Chromatographic methods for determination of neopterin in urine

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ABSTRACT:

Neopterin is considered to be a non-specific marker for the activation of the human immune system. It is synthesized by monocytes/macrophages in the immune response caused by T cells. The physiological role of neopterin and its derivatives is based on the modulation of macrophage cytotoxicity by enhancing the activity of reactive oxygen species under certain conditions. The concentration of neopterin is regarded as the evaluation parameter of oxidative stress immunologically induced. High concentrations of neopterin were observed in biological fluids of patients with bacterial infection, viral, autoimmune diseases and cancer and vascular diseases. At the proper neopterin elimination from the body, its concentrations in urine correlate to those in the serum or plasma. Measuring the concentration of neopterin is important in monitoring of the course of immunological activity in disease. In special cases, the determination of neopterin concentration was used in the differential diagnosis. This paper describes various methods for determining neopterin in urine primarily by high performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA). HPLC techniques are predominant due to the possibility of modifying the chromatographic system and other parameters. The combination of these methods may be useful in developing new analytical procedures for determining neopterin in urine.

Chromatograficzne metody oznaczania neopteryny w moczu

Słowa kluczowe: neopteryna, pterydyny, chromatografia cieczowa, ELISA

STRESZCZENIE:

Neopteryna, uważana za niespecyficzny marker aktywacji ludzkiego układu odpornościowego, syntezowana jest przez monocyty/makrofagi w odpowiedzi immunologicznej wywołanej przez limfocyty T. Fizjologiczna rola neopteryny oraz jej pochodnych polega na modulowaniu cytotoksyczności makrofagów poprzez zwiększanie aktywności reaktywnych form tlenu w określonych warunkach. Stężenie neopteryny jest uznawane za parametr oceny stresu oksydacyjnego wywołanego stanem zapalnym, a jej wysokie stężenia odnotowano m.in. w płynach biologicznych osób z infekcją bakteryjną, wirusową, chorobami autoimmunologicznymi i nowotworowymi oraz z chorobami układu krwionośnego. Przy prawidłowej eliminacji neopteryny z organizmu jej stężenie w moczu jest skorelowane z oznaczanymi w surowicy lub w osoczu. Oznaczanie stężenia neopteryny ma istotne znaczenie w monitorowaniu aktywności immunologicznej wielu chorób. W szczególnych przypadkach oszacowanie jej stężenia w materiale biologicznym znalazło zastosowanie w diagnostyce różnicowej.
W pracy zostały opisane różne metody oznaczania neopteryny w moczu, głównie z zastosowaniem wysokosprawnej chromatografii cieczowej (HPLC) i kompetycyjnego testu immunoenzymatycznego (ELISA). Dominują głównie techniki chromatograficzne (HPLC), ze względu na możliwość modyfikacji układu chromatograficznego oraz innych parametrów. Zestawienie tych metod może być pomocne w opracowaniu nowych procedur analitycznych oznaczania neopteryny w moczu.

The pterins are substances from the group of compounds known as the pteridines, which play an important role in the biochemical synthesis of vitamins and cofactors, for example riboflavin or tetrahydrobiopterin. The pteridines are divided into two groups of compounds: the pterins with 2-amino-4-oxo structure and lumazines with 2,4-dioxo structure. In addition, the pterins group can be divided into two subgroups, those with a complex structure (coupled with p-aminobenzoate or glutamate) and those with the simple structure (non-conjugated), characterized by an aliphatic chain in the 6 or 7 position of the ring. Neopterin, which is 2-amino-4--hydroxy-6-(1,2,3-dihydroxypropyl) pteridine, belongs to non-conjugated pterins, characterized by a bicyclic structure consist of linked pyrimidine and pyrazine rings (Fig. 1) [1-3].

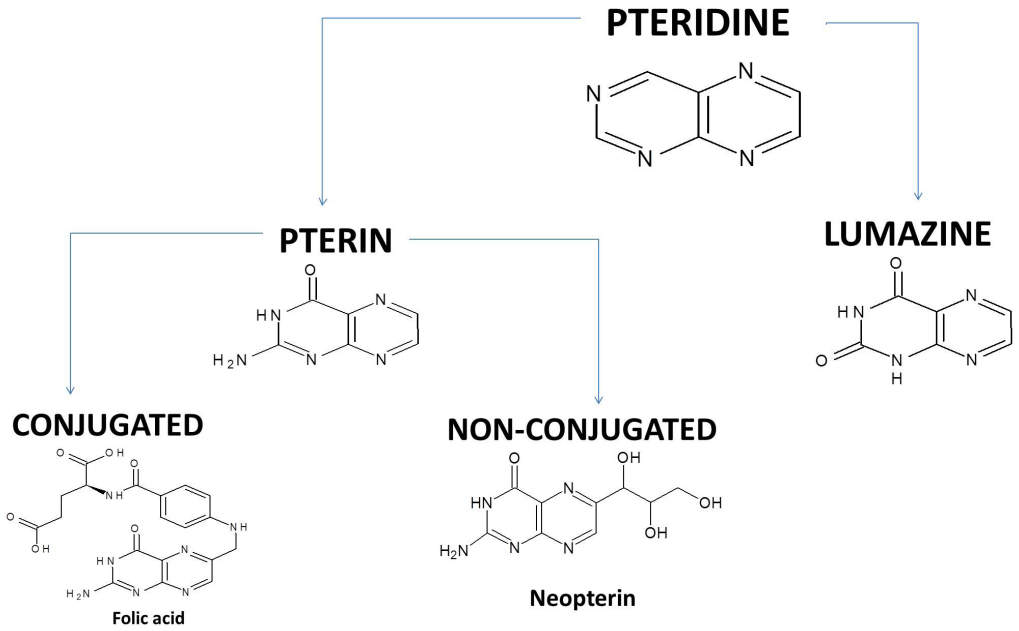

Figure 1. The pteridins division due to the ring structure [1]

It has been shown from the earlier published data, that pteridines have already been isolated from butterfly wings in 1889, but they received their name in 1936. The name „pteridine" comes from the Greek word „pteron" which means „wing" and is linked to the place of occurrence of this substance as a dye. Neopterin has been isolated in the 1960s from larvae and adult bees and royal jelly. Initially, it was called „novapterin" to emphasize the fact of obtaining a new molecule from honeybees belongs to the pteridines group. Finally, it has given the prefix „neo" to signify the start of a new era of discoveries in the study of this class of compounds. In 1967, 25 mg of neopterin was isolated from 500 liters of human urine, and relatively elevated levels in urine was detected in mice with Ehrlich carcinoma in later years. Then, a similar situation was observed in patients urine with malignancies. In 1981, for the first time thesis about the immune origin of neopterin was appeared [2, 4].

Currently neopterin is considered to be one of the markers of cellular immune response. Elevated concentrations of neopterin due to various pathogens has been detected in serum, plasma, urine, cerebrospinal fluid, saliva, pancreatic and gastric juice. A viral and bacterial infections, infections caused by intracellular parasites, autoimmune diseases, malignant tumors, inflammation after transplant rejection are states related with increased activity of macrophages [5].

Lymphocytes being a basic of specific immune response by a contact with an antigen secrete cytokines also including interferon γ. Stimulated by interferon γ, monocytes/macrophages produce neopterin. Biosynthesis of neopterin is started with the guanosine triphosphate (GTP) conversion into the 7,8-dihydroneopterin triphosphate influenced by GTP-cyclohydrolase-I. In contrast to other cells such as fibroblasts and endothelial cells, human monocytes/macrophages contain relatively small amounts of 6-pyruvoyl-tetrahydropterin synthase (PTPS). PTPS enables the transformation of 7,8-dihydroneopterin triphosphate to 5,6,7,8-tetrahydrobiopterin (BH4). For this reason, it is possible to detect only biopterin derivatives in the supernatants obtained from cells other than monocytes/macrophages. Instead of BH4 producing, macrophages/monocytes accumulate 7,8-dihydroneopterin triphosphate which synthesize neopterin after dephosphorylation and oxidation. It is assumed that the main stimulator for the neopterin production is interferon γ (Fig. 2) [1-8].

During the disease process the concentration of neopterin is closely associated with the running of these disease. In the laboratory diagnosis the monitoring of neopterin in body fluids is used to

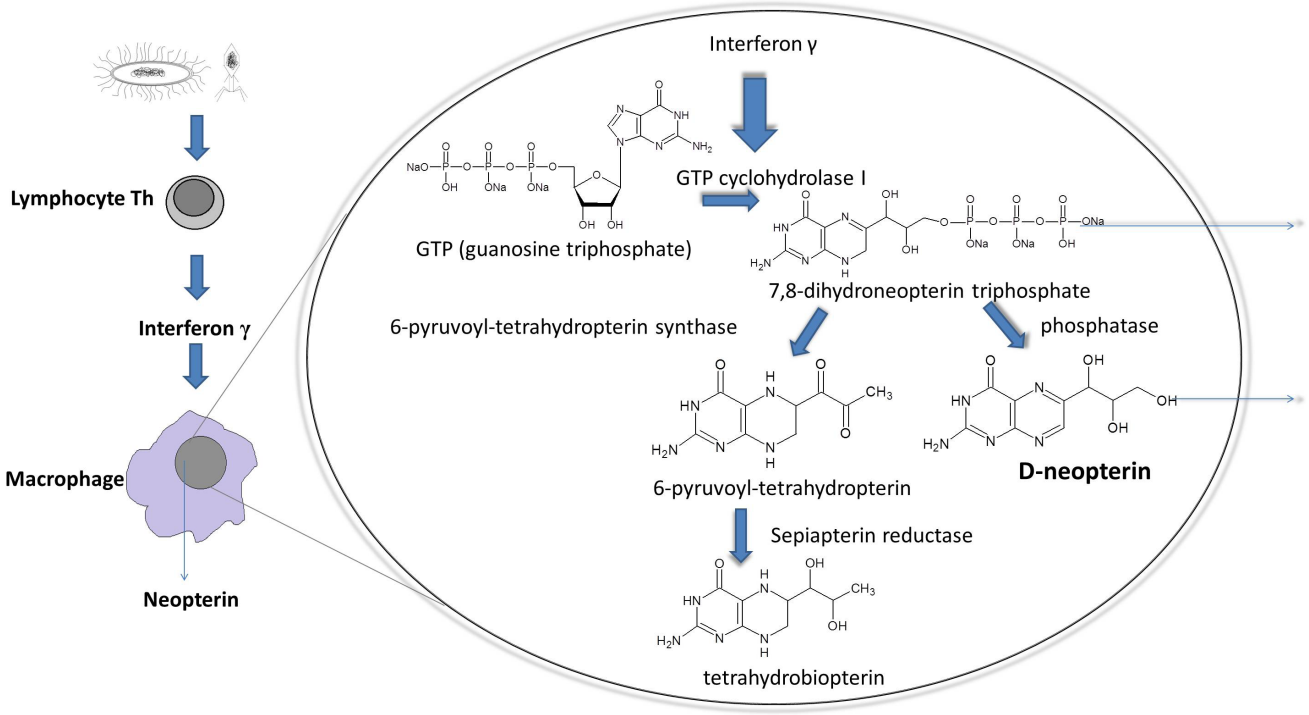

Figure 2 Synthesis of neopterin [1, 5, 9]

assess the involvement of the human immune system during inflammation, and as a parameter showing the progress of the disease. Neopterin is chemically stable in body fluids and it can be excreted by glomerular filtration. Changes of neopterin concentration in urine are correlated with changes in serum levels unless the renal failure occur [3-5, 9-11].

1. PHYSICOCHEMICAL PROPERTIES

Neopterin is slightly soluble in water, whereas is well dissolved in dimethyl sulfoxide (DMSO). In the water environment neopterin behaves as a weak acid. Because of its sensitivity to sunlight, especially in the UV-A range, it should be keep out of the light. Due to aromatic structure neopterin has fluorescent properties which allows it to be detected by spectrophotometric techniques. The optimum wavelength at which the excitation of fluorescence occur is 353 nm. Frequently, the maximum emission band of radiation is reported as a wavelength of 438 nm. Neopterin is usually determined in conversion to the creatinine concentration unit, a commonly used parameter of kidney function.

Neopterin and 7,8-dihydroneopterin which is released simultaneously in the immune response, due to the structural similarity have similar chromatographic properties and UV spectra. Consequently, using techniques of absorption it is possible to easily detect the total amount of both pterins. However, if neopterin in body fluids is chemically stable and suitable for routine laboratory analysis, but its reduced derivative in the presence of oxygen is readily degradable. More stringent criteria for sample preparation and for determination 7,8-dihydroneopterin limit its usefulness for clinical diagnosis [12-14].

2. CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF NEOPTERIN

Methods for determining the pteridines are generally multi-stepping and time-consuming, thus their use on a large scale is limited. Primarily, immunological techniques require small amounts of samples, but their compilation for neopterin is expensive and affected by the phenomenon of cross-reactivity. Other analytical methods are based largely on the chromatographic techniques which they usually require sample preparation. Among the published methods dominate procedures using high performance liquid chromatography with fluorescence and UV detectors.

To one of these methods has been developed by Tomandl et al., which uses a multi-stepping process of sample preparation comprising among others: hydrolysis of urine, the solid phase extraction procedure (SPE) with a Separon SGX C18 columns, elution by a mixture of ammonium phosphate (pH = 6) and acetonitrile (100:5, v/v), evaporation under nitrogen atmosphere and dissolving the sample in 0.1 ml of water. The chromatographic analysis was performed on a column Purospher RP-18 (250 x 4 mm, 5 µm) and pre-column (4 x 4 mm) with the same stationary phase. The mobile phase was phosphate buffer pH = 3 (solvent A) and a mixture of phosphate buffer pH = 3 with acetonitrile (100:15, v/v) (solvent B). The separation of the analytes was performed using a gradient program with 0.8 ml/min flow rate. Neopterin was determined by the fluorescence detector (λ_{em}= 450 nm, λ_{ex}= 355 nm). The authors of this method by optimizing the SPE procedure obtained satisfactory recovery of neopterin 99.5 ± 2.5%. Simultaneously, they consider these method suitable for routine measurements of neopterin in urine [15].

Llanos et al., pay attention to the fact that the pteridines determination is difficult because of their sensitivity to light, low solubility and low concentration in biological fluids. The authors point out that the pteridines excreted into urine occur in a different oxidation state and only fully oxidized derivatives have good fluorescent properties. They use the method which based on the oxidation of the urine samples by solutions of I_2/I^- and $KMnO_4$ to indicate the ratio of neopterin to biopterin (NEO/BIO) and neopterin to creatinine (NEO/CREA). Biopterin is the oxidized form of 7,8-dihydrobiopterin (BH2) and 5,6,7,8-tetrahydrobiopterin (BH4) – important indicators of biological activity. In both cases, the pH of the samples was adjusted to 6.1. The best conversion pteridines to completely oxidized forms was obtained with I_2/I^- solution at 9.5 x 10^6 mol/l for 40 minutes and with $KMnO_4$ solution at 10^3 mol/l for 10 minutes. The samples were diluted in buffer tris(hydroxymethyl) aminomethane/hydrochloric acid (Tris/HCl, pH = 6.1). The chromatographic analysis was performed using a Zorbax Eclipse XDB-C18 column (250 x 4.6 mm, 5 µm) and mobile phase consisting

of 15 mmol/l Tris/HCl buffer at pH 6.1 (solvent A) and 15 mmol/l Tris/HCl buffer at pH 6.4 (solvent B). Separation of the mixture components was performed using gradient with 1.0 ml/min flow rate. The analytes were determined by a diode-array detector (DAD) (λ = 230 nm) and fluorescence detector (λ_{em} = 445 nm, λ_{ex} = 272 nm). The NEO/CREA and NEO/BIO ratios largely depend on physicochemical conditions of the performed oxidation process. Oxidation method by means solution of I_2/I^- gives relatively lower the NEO/BIO ratio, because in this case BH4 is oxidized mainly to pterin, not to biopterin [16].

The method developed by Jimenez Giron et al. limits the urine sample pretreatment to filtration through 0.2 µm nylon membrane, adding 0.1% solution of dithiothreitol (DTT) and 10-fold dilution with ultrapure water. Studies were performed using a high performance liquid chromatography equipped with a Zorbax Eclipse XDB-C18 column (250 x 4.6 mm, 5 µm). The mobile phase was aqueous solution of 0.1% formic acid and 2% acetonitrile (solvent A) and 0.1% acetonitrile solution of formic acid (solvent B). Gradient program was used, and the flow rate was set at 0.6 ml/min. Neopterin was determined using a mass spectrometer equipped with a single quadrupole. Electrospray ionization (ESI) with formation of positive ions mode was applied. Nitrogen was used as atomizing gas. The identification and the quantitative determination was performed in selected ion monitoring mode (SIM). The results of this study clearly indicate the advantage of 7,8-dihydroneopterin in compare to neopterin in urine. Sum of the concentrations of both forms to the creatinine concentration obtained by proposed method turned out to be larger than those obtained by initial oxidation. The main advantage of this method is its uncomplicated procedure and low consumption of organic solvents [17].

The method of determination neopterin in urine proposed by de Castro et al. uses a HPLC apparatus with UV detection. The urine samples were filtered through paper and diluted 100-fold with water. The chromatographic analysis was performed using an isocratic elution, and two joined reverse phase columns: 300 Aquapore OD (250 x 4.6 mm, 5 µm) and LiChrospher 100 RP 18 (4 x 4 mm, 5 µm). The mobile phase was 150 mM phosphate buffer at pH 4.0. The flow rate was 0.8 ml/min and the total run time was 20 min. Wavelength of detection was set on 353 nm. According to the authors opinion the 100-fold dilution and acidification of the mobile phase allowed to obtain good chromatographic profile, reduce interferences and time of the analysis. This method allowed to obtain an acceptable recovery in the range of 79.5 to 82%, accuracy 12% and a linear range between 100 and 1000 ng/ml [18].

Pterins are relatively polar substances, and therefore they are determinated generally on the reverse phase columns. According to Kośliński et al. alternative separation technique of pterins is hydrophilic interaction chromatography (HILIC). The authors tested three types of chromatographic columns and performed the optimization analysis of the selected conditions. Satisfying separation of analytes obtained with a column Luna HILIC (150 x 4.6 mm, 5 µm) and LiChrospher C8 60 RP select B (250 x 4.0 mm, 5 µm). The urine samples in which pH has been stabilized to value 7 by phosphate buffer were prepared in two ways: by treating them with I_2/I^- solution and $KMnO_4$ solution. The authors noticed that a long time oxidation samples by $KMnO_4$ may lead to lower signal of some pterins as a result of the degradation of the aromatic ring and consequently weakening the fluorescent properties. Influence of the mobile phase organic component to the retention time of each analyte was determined. They checked on tested columns the effect of the organic component of the mobile phase to the retention time of each pterin. The percentage of acetonitrile or methanol in ammonium acetate solution (pH 6, 10 mM) was modified. Using a HILIC column, higher acetonitrile content in the mobile phase prolonged pterins retention time, but also improved the selectivity ratio peaks. On the other hand higher methanol content did not cause better separation of the peaks on this column. They also examined the pH effect of mobile phase (in the range of 4 to 7) for the separation of analytes. For this purpose the pH of the eluent was changed by adding salt buffer: 10 mM ammonium acetate and 10mM Tris/NaCl. The best results for pterins separation on HILIC column Luna was obtained at pH = 6.5. For a LiChrospher C8 column pH = 7 was optimum. The authors also optimized the ionic strength of the eluent by changing the addition of suitable buffer salts. For most of the analyzed pterins including neopterin, the enhance of the ionic strength caused a slight and irregular increasing retention time. An expla-

nation of this relationship for hydrophilic interaction chromatography can be fact that the higher salt concentration allows to increase the volume of a liquid layer immobilized on the stationary phase surface. However, this hypothetical model has not been confirmed by direct experimental evidence. The detection was carried out at an emission wavelength of 444 nm and an excitation wavelength of 280 nm. Based on the obtained results, the authors for better method of sample preparation admit these using the alkaline I_2/I^- solution. The developed method allowed analysis of urine samples from patients with bladder cancer. The results of study indicate the possibility of correlation between pterins and the stage of tumor progression [19].

Tomsíková et al. developed chromatographic method for determination of neopterin, biopterin and their reduced derivatives. Ultra-high performance liquid chromatography (UHPLC) coupled with fluorescence detector (λ_{em} = 438 nm, λ_{ex} = 353 nm) and UV detector (λ = 253 nm) was used as analysis apparatus. According to the authors, the conventional determination method using reverse phase liquid chromatography causes problems in the form of a lack of retention times reproducibility or the obtaining asymmetric peaks. Therefore, increasingly ion chromatography and hydrophilic interaction chromatography for this purpose are used. The separation of pterins was performed on a BEH Amide column (100 x 2.1 mm, 1.7 um). But the preparation method of biological samples, according to the authors, carried out by only filtration and dilution. It was a fast and simple sample preparation stage, but did not guarantee a good selectivity of analytes separation. That is very interesting way of the urine samples purification method proposed by Tomsíková et al., takes into account solid phase extraction. Before the (SPE) procedure, urine samples were diluted 20-fold in 1% aqueous solution of DTT. The reduced form had the best stability at pH = 6.8, but the lowest loss of neopterin up to 24 h was observed in an acidic medium at pH = 3.8. As a SPE sorbent authors have chosen a mixed phase containing C8 with a group of cation exchange (DSC-MCAX). The eluent was 75% aqueous solution of acetonitrile containing the 1% (w/v) ammonia solution of DTT. The eluent has been chosen primarily because of its superior compatibility to mobile phase which was a mixture of acetonitrile and 50 mM ammonium acetate pH = 6.8 (85:15, v/v). Compatibility of the two mixtures allowed to skip the evaporation step and inject directly the sample into a camera apparatus after the SPE procedure. The developed method allowed to obtain recovery of neopterin in the range of 91,4-121,9%, 0,9-8,4% precission and the limit of quantification equal to 1 ng/ml [20].

The separation results obtained using planar chromatography (TLC – Thin Layer Chromatography) were much worse, but this technique used for the initial qualitative and semi-quantitative determinations of pterins is limited and still work on the use of two dimensional-2D TLC analysis of neopterin and biopterin as well as their reduced derivatives [21]. The mobile phase was a mixture of solvents (ethyl acetate : isopropanol : 25% NH_4OH) in a volume ratio of 3:4:3 as a result of the modification of mobile phase used by Tomsova et al. and the chromatographic plates with silica gel 60 F_{254}[22].

A common method for determining of neopterin in biological samples, especially in serum and urine to a lesser extent, serving for the research and diagnostic purposes is enzyme-linked immunosorbent assay (ELISA). The main advantages of using commercially available ELISA test is the ability to perform multiple measurements of concentrations in a short time, simple procedure not requiring large sample volume and high sensitivity and precision of this method. In a study conducted by Westermann et al. have been evaluated ELISA test delivered by IBL GmbH. The test was started from mixing 100 µl of urine with 200 µl dilution buffer. The resulting solution was incubated for 10 min. at temp. 95 to 100°C. The suspension was centrifuged and then introduced into microplate wells 10 µl of test solution, 100 µl of the enzyme conjugate and 200 µl of neopterin antibody, respectively. Microplate was shaken for 90 min. at room temperature at 500 rpm. The wells were washed with buffer and next 200 µl chromogenic substrate was added. Incubations were carried out at room temperature for 10 min., after which the enzymatic reaction was stopped for addition of a suitable stop reagent. The optical density was measured at 450 nm for 1 h. The sensitivity of the method developed for urine samples was comparable with the analytical sensitivity 0.18 ng/ml, obtained for serum samples. According to the authors, the described method because of its

simplicity, short time of implementation, good repeatability and accuracy is suitable for clinical analysis and empirical study [23].

3. SUMMARY

The determination of neopterin in urine has not only special predictive value for the course and progression of many diseases, but also may provide important information about efficacy of treatment therapy. Difficulties in the analysis of neopterin and other pterins are mainly due to its low content in urine. The most widely used techniques for determining the concentrations in urine are chromatographic techniques, particularly those using a fluorescence detector, as well as immunochemical techniques based on the commercially available tests. Immunoassay techniques do not require sample preparation, simultaneously with little effort and time they guarantee high repeatability. However, measurements by immunoassay techniques are subject to interference associated with the cross-reactivity of the antibodies used in relation to other pterins contained in biological material. Chromatographic methods with sample preparation step, which are often applied by the type of a chromatographic column and most often a fluorescence detector require larger volumes of biological material. An overview of the published articles was primarily due to describe and demonstrate different variants of neopterin determination in urine. Summary of the above-described methods of neopterin concentration measurement may be useful in study of biological material and also can provide a basis for the development of new, more advanced and sophisticated analytical procedures for determining this important biochemical parameter.

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