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DETERMINATION OF RETINYL PALMITATE IN OINTMENT BY HPLC WITH DIODE ARRAY DETECTION

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Abstract: A simple and rapid HPLC with diode array detection method was developed for the determination of retinyl palmitate present together with other active substances in an ointment. Chromatographic separation was performed on 100 RP-18 Lichrospher column of particle size 5 µm. The mobile phase was methanol:water (98:2, v/v) and flow rate was 2.0 mL/min in isocratic mode. Samples were analyzed for 30 min. Spectophotometric detection was conducted at 325 nm. Under these conditions, the method featured high sensitivity, good precision and comparability of results as proven by the method validation and statistical analysis of the results. The limits of detection and determination were 0.4317 mg/100 mL and 1.3081 mg/100 mL, respectively, recovery values were measured at three levels 80%, 100% and 120% and yielded 101.05%, 101.34% and 100.43%, respectively. The linearity range was checked from 2 mg/100 mL to 10 mg/100 mL. The precision and inter-day precision of the method was expressed by relative standard deviation value and did not exceed 1.68%.

Keywords: retinyl palmitate, HPLC, diode array detection, quantitative determination

Vitamins belong to a group of organic compounds which are essential in very small amounts for the normal growth, self-maintenance and functioning of human and animal bodies. They fulfill different specific and vital functions in metabolism, and their lack or excess produces specific diseases.

Vitamin A is a micronutrient, essential for biological processes such as vision, reproduction, cell growth and differentiation and embryonic development in most mammalian species. The term vitamin A describes a group of lipid-soluble compounds (retinyl esters) related metabolically to *all-trans* retinol. Vitamin A in its various forms functions as a hormone and it is also an essential component of the visual cycle. Deficiency of vitamin A leads to a variety of symptoms in humans, including dryness of the skin, eyes and mucous membranes, retarded development and growth and night blindness, an early symptom commonly used in diagnosis of vitamin A deficiency (1).

Vitamin A combined with other substances of natural origin, such as propolis extract and calendula extract and with broad-spectrum antibiotic bacitracin, accelerates growth of skin cells, protects from infections and purifies injuries from metabolic products.

In the literature, several methods have been proposed for the determination of vitamin A that include flow injection analysis (2), LC-MS (3), RP-HPLC with UV detection (4, 5), RP-HPLC with electrochemical detection (6), HPLC-isotope dilution mass spectrometry (7), and simple HPLC (8-13).

An extraction step prior to determination of vitamins is normally required as sample pretreatment.

The aim of this paper was the development and validation of new RP-HPLC method for the determination of vitamin A in the ointment containing in 100 g standardized calendula flower extract 9.0 g, standardized propolis extract 3.0 g, bacitracin 1.0 g, retinyl palmitate 0.03 g, anhydrous lanolin, flaxseed oil, white vaseline, solid paraffin and cholesterol.

EXPERIMENTAL

Chemicals and reagents

Retinyl palmitate 1,7 mIU/g – DSM Nutritional Products Ltd., lot no. UT05040160. Methanol, water and *n*-hexane of HPLC purity; all manufactured by Merck.

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Table 1. Results from system suitability analysis.

Injection No.	Retinyl palmitate			
	Peak area	Retention time	Theoretical plates	Peak symmetry
1	8258229	19.353	4810	0.909
2	8238033	19.507	4447	0.924
3	8171655	19.487	4510	0.909
4	8215072	19.547	4473	0.893
5	8159164	19.140	4607	0.908
Mean	8208430.6	19.407	4569.4	0.909
RSD	0.52%	0.86%	3.23%	1.21%

Instrumentation and chromatography

The HPLC system consisted of pump L-2130, autosampler L-2200, diode array detector L-2455 and thermostat L-2350 all manufactured by Merck-Hitachi.

Separation was achieved using LiChrosphere 100 RP-18 column (4.6 × 250 mm; 5 µm particles, Merck). The isocratic mobile phase pumped at a flow rate of 2 mL/min consisted of methanol and water (98:2 v/v) freshly prepared. Autosampler was used for the injection of samples. The injection volume was 20 µL and the wavelength for detection was 325 nm. All separations were performed at 40°C for 30 min.

Standard solutions

Stock standard solution of retinyl palmitate was prepared by dissolving 100 mg of drug in 100 mL of *n*-hexane. Standard solutions for linearity (2, 4, 6, 8, 10 mg/100 mL) were prepared by subsequent dilution using *n*-hexane.

Placebo solution

Placebo in the amount of 2 g was shaken with 20 mL of *n*-hexane for 30 min and filtered.

Specificity solution

Retinyl palmitate in the amount of 6 mg was dissolved in 100 ml of *n*-hexane.

Standard solution for quantification

Standard solution was prepared in *n*-hexane by dilution of stock solution of retinyl palmitate to obtain a concentration of 3 mg/100 mL.

Preparation

Ointment containing standardized calendula flower extract 9.0 g, standardized propolis extract 3.0 g, bacitracin 1.0 g, retinyl palmitate 0.03 g, anhydrous lanolin, flaxseed oil, white vaseline, solid paraffin, and cholesterol in 100.0 g was prepared in

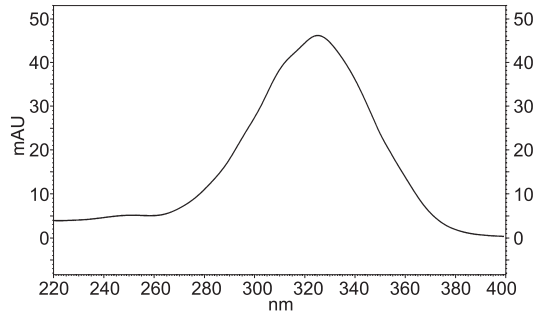

Figure 1. Absorption spectrum of retinyl palmitate

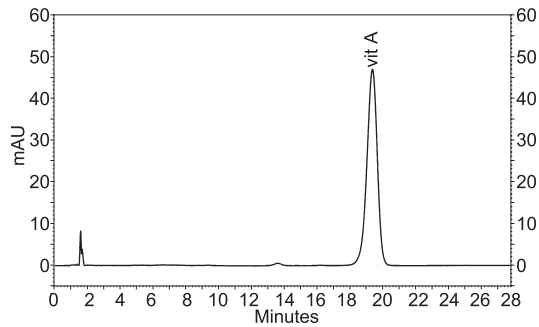

Figure 2. Chromatogram of retinyl palmitate standard solution

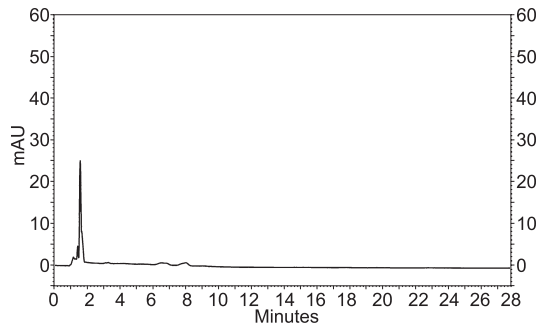

Figure 3. Chromatogram registered for placebo solution

Table 2a. Results from method validation.

Parameter	Retinyl palmitate
RT (n = 5)	19.40 min RSD = 0.86%
Linearity	P = -303 ×10³ + 1440 ×10³ c r = 0.9994 S_e = 1883 × 10²
Linearity range [mg/100 mL]	2 – 10
LOD [mg/100 mL]	0.4317
LOQ [mg/100 mL]	1.3081
Precision [area] RSD	from 3860361 to 4021299 mean 3941439.2 RSD = 1.46% from 8084304 to 8285621 mean 8187418 RSD = 1.12% from 12712714 to 13158456 mean 13041472 RSD = 1.40%
Inter-day precision [area] RSD	from 3847483 to 3944292 mean 3903552.2 RSD = 1.17% from 8040959 to 8355905 mean 8183126.2 RSD = 1.35% from 13064434 to 13638367 mean 13466021.2 RSD = 1.68%
Mean recovery n = 3 80% level 100% level 120% level	 101.05% 101.34% 100.43 %

accordance with the Polish Pharmacopoeia (FP VII). The constituents were weighed with an accuracy of 0.1 mg.

Preparation of solution

The solution was prepared by weighing 1 g of ointment with the accuracy of 0.1 mg and shaking with 10 mL of *n*-hexane in 25 mL flask in an ultrasonic bath for 10 min. The solution was filtered through hard filter paper.

Method validation

The method was validated according to the guidelines set on the International Conference on Harmonisation (ICH) for the validation of analytical procedures (14). The parameters which were used to validate the method of analysis were: system suitability, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, intermediate precision, accuracy, specificity, robustness and stability of solutions.

System suitability

The system suitability parameters: area repeatability, symmetry factor and number of theoretical plates were calculated.

Linearity

Linearity was determined as the relationship of peak areas to concentration. The chromatograms of retinyl palmitate standard solutions were recorded and the changes of peak areas were analyzed within the concentrations of 2 – 10 mg/100 mL. The results were analyzed using the linear regression method. The regression plot, regression equation and the correlation coefficient are indicative of linearity.

Limits of detection and quantitation

Using standard deviation and slope of a straight line coefficient, the values of LOD and LOQ were determined using the following equations: LOD = 3.3×S_e/a, LOQ = 10×S_e/a, where: S_e = standard error of the estimate, a = slope of a straight line.

Precision and inter-day precision

The precision was checked at three levels. Three stock solutions were prepared by weighing 0.3, 0.6 and 0.9 g of retinyl palmitate and dissolving in 100 mL of *n*-hexane. 1 mL of each solution was diluted to 100 mL

Table 2b. Results from method validation. Robustness.

Parameter				
Oven temperature [°C]	RT [min]	Peak area	Theoretical plates	Peak symmetry
36°C	21.467	8145877	3971	0.965
40°C	19.407	8208430	4569	0.909
44°C	17.873	8091129	4607	0.946
Flow rate [mL/min.]	RT min	Peak area	Theoretical plates	Peak symmetry
1.8	21.807	8980563	4527	0.964
2.0	19.407	8208430	4569	0.909
2.2	18.113	7032939	4523	0.959

Table 3. Stability studies of retinyl palmitate stock solution.

Time	Temperature 8°C		Room temperature	
	Peak area	[%]	Peak area	[%]
0	9228980 9241322 9089099 mean 9186467	100,00	9820004 9920637 10033711 mean 9924784	100,00
24 h	9100467 9147231 9252711 mean 9166803	99,79	9891139 9843768 9736271 mean 9823726	98,98
48 h	9092498 9140627 9149305 mean 9127477	99,36	9866924 9757005 9861983 mean 9828637	99,03
7 days	9124002 9141039 9209299 mean 9158113	99,69	9645414 9802678 9829530 mean 9759207	98,33

Table 4. Results of quantitative HPLC analyses of retinyl palmitate in the ointment.

Retinyl palmitate concentration mg/100 mL			
Sample no.	n = 3	oncentration	
1	mean	2.838	Declared content 3 mg/100 mL
	RSD %	0.299	
2	mean	2.794	
	RSD %	0.340	

with *n*-hexane and five measurements were carried out for each solution. The precision was expressed as the consistency of results from repeated analyses. Peak areas were used to evaluate method precision.

The same methodology was applied for checking inter-day precision, but chromatograms were registered the next day.

Accuracy

Accuracy was determined by quantitative determination of retinyl palmitate added to model mixture at three levels 80%, 100% and 120%. Recovery was calculated on the basis of determined content of retinyl palmitate to weighed amount added to model mixture.

Specificity

Specificity of the method was evaluated by comparing chromatograms registered for mobile phase, placebo solution and retinyl palmitate standard solution.

Robustness

Effect of flow rate variations (± 0.2 mL/min) and temperature of column oven (± 4°C) on the obtained results was checked.

Stability of solutions

Stability of stock solution was determined by the analysis of solutions that were stored at room temperature and in refrigerator at 8°C. Analysis was carried out for freshly prepared solution and after storage for 1, 2, and 7 days.

Quantitative analysis

Volumes of 20 µL standard and sample solutions were introduced onto the column five times. Isocratic elution was carried out under conditions specified by using mobile phase of the composition described above. Peak areas were registered and used for the calculation of retinyl palmitate content.

RESULTS AND DISCUSSION

The development of new, simple and precise methods for the determination of active substances is still an important problem due to creating new pharmaceutical formulations with complex matrix.

The chromatographic conditions were optimized and determination was performed on a 100 RP-18 Lichrospher column using a mobile phase consisting of methanol and water 98:2, v/v with flow rate of 2.0 mL/min in isocratic mode.

Analytical wavelength $\lambda = 325$ nm was chosen based on the absorption spectrum registered in UV with the application of DAD detector (Fig. 1).

The results of system suitability test for the determination of retinyl palmitate are presented in Table 1.

The obtained results met the acceptance criteria. Repeatability of peak area for five replicates described by RSD was 0.52%, repeatability of retention time for five replicates was described by RSD = 0.86%, the mean value of theoretical plates was 4569.4 (RSD = 3.23% for n = 5) and the mean value of peak symmetry was 0.909 (RSD = 1.21% for n = 5).

The applied mobile phase composition allowed suitable retention time of retinyl palmitate and good selectivity towards interference from the excipients and other components of the ointment was achieved (Fig. 2, 3). Standardized calendula flower extract, standardized propolis extract, bacitracin, anhydrous lanolin, flaxseed oil, white vaseline, solid paraffin and cholesterol did not give any peaks at the retention time of retinyl palmitate (19.4 min. under developed conditions).

The specificity of the proposed method demonstrates that the excipients present in an ointment do not interfere with the drug peak. Thus the proposed method may be useful for the quantitative determination of retinyl palmitate in the examined ointment.

Calibration curves were constructed using three series of standard retinyl palmitate solutions in the range 2 – 10 mg/100 mL. The equation of linear regression $P = -303 \times 10^3 + 1440 \times 10^3 c$ and statistical data $S_e = 1883 \times 10^2$ describe the relationship between peak area and concentration. The linearity of calibration curve was described by high value of correlation coefficient r = 0.9994.

Low values of LOD 0.4317 mg/100 mL and LOQ 1.3081 mg/100 mL indicate that the method is sensitive.

The precision and inter-day precision were checked at three levels. Statistical evaluation revealed that the method is highly precise, RSD was in the range 1.12% – 1.68%.

Recovery results, measured at three levels 80%, 100% and 120% were 101.05%, 101.34% nad 100.43%, respectively, which prove the suitability and accuracy of the proposed method.

Little changes of flow rate and column oven temperature did not cause any significant changes when analyzing peak areas, retention time, theoretical plates or peak symmetry (Tab. 2a, b).

Stability studies for stock solution revealed that no significant changes (0.31% after 7 days of storage at 8˚C and 1.67% after storage at room temperature) were observed for stock solution concentration (Tab. 3).

The developed and validated method has been applied for the quantitative determination of retinyl palmitate in the examined ointment. The obtained results are close to the declared content of retinyl palmitate in the ointment from 2.794 mg/100 mL to 2.838 mg/100 mL with RSD values 0.299% and 0.340%, respectively, for n = 3 (Tab. 4).

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