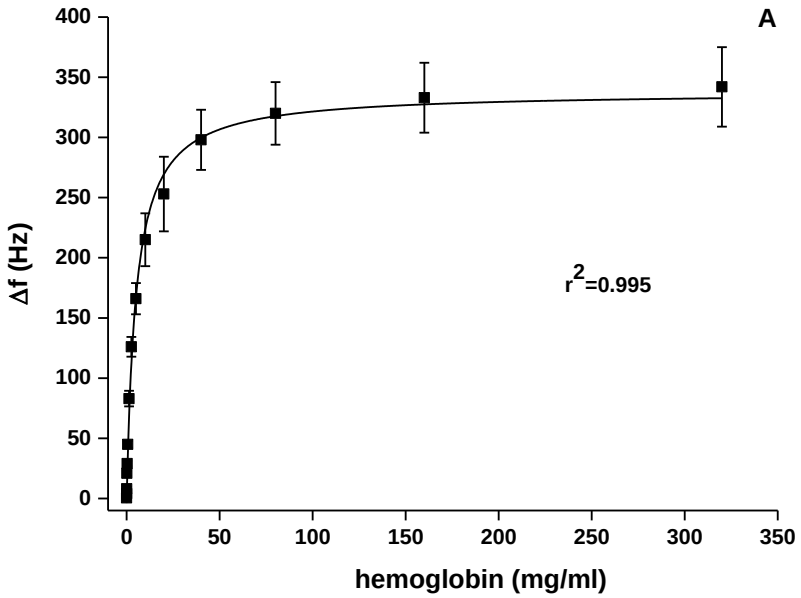
2.5. Statistics

The both biosensor and ELISA assays were performed in pentaplicate, standard deviation from the repeating measurements was calculated. Limit of detection was calculated according the rule that it is a point at calibration curve where signal to noise is equal to three (S/N = 3). Analysis of variance (ANOVA) on the probability levels 0.05 and 0.01 served for testing of statistical significance. Software Origin 9 (OriginLab Corporation, Northampton, MA, USA) served for data processing.

3. RESULTS AND DISCUSSION

Calibration scale of hemoglobin in PBS was tested by the both biosensor and ELISA method. The hemoglobin was prepared by two-fold serial dilution from the upper concentration 320 mg/ml. The individual concentrations for the calibration were equal to 0.0390, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00, 10.0, 20.0, 40.0, 80.0, 160 and 320 mg/ml and the scale of concentrations cower the both physiological ranges for plasma and blood hemoglobin levels. The calibration by biosensor is depicted as figure 2. In the figure 2, overall range of hemoglobin concentration and detailed range in the range up to 40 mg/ml where the biosensor is the most sensitive are depicted.

The results from the biosensor calibration and calibration of ELISA for the same samples were correlated mutually and the result is shown as figure 3. Regarding to the calibration, limit of detection was equal to 0.063 mg/ml and coefficient of determination r^2 was equal to 0.995. The limit of detection is deeply under expected physiological concentration of hemoglobin in blood and even in plasma [7]. The expected plasma concentration in heath individuals is around 0.120 mg/ml which is in the first third of exponential part of calibration curve. Because hemoglobin level increases in the plasma due to pathological processes up to several times, the assay by biosensor is good for the hemoglobin determination in plasma and it well cover expected ranges of hemoglobin that is typically under 0.3 mg/ml, moderately elevated level is up to 0.7 mg/ml and pathological processes are suspected when hemoglobin in plasma is above 0.7 mg/ml. Considering the biosensor sensitivity, the hemoglobin concentration 0.7 mg/ml is approximately equal to Δf signal 40 Hz. Though the assay appears well suitable for the determination of hemoglobin level in plasma, expected blood concentrations of hemoglobin (around 150 mg/ml) are in the flat part of calibration and blood samples should be diluted prior to use by this biosensor. The validation proved that the biosensor is fully comparable with the standard ELISA method but the assay is significantly simpler, faster and not demanding use of reagents or specific sample manipulation. These are the main advantages of the biosensor assay.



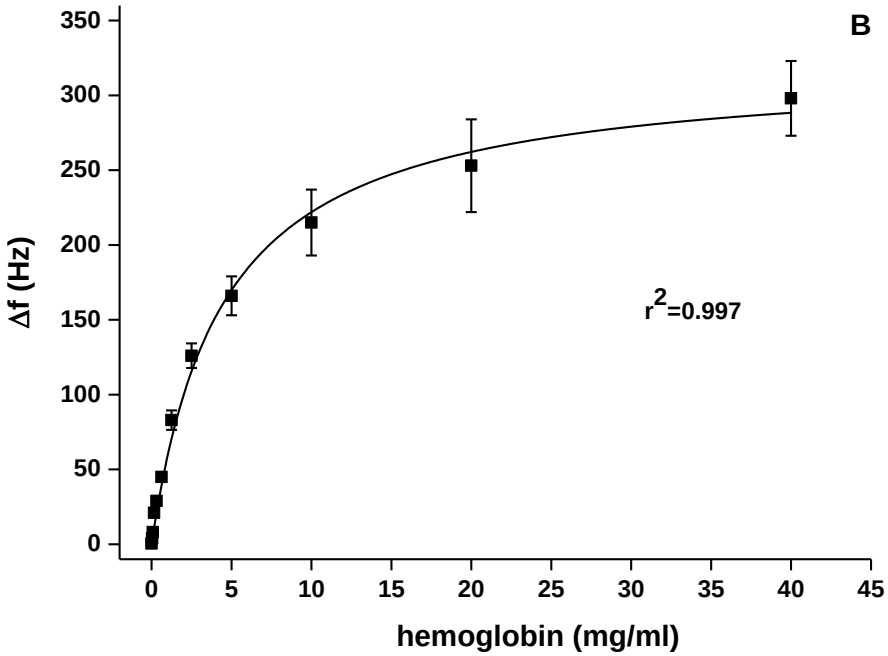


Figure 2. Calibration of QCM biosensor for hemoglobin assay for the range 0 – 320 mg/ml (part of the figure A) and 0 – 40 mg/ml (part of the figure B). Error bars indicate standard deviation for n = 5.

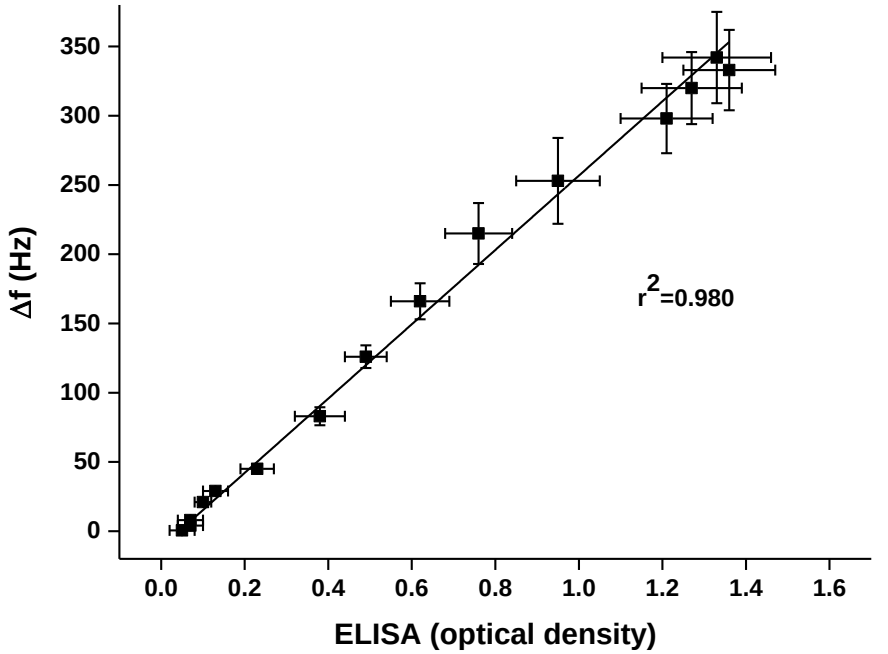


Figure 3. Validation of biosensor to ELISA method. The validation was achieved from calibrations of the samples containing hemoglobin in the concentrations 0.0390, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00, 10.0, 20.0, 40.0, 80.0, 160 and 320 mg/ml. Error bars indicate standard deviation for n = 5.

Human IgG and human serum albumin were tested as potential interferents because they occur as one of the most abundant plasma proteins [8,9]. Physiological concentration of IgG in plasma is approximately 7 – 16 mg/ml and it may exceed 70 mg/ml in the cases of hyperglobulinemia [10,11]. Physiological albumin plasma level is between 35 – 55 mg/ml and it decreases when liver damaged [12,13]. The both IgG and albumin were solved in PBS in concentrations 100 mg/ml which safely exceed physiological levels. The samples were processed the same way like the samples with hemoglobin and the results were compared with samples with hemoglobin above limit of detection for hemoglobin (in the calibration scale 0.0781 mg/ml and higher). The signal Δf was for the both IgG and albumin lower than 4 Hz and it was significantly (P = 0.01) lower than the signal for the signal for hemoglobin 0.0781mg/ml which was equal to 8.1±1.5 Hz.

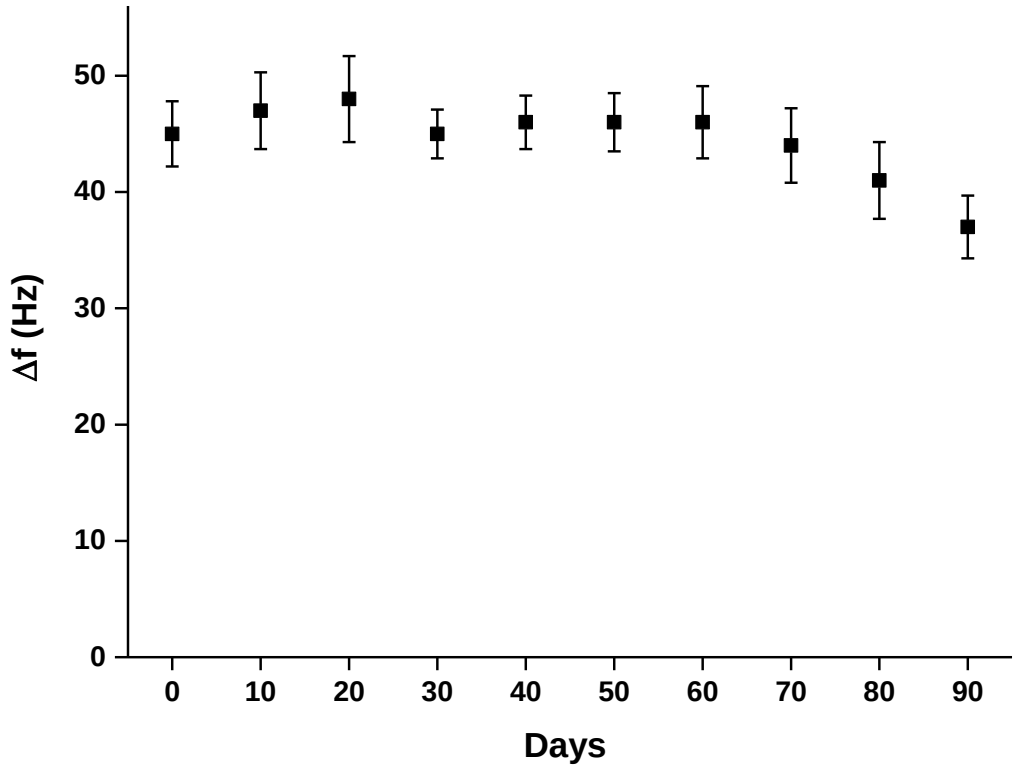


Figure 4. Biosensors long-term stability when sample containing hemoglobin 0.625 mg/ml analyzed. Error bars indicate standard deviation for n = 5.

The biosensors were also characterized for their long-term stability when stored in a dark box in laboratory conditions including room temperature (25 ºC). In a total 50 pieces of biosensors were prepared and five of them was used for analysis of hemoglobin 0.625 mg/ml the day of preparation and then every 10^{th} day. The biosensors were used only one time because repeated use is not supposed in clinical praxis due to low price of the biosensors and the fact that regeneration of biosensors for repeated use would be more expensive per one piece than the unit cost. Risk of false positive or negative results

would be also significantly increased. The finding about biosensor long-term stability can be learned from figure 4. It is obvious that the biosensor kept stability for at least 60 days. After that, mild decrease of sensitivity was observed and the outputting signal was 83 % of the initial one at the last interval of assay. The analytical parameters reached in this study including the limit of detection, long term stability and interference testing are summarized in table 1.

Table 1. Survey of the biosensor analytical parameters.

Limit of detection for hemoglobin	0.063 mg/ml
Drop of sensitivity after 60 / 90 days	not observed / 17 %
Coefficient of determination for calibration	r^2=0.995
IgG and albumin interference	no interference at concentration 100 mg/ml

Table 2. Comparison of methods for hemoglobin assay.

	Direct spectroscopy	ELISA	Biosensor
suitability for hemoglobin in blood	yes	yes	yes
suitability for free hemoglobin in plasma	no	yes	yes
portability, applicability outside laboratories	yes	no	yes
suitability to be performed without specific reagents	yes	no	yes

Piezoelectric biosensors are a suitable tool for a simple or even label free assays and they can be performed for various analytes with higher molecular weight typically above 1 kDa [14-19]. The biosensor described here is an original device and no comparable study on this issue was found in the

current literature. Standard analytical methods suitable for hemoglobin assay like immunochemistry and chromatography are based on other principles and they are significantly more elaborative and not suitable for a fast use outside from the standard laboratories. Direct spectroscopy of hemoglobin is an assay that can be performed in a label free mode and is also quite simple and have a broad use for blood assay but it is not sensitive enough to be applicable for plasma samples, the same can be told about non-invasive hemoglobin monitoring through skin that measure overall hemoglobin but are not able distinguish free hemoglobin and hemoglobin in erythrocytes [20-22]. The comparison of the biosensor based assay and standard methods is surveyed in table 2.

4. CONCLUSION

The described biosensor is a simple tool suitable for performance outside equipped laboratories. Plasma sample can be measured directly without any specific processing or adding of reagents which represents the major advantage comparing to the standard method. The assay by biosensor is readily for adaptation in clinical biochemistry praxis and it is highly competitive to the standard devices and protocols. Especially inexpensive QCM and used reagents, low price of measuring apparats (oscillation circuit and frequency counter) and no specific demands on staff because the assay does not process samples in an elaborative way. The limit of detection is low enough to cover all expected concentrations in plasma samples including the pathological one. Practical impact of findings presented here is expected.

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