UDC 557.161.2+612.017.1:616-097 doi: 10.15407/biotech9.02.028

# DEVELOPMENT AND VALIDATION OF IMMUNOENZYME TEST-SYSTEM FOR DETERMINATION OF 25-HYDROXYVITAMIN D IN BLOOD SERUM

*A. O. Mazanova*
*I. O. Shymanskyy*
*M. M. Veliky*

Palladin Institute of Biochemistry of NAS of Ukraine, Kyiv

*E-mail: ann.mazanova@gmail.com*

Received 18.02.2016

The present study was aimed at designing of immunoenzyme test-system for competitive determination of 25OHD in blood serum using polyclonal antibodies against 25OHD and biotin-streptavidin visualization technique. The validation parameters of test-system were determined. For development of test-system we used the preparation of specific antibodies against 25OHD with the titer 1:3000. The optimal concentration of competing agent (25OHD$_3$-LC-biotin) was found to be equal to 5 ng/ml. The standard calibration curve was built by using a set of seven calibrators. For the validation of test-system the following parameters were established: limit of detection, quantitative limit, cross-reactivity, intra- and inter-system coefficients of variation (Intra.CV, Inter.CV) and “matrix effect”. Constructed immunoenzyme test-system can be used successfully for 25OHD determination in blood serum samples.

***Key words:*** 25-hydroxyvitamin D$_3$, immunoenzyme analysis, polyclonal antibodies.

Vitamin D is a general name for a group of biologically active substances, the most important of which are cholecalciferol (vitamin D$_3$) and ergocalciferol (vitamin D$_2$). Chemically vitamin D belongs to secosteroids that are similar to steroid molecules except for the absence of a double bond between C$_9$ and C$_{10}$ carbon atoms in the ring B [1]. In humans, vitamin D$_3$ is formed from 7-dyhydrocholesterol by the action of UV radiation (290–315 nm). After photobiochemical synthesis, cholecalciferol binds to vitamin D-binding protein (DBP) and is transported to the liver for further conversion to 25-hydroxyvitamin D$_3$ (25OHD$_3$) [2].

Cholecalciferol, through its hormonally active form — 1,25(OH)$_2$D$_3$ (calcitriol), has a number of different pleiotropic effects in the organism, such as the regulation of calcium homeostasis, mineralization and remodeling of bone tissue, proliferation and differentiation of various cell types as well as modulation of the immune processes. Receptors for 1,25(OH)$_2$D$_3$ — VDR, and all the components of the D-endocrine system (enzymes that metabolize vitamin D$_3$), are found in various cell types. Decrease in bioavailability of vitamin D$_3$ due to insufficient dietary intake or deterioration of its metabolism in the body is known to be a major factor in the pathogenesis of osteoporosis. In recent years, large-scale epidemiological studies have revealed a correlation between vitamin D$_3$ deficiency and prevalence of infectious (viral infections, tuberculosis) [3–5], chronic inflammation (Crohn’s disease) [6] allergic (asthma) [7], autoimmune (multiple sclerosis, type 1 diabetes) [4, 8, 9], cardiovascular and neoplastic diseases [10–14].

The classic marker of cholecalciferol sufficiency is 25OHD level in the blood because its half-life is about 2–3 weeks and the serological samples (serum or plasma) can be stored frozen for a long period until analyzed [1].

25OHD level in the blood within 100–150 nmol/L (60–80 ng/ml) is considered to be physiologically normal. Its reduction to 50 nmol/L indicates vitamin D insufficiency, and to 20 nmol/L — vitamin D deficiency [12].

The measurement of 25OHD is challenging due to lipophilic nature of the compound that is bound to proteins in the blood and contained in nanomolar concentrations. Currently, there are several methods for 25OHD determination [15]: radioimmunoassay [16, 17], high performance liquid chromatography (HPLC), liquid chromatography combined with mass spectrometry (HPLC/LC-MS), different versions of immunoenzyme analysis using poly- or monoclonal antibodies against 25OHD or DBP [18].

The first immunological test systems were based on the usage of DBP and radiolabeled $^{3}$H-25OHD. Further use of antibodies against DBP was inappropriate, because such test systems also determined another metabolite of vitamin D — 24,25-dihydroxy D, thereby leading to false-positive results [19]. Therefore, radio competitive analysis has been developed based on antibodies against 25OHD using $^{125}$I-labeled 25OHD$_3$ [17].

HPLC/LC-MS is currently considered to be the most accurate method for 25OHD determination in serum samples. This method can identify all metabolites of vitamin D, including 25-hydroxyvitamin D. HPLC involves lipid extraction of vitamin D from blood sample, further chromatographic separation and UV-detection at 254–256 nm [20]. Despite the fact that HPLC is the “gold standard” of 25OHD measurement, it is not widely used in laboratory practice because of the complexity of sample preparation and expensive equipment.

In view of the aforementioned, the urgent task of biotechnology is to develop domestic test system that would allow reliable, rapid and inexpensive determination of 25OHD content in different serological samples.

The aim of the present study was to design the immunoenzyme test system for 25OHD measurement in serological samples by competitive method using biotin-streptavidin visualization technique and rabbit polyclonal antibodies against 25OHD [21]. To achieve this goal the following tasks were put forward: to optimize sorption conditions of rabbit polyclonal antibodies on the surface of polystyrene plates; to compare different regimes of competitive assay and determine conditions to achieve specificity; to build a standard calibration curve and validate the immunoenzyme test system by determining sensitivity of the system, limit of detection, quantitative limit, coefficients of variation (intra- and intersystem), cross-reactivity and “matrix effects”.

## Materials and Methods

*Salting out the fraction of serum IgG with ammonium sulfate ($(NH_4)_2SO_4$)*

Immunization of gray female rabbits was performed by using 25OHD$_3$-KLH conjugate as described previously [21]. Blood serum containing specific antibodies against 25OHD was centrifuged at 3 000 g for 30 min at temperature + 4 °C. Supernatant was transferred to a clean tube. The necessary amount of saturated ammonium sulfate solution (4.1 M at + 25 °C) was added to a final concentration of 50% and kept overnight at + 4 °C. The precipitate was centrifuged for 30 min at 3 000 g. Supernatant was discarded but the remaining 1 ml of supernatant above the obtained residue was left to prevent drying. Antibodies were stored at + 4 °C [22].

*Purification of the IgG fraction precipitate by dialysis*

Before the procedure, dialysis bag with pore diameter of 6 mm and MWCO (molecular weight cutoff) — 14 000 Da (Sigma, USA) was boiled 3–5 min in 100 mM EDTA solution. After drying, 0.5 ml of the precipitate was placed in the bag. The first phase of the dialysis was performed against phosphate buffer solution (PBS, pH 7.4) during 3 hours at +4 °C. The buffer solution was replaced with the fresh one and dialyzed overnight at +4 °C. After the third replacement of the buffer solution and the dialysis during 3 h, phosphate buffer solution was changed with 50% glycerol in PBS. Immunoglobulins were dialyzed three times with the substitution of glycerol solution in regime overnight-three hours-overnight at + 4 °C. The resulting protein concentration was measured and antibodies were stored at −20 °C.

*Assessment of antibody titer against 25OHD by indirect immunoenzyme analysis (ELISA)*

25OHD$_3$-ovalbumin conjugate, dissolved in phosphate buffer (PBS, pH 7.4; 1 mg/100 ml), was placed into polystyrene 96-well flat-bottomed plates (Grainer Microlon®) and incubated overnight at +4 °C [21]. After incubation the plate wells were washed three times with 200–300 μl of Tween-phosphate buffer (PBST, PBS + 0.1% Tween 20). Thereafter, the wells were washed after each round of incubation by the same scheme. Free binding sites were blocked by adding to each well 300 μl of PBST and incubated for 1 h at + 37 °C.

To assess the titer of serum antibody the dilutions were made in the range of 1:500–1:64000. 100 μl of each serum dilution were placed in two parallel wells and incubated during 1 h at + 37 °C. Then, secondary antibodies, conjugated with horseradish peroxidase (1:3 000, 100 ml per well; Sigma, USA), were added and incubated for 1 hour at + 37 °C. Measurement of the specific "antigen-antibody" interaction was conducted by color reaction using ABTS [2,2′-azino-bis- (3-ethylbenzothiazoline-6-sulfonic-acid) diammonium salt; Sigma, USA]. 100 μl of stock solution, contained 2.5 mg of ABTS in 5 ml Na-citrate buffer (50 mM, pH 5.0) and 5 μl of 30% $H_2O_2$, was added per well. Detection was performed on the reader ER-500 (BioRad) at the wavelength of 405 nm within 30 min.

To control the specificity of the immune response parallel analysis of control sera was conducted. In addition, for cross-reactivity monitoring the unconjugated keyhole limpet hemocyanin from *Megatura cranulata* (KLH) and white egg albumin (OVA) were used.

*Preparation of the components of immunoenzyme test system for 25OHD determination*

25OHD$_3$-LC-biotin (ImmunDiagnostik, Germany) was used as a competing agent for the immunoenzyme analysis. It was diluted in 98% ethanol to a final concentration of 1 mg per 0.5 ml of ethanol.

The standard calibrators of 25OHD$_3$ were prepared from the stock solution, which contained 1 mg 25OHD$_3$ (Sigma, USA) in 1 ml of 98% ethanol.

As cross-reactants, 1,25(OH)$_2$D$_3$ and cholecalciferol (Sigma, USA) were used. The stock solutions contained 50 ng of 1,25(OH)$_2$D$_3$ in 1 ml of 96% ethanol and 1 mg of vitamin D$_3$ in 0.5 ml of 96% ethanol.

## Results and Discussion

Determination of 25OHD in blood serum is an important task for the diagnosis of vitamin D insufficiency/deficiency in the body or, rarely, vitamin D intoxication [23]. Immunoenzyme test systems allow determine rapidly and reliably the contents of various low-molecular biologically active compounds in the diagnostic or biochemical laboratory. The development of immunoenzymatic kits for 25OHD determination is accompanied by a number of difficulties, such as follow:

– 25OHD is hydrophobic compound, which makes it impossible to work with aqueous solutions;

– lipophilic nature of the compound suggests that 25-hydroxyvitamin D is bound to proteins, in particular vitamin D-binding protein (DBP);

– antibodies raised against 25OHD can bind different metabolites of vitamin D, 1,25(OH)$_2$D, 24,25(OH)$_2$D, 3-epi-25D, thereby leading to false positive/negative results;

– total level of vitamin D comprises two components: vitamin D$_3$ and vitamin D$_2$. Therefore, a test system should be developed to provide the measurement of these two metabolites [18].

Because of undesirability of using radioactively labeled 25OHD as a competitor, there have been developed a number of test systems based on rabbit polyclonal, and after the invention of hybrid technologies — monoclonal antibodies against 25OHD. We have chosen rabbit polyclonal antibodies for the detection 25-hydroxyvitamin D. Previously we have synthesized immunoconjugate, carried out immunization of rabbits, received antiserum and characterized polyclonal antibodies against 25OHD [21].

The procedure of salting out of globulin fraction was conducted at the first stage of this work. For this purpose, a saturated solution of ammonium sulfate was used. Immunoglobulins obtained were dialyzed against PBS, pH 7.4 and stored in 50% glycerol solution at −20 °C.

After salting out and purification, the protein concentration in the resulting suspension was 24 mg/ml. Indirect immunoenzyme analysis was then performed to evaluate the specific antibody titer against 25OHD. Fig. 1 shows that after purification procedure, the antibodies against 25OHD were able to detect reliably this compound. It was also shown that they do not exhibit cross reactivity with protein carrier of comparison conjugate — ovalbumin.

Thus, these results suggest that obtained polyclonal antibodies can be successfully used to construct immunoenzyme test-systems for 25OHD determination.

The next phase of the study was to select and optimize the conditions of antibodies sorption on the surface of polystyrene plates.

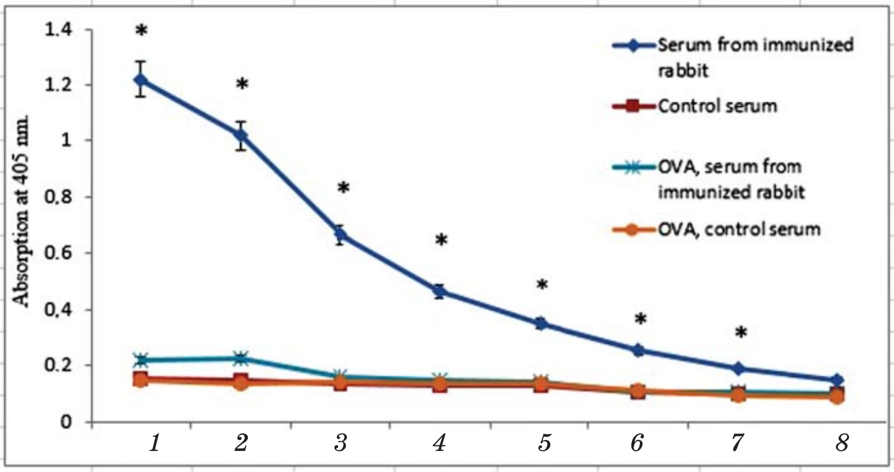


*Fig. 1.* **Antibodies titer against 25OHD from blood serum of rabbits, immunized with 25OHD$_3$-KLH conjugate, after salting out and purification by dialysis in dilution series:**
*1* — 1/500; *2* — 1/1 000; *3* — 1/2 000; *4* — 1/4 000; *5* — 1/8 000;
*6* — 1/16 000; *7* — 1/32 000; *8* — 1/64 000;
* $P < 0.05$ vs. control

For this purpose, the Grainer Micrlon® plates with a sorption capacity of 600 ng/cm$^2$ were used.

Purified antibodies were dissolved in PBS, pH 7.4 to a final concentration of 10 μg/ml and transferred 100 μl per well [24]. The plate was incubated overnight at + 4 °C. [25]. The wells were washed three times using a Tween-phosphate buffer pH 7.4 (PBST, PBS + Tween 20, 0.05% , 200 μl per well).

During immunoenzyme analysis it is important to block free binding sites after antibodies sorption on the surface of polystyrene. Several types of blocking solution are widely used nowadays [26]:

1. Phosphate buffer containing 1–5% of bovine serum albumin (BSA). Working with this blocking solution can lead to unwanted cross-reactivity of polyclonal antibodies with BSA. Moreover, the permanent use of BSA is expensive that will not reduce the cost of the developed test system.

2. Phosphate buffer containing skim milk at a concentration of 0.1–0.5% . This solution is unsuitable for those test systems, which are based on biotin-avidin/streptavidin binding.

3. Buffer system containing casein. This solution is also unusable for systems, in which the reaction of biotin-avidin/streptavidin coupling is utilized.

4. 5–10% solution of normal serum. The main disadvantage of this solution is the presence of blocking IgG, which may reduce the specific signal.

5. Phosphate buffer containing Tween 20 at a concentration of 0.05–0.1% .

After analyzing the characteristics of different buffer systems, we have chosen Twin phosphate buffer as it contains no additional components that may cross-react with antibodies in the system, and it is not expensive as well. That is why, 300 μl of PBS + Tween 20, 0.05% , were added to each well to block free binding sites. The plate was incubated for 1 hour at + 37 °C.

For our immunoenzyme test system a competitive version of ELISA was selected. It is based on competing between 25OHD of serum sample with 25OHD$_3$-LC-biotin added to each well. A pair of avidin (egg protein)/ biotin (water-soluble vitamin) is widely used in ELISA test systems. Avidin is tetrameric protein that contains four identical subunits, each of which can bind one molecule of biotin. The biotin-avidin couple withstands high salt concentration in incubation solution, sharp fluctuations in pH or the presence of high concentrations of such chaotropic agents as guanidine hydrochloride in the medium. In addition, biotin molecule can be effectively conjugated with proteins as well as with other molecules, including 25OHD$_3$, while avidin molecule easily forms conjugates with enzymes such as horseradish peroxidase (HRP) [27].

In recent years, streptavidin, the protein obtained from *Streptomyces avidinii*, is increasingly used instead of avidin. Streptavidin was shown to be less sensitive to pH changes and it can be obtained not only from natural producers, but also by recombinant technique. Compared with the first one, recombinant streptavidin has smaller size and isoelectric point within a neutral pH 6.8–7.5.

Therefore, a specific interaction between competitor conjugate, composed of biotin ($25OHD_3$-LC-biotin), and streptavidin coupled to horseradish peroxidase was used for signal detection in the present study. Horseradish peroxidase enzyme is the most suitable candidate for the signal detection in ELISA systems due to its high specificity and the possibility to use different substrates: OPD (*o*-phenylenediamine dihydrochloride), TMB (3,3′,5,5′-tetramethylbenzidine), ABTS, etc. [28].

Once the antibodies were adsorbed on the polystyrene plate and free binding sites were blocked as described above, 200 µl of $25OHD_3$-LC-biotin solution (in PBS), containing 200, 400, 600, 800, 1 000, 1 500 pg, were added to the wells [29]. Figure 2 shows the presence of the linear section of the curve in the concentration of $25OHD_3$-LC-biotin ranging from 200 to 1 000 pg/200 µl. This reflects proportional dependence of immune specific interaction on the accessibility of free antibody binding sites that were completely filled at concentrations of biotinylated $25OHD_3$ higher than 1 000 pg/ 200ml (saturation curve plateau). Thus, we have subsequently used $25OHD_3$-LC-biotin at a concentration of 1 000 pg/200 µl as a competing agent for the immunoenzyme system.

For the validation of any test system a number of standard parameters should be used, such as [30]: 1) selectivity; 2) cross-reactivity; 3) sensitivity; 4) stability; 5) reproducibility; 6) construction of the calibration curve.

Calibration (standard) curve shows the intensity of signal which depends on the concentration of analyte in the incubation medium. This dependence should be reproducible and uninterrupted.

The samples for the building of calibration curve were prepared in the same buffer as other reagents. For each measurement with the usage of 96-well plate the separate calibration curve was built. The standard calibration curve for the determination of 25OHD is a graph of the extinction (Y-axis) and the concentration of the calibrator $25OHD_3$ (X-axis); or the dependence of $B/B_0$ (Y-axis) on the concentration of the calibrator $25OHD_3$ (X-axis). The value $B/B_0$ is calculated as (extinction of calibrator (sample)/extinction of zero calibrator $C_0$)×100% . In addition, there is another way to build calibration curve, when the values of both the X-axis and the Y-axis are expressed logarithmically. Such approach is useful to reach maximum linearization of the standard curve, especially in the range of low concentrations of the calibrators. While this method makes it possible to assess the linearity of the calibration curve, however, it is rather inconvenient for further calculations of the measurement results [31].

There are several standard characteristics of the system, which can be estimated on the basis of calibration curve: 1) detection limit (relative sensitivity of the system); 2) quantitative limit; 3) linearity; 4) range of concentrations that may be measured by the imminoenzyme test system.

Taking into consideration the fact that the normal concentration of 25OHD in blood serum is 60–80 ng/ml (100–150 nmol/L) the following concentrations of this metabolite were selected to build the calibration curve: 1.25, 2.5, 10, 35, 70 and 150 ng/ml [2].

Fig. 3 depicts the dependence of extinction on the analyte concentration in the sample as well as a standard calibration curve, which exhibits a relationship between the concentration of the competitor in the sample and the ratio $B/B_0$. This relationship has

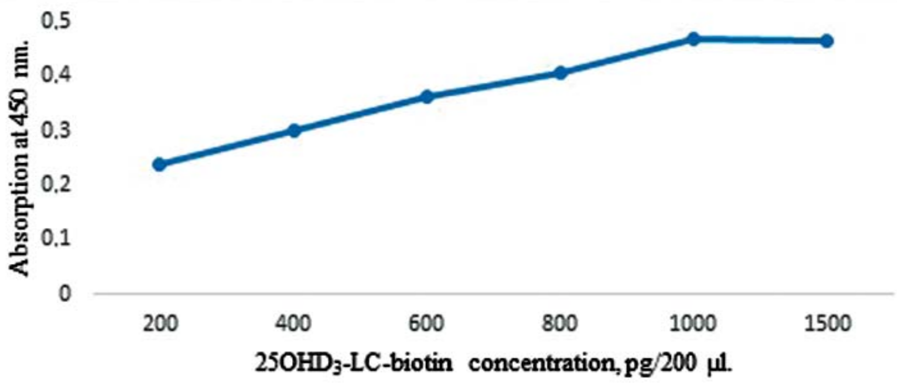


*Fig. 2.* **Selection of the optimal concentration of competing agent $25OHD_3$-LC-biotin**

| Concentration of $25OHD_3$ calibrator, ng/ml | Adsorption at 450 nm |
|---|---|
| 0 | 1.790 |
| 1.25 | 1.450 |
| 2.5 | 1.089 |
| 10 | 0.920 |
| 35 | 0.752 |
| 70 | 0.567 |
| 150 | 0.340 |

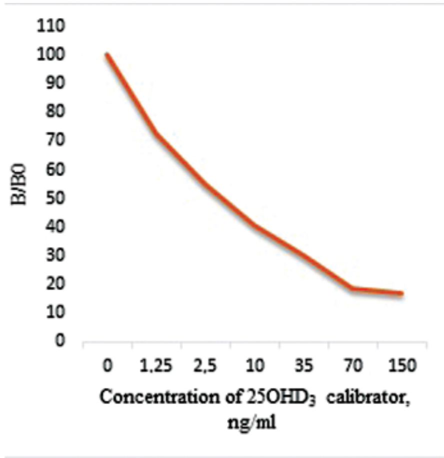


*Fig. 3.* **Standard calibration curve for 25OHD determination**

an inverse character, which is expressed in reducing the value of extinction with increasing number of competing agent in the well.

The limit of detection was determined by the formula: DL = 3.3 s/a, where s –standard deviation (SD) values of zero calibrator; a — the slope of the standard curve to the axis X.

SD- 0.170465; a = 0.2456; DL = 2.3 ng/ml.

The quantitative limit was determined by the formula: QL = 10s/a, where s — standard deviation (SD) values of zero calibrator; a — slope to the axis X.

SD- 0.170465; a = 0.2456; QL = 6.9 ng/ml.

One of the important parameters for the validation of immunoenzyme test system is the lack of "matrix effect" [32]. "Matrix effect" is an impact of sample components (serum or plasma) on the measurements. This parameter is defined as the difference between the extinctions of a sample diluted in a standard buffer solution (calibrator) and a sample of serum or plasma. It is known that serum contains a variety of proteins that can block the binding sites in the immunoglobulin molecules, thus leading to erroneous measurements. Additionally, the "matrix effects" may be manifested as increasing "noise" in the measurements.

Serial dilutions of major blood protein fractions are usually carried out to determine the "matrix effect". In this study we tested the effect of hemoglobin and bilirubin on the efficiency of 25OHD measurements using developed immunoenzyme system [33].

The normal concentration of hemoglobin in the blood is: for men — 120–180 g/l; for women — 110–160 g/l; for children — 110–160 g/l; for pregnant women — 95–150 g/l. However, for non-hemolyzed specimens that must be used in immunoenzyme analysis, the upper limit of the free hemoglobin concentration is 0.5 g/l. We have selected a range of hemoglobin concentrations 0.04–5 g/l to be tested. It was shown that hemoglobin in the concentration of 0.04 g/l does not inhibit a signal, while it reduces extinction value by 30% in the concentration of 5 g/l.

As for bilirubin, its normal concentration in the blood does not exceed 0.01 g/l (for total fraction). For testing we have selected a range of bilirubin concentrations 0,07–10 g/l. It was shown that the presence of 0.07 g/l of bilirubin in the incubation medium does not affect a signal strength and only significant (500-fold) increase in bilirubin concentration, up to 5 g/l, reduced the extinction by 50%.

Thus, we can conclude the absence of "matrix effect" elicited by both hemoglobin and bilirubin if non-hemolyzed serum samples are used.

Three types of most common errors are known to be associated with the immunoenzyme measurements: random errors or, in other words, a variation of measurement. They are expressed as standard deviation, relative error or coefficient of variation and calculated by the formula: RSD (relative standard error) = CV (coefficient of variation) = SD/mean 100%; system errors, which occurrence depends on the accuracy of analysis procedures; gross errors that result from equipment malfunction or denaturation of reagents.

If in the case of gross and systematic errors there are no reliable parameters of

*Table 1.* **Intra-system coefficient of variation for the serum samples of normal rats**

| | Control 1 | Control 2 | Control 3 | Control 4 | Control 5 |
|---|---|---|---|---|---|
| Extinction 1 | 0.508 | 0.601 | 0.668 | 0.657 | 0.657 |
| Extinction 2 | 0.600 | 0.493 | 0.796 | 0.639 | 0.701 |
| Extinction 3 | 0.554 | 0.552 | 0.734 | 0.648 | 0.69 |
| Medium mean | 0.554 | 0.549 | 0.732 | 0.648 | 0.682 |
| SD | 0.03 | 0.04 | 0.05 | 0.007 | 0.018 |
| Intra.CV | 5% | 7% | 7% | 1% | 2% |

their characteristics and correction of these errors strongly depends on the accuracy of the researcher and the maintenance of the equipment and reagents in working condition, the random errors can be successfully characterized by calculating the coefficients of variation (CV).

According to the guidelines of the International Conference of Harmonization (ICH, Japan, US and EU) related to optimization and validation of ELISA test kits, there are two parameters characterized by the coefficient of variation [31]:

1. Frequency or intra-system coefficient of variation (Intra.CV);
2. Reproducibility or inter-system coefficient of variation (Inter.CV).

For our analysis, we obtained serum from normal male Wistar rats. Table 1 shows that the frequency or Intra.CV for five samples is within the range from 1% to 7%, while the maximum allowable value is 10%. Inter-system coefficient of variation (Inter.CV) was no more than 7% (data not shown).

In addition to 25-Hydroxyvitamin $D_3$ other metabolites of cholecalciferol, such as $25OHD_2$, $1,25(OH)_2D$, $24,25(OH)_2D$ and vitamin D itself are present in serological samples [18]. In view of this, to validate the immunoenzyme system we conducted series of tests for cross-reactivity of antibodies against 25OHD with cholecalciferol and $1,25(OH)_2D_3$ (calcitriol) used in the concentration range of 5–800 ng/ml and 25–1 000 pg/ml respectively. Table 2 demonstrates the percentage of cross-reactivity of polyclonal antibodies against 25-Hydroxyvitamin D with the aforementioned compounds.

It was shown that rabbit polyclonal antibodies, which were used in the developed immunoenzyme test system to determine the concentration of 25OHD in serum samples, hardly reacted with cholecalciferol, while cross-reactivity to calcitriol reached the value of 10%. It is an acceptable threshold that cannot make any significant impact on the analysis because calcitriol in blood serum appears within picomolar concentrations, whereas 25OHD is detected in nanograms.

*Table 2.* **Percentage of the immunoenzyme system cross-reactivity with different metabolites of vitamin $D_3$**

| Analyte | Percentage of cross-reactivity |
|---|---|
| Cholecalciferol ($D_3$) | 2.5% |
| 25-Hydroxyvitamin $D_3$ | 100% |
| $1,25(OH)_2D_3$ | 10% |

Thus, in this experimental research we optimized the conditions of rabbit polyclonal anti-25OHD antibodies sorption on the surface of polystyrene plate. After comparing different modes of competition reaction, optimal concentration of a competing agent — 25-hydroxyvitamin $D_3$-LC-biotin was found to be 1 000 pg/200 µl. The developed immunoenzyme test system was validated by the following parameters: the limit of 25OHD detection (2.5 ng/ml); quantitative limit that was equal to 6.9 ng/ml of 25OHD and coefficients of variation (Intra.CV-5-7%, Inter.CV-7%). It was shown that the immunoenzyme test system has no "matrix effect" to hemoglobin and bilirubin provided using non-hemolyzed specimens. In addition, it was determined that the reaction of rabbit polyclonal antibodies with other metabolites of vitamin $D_3$ is not significant, that exclude false positive/negative results during the analysis.

## REFERENCES


1. *Carter G.* Accuracy of 25-hydroxyvitamin D assays: confronting the issues. *Cu. Drug targets*. 2011, V. 12, P. 19–28. doi: 10.2174/138945011793591608.
2. *Holick M.* The vitamin D deficiency pandemic and consequences for nonskeletal health: Mechanisms of action. *Mol. Asp. Med.* 2008, 29 (6), 361–368. doi: 10.1016/j.mam.2008.08.008.
3. *White J. H.* Regulation of intracrine production of 1,25-dihydroxyvitamin D and its role in innate immune defense against infection. *Arch. Biochem. Biophys.* 2012, 523 (1), 58–63. doi: 10.1016/j.abb.2011.11.006.
4. *Li H., Xie H., Fu M., Li W., Guo B., Ding Y., Wang Q.* 25-hydroxyvitamin $D_3$ ameliorates periodontitis by modulating the expression of inflammation-associated factors in diabetic mice. *Steroids*. 2012, 78 (2), 115–120. doi: 10.1016/j.steroids.2012.10.015.
5. *Barchetta I., Carotti S., Labbadia G., Gentilucci U. V., Muda A. O., Angelico F., Silecchia G., Leonetti F., Fraioli A., Picardi A., Morini S., Cavallo M. G.* Liver vitamin D receptor, CYP2R1, and CYP27A1 expression: relationship with liver histology and vitamin $D_3$ levels in patients with nonalcoholic steatohepatitis or hepatitis C virus. *Hepatology*. 2012, 56 (6), 2180–2187. doi: 10.1002/hep.25930.
6. *Cantorna M. T.* Vitamin D, multiple sclerosis and inflammatory bowel disease. *Arch. Biochem. Biophys*. 2012, 523 (1), 103–106. doi: 10.1016/j.abb.2011.11.001.
7. *Luong Kv., Nguyen L. T.* The role of vitamin D in asthma. *Pulm. Pharmacol. Ther.* 2012, 25 (2), 137–143.
8. *Takiishi T., Gysemans C., Bouillon R., Mathieu C.* Vitamin D and diabetes. *Rheum. Dis. Clin. North Am*. 2012, 38 (1), 179–206. doi: 10.1016/j.rdc.2012.03.015.
9. *Stivelman E., Retnakaran R.* Role of vitamin D in the pathophysiology and treatment of type 2 diabetes. *Curr. Diabetes Rev*. 2012, 8 (1), 42–47.
10. *Bennett R. G., Wakeley S. E., Hamel F. G., High R. R., Korch C., Goldner W. S.* Gene expression of vitamin D metabolic enzymes at baseline and in response to vitamin D treatment in thyroid cancer cell lines. *Oncology*. 2012, 83 (5), 264–272.
11. *Fleet J. C., DeSmet M., Johnson R., Li Y.* Vitamin D and cancer: a review of molecular mechanisms. *Biochem J*. 2012, 441 (1), 61–76. doi: 10.1042/BJ20110744.
12. *Seggese G., Vierucci F., Boot A. M., Czech-Kowalska J., Weber G., Camargo Jr C. A, Mallet E., Fanos M., Shaw N. J., Holick M. F.* Vitamin D in childhood and adolescence: an expert position statement. *Eur. J. Pediatr*. 2015, 174 (5), 565–576. doi: 10.1007/s00431-015-2524-6.
13. *Muscogiuri G., Mitri J., Mathieu Ch., Badenhoop K., Tamer G., Orio F., Mezza T., Vieth R., Colao A., Pittas A.* Vitamin D as a potential contributor in endocrine health and disease. *Eur. J. Endocrinol*. 2014, V. 171, P. 101–110. doi: 10.1530/EJE-14-0158.
14. *Hossein-nezhad A., Holick M. F.* Vitamin D for Health: A Global Perspective. *Mayo Clin. Proc*. 2013, 88 (7), 720–755. doi: 10.1016/j.mayocp.2013.05.011.
15. *He Ch., Gleeson M., Fraser W. D.* Measurement of circulating 25-hydroxy vitamin d using three commercial enzyme-linked immunosorbent assay kits with comparison to liquid chromatography: tandem mass spectrometry method. *ISRN Nutr*. 2013, V. 2013, P. 1–7. doi: 10.5402/2013/723139.
16. *Hollis B. W., Napoli J. L.* Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. *Clin. Chem*. 1985, 31 (11), 1815–1819.
17. *Wootton A. M.* Improving the Measurement of 25-hydroxyvitamin D. *Clin. Biochem. Rev*. 2005, 26 (1), 33–36.
18. *Zerwekh J. E.* Blood biomarkers of vitamin D status. *Am. J. Clin. Nutr*. 2008, V. 87, P. 1087–1091.
19. *Holick M. F.* Vitamin D status: measurement, interpretation, and clinical application. *Ann. Epidemiol*. 2009, 19 (2), 73–78. doi: 10.1016/j.annepidem.2007.12.001.
20. *Bauman V. K.* Biochemistry and physiology of vitamin D. *Riga*. 1989, 324 p. (In Russian).
21. *Mazanova A. O., Shymanskyy I. O., Petukhov D. M., Drobot L. B., Veliky M. M., Komisarenko S. V.* Synthesis of 25-hydroxovitamin $D_3$ conjugate with keyhole limpet hemocianin and obtaining of immune sera. *Biotechnol acta*. 2015, 8 (3), 45–55. doi: 10.15407/biotech8.03.045. (In Ukrainian).
22. *Harlow E., Lane D.* Antibodies. A laboratory manual. *Cold Spring Harbor Laboratory*. 1988, 726 p.
23. *Hossein-nezhad A., Holick M. F.* Vitamin D for Health: A Global Perspective. *Mayo Clin. Proc*. 2013, 88 (7), 720–755. doi:10.1016/j.mayocp.2013.05.011;1.
24. ELISA sample preparation guide Available at: http://www.abcam.com/?pageconfig=resource&rid=14666.
25. ELISA Development Guide Available at: http://www.origene.com/assets/documents/antibody_lysate/ELISA Protocol.pdf\npapers2://publication/uuid/8D251E08-B97E-4757-AD67-AFC91E2C9F5A\npapers2://publication/uuid/A0188924-E7E4-4817-B8AF-F777D9B2339E.
26. *Daven S.* Technical Guide for ELISA — Protocols — Troubleshooting. *KPL Inc*. 2009, 40 p.

27. *Crowther J. R.* The ELISA Guidebook. *N. J.: Humana Press.* 2001, 446 p.
28. Thermo Scientific Pierce Protein Assay Technical Handbook Version 2 Table of Contents. *U. S.: Thermo scientific.* 2010, 42 p.
29. *Holick M. F., Ray R.* Labeled vitamin D compounds and the use thereof. U.S. *Patent* 2002/ 0107411 A1, August 8, 2002.
30. *Booth B., Kadavil J.* Draft Guidance for Industry Bioanalytical Method Validation. *U. S.: Biopharmaceutics.* 2013, 34 p.
31. *Wakabayashi K.* ELISA -A to Z. Available at: http://www.sartorius-stedim.com.tw/Attachment/FCKeditor/Product/file/PDF/lab/filter&membran/filter.pdf\npapers2://publication/uuid/39532C5D-CAEB-429E-82C6-81FCA27 (accessed 2008).
32. Calculating and evaluation ELISA data Available at: http://www.abcam.com/protocols/calculating-and-evaluating-elisa-data.
33. *Wallace A. M., Gibson S., de la Hunty A., Lamberg-Allardt C., Ashwell M.* Measurement of 25-hydroxyvitamin D in the clinical laboratory: Current procedures, performance characteristics and limitations. *Steroids.* 2010, 75 (7), 477–488. doi: 10.1016/j.steroids.2010.02.012.


## РОЗРОБЛЕННЯ ТА ВАЛІДАЦІЯ ІМУНОЕНЗИМНОЇ ТЕСТ-СИСТЕМИ ДЛЯ ВИЗНАЧЕННЯ 25-ГІДРОКСИВІТАМІНУ D У СЕРОЛОГІЧНИХ ЗРАЗКАХ


*А. О. Мазанова*
*І. О. Шиманський*
*М. М. Великий*

Інститут біохімії ім. О.В. Палладіна НАН України, Київ

*E-mail: ann.mazanova@gmail.com*



Метою роботи було розроблення імуноензимної тест-системи для конкурентного визначення 25OHD у серологічних зразках з використанням поліклональних антитіл проти 25OHD і біотин-стрептавідинового способу візуалізації. Для розроблення тест-системи було використано специфічні антитіла проти 25OHD з титром 1:3000. Встановлено оптимальну концентрацію конкуруючого агента ($25OHD_3$-LC-біотину), яка становила 5 нг/мл, та побудовано стандартну калібрувальну криву з використанням набору із семи калібраторів. Для валідації тест-набору було визначено низку стандартних параметрів: ліміт детектування, кількісний ліміт, крос-реактивність, внутрішньо- та міжсистемний коефіцієнти варіації (Intra.CV, Inter.CV), «матричний ефект». Отримані результати показують, що створену тест-систему можна успішно використовувати для визначення 25OHD у серологічних зразках.



***Ключові слова:*** 25-гідроксивітамін $D_3$, імуноензимний аналіз, поліклональні антитіла.


## РАЗРАБОТКА И ВАЛИДАЦИЯ ИММУНОЭНЗИМНОЙ ТЕСТ-СИСТЕМЫ ДЛЯ ОПРЕДЕЛЕНИЯ 25-ГИДРОКСИВИТАМИНА D В СЕРОЛОГИЧЕСКИХ ОБРАЗЦАХ


*А. А. Мазанова*
*И. А. Шиманский*
*Н. Н. Великий*

Институт биохимии им. А.В. Палладина НАН Украины, Киев

*E-mail: ann.mazanova@gmail.com*



Целью работы была разработка иммуноэнзимной тест-системы для конкурентного определения 25OHD в серологических образцах с использованием поликлональных антител против 25OHD и биотин-стрептавидинового способа визуализации. Для разработки тест-системы были использованы специфические антитела против 25OHD с титром 1: 3000. Подобрана оптимальная концентрация конкурирующего агента ($25OHD_3$-LC-биотина), которая составляла 5 нг/мл, и построена стандартная калибровочная кривая с использованием набора из семи калибраторов. Для валидации тест-набора был определен ряд стандартных параметров: лимит детектирования, количественный лимит, кросс-реактивность, внутри- и межсистемный коэффициенты вариации (Intra.CV, Inter.CV), «матричный эффект». Полученные результаты показывают, что созданная тест-система может успешно использоваться для определения 25OHD в серологических образцах.



***Ключевые слова:*** 25-гидроксивитамин $D_3$, иммуноэнзимный анализ, поликлональные антитела.