

Development and Validation Of A Stability-Indicating Uplc Method For Simultaneous Estimation Of Tretinoin And Mequinol in Topical Formulation

Ajinkya V. Gulhane^{1*}, Prof. (Dr) Neelima Naneriya²

¹Research Scholar, Department of Pharmacy, Oriental University, Indore (M.P)-453555

²Professor & Supervisor, Department of Pharmacy, Oriental University, Indore (M.P)-453555

Corresponding Author, Ajinkya V. Gulhane, Email ID: ajinkyagulhane1@gmail.com

ABSTRACT:

Background: Tretinoin and mequinol are used in topical formulations and require reliable analytical procedures for their simultaneous quantitative determination. The present study aimed to develop and validate a simple, precise, accurate, and stability-indicating UPLC method for simultaneous estimation of tretinoin and mequinol in topical formulation.

Methods: Chromatographic separation was achieved using an Inertsil C18 column (250 × 4.6 mm, 5 µm) with methanol and phosphate buffer (pH 3.0) in the ratio of 70:30 (v/v) as the mobile phase. The flow rate was maintained at 1.0 mL/min, injection volume was 20 µL, and detection was performed at 325 nm under isocratic conditions. The method was validated for system suitability, specificity, linearity, precision, accuracy, sensitivity, robustness, and ruggedness. Linearity was evaluated over the concentration range of 10–50 µg/mL for both analytes. Forced degradation studies were performed under acidic, alkaline, oxidative, thermal, and photolytic stress conditions. The applicability of the method was further demonstrated through analysis of a marketed topical formulation.

Results: The developed method provided satisfactory chromatographic separation of mequinol and tretinoin, with mean retention times of approximately 2.9 and 5.7 min, respectively. The method demonstrated good linearity, with coefficients of determination (R^2) of 0.9991 for mequinol and 0.9994 for tretinoin. System precision showed %RSD values of 0.114% and 0.133%, while method precision showed %RSD values of 0.456% and 0.703% for mequinol and tretinoin, respectively. The mean recoveries were approximately 100% for both analytes. The LOD/LOQ values were 0.586/0.943 µg/mL for mequinol and 0.215/0.892 µg/mL for tretinoin. The method remained unaffected by deliberate variation in flow rate. Forced degradation generated additional degradation-related peaks that were chromatographically separated from the principal analyte peaks. Tretinoin showed greater susceptibility to photolytic, oxidative, and alkaline stress, whereas mequinol exhibited comparatively lower degradation under the investigated conditions.

Conclusion: A reliable and stability-indicating UPLC method was successfully developed for simultaneous estimation of mequinol and tretinoin. The method exhibited satisfactory specificity, linearity, precision, accuracy, sensitivity, and robustness and was successfully applied to the analysis of a marketed topical formulation. The developed procedure can therefore be used for routine quality-control analysis and stability assessment of topical formulations containing mequinol and tretinoin.

Keywords: Mequinol; Tretinoin; UPLC; Method Development; Method Validation; Forced Degradation; Stability-Indicating Method; Topical Formulation; Precision; Accuracy.

INTRODUCTION:

Topical drug delivery is widely used in dermatological therapy because it provides direct application of active pharmaceutical ingredients to the affected site and can improve therapeutic effectiveness. The quality and stability of topical formulations depend on accurate control of the incorporated drug substances. Therefore, reliable analytical methods are essential for their identification, assay determination, and stability evaluation.¹ Tretinoin is a vitamin A derivative widely used in topical treatment of acne and other dermatological conditions. It promotes epidermal cell turnover and regulates cellular differentiation. However, tretinoin is susceptible to degradation, particularly under light, oxidative, and thermal conditions, which necessitates appropriate analytical monitoring during formulation development and storage.

Mequinol (4-methoxyphenol) is a topical depigmenting agent that reduces melanin formation by interfering with melanogenesis. Combination formulations containing mequinol and tretinoin are used in the management of hyperpigmentation. Since these two drugs are present at different concentrations and have different physicochemical properties, their simultaneous estimation requires a selective and sensitive analytical method.

UPLC is a powerful chromatographic technique offering high separation efficiency, reproducibility, and relatively rapid analysis. A stability-indicating method should additionally be capable of separating the active ingredients from formulation components and degradation products generated under different stress conditions. Forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions are therefore important for establishing the stability-indicating capability of an analytical procedure.²

The present study was undertaken to develop and validate a stability-indicating UPLC method for simultaneous estimation of mequinol and tretinoin in a topical formulation. The developed method was optimized and validated for specificity, linearity, accuracy, precision, sensitivity, robustness, and ruggedness. Forced degradation studies were performed to evaluate the degradation behavior of both analytes, and the validated method was subsequently applied to the analysis of a marketed topical formulation. The developed procedure provides a suitable approach for routine quality-control and stability assessment of formulations containing mequinol and tretinoin.

MATERIALS AND METHODS:

MATERIALS:

Tretinoin and mequinol were obtained as gift samples from Topclass Enterprises LLP, New Delhi, India. Methanol and other solvents of HPLC grade were procured from Merck Life Science Pvt. Ltd., Mumbai, India. Phosphate buffer components and other analytical-grade reagents used during method development and validation were obtained from certified commercial suppliers. Ultrapure water generated using a Milli-Q® water purification system (Merck Millipore, Bedford, MA, USA) was used throughout the analytical studies. The marketed topical formulation containing mequinol and tretinoin was procured from a commercial source and used for applicability testing of the developed method. All reagents and solvents were of appropriate analytical or chromatographic grade.

Instrumentation and Chromatographic Equipment

Chromatographic analysis was carried out using a Shimadzu LC-2010 series liquid chromatographic system equipped with an autosampler and LC-UV-2075 UV–visible detector. The system was operated and data were processed using LC solution software (LC 3D). An Intersil ODS-3V C18 column (250 × 4.6 mm, 5 µm; GL Sciences Inc., Japan) was used for chromatographic separation. An electronic analytical balance (Elder) was used for accurate weighing, while an ultrasonic bath (Prima Instruments) was employed for dissolution and homogenization of standard and sample solutions.³⁻⁵

Preparation of Mobile Phase

The mobile phase was prepared by mixing methanol with phosphate buffer adjusted to pH 3.0 in a ratio of 70:30 (v/v). The mixture was thoroughly homogenized and degassed before use.

Preparation of Standard Stock Solution

Tretinoin (10 mg) and mequinol (20 mg) were accurately weighed and transferred to a 10 mL volumetric flask. Approximately 7 mL of diluent was added, followed by sonication until complete dissolution. The solution was then diluted to volume with the same diluent to obtain Stock Solution I.⁶⁻⁷

A 5 mL aliquot of Stock Solution I was transferred to a 25 mL volumetric flask and diluted to volume with diluent to obtain Stock Solution II. Subsequently, 1.5 mL of Stock Solution II was transferred to a 10 mL volumetric flask and diluted to the mark with diluent to obtain Stock Solution III, which was used for subsequent analytical studies.

Development and Optimization of the Chromatographic Method

The chromatographic method was developed by evaluating different mobile-phase compositions to obtain satisfactory separation of tretinoin and mequinol. Methanol:water at 40:60 and 80:20 (v/v) and methanol:phosphate buffer (pH 3.0) at 50:50 (v/v) were initially investigated. The chromatographic responses obtained under each condition were evaluated with respect to separation and peak characteristics.⁸⁻¹⁰

Based on the observed chromatographic performance, methanol:phosphate buffer (pH 3.0) at 70:30 (v/v) was selected as the optimized mobile phase for simultaneous estimation of tretinoin and mequinol.

Optimized Chromatographic Conditions

The optimized method employed an Intersil ODS-3V C18 column (250 × 4.6 mm, 5 µm) with methanol:phosphate buffer (pH 3.0) in the ratio of 70:30 (v/v) as the mobile phase. Detection was performed using an LC-UV-2075 UV–visible detector. The autosampler was capable of delivering injection volumes up to 20 µL, and chromatographic data were acquired and processed using LC solution software.¹¹⁻¹²

Method Validation

The developed chromatographic method was systematically validated to establish its suitability for the simultaneous quantitative estimation of tretinoin and mequinol in topical formulations. The validation was performed with respect to system suitability, specificity, linearity and range, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness, and ruggedness. The analytical performance characteristics were evaluated in accordance with the applicable ICH recommendations for analytical procedure validation.¹³⁻²⁰

System Suitability

System suitability testing was performed to verify the adequacy and consistency of the chromatographic system before undertaking the validation experiments. A freshly prepared mixed standard solution containing tretinoin and mequinol was injected six consecutive times under the optimized chromatographic conditions using a 20 µL injection volume. The chromatographic responses were evaluated in terms of retention time, peak area, tailing factor, and theoretical plate count for both analytes. The obtained results were assessed for consistency among replicate injections to confirm satisfactory system performance and chromatographic reproducibility.

Linearity and Range

The linearity of the analytical response was evaluated over the concentration range of 10–50 µg/mL for tretinoin and mequinol. Five concentration levels covering the selected analytical ranges were prepared by appropriate dilution of the standard stock solutions. Each concentration level was injected in six replicate determinations under the optimized chromatographic conditions.

The peak areas corresponding to tretinoin and mequinol were recorded, and the mean peak area was calculated for each concentration level. Calibration curves were constructed by plotting mean peak area versus the corresponding analyte concentration. Linear regression analysis was performed to obtain the regression equation and correlation coefficient. The linearity results were used to establish the proportional relationship between analyte concentration and chromatographic response within the selected range.

Specificity

Specificity was investigated to demonstrate the ability of the developed method to selectively estimate tretinoin and mequinol in the presence of potentially interfering components. Blank solution, individual standard solutions, and a mixed standard solution containing 20 µg/mL each of tretinoin and mequinol were analyzed under the optimized chromatographic conditions.

The resulting chromatograms were examined for any interfering peaks at the retention times of the analytes. Where applicable, peak-purity characteristics were also evaluated to confirm the homogeneity of the analyte peaks. The absence of significant interference at the respective retention times was considered evidence of adequate specificity of the method.

Precision

Precision was evaluated to determine the degree of agreement among repeated analytical measurements. The precision study included system precision, method precision, intraday precision, and inter-day precision.

System Precision

System precision was assessed by injecting a mixed standard solution containing 30 µg/mL each of tretinoin and mequinol six consecutive times. The retention time and peak area of each analyte were recorded for all injections. The mean, standard deviation (SD), and percentage relative standard deviation (%RSD) were calculated to determine the repeatability of the chromatographic system and detector response.

Method Precision

Method precision was evaluated using six independently prepared sample solutions containing tretinoin and mequinol at a concentration of 30 µg/mL. Each preparation was analyzed according to the proposed analytical procedure. The assay results obtained from the individual preparations were used to calculate the mean, SD, and %RSD for both analytes. The resulting variability was used to assess the repeatability of the complete analytical procedure.

Intraday Precision

Intraday precision was determined by analyzing the analytes at three concentration levels of 10, 30, and 50 µg/mL within a single day. The prepared solutions were analyzed at approximately 2-h intervals over a 12-h period under the optimized chromatographic conditions. The mean, SD, and %RSD were calculated at each concentration level for tretinoin and mequinol to assess the variability of the analytical response during the same analytical day.

Inter-Day Precision

Inter-day precision was performed to assess the reproducibility of the method on different days. Solutions corresponding to 10, 30, and 50 µg/mL were analyzed on three different days under the established chromatographic conditions. The mean, SD, and %RSD were calculated for each concentration level and analyte. The results were used to assess the day-to-day variability of the proposed method.

Accuracy

The accuracy of the developed method was determined using the standard addition technique. Known quantities of tretinoin and mequinol reference standards were added to predetermined quantities of the respective analytes at three concentration levels corresponding to 80%, 100%, and 120% of the nominal concentration.

The spiked samples were processed and analyzed using the proposed chromatographic procedure. The amount of drug recovered at each level was calculated, and the mean percentage recovery and %RSD were determined. Accuracy was expressed as the closeness between the amount of analyte recovered and the corresponding amount added.

Percentage recovery was calculated using:

$$\% \text{ Recovery} = A/B+C \times 100$$

Where, A represents the total amount of drug determined after standard addition, B represents the amount of drug originally present in the sample, and C represents the amount of reference standard added. The mean percentage recovery and %RSD were calculated for each concentration level to assess the accuracy of the proposed method.

Limit of Detection and Limit of Quantification

The sensitivity of the developed analytical method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) for tretinoin and mequinol. The calculations were based on the standard deviation of the analytical response and the slope of the respective calibration curves.

The LOD and LOQ were calculated using the following equations:

$$\text{LOD}=3.3 \times \text{SD}/S$$

$$\text{LOQ}=10 \times \text{SD}/S$$

Where, σ is the standard deviation of the response and S is the slope of the corresponding calibration curve.

Robustness

Robustness was evaluated to determine the ability of the analytical method to remain unaffected by deliberate, small variations in selected chromatographic conditions. A pre-analyzed solution containing 30 $\mu\text{g/mL}$ mequinol and 20 $\mu\text{g/mL}$ tretinoin was subjected to analysis under modified flow-rate conditions.

The flow rate was deliberately varied to 0.8 and 1.2 mL/min from the optimized condition. The chromatographic responses obtained under each altered condition were evaluated by monitoring relevant system suitability parameters and assay response. The results were compared with those obtained under the optimized chromatographic conditions to determine the influence of the flow-rate variation on method performance.

Ruggedness

Ruggedness was investigated to evaluate the reproducibility of the analytical response under the specified temperature conditions. Tretinoin and mequinol solutions at 10, 20, and 30 $\mu\text{g/mL}$ were analyzed at 25°C, 37°C, and 60°C. The corresponding chromatographic peak areas were recorded, and the %RSD was calculated for each analyte and concentration level.

The results obtained under the different temperature conditions were compared to assess the consistency of the analytical response and the sensitivity of the method to the specified experimental variation.

Forced Degradation Studies

Forced degradation studies were performed to evaluate the degradation behavior of tretinoin and mequinol under predetermined stress conditions and to establish the stability-indicating capability of the developed UPLC method. The drug substances were subjected to acidic, alkaline, oxidative, thermal, and photolytic stress conditions. After completion of each stress treatment, the samples were appropriately diluted with the selected diluent and analyzed under the optimized chromatographic conditions. The chromatograms of stressed samples were compared with those of unstressed standards to evaluate changes in analyte response and the formation of additional degradation-related peaks.¹⁷⁻²²

Acidic degradation

Approximately 100 mg each of tretinoin and mequinol was accurately weighed and transferred separately into suitable volumetric flasks. Methanol was added and the drugs were dissolved with the aid of sonication. The resulting solutions were subjected to acidic stress using hydrochloric acid and maintained at 55–60°C for 2 h in a water-bath shaker. After completion of the stress treatment, the solutions were neutralized with sodium hydroxide and suitably diluted with the selected diluent to obtain a final concentration of 30 $\mu\text{g/mL}$ of each analyte. The prepared solutions were analyzed by UPLC, and the chromatograms were examined for degradation of the parent compounds and formation of additional peaks.

Alkaline degradation

For alkaline degradation, approximately 100 mg each of tretinoin and mequinol was accurately weighed and dissolved in methanol. The solutions were treated with sodium hydroxide and maintained at 55–60°C for 2 h using a water-bath shaker. Following the stress treatment, appropriate aliquots were withdrawn and diluted with the selected diluent to obtain a final concentration of 30 $\mu\text{g/mL}$ of each analyte. The samples were subsequently analyzed by UPLC, and the resulting chromatograms were evaluated for degradation and the appearance of additional chromatographic peaks.

Oxidative degradation

Oxidative degradation was investigated by treating approximately 100 mg each of tretinoin and mequinol with hydrogen peroxide. The solutions were maintained at 55–60°C for 2 h to promote oxidative stress. Following treatment, suitable aliquots were withdrawn and appropriately diluted with the selected diluent to obtain final concentrations of 30 µg/mL mequinol and 10 µg/mL tretinoin. The samples were analyzed using the developed UPLC procedure, and the chromatographic profiles were examined for reduction in the principal analyte peaks and the generation of degradation-related responses.

Thermal degradation

Thermal degradation was evaluated by exposing approximately 100 mg each of tretinoin and mequinol to 55–60°C for 3 days in an oven. Samples were withdrawn at predetermined intervals and appropriately diluted with the selected diluent. Test solutions containing 30 µg/mL of each analyte were prepared and analyzed by UPLC. The chromatographic responses were compared with those of the corresponding unstressed solutions to assess the extent of thermally induced degradation.

Photolytic degradation

The photolytic stability of tretinoin and mequinol was investigated by exposing approximately 100 mg of each drug to ultraviolet radiation for 48 h. Samples were withdrawn at predetermined intervals and diluted appropriately with the selected diluent to obtain a final concentration of 30 µg/mL of each analyte. The samples were analyzed by the developed UPLC method, and the chromatograms were examined for changes in the analyte peaks and formation of additional degradation products.

Assay of Marketed Formulation

The applicability of the developed UPLC method was demonstrated by determining tretinoin and mequinol in a commercially available topical formulation, Melalong AD Cream (15 g; Ajanta Pharma Ltd.), containing 2% w/w mequinol and 0.01% w/w tretinoin.

Approximately 1.0 g of the cream was accurately weighed into a 10 mL volumetric flask. Approximately 7 mL of the selected diluent was added, and the mixture was sonicated for 15–20 min with intermittent shaking to facilitate extraction of both analytes from the semisolid formulation matrix. The solution was allowed to cool and then diluted to volume with the selected diluent and mixed thoroughly. The resulting extract was passed through a 0.45 µm membrane filter.

Suitable aliquots of the filtrate were further diluted to bring the concentrations of tretinoin and mequinol within their respective validated linearity ranges. The prepared solutions were analyzed under the optimized chromatographic conditions. Identification of the analytes was based on comparison of their chromatographic retention times with those of the corresponding reference standards.

The concentrations of tretinoin and mequinol were calculated from their respective calibration equations. The assay results were expressed as percentage of the labeled amount and amount of drug present per 15 g tube. Based on the declared composition, the theoretical contents were 300 mg of mequinol and 1.5 mg of tretinoin per 15 g tube.¹⁸⁻²³

RESULTS AND DISCUSSION:

Chromatographic method development and optimization

The chromatographic conditions were optimized systematically to obtain satisfactory separation and reproducible responses for tretinoin and mequinol. Different mobile-phase compositions were evaluated while maintaining the C18 column, isocratic elution, 20 µL injection volume, 1.0 mL/min flow rate, ambient column temperature, and detection at 325 nm. The initial methanol:water composition (40:60, v/v) produced poorly defined peaks with an unstable baseline. Increasing the methanol proportion to 80:20 (v/v) did not provide adequate separation and resulted in unsatisfactory peak characteristics. Methanol:phosphate buffer (pH 3.0) at 50:50 (v/v) improved peak shape; however, adequate separation of the two analytes was not achieved. Further optimization using methanol:phosphate buffer (pH 3.0) at 70:30 (v/v) resulted in satisfactory chromatographic separation with well-defined peak characteristics and an acceptable baseline. Therefore, this composition was selected as the optimized mobile phase for subsequent validation studies.

Table 1. Optimization of Chromatographic Conditions

Trail	Mobile phase	Flow rate (mL/min)	Injection volume	Detection wavelength	Observation	Decision
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1	Methanol:Water (40:60)	1.0	20 µL	325 nm	Poorly defined peaks and unstable baseline	Rejected
2	Methanol:Water (80:20)	1.0	20 µL	325 nm	Inadequate separation and unsatisfactory peak shape	Rejected
3	Methanol:Phosphate buffer pH 3.0 (50:50)	1.0	20 µL	325 nm	Improved peak shape but inadequate separation	Rejected
4	Methanol:Phosphate buffer pH 3.0 (70:30)	1.0	20 µL	325 nm	Satisfactory separation and peak characteristics	Selected

Evaluation of Chromatographic Trials

The chromatographic responses obtained during method optimization are summarized in Table 1. In Trial 1, using methanol:water (40:60, v/v), mequinol and tretinoin showed retention times of 4.590 and 9.169 min, respectively. Although both analytes produced detectable responses, the chromatographic profile was associated with an unsatisfactory baseline and inadequate peak characteristics.

With an increased methanol proportion in Trial 2 [methanol:water (80:20, v/v)], the retention times decreased to 3.043 min for mequinol and 4.120 min for tretinoin. However, satisfactory separation and peak characteristics were not achieved. In Trial 3, replacement of water with phosphate buffer at pH 3.0 [methanol:phosphate buffer, 50:50 (v/v)] substantially reduced the retention times to 1.735 and 2.132 min for mequinol and tretinoin, respectively, with improved peak responses. Nevertheless, the separation between the analytes remained inadequate.

Further optimization of the mobile-phase composition to methanol:phosphate buffer (pH 3.0), 70:30 (v/v) resulted in the selected chromatographic condition. Under this condition, mequinol and tretinoin exhibited retention times of 2.957 and 5.743 min, respectively, with peak areas of 542187 and 658421 µV·sec. The corresponding peak heights were 55348 and 57146 µV, respectively. The resulting chromatographic profile showed well-defined and adequately separated analyte peaks and was therefore selected for subsequent method validation.

Table 2. Chromatographic Response During Method Optimization

Trial	Analyte	Retention time (min)	Peak area (µV·sec)	Peak height (µV)	Observation
1	Mequinol	4.590	318628	6453	Unsatisfactory chromatographic profile
1	Tretinoin	9.169	350194	7493	Unsatisfactory chromatographic profile
2	Mequinol	3.043	310553	13620	Inadequate separation
2	Tretinoin	4.120	178953	4722	Inadequate separation
3	Mequinol	1.735	535822	56372	Improved response; inadequate separation
3	Tretinoin	2.132	675603	56980	Improved response; inadequate separation
4	Mequinol	2.957	542187	55348	Selected condition
4	Tretinoin	5.743	658421	57146	Selected condition

The optimized chromatographic condition provided distinct peaks for both analytes, with mequinol eluting at 2.957 min and tretinoin at 5.743 min. The difference in retention behavior between the two compounds provided sufficient chromatographic separation for simultaneous analysis. The relatively sharp and well-defined peaks, together with the satisfactory detector response, supported the suitability of the selected chromatographic conditions for subsequent validation experiments.

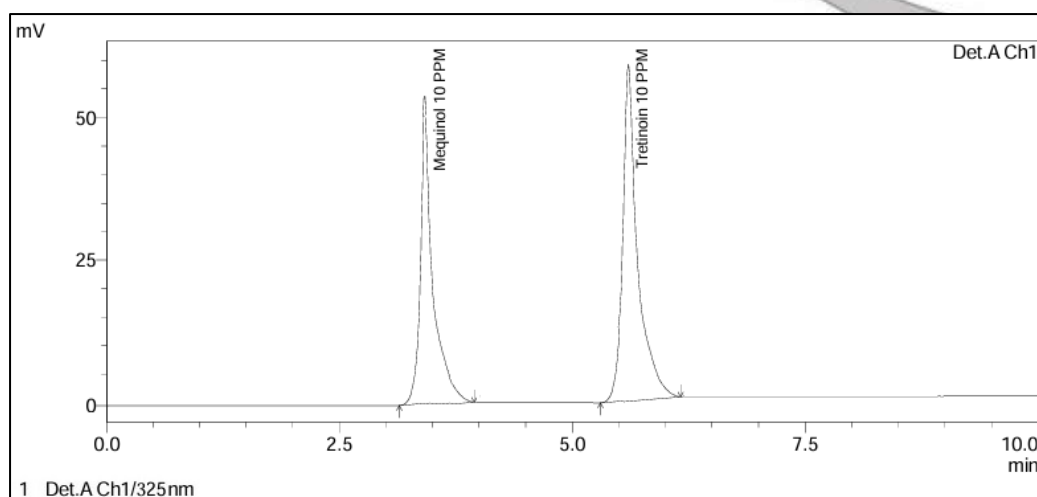


Figure 1. Optimized UPLC chromatogram of mequinol and tretinoin under the selected chromatographic conditions.

Method Validation

The optimized analytical method was evaluated for system suitability, specificity, linearity and range, precision, accuracy, robustness, ruggedness, LOD, LOQ, solution stability, and applicability to the marketed formulation. The validation studies were performed to establish the reliability, reproducibility, and suitability of the method for simultaneous determination of mequinol and tretinoin in the topical formulation.

System Suitability

System suitability was assessed by six consecutive injections of a mixed standard solution containing mequinol and tretinoin under the optimized chromatographic conditions. The retention times, peak areas, theoretical plate counts, and tailing factors were evaluated for both analytes.

For mequinol, the mean retention time was 2.915 ± 0.041 min, with a %RSD of 1.41%, while tretinoin showed a mean retention time of 5.758 ± 0.077 min, with a %RSD of 1.34%. The mean peak areas were 834275.67 and 696058.33 $\mu\text{V}\cdot\text{sec}$ for mequinol and tretinoin, respectively, with very low variability (%RSD of 0.022% and 0.016%, respectively). Theoretical plate counts were 2923 ± 52 for mequinol and 3514 ± 63 for tretinoin, indicating satisfactory column efficiency. The mean tailing factors were 1.546 and 1.554 for mequinol and tretinoin, respectively, demonstrating acceptable peak symmetry. Overall, the low variability in chromatographic responses confirmed satisfactory system performance and repeatability of the optimized analytical system.

Table 3. System Suitability Results for Mequinol and Tretinoin

Parameter	Mequinol	Tretinoin
Retention time (min)	2.915 ± 0.041	5.758 ± 0.077
Retention time %RSD	1.41	1.34
Peak area ($\mu\text{V}\cdot\text{sec}$)	834275.67 ± 181.77	696058.33 ± 112.10
Peak area %RSD	0.022	0.016
Theoretical plates (N)	2923 ± 52	3514 ± 63
Theoretical plates %RSD	1.79	1.78
Tailing factor	1.546 ± 0.003	1.554 ± 0.003
Tailing factor %RSD	0.21	0.20

Note: Values represent six replicate injections; mean $\pm$ SD.

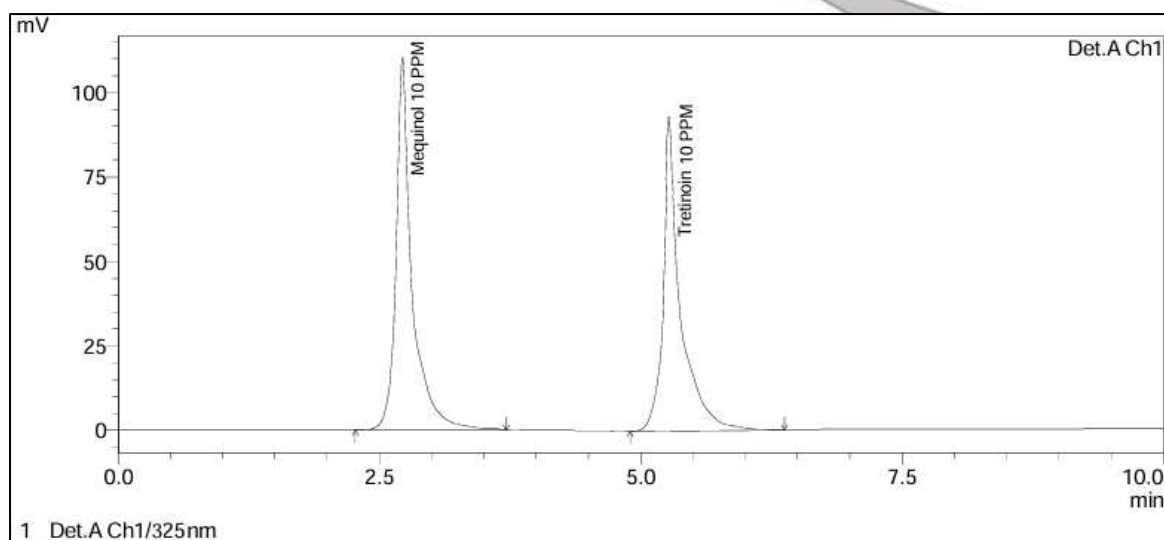


Figure 2. System suitability chromatogram of Mequinol & Tretinoin Mixture (10 µg/ml) 1

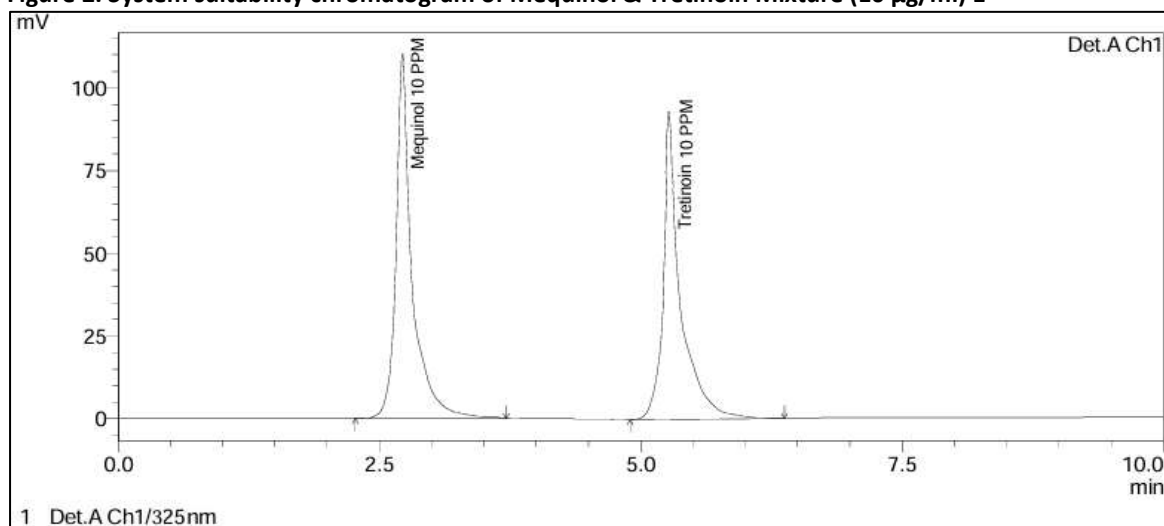


Figure 3. System suitability chromatogram of Mequinol & Tretinoin Mixture (10 µg/ml) 2

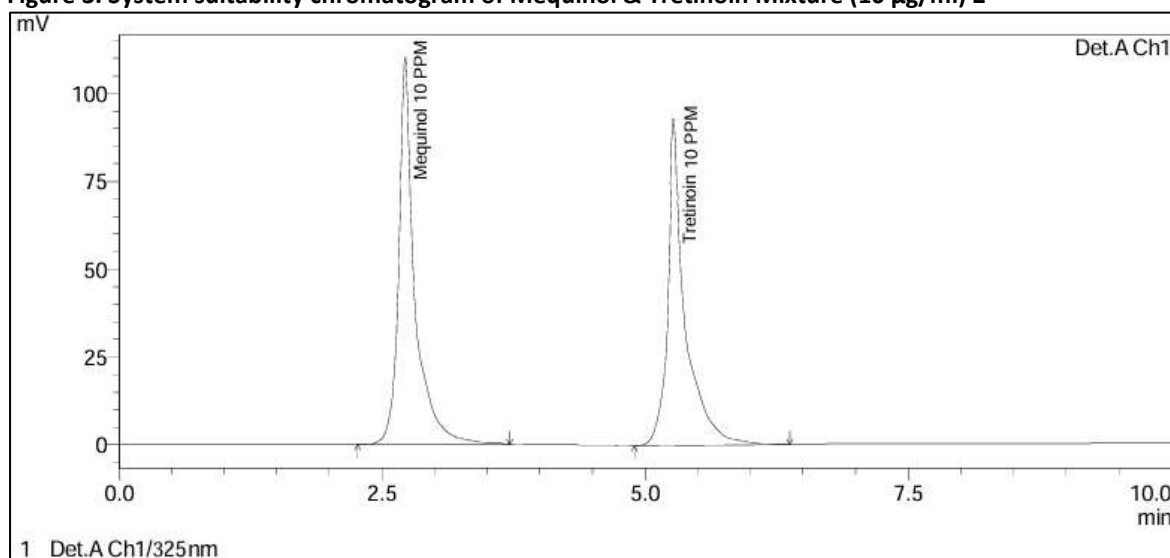


Figure 4. System suitability chromatogram of Mequinol & Tretinoin Mixture (10 µg/ml) 3

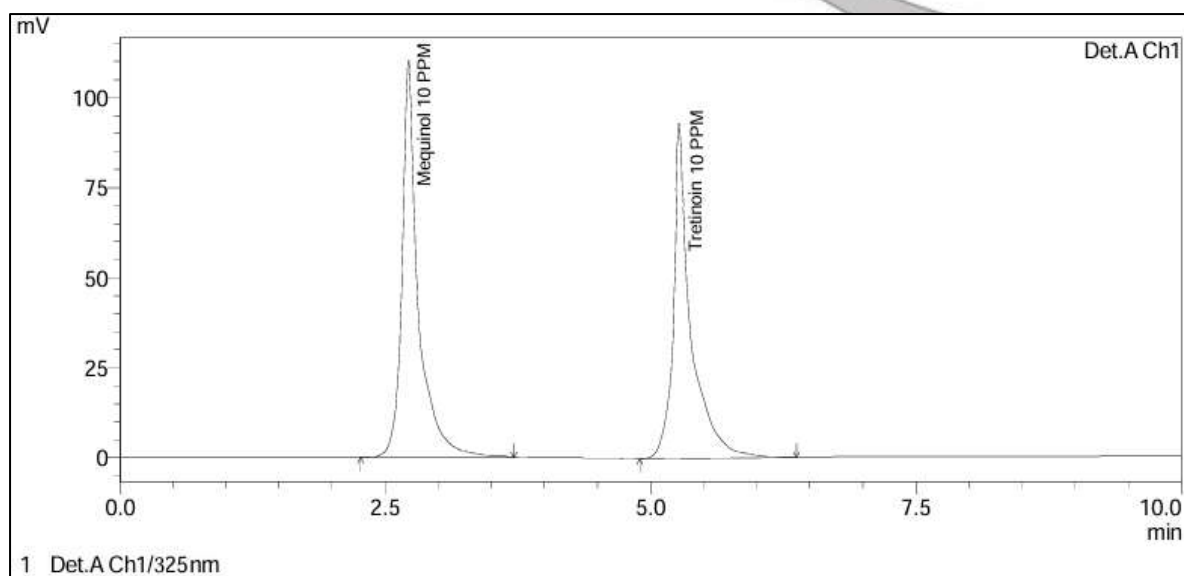


Figure 5. System suitability chromatogram of Mequinol & Tretinoin Mixture (10 µg/ml) 4

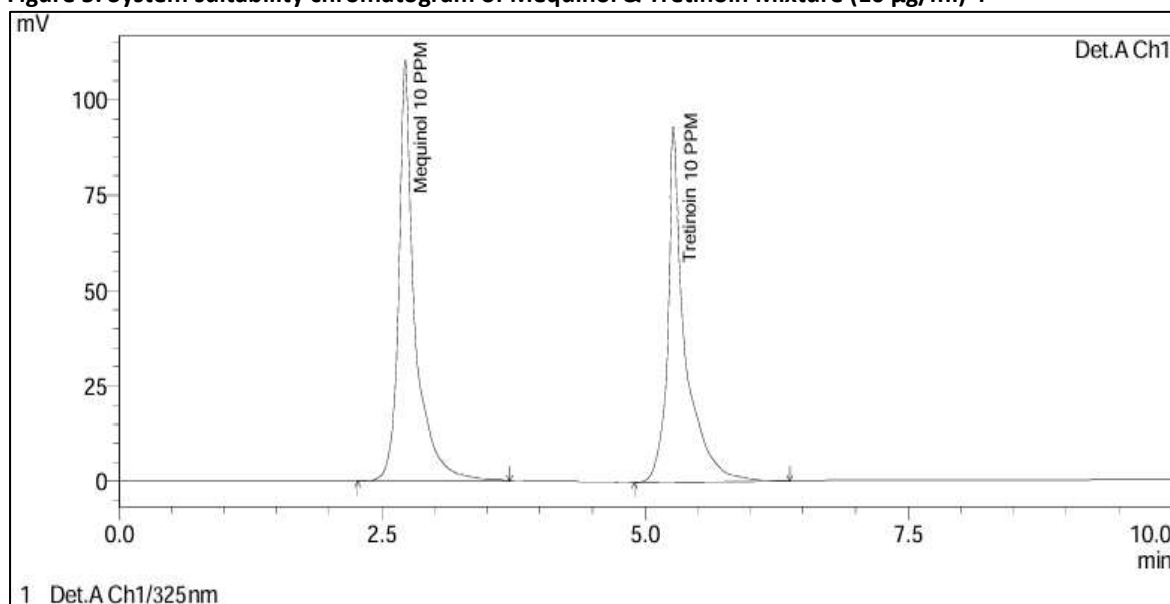


Figure 6. System suitability chromatogram of Mequinol & Tretinoin Mixture (10 µg/ml) 5

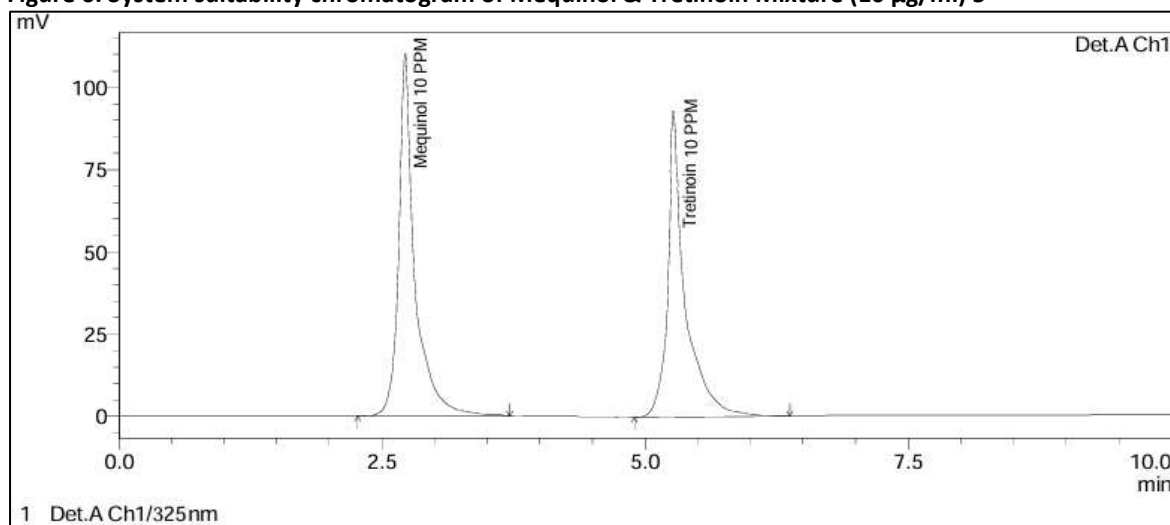


Figure 7. System suitability chromatogram of Mequinol & Tretinoin Mixture (10 µg/ml) 6
Specificity

Specificity was evaluated by comparing chromatograms obtained from the blank, standard solutions, and formulation sample solutions. No additional chromatographic peaks were observed at the retention times of mequinol and tretinoin in the blank chromatogram, indicating the absence of significant interference from the formulation matrix. The peak-purity profiles of the mequinol and tretinoin peaks in the standard and sample preparations demonstrated homogeneous chromatographic peaks. The well-resolved analyte peaks and absence of interfering responses confirmed the specificity of the developed method for simultaneous determination of mequinol and tretinoin in the topical formulation.

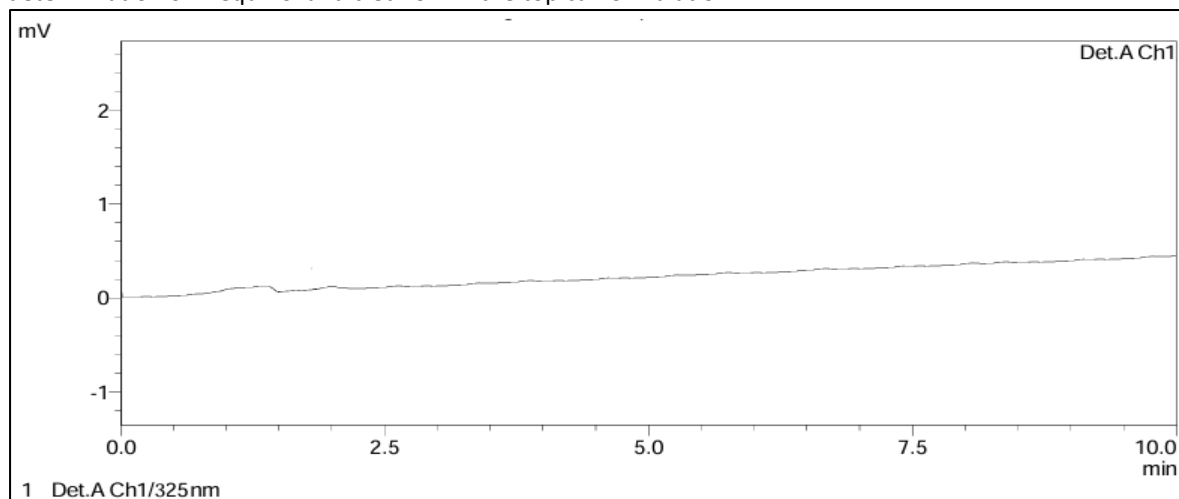


Figure 8. Blank chromatogram for specificity assessment.

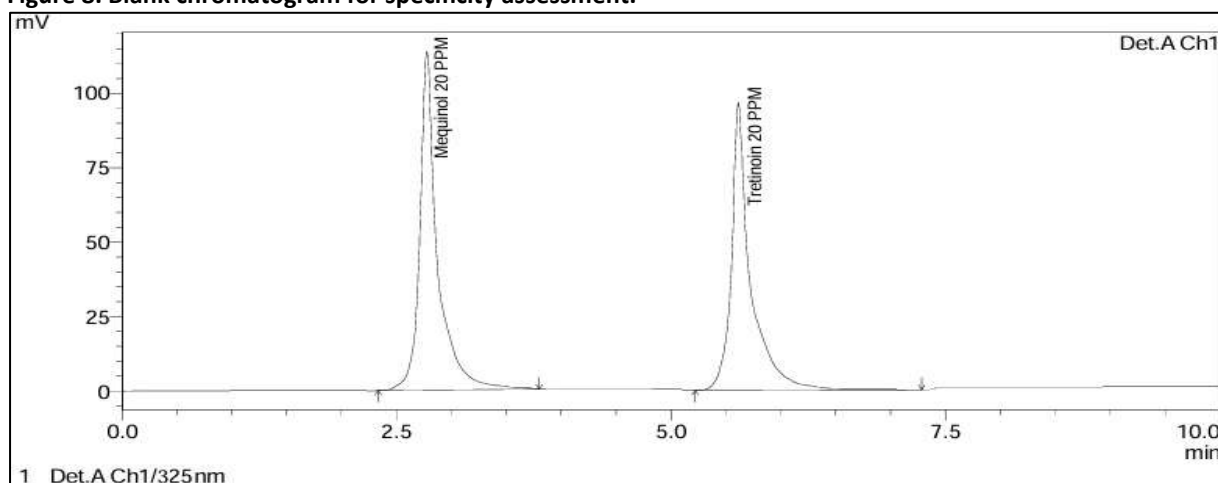


Figure 9. Peak-purity chromatogram of mequinol and tretinoin standard solution (20 µg/mL).

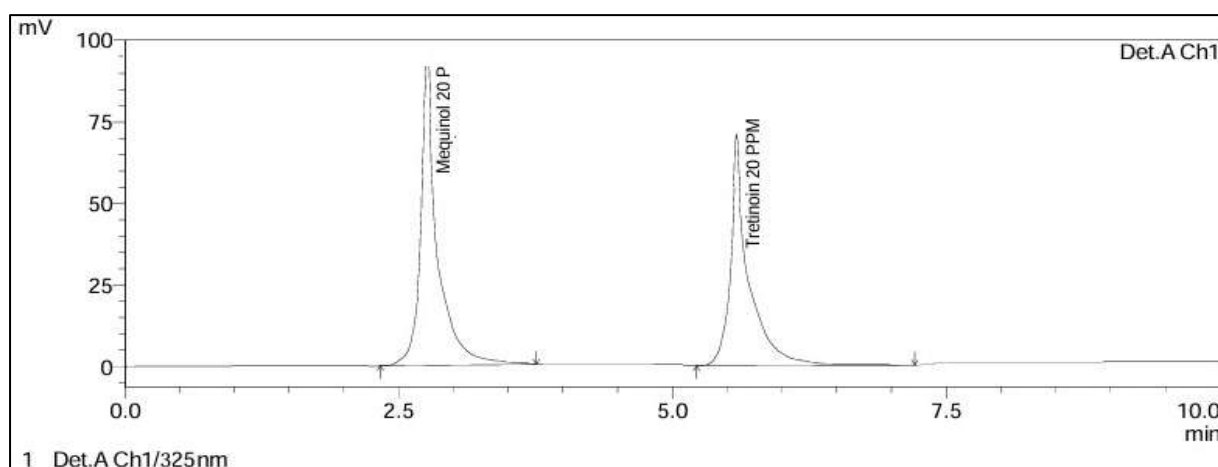


Figure 10. Peak-purity chromatogram of mequinol and tretinoin sample solution (20 µg/mL).

Linearity and Range

The linearity of the method was evaluated over the concentration range of 10–50 µg/mL for both mequinol and tretinoin. A progressive increase in peak area was observed with increasing analyte concentration. The calibration data showed excellent linear correlation, with correlation coefficients (r^2) of 0.9991 for mequinol and 0.9994 for tretinoin.

For mequinol, the regression analysis gave a slope of 51,835 and an intercept of 298,662, whereas the corresponding values for tretinoin were 53,903 and 174,371, respectively. The high coefficients of determination indicated a strong linear relationship between analyte concentration and chromatographic response over the investigated range. These findings demonstrate the suitability of the developed method for quantitative estimation of both analytes within the selected concentration range.

Table 4. Linearity Data for Mequinol and Tretinoin

Concentration (µg/mL)	Mean peak area – Mequinol	Concentration (µg/mL)	Mean peak area – Tretinoin
10	834357	10	696144
20	1324997	20	1284015
30	1851763	30	1786283
40	2337635	40	2315063
50	2919679	50	2875756
Slope	51,835	Slope	53,903
Intercept	298,662	Intercept	174,371
r^2	0.9991	r^2	0.9994

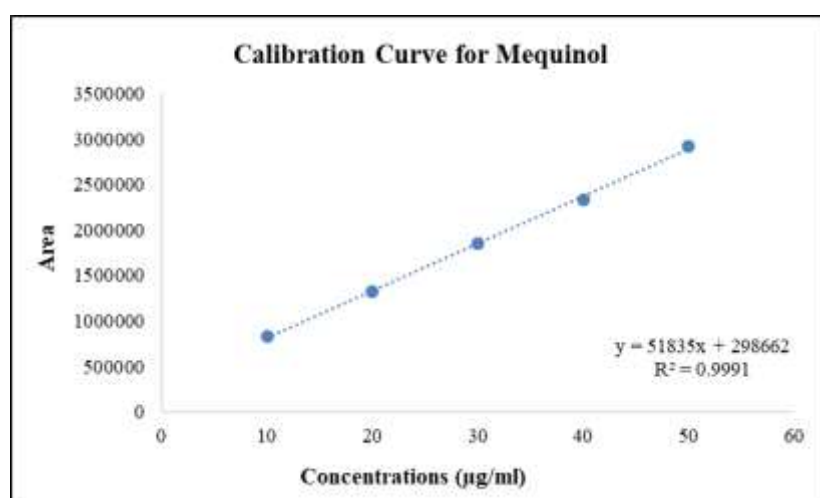


Figure 11. Calibration curve of mequinol over the concentration range of 10–50 µg/mL.

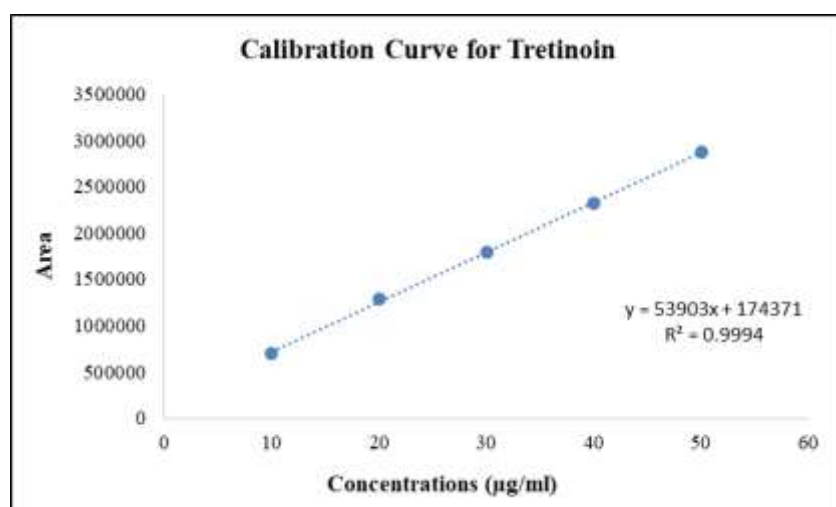


Figure 12. Calibration curve of tretinoin over the concentration range of 10–50 µg/mL.

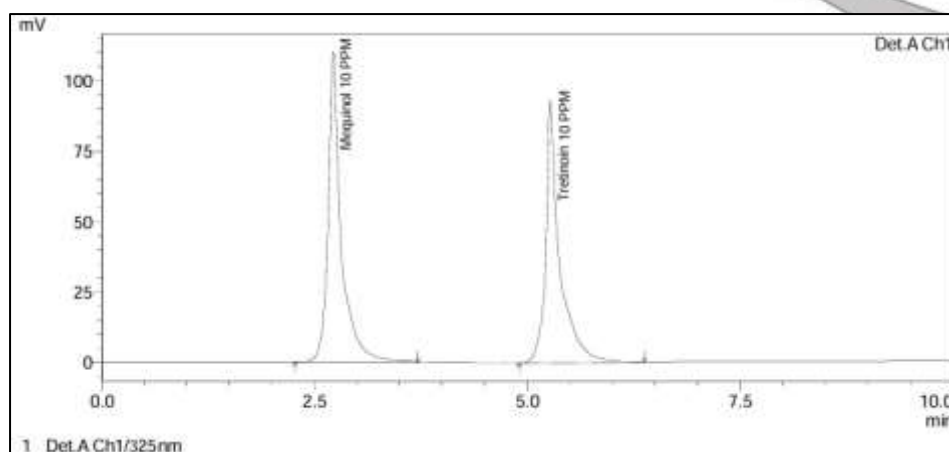


Figure 13. Standard Chromatogram for Linearity (Mequinol & Tretinoin 10 µg/ml)

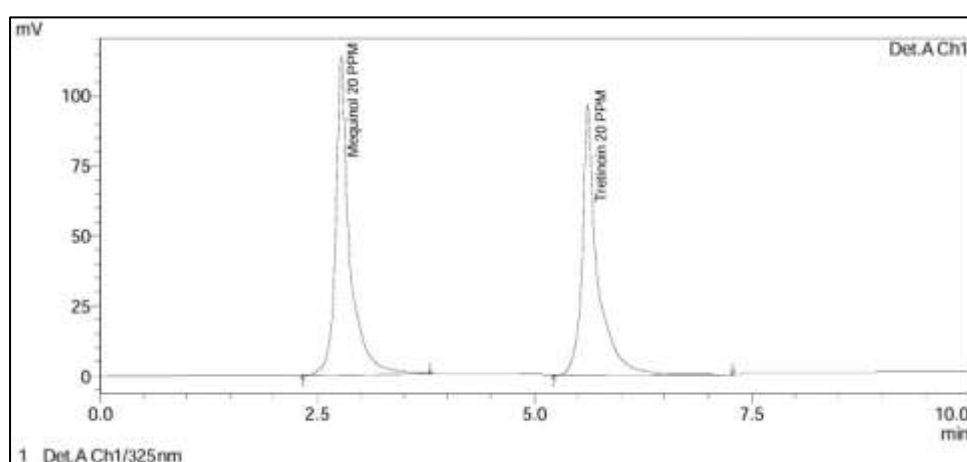


Figure 14. Standard Chromatogram for Linearity (Mequinol & Tretinoin 20 µg/ml)

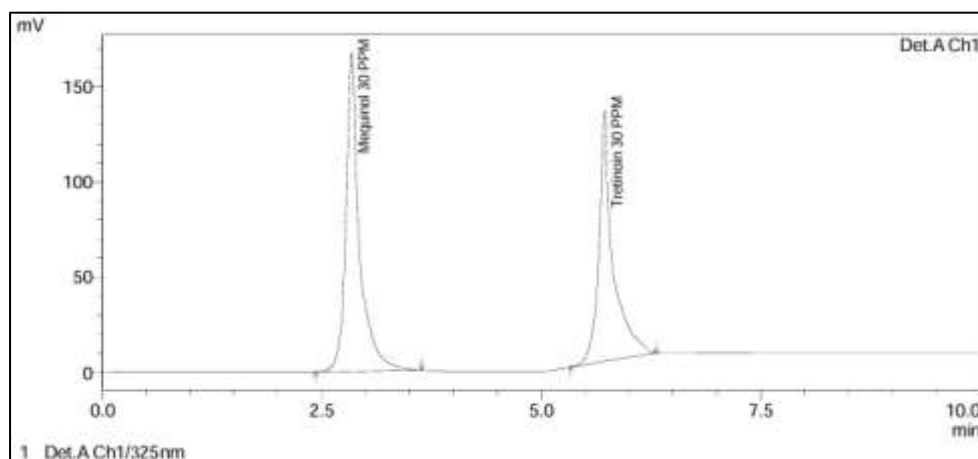


Figure 15. Standard Chromatogram for Linearity (Mequinol & Tretinoin 30 µg/ml)

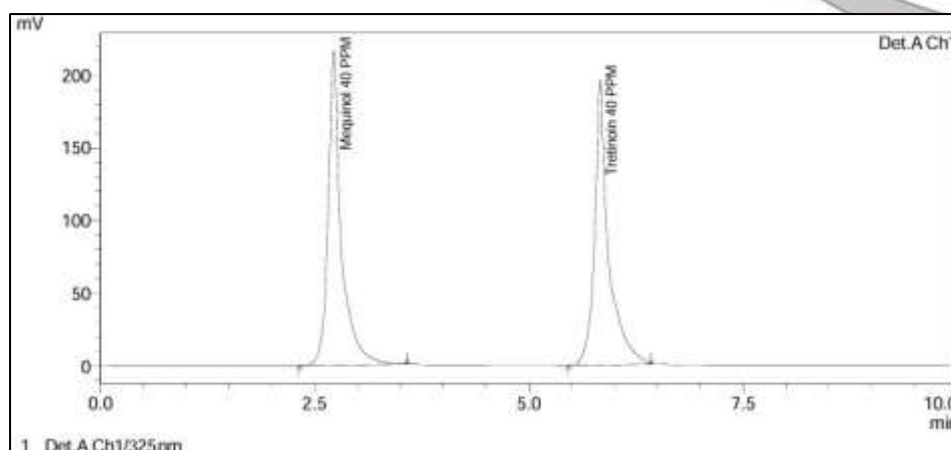


Figure 16. Standard Chromatogram for Linearity (Mequinol & Tretinoin 40 µg/ml)

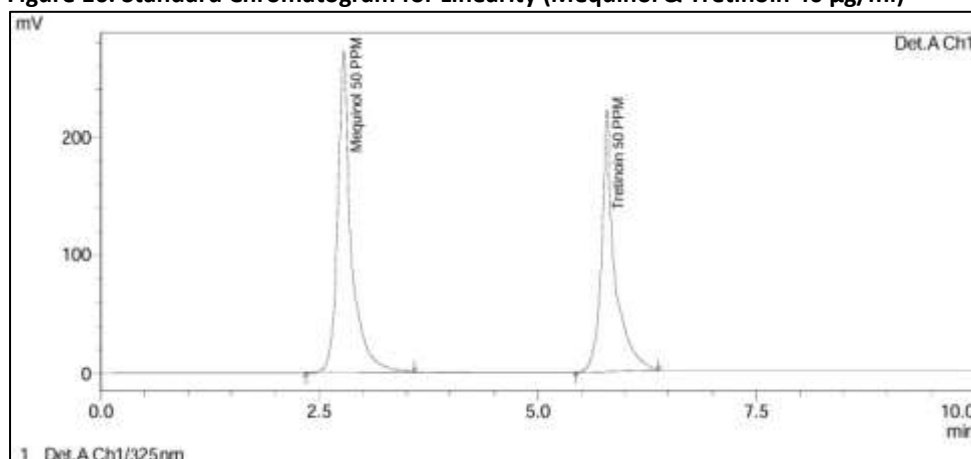


Figure 17. Standard Chromatogram for Linearity (Mequinol & Tretinoin 50 µg/ml)

Precision

a) System Precision

System precision was evaluated by injecting the mixed standard solution of mequinol and tretinoin six consecutive times under the optimized chromatographic conditions. The peak areas obtained for both analytes showed minimal variation across the six injections. The mean peak areas were 1,852,307 and 1,787,627 µV·sec for mequinol and tretinoin, respectively, with %RSD values of 0.114% and 0.133%. Both values were well below the predefined acceptance criterion of 2.0%, demonstrating excellent repeatability of the chromatographic system. The low variability in peak response confirms consistent injection performance and stable chromatographic conditions during analysis.

Table 5. System Precision Data for Mequinol and Tretinoin

Injection No.	Mequinol Peak Area	Tretinoin Peak Area
1	1,851,763	1,786,283
2	1,850,428	1,789,516
3	1,856,145	1,786,317
4	1,851,364	1,785,862
5	1,850,874	1,791,639
6	1,853,267	1,786,146
Mean	1,852,307	1,787,627
SD (±)	2,117.6	2,387.2
%RSD	0.114	0.133
Acceptance criterion	%RSD ≤ 2.0%	%RSD ≤ 2.0%

b) Method Precision

Method precision was determined by analyzing six independently prepared sample solutions under the

optimized chromatographic conditions. The assay results for mequinol ranged from 99.26% to 100.37%, while the assay values for tretinoin ranged from 99.37% to 101.34%. The mean assay values were 99.89% for mequinol and 100.28% for tretinoin. The corresponding %RSD values were 0.456% and 0.703%, respectively, which were below the predefined acceptance criterion of 2.0%. These findings demonstrate good repeatability of the complete analytical procedure, including sample preparation and chromatographic analysis.

Table 6. Method Precision Data for Mequinol and Tretinoin

Sample No.	Mequinol Assay (% w/w)	Tretinoin Assay (% w/w)
1	100.21	100.52
2	99.26	101.34
3	100.37	99.37
4	100.24	100.28
5	99.85	99.64
6	99.46	100.54
Mean	99.89	100.28
SD (±)	0.455	0.705
%RSD	0.456	0.703
Acceptance criterion	%RSD ≤ 2.0%	%RSD ≤ 2.0%

c) Intraday Precision

Intraday precision was assessed at three concentration levels, namely 10, 30, and 50 µg/mL, for both analytes. For mequinol, the %RSD values were 0.703%, 0.410%, and 0.118% at 10, 30, and 50 µg/mL, respectively. For tretinoin, the corresponding %RSD values were 0.217%, 0.270%, and 0.112%. The low %RSD values across all concentration levels indicate satisfactory repeatability of the method within a single analytical day.

Table 7. Intraday Precision Data for Mequinol and Tretinoin

Analyte	Concentration (µg/mL)	Mean Peak Area	SD (±)	%RSD
Mequinol	10	840,750	5,912.59	0.703
Mequinol	30	1,857,444	7,619.86	0.410
Mequinol	50	2,979,867	3,534.08	0.118
Tretinoin	10	695,315*	1,510.76	0.217
Tretinoin	30	1,790,604	4,839.51	0.270
Tretinoin	50	2,878,946	3,212.93	0.112

d) Inter-day Precision

Inter-day precision was evaluated at 10, 30, and 50 µg/mL over three different days. The %RSD values for mequinol were 0.836%, 0.249%, and 0.129%, whereas those for tretinoin were 0.126%, 0.151%, and 0.089% at the respective concentration levels. The observed low variability across different analytical days demonstrates satisfactory intermediate precision and indicates that the method provides consistent responses under normal day-to-day analytical variations.

Table 8. Inter-day Precision Data for Mequinol and Tretinoin

Analyte	Concentration (µg/mL)	Day 1 Area	Day 2 Area	Day 3 Area	Mean Peak Area	SD (±)	%RSD
Mequinol	10	833,689	845,327	846,382	841,799.33	7,043.54	0.836
Mequinol	30	1,857,862	1,848,637	1,853,954	1,853,484.34	4,630.41	0.249
Mequinol	50	2,978,687	2,983,087	2,986,371	2,982,715	3,855.48	0.129
Tretinoin	10	696,422	697,438	698,179	697,346.41	882.08	0.126
Tretinoin	30	1,793,415	1,790,386	1,795,813	1,793,204.67	2,719.61	0.151
Tretinoin	50	2,882,674	2,877,625	2,879,839	2,879,839.32	2,581.03	0.089

Overall, the precision studies demonstrated low variability in chromatographic response and assay results for both mequinol and tretinoin. System precision, method precision, intraday precision, and inter-day precision produced %RSD values below the predefined acceptance criterion of 2.0%. The results therefore demonstrate satisfactory repeatability and intermediate precision of the developed UPLC method for simultaneous estimation of mequinol and tretinoin in the investigated topical formulation.

Accuracy (Recovery Study)

Accuracy of the developed UPLC method was evaluated by the standard addition technique at three concentration levels corresponding to 80%, 100%, and 120% of the nominal test concentration. Each level was analyzed in triplicate, and the percentage recovery was calculated from the amount added and amount recovered. For mequinol, the mean recoveries were 100.00%, 100.14%, and 100.05% at the 80%, 100%, and 120% levels, respectively. For tretinoin, the corresponding mean recoveries were 100.00%, 99.85%, and 99.97%. The overall mean recovery was 100.06% for mequinol and 99.94% for tretinoin. The low %RSD values observed at all recovery levels indicate good accuracy and consistency of the developed method for simultaneous estimation of both analytes.

Table 9. Accuracy/Recovery Data for Mequinol

Recovery level	Set	Amount added (µg/mL)	Amount found (µg/mL)	% Recovery	Mean ± SD	%RSD
80%	1	10	9.998	100.02		
	2	10	10.001	99.99	100.00 ± 0.018	0.018
	3	10	10.000	100.00		
100%	1	20	19.994	100.03		
	2	20	20.003	99.98	100.14 ± 0.232	0.232
	3	20	19.919	100.40		
120%	1	30	30.013	99.95		
	2	30	29.974	100.09	100.05 ± 0.083	0.083
	3	30	29.967	100.11		

Table 10. Accuracy/Recovery Data for Tretinoin

Recovery level	Set	Amount added (µg/mL)	Amount found (µg/mL)	% Recovery	Mean ± SD	%RSD
80%	1	10	9.998	100.02		
	2	10	10.001	99.98	100.00 ± 0.020	0.020
	3	10	10.001	99.99		
100%	1	20	19.962	100.19		
	2	20	20.033	99.83	99.85 ± 0.335	0.335
	3	20	20.097	99.51		
120%	1	30	30.008	99.97		
	2	30	29.990	100.03	99.97 ± 0.060	0.060
	3	30	30.026	99.91		

Limit of Detection and Limit of Quantitation (LOD & LOQ)

The sensitivity of the developed method was evaluated by determining the limit of detection (LOD) and limit of quantitation (LOQ) for mequinol and tretinoin. The LOD was found to be 0.586 µg/mL for mequinol and 0.215 µg/mL for tretinoin, whereas the corresponding LOQ values were 0.943 µg/mL and 0.892 µg/mL, respectively. The low LOD and LOQ values indicate that the developed method possesses adequate sensitivity for the detection and quantification of mequinol and tretinoin at low concentration levels.

Robustness

The robustness of the developed UPLC method was investigated by deliberately varying the flow rate from the optimized condition of 1.0 mL/min to 0.8 and 1.2 mL/min. The effect of these changes was assessed by monitoring retention time, peak area, theoretical plate count, and tailing factor for both analytes. At both modified flow rates, the chromatographic parameters showed relatively low variability, with %RSD values remaining below 2.0%. These observations indicate that minor variation in flow rate did not substantially affect the chromatographic performance of the method, demonstrating its robustness under the investigated conditions.

Table 11. Robustness Data for Mequinol and Tretinoin

Flow rate (mL/min)	Analyte	Mean RT (min)	Mean Peak Area	Mean Theoretical Plates	Mean Tailing Factor	%RSD RT	%RSD Area	%RSD Plates	%RSD Tailing
0.8	Mequinol	3.178	1,870,180	2,967.13	1.542	1.550	0.722	0.517	1.038
0.8	Tretinoin	6.035	1,277,123	3,477.52	1.655	1.585	0.839	0.605	1.469
1.2	Mequinol	2.772	1,868,722	2,527.29	1.560	1.381	0.841	1.039	0.739
1.2	Tretinoin	5.610	1,279,108	3,460.22	1.669	1.312	1.011	0.287	0.988

Ruggedness

Ruggedness was evaluated by examining the analytical response of mequinol and tretinoin under different temperature conditions. The standard solutions were analyzed at 25°C, 37°C, and 60°C, and the resulting peak areas and variability were evaluated. For mequinol, the %RSD values were 0.242%, 0.797%, and 1.706% at 25°C, 37°C, and 60°C, respectively. For tretinoin, the corresponding values were 0.459%, 1.424%, and 1.584%. All observed %RSD values remained below 2.0%, indicating low variability in chromatographic response under the investigated temperature conditions.

Table 12. Ruggedness Data for Mequinol

Temperature	Peak Area 1	Peak Area 2	Peak Area 3	Mean Peak Area	SD	%RSD
25°C	836,575	835,421	832,639	834,878	2,023.34	0.242
37°C	1,336,186	1,357,621	1,345,698	1,346,502	10,740.1	0.797
60°C	1,823,756	1,886,357	1,863,874	1,857,996	31,711.8	1.706

Table 13. Ruggedness Data for Tretinoin

Temperature	Peak Area 1	Peak Area 2	Peak Area 3	Mean Peak Area	SD	%RSD
25°C	695,248	692,386	698,763	695,466	3,194.07	0.459
37°C	1,223,961	1,242,673	1,259,337	1,241,990	17,697.9	1.424
60°C	1,775,226	1,719,856	1,747,558	1,747,547	27,685	1.584

Forced Degradation Study

Forced degradation studies were performed under acidic, alkaline, oxidative, thermal, and photolytic stress conditions to investigate the degradation behavior of mequinol and tretinoin and to establish the stability-indicating capability of the developed UPLC method. The stressed samples were analyzed under the optimized chromatographic conditions and compared with the unstressed drug mixture. Additional chromatographic peaks were observed at retention times different from those of the parent analytes and were designated as degradation products (DP1, DP2, and DP3). The degradation products were adequately separated from the principal peaks of mequinol and tretinoin.

Mequinol exhibited degradation of 6.00%, 6.92%, 4.40%, 3.97%, and 4.51% under acidic, alkaline, oxidative, thermal, and photolytic conditions, respectively. Tretinoin showed comparatively greater degradation under all investigated stress conditions, with degradation values of 10.05%, 11.96%, 15.00%, 6.85%, and 17.97%, respectively. Among the investigated conditions, photolytic stress produced the highest degradation of tretinoin (17.97%), followed by oxidative (15.00%) and alkaline (11.96%) conditions. For mequinol, the highest degradation was observed under alkaline conditions (6.92%), followed by acidic conditions (6.00%). The relatively lower degradation observed under thermal conditions indicates comparatively limited degradation of both analytes under the investigated thermal stress condition.

The retention times of the principal mequinol and tretinoin peaks remained distinguishable from the additional degradation-related peaks under all stress conditions. The ability of the method to separate the parent analyte peaks from the degradation products demonstrates its suitability for stability-indicating analysis of mequinol and tretinoin in the investigated topical formulation.

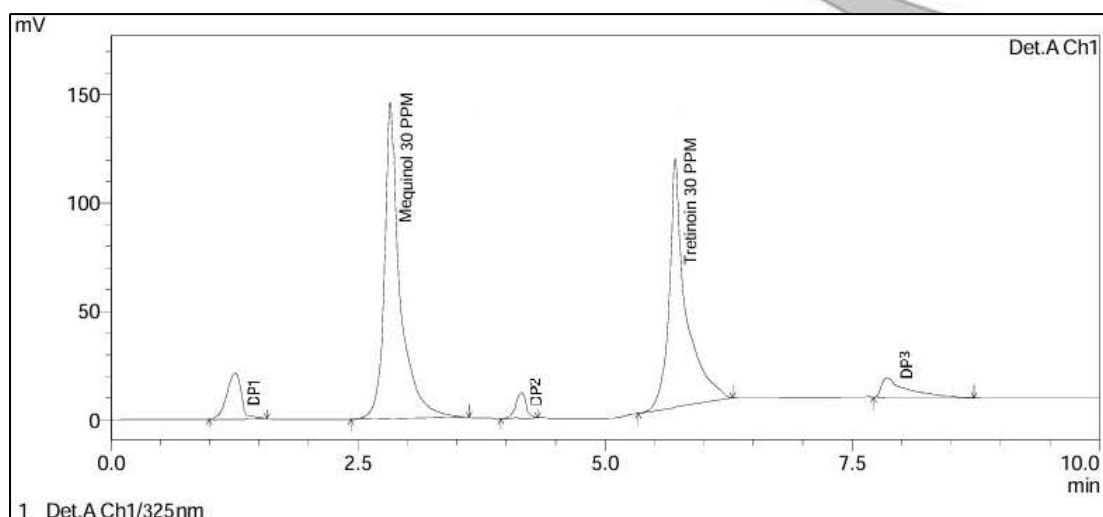


Figure 18. UPLC chromatogram of acid-degraded Mequinol and Tretinoin mixture (30 µg/mL).

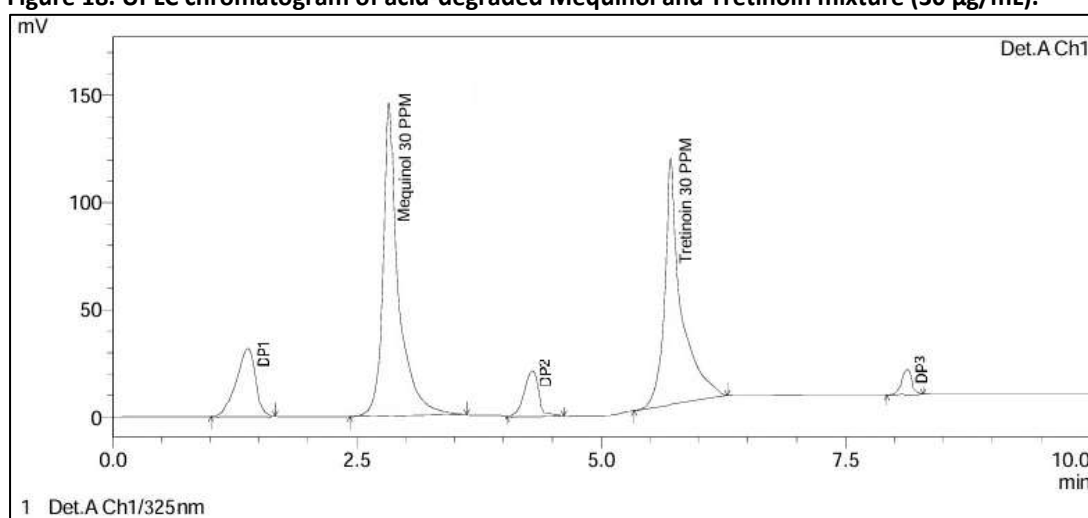


Figure 19. UPLC chromatogram of base-degraded Mequinol and Tretinoin mixture (30 µg/mL).

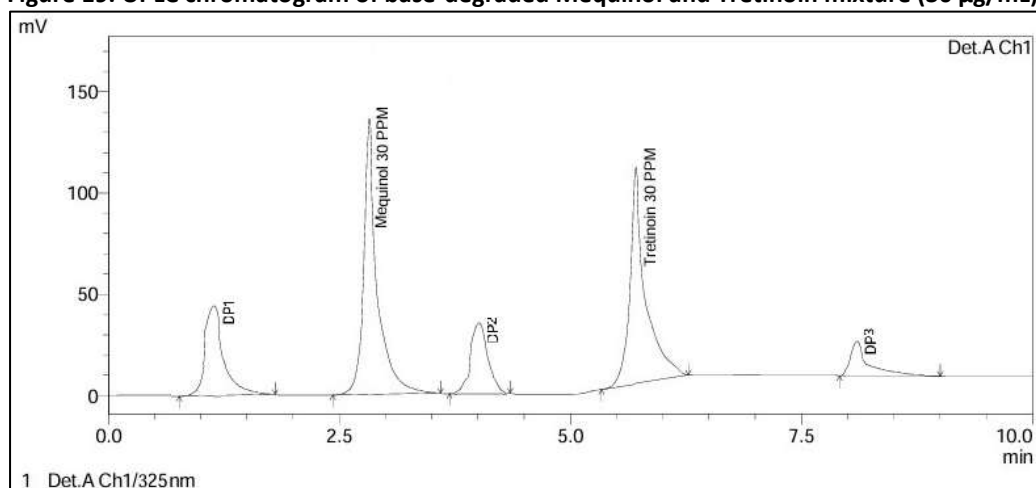


Figure 20. UPLC chromatogram of oxidatively degraded Mequinol and Tretinoin mixture (30 µg/mL).

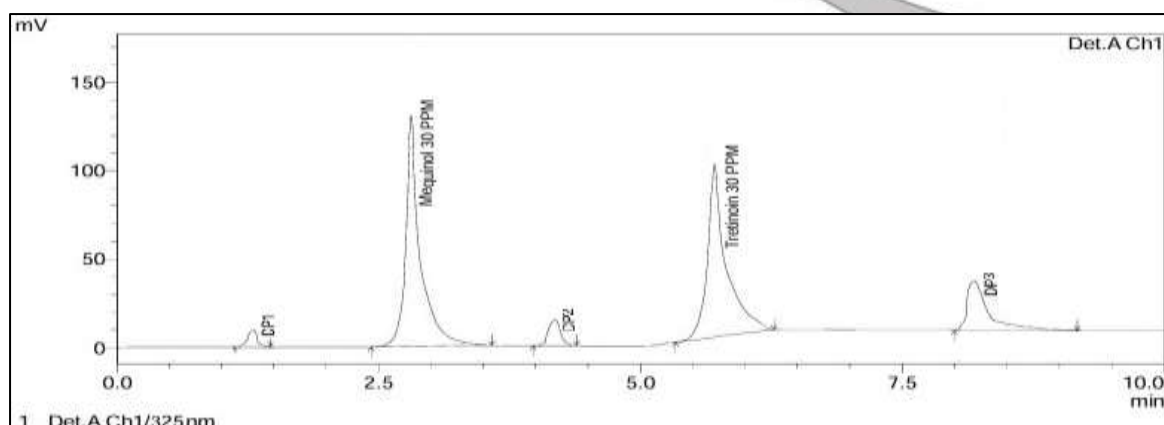


Figure 21. UPLC chromatogram of thermally degraded Mequinol and Tretinoin mixture (30 µg/mL).

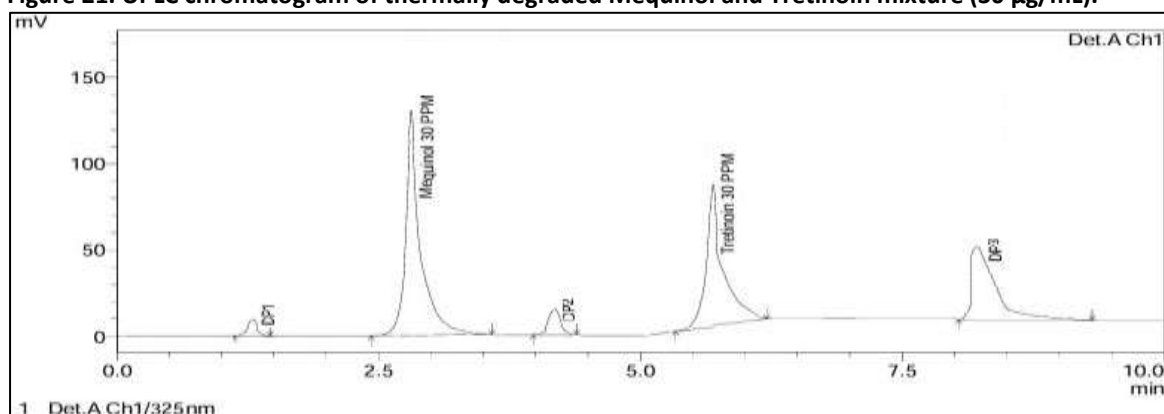


Figure 22. UPLC chromatogram of photolytically degraded Mequinol and Tretinoin mixture (30 µg/mL).

Analysis of Marketed Formulation by UPLC

The applicability of the developed UPLC method was evaluated by analyzing a marketed topical formulation, Melalong AD Cream, containing mequinol and tretinoin. The formulation was analyzed using the optimized chromatographic conditions, and the assay was calculated by comparing the sample response with that of the corresponding reference standards.

The formulation contained 2% w/w mequinol and 0.01% w/w tretinoin, corresponding to labeled amounts of 300 mg and 1.5 mg, respectively, in a 15-g tube. The calculated sample concentrations were 20.00 µg/mL for mequinol and 9.59 µg/mL for tretinoin. The corresponding assay values were 100.78% for mequinol and 99.32% for tretinoin. The calculated amounts per 15-g tube were 302.34 mg for mequinol and 1.48 mg for tretinoin.

The close agreement between the measured analyte responses and the corresponding reference standards, together with the absence of significant interference from the formulation matrix, demonstrates the applicability of the developed method to the simultaneous quantitative analysis of mequinol and tretinoin in the marketed topical formulation. The method was therefore suitable for assay determination of the two analytes present at markedly different concentrations within the formulation.

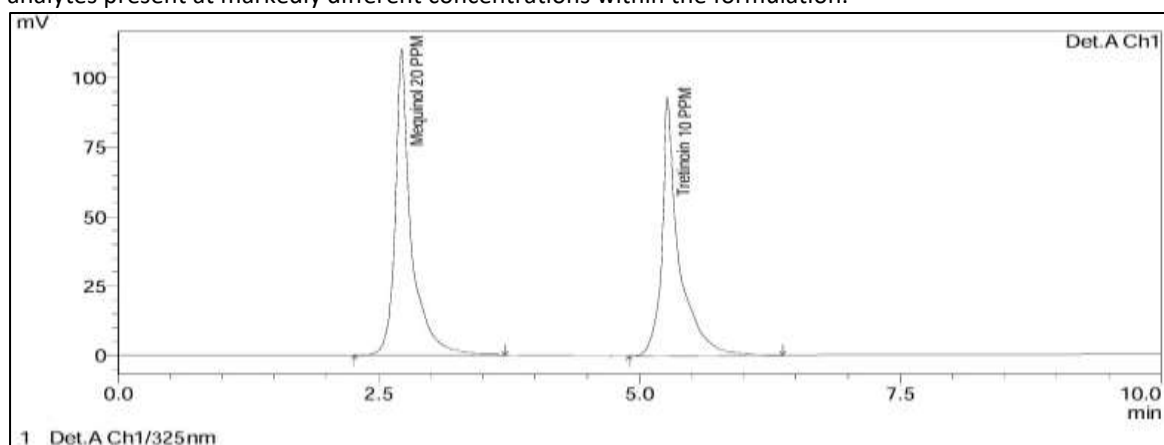


Figure 23. UPLC chromatogram of Melalong AD Cream showing simultaneous estimation of mequinol and tretinoin.

CONCLUSION:

A simple, precise, accurate, robust, and stability-indicating UPLC method was successfully developed for the simultaneous estimation of mequinol and tretinoin in topical formulations. The optimized chromatographic conditions using an Inertsil C18 column with methanol and phosphate buffer (pH 3.0) in the ratio of 70:30 (v/v), at a flow rate of 1.0 mL/min and detection at 325 nm, provided satisfactory separation of the two analytes within a practical analysis time.

The developed method demonstrated excellent linearity over the concentration range of 10–50 µg/mL for both mequinol and tretinoin, with high coefficients of determination. The low %RSD values obtained during system precision, method precision, intraday precision, and inter-day precision studies confirmed the reproducibility of the analytical procedure. The recovery results demonstrated satisfactory accuracy, while the low LOD and LOQ values confirmed adequate sensitivity. Robustness studies further demonstrated that minor variation in flow rate did not adversely affect the chromatographic performance.

Forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions produced additional degradation-related peaks while maintaining separation of the principal analyte peaks from the degradation products. Tretinoin showed comparatively greater susceptibility to photolytic and oxidative stress, whereas mequinol exhibited relatively lower degradation under the investigated conditions. The ability of the method to resolve degradation-related components from the analyte peaks supports its application as a stability-indicating analytical procedure.

Finally, successful application to the marketed topical formulation demonstrated the practical utility of the method for simultaneous quantitative determination of mequinol and tretinoin in the presence of formulation excipients. Overall, the developed UPLC method provides a suitable analytical approach for routine quality-control testing and stability evaluation of topical formulations containing mequinol and tretinoin.

FUNDING:

This research did not receive any specific grant.

CONFLICT OF INTEREST:

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION:

All authors contributed equally to the study and manuscript preparation.

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