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Research Article

DEVELOPMENT AND VALIDATION OF NEW RP-HPLC METHODS FOR STABILITY STUDY OF FAT SOLUBLE VITAMINS

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ABSTRACT

Objective- A simple reversed phase HPLC method was developed for the determination of Vitamin A and Vitamin E present in bulk and pharmaceutical dosage forms.

Methods- An Alltech Prevail-C_{18} 150 X 4.6 mm, 5 µm column with mobile phase Acetonitrile: Methanol (75:25) was used. The flow rate was 1.0 ml/min and effluent was monitored at 220 nm.

Results-The retention times were 3.41 min and 8.74 min for vit.A and vit.E respectively. The linearity range was found to be 1-200 µg/ml for vit.A and 1-500 µg/ml for vit.E respectively.

Conclusion-The proposed method was validated for linearity, precision and accuracy. The stability of these vitamins was studied for the period of two month.

Keywords: Vitamin A, Vitamin E, HPLC, Precision.

INTRODUCTION

Vitamin A is an essential human nutrient. It exists not as a single compound, but in several forms. In foods of animal origin, the major form of vitamin A is an alcohol (retinol), but can also exist as an aldehyde (retinal), or as an acid (retinoic acid). Precursors to the vitamin (a provitamin) are present in foods of plant origin as some of the members of the carotenoid family of compounds. All formals of vitamin A have a Beta-ionone ring to which an isoprenoid chain is attached. This structure is essential for vitamin activity. Retinol, the animal form of vitamin A, is a yellow, fat-soluble, vitamin with importance in vision and bone growth. Vitamin E is a fat-soluble vitamin that exists in eight different forms. Each form has its own biological activity, which is the measure of potency or functional use in the body. Alpha-tocopherol (α-tocopherol) is the name of the most active form of vitamin E in humans. It is also a powerful biological antioxidant. Vitamin E in supplements is usually sold as alpha-tocopheryl acetate, a form of alpha-tocopherol that protects its ability to function as an antioxidant. The synthetic form is labeled "D, L" while the natural form is labeled "D". The synthetic form is only half as active as the natural form.

An extensive literature survey revealed the availability of many methods which includes simultaneous assessment of the status of lipid-soluble vitamins; retinol, α-tocopherol, 25 hydroxyvitamin D3 and 24, 25 dihydroxyvitamin D3 in serum of blood donors [1] and other analytical methods [2-10]. In the present research work, an attempt was made to develop a simple and precise analytical method for the determination of Vitamin A and Vitamin E, respectively.

MATERIALS AND METHODS

Instrumentation

Quantitative HPLC was performed on a Waters Separation Module 2695, with an auto injector, and Waters 2696 PDA detector. The output signal was monitored and integrated using Empower software. An Alltech Prevail-C18 150 X 4.6 mm, 5 µm column was used.

Reagents used

Acetonitrile HPLC grade (Fisher scientific)

Methanol HPLC grade (Fisher scientific)

Optimization of the method

To ascertain the maximum wavelength, λ_{max} of the proposed method, the drug solution (50 µg/ml) was scanned between the wavelength ranges of 200 – 380 nm. The λ_{max} was found to be 220nm and selected 220 nm so that we can get symmetrical peaks without interferences. To develop a suitable and robust HPLC method for the determination of vit.A and vit.E, different mobile phases methanol: water, Acetonitrile:water, Acetoniotrile:Methanol: were used in different compositions of the mobile phases (30:70, 40:60, 50:50, 70:30, 80:20) at different flow rates (1.0, 1.2, 1.5, 1.8 ml/min). The mobile phase Acetonitrile: Methanol in the ratio of 75:25 at flow rate of 1.0 ml/ min gave sharp peaks with minimum tailing and good resolution. Vitamin A, vitamin E were eluted at retention times around 3.41, 9.07 respectively with symmetric peak shape. Optimized chromatographic conditions were shown in table 1.

Table 1: Optimized chromatographic conditions

Parameters	Method
Stationary phase (column)	Alltech Prevail-C18 150 X 4.6 mm, 5 µm
Mobile Phase	Acetonitrile:Methanol(75:25)
Flow rate (ml/min)	1.0 ml
Run time (minutes)	15
Column temperature (°C)	Ambient
Volume of injection loop (µl)	10
Detection wavelength (nm)	220
Drugs RT (min)	3.41 Vit. A 9.07 Vit E

RESULTS AND DISCUSSION

Preparation of standard drug solution

Stock solution of the drugs (pure) prepared by dissolving 25 mg and 2.5mg each of Vit.E and Vit A respectively in 100ml volumetric flasks to this 70ml of mobile phase was added and sonicated for 15 minutes then the volume was made up to 100 ml with mobile phase to give final concentration of 250ppm and 25ppm of vit. E and vit. A respectively.

Preparation of sample drug solution

The sample solution was prepared by taking tablets. The twenty tablets were powdered and powder equivalent to average wt. of 20 tablets was

taken in 100 ml volumetric flask and this 70ml of mobile phase was added, sonicating up to 15 min and the final volume was made to 100 ml mobile phase. This solution was filtered through a 0.45µm and and further analyzed by using above mention HPLC condition.

Procedure for calibration curve:

The contents of the mobile phase were filtered before use through Whattman filter paper, and pumped from the respective solvent reservoirs to the column at a specified flow rate. Prior to injection of the drug solutions, the column was equilibrated for at least 60 min with the mobile phase flowing through the systems. Ten micro liters of each of standard and sample solutions were injected into the HPLC system for three times to get the chromatograms. The retention time, were recorded. A graph was plotted by taking conc. on x- axis and peak area on y axis. The linearity was found to be in between 1-200 µg/ml for vit.A and 1-500 µg/ml for vit.E respectively. The linearity range and linearity graphs were shown in table 2 and figure 1 & 2 respectively.

Table 2: Linearity of vitamin A and vitamin E

Concentration in ppm	Area under curve			
	Average	Injection 1	Injection 2	Injection 3
1	12322	12149	12458	12359
5	59944.67	60315	59268	60251
10	122089	121524	123856	120887
20	241910	241568	242597	241565
50	621313	612571	625489	625879
100	1202640	1198461	1204562	1204897
200	2367644	2345675	2355689	2401567

Concentration in ppm	Area under curve			
	Average	Injection 1	Injection 2	Injection 3
10	1386673.7	1391254	1381521	1387246
20	2862000.3	2864597	2864913	2856491
50	7013667.7	7041253	7054129	6945621
100	14059113	14025781	14125879	14025678
150	20495313	20985461	20345619	20154859
250	34647830	34812659	34561918	34568912
500	69210928	68594879	69584925	69452979

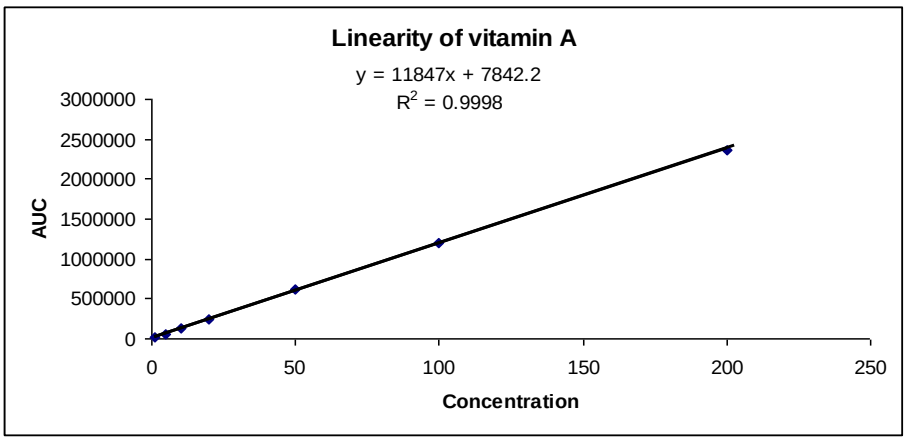

Fig. 1: Linearity Graph of Vitamin A

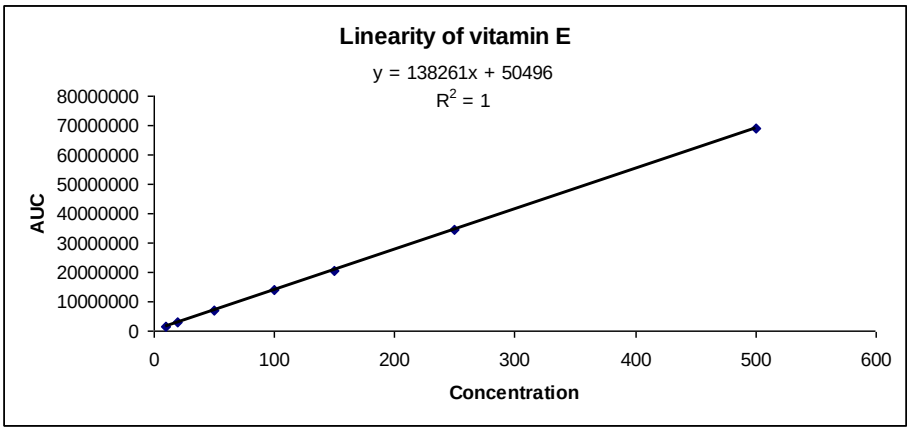

Fig. 2: Linearity Graph of Vitamin E

Analysis of Formulations

The amount of drugs present in each pharmaceutical formulation was calculated through peak area of drugs by using the standard calibration curve (concentration in µg/ml was taken on x-axis and peak area ratio on y-axis). The results were shown in table 3. A typical chromatogram of vit.A and vit.E in bulk and formulation was shown in figure 3 & 4 respectively -. The present work is to multivitamin tablets with different binder level, the binders are Plasdone S-630, Kollidon VA-64, HPC and Starch 1500. This study was undertaken to estimate key vitamins Assay percentage.

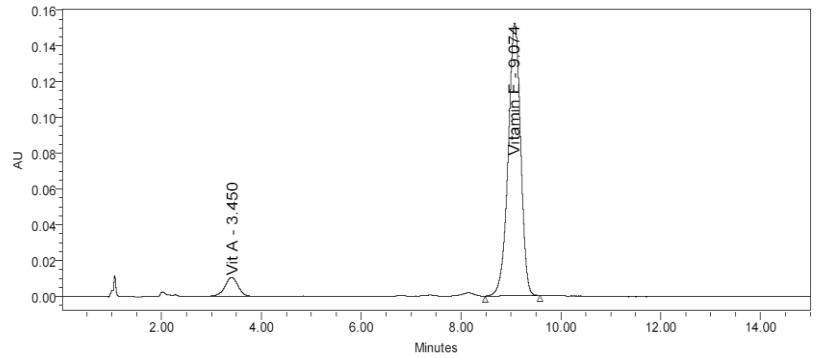

Fig. 3: Typical chromatogram of Vitamin A and Vitamin E (Standard Drug)

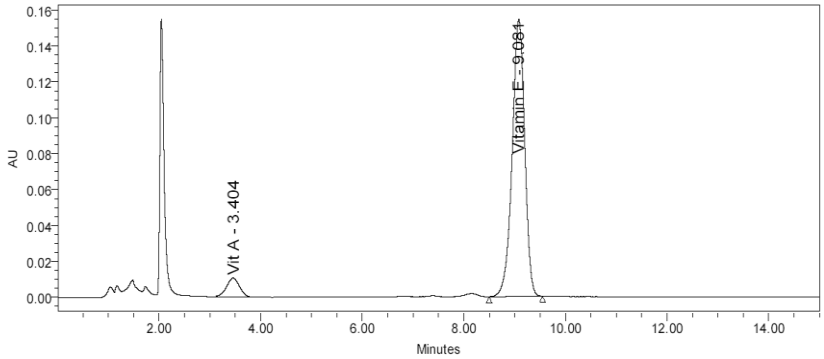

Fig. 4: Typical chromatogram of Vitamin A and Vitamin E (Formulation)

Table 3: Assay of Vitamin A & E zero day

Vitamin A&E Zero day			
Serial No.	**Sample Details**	**% Assay vit.A**	**% Assay vit.E**
1	Control	98.55	99.15
2	Plasdone S-630	99.12	99.54
3	HPC	99.23	99.43
4	VA-64	100.46	99.23
5	Starch 1500	99.53	99.25

Table 4: Assay of Vitamin A & E one month

Vitamin A&E one month 25° 60RH			
Serial No.	**Sample Details**	**% Assay vit.A**	**% Assay vit.E**
1	Control	98.23	100.25
2	Plasdone S-630	99.59	100.88
3	HPC	101.29	101.59
4	VA-64	100.54	100.56
5	Starch 1500	99.66	100.36
Vitamin A&E one month 30° 65RH			
Serial No.	Sample Details	% Assay vit.A	% Assay vit.E
1	Control	99.33	99.56
2	Plasdone S-630	100.52	100.36
3	HPC	100.65	100.57
4	VA-64	99.11	100.74
5	Starch 1500	98.44	99.45
Vitamin A&E one month 40° 75RH			
Serial No.	Sample Details	% Assay vit.A	% Assay vit.E
1	Control	96.35	99.96
2	Plasdone S-630	99.82	100.11
3	HPC	100.42	100.84
4	VA-64	98.85	100.54
5	Starch 1500	97.34	100.84

Table 5: Assay of Vitamin A & E second month

Vitamin A&E second month 25° 60RH			
Serial No.	**Sample Details**	**% Assay vit.A**	**% Assay vit.E**
1	Control	98.59	100.38
2	Plasdone S-630	97.55	101.55
3	HPC	104.67	104.37
4	VA-64	99.34	102.01
5	Starch 1500	97.12	101.55
Vitamin A&E second month 30° 65RH			
Serial No.	Sample Details	% Assay vit.A	% Assay vit.E
1	Control	96.11	101.34
2	Plasdone S-630	98.77	102.38
3	HPC	98.13	104.08
4	VA-64	100.27	101.12
5	Starch 1500	98.89	102.64
Vitamin A&E second month 40° 75RH			
Serial No.	Sample Details	% Assay vit.A	% Assay vit.E
1	Control	97.65	101.34
2	Plasdone S-630	99.01	102.09
3	HPC	96.34	103.56
4	VA-64	96.28	102.67
5	Starch 1500	96.44	101.89

Method validation parameters

Linearity

The linear fit of the system was illustrated graphically. Least square regression analysis was carried out for the slope, intercept and correlation coefficient. The results are presented in table 2 (b).

Precision

The precision of each method was ascertained separately from the peak area obtained by actual determination of six replicates of a fixed amount of drug. The percent relative standard deviation and percentage range of errors (at 0.05 and 0.01 confidence limits) were calculated for vit.A and vit.E and were presented in the table. The precision of the assay was also determined in terms of intra-and inter-day variation in the peak areas for a set of drug solutions on three different days. The intra-and inter-day variation in the peak area of the drug solution was calculated in terms of %RSD and the results are presented in the table 6.

Accuracy

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of vit.A and vit.E bulk samples of along within the linearity range were taken and added to the pre-analyzed formulation. From that percentage recovery values were calculated. The results were shown in table 7.

Table 6: Precision of the proposed method

Vitamin A STD		
S. No.	**RT**	**AUC**
1	3.412	614565
2	3.421	612549
3	3.422	609856
4	3.425	609034
5	3.425	606798
6	3.421	608715
AVG	3.42	610252.83
STDEV	0.00	2821.74
%RSD	0.14	0.46

Vitamin E STD		
S. No.	**RT**	**AUC**
1	9.071	33894561
2	9.078	33265948
3	9.075	33164578
4	9.074	33124578
5	9.072	33461569
6	9.079	33791934
AVG	9.07	33450528.00
STDEV	0.00	327391.46
%RSD	0.04	0.98

Table 7: Accuracy study of the proposed method

Sample ID	**Conc. of drug (mcg/ml)**				**%Recovery of pure drug**		**Statistical Analysis**		
	Pure drug		**Formulation**						
	Vit.A	**Vit.E**	**Vit.A**	**Vit.E**	**Vit.A**	**Vit.E**		**Vit.A**	**Vit.E**
S_1: 80 %	20	200	25	250	98.81	100.01	Mean	99.22	99.93
S_2: 80 %	20	200	25	250	99.41	99.86	SD	0.35	0.07
S_3: 80 %	20	200	25	250	99.45	99.91	%RSD	0.36	0.07
S_4: 100%	25	250	25	250	98.22	99.92	Mean	98.23	100.11
S_5: 100%	25	250	25	250	98.24	100.33	SD	0.01	0.20
S_6: 100%	25	250	25	250	98.22	100.10	%RSD	0.01	0.20
S_7: 120%	30	300	25	250	99.41	100.16	Mean	99.49	100.02
S_8: 120%	30	300	25	250	99.45	99.81	SD	0.0094	0.18
S_9: 120%	30	300	25	250	99.61	100.10	%RSD	0.0094	0.18

System suitability parameter

System suitability parameters can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. (Or) The USP (2000) defines parameters that can be used to determine system suitability prior to analysis. The system suitability parameters like Theoretical plates (N), Resolution (R), Tailing factor (T), LOD (µg/ml) and LOQ (µg/ml) were calculated and compared with the standard values to ascertain whether the proposed RP-HPLC method for the estimation of vit.A and vit.E in pharmaceutical formulations was validated or not. The results were shown in table 8.

Table 8: System suitability parameters

S. No.	Parameters	Vitamin A	Vitamin E
1	Theoretical plates (N)	4652	5643
2	Resolution (R)	3.54	3.27
3	Tailing	1.25	1.28
4	LOD(ppm)	0.04	0.06
5	LOQ(ppm)	0.12	0.18

RESULTS AND DISCUSSION

From the linearity table 2, it was found that the drug obeys linearity within the concentration range of 1-200 µg/ml for vit.A and 1-400 µg/ml for vit.E. From the results shown in precision table- 6, it was found that % RSD is less than 2%; which indicates that the proposed method has good reproducibility. From the results shown in accuracy table- 7, it was found that the percentage recovery values of pure drug from the pre-analyzed solutions of the formulations were in between 95-105%, which indicates that the method is accurate and also reveals that the commonly used excipients and additives present in the pharmaceutical formulations were not interfering the proposed method. The system suitability parameters also reveal that the values were within the specified limits for the proposed method. The proposed method stability was analyzed for the period of two months with different temperature and humidity conditions and the results are depicted in the table 4 & 5, respectively.

CONCLUSION

The proposed method was found to be simple, precise, accurate and rapid for simultaneous determination of vit.A and vit.E from pure and its dosage forms. The mobile phase is simple to prepare and economical. The sample recoveries in formulation was in good agreement with their respective label claims and they suggested non – interference of formulation excipients in the estimation. Hence, the method can be easily and conveniently adopted for routine analysis of vit.A and vit.E in combined dosage forms and can also be used for dissolution or similar studies. It was found that the assay percentage of fat soluble vitamins remained unchanged when compare to day zero values and are within the range.

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