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"ANALYTICAL METHOD VALIDATION OF VITAMIN-A ACETATE IN SUNFLOWER BY LIQUID CHROMATOGRAPHY METHOD (RP-HPLC)"

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ABSTRACT

A simple, efficient and reproducible RP-HPLC method for the determination of Vitamin A acetate in sunflower oil has been developed and validated. The Chromatographic Separation was carried out on C18 Column (inertsil ODS) column using the mobile phase consists of Methanol: Acetonitrile (70:30). The mobile phase was flowed at the rate of 1.0 ml/min and effluent was detected at 325 nm. The retention times of Vitamin A acetate was 2.928 min. The method was validated according to ICH guidelines and the acceptance criteria for system suitability, specificity, linearity, precision, robustness and ruggedness were met in all cases. The method was linear in the range of 0.05-10 ppm of the operating concentration of Vitamin A acetate (r2 = 0.998)). The percentage relative standard deviation for Intermediate precision was found to be less than 2.0%. Hence, the method could be successfully applied for routine analysis of Vitamin A acetate from Sunflower oil.

Introduction:

Vitamin A Acetate or Retinol and derivatives of retinol that play an essential role in metabolic functioning of the retina, the growth of and differentiation of epithelial tissue, the growth of bone, reproduction, and the immune response. Dietary vitamin A is derived from a variety of carotenoids found in plants. It is enriched in the liver, egg yolks, and the fat component of dairy products. Retinyl acetate (retinol acetate, vitamin A acetate) is a natural form of vitamin A which is the acetate ester of retinol. Vitamin A is critical for vision as an essential component of rhodopsin, a protein that absorbs light in the retinal receptors, and because it supports the normal differentiation and functioning of the conjunctival membranes and cornea. Vitamin A also supports cell growth and differentiation, playing a critical role in the normal formation and maintenance of the heart, lungs, kidneys, and other organs. It has potential

antineoplastic and chemo preventive activities.

Analytical chemistry is a branch of chemistry that deals with the separation, identification and determination of components in a sample. In this present work, an attempt was made to develop a simple, feasible and simultaneous determination of Vitamin A acetate in a sunflower by RP-HPLC. A simple reversed phase HPLC method was developed for the determination of Vitamin A in sunflower.

Materials and Methods:

Experimental:

Chemicals:

Acetonitrile (HPLC Grade), Methanol (HPLC Grade), Ethanol selected as solvent for developing spectral characteristics of the oil. The selection was made after assessing the solubility of oil in different solvents. Sunflower oil was selected for evaluating Vitamin A Acetate.

Instrument Tools:

HPLC (Dionex Ultimate 3000) instrument with in line degasser, auto sampler, Injector (100uL capacity), column oven temperature compartment and UV-visible detector. Data acquisition performed on Chromeleon software, separation and quantitation were made on C18 column (Inertsil ODS) 4.6×250mm (5um particle size). The detector was set at 325 nm.

PREPARATION OF STANDARD VITAMIN A (Acetate):

Weight and transfer accurately 10mg of vitamin A acetate in10ml volumetric flask. Add 2ml of ethyl acetate into it; mix well and vortex it for 1-2 minute. Add diluent up to 80% capacity of flask; mix well and sonicate it for 10-12 minute with vigorous shaking.

Chromatographic Conditions:

Selection and optimization of wavelength:

Many HPLC trials were carried out for the selection and optimization of wavelength. The suitable wavelength i.e. 325 nm was selected.

Separation Variables:

In this trial chromatographic condition shown in table no. 1. Methanol & Acetonitrile in the ratio 70:30%v/v gives good resolution of peaks with acceptable peak symmetry, as compare to other mobile phases. Methanol & Acetonitrile in the ratio of 70:30.

Table no 1: Selection of separation variable:

Parameter	**Value**
Column	C18 Column (inertsil ODS)
Dimensions	4.6×250mm
Particle size	5 um
Mobile phase	Methanol: Acetonitrile
Flow rate	1ml/min
Retention time	30 min
Column temperature	Ambient
Injection volume	20 ul
Detection wavelength	325 nm

PREPARATION OF STANDARD VITAMIN A (Palmitate):

Weight and transfer accurately 10mg of vitamin A Palmitate in10ml volumetric flask. Add 2ml of ethyl acetate into it; mix well and vortex it for 1-2 minute. Add diluent up to 80% capacity of flask; mix well and sonicate it for 10-12 minute with vigorous shaking.

PREPARATION MIX STANDARD:

Pipette out accurately 1ml of standard vitamin A (Acetate) & 1ml of standard Vitamin A (Palmitate) transfer it into 10ml volumetric flask; make up volume up to mark with diluent. Mix well and filter using 0.45 nylon filter & use for injection.

PREPARATION OF SAMPLE

Weight and transfer accurately 1gm of sample into 10ml volumetric flask. Add Diluent up to 80% capacity of flask; mix well and sonicate it for 10-12with vigorous shaking; make up to mark with Diluent.

Table No 2: Validation parameters to be tested for HPLC method

Sr.no.	Characteristics
1.	Linearity
2.	Limit of Detection
3.	Limit of Quantification
4.	Specificity
5.	Selectivity
6.	Accuracy (recovery)
7.	Precision (Repeatability)
8.	Intermediate Precision
9.	Reproducibility (within lab)
10.	Ruggedness

RESULTS AND DISCUSSION

All of the analytical validation parameters for the proposed method were determined according to International Conference on Harmonization (ICH) guidelines

Specificity and System Suitability

The specificity of the HPLC method is illustrated in Fig. 1 where complete separation of Vitamin A acetate was noticed in presence of excipients used in the sunflower oil. The retention time of sample and standard peaks were comparable. In addition, there were no any interfering peaks eluted at the retention time of analytes from the blank and placebo solution. In peak purity analysis with PDA detector, purity angle was always less than purity threshold for the analyte. This showed that the peaks of analyte were pure and excipients in the formulation do not interfere with the analyte. The results were shown in Table No.2. The resolutions between the peaks were found more than 2.0. Hence the peaks were well separated. The results were given in the Table No.3.

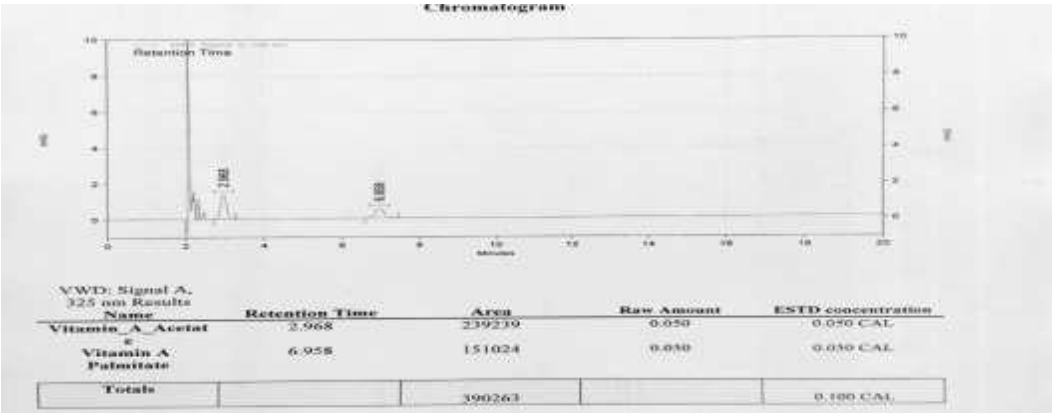

Fig. No. 01: Chromatogram of vitamin-A acetate from sunflower oil

1. Linearity:

The linearity experiment will be performed using 8 calibration standards. The plot of peak area versus concentration for the analyte.

Table no. 3: Specificity for Vitamin A acetate

Vitamin	Type	Retention time (min)	Area	Purity angle	Purity threshold
Vitamin-A acetate	Sample	2.968	239239	1.236	1.752
	Standard	2.885	238568	1.138	1.738

Table no. 4: System Suitability of Vitamin A acetate

Conc.1ug/ml	RT	Area	Asymmetry
R1	2.978	467898	0.987
R2	2.973	457540	0.964
R3	2.977	452594	0.954
R4	2.977	452594	0.954
R5	2.973	457540	0.964
R6	2.977	452594	0.954
Mean	**2.975**		
SD	**0.002**		
%RSD	**0.077**		

Linearity and Range

The linearity of this method was determined at eight concentration levels from 0.05-10 ppm of the operating concentration for Vitamin A acetate and it was shown in the table no. 05. The plot of peak area of each sample against respective concentration of Vitamin A acetate were found to be linear in the range of 0.05-10 ppm of the operating concentration. Correlation co –efficient of the standard curve was found to be 0.9999 for Vitamin A acetate. It observed that correlation coefficient and regression analysis are within the limits.

Table no. 5: Linearity of Response for Vitamin A acetate

Sr. no	Vitamin A Acetate		Corr-coefficient	
	nc. of std (ppm)	Area		
1)	0.05	239239	0.9999	**NLT 0.990**
2)	0.1	474226		
3)	0.2	958821		
4)	0.5	2303555		
5)	1	4439843		
6)	2	9151805		
7)	5	21925372		
8)	10	43502666		

Y=4E+6x+134838

Precision The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous sample under the prescribed conditions.

1. **Repeatability**:

Table no. 6: Repeatability study

Sr. no.	Type	Conc.	Conc. Found			Avg	SD	RSD
			R1	R2	R3			
1	Vitamin - A acetate	1	0.98	0.96	0.95	0.963	0.014	1.418
2		5	4.95	4.95	4.93	4.947	0.010	0.209
3		10	9.97	9.99	9.97	9.975	0.031	0.309

1.1 Intermediate Precision:

Table no. 7: Day to Day variation

Sr.no	Days	Conc. 2 ug/ml			Mean	SD	RSD
		R1	R2	R3			
1	Day 1	1.90	1.94	1.92	1.92	0.012	0.608
2	Day 2	1.91	1.93	1.92	1.92	0.012	0.608
3	Day 3	1.94	1.93	1.92	1.93	0.009	0.463

ii) Analyst to Analyst variation:

Table no. 8: Analyst to Analyst variation

Sr.no	Conc at µg/ml	Conc. found		Mean	SD	RSD
		Analyst 1	Analyst 2			
1	1	0.98	0.95	0.96	0.014	1.418
2	2	1.90	1.94	1.92	0.013	0.692

Reproducibility

Examines the precision between laboratories and is often determined in collaborative studies. Reproducibility data for Vitamin A acetate was shown in Table 8. This indicated that method was highly precise.

Table no. 9: % recovery of amount by developed method

Sr. no.	Type	Conc. Ug/ml	% Recovery Amt in Replicates						Avg
			R1	R2	R3	R4	R5	R6	
1)	Vitamin	1	98.00	96.00	95.00	96.00	95.00	98.00	96.33
2)	-A	5	99.00	99.00	98.60	99.00	99.20	98.80	98.93
3)	acetate	10	99.70	99.90	99.70	99.20	100.1	99.90	99.75
								Mean	98.33
								SD	1.45

ROBUSTNESS

Measure of methods capacity to remain unaffected by small, but deliberate variation in method. **Change in the ratio of solvents in the mobile phase (±2.0%)** Three sample preparations were analyzed as per the methodology by changing the ratio solvents in the mobile phase by means of ±2.0.The robustness data Vitamin A acetate by changing the ratio of solvents in the mobile phase. It was shown in Table No.9. It was observed that there were no marked changes in the chromatograms and the % difference in assay value between the Precision and Robustness studies were found not more than 2.0% which demonstrates that the proposed method is robust.

Table no. 10: Robustness - Change in the ratio of solvents in the mobile phase (±2.0%)

Vitamins	Method	% Label Claim	Precision Result	% Difference
Vitamin-A acetate	Low organic	102.5	102.2	0.3
	High organic	101.6		0.6

Low organic: Methanol: Acetonitrile: 68:32

High organic: Methanol: Acetonitrile: 72:28

RUGGEDNESS

Six sample preparations were analysed as per the methodology by a different analyst on a different instrument on a different day. The ruggedness data of Vitamin A acetate was shown in Table no.10 . It was observed that there were no marked changes in the chromatograms, which demonstrates that the proposed method is rugged.

Table no. 11: Ruggedness data for Vitamin A acetate

S.No.	Sample Name	Area	Drug Recovery (%)
1.	Sample -1	125787	102.9
2.	Sample -2	126213	102.4
3.	Sample -3	126145	102.4
4.	Sample -4	125465	101.8
5.	Sample -5	126532	102.7
6.	Sample -6	124985	101.4
		Mean	**102.2**
		Standard deviation	**0.46**
		RSD %	**0.5**

CONCLUSION

The Proposed study describes a simple, feasible and sensitive reverse-phase high- performance liquid chromatographic method for the quantitative determination of Vitamin A acetate in sunflower oil. The method was validated as per ICH guidelines and found to be simple, specific, linear and precise. Therefore the proposed method can be successfully used for the routine analysis of Vitamin A acetate in sunflower oil without any interference.

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