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Development and validation of stability indicating HPLC–PDA method for the simultaneous determination of benzoyl peroxide and tretinoin in topical dosage form

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Abstract

Background Acne vulgaris is a very dangerous skin disease leads to psychological disorders. Benzoyl peroxide and tretinoin in combination with topical dosage are used to improve the appearance of acne-prone skin. The study aimed to develop and validate an HPLC–PDA method for the simultaneous estimation of both drugs. Intentional modifications were implemented in the analytical method to get better optimum conditions. The final method was chosen for the reverse phase chromatographic separation by using the C18 column as a stationary phase and 0.01 M phosphate buffer adjusted pH 2.5 mixed with acetonitrile (25:75 v/v) as a mobile phase. The optimized conditions were 1.5 mL/min flow rate, 30 °C column temperature, 5 °C autosampler temperature, and 20 µL injection volume. The wavelength was chosen for detection of Benzoyl peroxide at 272 nm and Tretinoin at 353 nm by utilizing a PDA detector. All standard and sample solutions were made in methanol.

Results The developed method exhibited peak retention times of 2.94 min for Benzoyl peroxide and 11.34 min for Tretinoin. This analytical method was proven to be robust, linear in calibration curve, accurate, precise, and specific. The forced degradation studies results showed that a high degree of specificity was obtained by separating both analytes from produced impurities completely.

Conclusions The developed analytical method is fast, economic and stability indicating. It is useful for routine pharmaceutical analysis where the combination of benzoyl peroxide and tretinoin is formulated for their quality and safety.

Keywords Benzoyl peroxide, Tretinoin, HPLC, Method validation, Stress studies

Background

Acne vulgaris, papules, and pustules are skin diseases with adverse effects including scarring and hyperpigmentation leading to severe psychological distress [1–3]. Acne can be as non-inflammatory lesions, burns, or a combination of both, and most commonly affects the face, yet additionally the back and chest [4]. Tretinoin ((2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl) nona-2,4,6,8-tetraenoic acid) a type of carboxylated retinoic acid that is an adjunct of vitamin A. In addition, it is particularly sensitive to light, intensity, and oxygen in indoor air [5, 6]. Benzoyl peroxide (benzoyl benzenecarboperoxoate) is an organic peroxide in which two benzoyl groups are bridged through a peroxide interface. It quickly decomposes into benzoic acid and hydrogen peroxide and forms free radicals that oxidize the proteins of the bacterial cell layer,

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exhibiting antiseptic activity [7, 8]. Both drugs in combination are used for the treatment of certain skin diseases such as acne vulgaris, psoriasis, and photoaging [4]. The chemical structure of both drugs is shown in Fig. 1.

In the United States pharmacopeia [9], the titration methods of both drugs are available while in semisolid dosage forms, HPLC methods are used for alone drug determination and no combination method is available. By the survey of the literature, a few methods of Benzoyl peroxide [10–12] and Tretinoin [13–17] were reported. One UV spectrophotometric method was reported for the simultaneous estimation of Tretinoin and Benzoyl peroxide in bulk and semisolid dosage form [18]. Although, the UV spectrophotometric method is useful for routine analysis because is fast and economic but this method is not stability indicating that the product is sensitive to temperature, and light and produces impurities. Because this method does not access the generation of impurities in the drug product. Another RP-HPLC analytical method was reported for the simultaneous determination of three components (spironolactone, benzoyl peroxide, and Tretinoin) [19]. This method also not discuss about stability indicating studies. So, according to our knowledge, there is a need for stability indicating the HPLC–PDA method for simultaneous estimation of Tretinoin and Benzoyl peroxide. Hence, our aim of the study was to develop and validate the stability indicating HPLC–PDA method for simultaneous estimation of both drugs in a topical dosage form for their quality and safety as per ICH guidelines [20].

Methods

Materials and chemicals

Benzoyl peroxide (BPO) was obtained from Cambrex Karlskoga, Sweden while Tretinoin (TRN) was obtained from Chongqing huapont shengchem pharmaceuticals, China. The Twyneo (Benzoyl peroxide 3% and Tretinoin 0.1%) cream was purchased online. All the commercial grade excipients used for the method validation study were kindly gifted by Horizon Healthcare, Pakistan. We used Honeywell-made HPLC-grade acetonitrile, methanol, and water. Potassium dihydrogen phosphate, orthophosphoric acid, and all other chemicals were used of analytical grade, manufactured by Sigma Aldrich. The mobile phase, standards, and samples were filtered through 0.22 µm polyamide membrane filters.

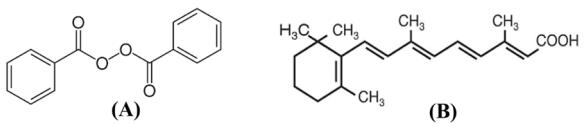

Fig. 1 Chemical structures of **A** Benzoyl peroxide, **B** Tretinoin

Chromatographic system

Shimadzu HPLC quaternary gradient LC-10AD equipped with coolant autosampler, column oven, and photodiode array multi-channel detector was used for the whole study. Chromatographic separation was accomplished by the reverse phase isocratic method using SHIMPAC® octadecyl silane C18 column, 4.6 mm internal diameter, 15 cm length, and 5 µm particle size. The final mobile phase contains a 25% volume of 0.01 M solution of potassium phosphate buffer in distilled water adjusted pH 2.5 with orthophosphoric acid and 75% volume of acetonitrile. The flow rate of the mobile phase was 1.5 mL/min, injection volume 20 µL, autosampler coolant temperature 4 °C, and the column temperature was set at 30 °C.

Preparation of standard solution

The stock standard solution of TRN drug substance was prepared by transferring 50.0 mg in a 100 mL amber-colored or low actinic volumetric flask, dissolved in methanol by using ultra sonicator, and dilute to volume. The final standard solution was prepared by transferring 30.0 mg of BPO in another 100 mL amber-colored volumetric flask, dissolved in 40 mL methanol, adding 2 mL of TRN stock solution, and mix well then diluted to volume with methanol. Hence the target concentrations were for BPO (0.3 mg/mL) and TRN (0.01 mg/mL).

Preparation of sample solution

Mix the contents of three creams, and transfer 1.0 g accurately weighed quantity of cream (nominally equivalent to 30 mg of BPO and 1 mg of TRN) into 100 mL amber-colored volumetric flask, dissolve the active ingredients by using ultra sonicator and dilute to volume. Finally, filter this solution through 0.22 µm polyamide membrane filter before injection.

The stability of the solutions was evaluated for 24 h at intervals of 0, 6, 12, and 24 h while the standard and sample solutions were both maintained in the refrigerator at 2 °C to 8 °C, protected from light.

Preparation of placebo solution

The placebo solution was prepared by transferring the excipients (anhydrous citric acid; 2.5 mg, butylated hydroxytoluene; 1 mg, carbomer homopolymer type C; 1 mg, cetrimonium chloride; 0.5 mg, cetyl alcohol; 70 mg, cyclomethicone; 10 mg, edetate disodium; 1 mg, glycerin; 20 mg, imidurea; 1 mg, (S)-lactic acid; 1 mg, macrogol stearate; 25 mg, mono and di-glycerides; 10 mg, polyquaternium-7; 10 mg, silicon dioxide; 1 mg, squalane; 20 mg, tetraethyl orthosilicate; 1.5 mg, white wax; 20 mg, and purified water; 765 mg) into 100 mL beaker. Add methanol, stir by magnetic stirrer, and heat at 50 °C for

15 min then transferred to 100 mL volumetric flask make up the volume to mark with methanol. This solution was sonicated for 20 min with intermittent shaking then filtered through 0.22 µm polyamide membrane filter before injection.

Preparation of solutions for stress studies

Stress study or Forced degradation involved the submission of samples under various environmental conditions like acid, alkali, oxidative, photolytic, and thermal with humidity to verify what are the degradation products. The control standard solution was prepared as described above. 5 mL of 1 N solution of hydrochloric acid for acidic stress, 5 mL of 1 N solution of sodium hydroxide for alkali stress, and 5 mL of 3% v/v solution of hydrogen peroxide for oxidative stress was separately added in separate samples and separate placebo solutions, then diluted with methanol to volume. All samples were kept at room temperature for 24 h. Photolytic stress was applied by keeping the sample in Laminar flow hood under UV light for 24 h. Thermal and humidity stress was applied at two different conditions for 24 h, one is at room temperature and other is 40 °C temperature with 75% relative humidity in climatic chamber.

Results

Development of analytical method and system suitability

Various deliberate changes were done to get a better-optimized condition of the chromatographic system. Firstly, we used methanol as an organic modifier but the asymmetry and theoretical plates of the BPO peak were obtained at 1.43 and 1790 which was poor, also the retention time of the TRN peak was obtained at 23.5 min, which was far. Then we consider the acetonitrile with a ratio of 50% which provided the retention time of TRN 23.69 min and BPO 6.91 min with less theoretical plates value, finally, we selected 75%. As per above said chromatographic system parameters, we injected the six replicates of the standard solution and got the system suitability as shown in Table 1.

The detection wavelength was chosen for BPO 272 nm and TRN 353 nm by scanning from 200 to 500 nm as shown in Fig. 2.

Stability of solutions

Solutions stability studies were assessed by keeping the standard and sample solution at 2 – 8 °C for 24 h and analyzed by comparing it with a freshly prepared standard solution at time intervals of 0, 6, 12, and 24 h. The results are provided in Table 2.

Method validation

Specificity

The specificity was done by injecting standard, sample, blank, and placebo solutions. The average peak purity index for the TRN sample was observed at 1.0000 and for BPO 0.99994 indicated no interference of any impurity. Also, there was no interference of diluent and placebo was observed in the retention time of both drugs. The chromatograms of specificity are shown in Fig. 3. The forced degradation studies revealed no interference of generated impurities in the R_t of main analytes peaks as shown in Fig. 3.

Linearity with limit of quantification and detection

The Linearity of analytical method was analyzed by preparing standard solutions in the range of 70–130% for both drugs. The concentration range for BPO (0.21 – 0.39 mg/mL) and for TRN (0.007–0.013 mg/mL) was produced. The quantification and detection limits were calculated by using the standard deviation of intercept and slope of calibration curve from regression data. The calibration curves for both drugs are shown in Fig. 4. The regression data are provided in Table 3.

Table 1 System suitability results

System suitability parameters	*Results		Acceptance criteria [9]
	BPO	TRN	
Retention time (R_t)	2.94 ± 0.05 min	11.34 ± 0.1 min	–
Peak asymmetry (T_f)	1.060 ± 0.05	1.005 ± 0.01	0.8–2.0
No. of theoretical plates (N)	6109 ± 200	12,373 ± 300	≥ 2000
Resolution (R)	–	30.119 ± 0.5	≥ 2
Peak purity Index	0.99997	1.00000	≥ 0.999
%RSD of peak areas	0.201	0.365	≤ 2.0%

*All the measurements were taken in six replicates

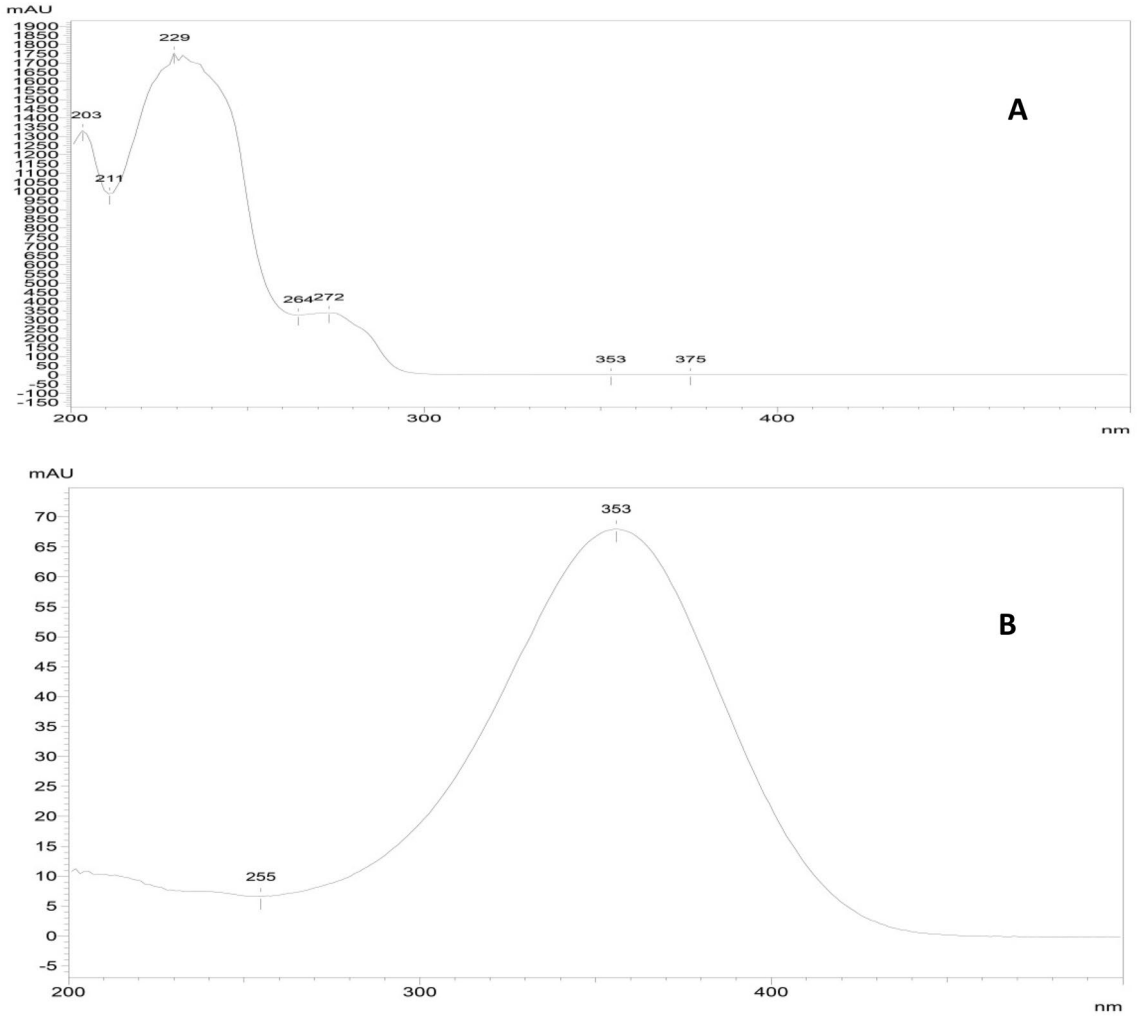

Fig. 2 UV Spectrum of **A** Benzoyl Peroxide, **B** Tretinoin

Table 2 Solution stability results

Time intervals (hours)	*Results (%)			
	BPO		TRN	
	Standard	Sample	Standard	Sample
Initial	100.21	99.86	99.89	99.78
6	99.69	99.34	99.56	99.89
12	99.38	100.38	99.52	100.54
24	99.23	99.21	99.19	99.02

*All the measurements were taken in three replicates

Accuracy

Accuracy was assessed by spiking both drug substances in placebo solution approximately at 80%, 100%, and 120% spiked levels. Three replicates of each solution were injected as per above said method and percent recovery with percent relative standard deviation between the spiked levels is calculated as shown in Table 4.

Precision

The system precision was performed by injecting six replicates of standard solution as described in the system suitability results. In method precision, repeatability was analyzed by preparing six sample solutions on the same day while the reproducibility was assessed by preparing six sample solutions by two analysts on three consecutive days and three replicates of each preparation were injected. The average percent recovery and relative standard deviation is calculated as provided in Table 5.

Robustness/rigidness

Robustness was performed by deliberating the few variations in instrumental and method parameters such as

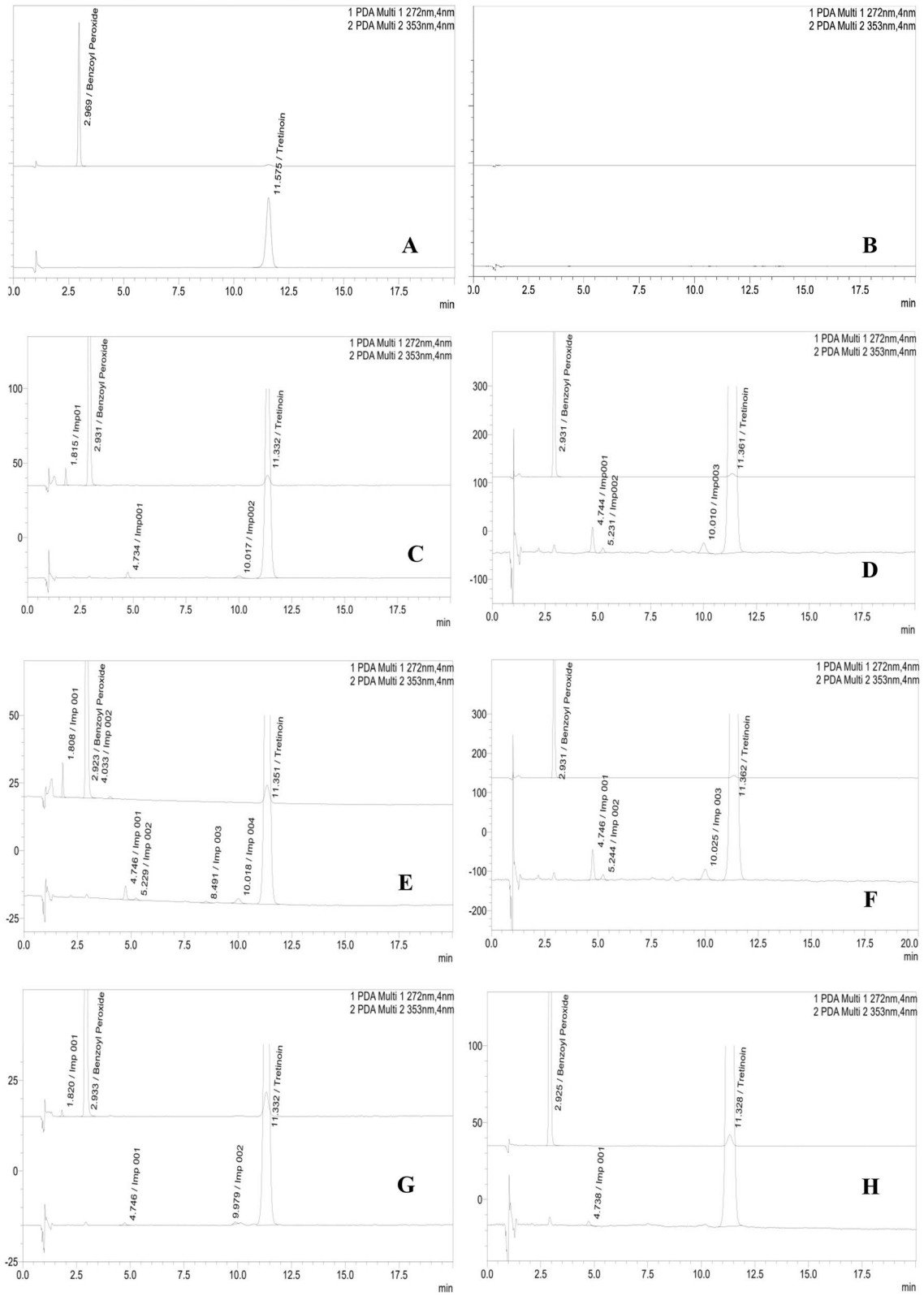

Fig. 3 Specificity chromatograms **A** standard solution, **B** Placebo solution, **C** acidic stress, **D** alkali stress, **E** oxidative stress, **F** photolytic stress, **G** thermal & humidity, **H** room temperature

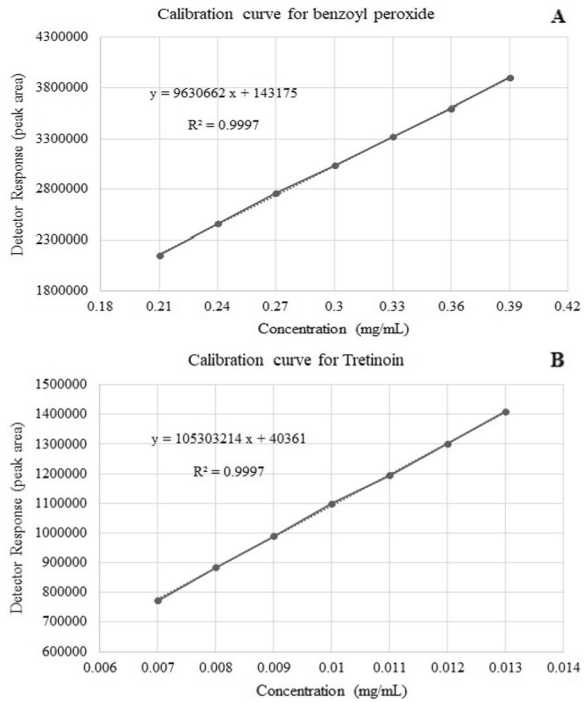

Fig. 4 Calibration Curves **A** Benzoyl peroxide, **B** Tretinoin

Table 3 Regression data

Parameters	Regression data	
	BPO	TRN
Correlation coefficient (R^2)	0.9997	0.9997
Slope (m)	9,630,662	105,303,214
Intercept (C)	143,175	10,361
Standard deviation of intercept (SDI)	58,176	21,842
Limit of quantification (10 × SDI/m)	0.06041 mg/mL	0.00207 mg/mL
Limit of detection (3.3 × SDI/m)	0.01993 mg/mL	0.00068 mg/mL

variation in column, wavelength, column temperature, auto sampler temperature and organic modifier in mobile phase. The robustness results are provided in Table 6.

Forced degradation studies

Forced degradation studies were employed by applying various environmental conditions like acid, alkali, oxidative, photolytic, and thermal with humidity on laboratory-prepared samples as well as commercial samples. The chromatograms of stress studies are provided in Fig. 3 while the results are shown in Table 7.

Discussion

The review of the literature indicated that there was no stability-indicating liquid chromatographic method available in a combination of benzoyl peroxide and tretinoin [10–19]. So, according to our aim, we developed and validated the stability-indicating liquid chromatographic-PDA method for simultaneous determination of both drugs. The summary of results indicated that the method was developed by optimizing the various suitability parameters and finally selected parameters provided the method and system suitability results as shown in Table 1. The solution stability results are provided in Table 2 showed the solutions were stable at 2–8 °C for 24 h. The method validation data indicated the specificity results in Fig. 3 provided, that there was no interference of excipients, diluent and impurities observed at the retention time of both analytes. Regression data in Table 3 provided the linearity of the method having a correlation coefficient for both analytes 0.9997, measurable quantification limits, and detection limits. Accuracy results in Table 4 revealed the obtained recovery reflects the spiked level and average recovery BPO 101.52%, TRN 99.88%. The precision data in Table 5 showed the percent relative standard deviation for repeatability of BPO 0.70% and TRN 0.94% while for reproducibility of BPO 0.52% and TRN 0.76%, indicating the method was repeatable and reproducible. Various deliberate changes were done in the robustness study, and the provided results in Table 6 indicated the using of octylsilyl-silane C8 column cause increasing the retention time of BPO while decreasing the TRN. It also given the less theoretical plates value

Table 4 Accuracy results

Analyte	Spike level (%)	Quantity added in 100 mL (mg)	Quantity recovered in 100 mL (mg)	Recovery (%)	Average recover; RSD (%)
BPO	80	24.0	24.53	102.21	101.52; 0.60
	100	30.0	30.38	101.27	
	120	36.0	36.39	101.08	
TRN	80	0.80	0.796	99.50	99.88; 0.42
	100	1.00	0.998	99.80	
	120	1.20	1.204	100.33	

Table 5 Precision results

Analyte	Sample	*Recovery (%)			
		Repeatability	Reproducibility		
			Day 1	Day 2	Day 3
BPO	1	99.79	99.90	100.53	99.67
	2	99.23	100.25	99.15	99.99
	3	100.69	100.25	99.98	100.03
	4	99.31	100.13	101.10	99.76
	5	98.58	100.09	99.40	100.34
	6	99.53	100.03	98.92	99.36
Average recovery; RSD (%)		99.52; 0.70	99.94; 0.52		
TRN	1	98.60	99.92	101.42	99.67
	2	98.63	100.29	100.04	99.89
	3	99.43	99.96	101.40	99.66
	4	99.64	100.26	101.66	99.26
	5	98.99	100.00	99.90	99.63
	6	97.04	100.01	99.77	98.55
Average Recovery; RSD (%)		98.72; 0.94	100.07; 0.76		

* All the measurements were taken in three replicates

Table 6 Robustness results

Variations*	Deliberate changes	Retention time (R_t) min		Theoretical plates (N)		Tailing factor (T_f)		Resolution (R)	Peak purity index		Peak area		% Recovery	
		BPO	TRN	BPO	TRN	BPO	TRN	TRN	BPO	TRN	BPO	TRN	BPO	TRN
Column	C18	2.94	11.34	6096	12,251	1.051	0.996	30.121	0.9999	1.0000	3,102,197	1,097,689	99.87	100.33
	C8	3.84	9.87	3425	7763	1.213	1.105	21.011	0.9987	0.9997	3,589,971	1,358,892	98.56	97.52
Wavelength ± 2 nm	270 and 351 nm	2.95	11.34	6214	12,306	1.016	0.986	30.119	0.9999	1.0000	3,956,699	1,129,654	99.56	100.87
	274 and 355 nm	2.94	11.35	5948	12,159	1.094	0.991	30.131	0.9999	0.9999	2,462,467	1,048,597	99.12	99.62
Column Temperature ± 5 °C	25 °C	2.99	11.84	6090	12,416	1.061	0.994	30.351	0.9999	1.0000	3,126,890	1,136,910	99.69	100.12
	35 °C	2.81	10.12	5718	11,869	1.059	0.977	29.811	0.9997	0.9999	3,027,889	1,076,158	98.06	99.15
Auto sampler temperature	15 °C	2.93	11.33	5987	12,051	1.045	1.004	30.112	0.9995	0.9997	3,044,125	1,078,569	97.89	98.02
	25 °C	2.92	11.34	6012	12,284	1.036	0.997	30.120	0.9993	0.9996	3,002,145	1,066,935	95.87	97.01
Organic modifier	ACN 50%	6.91	23.69	3874	6524	1.121	1.091	42.980	0.9997	0.9999	4,362,514	1,763,698	99.45	99.41
	MeOH 75%	5.21	23.51	1790	3452	1.431	1.236	47.991	0.9991	0.9999	4,552,365	1,813,987	98.54	97.23

*All the results were taken from the triplicate measurements, BPO (Benzoyl peroxide), TRN (Tretinoin)

Table 7 Stress studies results

Stress conditions		% Mean recovery; ± RSD*		Extent of degradation	
		BPO	TRN	BPO	TRN
Acidic	1 N HCl	88.12 ± 0.12	95.22 ± 0.27	Substantial	Slight
Alkali	1 N NaOH	96.38 ± 0.11	87.91 ± 0.37	Slight	Substantial
Oxidative	3% H_2O_2	92.33 ± 0.08	89.33 ± 0.54	Slight	Substantial
Photolytic	254 nm	98.54 ± 0.04	92.07 ± 0.24	None	Slight
Thermal and humidity	Room Temperature 25 °C and 60% RH	97.33 ± 0.22	96.0 ± 0.15	None	None
	Climatic Chamber 40 °C and 75% RH	94.14 ± 0.37	95.36 ± 0.25	Slight	None

*All the measurements were taken from three replicates

for both analytes and peak purity index for BPO. The variation in wavelength, column temperature having no significant effect except peak areas. Variation in auto sampler temperature revealed the decreasing of recovery. The change in organic modifier cause increasing the tailing factor and decreasing the theoretical plates value for both analytes. The stress studies showed that BPO was observed unstable against acidic environment and produce impurity at R_t 1.815 min as shown in Fig. 3C while TRN also influenced and generate the impurities as shown in Fig. 3C. In alkali environment the BPO also generate the impurity at R_t 1.821 min but as compared to acidic environment it was less while TRN generate three impurities at R_t 4.74, 5.23 and 10.01 min indicated it is sensitive to alkali conditions as shown in Fig. 3D. In oxidative environment, BPO slightly degraded while TRN produce impurities at R_t 4.78, 5.21 and 10.04 as shown in Fig. 3E. In photolytic conditions TRN reduced and shows impurities as shown in Fig. 3F. In thermal and humidity environment there were little bit degradation of both analytes observed. The summery of degradation studies shows that BPO is unstable against acidic while TRN is unstable against alkali and oxidative conditions. The BPO is stable at photolytic while TRN is stable in an acidic medium, but both are stable in a mild thermal humidity medium. But the all generated impurities were separated and shows no interaction in the R_t of main analytes provided the selectivity and stability—indicating activity of method.

Conclusion

The developed and validated analytical stability-indicating liquid chromatographic method for simultaneous determination of benzoyl peroxide and tretinoin is specific, accurate, precise, linear, and robust observed. It is the simple and fast isocratic method that can be used in routine pharmaceutical analysis where the combination of benzoyl peroxide and tretinoin formulated.

Abbreviations

HPLC	High performance liquid chromatography
PDA	Photodiode array
RP	Reverse phase
UV	Ultraviolet
ICH	International conference on harmonization
BPO	Benzoyl peroxide
TRN	Tretinoin
R_t	Retention time
T_f	Tailing factor
N	Number of theoretical plates
R	Resolution
RSD	Relative standard deviation
R^2	Correlation coefficient
m	Slope
C	Intercept
SDI	Standard deviation of intercept
RH	Relative humidity
RT	Room temperature

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Author contributions

MU conceptualized and collected the necessary data from literature. MU designed the methodology and TA performed the whole experiment; both are responsible for the data curation. MU and TA collaboratively wrote the manuscript draft. MU proof read the manuscript. The authors declare that they have read and agreed to the published version of the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors declare no conflict of interest.

Competing interests

The authors declare no conflict of interest regarding this publication.

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