ORIGINAL ARTICLE

DoE-based RP-HPLC Method Development and Validation for Estimation of Preservative Phenoxyethanol in the Presence of Adapalene in an Anti-acne Formulation Using Principles of White Analytical Chemistry

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ABSTRACT

Introduction: A simple and economical reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated as per ICH guidelines to determine Phenoxyethanol in the presence of Adapalene. This study aimed to create a robust and environment friendly method for simultaneous analysis of these drugs in pharmaceutical formulations. **Methods:** The method employed C18 column as a stationary phase, with Acetonitrile: Water as a mobile phase at a flow rate of 1.2 mL/min and a detection wavelength of 272 nm. Validation parameters included linearity, sensitivity, accuracy, and precision. A Design of Experiments (DoE) approach, specifically the Box-Behnken design, was used to evaluate the effect of Critical Method Variables (CMVs) flow rate, wavelength, and acetonitrile proportion on Critical Analytical Attributes (CAAs) retention time, tailing factor, and theoretical plates. The Red, Green, Blue (RGB) model used for White analytical Chemistry (WAC) assessment of published and proposed method. **Results:** The method demonstrated excellent linearity and sensitivity for both compounds, with high accuracy and precision under varied conditions. Robustness was confirmed through the DoE approach, minimal variability observed in key chromatographic parameters. The WAC score was calculated for published method (72.33) and proposed method (88.33). **Conclusion:** The developed RP-HPLC method was simple, economical, and robust, suitable for routine quality control analysis of Adapalene and Phenoxyethanol. Its usability, validity, cost and time effectiveness evaluated by WAC approach enhances its applicability as a sustainable method for pharmaceutical quality assurance.
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INTRODUCTION

Acne vulgaris is a predominant dermatological condition characterized by the presence of comedones (pimples, blackheads, and whiteheads) and sometimes cysts. It involves chronic inflammation of the pilosebaceous unit, comprising the hair follicles, hair shaft, and sebaceous glands. Topical retinoids, derivatives of vitamin A, are frequently prescribed for its management (1). A novel formulation, Retin-A Micro, has been developed to ameliorate acne symptoms. Tretinoin, a member of the retinoid class, has been utilized in acne treatment for an extended period, demonstrating efficacy but potentially causing skin irritation, which may affect patient adherence (2). Adapalene (AD), a synthetic analogue of a vitamin A-like compound (naphthoic acid), exhibits low solubility in water but is soluble in tetrahydrofuran and ethanol (3). As a retinoid, it is widely used in topical formulations to manage acne by modulating inflammation, keratinization, and cellular turnover (4). Adapalene is recognized in several pharmacopoeias, including the British and the United States (5,6). Preservatives are integral to topical acne treatments to prevent microbial contamination. Methylparaben, propylparaben, and phenoxyethanol are commonly employed for this purpose (7,8). These agents inhibit the growth of fungi, bacteria, and yeast, ensuring product stability. Notably, these preservatives are also utilized in other industries, such as ink and insect repellent manufacturing (9).

According to the literature review, there are limited analytical techniques for analyzing adapalene (AD) in gel formulation, primarily UV derivative spectroscopy (10) and LCMS/MS (11,12). A single study has reported the use of HPTLC for this purpose (13). Researchers had also published HPLC method for estimation

of AD in pharmaceutical gel formulation (14-17). Additionally, conductometry (18) and Fluorimetry (19) have been employed for AD estimation. Authors have also published HPTLC method for estimating AD and phenoxyethanol (PE), including stability studies (20). A complex HPLC method using gradient elution and tetrahydrofuran (THF) as part of the mobile phase has been documented for AD and PE determination (21).

However, THF is carcinogenic and poses significant health and environmental risks, including skin and eye irritation, respiratory issues, and potential liver and kidney damage. The Occupational Safety and Health Administration (OSHA) has given the airborne permissible exposure limit of 200 ppm averaged over an 8-h work shift. Therefore, use of THF as part of RP–HPLC mobile phase is hazardous for the researchers working on it (22-24). Consequently, there is a need for novel, ecofriendly analytical method to determine AD in the presence of PE. Along with greenness profile, cost and time efficiency of the analytical method play vital role in analysis.

2. Materials and Methods

2.1 Materials: Standard samples of AD (purity >98%) and PE (purity >98%) were kindly supplied as a gift sample by Cipla, Mumbai. HPLC grade solvents (Merck chemicals) and Analytical grade reagents (Loba chemicals) were used for analysis. Marketed Addiff Gel (0.1 % w/w Adapalene and 0.25% w/w PE) was used for analysis.

2.2 Instrument and Software: Electronic balance (Shimadzu-AUX 220) and Ultra Sonicator (Dakshin), HPLC system (Cyberlab LC-100) equipped with pumps, Shimadzu C18 reverse phase column (150 x 4.6 mm, 5 microns), UV Detector and WC-100 Workstation software were used.
AGREE calculator for greenness assessment and Design Expert software (Stat-Ease, trial version 8.0) were used for Analytical QBD.

2.3 Method Development

2.3.1 Preparation of standard AD and PE stock solution

After weighing 10 mg of AD it was dissolved in the least amount of THF. Following five minutes of sonication, the flask (10 ml) was filled to the appropriate volume with the same solvent yielding 1000µg/ml of AD (Stock solution A). Suitable dilutions were made using acetonitrile (ACN) to get final concentration of 20 µg/ml. A solution containing 10,000 µg/ml of PE (Stock solution B), was obtained by dissolving 0.1 ml of PE in a few ml of THF and the final volume (10 ml) was adjusted with the same solvent. To obtain a solution with 1000 µg/ml of PE, 1 ml of the stock solution B was diluted to 10 ml with ACN. To achieve a final concentration of 50 µg/ml of PE, the solution was further diluted with ACN.

2.3.2 Preparation of mixed standard solutions

A series of mixed standard solutions were prepared by transferring and mixing 0.1-0.6 ml of stock solution A and 0.25-1.5 ml of stock solution B respectively, volumes were made up to the mark (10 ml) using ACN.

2.3.3 Selection of column

Analysis for isocratic elution was performed on an analytical column Shimadzu C18 (150 x 4.6 mm, 5 microns) and kept at 30 ◦C.

2.3.4 Optimization of mobile phase

The mobile phase containing different proportions of acetonitrile and water was tried.

2.3.5 Injection volume

Sample volume of 20 µl was injected into the chromatographic system.

2.3.6 Optimization of flow rate and run time

The flow rates such as 1 ml/min, 1.2 ml/min, and 1.3 ml/min were tried for analysis. The optimization of all parameters was done by using 5, 10, and 15 minutes as a run time for analysis.

2.3.7 Selection of wavelength for analysis

The wavelength of detection was selected based on literature and the UV-visible spectrum of drugs.

2.3.8 Analysis of marketed formulation

Gel equivalent to 1 mg AD and 2.5 mg PE was transferred to a 10 ml volumetric flask and dissolved in the Tetrahydrofuran. The flask was sonicated for 15 min to give a solution containing 100 µg/ml of AD and 250 µg/ml of PE. Further dilutions were made with the ACN to get a solution containing 20 µg/ml of AD and 50 µg/ml of PE.

2.4 Method Validation (25,26)

Method was validated for linearity, range, detection and quantitation limits, accuracy and precision as per ICH guideline ICH Q2(R1).

2.4.1 System suitability test

The system suitability test was carried out by injecting five replicates of the mixed standard solution. The parameters like retention time, tailing factor, theoretical plates and peak purity were measured to evaluate the resolution and reproducibility of the system.

2.4.2 Solution stability (27)

The stability of the standard and sample solution was studied for 24 h ambient temperature.

2.4.3 Robustness determination using DOE (28-32)

The robustness was determined by changing wavelength of analysis, mobile phase compositions, flow rate and analyzing the test solution and by applying the DoE approach as per ICH Q8 (R2) guideline.

The Critical Method Variable (CMV) were optimized using Box-Behnken method and DOE based Response Surface Modelling. The Box-Behnken method was selected due to its flexibility covering all points in fewer runs and applied to optimize the HPLC separation by gaining a better understanding of the factor's main and interaction effects. It included seventeen experimental runs including five center points. Selected three (CMVs) were detection wavelength (±2nm), flow rate (±0.1 ml/min) and ACN concentration (± 5 ml). Selected (CAAs) on which significant effects observed were retention time, theoretical plate, tailing factor of both the drugs.
Based on the tailing factor, theoretical plate, and retention time of AD and PE the model that best fit was quadratic. The analysis of variance (ANOVA) data obtained was validated by the quadratic model using Design Expert software (trial version 8.0).

2.5 Application of White Analytical Chemistry approach (33-41)

The published and proposed RP-HPLC methods were evaluated using the White Analytical Chemistry (WAC) framework, which combines analytical performance, environmental sustainability, and practical usability. The Red model, based on ICH Q2(R1), assessed validation efficiency including accuracy, precision, linearity, and robustness. The Green model used AGREE software to evaluate compliance with the 12 principles of green chemistry including the sample size and number, quantities and toxicity of reagents, waste generation, energy requirements, procedural steps, miniaturization and automation, while the Blue model measured cost-effectiveness, time, and operational simplicity.

3. Result

3.1 Optimized chromatographic conditions

Separation between the two drugs with 9.144±0.2 Resolution factor (fig 1(a) (b)) was achieved by using Shimadzu C18 Column (150 x 4.6 mm, 5 micron) with ACN: Water (8:2, v/v) as mobile phase at a flow rate of 1.2 mL/min, and a runtime of 10 minutes. The detection was performed at 272 nm with a bandwidth of 2 nm, and the injection volume was 20 µL.

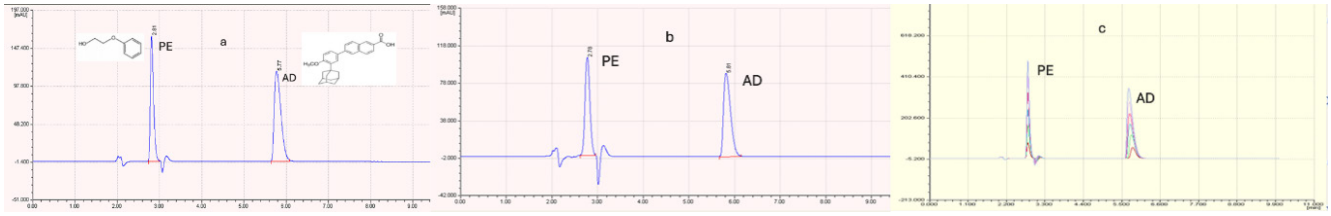

Fig. 1: Chromatogram of 1a standard mixture, 1b Formulation, 1c Overlay chromatogram of physical mixture for linearity

The retention time was found to be 2.78±0.045 and 5.81±0.096 min, for AD and PE respectively. The number of theoretical plates were 5779.6±73.19237 and 2486.1±19.9226, the tailing factor was 1.796±0.013115 and 0.736±0.010 for AD and PE respectively.

3.2 Analysis of marketed formulation

Analysis of marketed formulation was done by the proposed method. The % drug contents were found to be 100.2391±1.04 and 101.6428±1.51 for AD and PE respectively (Table I).

Table I: Results of method validation.

Parameters	AD	PE
Assay	100.2391±1.04;1.04	101.6428±1.51;1.49
% Drug Content ±SD;%RSD		
Linearity		
Linearity range	10-60 µg/ml	25-150 µg/ml
Linear regression equation	y=7031x- 15974	y=1728x+3989
Slope	7031.7	1728.2
Intercept	15974	3989
Correlation coefficient (R2)	0.999	0.996

CONTINUE

Table I: Results of method validation. (CONT.)

Parameters	AD	PE
Precision		
(a)Repeatability studies (n=6)	99.39±1.689; 1.700	2100±1.545; 1.545
%Drug Content ±SD; %RSD		
(b) Inter-day precision studies(n=3)	100±1.909; 1.909	101.8±1.939; 1.905
%Drug Content ±SD; %RSD		
Accuracy (% recovery) ± SD;%RSD		
80%	100.5±2.004; 1.994	100.5±1.814; 1.804
100 %	102.7±2.050; 1.995	101.3±1.886; 1.860
120%	102.7±1.902; 1.852	100±2.010; 2.00
Limit of Detection (LOD) (µg/ml)	0.901	1.48
Limit of Quantification (LOQ) (µg/ml)	2.73	4.51

3.3Method Validation

3.3.1 Linearity and Range

The linearity study showed linear relationship between concentration and response with R2 value of 0.999 (Fig 1(c)).

3.3.2 LOD and LOQ

LOD and LOQ values were calculated for the proposed method. Lower limit values indicated in t Table I showed sensitivity of the method.

3.3.3 Accuracy and Precision

The accuracy of the proposed method was performed by recovery studies. % RSD values of accuracy and precision were found to be less than 2 confirmed the method was accurate and precise (Table I).

3.3.4 System suitability

The proposed method was checked for system suitability parameters and it was found that all the parameters were within the acceptance criteria.

3.3.5 Robustness

DoE was applied to check the robustness. The software based Response Surface Analysis suggested 2 factor interaction and a quadratic model. From the ANOVA analysis with p-value <0.05 the main, interaction and quadratic effect for the method variable were found to be significant. The robustness was determined as an adequate response to noise ratio. The ratio obtained is greater than 4 concluding the robust nature of the developed method. A coefficient of variation (% CV) was less than 10 % as desirable and predicted R2 was in reasonable agreement with the adjusted R2 value (>0.9). Perturbation plots were developed for the predicted model. The design model showed that the change in ACN concentration, wavelength, and flow rate showed significant effects on the tailing factor, theoretical plate, and retention time of AD and PE. The yellow region of the overlay plot showed the method was highly robust Fig.3. Perturbation plots showed less than 6% standard error for all three independent factors.

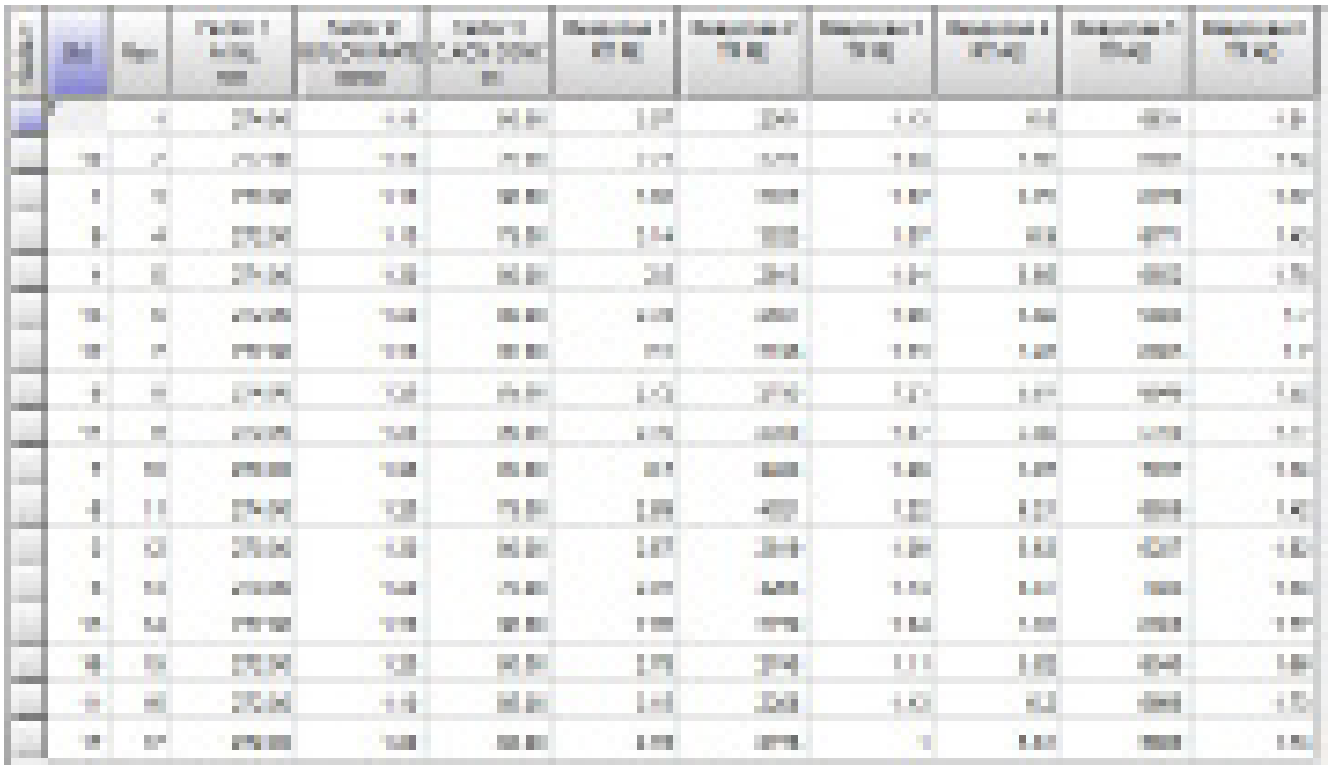

Fig.2: Design Model including 17 runs with 3 factors and 6 responses

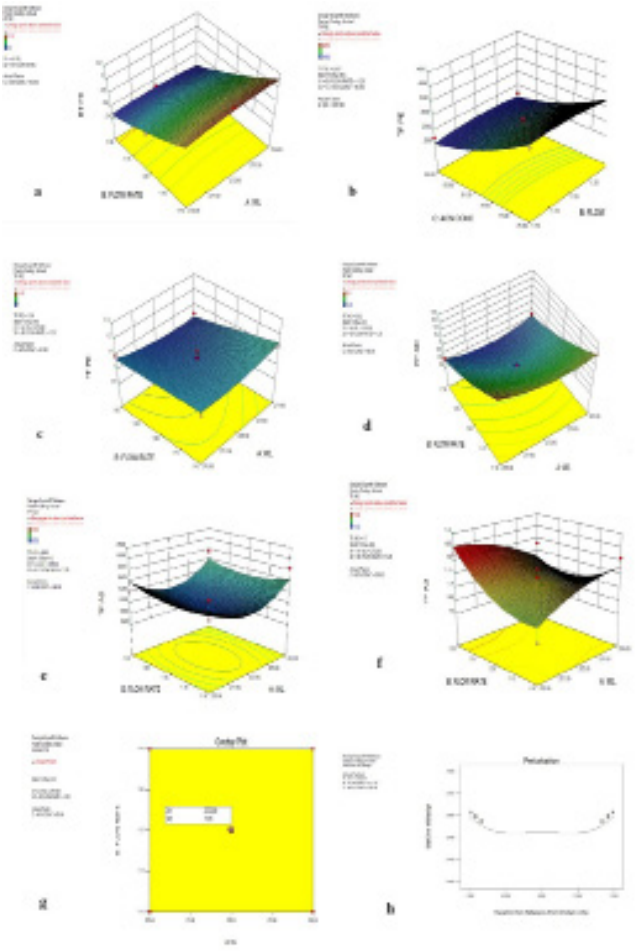

Fig.3 3D Plots of response factors. 3a. Effect on the retention time of PE 3b. Effect on the theoretical plate of PE 3c. Effect on the tailing factor of PE 3d. Effect on retention Time of AD 3e. Effect on the theoretical plate of AD
3f. Effect on a tailing factor of AD 3g. Overlay plot of independent factors 3h. Perturbation plots for independent Factors

3.3.6 Solution stability

The stability study was performed on the 20 µg/ml solution of AD and 50 µg/ml solution of PE. The prepared solution was found to be stable for 24 hours at ambient temperature.

3.4 Whiteness Profile Assessment

Red model score for the published RP-HPLC method was found to be 95, while the proposed method obtained a score of 100. The Green model score, based on AGREE software evaluation and solvent safety, was 52 for the published method and 70 for the proposed method Fig. (4A and 4B). For the Blue model, the published method scored 70, whereas the proposed method scored 95. The overall WAC score, calculated as the average of the RGB score, was 72.33 and 88.33 out of 100 for the published and proposed method respectively (Table III).

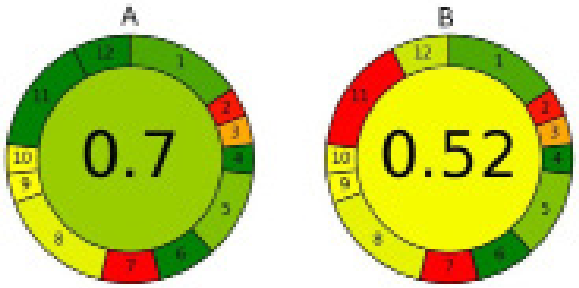

Fig.4 Result of greenness assessment. 4A) Proposed method 4B) Published method

Table III: White Analytical Chemistry-Based Assessment

Principles of white analytical chemistry	Previous RP-HPLC Method (AD + PE + MHB)	Proposed RP-HPLC Method (AD + PE)
R1 – Application and Scope	Simultaneous estimation of AD, PE, and MHB	Simultaneous estimation of AD and PE with Analytical QBD
R2 – LOD and LOQ	4–40 µg/mL linearity for all 3 compounds	LOD/LOQ within ICH Q2(R1) limits
R3 – Precision	%RSD < 2.0% for all analytes	%RSD < 2.0% for both analytes
R4 – Accuracy	Recovery 98.0%–98.6%	Recovery 98–102%
Red Model Score	95	100
AGREE Score	0.52	0.70
Solvent Hazard / Eco-toxicity	Uses THF and buffer, high toxicity	ACN-water only, safer solvents, Uses THF only for Stock solution
Waste Generation	Gradient elution, more solvent	Isocratic, less solvent used
Green Model Score	52	70
B1 – Time Efficiency	Run time >10 min	Run time ~10 min
B2 – Cost Efficiency	High cost (THF, buffer, ACN)	Economical solvents (ACN-water)
B3 – Requirements	Gradient system, buffer, toxic solvents	Standard isocratic system
B4 – User Friendliness	Moderate; not ideal for routine use	High; suitable for regular QC
Blue Model Score	70	95
White Analytical chemistry based assessment		
WAC Score (Avg of R+G+B)	Average of (95 + 52 + 70) / 3 = 72.33	Average of (100 + 70 + 95) / 3 =88.33
WAC Status	Good white	Excellent white

WAC status, poor white (score = 0–25), average white (score = 26–50), good white (score = 51–75), excellent white (score = 76–100), extraordinary white (score >100).

Discussion

Phenoxyethanol is more polar compared to adapalene, so it was eluted out at a faster rate than AD with retention time of 2.84±0.045 min. The values of number of theoretical plates (>2000), tailing factor (<2) and resolution (>2) were found within the acceptance criterion (5). Developed method was validated as per ICH guidelines Q2 (R1) was found to be sensitive, accurate, precise and robust.

The DoE reported that an increase in flow rate decreases the retention time and there is no effect of wavelength on retention time with constant ACN concentration for both the drugs Fig. 3 (a), (d). Fig. 3(b), (e) showed with increase in concentration of ACN there is a decrease in the number of theoretical plates of PE at a constant wavelength 272 nm. Fig. 3(c), (f) showed no linear relationship between all the parameters with the tailing factor of PE. Confirmation report (Table II) supported the accurate selection of experimental conditions for separation of analytes. As, the response of analytes remained unaffected by deliberate changes in the operational parameters indicated the method was robust. The analytical control space was identified.
For the evaluation of validation efficiency, the Red model-based assessment was applied using four parameters (R1 to R4) in accordance with ICH Q2(R1) guidelines. The published RP-HPLC method obtained a Red model score of 95 for the estimation of Adapalene, Phenoxyethanol, and Methylparaben. The proposed RP-HPLC method, which employed a single chromatographic condition and incorporated an Analytical Quality by Design (AQbD) approach for the simultaneous estimation of Adapalene and Phenoxyethanol, received a Red model score of 100.

Table II: DoE confirmation report

Factor	Name	Level	Low Level	High Level	SD
A	Wavelength	272	270	274	0.000
B	Flow Rate	1.20	1.10	1.30	0.000
C	ACN Conc.	80	75	85	0.000
Two sided	Confidence	=95%	n=1		

Response	Prediction	Experimental output	SD	SE (n=1)	95% PI Low	95% PI High
RT PE	2.78	2.78	0.025	0.027	2.7141	2.845
TP PE	2680	2707	224.8	246.3	2097.4	3262.5
TF PE	1.056	1.06	0.066	0.072	0.8834	1.227
RT AD	5.816	5.815	0.0099	0.010	5.791	5.841
TP AD	5928	5884	223.4	244.7	5349.2	6506.7
TF AD	1.728	1.70	0.0787	0.086	1.532	1.932

1) Retention time of PE 2) Theoretical plate of PE 3) Tailing factor of PE
4) Retention time of AD 5) Theoretical plate of AD and 6) Tailing factor AD
SE=Standard Error PI= Prediction Interval

The proposed simultaneous RP-HPLC analysis of AD and PE was carried out using acetonitrile and water (less volatile, biodegradable, safe, and green solvent than THF). Hence, the proposed RP-HPLC method has obtained a score of 70 out of 100 using a Green model-based assessment. The published method utilized gradient elution and required multiple reagents, including costly organic solvents such as tetrahydrofuran and acetonitrile, resulting in higher resource consumption and longer analysis time. In contrast, the proposed method employed a single isocratic chromatographic condition and used a simpler mobile phase composed of acetonitrile and water, which reduced both operational complexity and solvent usage. Hence, the proposed method has obtained 90 out of 100 for cost efficiency, time efficiency, and user-friendliness. The WAC score was calculated as the average of the Red, Green, and Blue (RGB) model scores. The published method, used for the estimation of Adapalene, Phenoxyethanol, and Methylparaben, showed a WAC score of 72.33 out of 100, indicating a good white method. In comparison, the proposed method, developed for the simultaneous estimation of Adapalene and Phenoxyethanol, achieved a higher WAC score of 88.33 out of 100, which classifies it as an excellent white method. The better score reflects its greener nature, lower cost, shorter analysis time, and alignment with ICH Q2 (R1) guidelines.

Conclusion

To assess the quantities of AD and PE, a simple and moderately priced RP-HPLC method was developed and validated using ICH Q2 (R1) recommendations. The developed RP-HPLC method was found to be accurate and precise. Applying the DoE strategy, yielded data indicated this procedure was robust. WAC assessment score demonstrated ecofriendly, safe, time and cost effective character of method. This RP-HPLC method was proposed as a good approach for measuring AD and PE simultaneously in regular quality control analysis of combination medication formulations.

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