e-ISSN: 0975-1556, p-ISSN:2820-2643

Available online on www.ijpcr.com

International Journal of Pharmaceutical and Clinical Research 2022; 14(11); 542-552

Original Research Article

Stability Indicating HPLC Method Development for a Marketed Retinol Acetate

M. K. Velhal, A. N. Hasnain, N. V. Nibe, R. R. Todkari, A. G. Alhat

Trinity College of Pharmacy, Pune, Maharashtra

Received: 03-10-2022 / Revised: 05-11-2022 / Accepted: 20-11-2022
Corresponding author: Anjum. N. Hasnain
Conflict of interest: Nil

Abstract

A rapid, sensitive, and accurate stability indicating high performance liquid chromatography for the determination of retinol acetate from the marketed sample was developed. Retinol acetate is a vitamin A acetate, useful in normal eye vision, also acts as a supportive anticancer, which works by binding to glycocalyx of colon slowing or stopping the growth of cancer cells. The chromatographic separation was performed on a HPLC system consisted of Plus intelligent LC pump® PU-2080 from Agilent, Germany equipped with a Agilent®UV-2075 Intelligent UV-Visible detector, an injector Rheodyne®7725 (Rheodyne, Cotati, CA, USA), along with Agilent chromapass chromatography data system software (Version 1.8.6.1). Column Purospher Star 5µm Agilent® RP C18 XDB (4.6 mm × 150 mm) using a sonicated, degassed mobile phase containing solvent A: acetonitrile and solvent B: methanol in ratio (89:11v/v) having pH 3.5 with a flow rate of1 mL/min. The elution was detected by a uv-visible detector at 360 nm. The total chromatographic runtime is 20.0 min with a retention time for sample BASF stabilized sample and standard Sigma Aldrirch internal standard was of 8.05 and 8.2 min, respectively. The method was validated over a dynamic linear range of 10-50µg/mL for retinol acetate with a correlation coefficient (r^2) 0.999. The forced degradation study also revealed the susceptibility (sensitivity) of a drug towards heat, acid, base, hydrogen peroxide, light and photolytic degradation. This indicated photosensitivity and temperature sensitivity of the drug substance

Keywords: HPLC, High performance Liquid Chromatography, validation, Retinol acetate, Anticancer, Mobile phase, Retention time, Forced Degradation

Introduction

People consume dietary supplements to improve their health, immunity, body requirements. Vitamin A is necessary for many essential processes of life such as: normal eye vision functions, 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 [1,2]. 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.

Retinol acetate is a naturally occurring fatty acid ester form of retinol (Vitamin-A) with potential antineoplastic and

chemopreventive activities. It binds to and activates retinoic acid receptors (RARs) and retinoid X receptors (RXRs) retinoid receptors, which are rich in colon epithelial cell layers, inducing cell differentiation and decreasing cell proliferation, tumor growth. Use of retinol acetate is also referenced as an immunomodulatory agent via antioxidant mechanism [3-5]. Retinol acetate is included in official pharmacopoeias viz. Indian Pharmacopoeia, United States Pharmacopoeia, European Pharmacopoeia and British Pharmacopoeia.

HPLC methods for detection of retinol acetate are referenced in pharmacopoeias as well as in the literature [5]. HPLC test method described in the literature was not sufficiently selective to separate retinol acetate and its additives when the chromatogram was developed. Limitations were observed with respect to applicability of those methods for the analysis of drug substance and the drug products mentioned in this thesis. This fact has led to development of a HPLC test method which was able to detect retinol acetate and its additives or the impurities in the drug product [6-8].

Hence, prominence was given on developing a suitable new and robust HPLC method for detection of retinol acetate and its degradation products using UV detector. Selection of column and mobile phase was done as per literature, in which properties of retinol acetate like its nature, log P value 4.1, pKa value 0.667, isoelectric point 4.3 to 4.7 and partition coefficient 0.01- 0.02 played an important role [6-9].

2. Materials and Method

2.1 Chemicals and Reagents

Retinol acetate®325 GFP was obtained as a gift sample from BASF Ltd., India., standard retinol acetate from Sigma Aldrich was used as a reference standard. Analytical reagents (A.R.) grade solvents like ethanol (99%), trimethyl silane (TMS); High Performance Liquid Chromatography (HPLC) grade solvents like acetonitrile, methanol were purchased from S.D. Fine Chemicals Ltd. Mumbai, India. Double distilled water was obtained from Millipore system, USA was used throughout analysis, 0.45µ membrane filters were purchased from Pall Life Science, India. All other chemicals used were of analytical grade unless otherwise indicated.

2.2 Instruments (Apparatus)

Electronic balance (AY-120) Shimadzu Ltd., Japan, RP- HPLC (Model- PU2080) from Agilent Ltd., Germany HPLC system consisted of Plus intelligent LC pump® PU-2080 from Agilent, Germany equipped with a Agilent®UV-2075 Intelligent UV-Visible detector, an injector Rheodyne®7725 (Rheodyne, Cotati, CA, USA), along with Agilent chromapass chromatography data system software (Version 1.8.6.1), Column Purospher Star 5µm Agilent®RP C18 XDB (4.6 mm × 150 mm), amber coloured lamp, glassware's, amber coloured flasks.

2.3 High Performance Liquid Chromatography (HPLC) method development

Different HPLC methods were developed and validated in the research papers [8,9]. HPLC methods for detection of retinol acetate are referenced in pharmacopoeias as well as in the literature [10,11]. HPLC test method described in the literature was not sufficiently selective to separate retinol acetate and its additives when the chromatogram was developed. Limitations were observed with respect to applicability of those methods for the analysis of drug substance and the drug products mentioned in this thesis. This fact has led to development of a HPLC test method which was able to detect retinol acetate and its additives or the impurities in the drug product.

Hence, prominence was given on developing a suitable new and robust HPLC method for detection of retinol acetate and its degradation products using UV detector.

2.4 Description and procedure for HPLC analytical method

The HPLC system solvent ports, injector ports, columns were flushed with the acetonitrile: methanol (89:11, %v/v) mobile phase and the column as well as the system were allowed to equilibrate before the analysis.

2.5 Preparation of standard and stock solutions

Retinol acetate was used as a working / reference standard. A stock solution of retinol acetate (1000 µg/ml) was prepared by dissolving accurately weighed 10 mg of retinol acetate in10 ml volumetric amber coloured volumetric flask using 50:50 v/v of ethanol: water. Standard solutions were prepared by dilution of the stock solution with same solvent to give solutions containing retinol acetate in the concentration range of 10-50 µg/ml. All solutions were prepared in amber coloured flask and amber lamp was used throughout was used throughout the experimentations.

2.6 Blank preparation

Ethanol /water (50/50, v/v) which was used in preparation of standard stock solution was used as a blank solution.

2.7 Chromatographic parameters

Instrument	HPLC system consisted of Plus intelligent LC pump® PU-2080 from Agilent, Germany equipped with a Agilent®UV-2075 Intelligent UV-Visible detector, an injector Rheodyne®7725 (Rheodyne, Cotati, CA, USA), along with Agilent chromapass chromatography data system software (Version 1.8.6.1)
Column	Column Purospher Star 5µm Agilent®RP C18 XDB (4.6 mm × 150 mm)
Flow rate	1.0 ml /minute
Injection volume	20 µl
Wavelength	326 nm
Run time	20 minutes
Temperature	Room temperature 25 ± 2ºC

2.8 Sample preparation for assay and degradation products

Sample was prepared using stock solution with further dilution in ethanol /water (50/50, % v/v) as a vehicle in the 10 ml volumetric amber coloured flask.

2.9 System suitability procedure

The chromatographic system was set and injected blank (ethanol /water (50/50, % v/v) single), standard preparation (six replicates) and recorded the chromatograms to evaluate the system suitability parameters.

The system was considered suitable for analysis if and only if, the retention time of retinol acetate peak was about more than 3 minutes and based on following criteria. It is shown in the observation table 1 below.

Table 1: System suitability for RP-HPLC

Parameter	Value
Resolution	Resolution >1
Tailing factor	Tailing factor 0.9 - 1.1
Column efficiency (as theoretical plates)	Column efficiency > 1000

2.10 Forced degradation of retinol acetate (Stability indicating HPLC method development)

To study the degradation pattern and ability of method to separate the peaks of drug substance, the additives and the degradants, as per ICH guidelines forced degradation

studies were carried out [12-16]. This was performed by stressing placebo and test simultaneously under the following maximum stress conditions. Sample was subjected to various forced degradation conditions to provide an indication of the stability indicating property.

The working solution for the studies were prepared by subjecting 1ml of 10 µg/ml which was prepared by diluting stock solution with 50:50 % v/v of ethanol: water system. During initial studies, the samples were treated with acid, alkali and hydrogen peroxide with heating on a water bath for an hour. However, all the samples exhibited complete degradation. Hence, the sample solutions without application of heat were treated for 5 minutes and were analyzed with RP-HPLC, using the method discussed above.

2.11 Alkali degradation

1ml of 10 µg/ml of retinol acetate solution was taken in a 10 ml volumetric flask. 1ml of 0.1N NaOH was added to it and neutralized by addition of 0.1N HCl. Volume was made with diluent, ethanol/ water (50/50%v/v). This neutralized solution was injected in triplicate and chromatogram was run as described previously. An equivalent amount of placebo was treated in a similar manner and analysed as per the proposed method.

2.12 Oxidative degradation (Hydrogen peroxide-induced degradation)

1ml of 10 µg/ml of retinol acetate solution was taken in a 10 ml volumetric flask. 1 ml of 30% hydrogen peroxide (H_2O_2) was added to it and volume was made with diluents ethanol/ water (50/50,% v/v), heated for 5 minutes at 80°C. This solution was injected in triplicate and chromatogram was run as described previously. An equivalent amount of placebo was treated in a similar manner and analysed as per the proposed method.

2.13 Method validation

The purpose of the method validation was to assure that the analytical method for retinol

acetate by HPLC was under control and gave consistent and repetitive results. The analytical method employed herein is described in the previous section.

2.14 Precision

For system precision, repeatability of the sample application and measurement of peak areas

was carried out using six replicates of the 10 µg/ml and expressed in terms of percent relative standard deviation (% RSD) and standard error (SE).

2.15 Limit of detection and limit of quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank sample consisting of 50:50,% v/v (ethanol: water) was injected six times by the same method as explained above. The LOD and LOQ for retinol acetate were estimated at a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively.

2.16 Accuracy and recovery studies

Accuracy determination was carried out at 10 µg/ml, 30 µg/ml and 50 µg/ml of retinol acetate concentration. The percentage recoveries for retinol acetate were calculated. At each level of the amount, six determinations were performed both, intra-day and inter-day and was expressed in terms of % relative standard deviation (RSD) and standard error (SE). This was also done to check for the recovery of a drug at different levels in the formulation.

2.17 Linearity

Linearity test solutions for retinol acetate were prepared by diluting the stock solution with ethanol: water (50:50, %v/v) to the required concentrations range of 10 to 50 µg/ml. The calibration curves were drawn by plotting the peak areas of retinol acetate versus the corresponding concentration.

The slope and *Y*-intercept of the calibration curve was calculated.

2.18 Solution stability

The solution stability of the drug sample was carried out by leaving a spiked sample (10 µg/ ml) solution in a tightly capped volumetric flask at room temperature for 24 hours. Content of solution was determined at 24 hours intervals by the procedure as described previously. This solution was kept in an amber coloured container at room temperature, protected from light and heat.

3. Results and discussion

3.1 Development of an optimum mobile phase

Initially, method referenced in the literature by Tee and Khor *et al*. 1999 was adopted. According to these methods, the mobile phase of ethyl acetate: water (82:18, % v/v) and methanol: water (88:12, % v/v) were used with Purospher Star®250 x 4.6 mm HPLC cartridge with RP18 (C18) (5µm) column. Using these methods, asymmetrical peaks with tailing were obtained, retention time was also changed in successive trials without reproducible results with the given mobile phase system.

Hence, the emphasis was given on developing a suitable mobile phase and in turn a new and robust HPLC stability indicating method for detection of retinol acetate and its degradation products using UV detector. Due to embedded, stabilized form of retinol acetate solubility

Accordingly, new mobile phase composition of acetonitrile: methanol (89:11, % v/v) was used for further studies. Standard chromatogram obtained by this mobile phase has shown a prominent peak of retinol acetate with better resolution with symmetrical peak shape and also additional peaks of additives present in the sample which is shown below due to the excipients added. By various trials, the results obtained with the HPLC method were found reproducible, thus this mobile phase was considered as optimum for RP-HPLC. Drug has a retention time of 8.058 minutes with > 0.885 symmetry. Standard chromatogram (figure: 1) with standardization graph of various concentration with area (figure: 2) is shown below. As it is shown by graphs and data, a linear relationship was observed over a concentration range 10-50 µg/ml (n=3) with respect to the peak area, thus making it possible to differentiate between different concentrations. No significant difference was observed in the slopes of standard curves. (ANOVA, $P < 0.0001$).

3.2 Forced degradation study

During forced degradation study, the chromatograms degradation study, the chromatograms of the samples treated with acid, base and hydrogen peroxide showed well resolved peaks for retinol acetate as well as for the degradation products. Thus, the system was found to be suitable. Samples treated with acid, base and hydrogen peroxide with heat in water bath for an hour got degraded. Hence, samples solutions were treated for 5 minutes without heat and analyzed with HPLC method. Also for thermal degradation study, sample solution with 5 minutes of water bath heating at 80°C was used in RP-HPLC.

3.2.1 Acid induced degradation product

Total degradation of product under mild acidic conditions (0.1N HCl) with heating was observed. With 0.1N HCl without heating, standard drug peak along with several degradation peaks were observed. The presence of additional degradation peaks at mild conditions confirmed the vulnerability of drug to degradation in acidic conditions (figure: 3).

3.2.2 Base induced degradation product

Total degradation of product was observed under mild basic conditions (0.1N NaOH) with
heating. With 0.1N NaOH without heating, the standard drug peak along with several degradation peaks would be observed. The chromatograms of the base (0.1N NaOH) degraded sample showed additional peaks at 1.89, 11.79, 17.77 minutes. The presence

of additional degradation peaks at mild conditions confirmed the vulnerability of drug to degradation in basic conditions (figure: 4).

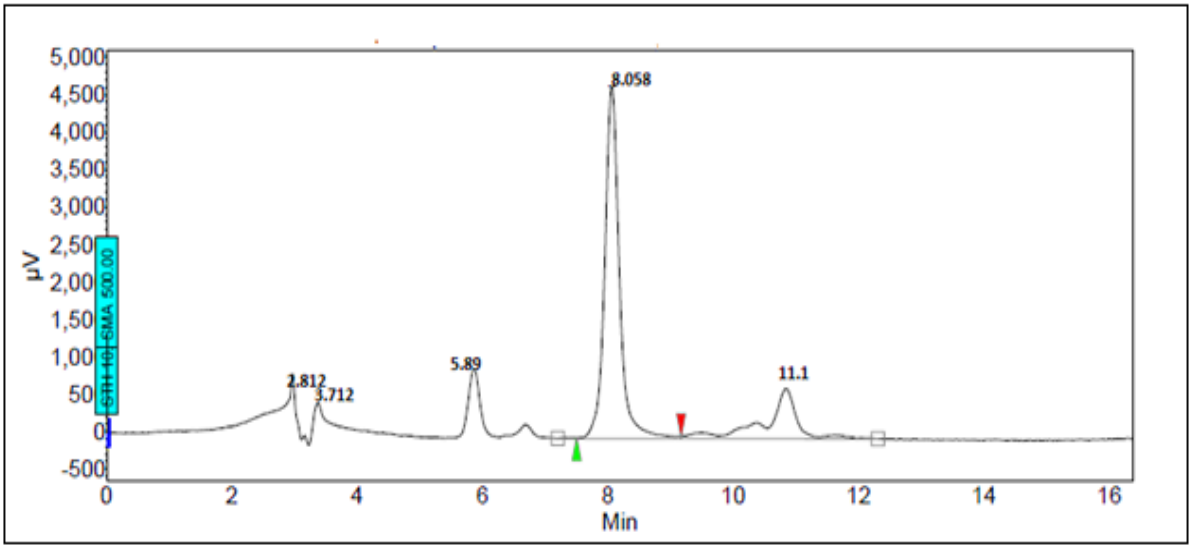

Figure 1: Standard chromatogram of retinol acetate by RP-HPLC

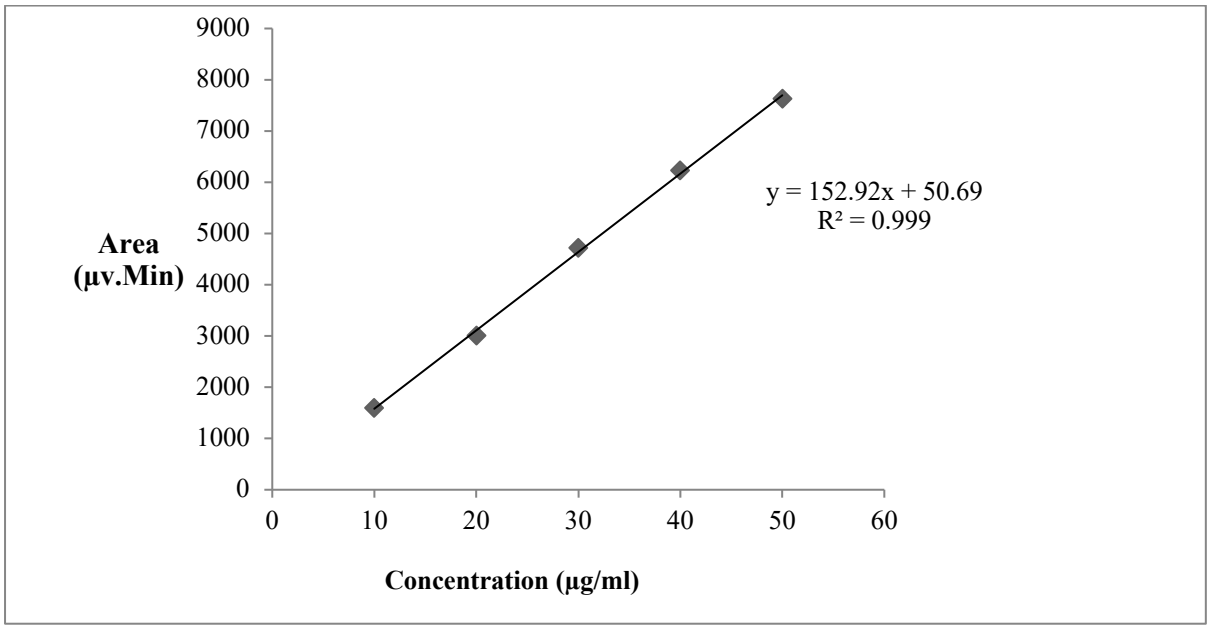

Figure 2: Standardization of retinol acetate by RP-HPLC

Table 2: Acid, base, oxidation, photo (sunlight), thermal degradation study by HPLC

Type of degradation	Time for reaction (minutes)	Intact drug(%)	Retention time (minutes) of degradation product
Acid	5	47.64	3.3, 6, 6.74
Base	5	91	1.89, 11.79, 17.77
Oxidation	5	72	3.32
Photo (sunlight)	15	20	2.5, 3.5, 5.45, 6.5, 10.56, 11.31
Thermal	5	93.25	5.92, 6.79, 10.47, 11.12

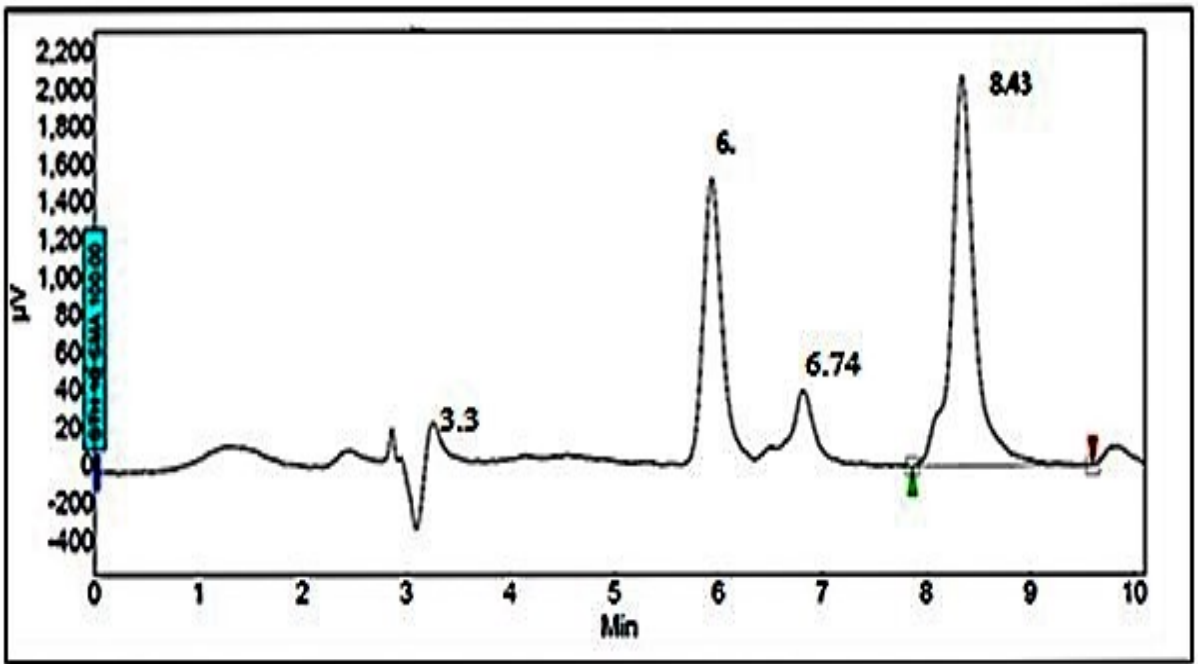

Figure 3: Chromatogram of acid induced degradation

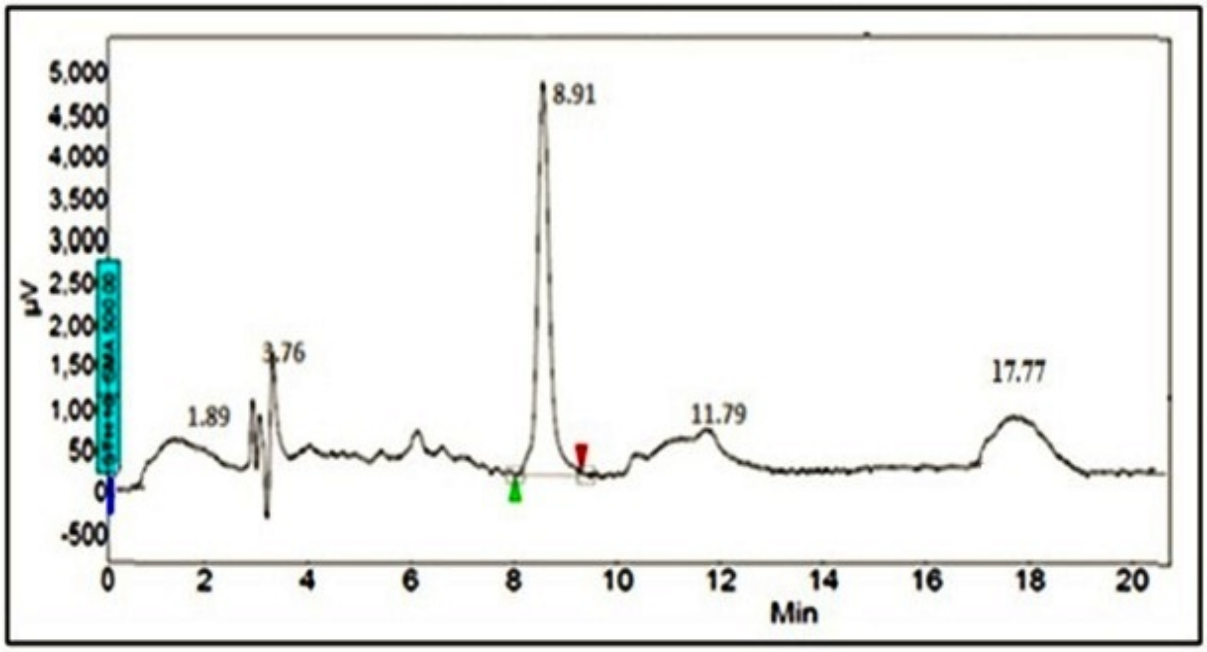

Figure 4: Chromatogram of base induced degradation with water bath heat for 5 minutes

3.2.3 Hydrogen peroxide-induced degradation product

The chromatogram of the sample of retinol acetate treated with 30% (v/v) hydrogen peroxide on water bath for 5 minutes showed additional peak at 3.32 minutes, indicated instability in presence of hydrogen peroxide (figure: 5).

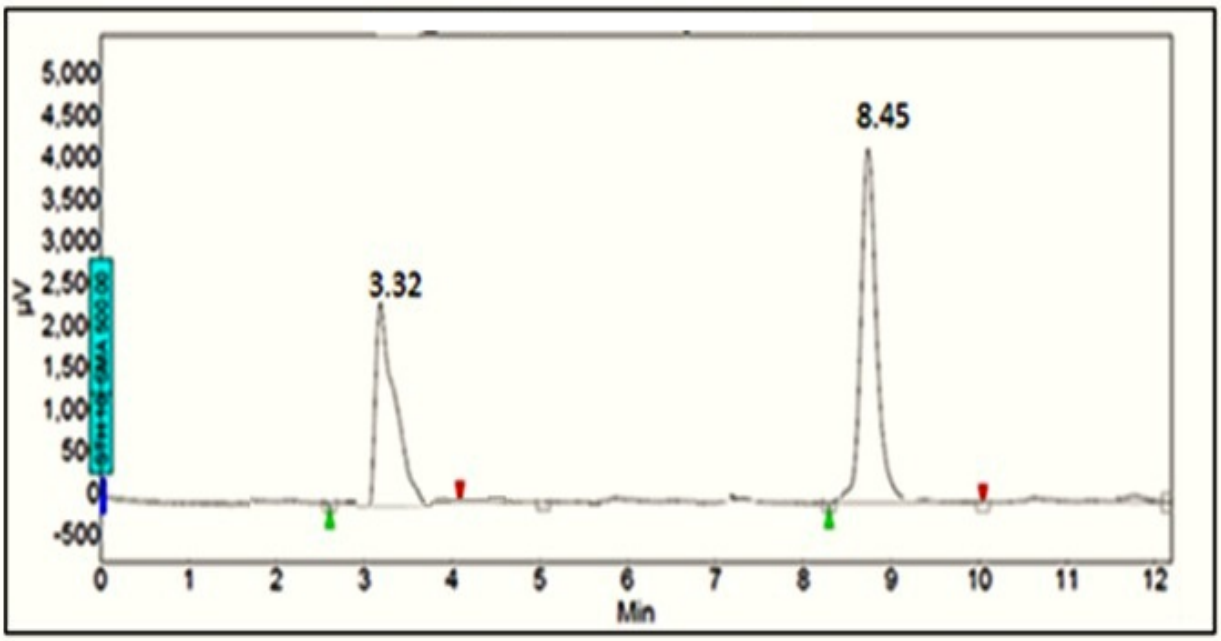

Figure 5: Chromatogram of hydrogen peroxide-induced degradation

3.2.4 Photodegradation product

The chromatogram of the sample of retinol acetate exposed to direct sunlight for 15 minutes showed additional peaks at 2.5, 3.5, 5.45, 6.5, 10.56, 11.31 minutes. This indicated photo-instability of a drug (figure: 6).

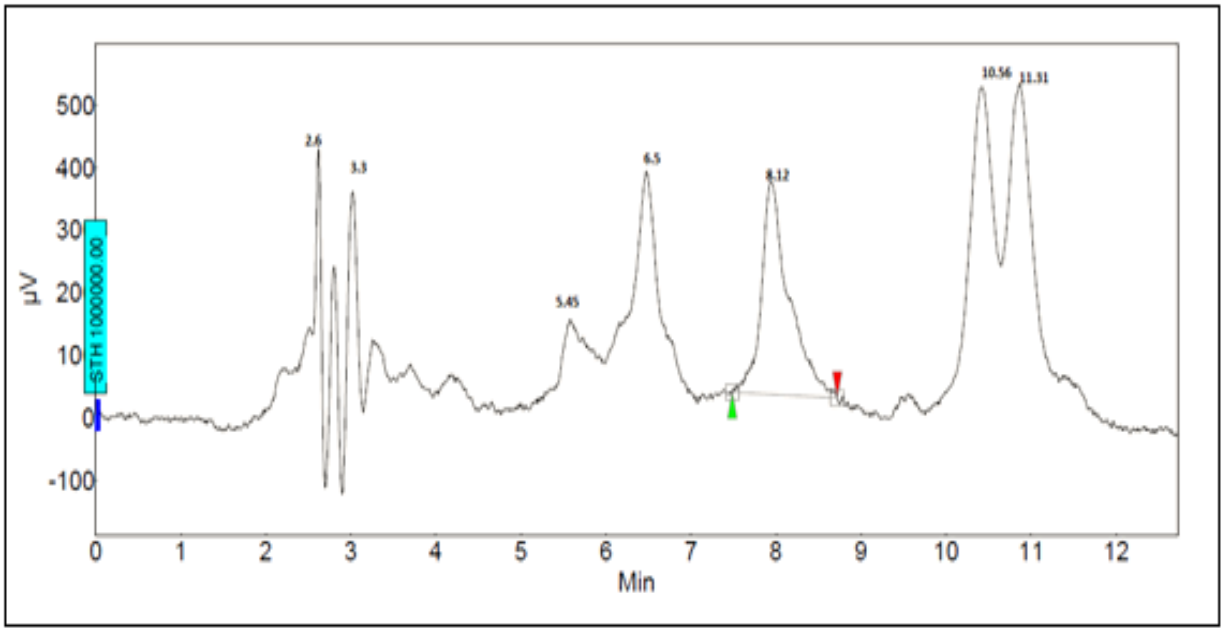

Figure 6: Chromatogram of photodegradation

3.2.5 Thermal degradation product

The chromatogram of the sample of retinol acetate heated at 85ºC for 5 minutes on water bath have showed additional peaks at 5.92, 6.79, 10.47, 11.12 minutes, indicated thermal instability of the drug as shown in figure 7. The forced degradation studies revealed susceptibility of retinol acetate towards heat, hydrogen peroxide, acid, light and photolytic conditions.

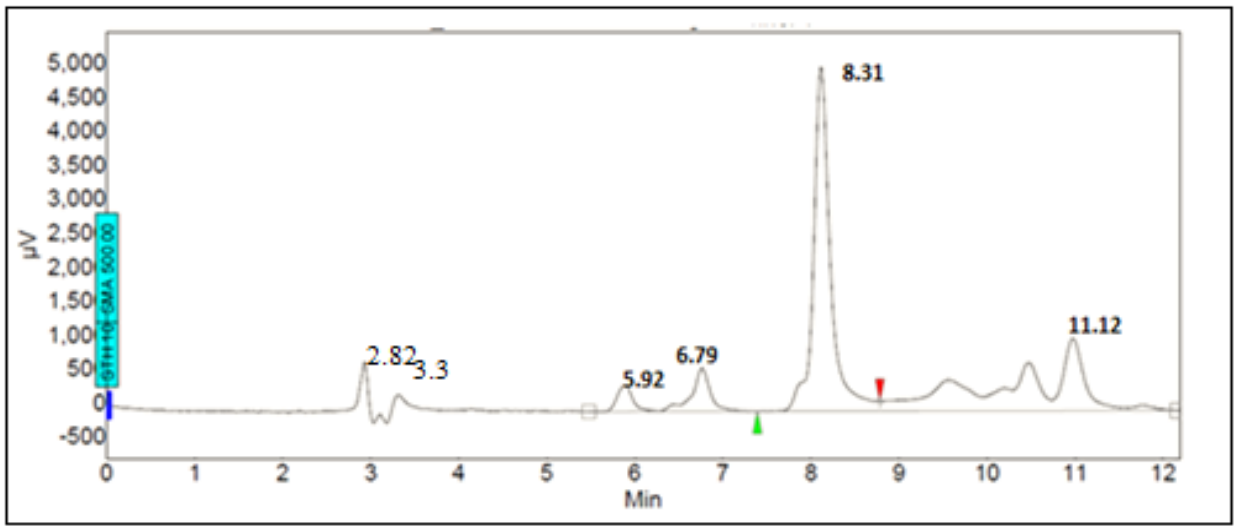

Figure 7: Chromatogram of thermal induced degradation

3.2.6 Validation of the method

3.2.6.1 Precision

For this 10 µg/ml solution was prepared to determine if the test method presents an acceptable repeatability. Obtained results were as follows:

% RSD for sample application (10 µg/ ml) by measurement of peak area was found 1.2704 and 1.0594 for intra-day inter-day respectively. Standard errors were found 0.07 and 0.03%, respectively. The measurement of the peak area thus showed lower values of % relative standard deviation (RSD) < 2 and very lower values of standard error (SE) < 0.1 for inter and intra-day variation. As is shown in the observations table 3; although repeatability was variable, by taking into account low concentration obtained satisfactory results, precision of the method was established.

Table 3: Intra-day and inter-day precision of HPLC method (n=6)

Drug added (µg)	Intra-day Precision			Inter- day Precision		
	Drug recovered (µg)	Drug recovered (%)	RSD (%)	Drug recovered (µg)	Drug recovered (%)	RSD (%)
10	9.91	99.10	1.2704	9.89	98.9	1.0594

3.2.6.2 Limit of detection (LOD) and limit of quantification (LOQ)

Signal-to-noise ratio of 3:1 and 10:1 were considered as LOD and LOQ and were found to be 0.0651 µg/ml and 0.217 µg/ml respectively. This indicated adequate sensitivity of the method.

3.2.6.3 Accuracy and recovery studies

Accuracy of the method was determined by spiking known amount of retinol acetate solution in triplicate at low, medium and high levels of the specified limit. The recoveries for three dilution samples of retinol acetate were calculated. Samples were analysed as per the proposed method and the results obtained are tabulated below in the observation table 4. Recovery of the investigated components ranged from 99.37 % to 98.38 % for intra-day and 99.51% to 98.72% for inter-day. Their % RSD are shown in the observation table 3.10 and standard error were found to be 0.07 % and 0.03% for 10 µg for intra and inter-day, 0.08 and 0.073% for 30 µg for intra and inter-day and 0.09 and 0.093%, for 50 µg for intra and inter- day.

Table 4: Intra-day and inter-day accuracy of RP-HPLC method

Drug added (µg)	Intra-day accuracy			Inter-day accuracy		
	Drug recovered (µg)	Drug recovered (%)	RSD (%)	Drug Recovered (µg)	Drug Recovered (%)	RSD (%)
10	9.91	99.10	1.27041	9.89	98.9	1.0594
30	29.81	99.37	1.38927	29.85	99.51	1.3134
50	49.19	98.38	1.64286	49.36	98.72	1.6197

3.2.6.4 Linearity

Linearity was determined in the concentration range of 10 to 50 µg/ml. The area response against the corresponding concentration is provided in the figure 3.14. As it is shown by graph and data, a linear relationship was observed over a concentration range 10-50 µg/ml (n=3) with respect to the peak area, thus making possible to differentiate for different concentrations. Squared correlation coefficient (R^2) value 0.999 was well within the limit.

4. Conclusion

A HPLC method development specific to the pro retinol acetate sample was developed and validated as per the ICH guidelines. The forced degradation study also revealed the susceptibility (sensitivity) of a drug towards heat, acid, base, hydrogen peroxide, light and photolytic degradation. This indicated photosensitivity and temperature sensitivity of the drug substance and hence it was advised to keep all the sample solutions in amber coloured container at room temperature, protected from light and heat.

Thus the developed HPLC technique was linear, precise, specific and accurate for the determination of retinol acetate. From the studies and obtained results, it was also evident that the test method was robust to differentiate the drug and the related substances peaks (especially degradation products) and thus demonstrated its usefulness as a stability-indicating method. Thus overall, standardization of given sample of retinol acetate®325 GFP from BASF Ltd., India was done successfully by various physiochemical and spectroscopic methods. Results were complied with the certificate of analysis and literature reports.

References

1. Goodman DS. Vitamin A and retinoids in health and disease. N. Engl J Med 1984, 310,1023-31.
2. Machlin, L.J. ed. Handbook of Vitamins, Marcel Dekker, Inc., New York, 1991.
3. Vitamin A. Micronutrient Information Center, Linus Pauling Institute, Oregon State University, Corvallis. January 2015. Retrieved 6 July 2017.
4. Vitamin A. MedlinePlus, National Library of Medicine, US National Institutes of Health. 2 December 2016.
5. Vitamin A. Office of Dietary Supplements, US National Institutes of Health. 31 August 2016.
6. https://go.drugbank.com/drugs/DB00162
7. https://pubchem.ncbi.nlm.nih.gov/compound/Retinol
8. USP29, Momograph of Vitamin A 2005
9. European Pharmacopeia, Vitamin A monograph. 10th edition; 2019.
10. Scientific committee on consumer safety, SCCS, Opinions on Vitamin A, SCCS/1576/16 Final version of 6 October 2016 Corrigendum on 23 December 2016
11. Genestar, C., Grases, F. Determination of Vitamin A in pharmaceutical preparation by high-performance liquid chromatography with diode-array detection, Chromatographia 1995; 40 (3-4): 143-146.
12. S E Tee Jr, S C Khor Simultaneous determination of retinol and α-tocopherol by high pressure liquid chromatography in micro-volumes of

serum Malays J. Nutr. 1999 Dec; 5(1): 21-30.
13. Ake, M., Fabre, H., Malan, A, K., Mandrou B. Column liquid chromatography determination of vitamins A and E in powdered milk and local flour: a validation procedure. J. Chromatogr.1998, A 826(2): 183-9. 5.
14. Satoshi Yokota, Shgeru Oshio, A simple and robust quantitative analysis of retinol and retinyl palmitate using a liquid chromatographic isocratic method, Journal of food and drug analysis 2018; 26.
15. R. Prabakaran, A. Chenthilnathan and S. Vikraman Validation of simultaneous determination of vitamin A acetate and vitamin E acetate in multivitamin tablets by RP-HPLC International Journal of Pharmaceutical, Biological and Chemical Sciences (IJPBCS). Apr-Jun 2014; 3(2): 27-34.
16. International Conference on Harmonization (ICH), Stability testing of new drug substances and products Q1A (R2), Geneva, Switzerland, (2003).
17. International Conference on Harmonization (ICH), Validation of analytical procedures: Text and methodology Q2 (R1), Geneva, Switzerland. 2005.