Validation of a High-Performance Liquid Chromatography method for the determination of vitamin A, vitamin D_3, vitamin E and benzyl alcohol in a veterinary oily injectable solution

Validarea unei metode de cromatografie lichidă de înaltă performanță pentru determinarea vitaminei A, vitaminei D_3, vitaminei E și alcoolului benzilic într-o soluție injectabilă uleioasă de uz veterinar

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Key words: *vitamin A, vitamin D3, vitamin E, validation, HPLC*
Cuvinte cheie: *vitamina A, vitamina D3, vitamina E, validare, HPLC*

Abstract

A new simple, rapid, accurate and precise high – performance liquid chromatography (HPLC) method for determination of vitamin A, vitamin D_3, vitamin E and benzyl alcohol in oily injectable solution was developed and validated. The method can be used for the detection and quantification of known and unknown impurities and degradants in the drug substance during routine analysis and also for stability studies in view of its capability to separate degradation products. The method was validated for accuracy, precision, specificity, robustness and quantification limits according to ICH Guidelines. The estimation of vitamin A, vitamin D_3, vitamin E and benzyl alcohol was done by Waters HPLC system manager using gradient pump system. The chromatographic conditions comprised a reverse-phased C18 column (5 µm particle size, 250 mm×4.6 mm i.d.) with a mobile phase consisting of tetrahydrofurane, acetonitrile and water in gradient elution. The flow rate was 0.8 ml/min and 2.0 ml/min. Standard curves were linear over the concentration range of 16.50 µg/ml to 11.00 mg/ml for vitamin A, 10.05 µg/ml to 6.70 mg/ml for vitamin E, 0.075 µg/ml to 0.050 mg/ml for vitamin D_3 and 1.25 mg/ml to 5.00 mg/ml for benzyl alcohol. Statistical analyses proved the method was precise, reproducible, selective, specific and accurate for analysis of vitamin A, vitamin D_3, vitamin E, benzyl alcohol and impurities.

Rezumat

A fost realizată și validată o metodă de cromatografie lichidă de înaltă performanță simplă, rapidă și precisă pentru determinarea vitaminei A, vitaminei D_3, vitaminei E și alcoolului benzilic într-o soluție injectabilă uleioasă. Metoda poate fi utilizată pentru detectarea și cuantificarea impurităților cunoscute și necunoscute și degradate în substanțe medicamentoase în timpul analizei de rutină cât și pentru studiile de stabilitate în ceea ce privește capacitatea de separare a produșilor de degradare. Metoda a fost validată pentru acuratețe, precizie, specificitate, robustețe și limite de cuantificare conform Ghidurilor ICH. Evaluarea vitaminei A, vitaminei D_3, vitaminei E și acoolului benzilic s-a realizat prin sistemul de purificare a apei HPLC prin utilizarea sistemul de pompare a gradientului. Condițiile cromatografice cuprind o coloană C18 cu fază inversă (dimensiunea particulelor de 5 µm, 250 mm×4.6 mm i.d.) cu o fază mobilă constând din tetrahidrofuran acetonitril și apă în eluție de gradient. Debitul a fost de 0,8 ml/min și 2,0 ml/min. Curbele standard au fost liniare depășind intervalul de concentrație de 16,50 µg/ml până la 11 mg/ ml pentru vitamina A, 10,05 µg/ml la 6,70 mg/ml pentru vitamina E, 0,075 µg/ml la 0,050 mg/ml pentru vitamina D_3 și 1,25 mg/ml la 5,00 mg/ml pentru alcoolul bezilic. Analizele statistice au arătat că metoda a fost precisă, reproductibilă. selectivă, specifică și exactă pentru analiza vitaminei A, vitaminei D_3, vitaminei E, alcoolului benzilic și impurităților.

1. Introduction

Vitamins are essential micronutrients needed by the body in small amounts for unique roles in maintaining normal cell function, growth and development.

Vitamins presented in this method (vitamin A, vitamin D_3 and vitamin E) are fat-soluble vitamins, which means that they are stored in fat tissue after the body uses what it needs.

This can lead to the accumulation of fat-soluble vitamins in the body if there are large intakes of drugs containing them.

Fat-soluble vitamins stored in the body lead to hypervitaminosis, a potentially dangerous condition. This problem does not arise in the case of water-soluble vitamins, as the excess is eliminated quickly through urine.

Quantitative analysis of multivitamin compositions is still a challenge for analytical chemists. One of the main difficulties is related to the highly reactive nature of vitamins and quantitative isolation of each component [1].

The analytical procedure for the estimation of vitamins is very laborious and long-lasting [2-6].

Several methods have been proposed for the determination of water-soluble and fat-soluble vitamins in the same chromatographic run [7, 8].

However, there is no indicating HPLC method that allows the separation, assay and purity determination of vitamin A, vitamin D_3, vitamin E and benzyl alcohol in an oily injectable solution in one chromatographic run. The main difficulties in developing the method were the separation of benzyl alcohol from phenol and the long elution time for vitamin A.

Vitamin A is usually separated in a normal phase chromatographic column, as it is fat-soluble, but by using tetrahydrofurane in the mobile phase composition, the separation was possible on a reverse phase column.

Phenol and benzyl alcohol were separated by using a mobile phase consisting of water and acetonitrile (50:50) which allowed for a resolution higher than 1.5 between them.

The method was validated for: accuracy, precision, specificity, robustness and quantification limits according to ICH Guidelines [9, 10].

The aim of the present work is to develop an accurate, specific, reproducible method for determination of vitamin A, vitamin D_3, vitamin E and benzyl alcohol in the same chromatographic condition.

2. Materials and methods

2.1. Chemicals and reagents

Vitamin A was obtained from USP, vitamin D_3, vitamin E and benzyl alcohol were obtained from EDQM. Acetonitrile, n-hexane and tetrahydrofurane, all of reagent grade, were obtained from Merck. Purified water was also used.

2.2. Apparatus

The apparatus used was HPLC Waters separation modules 2695 consisted of auto injector and Waters 2696 PDA detector. The column used was Thermo BDS Hypersil – C18, 250 mm x 4.6 mm, 5 µm particle size.

2.3. Chromatographic conditions

Chromatographic separation was achieved at 25 °C on a reversed phase column using a mobile-phase consisting of solvent A (tetrahydrofurane), solvent B (acetonitrile), and solvent C (water). Gradient elution was performed slowly from 0 to 5 minutes 50% B and 50% C; 5-15 minutes 100% B; 15-28 minutes 25% A and 75% B; 28-35 minutes 50% B and 50% C. The flow rate was kept at 0.8 ml/min from 0-5 minutes and 28-30 minutes and 2.0 ml/min from 5-28 minutes and detection was performed at 265 nm for vitamin D_3, benzyl alcohol and related substances for vitamin D_3, 285 nm for vitamin E and related substances for vitamin E and 325 nm for vitamin A and related substances for vitamin A. The injection volume was 1 µl for the standards and samples of vitamin A and 50 µl for the standards and samples of vitamin E, vitamin D_3 and benzyl alcohol.

2.4. Method Validation

Method validation parameters studied were limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy,

precision, repeatability, robustness and specificity.

The LOQ was defined as the lowest concentration that could be determined with acceptable accuracy and precision. The LOD and LOQ were determined from signal to noise response.

The linearity was determined by calibration curve. For the construction of calibration curve, five calibration standard solutions were prepared for assay and five calibration standard solutions were prepared for purity. The precision and repeatability were estimated by assaying six replicate samples on day-1 and day-2. The accuracy was evaluated by the recovery determination.

The specificity was evaluated by force degradation of the samples. The samples were subjected to acid and basic degradation, thermal degradation and hydrogen peroxide degradation.

2.5. Preparation of Solution

Standard preparation: A standard solution for assay of vitamin A, vitamin E and vitamin D_3 was prepared by accurately transferring 55.0 mg vitamin A RS, 33.5 mg vitamin E RS, 25.0 mg benzyl alcohol and 1 ml of vitamin D_3 standard stock solution (0.25 mg/ml vitamin D_3) into 10 ml volumetric flask. Dissolve and dilute to volume with hexane. A standard solution for purity was prepared by diluting standard solution for assay to give a final concentration of 55.0 ppm vitamin A, 33.5 ppm vitamin E and 0.25 ppm vitamin D_3

Sample preparation: The sample solution was prepared by accurately transferring 1.0 ml oily solution with a syringe into a 10 ml volumetric flask. Dilute to volume with hexane.

3. Results and discussion

LOD and LOQ: Limit of detection and Limit of quantification were determined.

The results are shown in Table 1.

Table 1

LOD and LOQ results

Vitamin	LOD	LOQ
Vitamin A	0.160 µg/ml	0.533 µg/ml
Vitamin D3	0.0006 µg/ml	0.0021 µg/ml
Vitamin E	0.090 µg/ml	0.301 µg/ml

Linearity: The method was found to be linear for the concentration range described.

When average peak areas were plotted against concentration levels, good correlation coefficients (r^2) were obtained.

The results are shown in Table 2.

Table 2

Correlation coefficients for the two compounds

Vitamin / component	Correlation coefficient r^2	
	Assay	Related substances
Vitamin A	0.9994	0.9993
Vitamin D_3	0.9996	0.9994
Vitamin E	0.9995	0.9999
Benzyl alcohol	0.9997	0.9998

Accuracy: The current method is valid and accurate. Accuracy was evaluated by the recovery determination.

The method's accuracy was determined by spiking working standards of vitamin A, vitamin D_3, vitamin E and benzyl alcohol into placebo at different concentration levels: 80, 100 and 120% of target concentration of each of the compounds for purity and assay.

The absolute recovery was determined in triplicate by direct comparison of peak area from standard versus sample. The data was analyzed statistically by calculating RSD.

The results are shown in Table 3.

Table 3

Accuracy results

Vitamin / component	Recovery (%)	
	Assay	Related substances
Vitamin A	95.0-101.7	95.2-104.3
Vitamin D_3	99.1-102.6	97.4-102.8
Vitamin E	96.3-101.5	97.6-100.6
Benzyl alcohol	97.9-100.5	-

Precision: Precision was measured in terms of repeatability of application and measurement data. In order to evaluate the method's capability to produce similar results on repetitive test for nominal concentration, six individual samples from vitamin A, vitamin D_3, vitamin E and benzyl alcohol were tested separately.

It showed low relative standard deviations for the four compounds. Intermediate precision of the method was carried out in six different sample preparations by a different analyst, in two different days.

The value of RSD is below 2% for the two compounds; intermediate precision of the method is established.

The results are shown in Table 4.

Table 4

Precision results

Vitamin / component	Repeatability, %RSD	Intermediate precision, %RSD
Vitamin A	1.6	0.2
Vitamin D_3	1.4	0.4
Vitamin E	0.7	0.8
Benzyl alcohol	1.1	0.9

Specificity: The specificity of the method was ascertained by analyzing spiked samples under stress condition. The specificity has been investigated to demonstrate that there is no interference between excipients, active ingredients and degradation compounds that may be present in samples.

The stressed condition samples are evaluated relative to the control sample with respect to assay and degradation (%).

The presence of other ingredients in the formulations did not cause any interference with the vitamin A, vitamin D_3 and vitamin E.

Robustness

Robustness of the method was determined by analyzing the same standards at normal operating conditions and also by changing some analytical conditions such as detection wavelength, mobile phase composition and debit.

The results obtained showed that the method is robust. The results are shown in Table 5.

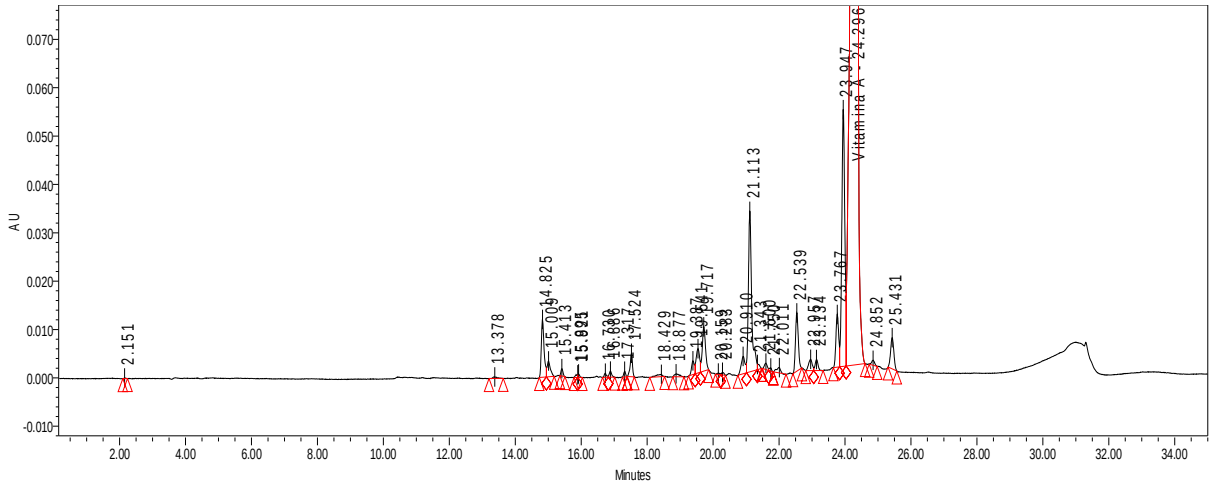

Fig. 1 Chromatogram of the acid-exposed sample

Table 5

Robustness parameters and resolution

Parameter	*Variation*	*Resolution*	
		Between benzylic alcohol and phenol	**Between vitamin D_3 + previtamin D_3 and vitamin E**
Aqueous solvent in mobile phase composition on the isocratic elution	+10% (55 : 45)	2.6	1.7
	-10% (45: 55)	1.7	1.9
Debit of the HPLC method	+ 0.2 ml/min (1.0 ml/min)	2.1	1.9
	-0.2 ml/min (0.6 ml/min)	2.1	1.8
Wavelength change	+ 2 nm (267 nm)	1.7	2.1
	- 2 nm (263 nm)	**1.7**	**2.1**

Conclusion

The aim of this study was to develop a selective and sensitive HPLC method for the rapid detection of vitamin A, vitamin D_3, vitamin E and benzyl alcohol in the same chromatographic conditions. The proposed method was found to be rapid, accurate, repeatable, specific and robust.

This method was applied for the analysis of the drug product in marketed formulations and could be used for the routine analysis of the drug product.

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