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DEVELOPMENT AND VALIDATION OF A VISIBLE SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF TRETINOIN IN BULK AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT
Background: Tretinoin is a widely used dermatological agent, and its accurate quantification is essential for quality control in pharmaceutical formulations. Existing UV-spectrophotometric methods often lack the sensitivity and simplicity required for routine analysis. **Methodology:** A simple, rapid, and cost-effective visible spectrophotometric method was developed for the determination of tretinoin in bulk and 0.05% cream formulations. The method is based on the formation of a stable ion-pair complex between tretinoin and methylene blue in an alkaline medium (pH 9.8). The resulting complex exhibits maximum absorbance at 650 nm. Method validation was carried out in accordance with ICH Q2(R1) guidelines. **Results and Discussion:** The method showed linearity in the concentration range of 2–10 µg/mL, with a correlation coefficient (r^2) of 0.999. The limit of detection (LOD) and limit of quantification (LOQ) were found to be **0.0434** µg/mL and **0.1316** µg/mL, respectively. Precision and accuracy results were within acceptable limits. Recovery ranges for 0.05% tretinoin cream formulations are 98.7% to 101.1%, confirming the method's suitability for real-sample analysis. Compared to existing UV methods, this approach offers enhanced sensitivity and operational simplicity. **Conclusion:** The proposed spectrophotometric method is a reliable and sensitive alternative for the routine quantification of tretinoin in pharmaceutical formulations. Its simplicity, cost-effectiveness, and compliance with regulatory validation standards make it well-suited for quality control laboratories.

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INTRODUCTION

Bioactive compounds are present in both plant and animal products and can also be synthesized artificially. Among these, drugs constitute a significant class of bioactive substances. The estimation of such compounds is often performed using simple and economical methods based on reactions with suitable chromogenic reagents. Over-the-counter (OTC) skin care medications are generally categorized into products for dry skin, acne, sunburns, sun protection, and foot care. Acne, primarily resulting from hormonal fluctuations, is particularly common during adolescence, affecting approximately 80–90% of teenagers. Tretinoin, a retinoid used to treat acne, promotes the replacement of old skin cells with new ones. Combination therapies involving topical clindamycin and tretinoin have demonstrated efficacy in the management of acne vulgaris, a prevalent dermatological condition [1].

A comprehensive review of the literature reveals that various analytical methods have been employed to quantify tretinoin (TTN). Chromatographic techniques such as high-performance liquid chromatography (HPLC) [2–6], reverse-phase HPLC (RP-HPLC) [7–12], liquid chromatography (LC) [14, 27], ultra-performance liquid chromatography (UPLC) [13, 27], and gas chromatography (GC) have been extensively reported. In addition, spectroscopic methods including UV-spectrophotometry [17–22, 27] and UV-derivative spectroscopy [23–25, 27] have been explored. Studies on the phototoxicity of tretinoin have also been documented [16, 27]. Most of these analytical techniques require sophisticated instrumentation that is expensive, demanding extensive maintenance, and often inaccessible to many routine laboratories. As an alternative, visible spectrophotometry offers several advantages, including simplicity, rapid analysis, cost-effectiveness, and ease of use, making it particularly suitable for routine quality control analysis. Importantly, this approach emphasises reaction sensitivity over the sophistication of the instrumentation. However, very few visible spectrophotometric methods were reported in the literature for the determination of Tretinoin [28].

The present study aims to develop and validate a simple, precise, and cost-effective visible spectrophotometric method for estimating tretinoin in bulk drug and pharmaceutical formulations using methylene blue (MB) as a chromogenic reagent. Compared to bromocresol green and safranine-O, methylene blue has better extractability in chloroform with high colour intensity, superior molar absorptivity, and more stable ion-pair complex formation. The chemical structure of tretinoin is shown in Figure 1.

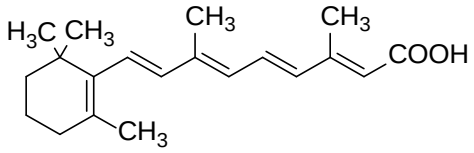


Figure 1: Chemical structure of tretinoin

("2E,4E,6E,8E)-3, 7-Dimethyl-9-(2,6,6-trimethylcyclo-hex-1-enyl) nona-2,4,6,8-all-trans-tetraenoic acid") [28]

MATERIALS AND METHODS

Instrumentation

A Shimadzu UV-1800 double-beam spectrophotometer was used to measure optical density with precision and accuracy. pH measurements of the sample solutions were conducted using a digital pH meter (Equiptronics, India). The weight of each substance was determined using a Dhona 200 D electronic balance (India).

Chemicals and Reagents

Tretinoin formulations used in this study included Retino-A (Ethnor Janssencilag) and Airol (Piramal Healthcare). The bulk drug sample of tretinoin was procured from Biophore India. Additionally, Eudyna (German Remedies) and Avita (Bertek Pharmaceutical Inc.) were obtained from a registered pharmacy. Analytical-grade reagents used in this study included methylene blue (Fluka), borax, sodium hydroxide, and chloroform (Qualigens, Mumbai, India).

To prepare the methylene blue solution, 10 mg of methylene blue (Fluka; 0.01%, 3.12×10^{-4} M) was dissolved in 100 mL of distilled water. The solution was then washed with chloroform to remove any chloroform-soluble impurities. A buffer solution with a pH of 9.8 was prepared by mixing 15 mL of 0.1 M NaOH and 50 mL of 0.025 M borax solution in 100 mL of distilled water [27].

Bulk Sample Solution

For the bulk drug sample, a stock solution was prepared by dissolving 100 mg of tretinoin in 100 mL of chloroform. A working standard solution of tretinoin at 40 µg/mL was prepared by diluting the stock solution with chloroform.

Formulations

A solution of 50 mg of tretinoin cream was prepared by dissolving it in 30 mL of aqueous methanol (3:1). Three separate

25.0 mL portions of chloroform were used to extract the solution, which was then filtered. The filtrate was dried over 5 g of anhydrous Na_2SO_4 and filtered again. The resulting chloroform extract was diluted to a final volume of 200 mL with chloroform, yielding a stock solution at 250 µg/mL. The stock solution was further diluted to achieve a final concentration of 40 µg/mL.

Analytical Wavelength Selection

The visible spectrum (350–750 nm) was scanned for the sample solution against a reagent blank to determine the optimal analytical wavelength. The sample solution was prepared by combining a fixed quantity of tretinoin, buffer, methylene blue (MB), a basic dye, and other related variables, as per the experimental procedure. A wavelength of 650 nm was selected for analysis, as it corresponds to the maximum absorbance observed in the absorption spectra of the colored species formed by ion-pair complexation between tretinoin and methylene blue (Figure 2).

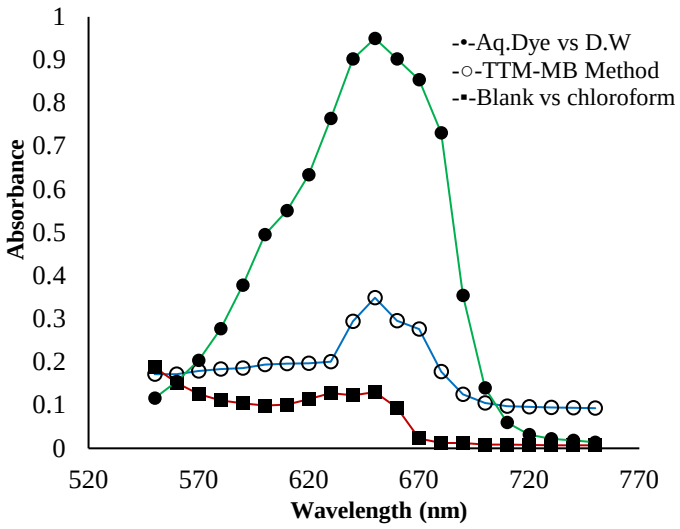


Figure 2: Absorption spectrum of the TTN- MB method

Method Development

To develop the analytical method, a series of solutions was prepared by transferring aliquots (0.5 to 2.5 mL) of the standard drug solution (40 µg/mL) into 40.0 mL separating funnels. To each aliquot, 1.0 mL of buffer solution (pH 9.8) and 2.0 mL of a 3.12×10^{-4} M safranine solution were added. The total volume of each solution was then adjusted to 15.0 mL using distilled water, and 10.0 mL of chloroform ($CHCl_3$) was added. The contents of the separating funnel were shaken for 2 minutes to ensure proper mixing. After separation of the two layers, the absorbance of the organic layer was measured at 650 nm against a reagent blank. A decrease in optical density was observed after 60 min, indicating the dissociation of the coloured complex. The concentration of tretinoin in the sample was determined from the calibration curve, which relates optical density to drug concentration (Figure 3).

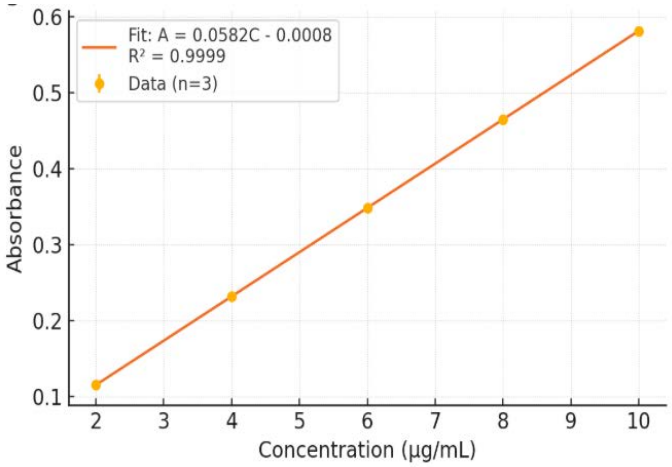


Figure 3. Beer's law plot of (TTN- MB) Method

RESULTS AND DISCUSSION

Optimum Conditions

Various experimental parameters were investigated to determine the optimal conditions for the spectrophotometric analysis of tretinoin. Factors such as the concentration of methylene blue (MB) (basic dye), ranging from 0.31 to 0.94×10^{-4} M, the volume of the extracting solvent, and the stability of the ion-pair complex were evaluated. The method was found to yield the best results under the following conditions: 1.0 mL of borax buffer (pH = 9.8), 2.0 mL of methylene blue (0.624×10^{-4} M), and 2 minutes of agitation time at temperatures ranging from 28°C to 20°C. After these conditions were met, the ion-pair complex remained stable for up to 60 minutes, during which time the absorbance was stable. However, beyond this time period, a progressive decrease in absorbance was observed, indicating the dissociation of the ion-pair complex. These findings are summarised in Table 1.

Since the molar absorptivity of the ion-pair complex decreases if pH increases beyond 9.8, at high volume of dye (> 2.0 mL) and the shaking time is more than 5 minutes, the optimum conditions, such as pH 9.8, dye volume of 2.0 mL, and shaking time of 5 minutes, were chosen. The effect of the duration of the association complex formation (1–60 minutes), the color intensity of the generated species, and the ratio of aqueous to $CHCl_3$ solvent on the stability of the complex were also studied [26].

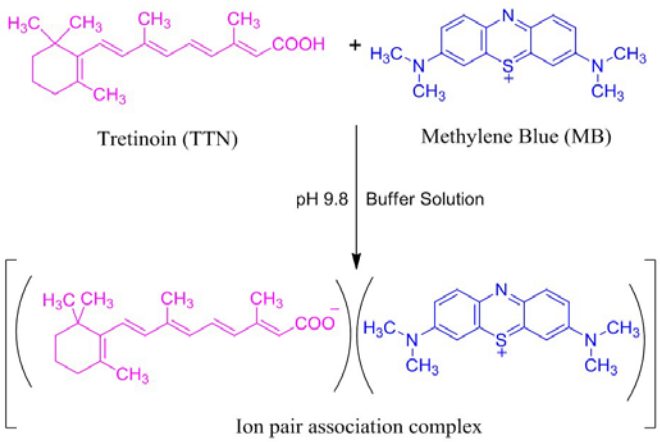
Table 1: Optimum conditions of the proposed method

Parameter	Conditions	Optimum range
λmax (nm)	650	640 – 660
pH(0.025 M borax solution and +0.1M NaOH solution buffer solution)	pH = 9.8	9.0 – 10.0
Volume of buffer solution	1.0 ml	0.5 – 1.5 ml
Volume of basic dye (methylene blue)	2.0 ml	1.0 – 2.5 ml
Organic solvent used for the extraction of the ion pair association complex	$CHCl_3$	$CHCl_3$
Ratio of the organic to the water phase	2 : 3	2 : 3
Shaking time	5 min.	3 – 8 min.
Temperature	Laboratory Temp.	Lab. Temp.(28+2ºC)
Stability of the coloured species	5 min.	1 – 60 min.
Molar absorptivity(ϵ_{max})	1.7453×104 L $mol^{-1}cm^{-1}$	--
Range of linearity bounds	2-10 µg mL^{-1}	--
LOD, or limit of detection	4.342×10-2 µg mL^{-1}	--
LoQ, or limit of quantification	1.316×10-1 µg mL^{-1}	--
Standard error of estimation (Se)	7.303×10^{-4}	--
Sandell's Sensitivity	1.721×10^{-2}	--

Low absorbance values at pH < 9 and pH > 10

Mechanism of Ion-Pair Association Complex

The ion-pair association complex between tretinoin (TTN) and methylene blue (MB) is formed through the interaction of the carboxyl group on the side chain of tretinoin and methylene blue in an alkaline solution [28]. The mechanism of ion-pair formation is explained in light of previous studies. The positively charged Methylene blue (MB) interacts electrostatically with the negatively charged carboxylate anion on the tretinoin molecule. This electrostatic attraction results in the formation of a stable ion-pair complex, which behaves as a single entity. The most probable sequence of this reaction mechanism is illustrated in Scheme 1.



Scheme 1: Mechanism of ion pair association complex formation reaction for the proposed method (TTN- MB Method)

Validation of Analytical Data

The proposed TTN–MB spectrophotometric method was developed and validated in accordance with ICH Q2(R1) guidelines [27]. Key validation parameters, including slope (b), intercept (a), linear correlation coefficient (r), and intraday precision (%RSD), were statistically evaluated. The maximum absorbance (λ_{max}) was observed at 650 nm, and the molar absorptivity (ε_{max}) was calculated as 1.7453×10^4 L mol^{-1} cm^{-1}.

The method shows a 33% increase in molar absorptivity compared to previous visible methods (1.74×10^4 vs 1.31×10^4 L mol^{-1} cm^{-1}). The method exhibited excellent linearity within the concentration range of 2–10 µg mL^{-1}, with a correlation coefficient (r^2) of 0.9999, confirming the reliability of the calibration curve over the tested range.

Limits of detection (LOD) and quantification (LOQ) were determined from the standard deviation of the response and the slope, indicating the method's high sensitivity. Method precision was assessed by analyzing six replicates (n = 6) under optimized conditions, and results were expressed as %RSD.

Accuracy was evaluated through recovery studies by spiking known quantities of tretinoin at three concentration levels into pre-analyzed formulations. The percentage recovery ranged from 99.5% to 99.88%, with standard deviations between ±0.7

and ±1.2, confirming the method's accuracy and reproducibility. Statistical comparison using Student's *t*-test and *F*-test demonstrated no significant differences between the proposed method and a reference UV spectrophotometric method. At a 95% confidence level, the calculated *t*- and *F*-values remained within acceptable limits (2.57 and 5.05, respectively), validating the equivalence and robustness of the developed method. The sensitivity of the present visible spectrophotometric method is comparable to that of the HPLC method. Detailed results are presented in Tables 1–3.

Table 2: Validation parameters

Validation parameter	TTN-MB Method
Slope(b)	0.0582
Standard deviation on slope(Sb)	0.0001155
Intercept(a)	-0.0008
Standard deviation on intercept(Sa)	0.0007659
Linear correlation coefficient(r)	0.9999
Intra -day precision(%RSD)*	0.62
Inter- day precision(%RSD)*	0.70
0.01 Level of confidence limits	0.649
0.05 Level of confidence limits	1.018

Table 3: Assay of TTN in pharmaceutical formulations

Formulation$^{\theta}$, Proprietary name & Pharmaceutical company concerned	Labelled Amount (g)	Quantity determined using the suggested approach (g)●	95 percent Confidence intervals F -Test@	95 percent confidence intervals t-Test$	The amount discovered using the UV absorption method (mg) [29]	% Recovery by developed method*
Cream (A Ret-HC) Shalaks pharmaceuticals	20	19.9±0.23	2.33	0.72	19.95±0.15	99.50±1.16
Cream (Retinol-A) Janssen-Cilag Pharmaceuticals	20	19.98±0.14	4.7	0.19	19.99±0.06	99.88±0.7
Cream (Airol) Piramal Healthcare	20	19.90±0.22	1.05	0.09	19.91±0.22	99.52±1.09
Cream(Avita) Bertek Pharmaceutical Inc.	20	19.93±0.12	1.05	0.08	19.94±0.12	99.66±0.60

● Average value of six observations. @ At a 95% confidence level, the tabulated F-value is 5.05.

***** Average value of three measurements.

$ The calculated t-value is 2.57 with a 95% confidence interval. The suggested approach was shown to be more sensitive in terms of molar absorptivity, linear correlation coefficient (r), LoD, and LoQ values, compared with the literature technique [28]. Percentage recovery and relative standard deviation (%RSD) were used to assess precision and accuracy. The findings for the created method's RSD and recovery % were relatively comparable to those reported in previous literature. Table 4 summarizes the findings.

Table 4: Comparison of the proposed method with the literature (20) method

Reagent used	Iodine (I2)	Methylene blue(MB)
Wavelength(λmax) nm	295	650
Molar absorptivity(ϵmax) L mol^{-1} cm^{-1}	1.31×104	1.7453×104
Limits of linearity range(µg mL^{-1})	9.04 – 29.71	2-10
Linear correlation coefficient (r)	0.9974	0.9999
Relative standard deviation (%RSD)	1.95-0.88	Intraday(0.62) Interday(0.70)
% Recovery	97.84 – 102.80	99.5 – 99.88
Limit of detection(LoD) µg mL^{-1}	4.35	0.00434
Limit of quantification(LoQ) µg mL^{-1}	13.17	0.1316
Method	Zayed MA and Abdel-Basset MH (20)	Present method

The developed method demonstrates comparable or superior performance to existing UV and visible assays, particularly in terms of sensitivity, molar absorptivity, reagent cost, and operational simplicity.

Table 5: Intermediate precision (inter-day and analyst-to-analyst variability) for the TTN–MB method

Parameter	Day / Analyst	Mean Absorbance	SD	%RSD	Remarks
Inter-day Precision	Day 1(n = 6)	0.351	0.0021	0.60	Acceptable
	Day 2(n = 6)	0.349	0.0024	0.69	Acceptable
	Overall Inter-day %RSD	—	—	0.70	Meets ICH limits
Analyst-to-Analyst Precision	Analyst A(n = 6)	0.352	0.0023	0.65	Acceptable
	Analyst B(n = 6)	0.348	0.0026	0.78	Acceptable
	Overall Analyst Variation %RSD	—	—	0.82	No significant analyst variability

Intermediate precision was statistically demonstrated, with overall inter-day %RSD of 0.70% and analyst-to-analyst variation of 0.82%, confirming good reproducibility of the TTN–MB method.

Table 6: Robustness evaluation of the TTN–MB method under deliberate variations

Robustness Parameter	Condition Varied	Mean Absorbance	SD	%RSD	Conclusion
pH variation	pH 9.6	0.352	0.0029	0.82	Robust
	pH 10.0	0.350	0.0030	0.86	Robust
Dye volume variation	1.8 mL	0.348	0.0031	0.89	Robust
	2.2 mL	0.351	0.0033	0.94	Robust
Shaking time	4 min	0.349	0.0027	0.77	Robust
	6 min	0.351	0.0028	0.80	Robust

Robustness results indicate that small deliberate variations in pH, dye volume, and shaking time produced %RSD values below 1%, confirming that the TTN–MB method remains stable and reliable under routine analytical conditions.

CONCLUSION

The present study demonstrates the development and validation of a simple, sensitive, and cost-effective visible spectrophotometric method (TTN–MB system) for the quantitative estimation of tretinoin in both bulk and pharmaceutical cream formulations. The method, based on the formation of a stable ion-pair complex with methylene blue in an alkaline medium, exhibited excellent linearity, low detection limits, and high recovery rates, indicating strong analytical performance and robustness. In contrast to more complex and instrument-intensive techniques such as HPLC or LC-MS, the proposed method is very useful for laboratories with limited resources. However, the method is primarily suitable for pure and formulated tretinoin and may not resolve individual degradation products or complex mixtures. Future studies could explore its integration with hyphenated techniques to broaden its scope. Future work may include coupling with derivative spectroscopy or combining with HPLC to evolve a complete stability-indicating protocol.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Manikya S. Thuttagunta was responsible for the experimental work and preparation of the first draft of the paper. Ramakrishna Karipeddi is responsible for the idea, supervision, review, and editing of the paper. Surekha Pinninti & Santosh Kumar Nadikatla are responsible for collecting the literature and fine-tuning the manuscript. Santosh Kumar Badampudi is responsible for reviewing and editing the final manuscript.

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