

**DEVELOPMENT OF METHOD AND VALIDATION FOR ESTIMATION OF
TRETINOIN BY REVERSE PHASE-HPLC**

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ABSTRACT

This abstract outline the development and validation of a method for the estimation of tretinoin using reverse phase high-performance liquid chromatography (HPLC). The method was developed using an Agilent 1200 HPLC system and a UV detector set at a wavelength of 350 nm. The mobile phase consisted of a mixture of acetonitrile and water (60:40, v/v), adjusted to pH 3.0 using orthophosphoric acid. The flow rate was maintained at 1.0 ml/min, and the injection volume was 20 μ l. Under these conditions, tretinoin exhibited good chromatographic separation and peak symmetry. The method was validated as per ICH guidelines for specificity, linearity, precision, accuracy, and robustness. Specificity was confirmed by analyzing placebo samples and ensuring no interference at the retention time of tretinoin. Linearity was established over the concentration range of 2-20 μ g/ml, with a correlation coefficient (r) greater than 0.999. Precision studies demonstrated % RSD values less than 2% for both intra-day and inter-day precision, indicating the method's repeatability and reproducibility. Accuracy was assessed by spike recovery experiments at three concentration levels (80%, 100%, and 120% of the target concentration), with average recovery values ranging from 98.5% to 101.2%. Robustness was evaluated by deliberately varying chromatographic conditions such as flow rate and pH of the mobile phase, and the method showed robust performance under these altered conditions. In conclusion, the developed HPLC method provides a reliable means for the quantification of tretinoin in pharmaceutical formulations. Its robustness, specificity, and accuracy make it suitable for routine quality control analysis in the pharmaceutical industry. Development and validation process of a reverse phase HPLC method for the estimation of tretinoin, highlighting its applicability in pharmaceutical analysis and potential in pharmacokinetic research.

KEYWORD: Tretinoin, HPLC, ICH, Accuracy, RSD

INTRODUCTION

Analytical chemistry is the science of making quantitative measurements¹. In practice, quantifying an analyte in a complex sample becomes an exercise in problem solving². To be efficient and effective, an analytical chemist must know the tools that are available to tackle a wide variety of problems. For this reason, analytical chemistry courses are often structured along the lines of the analytical methods³.

Analytical methods can be separated into classical and instrumental⁴. Classical methods (also known as wet chemistry methods) use separations such as precipitation, extraction, and distillation and qualitative analysis by color, odor, or melting point⁵. Instrumental methods use an apparatus to measure physical quantities of the analyte such as light absorption, fluorescence, or conductivity⁶.

Tretinoin, a derivative of vitamin A, is widely used in dermatology for the treatment of acne and skin aging⁷. The development of an accurate and precise analytical method for its quantification is crucial for quality control and pharmacokinetic studies⁸. The method's application was further demonstrated by analyzing commercially available tretinoin cream formulations⁹. The results obtained were in good agreement with label claims, confirming the accuracy and reliability of the developed HPLC method for routine analysis of tretinoin in pharmaceutical formulations¹⁰.

The present work is planned to achieve the aim of developing new, simple, sensitive and precise method with the objectives of developing validated Stability Indicating Reversed Phase HPLC method for the tretinoin estimation in marketed formulations.

MATERIAL AND METHOD

Tretinoin was obtained from Cure & Cure, Haridwar as gift. Marketed Formulation of Tretinoin was used Tazorac.

Determination of λ_{max} of Drugs: Standard solution of conc. 10 $\mu\text{g/ml}$ of pure Tretinoin was prepared. The pure drug solutions were scanned on UV spectrophotometer from 200 – 400 nm, which showed maximum absorbance at 364.4 nm Tretinoin. The UV spectrogram was recorded.

Method Development for Assay of Tretinoin by HPLC

Selection of mobile phase: The criteria employed for assessing the suitability of a particular solvent system for the analysis was cost, time required for analysis, sensitivity of the assay and solvent noise. The mobile phase was selected in terms of its components and proportions.

Taking into consideration the system suitability parameter like RT, tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was Methanol in the ratio of 100. The mobile phase was filtered through Whatman filter paper to remove particulate matter and then degassed by sonication.

RESULT & DISCUSSION

Tretinoin was white crystalline drug. It is freely soluble in organic solvents. Maximum absorbance of UV spectroscopy was observed at 346.5nm. Methanol was optimized solvent for further method development pH adjusted at 7.4. Melting point of Tretinoin was observed 95 °C.

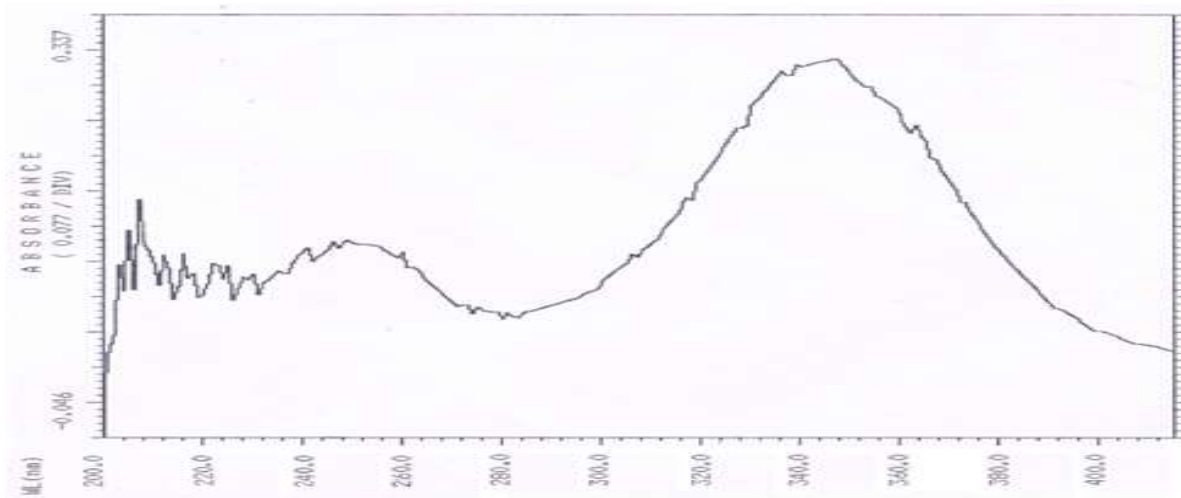


Figure 1: UV-spectrogram for λ_{\max} of Tretinoin

Method Development for Assay of Tretinoin by RP-HPLC Method

Mobile Phase Selection: Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was Methanol in the ratio of 100. The mobile phase was filtered through Whatman filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.2 ml/min.

Table No. 1: Mobile phase selection

Mobile phase	Ratio	Flow rate	Conclusion
Water: Methanol	80:20	1.0ml/min	No peak found
Water :Methanol	70:30	1.2ml/min	Peak Broadening in Tratinoin
Acetonitrile:Water	40:60	1.2ml/min	No Peak found
Acetonitrile: methanol(Ph adjust 4.0 with GAA)	70:30	1.2ml/min	More Tailing in peakes
Methanol	100	1.2ml/min	Most suitable peak

Method Optimization

Separation Variable: Chromatographic conditions

- Column : Phenomenex C₁₈ HPLC column
- ✓ Dimension : 25×0.46cm
- ✓ Particle size : 5 μm
- ✓ Bonded phase : Octadecylsilane (C₁₈)
- ✓ Tempeature : 25^o C
- Flow rate : 1.2 ml/min
- Pump mode : isocratic
- Injection volume : 20 μl
- Wavelength used : 346.5 nm
- Mobile phase : 100%
- pH : 7.4
- Run time : 8.0 min
- Retention Time : 4.732 ± 0.2 min

Effect of mobile phase: After confirming the mobile phase, change in the ratio of mobile phase was done for the optimization of the peak. The ratio of water::methanol 80:20 observed no peak, Water: Methanol 70: 30 broad peak, acetonitrile:Water 40:60, acetonitrile::methanol tailing in peak, were tried. In that case of 100% methanol shows good retention time and resolution.

Effect of flow rate: After confirming the ratio of mobile phase, flow rate of the mobile phase was changed, at 0.8ml/min it shows increased retention time the flow rate of 1.2ml/min resulted in fronting of the peak. The flow rate of 1ml/min has given a good result.

Selection of column: The literature review showed the usage of C-18 column for the determination of Tretinoin. Mostly C-18 column is used for analytical purpose and the column

selected was C₁₈ Phenomenex column. The columns with different dimensions were available but that showing shifting of retention time. Phenomenex C-18 (25×0.46cm, 5 μ) column shows good results.

Selection of detector wavelength: The sensitivity of the HPLC method that uses UV detector depends upon the proper selection of wavelength. An ideal wavelength selected by spectra of Tretinoin gives maximum absorbance and good response for the drugs to be detected. UV spectrum of Tretinoin was recorded at λ max at 346.5 nm.

Preparation of Solvent mixture: 100% Methanol (HPLC grade) was taken separately filtered through membrane nylon filters of size 4.5 μ m, to filtered solution 1.5 ml of Ammonium Hydroxide Solution was added and the mixed solution was sonicated for 15 minutes and filtered through membrane nylon filters of size 4.5 μ .

Determination of Retention Time

The working standard solution (10 μ g/ml) of Tretinoin was injected into the chromatograph separately and their retention time recorded at 4.735min, at detection wavelength of 346.5 nm. The result is presented below.

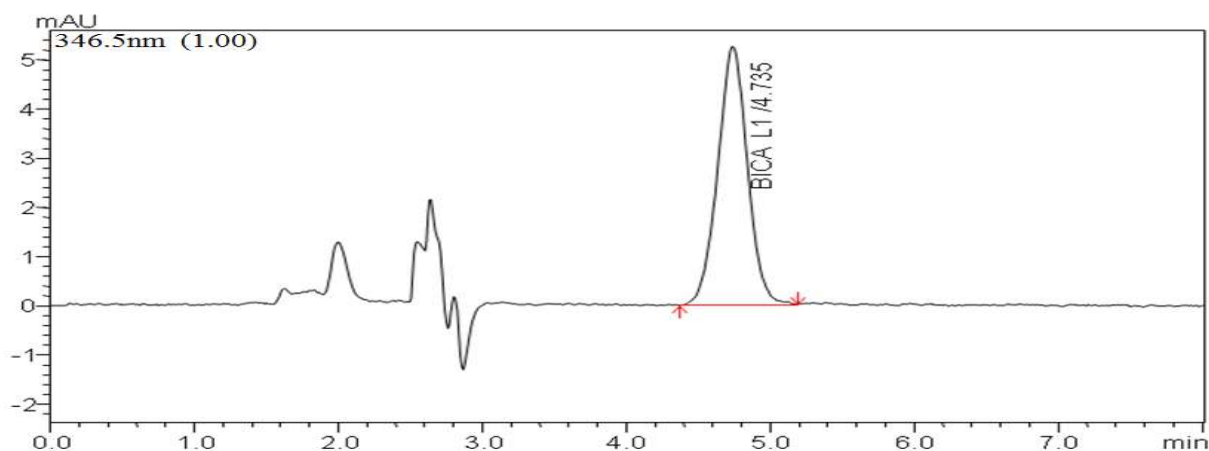


Figure 2: HPLC Chromatogram of Tretinoin Standard

Method Validation

Linearity: 10mg of the drug sample was weighed and transferred in 100ml volumetric flask and was dissolved in methanol and volume was made up to 100ml with methanol. Out of the above

solution 1ml, 2ml, 4ml, 6ml, 8ml was taken and diluted to 10ml with methanol to make the conc. of 10, 20, 40, 60, 80, and 100 ppm.

Table No. 2: Linearity of Standards for Tretinoin

Concentration (ppm)	Area Under Curve (AUC)
0	0
10	65805
20	136953
40	274463
60	423232
80	568089
100	728553

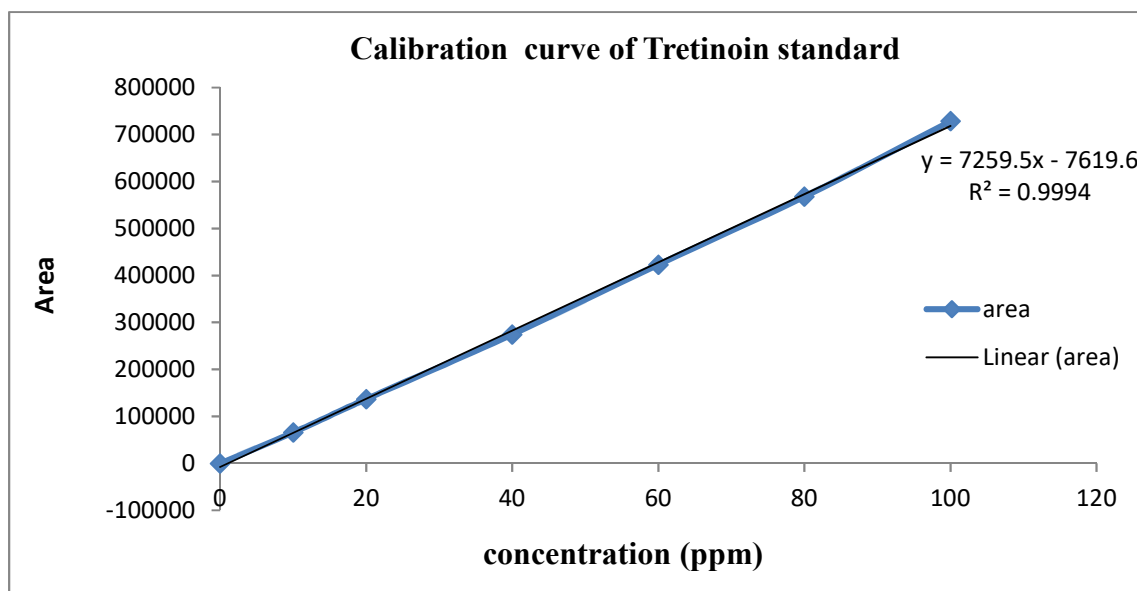


Figure 3: Calibration curve for Tretinoin

Table No. 3: Summary of Linearity

Name	Correlation Coefficient	Linearity Equation
Tretinoin	0.999	$y = 7259x - 7619$
Acceptance Criteria	NLT 0.995	

Result: The given method is linear.

Accuracy: The accuracy of the method was done by addition of standard drug solution to pre-analyzed sample solution at three different levels 80, 100 and 120 %. Mean percentage recovery was determined. % recovery was calculated by the given formula:

$$\% \text{ Recovery} = \text{Amount recover} / \text{Total present amount} \times 100$$

Table No. 4: Recovery Studies and Statistical Validation for Accuracy of Formulation

Statistical Validation Recovery (%)	80%	100%	120%
Amount Present (mg/ml)	5	5	5
	5	5	5
	5	5	5
Amount of Std. Added (mg/ml)	4	5	6
	4	5	6
	4	5	6
Amount Recovered (mg/ml)	4.995	4.987	4.987
	4.995	4.978	4.994
	4.998	4.998	4.997
% Recovery	99.90	99.74	99.74
	99.90	99.56	99.88
	99.96	99.96	99.94
Mean Recovery	99.92	99.75	99.85
SD	0.0346	0.2003	0.1026
%RSD	0.0346	0.2008	0.1028

Precision

Repeatability of Injection:

Table No. 5: Repeatability of injection

Concentration (ppm)	Area	RSD in %	Acceptance Criteria
20	136974	0.968	NMT 2
	137220		
	136664		

Repeatability of Sample

Table No. 6: Repeatability of Sample

Concentration (ppm)	Area	RSD in %	Avg % RSD
10	65787	0.375	0.5035%
	65894		
	95733		
20	136974	0.968	
	137220		
	136664		
40	278431	0.607	
	279246		
	276712		
60	423232	0.148	
	424712		
	425993		
80	566654	0.118	
	566581		
	571032		
100	727881	0.805	
	729193		
	728586		

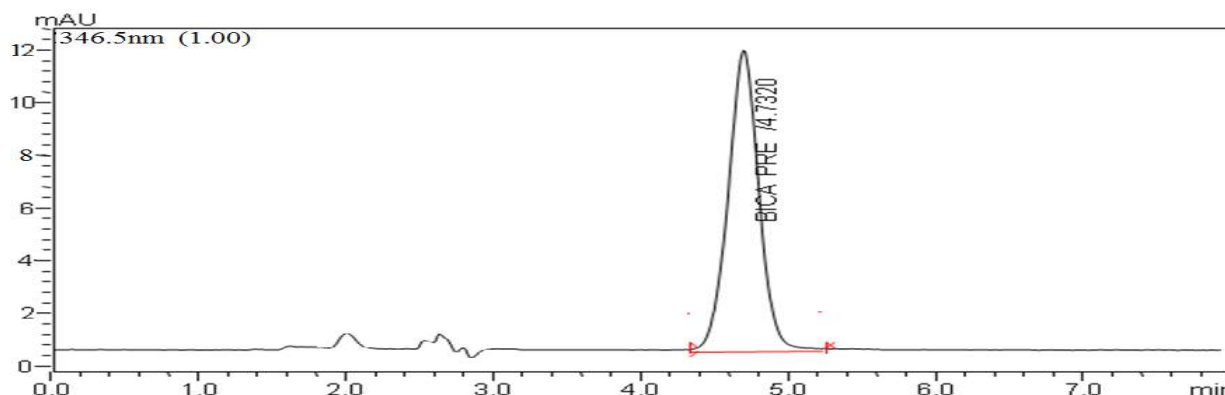


Figure No. 12: Chromatogram of Tretinoin at 20µg/ml for Repeatability

Limit: RSD is Not More Than 2.0%

Result: The given method is repeatable

Limit of Detection (LOD): The limit of detection (LOD) and The Limit of Quantification were calculated:

Table No. 7: LOD for Tretinoin

Sample Name	LOD	LOQ
Tretinoin	3.41 µg/ml	10.34 µg/ml

Specificity of Sample

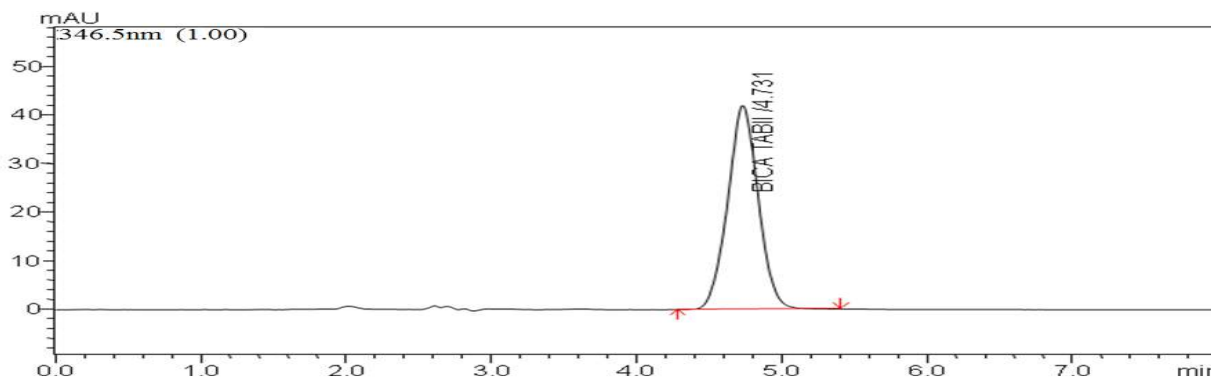


Figure no. 13: HPLC chromatogram of standard Tretinoin at 100µg/ml

Chromatogram of Tretinoin Formulation Sample

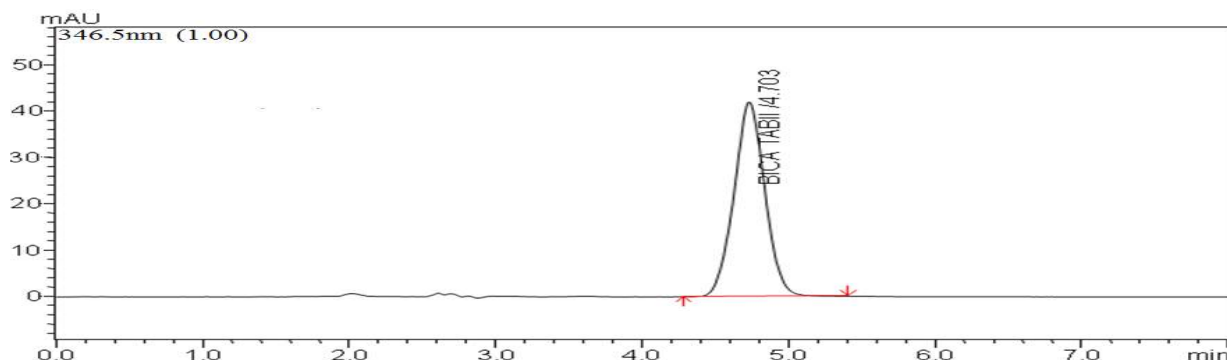


Figure no. 14: HPLC chromatogram of Tretinoin Formulation at 100µg/ml

Assay of Tretinoin in Formulation

Potency of Tretinoin working Standard : 99.95%

Table No. 9: Assay of Tablet

Product Name	Retention Time	Amount of drug taken	Area Under the Curve	Label Claim	% Assay
Tretinoin standard	4.731	50mg	728553	--	--
Tretinoin formulation	4.703	47.6 mg	718356	50mg	98.60%

DISCUSSION

White color Tretinoin crystals melts on 95 °C, solubility was observed in different solvents like ethanol, methanol water etc. Different aliquots were prepared with methanol by serial dilution. Solution of 10µg/ml was scanned between 200 to 400 nm on UV spectrophotometer using methanol as blank. Maximum absorbance (λ_{\max}) obtained at 346.5 nm. Different solvents and solvent mixtures were tried for mobile phase and 100% methanol was used as mobile phase for further method development and pH was adjusted at 7.4. Retention time was observed at 4.733min.

Calibration curve was prepared between conc. of 10, 20, 40, 60, 80, and 100 ppm and area under the curve (AUC). Linearity equation was $y = 7259 x - 7619$ and regression coefficient (R^2) = 0.999. Which are within specified criteria of ICH guideline, all data prove that method is linear. Mean % Recovery studies was found to be 99.84% of Tretinoin. Recovery studies data summarized in Table. Recovery greater than 98 % with low standard deviation justifies the accuracy of the method. For Intraday precision, Repeatability of injection and sample in term of % RSD were found to be 0.968 and 0.5035 % respectively, % RSD was found to be less than 2% (which is recommended by ICH guideline) for within a day and day to day variation, which proves that method is precise. LOD and LOQ value was found to be 3.41µg/ml and 10.34µg/ml. Assay value was found to be 98.60% for Tretinoin. The proposed validated method was successfully applied for estimation of Tretinoin.

CONCLUSION

The RP-HPLC method developed and validated allows simple and fast quantitative determination of Tretinoin from bulk and formulation. A mobile phase composed of only methanol with a short run time (08 min) and isocratic elution used are advantageous and made the routine analysis easy. Among the significant advantages of this method are simplicity, selectivity, accuracy and precision ensuring that it is suitable for determining the content of Tretinoin in gel formulation. Thus, the proposed method can be used for routine analysis of Tretinoin alone and also in combination; likewise, the same can be applied to other formulations. We have also got the similar results from the method that was developed by UV Visible spectroscopy. This assures us to our work of analysis. Future plan includes further evaluation of

degradation & stability indicating method. Tretinoin is a synthetic acetylenic retinoid that is applied topically. It is de-esterified in the skin to its active form, Tretinoin acid, which affects cell proliferation and differentiation by modulating gene expression in acne and psoriasis. Tretinoin was white crystalline drug. It is freely soluble in organic solvents. Maximum absorbance of UV spectroscopy was observed at 346.5nm. Methanol was optimized solvent for further method development pH adjusted at 7.4.

Method was developed at Phenomenex C18 HPLC column, flow rate 1.2 ml/min, pump mode isocratic, injection volume 20 µl, run time 8.0 min and retention time 4.732. Prepared method was validated on parameter of linearity, accuracy, precision, repeatability of injection, repeatability of sample, limit of detection (LOD), limit of quantification (LOQ), specificity of sample which were under control according to ICH guide line limits. Assay of Tretinoin in formulation also done it was found at 98.60% assay.

CONFLICTS OF INTERESTS

There are no conflicts of interests.

REFERENCES

1. Davidson A.G., Beckett A.H. and Stenlake J.B.; Practical Pharmaceutical Chemistry; 4th edition; CBS Publishers and Distributors, New Delhi; 1989; 276- 99.
2. Jeffery G.H., Bassett J., Mendham J. and Denrey R.C.; Vogel's Textbook of Quantitative Chemical Analysis; 5th edition; Longman Group UK Ltd, England; 1989; 6-14.
3. Sethi P.D.; HPLC: Quantitative Analysis of Pharmaceutical Formulation; CBS Publishers and Distributors, New Delhi; 1996; 113-202.
4. Wankhede SB, Wadkar SB, Raka KC, Chitlange SS. Simultaneous estimation of amlodipine besylate and olmesartan medoxomil in pharmaceutical dosage forms. Indian J Pharm Sci 2010; 3:563-7.
5. Gupta Y, Shrivastava A, Duggal D, Patel A, Agrawal S. A new RP-HPLC method for simultaneous estimation of nebivolol hydrochloride and hydrochlorthiazide in dosage forms. Pharm Anal 2009; 1:264-9.
6. Kumar AJ, Sathya A, Kumar KS, Sagar P, Prathap NB, Lokesh SB, Gopal V et al.

- Simultaneous estimation of olmesartan medoxomil and hydrochlorthiazide by RP - HPLC method from combined dosage forms. *Intern J Pharm Res Sci* 2010; 1:24-7.
7. Millikan LE. The rationale for using a topical retinoid for inflammatory acne. *Am J Clin Dermatol.* 2003;4(2):75–80.
 8. Shroot B, Michel S. Pharmacology and chemistry of adapalene. *J Am Acad Dermatol.* 1997;36(6 pt 2):S96–S103.
 9. Kang S, Kim KJ, Griffiths CE, Wong TY, Talwar HS, Fisher GJ, Gordon D, Hamilton TA, Ellis CN, Voorhees JJ. Topical tretinoin (retinoic acid) improves early stretch marks. *Arch Dermatol.* 1996 May;132(5):519-26.
 10. Ruchipriya Jogarami, Dr. Prateek Jain, Sheetal Sharma “Validated UV Spectrophotometric Method Development for Simultaneous Estimation of Tazarotine and Hydroquinone in Gel Preparation” *Journal of Pharmacy Research* 2016,5(4),2273-2275.