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HPLC-UV Method Development and Validation for Vitamin D_3 (Cholecalciferol) Quantitationin Drugs and Dietary Supplements

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Abstract

Introduction. An inadequate diet and living in the northern regions can lead to a lack of vitamin D_3 and the development of diseases, including a decrease in immunity. To compensate for the lack of vitamin D, vitamin drugs are used that contain vitamin D in one of its active forms (usually in the form of cholecalciferol, vitamin D_3).

Aim. To develop and validate HPLC-UV method for the determination of vitamin D_3 in vitamin drugs and to evaluate the content of cholecalciferol in selected drugs anddietary supplements presented in the Russian Federation.

Materials and methods. Determination of vitamin D_3 was carried out by HPLC with UV detection at a wavelength 266 nm. Sample preparation of vitamin drugs was carried out by extraction with methanol (for liquid dosage forms based on aqueous or triglyceride solutions) and extraction with an aqueous-methanol solution (for solid dosage forms based on water-soluble substances with vitamin D_3) in a ratio of 2 to 8 (water-methanol).

Results and discussions. The analysis methodology for the parameter "Vitamin D_3 (cholecalciferol) content" in vitamin dosage forms by HPLC was validated according to the following validation parameters: specificity; accuracy; precision; linearity; range.

Conclusion. The analysis methodology for the parameter "Vitamin D_3 (cholecalciferol) content" in vitamin dosage forms by HPLC was developed. The method was validated according to the following validation parameters: specificity; accuracy; precision; linearity; range. The range of the method was 9,5–38 μg/ml. The method was used to determine vitamin D_3 in vitamin drugs based on water-soluble forms of vitamin D_3, in the form of aqueous solutions and form of fatty acids triglyceridessolutions.

Keywords: vitamin D_3, cholecalciferol, HPLC, drugs, dietary supplements, validation

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Maria N.Bogachuk, Maria A. Paleeva, Yuri V. Medvedev developed and validated a method for the determination of vitamin D_3 and carried out a study of samples. Stanislav A. Kulakov, Igor E. Shokhin, Evgeniya A. Malashenko carried out statistical processing and interpretation of the results. All authors participated in the discussion of the results and the writing of the text of the article.

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Разработка, валидация и применение методики количественного определения витамина D_3 (холекальциферола) методом ВЭЖХ с УФ-детектированием для анализа лекарственных средств и биологически активных добавок к пище

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Резюме

Введение. Недостаточный рацион питания и проживание в северных регионах могут способствовать развитию дефицита витамина D и, как следствие, ряда заболеваний, приводящих к снижению иммунитета. В связи с этим уже достаточно долгое время применяются витаминные лекарственные препараты, содержащие витамин D в одной из его активных форм (чаще, в виде холекальциферола, витамина D_3).

Цель. Разработать и валидировать методику определения витамина D_3 в витаминных ЛП методом ВЭЖХ с диодноматричным детектированием, с последующим определением содержания холекальциферола в ряде лекарственных средств и биологически активных добавок к пище (БАД к пище), обращающихся на рынке РФ.
Материалы и методы. Определение витамина D_3 проводили методом ВЭЖХ с УФ-детектированием при длине волны 266 нм. Пробоподготовка витаминных лекарственных препаратов заключалась в экстракции метанолом (для жидких ЛФ на основе водных растворов и растворов триглицеридов) и водно-метанолным раствором (2 : 8 вода – метанол) – для твердых лекарственных форм на основе водорастворимых субстанций с витамином D_3.
Результаты и обсуждения. Была проведена валидация методики показателя «Количественное определение витамина D_3 (холекальциферола)» в витаминных препаратах методом ВЭЖХ по следующим параметрам: специфичность, правильность, линейность, диапазон применения, прецизионность.
Заключение. Разработана методика определения показателя «Содержание витамина D_3 (холекальциферола)» в витаминных лекарственных формах методом ВЭЖХ. Методика была валидирована по следующим валидационным параметрам: специфичность, правильность, линейность, диапазон применения, прецизионность. Показано, что результаты валидации удовлетворительны по всем указанным критериям. Диапазон применения методики 9,5–38 мкг/мл. Данная методика была использована для определения витамина D_3 в витаминных лекарственных препаратах на основе водорастворимых субстанций витамина D_3 в виде водных растворов и растворов триглицеридов жирных кислот.

Ключевые слова: витамин D_3, холекальциферол, ВЭЖХ, лекарственные средства, БАД, валидация

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. М. Н. Богачук, М. А. Палеева, Ю. В. Медведев разработали и валидировали методику определения витамина D_3 и провели исследование образцов. С. А. Кулаков, И. Е. Шохин, Е. А. Малашенко проводили статистическую обработку и интерпретацию результатов. Все авторы принимали участие в обсуждении результатов и написании текста статьи.

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INTRODUCTION

Vitamin D at the modern level of biochemistry development does not belong to vitamin in the classic understanding of the concept. It is considered as the steroid pro-hormone which is converted in the body to active metabolites [1, 2]. It is known that vitamin D have two delivery pathways to the human body (vitamins D_2 and D_3), and as the result of the synthesis in the skin exposed to the ultraviolet irradiation (vitamin D_3). Poor diet and life in northern regions may promote vitamin D deficiency and, as the result, some diseases leading to the immune deficiency. Due to that, vitamin products containing vitamin D in one of its active forms have been long used (more often, as cholecalciferol, vitamin D_3) (figure 1) [3].

Vitamin D_3 is contained in various dosage forms, drug products and dietary supplements as water soluble substances (for example, vitamin D_3 100 SD/S by DSM Nutritional Products Ltd., Switzerland) or fat soluble forms dissolved in clean or water-alcohol solutions or vegetative oil/fish fat [4]. The Russian market of vitamin products containing D_3 has rapidly expanded. It is especially evident on the example on vitamin dietary supplements including the ones with cholecalciferol [5]. Moreover, manufacturers

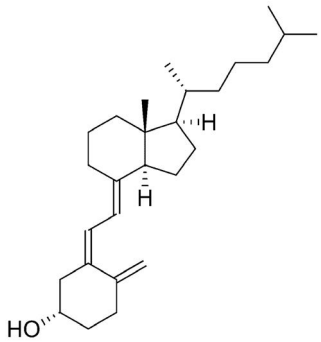

Figure 1. Structural formula of vitamin D_3 (cholecalciferol)

of specialized food products strive to keep up with the time, and the number of products enriched with D_3 is also developing.

Cholecalciferol is rather unstable in the light and the medium rich in oxygen. Due to that, multistage sample preparation may promote its destruction and isomerization to sterins [1, 6–8]. The classic approach for such objects is described in GOST 32043-2012. Samples are prepared by saponification with aqueous-alcohol solution of potassium hydroxide, extraction – with hexane and

further determination of vitamin D (in forms D_2 and D_3) – with the method of normal phase chromatography [9].

As water soluble substances and pure or aqueous-alcohol solutions of triglycerides are used for manufacture of vitamin products containing vitamin D, then to reduce vitamin D on the stage of sample preparation, we skip saponification stage starting immediately with cholecalciferol with the corresponding extractant. Moreover, the reversed phase system is used for vitamin D_3 chromatography D_3 [4].

The present study shows the development, validation of the method for cholecalciferol assay in monovitamin products (tablet and liquid dosage forms) and the use of the method for quantification of cholecalciferol in some drug products and biologically active dietary supplements (food supplements) circulating on the market of the Russian Federation.

Study objective. To develop and validate a method for determination of vitamin D_3 in vitamin products with HPLC combined with diode array detection, then to evaluate cholecalciferol contents with the method in some drug products and biologically active dietary supplements (food supplements) circulating on the market of the Russian Federation.

MATERIALS AND METHODS

Vitamin D_3 was determined with the following equipment:

Main equipment:

- high performance liquid chromatograph Agilent 1100 with diode matrix detector (s/n DE40521679/ DE40915278) (USA);
- electronic laboratory balance GH-120 I accuracy class, 0.1 mg, up to 120 g (s/n 15101394) (A&D Company Ltd., Japan);
- variable volume one-channel pipette dispenser Eppendorf Research 500–5000 µl (s/n 4146697) (Eppendorf, Germany);
- one channel variable volume dispenser Eppendorf Research 100–1000 µl (s/n 1581088) (Eppendorf, Germany).

Ancillary equipment:

- laboratory water purification system Milli-Q (s/n F8KN02139A) (Millipore, France);
- ultrasonic bath Bandelin RK 31 (s/n: 329.00072514.029) (Bandelin electronic, Germany);
- water bath STEGLER WB-2 with possible fixation of constant temperature;
- Vortex type vibration shaker (ELMI, Latvia);
- centrifuge 5424 Eppendorf (maximal rotation up to 20000 rot/sec) s/n 16934300 (Eppendorf, Germany);
- glass beakers 500 ml;
- volumetric flasks of class "A" 10 ml;
- ceramic mortar and pestle.

Reagents and solutions

The following reference samples and chemical reagents were used for the study: cholecalciferol reference sample (vitamin D) (Sigma-Aldrich, USA, CAS № 67-97-0, lot. 3LRAB2929); substance cholecalciferol 100 SD/S (DSM Nutritional Products GmbH, Germany, batch № 11c/2397-10/0319); acetonitrile (Panreac Quimica S.L.U, Spain, class HPLC-gradient № 361881.1612, valid up to 06.2023); formic acid 99 %, № 270480010 (Acros Organics, Finland, valid up to 20.04.2021); methanol (TH "CHIMMED" LLC, Russia, purity class "chemically pure"), valid up to 06.2021; purified water.

Study objects

For the assay of cholecalciferol with developed method in real samples, the following vitamin drug products: "Aquadetrim", aqueous solution 10 ml by JSC "Medana Pharma" (valid up to 04.2023, batch № 050420); "Aquadetrim", water soluble tablets by JSC "Akrikhin" (valid up to 04.2022, batch № 170420). Vitamin dietary supplements were also investigated: "Ultra-D", chewable tablets by "Pharmia Oy" (valid up to 05.12.2022, batch № 1913870002); "Detrimax 1000 IU", tablets by "Eagle Nutritionals Inc" (valid up to 02.2022, batch № WJ141); "Detrimax 2000 ME", tablets by "Grokam JBL Sp.z.o.o" (valid up to 11.02.2023, batch № 260220).

Preparation of samples and solutions

Mobile phase (MP). As MP, the mixture of acetonitrile and 0.1 % formic acid solution in percentage (70:30) in gradient elution. To prepare 0.1 % formic acid solution about 400 ml of purified water, 1 ml of formic acid was added to a volumetric flask 1000 ml and mixed. Then the resulting solution was made up to the mark with purified water and mixed. The solution was kept for 30 days.

Stock and working solutions of the reference sample (RS). About 20 mg of cholecalciferol (an accurate weigh) was transferred to a volumetric flask 100 ml, about 40 ml of methanol was added and mixed till the substance was fully dissolved. Then the solution was made up in the flask to the mark with methanol and mixed (stock solution).

5.0 ml of the stock solution was placed to a volumetric flask 100 ml, the solution was made to the mark with methanol and mixed.

The solutions were used within 10 days after the preparation in the storage conditions in the freezer at temperature not above –20 °C and wrapping of seals and the flask neck with paraffin, and the flask itself – with foil.

Extraction. B The product (50–500 mg) was weighed into a volumetric flask 10 ml. Then 2 ml of purified water was added, sealed and thoroughly mixed on the vibration shaker. The flasks were placed to water bath for 10 minutes at temperature not above 60 °C. Then 5 ml of methanol was introduced to the flask, the resulting solution was sealed and shaken, if necessary was placed to water batch at temperature not above 60 °C, then was made up with methanol to the mark and mixed for at least 20 times.

For liquid samples representing aqueous solutions and glyceride solutions with introduced cholecalciferol, the product was weighed into the volumetric flask (50–500 mg). Then 5 ml of methanol and mix thoroughly on vibration shaker. Then it was made up with methanol to the mark. It was mixed for at least 20 times.

Then the aliquot was transferred to Eppendorf vials 2 ml and centrifuged at 14000 rot/sec. for 10 minutes or filtered through a syringe filter to a vial. The resulting solutions were analyzed freshly prepared.

Conditions for the analysis with HPLC and UV-detection

Conditions for the analysis with HPLC and UV-detection were selected during the experiment and given in table 1.

RESULTS AND DISCUSSION

Method development

When the assay method was developed, it was found that retention times of natural tocoferols used antioxidants and vitamin D stabilizers in substances for manufacturer of vitamin products and determined D_3 overlayed on columns with octadecyl silica gel sorbent. While a column with polyaromatic carbohydrates as the sorbent (ZORBAX Eclipse PAH Narrow Bore 2.1 × 150 mm, 3.5 µm) allowed to separate all matrix components [10].

Isocratic elution regime on the column with polyaromatic carbohydrates did not allowed to get satisfactory results of efficiency of separation of the matrix components [number of theoretic plates (TP) was less than 5 thousand], the gradient elution regime allowed to increase TP up to 20 thousand and more.

The selected gradient elution regime was shown in table 2.

Since vitamin D_3 has a specific spectrum and its characteristic absorption maximum (266 nm), and its concentration in the LP is quite high, the UV detector is quite suitable for recording the vitamin D_3 signal.

Table 1. Conditions for the chromatographic determination of vitamin D_3

Parameter	Value
Mobile phase	A mixture of acetonitrile and 0.1 % formic acid solution in percentage (70:30) in gradient elution
Mobile phaseflowrate	0,8 ml/min
Chromatographic column	ZORBAX Eclipse PAH Narrow Bore 2.1 × 150 mm, 3,5 µm
Column temperature	40 °C
Injected sample volume, µl	2
Detection wavelength, nm	266
Retention time, min t_R	12.4
Total analysis time, min	15

Table 2. Gradient elution mode

Chromatography time, min	Mobile phase A (0.1 % formic acid solution), %	Mobile phase B (acetonitrile),%
0	30	70
2	30	70
10	0	100
12	0	100
14	30	70
15	30	70

Method validation

As vitamin D_3 has a specific spectrum and typical absorption maximum (266 nm), and its concentration in a drug product is rather high, then the UV-detector was rather suitable for recording of vitamin D_3 signal. The method for parameter "Assay of vitamin D_3 (cholecalciferol)" in vitamin products was validated with HPLC based on General Pharmacopeial monograph 1.1.0012.15 "Validation of analytical methods" of the State Pharmacopeia of the Russian Federation, edition XIV, Guidance on Analytical methods of the Eurasian Economic Union dated 17.07.2018 and International Group Recommendations 61-2010 by the following parameters:

- specificity;
- accuracy;
- linearity;
- range;
- precision.

Specificity

Specificity was confirmed by the comparison of chromatograms of the diluent, placebo solution, reference solution and test solution containing all product components [11]. Specificity of the analytical method was proven if the following conditions were met:

- ✓ The chromatographic system requirements should be fully met.
- ✓ Diluent and placebo components should not interfere with cholecalciferol assay in the solution (figures 2–4).
- ✓ Placebo and cholecalciferol peaks had a resolution of over 1.5 and (or) change the result of cholecalciferol determination for less than 0.5 % [12].

According to the results, there are no peaks with retention times typical for cholecalciferol on the diluent and placebo chromatograms.

Efficiency of the chromatographic column for reference solution calculated by cholecalciferol peak was more than 35 000 theoretical plates; tailing factor of cholecalciferol peak – less than 1.00.

Specificity of the analytical method was proven as the requirements for the system suitability were met, placebo and diluent components do not preclude the assay of cholecalciferol in the solution.

Linearity

Solution of the concentrated reference sample. About 19.0 mg (accurate weigh) of cholecalciferol reference sample was transferred to a volumetric flask 100 ml, 50 ml of methanol was added, sonicated for 5 min, the solution was made up to the volume with the same solution to the mark and mix (reference solution).

Preparation of calibration reference solutions

Test calibration solutions were prepared by dilution of concentrated reference solution with methanol in volumetric flasks. Final concentrations of the solution were: 38 µg/ml, 31.7 µg/ml, 27.1 µg/ml, 23.8 µg/ml, 21.2 µg/ml, 19 µg/ml and 9.5 µg/ml.

Solutions were prepared for determination of linearity with cholecalciferol reference solution (Sigma-Aldrich, USA). The reference example is given in figure 5.

Acceptability criteria:

- ✓ Linearity dependence between the analytical signal and cholecalciferol concentration.
- ✓ Correlation coefficient $r_{XY} \geq 0.99$ [13].

7 samples of calibration solutions with concentrations 38 µg/ml, 31.7 µg/ml, 27.1 µg/ml, 23.8 µg/ml, 2.2 µg/ml, 19 µg/ml and 9.5 µg/ml were analyzed. Each solution was chromatographed 4 times. They were graduated with the method of absolute calibration. The calibration dependence between the ratio of cholecalciferol peak area and its concentration was described by equation $y = bx + a$. Using the obtained values, calibration plots were made. Calibration plots are given in figure 6 (table 3).

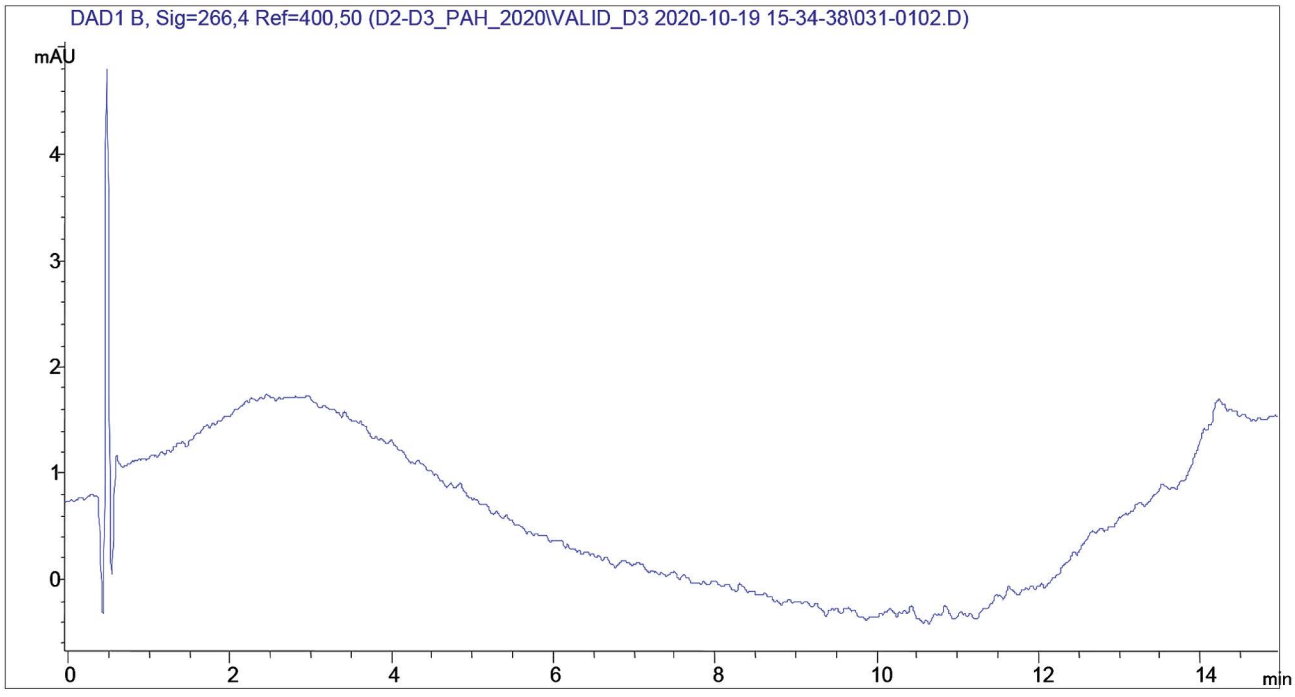

Figure 2. Chromatogram of the extractant (methanol)

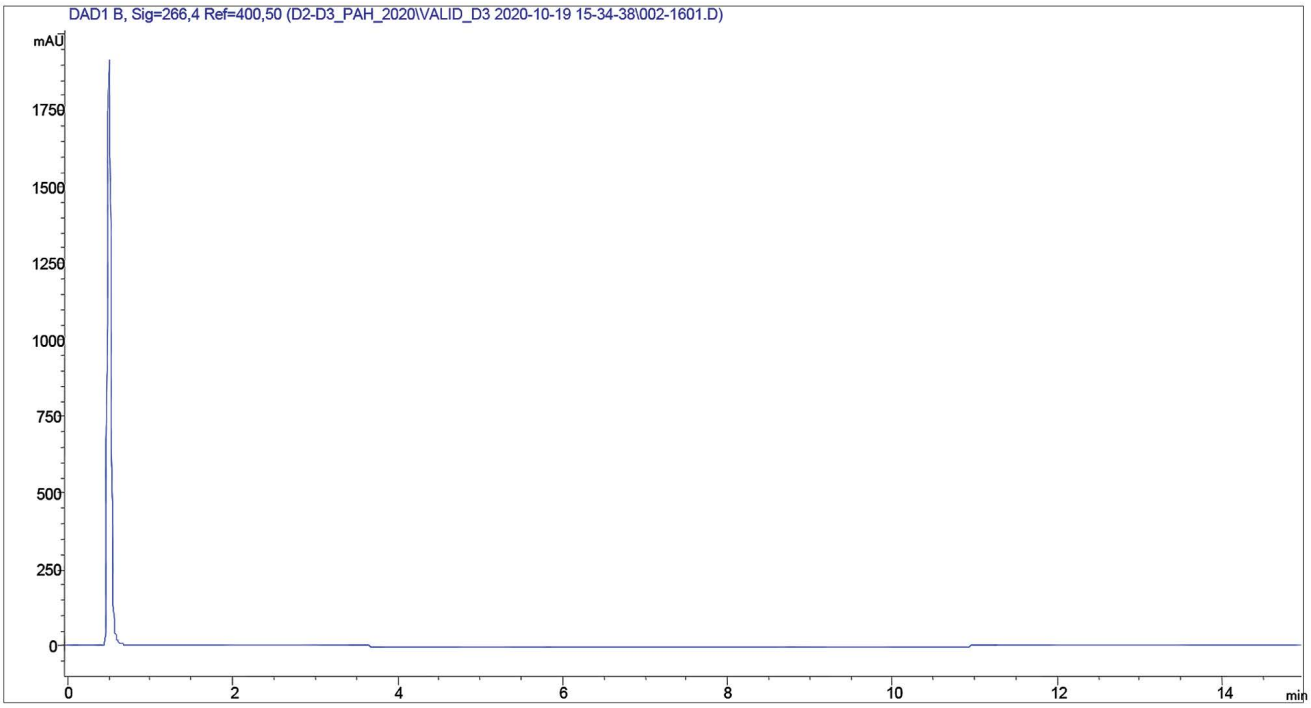

Figure 3. **Chromatogram of dry placebo (tablet mixture Akvadetrim, produced by LLCAkrikhin)**

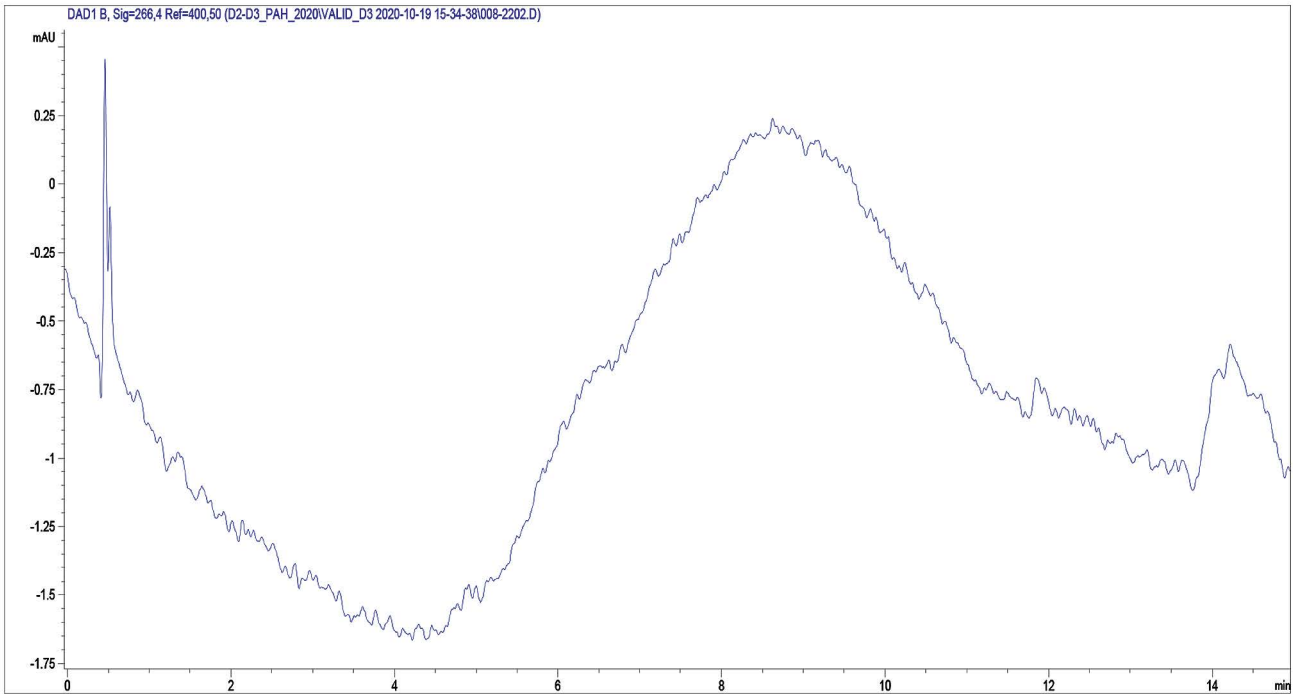

Figure 4. **Chromatogram of liquid placebo (purified water)**

Correlation coefficient for the calibration plot r_{xy} = 0.9990 which corresponded to the established normal value (at least 0.9900).

Using empirically measured values of variable *y* for the assigned values of argument *x*, coefficients *a* and *b* were calculated with the regression analysis:

Angle coefficient (*b*)	7.668
Free member (*a*)	–3.3757

The obtained results met the acceptability criterion "Linearity".

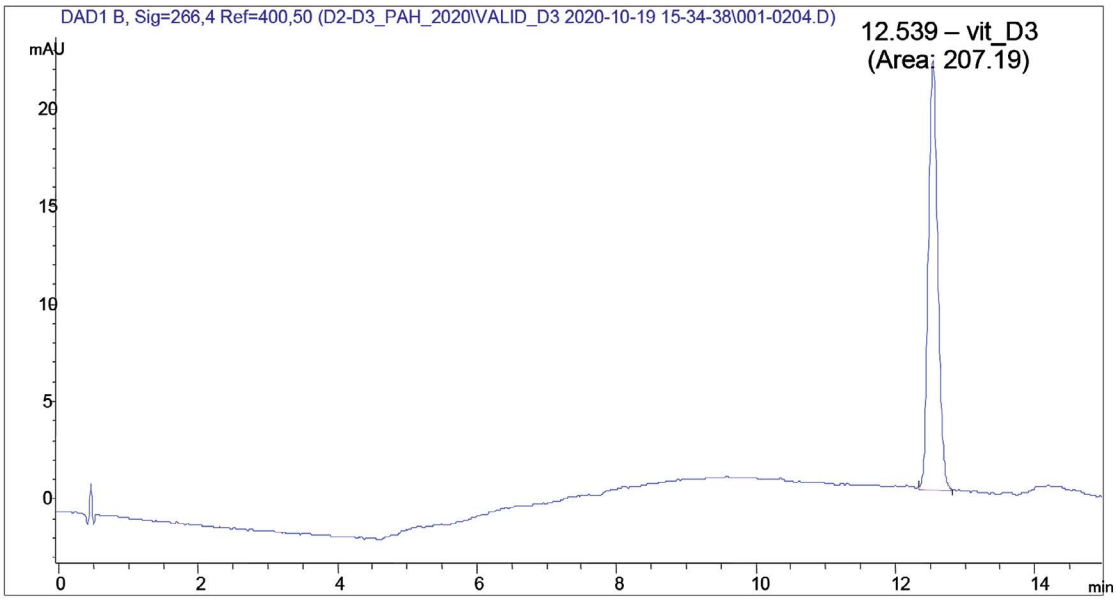

Figure 5. **Chromatogram of a standard sample (27.1 ng/μL)**

Accuracy

To check accuracy of the developed method, test samples obtained with introduction of 5 various concentrations of cholecalciferol 100 SD/S to liquid placebo (purified water) and dry placebo (tablet mixture "Aquadetrim" by "Akrikhin" LLC.

Test samples were prepared in accordance with section "Preparation of samples and solutions".

Test samples were prepared as follows: about 1.0 of placebo (mixture of all components except for vitamin D_3 provided by the customer and purified water as liquid placebo) was transferred to volumetric flasks 10 ml, various weighs of cholecalciferol substance 100 SD/S were added, then 2 ml of purified water was added, flasks were sealed, mixed on the vibration shaker and put to water bath for 10 min at temperature not above 60 °C. Then 5 ml of the diluent (methanol), sealed and shaken on the vibration shaker, if necessary, it was transferred to water bath at temperature not above 60 °C for not more than 10 min and made up with methanol to the mark. Then it was mixed for at least 20 times. The resulting solutions were analyzed in accordance with the analysis methoda. Based on the results, cholecalciferol concentrations were calculated in test samples, and the shift of the results in relation to reference values of contents of the introduced reference samples using Excel table.

Table 3. **Information for evaluating the linear dependence of peak areas on concentrations**

Content, ng	Concentration of a standard sample, μg/ml	Concentration of the standard sample (corrected for the purity of the standard), μg/ml	Peak area				Average peak area
			S_1	S_2	S_3	S_4	
19	9.50	9.47	72.14	71.69	71.89	71.21	71.73
38	19.00	18.94	141.66	137.19	141.22	141.04	140.28
42.4	21.20	21.14	153.03	155.24	156.55	156.50	155.33
47.6	23.80	23.73	176.86	172.69	176.06	176.82	175.61
54.2	27.10	27.02	207.01	209.70	210.63	208.66	209.00
63.4	31.70	31.60	240.42	240.77	238.41	237.23	239.21
76	38.00	37.89	290.49	296.44	291.21	285.10	290.81

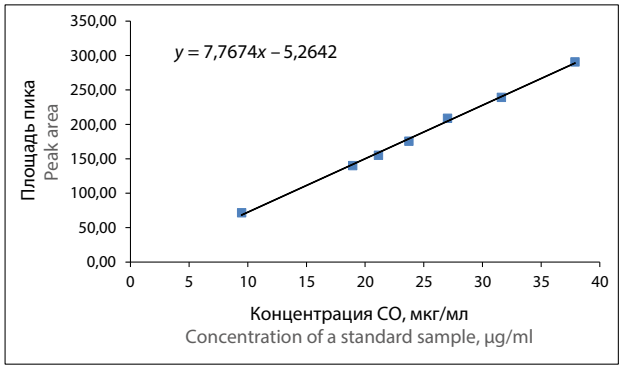

Figure 6. Calibration curve of standard cholecalciferol solutions

The analysis was performed within 1 working day by one analytic. The recovery values and mean recovery percentage were calculated for obtained concentration values.

The obtained results meet the acceptability criteria by parameter "Accuracy": mathematic hypothesis of reference cholecalciferol contents added to reference samples accepted in relative units as 100 %, is not beyond confidence intervals of the mean recovery value (96.96; 101.56) (table 4).

The results meet the acceptability criteria by parameter "Accuracy": mathematic hypothesis of contents of reference cholecalciferol added to reference samples accepted in relative units as 100 % is not beyond confidence intervals of the mean recovery value (99.07; 100.87) (table 5).

Table 4. The results of calculating the accuracy on placebo dry (an example of a typical chromatogram is shown in Figure 7)

Number of results	15
Mean Z, %	99.26
Dispersion (S^2)	17.15
Mean square deviation (СКО, σ)	4.14
Number of degrees of freedom, *f*	2.15
The critical value of the Student's t-test for $P = 0.95$	14
The coefficient of variation (CV, %)	4.17
Standard error of the mean (SEM)	1.07
Confidence interval (±ΔZ), %	2.30
Lower limit of the confidence interval, %	96.96
Upper limit of the confidence interval, %	101.56

Table 5. Results of calculating the accuracy on placebo liquid (an example of a typical chromatogram is shown in Figure 8)

Number of results	15
Mean Z, %	99.97
Dispersion (S^2)	2.63
Mean square deviation (СКО, σ)	1.62
Number of degrees of freedom, *f*	14
The critical value of the Student's t-test for $P = 0.95$	2.15
The coefficient of variation (CV, %)	1.62
Standard error of the mean (SEM)	0.42
Confidence interval (±ΔZ), %	0.90
Lower limit of the confidence interval, %	99.07
Upper limit of the confidence interval, %	100.87

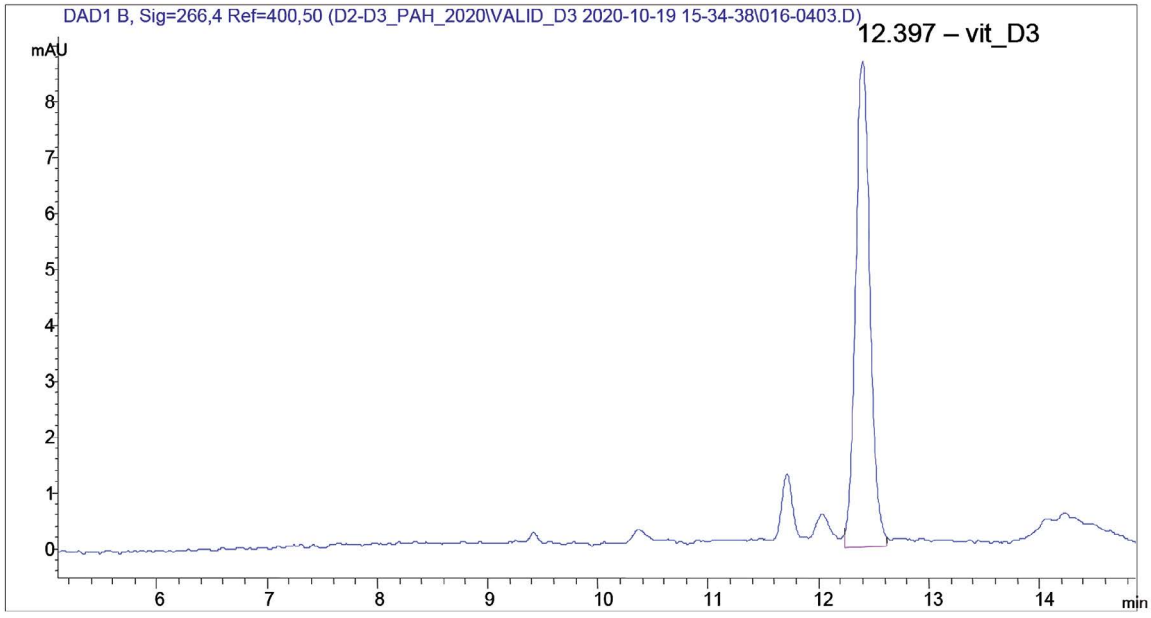

Figure 7. Chromatogram of dry placebo with added cholecalciferol substance 100 SD/S

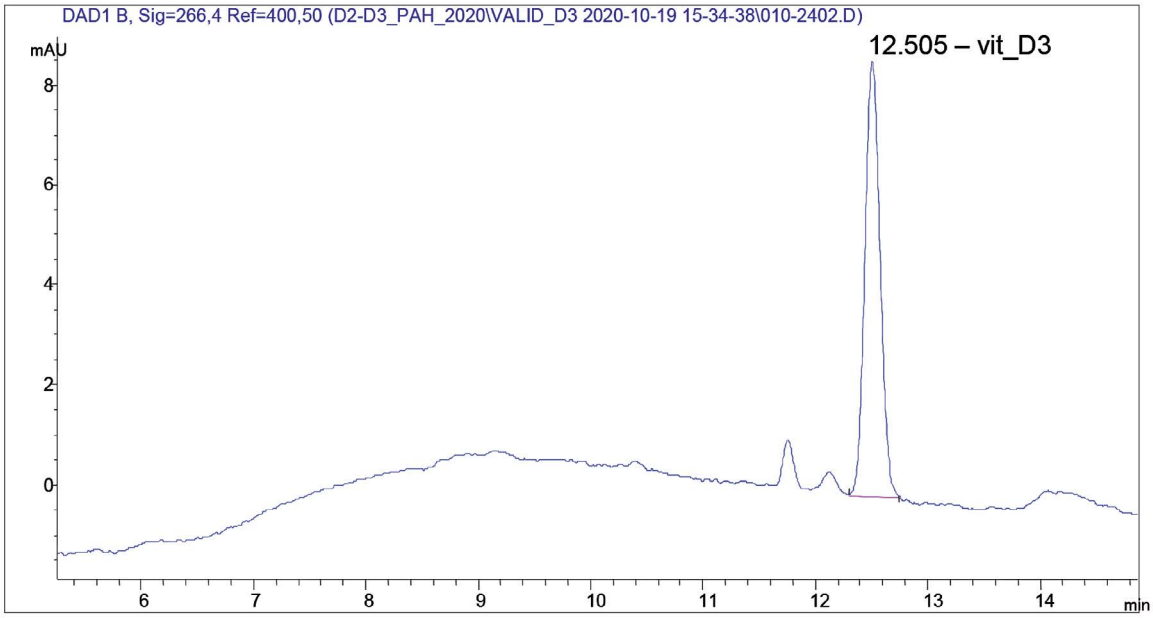

Figure 8. **Chromatogram of liquid placebo with added cholecalciferol substance 100 SD/S**

Range

In accordance with the data, the validated method provides accuracy of results and linear dependence of the analytical signal and solution concentrations for at least in the interval 0.0095 ÷ 0.038 ml/ml. The interval is the range for the method application based on the validation results.

Precision

The method precision was evaluated in accordance with the International Group Recommendations 61-2010 in repeatability conditions, i. e. when the analysis was performed by one chemist within a short period of time using the same equipment, as well as, in the conditions of intralaboratory precision when a sample of the same product batch at the second time was analyzed. In both cases, six homogeneous product samples with stable composition were analyzed.

Repeatability

It was determined on 6 test solutions for each drug products ("Aquadetrim", soluble tablets and "Aquadetrim", solution). 6 test solutions of both products ("Aquadetrim", soluble tablets and "Aquadetrim", solution) were prepared with the method described above. Each solution was chromatographed thrice, reference solution of cholecalciferol was chromatographed for 5 times.

The analysis was performed within 1 working day by one analytic. Values of the mean arithmetic and dispersion of results were calculated for the values (tables 6 and 7).

Table 6. **The results of calculating the precision for cholecalciferol in the drug "Akvadetrim" soluble tablets**

Samplecode	Weighedamount, mg	Peak area	Found, µg/g	Average content for samples $\bar{x}$, µg/g	Dispersion of results, S^2
AS-1	907.7	105.1	151.4	150.7	0.52
	907.7	104.1	150.0		
	907.7	104.6	150.7		
AS-2	653.4	76.74	153.6	155.2	6.37
	653.4	76.9	153.9		
	653.4	79	158.1		
AS-3	798	92.74	152.0	150.3	2.32
	798	91.3	149.6		
	798	91	149.1		
AS-4	717.2	82.67	150.8	150.1	0.75
	717.2	81.76	149.1		
	717.2	82.46	150.4		
AS-5	793	97.9	161.5	159.2	9.43
	793	94.4	155.7		
	793	97.25	160.4		
AS-6	967	100.5	135.9	134.6	1.51
	967	99.4	134.4		
	967	98.7	133.5		

Table 7. The results of calculating the precision for cholecalciferol in the drug "Akvadetrim" solution

Samplecode	Weighedamount, mg	Peak area	Found, µg/g	Average content for samples $\bar{x}$, µg/g	Dispersion of results, S^2
AL-1	400.5	117.85	384.8	392.0	46.4
	400.5	122	398.4		
	400.5	120.3	392.8		
AL-1	439.8	130.44	387.9	387.4	1.6
	439.8	130.6	388.4		
	439.8	129.8	386.0		
AL-3	501.2	139.2	363.2	367.2	13.0
	501.2	141.05	368.1		
	501.2	141.9	370.3		
AL-4	509.2	140.96	362.1	363.4	9.8
	509.2	142.87	367.0		
	509.2	140.6	361.1		
AL-5	458.8	131.83	375.8	377.6	6.4
	458.8	132.1	376.6		
	458.8	133.48	380.5		
AL-6	423.4	124	383.0	380.6	6.1
	423.4	122.4	378.1		
	423.4	123.2	380.6		

In accordance with cl. 5.2.1 of IGR 61, the mean arithmetic and dispersions of results of a single analysis obtained in repeatability conditions were calculated. The homogeneity of dispersion distributions was evaluated with the Cochrane test. The estimated values for confidence probability did not exceed its critical limit for confidence probability $P = 0.95$ and number of degrees of freedom $f = 17$.

Homogeneity of dispersion distributions was evaluated with Cochrane test. The estimated test value did not exceed its critical limit for confidence probability $P = 0.95$ and number of degrees of freedom $f = 17$.

Therefore the convergence between two parallel determinations obtained with the method should not in 19 out of 20 cases:

- 3.46 % for solid dosage forms;
- 2.74 % for liquid dosage form.

Intralaboratory precision

Intralaboratory precision was evaluated based on the results of cholecalciferol determination in the same samples which were used for evaluation of repeatability ("Aquadetrim", soluble tablets and "Aquadetrim" solution) another day, 6 weighs of each drug product. The contents of cholecalciferol in product "Aquadetrim", soluble tablets was 156 µg/1g (12.5 µg/tabl.); in product "Aquadetrim", solution – 375 µg/ml. Each solution was chromatographed trice, reference solution of cholecalciferol was chromatographed for 5 times.

Mean arithmetic values of individual determinations obtained by one chemist at various days were calculated for obtained concentration values.

In accordance with cl. 5.2.2.1 of IGR 61, mean values of individual determinations were checked for outliers by the Grubbs test. As results, mean values of determination of samples AT-2 and AT-5 at the first day of determination were rejected from further calculations.

Based on accepted results, a random MSD of the results obtained in the conditions of intralaboratory precision was calculated:

$$\sigma_R = \sqrt{\frac{\sum_{l=1}^{L}(X_l - \bar{X})^2}{L-1} + \left(\frac{1}{2} - \frac{1}{N}\right)\cdot\sigma_r^2} = 8.06,$$

where N – number of samples involved in precision evaluation.

Mean MSD of intralaboratory precision was:

$$\sigma_{R,\%} = 5.4\ \%.$$

The value of intralaboratory precision of the method as reproducibility limit R was calculated by equation:

$$R = Q(P, n)\,\sigma_R = 22{,}3,$$

$$R_{\%} = 15.0\ \%.$$

Based on the evaluation by the Grubbs test, the mean value of determination of AL-1 sample at the first day of the determination was rejected from further calculations.

Based on the accepted results, random MSD of the results obtained in the conditions of intralaboratory precision.

$$\sigma_R = \sqrt{\frac{\sum_{I=1}^{L}(X_I - \bar{X})^2}{L-1} + \left(\frac{1}{2} - \frac{1}{N}\right)\cdot\sigma_r^2} = 10.9.$$

Relative MSD of intralaboratory precision was:

$$\sigma_{R,\%} = 2.9\ \%.$$

The value of intralaboratory precision of the method as reproducibility limit *R* was calculated by equation:

$$R = Q\,(P, n)\,\sigma_R = 30.3,$$

$$R_{\%} = 8.0\ \%.$$

Therefore the convergence between mean results of two series of determinations performed with the method in the conditions of intralaboratory precision should not exceed in 19 out of 20:

- 15.0 % for solid dosage forms;
- 8.0 % for liquid dosage form.

The developed and validated method was used for the analysis of the following dosage forms (drug products and biologically active dietary supplements):

Drug products:

1. "Aquadetrim", aqueous solution 10 ml by JSC "Medana Pharma" (valid up to 04.2023, batch № 050420).
2. "Aquadetrim", water soluble tablets by JSC "Akrikhin" (valid up to 04.2022, batch № 170420).

Biologically active dietary supplements:

1. "Ultra-D" chewable tablets "Pharmia Oy" (valid up to 05.12.2022, batch № 1913870002).
2. "Detrimax 1000 IU", tablets by "Eagle Nutritionals Inc", (valid up to 02.2022, batch № WJ141).
3. "Detrimax 2000 IU" tablets by "Grocam JBL Sp.z.o.o" (valid up to 11.02.2023, batch № 260220).

All samples were tested within one analytical cycle which allowed to reduce intralaboratory variability of the investigation results. System suitability met the normal values. Chromatograms of both solid and liquid dosage forms did not show peaks interfering with the analysis. Therefore the test results may be considered significant within the established validation characteristics.

The analysis results were summarized in table 8, standardized by parameter contents μg/g for solid dosage forms and μg/ml for liquid dosage forms. The analysis for liquid dosage forms was performed as corrected by density ("Aqudetrim", aqueous solution – 1 g/ml).

The method error was calculated by equation:

$$\Delta = 1.96 \cdot СКО,$$

where MSD – mean square deviation of intralaboratory precision (MSD for liquid forms was 2.9 %, MSD for dry forms was 5.8 %).

Table 8. Results of quantitative determination of vitamin D_3 (cholecalciferol) in dosage forms (drugs and dietary supplements)

Drugname	Vitamin D_3 content in the drug (declared)	Vitamin D_3 content in 1 g (1 ml) of the drug (declared)	Vitamin D_3 content in 1 g (1 ml) of the drug (found)	Deviation (found/ declared), %
Ultra-D	25 μg/tablet (tablet weight 425 mg)	58.8 μg/g	52.6 μg/g	–10.5
Detrimax 1000 IU	25 μg/tablet	108.7 μg/1 g	106.1 μg/g	–2.4
Detrimax 2000 IU	50 μg/tablet	208.3 μg/1 g	202.4 μg/g	–2.8
"Aquadetrim" aqueous solution	15 000 IU / ml; 1 drop (33.3 μl) = = 500 IU (12.5 μg/33.3 μl) 375 μg/ml	375 μg/ml	370 μg/ml	–1.3
"Akvadetrim" water-soluble tablets	500 IU/tablet (12.5 μg/tablet)	156 μg/1 g	155 μg/g	–0.6

The method error for liquid forms (aqueous solutions) was ± 5.68 %, for dry dosage forms ± 11.37 %, respectively.

CONCLUSION

The method for determination of parameter "Contents of vitamin D_3 (cholecalciferol)" in vitamin dosage forms with HPLC was developed. The method was validated by the following validation parameters: specificity, accuracy, linearity, range, precision. It was shown that the validation results are satisfactory by all specified criteria. The range of the method is 9.5–38 µg/ml.

The results of the method validation and investigation of actual samples may be used for determination of vitamin D_3 in vitamin products based on water soluble substances of vitamin D_3 and as aqueous solutions.

Based on the assay results, it was established that:

1. Actual contents of vitamin D_3 for product "Aquadetrim", aqueous solution 10 ml by JSC "Medana Pharma" (valid up to 04.2023, batch № 050420), Actual contents of the vitamin was 370 µg/ml, and the deviation of the actual contents from the label claim was –1.3 %.
2. Actual contents of vitamin D_3 for "Aquadetrim", water soluble tablets by JSC "Akrikhin" (valid up to 04.2022, batch № 170420) was 155 мкг/г, and the deviation of the actual contents from the label claim was –0.6 %.
3. Actual contents of vitamin D_3 for biologically active dietary supplement "Ultra-D", chewable tablets by "Pharmia Oy" (valid up to 05.12.2022, batch № 1913870002) 58,8 µg/g, and the deviation of the actual contents from the label claim –10,5 %.
4. Actual contents of vitamin D_3 for biologically active dietary supplement "Detrimax 1000 IU", tablets by "Eagle Nutritionals Inc" (valid up to 02.2022, batch № WJ141) was 106.1 µg/g, and the deviation of the actual contents from the label claim –2.4 %.
5. Actual contents of vitamin D3 for biologically active dietary supplement "Detrimax 2000 IU", tablets, by "Grocam JBL Sp.z.o.o." (valid up to 11.02.2023, batch № 260220) was 202.4 µg/g, and the deviation of the actual contents from the label claim –2.8 %.

As a result of the studies, it was shown that for the determination of vitamin D_3, used in the form of a water-soluble substance, in medicines and dietary supplements for food, it is possible to use methods without carrying out the saponification stage. This provides tangible benefits, since allows you to avoid the loss of the active substance in the process of multi-stage sample preparation, because vitamin D_3 is unstable in the light and in an environment rich in oxygen.

REFERENCES

1. Thomson B. M., Cressey P. J. Determination of vitamin D in foods: Current knowledge and data gaps. Wellington: Ministry for Primary Industries; 2014. 39 p.
2. Mahmoodani F., Perera C. O., Fedrizzi B., Abernethy G., Chen H. Degradation studies of cholecalciferol (vitamin D3) using HPLC-DAD, UHPLC-MS/MS and chemical derivatization. *Food Chemistry*. 2017;219:373–381. DOI: 10.1016/j.foodchem.2016.09.146.
3. Vilms E. A., Dobrovolskaya E. V., Turchaninov D. V., Bykova E. A., Sokhoshko I. A. Provision of vitamin D in the adult population of Western Siberia: a population-based study. *Voprosy pitaniya = Problems of Nutrition*. 2019;88(4):75–82. (In Russ.) DOI: 10.24411/0042-8833-2019-10044.
4. Winuprasith T., Khomein P., Mitbumrung W., Suphantharika M., Nitithamyong A., McClements D. J. Encapsulation of vitamin D3 in pickering emulsions stabilized by nanofibrillated mangosteen cellulose: Impact on in vitro digestion and bioaccessibility. *Food Hydrocolloids*. 2018;83:153–164. DOI: 10.1016/j.foodhyd.2018.04.047.
5. Gammel I. V., Suvorova O. V., Zaporozhskaya L. I. *Analiz tendentsiy rossiyskogo rynka biologicheski aktivnykh dobavok k pishche* [Analysis of trends in the Russian market of biologically active food additives]. *Meditsinskiy al'manakh = Medical Almanac*. 2017;6(51):154–158. (In Russ.)
6. Perales S., Alegría A., Barberá R., Farré R. Review: Determination of vitamin D in dairy products by high performance liquid chromatography. *Food Science and Technology International*. 2005;11(6):451–462. DOI: 10.1177/1082013205060129.
7. Agilent Technologies 5990-8627 EN LC/MS/MS Determination of vitamin D in food. Available at: www.agilent.com/chem/QQQ. Accessed: 12.05.2021.
8. Afrozul H., Jaishen R., Laila O. A.-W., Dhabi A.. Routine HPLC Analysis of vitamin D3 and D2. *Chromsystems*. Available at: www.chromosystems.de. Accessed: 12.05.2021.
9. *GOST 32043-2012 Premiksy. Metody opredeleniya vitaminov A, D, E* [GOST 32043-2012 Premixes. Methods for the determination of vitamins A, D, E]. Available at: https://docs.cntd.ru/document/1200104849/titles/353F5U8. Accessed: 12.05.2021. (In Russ.)
10. Epshtein N. A. Chromatographic system suitability testing: influence of correction factors and late eluting peaks of im-

purities on the requirement for the signal-to-noise ratio. *Razrabotka i registratsiya lekarstvennykh sredstv = Drug development & registration*. 2019;8(1):108–112. (In Russ.) DOI: 10.33380/2305-2066-2019-8-1-108-112.

11. Sukhanova A. M., Perova I. B., Eller K. I., Rodionova G. M., Chernova S. V., Kuzina V. N. Development and Validation of Sibutramine Determination in Drug Products by Capillary Electrophoresis. *Razrabotka i registratsiya lekarstvennykh sredstv = Drug development & registration*. 2020;9(4):141–145. (In Russ.) DOI: 10.33380/2305-2066-2020-9-4-141-145.
12. Epshtein N. A. Validation of Chromatographic Methods: Checking the Peak Purity and the Specificity of Methods with Diode Array Detectors. *Razrabotka i registratsiya lekarstvennykh sredstv = Drug development & registration*. 2020;9(3):129–136. (In Russ.) DOI: 10.33380/2305-2066-2020-9-3-129-136.
13. Epshtein N. A. Validation of Analytical Procedures: Graphic and Calculated Criteria for Assessment of Methods Linearity in Practice. *Razrabotka i registratsiya lekarstvennykh sredstv = Drug development & registration*. 2019;8(2):122–130. (In Russ.) DOI: 10.33380/2305-2066-2019-8-2-122-130.