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International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

Available online at www.ijpsdronline.com

Research Article

A Stability Indicating Reverse Phase High Performance Liquid Chromatography Method for Simultaneous Estimation of Allantoin, Hydroquinone and Tretenoin in Cream Formulation

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ARTICLE INFO

Article history:

Received: 08 September, 2021

Revised: 06 February, 2022

Accepted: 19 February, 2022

Published: 30 March, 2022

Keywords:

Allantoin, Hydroquinone, RP-HPLC, Stability Indicating Method, Tretenoin.

DOI:

10.25004/IJPSDR.2022.140206

ABSTRACT

The present investigation deals with simple, sensitive, rapid, precise and accurate reverse phase high performance liquid chromatography (RP-HPLC) method developed and validated for simultaneous estimation of Hydroquinone, Allantoin and Tretenoin in a cream formulation. The chromatographic separation was achieved on a reversed-phase InertsilC_{18} (4.6mm I.D. × 250mm, 5µm) column using a mobile phase consisting of Buffer (pH 3.5) 0.05M Potassium dihydrogen ortho phosphate-Methanol (70:30% V/V) at a flow rate of 1-mL/min and UV detection at λ_{max} 223 nm. The method showed linearity with correlation coefficient of Hydroquinone, Allantoin and Tretenoin was 0.999, 0.999 and 0.999 over the range of 100–300 µg/mL, 50 to 150 µg/mL and 0.625 to 1.875 µg/mL, respectively. The mean recoveries were found to be 99.00 to 101.00% for all the components. The method was validated as per the ICH guidelines. The developed protocol was most accurate, repeatable, and detectable towards hydroquinone, allantoin and tretenoin in combination without any unwanted interference. When evaluated on various parameters like system suitability, precision, accuracy, linearity, robustness, stablity study, the method is efficient in separating the API from its degradants and can be utilized for analyzing the samples of hydroquinone, allantoin and tretenoin.

Introduction

A combine cream formulation of hydroquinone (2%), allantoin (1%) and tretenoin (0.012%) is available as cream formulation used in the treatment of Melasma disease.[1] Melasma is a common skin problem. The condition causes dark, discoloured patches on your skin.

Hydroquinone chemically benzene-1, 4-diol or quinol is an phenol type aromatic organic compound derived from benzene, having the chemical formula $C_6H_4(OH)_2$ (Fig. 1). Hydroquinone is a topical *depigmenting agents* agent used in hyper pigmentation conditions.[2] It produces lightening of your skin by decreasing the number of melanocytes present or produced in your skin.[3] Allantoin is 2, 5-dioxo-4-imidazolidinyl-urea Allantoin is a skin protectant which works as a moisturizer and relieves minor skin irritations.[4] Allantoin is also known as kerolytic molecules that remove warts, corns and horny layer of the skin. Tretenoin is a form of vitamin A that helps the skin to renew itself more quickly. It is used to treat Acne or skin disease such as wrinkles, dark spot and rough skin.

Hydroquinone and Allantoin is an official drug in USP while Tretenoin are official in both USP and BP. Literature revealed some analytical methods for the analysis of HQ either alone or in various combination[6,7] including spectrophotometry[8] and high performance liquid chromatography (HPLC).[9-14] Literature study reveals that no stability indicating reversed phase-high

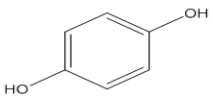

Fig. 1: Structure of Hydroquinone

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

performance liquid chromatographic (RP-HPLC) methods are available for the estimation of hydroquinone, allantoin and Tretenoin combine formulation.

The aim of this present study was to develop and validate a stability indicating RP-HPLC method for simultaneous estimation of allantoin, hydroquinone and tretenoin in cream formulation. The stability studies of pharmaceutical products are one of the very important parameter for development of new drugs as well as new formulations. The shelf-life prediction is a major role for the pharmaceutical product development of all the dosage forms and also it is utilized to determine the particular storage conditions and to suggest label instructions. Stability studies of pharmaceutical products ensuring the maintenance of product quality, safety and efficacy throughout the shelf life are considered as pre-requisite for the acceptance and approval of any pharmaceutical products.[5]

Material and Method

Chemicals and Reagents

Pharmaceutically pure samples of hydroquinone, allantoin and tretenoin were obtained as a gift sample from R.K. School of Pharmacy, Loba Chemical Private limited, Mumbai, and Abbott Pharmaceutical, Mumbai respectively. Acetonitrile and Methanol were obtained from Merck Specialties Private Limited, Mumbai and Molychem, Mumbai, respectively. HPLC grade water was obtained from Loba Chemie Pvt. Ltd., Mumbai and Astron Chemicals, Ahmadabad respectively.

Instrumentation

HPLC instrument having UV-Visible detector, Shimadzu: the separation was performed on InertsilC_{18} (4.6 mm I.D. × 250 mm, 5 µm).

Buffer Preparation (0.05M potassium dihydrogen ortho phosphate, pH-3.5)

6.8 gm potassium dihydrogen ortho phosphate reagent was taken into 1000 mL volumetric flask. Then 800 mL water was added to dissolve and pH 3.5 was adjusted with 1% Orthophosphoric acid. Finally, volume was made up to 1000 mL with water.

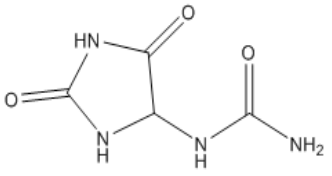

Fig. 2: Structure of Allantoin

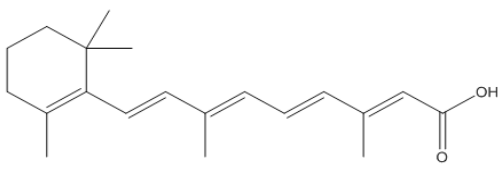

Fig. 3: Structure of Tretenoin

Preparation of Standard Solution

Accurately weighted quantity of hydroquinone 200 mg, allantoin 100 mg and tretenoin 12.5 mg was transferred into different 100 ml volumetric flask to get concentration of hydroquinone 2000 µg/mL, allantoin 1000 µg/mL, tretenoin 125 µg/mL.

Preparation of Combined Working Standard Solution

1-mL from hydroquinone, allantoin and tretenoin standard stock solution were taken in 10 mL volumetric flask and finally volume made up to 10 mL with mobile phase to get concentration hydroquinone 200 µg/mL, allantoin 100 µg/mL, tretenoin 1.25 µg/mL.

Result and Discussion

Chromatographic Condition

The Chromatographic Separation was achieved on reverse phase inertsilc_{18} (4.6 mm I.D. × 250 mm, 5 µm) column was used for Chromatographic Separation. Standard solutions of hydroquinone, allantoin and Tretenoin were injected in column with 20 µL micro-syringe. The chromatogram was run for appropriate minutes with mobile phase Buffer 0.05M potassium dihydrogen ortho phosphate (pH 3.5) Methanol in the ratio of 70:30 %V/V at a flow rate of 1-mL/min and UV detection at λ_{max} 223 nm. The chromatogram was stopped after separation achieved completely. Optimized Chromatographic Condition is shown in Table 1 and Fig. 4.

Method Validation

Method was validated as per ICH guidelines.[15]

System Suitability

The system suitability was assessed by triplicate analyses of the drugs at a concentration of 200, 100, 1.25 µg/mL of hydroquinone, allantoin and tretinoin, respectively. System suitability parameters were shown in Table 2.

Table 1: Optimised chromatographic conditions

Parameter	*Result*
Mobile Phase	Buffer 0.05M potassium dihydrogen ortho phosphate (pH 3.5) Methanol in the ratio of 70:30 %V/V
Column	Inertsilc_{18}
Flow Rate	1-mL/min
Wave length	223 nm
Injection Volume	20 µL
Retention Time	Hydroquinone: 3.9 min Allantoin: 6.3 min Tretenoin: 8.6 min
Run Time	10 minutes

Table 2: System suitability parameter

Retention Time	*Area*	*Height*	*Asymmetry*	*Efficiency*	*Resolution*
3.960	4129.144	593.185	1.346	7180	-
6.330	5678.481	470.835	1.419	6371	9.402
8.627	2138.884	140.765	1.382	7158	6.335

Table 3: Linearity data

Hydroquinone (2%)		*Allantoin (1%)*		*Tretenoin (0.0125%)*	
Conc. (µg/mL)	*Area*	*Conc. (µg/mL)*	*Area*	*Conc. (µg/mL)*	*Area*
100	2052.146	50	2822.120	0.625	1061.556
150	3089.538	75	4248.805	0.937	1600.348
200	4132.373	100	5682.945	1.250	2140.578
250	5148.993	125	7089.343	1.562	2667.745
300	6128.679	150	8438.344	1.875	3175.369

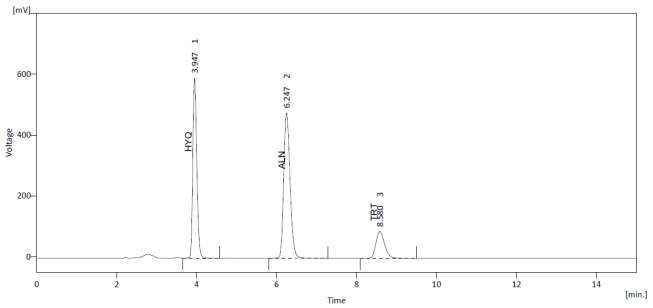

Fig. 4: Chromatogram of sample solution

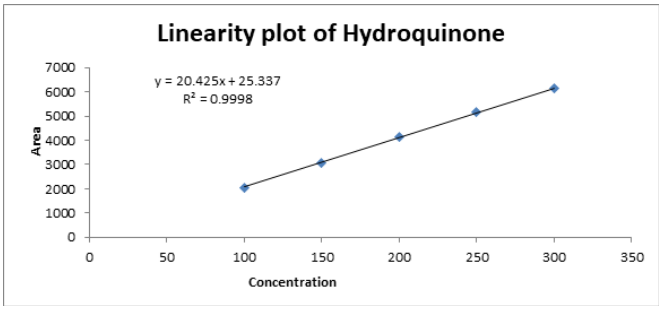

Fig. 5: Calibration curve hydroquinone X-axis: Concentration Y-axis: Area

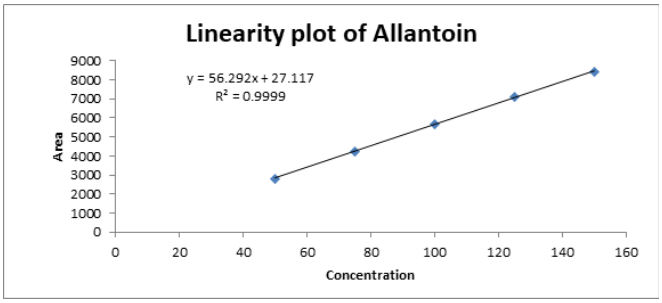

Fig. 6: Calibration curve allantoin X-axis: Concentration Y-axis: Area

Specificity

At the retention time of 3.9, 6.3 and 8.6 min, the proposed method was specific for the detection of hydroquinone, allantoin, tretinoin, respectively. There were no peaks at the retention time of hydroquinone, hydrocortisone, tretnoin.

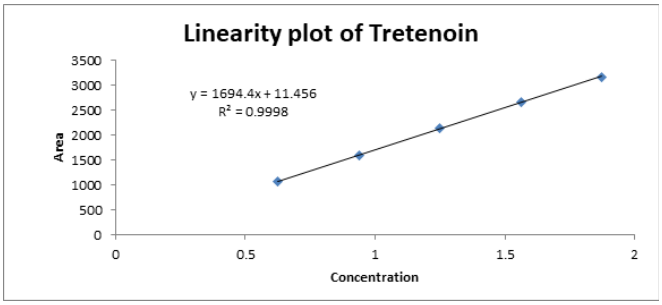

Fig. 7: Calibration curve tretenoin X-axis: Concentration Y-axis: Area

Linearity

Linearity was tested in the concentration range µg/mL for 100, 150, 200, 250, 300 for hydroquinone 50, 75, 100, 125, 150 for allantoin 0.62, 0.93, 1.25, 1.56, and 1.87 for tretinoin. All the solutions were measured six times in accordance with the ICH. The typical table of linearity are shown in Table 3 and calibration curve shown in Figs. 5 to 6.

Precision

For repeatability standard solution containing hydroquinone (200 µg/mL), allantoin (100 µg/mL) and tretinoin (1.25 µg/mL) were injected six times and area of peak were measured. For intraday precision, the solutions were analyzed six times on the same day and for interday precision, the solutions were analyzed six times on the different day and % RSD was calculated. Precision condition was shown in Table 4.

Accuracy

Recovery studies were carried out by applying the method to drug sample present in topical dosage form to which known amount of hydroquinone, allantoin and tretenoin corresponding to 80, 100, and 120% of label claim was added by standard addition method. Accuracy condition was shown in Tables 5 to 7.

Table 4: Precision

	Drug	*Concentration*	*Peak Area ± SD*	*%RSD*
Repeatability	Hydroquinone	200	4165.95 ± 0.773	0.769
	Allantoin	100	5691.95 ± 0.670	0.670
	Tretenoin	1.25	2095.78 ± 0.547	0.556
Intraday Precision	Hydroquinone	200	4127.63 ± 0.731	0.726
	Allantoin	100	5690.33 ± 0.601	0.600
	Tretenoin	1.25	2108.37 ± 0.560	0.560
Interday Precision	Hydroquinone	200	4161.66 ± 0.810	0.810
	Allantoin	100	5670.71 ± 0.660	0.660
	Tretenoin	1.25	2078.52 ± 0.560	0.510

Table 5: Accuracy data for Hydroquinone

Amt of hydroquinone present in sample (µg/mL)	*Amt of std hydroquinone added (µg/mL)*	*Total amount found (µg/mL)*	*%Recovery*	*Mean % recovery*	*SD*	*% RSD*
100	80	79.398	99.247	99.351	0.763	0.768
100	80	78.917	98.646			
100	80	80.129	100.161			
100	100	99.662	99.662	99.375	0.975	0.981
100	100	100.170	100.170			
100	100	98.286	98.286			
100	120	119.446	99.539	99.087	1.132	1.143
100	120	117.358	97.798			
100	120	119.907	99.923			

Table 6: Accuracy data for Allantoin

Amt of Allantoin present in sample (µg/mL)	*Amt of std allantoin added (µg/mL)*	*Total amount found (µg/mL)*	*% recovery*	*Mean % recovery*	*SD*	*% RSD*
50	40	39.710	99.275	99.322	0.901	0.908
50	40	39.378	98.445			
50	40	40.099	100.247			
50	50	49.839	99.678	99.585	0.722	0.725
50	50	50.128	100.256			
50	50	49.410	98.820			
50	60	59.748	99.581	99.230	0.942	0.949
50	60	58.898	98.163			
50	60	59.968	99.946			

Table 7: Accuracy data for tretenoin

Amt of tretenoin present in sample (µg/mL)	*Amt of std tretenoin added (µg/mL)*	*Total amount found (mean) (µg/mL)*	*% recovery*	*Mean % recovery*	*SD*	*% RSD*
0.625	0.5	0.498	99.543	99.452	1.096	1.102
0.625	0.5	0.492	98.313			
0.625	0.5	0.503	100.500			
0.625	0.625	0.624	99.919	99.563	1.132	1.136
0.625	0.625	0.628	100.474			
0.625	0.625	0.614	98.297			
0.625	0.75	0.748	99.794	99.511	0.832	0.836
0.625	0.75	0.739	98.574			
0.625	0.75	0.751	100.164			

Limits of Detection and Quantification (LoD and LoQ)

The LoD values were found to be 3.75, 1.80, 0.02 for hydroquinone, allantoin and tretinoin, respectively. The LoQ values were found to be 11.37, 5.44 and 0.07 for hydroquinone, allantoin and tretinoin, respectively. LoD and LoQ condition are shown in Table 8.

Robustness

It was measured by changing pH, Ratio of mobile phase and flow rate. The pH of mobile phase was set at ± 0.2, Ratio of Mobile phase was set ± 5 mL and flow rate was set at ± 0.2 mL/min. Solution of both the drugs was injected three times. Robustness condition was shown in Table 9.

Analysis of Marketed Formulation

Weight about 10 gm topical cream into a 100 mL volumetric flask. Add 60mL methanol and put this volumetric on water bath at 60°C for 15 minutes then allow cooling at room temperature. Shake for 15 minutes. Make up volume with methanol up to 100 mL. Filter this solution with what man filter paper no-1. So finally became hydroquinone-2000 µg/mL, allantoin-1000 µg/mL, and tretenoin-12.5 µg/mL. The results are shown in Table 10.

Force Degradation Studies

The drugs were intentionally degraded by treating with Acid, Base, Thermal, Oxidation and exposing to Sunlight condition. Degradation condition shown in Table 11 and Figs. 9–13.

Acid Degradation

For Acid Degradation for Blank, 2 mL 0.1N HCl and 2 mL 0.1N NaOH was taken and volume made up the volume 10 mL with mobile phase. For hydroquinone, allantoin

Table 8: LOD and LOQ

1. Parameters	2. Allantoin	3. Hydroquinone	4. Tretenoin
5. Linearity range (µg/mL)	6. 100–300 µg/mL	7. 50–150 µg/mL	8. 0.6–1.8 µg/mL
9. Correlation Co-efficient	10. 0.999	11. 0.999	12. 0.999
13. Slope Mean	14. 20.43	15. 56.29	16. 1694.41
17. SD of Intercept	18. 23.23	19. 30.64	20. 12.26
21. LOD	22. 3.75	23. 1.80	24. 0.02
25. LOQ	26. 11.37	27. 5.44	28. 0.07

Table 9: Robustness Data

Change in flow rate		*Buffer(pH4) Methanol /80:20*			*pH 4*	
Hydroquinone						
Level	Avg. Area ± SD	% RSD	Avg. Area ± SD	% RSD	Avg. Area ± SD	% RSD
+0.2	3920.75 ± 34.90	0.890	3908.60 ± 40.01	1.024	4107.04 ± 30.65	0.746
-0.2	4299.36 ± 37.19	0.865	4299.47 ± 43.93	1.022	4112.39 ± 42.16	1.025
Allantoin						
+0.2	5398.35 ± 40.79	0.756	5383.82 ± 40.41	0.751	5653.99 ± 51.17	0.905
-0.2	5901.31 ± 64.18	1.088	5924.16 ± 43.01	0.726	5653.44 ± 61.02	1.079
Tretenoin						
+0.2	2025.37 ± 20.79	1.026	2025.02 ± 20.18	0.997	2125.92 ± 25.72	1.210
-0.2	2221.20 ± 26.30	1.184	2225.33 ± 25.58	1.150	2128.26 ± 24.70	1.161

Table 10: Analysis of marketed formulation

Drug	*Label claim (%w/w))*	*Result (% w/w)*	*%Assay*	*Mean %Assay*	*%RSD*
Hydroquinone	2	2.029	101.437	100.639	0.872
	2	1.994	99.698		
	2	2.016	100.780		
Allantoin	1	1.008	100.768	99.959	0.991
	1	0.989	98.854		
	1	1.003	100.254		
Tretenoin	0.0125	0.012	99.959	97.791	0.744
	0.0125	0.012	97.059		
	0.0125	0.012	97.798		

Table 11: Result of degradation study

	Hydroquinone	*Allantoin*	*Tretenoin*
Condition	*% Drug degradation*		
Acid	22.74	17.46	10.50
Base	14.17	11.91	20.45
Oxidation	12.28	25.57	18.66
Photo	16.36	20.25	8.82
Thermal	10.99	13.89	10.12

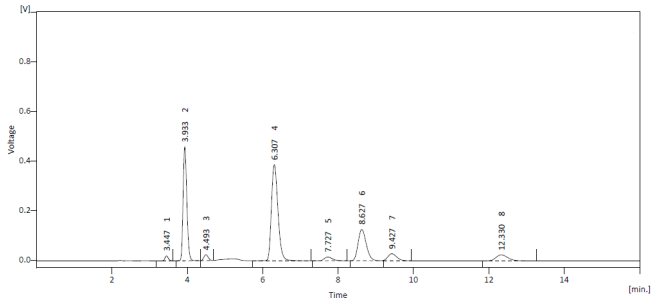

Fig. 8: Acid degradation study

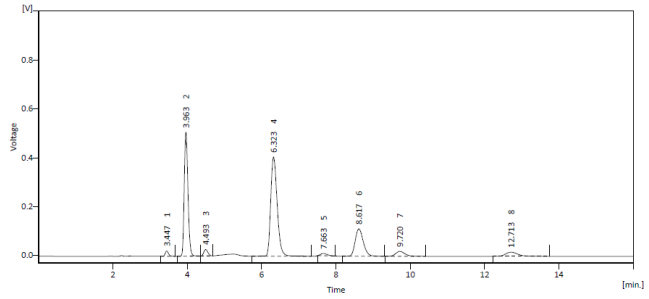

Fig. 9: Base degradation study

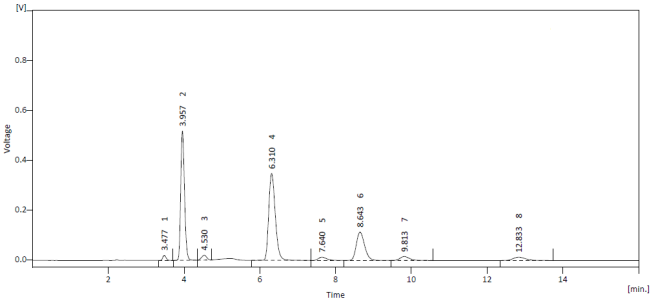

Fig. 10: Oxidative degradation study

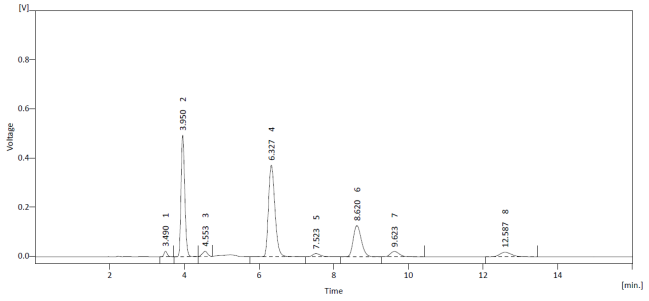

Fig. 11: Photo degradation study

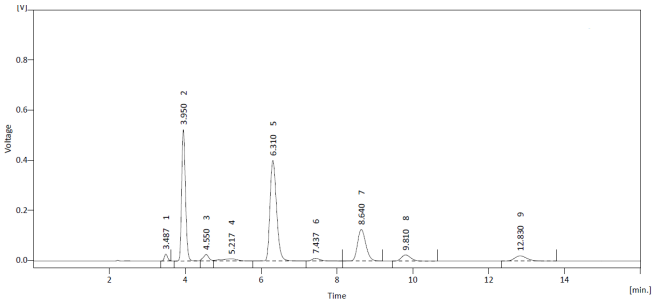

Fig. 12: Thermal degradation study

and tretenoin standard degradation, 1mL hydroquinone, allantoin and tretenoin stock solution was taken and 2 mL 0.1N HCl was added then kept for 5 hours and neutralize with 2 mL 0.1N NaOH to stop the degradation further. Finally, volume was made up to 10mL with mobile phase. For sample degradation also same procedure was followed.

Base Degradation

For Base Degradation blank preparation, 2 mL 0.1 N NaOH and 2 mL 0.1 N HCl was taken and volume makes up to 10 mL with mobile phase. For standard degradation, hydroquinone, allantoin and tretenoin were taken 1-mL from stock solution and 2 mL of 0.1N NaOH was added then kept for 3.5 hours and neutralize with 2 mL 0.1N HCl to stop the degradation further. Finally, volume was made up to 10 mL with mobile phase. For sample degradation also same procedure was followed.

Oxidation Degradation

For Oxidative Degradation blank preparation 2 mL of 3% H_2O_2 was taken and volume made up to 10 mL with mobile Phase. For hydroquinone, allantoin and tretenoin, 1-mL stock solution was taken and 3% H_2O_2 was added and kept for 3 hours. Finally volume was made up to 10mL with mobile phase. For sample degradation also same procedure was followed.

Thermal Degradation

For Thermal Degradation blank preparation, 2 mL mobile phase was kept at 105^0C and then volume made up to 10 mL with mobile phase. For hydroquinone, allantoin and tretenoin, 1-mL stock solution were taken and kept at 105^0C for 4.5 hours. Finally, volume was made up to 10mL with mobile phase. For sample degradation also same procedure was followed.

Sunlight Degradation

For Sunlight Degradation, 1-mL hydroquinone, allantoin and tretenoin were taken and kept at sunlight for 3.5 hours. Finally, volume was made up to 10 mL with mobile phase. For sample degradation also same procedure was followed.

Conclusion

The Results of our study indicate that the proposed stability indicating RP-HPLC Method is simple, rapid, precise and

accurate. This Method was developed and validated for the routine analysis of hydroquinone, allantoin and tretinoin in cream topical formulation. The result reveals that the proposed method could be successfully applied for the routine analysis and quality control of pharmaceutical dosage forms containing hydroquinone, allantoin and tretinoin. The mobile phase conditions were optimized so there was no interference from solvent and excipients. The mobile phase contains Buffer (pH 3.5) 0.05M Potassium Dihydrogen Ortho phosphate-Methanol in the ratio of 70:30% V/V at a flow rate of 1-mL/min was selected. To determine the appropriate wavelength for simultaneous estimation of hydroquinone, allantoin and tretenoin solution of these compounds in methanol were scanned in the range of 200 to 400 nm. From the overlay UV spectra it concluded that 223 nm was the most appropriate wavelength for analysis of all the drugs with suitable sensitivity. Statistical analysis proves that the method is repeatable and selective for the analysis of this cream formulation. The Method was found to be stable, as there was less degradation observed when the drug was stressed under accelerated conditions. It can therefore be concluded that use of the method can save much time and money and it can be used in small laboratories with very high accuracy and a wide linear range.

Acknowledgement

We would like to express our gratitude to R. K. School of Pharmacy, Loba Chemical Private limited, Mumbai, Abbott Pharmaceutical, Mumbai for providing the gift samples. We would also like to acknowledge School of Pharmacy, RK University, Rajkot and Department of pharmaceutical sciences, Saurashtra University, Rajkot for providing the necessary infrastructure required to carry out the present research work.

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HOW TO CITE THIS ARTICLE: Shilu R, Kapupara P. A Stability indicating Reverse Phase High Performance Liquid Chromatography Method for Simultaneous Estimation of Allantoin, Hydroquinone and Tretenoin in Cream Formulation. Int. J. Pharm. Sci. Drug Res. 2022;14(2):195-201. **DOI:** 10.25004/IJPSDR.2022.140206