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Validation of HPLC method for the determination of retinol in different dietary supplements

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Abstract

This paper proposes a simple HPLC method for the identification of vitamin A in dietary supplements, which does not imply thout saponification of the samples in order to preventing their degradation.

The method was applied to the determination of the retinol concentration in five commercial products containing known amounts of vitamin A, under the following chromatographic conditions: werw used C18 column, a mobile phase consisted of methanol/water 95:5 (v/v) and UV (DAD) set at 325 nm. The linearity range was established between 0.05-10 µg/mL for all-trans-retinoland prior to the analysis. The extraction was made performed with water:n-hexan 5:1 (v/v) prior to the analysis. The limit of quantification for trans-retinol was of 20 ng/mL and for retinyl acetate (internal standard) was of 50 ng/ml. Rapid determination of vitamin A by through the chromatographic method represents a good solution for its quantification in the different dietary supplements.

Keywords: retinol (vitamin A), dietary supplements, HPLC method.

1. Introduction

People consume dietary supplements to improve their health. However, the effectiveness of nutritional supplements is questionable since the effects of most of them have not been tested by scientific studies. Although the excessive consumption of most vitamins and minerals are not dangerous, some supplements are incriminated for having adverse effects at high doses. Use of approved dietary supplements are justified only if they are proven deficiencies as a result, the recommended doses must correspond to the physiological needs.

Vitamin A is necessary for many essential processes of life such as: metabolism, cell homeostasis, bone development in the growth process and embryo development, proper functioning of epithelial cells, it modulates the immune function and increases the resistance body to infectious diseases. These processes can be supported by all forms of vitamin A: retinol and retinyl esters, including β-carotene. Vitamin A cannot be synthesized by the human body. Daily requirement must be provided by diet under the preformed of retinol (retinyl esters) from the food of animal origin food and through provitamin A (carotenoids) from the vegetables products. Deficiency of vitamin A affects the vision, causing corneal

degeneration and dryness. In addition, excessive supplementation of vitamin A can lead to the toxic effects, including skin pathological changes, alopecia, anorexia, muscle and bone pain, and conjunctivitis.

Food intakes, the preparation and the storage methods, influence the stability vitamin A. Vitamin A degrades in the presence of oxygen and UV radiations, at high temperature and low pH values. In these conditions occur both, the isomerization of the *trans*-retinol, in a less potent biological form (the *cis*-retinol), and the hydrolysis of the retinyl ester, in a less stable chemical form, (the retinol). In the USA, the "Dietary Supplement Health and Education Act" (DSHEA) defines the following terms: „food supplement" and the „dietary *ingredient*" and „new dietary *ingredient*", such components of dietary supplements. In the category of the *ingredients* have been included one or more components of the following types of substances: vitamins, minerals, herbs, amino acids, various food substances (enzymes or tissues from organs or glands), concentrates, metabolites, extracts [11].

In Romania, the Health Ministerial Order no.1069/2007 approving the norms regarding dietary supplements partly regulates their production and circulation. Dietary supplements represent the dietary products which complete the normal diet. They are concentrated sources of nutrients or other substances, having a nutritional or physiological effect, alone or in combination, marketed as doses: capsules, lozenges, tablets, pills and other similar. Dietary supplements that contain vitamin A should meet the criteria laid down in the Directive 46/2004 on food supplements as amended according to the EC Directive 100/2008, respectively HG 685/2009 must be at least 15% of the Recommended Daily Allowance (RDA) - 800 μg Vitamin A [10].

Recent studies estimate that in the developed countries more than 30% of the population daily intakes various forms of dietary supplements, which do not present a scientific certification. Concerning the quality supplements, in a recent study realized in France taking into account 382 products, and focused on the determination of the nutrient levels (nutritional values, the vitamins and the minerals), of preservatives, additives, heavy metals, pesticides and labeling, it was found that 75% of the supplements were inappropriate, because they contained substances, plants or parts of plants unauthorized for human consumption. In addition, vitamins and minerals were added in higher concentrations than the recommended daily doses, the organo pesticides concentrations were not to be neglected, and the labeling was incomplete. Therefore we need a fast and reliable method for the determination of vitamins nutritional supplements throughout the production and the storage of these products [14].

To this end, the aim of this work is the development and the validation of a fast and selective method for the determination of the retinol in dietary supplements through HPLC method using the UV detection system with diode-array detection (DAD) and the validation of the working conditions.

2. Materials and Methods

The samples

For the study, there were chosen five commercial products containing various amounts of vitamin A as retinyl esters, denoted by A, B, C, D, E,usually consumed for the vitamin balancing of the body. These products were purchased from the local (Galati) pharmaceutical network .

The reagents used

In this study it was used as the reference Bui-Nguyen and Blanc method [13] for vitamin A adapted to the food. The Bui-Nguyen and Blanc methods were used as reference for food adapted vitamin A.In order to achieve the HPLC determinations, the following chemical reagents were necessary: all *trans*-retinol (Vitamin A) (Supelco Analytical, SUA), 99,99 % purity; retinyl acetate (internal standard) (Supelco Analytical, SUA), 99,99 % purity; 2,6-ditertbutyl-4-methylphenol (BHT) (Supelco Analytical, SUA), 99,99 % purity; methanol HPLC 99,99% analytical purity (Merck, Germania); *n*-hexane HPLC 99,99 % analytical purity, (Merck, Germania); deionized water (TKA, Thermo Electron, Germania).

Achieving internal standard

The quantitative determination of vitamin A from dietary supplements was made using the internal standard method. The retinyl acetate, a commonly reagent used in literature, was chosen as the internal standard for this study. A stock solution of 10% concentration in retinyl acetate was obtained by the dissolution of the retinyl acetate in the methanol/water 95:5 (v/v) binary solvent. The standard solution of 5 μg/mL concentration was prepared through the dilution of the working stock solution. The samples were prepared in flasks wrapped in aluminum foil and protected from the light. The stock solution was stored at -10°C, presenting a good chemical stability for 14 days.

The internal standard solution was analyzed under the same conditions.

The bioanalitycal measurements

The samples were dissolved in 50 mL distilled water and stirred with 10 mL of *n*-hexane with 0.05% BHT, as antioxidant, for 10 minutes in a separatory funnel. The fat-soluble vitamins were extracted three times, and the organic extracts were combined and evaporated to dryness under a nitrogen stream. The obtained residue was dissolved in 100 μL methanol/water 95:5 (v/v) mixture, to which it was added a certain volume of the standard solution of retinyl acetate (SI) and which was finally analyzed through HPLC chromatography.

The „Thermo Finnigan Surveyor" system with Photodiode Array Detector (DAD) and Chrom Quest 4.1. version software was used. The column was C18 (Thermo Scientific Hypersil GOLD) (250mm x 4.6 mm I.D.) with the thickness of the stationary phase particles of 5 μm. The isocratic mobile phase was methanol/water 95:5 (v/v) at a flow-rate of 1.5 mL/min. The retinol detection was performed at 325 nm and the volume of sample injected was of 20 μL.

The determination of linearity

In order to determine the linearity, a stock solution of 10% concentration in all *trans*-retinol was obtained through the dissolution in the methanol/water 95:5 (v/v) of the binary solvent. The obtained solution was stored at -10^{0}C in a flask covered with aluminum foil. The working standard solutions were prepared by dilution in methanol/water 95:5 (v/v) in order to obtain five solutions of different concentrations: 0.05; 0.5; 2; 4; 10 μg/mL. The samples were analyzed three times in the same chromatographic conditions.

Selectivity

In order to obtain different retention times (for a good resolution), different mobile phases were further tested: pure methanol, methanol/water 99:1(v/v), methanol/water 95:5(v/v), methanol/water 90:10 (v/v) and methanol/acetonitrile/water 75:20:5(v/v/v). In these experimental conditions were simultaneously analyzed different all trans-retinol and retinyl acetate concentrations (0.5-5 μg/ml).

The resolution separation

By using the methanol/water 95:5 (v/v) mixture as mobile phase, the resolution of the chromatographic separation determined as follows (Eq.1):

$$Rs = 1{,}18\frac{(t_2 - t_1)}{w_1 + w_2} \qquad (1)$$

where:
t_1, t_2 – the retention times of *trans*-retinol and retinyl acetate;
w_1, w_2 – the peak widths at half-height.

3. Results and Discussions

The naturally compounds that contain Vitamin A or synthetic analogues of the retinol are called „retinoids". The determination of these retinoids has a great importance considering their antioxidant effect against damaging free radicals and their significance in the biosynthetic pathway of glycoproteins and proteoglycans. The use of high performance liquid chromatographic techniques for the analysis of vitamin A provides the advantage of a fast and accurate method [1]. The increased consumption of vitamin supplements in recent years imposes finding some methods of the separation and the quantification of the retinol content. Knowing the level of the retinol in dietary supplements is very important in establishing the dose of vitamin A to be administered, thus, preventing the overdose and the occurrence of the toxicity.

The calibration curve was obtained by plotting the surface of the chromatographic retinol peaks on the ordinate, and the retinol concentration in the sample on the abscissa. The retinol concentration of the unknown samples was determined by applying the linear regression equation curve to areas ratio Y = 346 + 5368X, with a correlation coefficient $r^2 = 0.9995$ (see Fig.1).

The application of the HPLC method has been reported for the determination of retinol and retinyl esters in some pharmaceutical preparations, such as creams and tablets. Generally, it was used the saponification reaction in order to break the ester bonds, followed by the extraction of the samples before the injection into the HPLC systems. In these cases it was observed that the saponification is time-consuming and it can leads to the vitamin degradation [2].

Genestar and Grases, 1995 [1], evaluated the extraction with methanol, chloroform and chloroform/methanol (2:1). For the dissolution of vitamin A, the sample was vortexed and centrifuged for 1 min. The authors analyzed the pharmaceutical products Lit-Stop® capsules based on retinyl acetate. The manufacturer's stated concentration of vitamin A was of 2500 UI per capsule but the study found a mean value of 2760±220 UI.

Figure 2a shows the chromatogram of the all *trans*-retinol for a standard solution containing 2 µg/mL concentration of all *trans*-retinol (three-dimensional planar representation), obtained in the chromatographic conditions mentioned above.

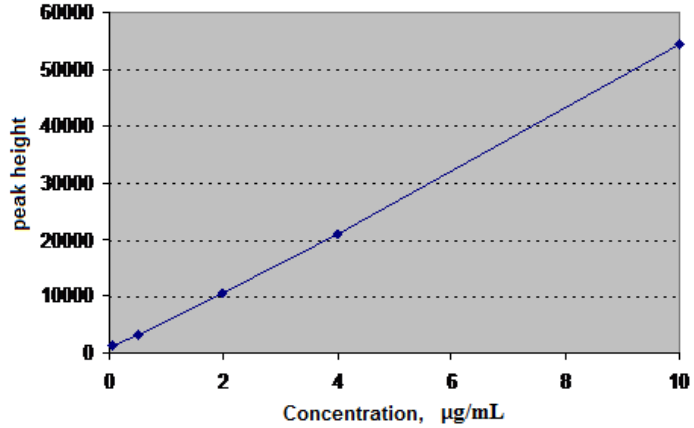
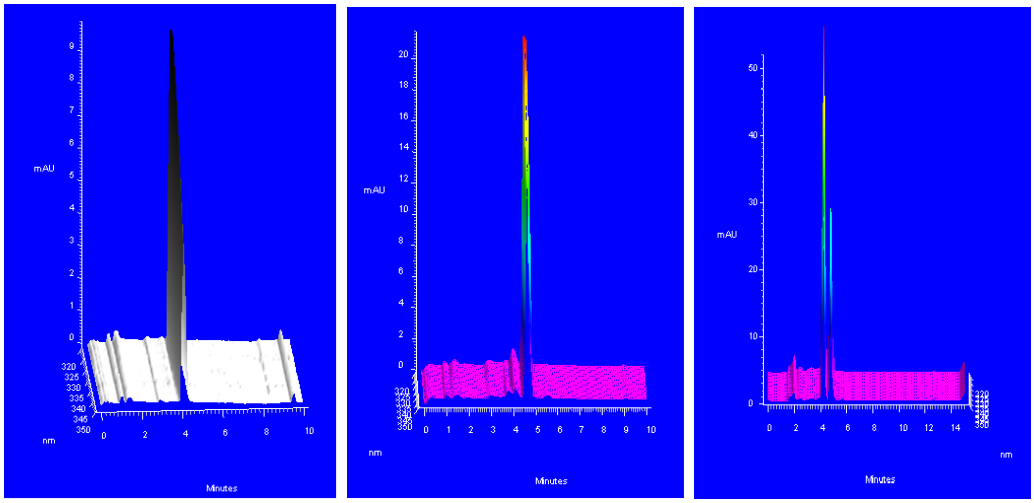


Figure 1. The calibration curve obtained in the case of all *trans*-retinol dissolved in the methanol/water 95:5 (v/v) mixtures, using all *trans*-retinol concentrations ranging between 0.05 and 10 µg/mL.



a) b) c)

Figure 2. Three-dimensional representations of the following chromatograms: **a)** all *trans*-retinol standard solution of 2 µg/mL concentration; **b)** internal standard based on retinyl acetate; **c)** mixture based on all *trans*-retinol and retinyl acetate (SI), containing 10 µg/mL all *trans*-retinol and 4 µg/mL retinyl acetate.

Table 1. Retention times (in minutes) obtained for some mixed mobile phases

Mobile phase	All trans-retinol min	Retinyl acetate min
Methanol	2,34	2,52
Methanol/water (99:1)	3,81	4,19
Methanol/water (95:5)	4,15	4,70
Methanol/water (90:10)	4,52	5,22
Methanol/acetonitrile/water (75:20:5)	2,49	2,85

Table 2 The average values of the retention times of retinol and retinyl acetate in pharmaceuticals products

	Average values	
Vitamin A sources	**t_R retinol**	**t_R retinol acetate**
A	4,413	4,98
B	4,607	5,208
C	4,478	5,07
D	4,49	5,072
E	4,553	5,152

The value obtained for the resolution of the separation, Rs = 1.82 shows a good separation of the two components, as the chromatograms in Fig.3 prove. The relative retention α_s, as a measure of the selectivity of the method, has an average value of 1.4 ± 0.2 determined for three times using different concentrations (0.5, 2 and 5 μg/mL) of retinol and retinol acetate.

The limit of quantification was evaluated as the concentration equal to five times the value of the signal-to-noise ratio (S/N). In this study, for S/N = 3, the limit of quantification was set at 20 ng/mL for all *trans*-retinol and at 50 ng/mL for retinyl acetate (SI). These results demonstrate that the analysis method can be use to determine vitamin A levels in samples wich contain very low concentrations (Table 1-2). The retention time t_R for retinol, in the main pharmaceutical products that mention the content of vitamin A in their composition, presents the value of 4.5082±0.033 (Table 3-4).

Zajac et al., 1999 [12] proposed the determination of the retinyl palmitate level in capsules and ointments through HPLC method performed with a LiChrospher RP-18 column, using the methanol as mobile phase, UV detection at 325 nm and retinyl acetate as internal standard. In the specified conditions, a linear dependence was obtained between the height of the peaks corresponding to the retinyl palmitate, in the range between 15 μg/mL to 175 μg/mL, $y = (75.5\pm5.3)\cdot x$, with $r^2 = 0.9985$. The HPLC method established the limit of detection of retinyl palmitate at 15 μg/mL.

In dietary supplements, vitamin A is added under the form of esters, in order to prevent the vitamin oxydation [2]. As a result, it becomes more difficult to determine the vitamin A level due to the vitamin instability during the sample preparation. If using the saponification reaction, a large number of samples and great volumes of solvent would be required, the method proving to be expensive and lengthy.

The second stage in the determination of retinol levels is the extraction from the saponified sample. The extraction with diethyl ether as solvent (alone or combined with oil) [3,4] dichloromethane/methanol [5] or hexane/toluene [6] is described in detail in literature, some authors obtaining satisfactory results with hexane, the most widely used of all solvents [7, 8, 9].

The last step is the separation process. Some authors have used the chromatographic separation with *n*-hexan/1-octanol [7] or ethyl acetate/hexane [9], but the best HPLC quantification is performed using acetonitrile [4] or methanol [5], which provides higher reproducibility [2]. Therefore, the main focus of the study was to develop a fast, specific and reliable method, for the quantification of the retinol in dietary supplements, by avoiding the saponification reaction. The method can be easily applied to the analysis of the quality control.

Dietary supplements are a current modality to ensure a balanced intake of nutrients, especially vitamins and minerals, although nutritionists claim that eating remains the best solution to provide the physiological needs of the body. In addition, quite permissive legislation authorizing and movement of these products may favor the reducing of health benefits to which the consumer aspires.

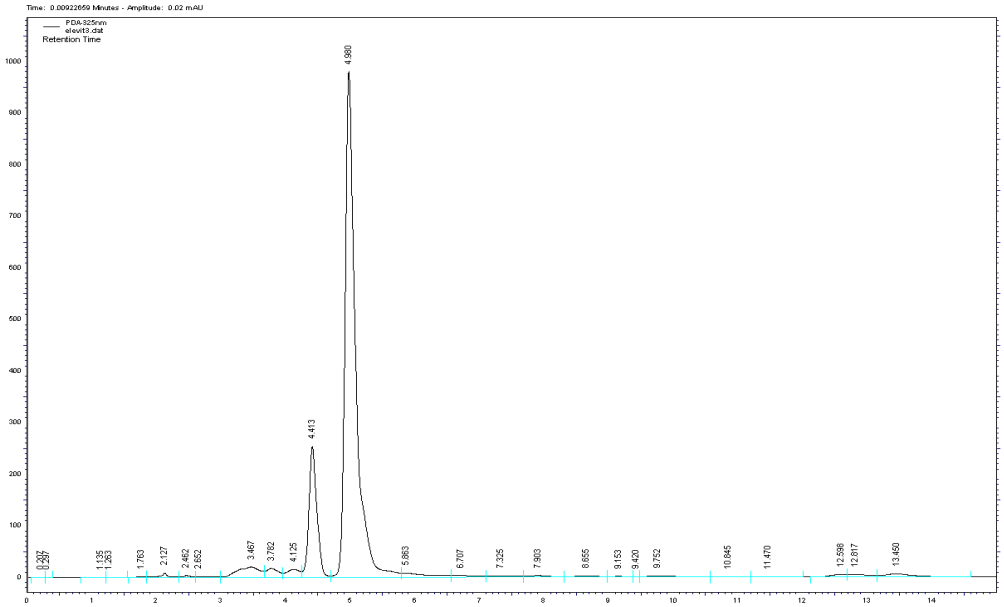


Figure 3. Chromatogram for A compound (t_R = 4,413) and retinyl acetat (t_R = 4,980)

Table 3. Descriptive statistics for the retention times of the retinol for the pharmaceutical products containing vitamin A

t_R Retinol	
Mean	4.5082
Standard Error	0.033222583
Median	4.49
Standard deviation	0.074287953
Sample variance	0.0055187
Kurtosis	-0.514042644
Skewness	0.160551333
Range	0.194
Minimum	4.413
Maximum	4.607
Sum	22.541
Count	5
Confidence level (95.0%)	0.092240677

Table 4. Descriptive statistics for the retention times of the retinyl acetate for the pharmaceutical products containing vitamin A

t_R Retinyl acetate	
Mean	5.0964
Standard Error	0.038978969
Median	5.072
Standard deviation	0.087159624
Sample variance	0.0075968
Kurtosis	-0.438944574
Skewness	-0.030341884
Range	0.228
Minimum	4.98
Maximum	5.208
Sum	25.482
Count	5
Confidence level (95.0%)	0.108222967

4. Conclusions

The analytical method described in this study is optimized for the determination of the retinol levels from dietary supplements. The UV detection after the separation of the retinoids through HPLC method provides the specificity of this analysis, since a small number of compounds are absorbed at a characteristic wavelength of retinoids. The HPLC method can be used to determine the vitamin A levels even in the dietary supplements wich contain very low concentrations. The products analyzed were consistent with the data reported in the prospectus.

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