PROSPECTS
IN PHARMACEUTICAL SCIENCES

Prospects in Pharmaceutical Sciences, 23(3), 84-96
https://prospects.wum.edu.pl/

Original Article

QUALITY BY DESIGN APPROACH IN HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF ABIRATERONE ACETATE AND PREDNISOLONE WITH GREENNESS ASSESSMENT

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Received: 21.01.2025 / Revised: 02.04.2025 /Accepted: 11.04.2025 / Published online: 05.08.2025

ABSTRACT

A Quality by Design (QbD) approach was employed to develop and optimize a novel and robust Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the simultaneous estimation of abiraterone acetate (ABTA) and prednisolone (PDS) in the pharmaceutical dosage form. The optimized method parameters were determined using a Central Composite Design (CCD) and included a run time of 8 minutes, the flow rate of 0.8 mL//min, and a column temperature of 25°C. The mobile phase comprised methanol and acetonitrile (90:10v/v) with detection at 260 nm. Retention times of 5.23 minutes for ABTA and 3.335 minutes for PDS were achieved, ensuring efficient separation with high resolution and minimal tailing. Method validation confirmed precision, accuracy, linearity, robustness, and system suitability in compliance with ICH guidelines, establishing the method's reliability for routine use. Green analytical chemistry assessment using GAPI, AGREE, HPLC-EAT, and BAGI tools highlighted the method's environmental sustainability, practicality, and safety. The development of this method supports efficient quality control by providing a precise, eco-friendly analytical solution for simultaneous drug analysis. Additionally, its adaptability for future pharmaceutical applications makes it a valuable tool for enhancing process efficiency and meeting sustainability goals.

KEYWORDS: Abiraterone acetate, Prednisolone, Quality by Design (QbD), High-Performance Liquid Chromatography (HPLC), Greenness

1. Introduction

Abiraterone acetate (ABTA), chemically known as [(3S,8R,9S,10R,13S,14S)-10,13-dimethyl-17-pyridin-3-yl-2,3,4,7,8,9,11,12,14,15-decahydro-1H-cyclopenta[a] phenanthrene-3-yl] acetate (Fig. 1), is a prodrug that is enzymatically converted into its active form, abiraterone. This active metabolite functions as a highly potent and selective inhibitor of cytochrome P450 17A1 (CYP17A1). The enzyme CYP17A1 is crucial for androgen biosynthesis since it catalyzes the transformation of pregnenolone and progesterone into androgen precursors. Abiraterone acetate reduces androgen receptor activation, a crucial element in the development of prostate cancer.

Prednisolone (PDS), chemically described as (8S, 9S,10R, 11S, 13S, 14S, 17R)-11-17—dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-7, 8, 9, 11, 12, 14, 15,16- octahydro- 6H-cyclopenta [a] phenanthrene-3-one) (Fig. 2) is a glucocorticoid used alongside abiraterone acetate to reduce the secondary effects of abiraterone such as hypertension, hypokalaemia, and fluid retention. Prednisolone helps to maintain normal corticosteroid levels in the body by delivering supplementary glucocorticoid action, lowering the likelihood of adverse effects, and enhancing treatment tolerance overall [1,2,3].

The literature has documented several HPLC methods for the analysis of abiraterone acetate alone [4-6], prednisolone alone [7-9], and abiraterone acetate and prednisolone combined with other pharmaceutical formulations and biological matrices [10-13]. However, a QbD-based RP-HPLC method for simultaneous measurement of abiraterone acetate and prednisolone has not yet been reported. Clinical investigations have proven that abiraterone acetate and prednisolone together significantly increase the survival rate and progression-free survival in individuals with CRPC. This therapeutic strategy emphasizes the importance of targeting both the androgen receptor system and the physiological implications inhibiting androgen production in the treatment of advanced prostate cancer [14]. HPLC

is widely regarded as a superior analytical technique due to its selectivity, making it particularly suitable for the simultaneous estimation of several active pharmaceutical ingredients in a mixture. Achieving optimal separation in HPLC depends on several controlled parameters, including flow rate, temperature, and mobile phase composition. In addition, the literature suggests that the stationary phase and detector parameters must be carefully considered during method development to achieve optimal performance. Traditionally, the trial-and-error or one-factor-at-a-time (OFAT) technique has been used to optimize HPLC processes. Analytical chemists use this strategy to modify one variable at a time using prior information. While this approach can provide stable operating circumstances, it frequently fails to discover truly optimal settings, potentially leading to robustness difficulties in the produced system [15, 16].

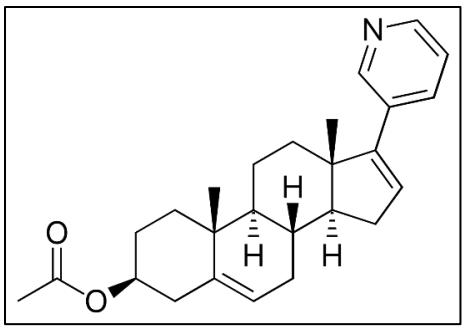


Fig. 1. Structure of abiraterone acetate

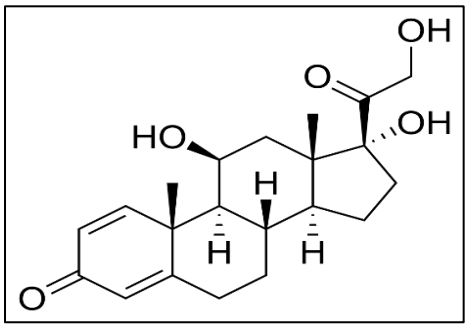


Fig. 2. Structure of prednisolone

The International Council for Harmonisation (ICH) describes Quality by Design (QbD) as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and control, based on sound science and quality risk management" [17]. Analytical QbD (AQbD) applies these ideas to the analytical process, allowing for the development of reliable and economical processes. AQbD focuses on identify ing critical method parameters and establishing a method-operable region (MODR) by accounting for various influencing factors. Factorial experimental designs, such as the central composite design (CCD) and Box-Behnken design (BBD), are widely employed in pharmaceutical analysis under AQbD. In this study, CCD was chosen because it is efficient and requires fewer design points and experimental runs. This approach establishes a clear link between causes and responses while producing statistically significant results [18]. The primary goal of AQbD is to detect potential failure modes, assure robustness, and create a design space that meets relevant system suitability requirements, hence facilitating continuous life cycle management [19]. The goal of this research is to use a QbD-based strategy to develop and optimize an HPLC method for the simultaneous measurement of prednisolone and abiraterone acetate in pharmaceutical dosage forms.

2. Methods

2.1. Materials

Abiraterone acetate was procured from Glenmark Pharmaceuticals, and prednisolone was obtained from Micro Labs Limited, Mumbai. The solvents utilized were HPLC grade, while all other chemicals and reagents were analytical grade.

2.2. Instrument and chromatographic conditions

The chromatographic analysis of the active pharmaceutical ingredients (APIs) was performed using the JASCO Extrema LC 4000 HPLC system, which is equipped with a photodiode array (PDA) detector and features variable gradient solvent management via its modular pump design. ChromNAV software was used for system control, data processing, and collection of chromatographic results. Separation was done using a Hypersil C18 column (250 mm × 4.6 mm, 5 µm particle size). Isocratic elution was used for efficiently separating abiraterone acetate (ABTA) from prednisolone (PDS). The mobile phase was composed of methanol and acetonitrile in a 90:10% v/v ratio, supplied at a flow rate of 0.8 mL/min. The run time was 8 minutes, with the column maintained at 25°C. The injection volume for the method was set at 10 µL.

2.3. Preparation of standard solution and working solution

In a 100 mL volumetric flask, precisely 100.0 mg of ABTA and 1.0 mg of PDS were added. After adding around 70 mL of methanol, the liquid was sonicated to completely dissolve the PDS and ABTA, and the volume was made up to the mark. To create the working solution, the aliquot of the stock solution was further diluted with the same solvent to achieve a final concentration of 100 µg/mL of ABTA and 1 µg/mL of PDS.

2.4. Selection of detection wavelength

Standard solutions of both drugs were analyzed with a UV spectrophotometer in spectrum mode, spanning the wavelength range of 200-400 nm, with methanol as the reference solvent. The two drugs, ABTA and PDS, exhibited their respective λmax at 254 nm and 243 nm. A detection wavelength of 260 nm was chosen as an isobestic wavelength, where an absorption spectrum of both drugs can cross with each other, facilitating simultaneous estimation for method development and validation.

2.5. HPLC method development by QbD approach

As demonstrated below, an Analytical Quality by Design (AQbD) technique was used in the development of the HPLC method.

2.6. Selection of quality target product profile (QTPP)

The key performance attributes needed to provide accurate, precise, and repeatable results are outlined in the Quality Target Product Profile (QTPP). The retention

time, theoretical plates, and tailing factor were determined to be crucial QTPP parameters for the proposed HPLC method.

2.7. Determination of critical quality attributes (CQA)

The performance of the procedure and the accuracy of the results are significantly influenced by Critical Quality Attributes (CQA). Run time, flow rate, and temperature were identified as critical variables for this strategy that need to be carefully controlled in order to maintain the QTPP within an acceptable range [20].

2.8. Factorial design

The central composite response surface technique served as the foundation for the experimental design. To optimize the chromatographic parameters, the experiment data were analyzed using the Design-Expert software® (Version 8.0.7.1). Run time (8–12 minutes), flow rate (0.6–1 mL/min), and temperature (20–30 °C) were used as independent variables in order to choose suitable chromatographic conditions (Table 1). For the best separation efficiency, however, the dependent parameters of resolution, theoretical plates, and tailing factor were selected. About 20 experimental runs were used to create the experimental design matrix for the three specified variables at two levels.

Table 1. Coded value for independent variables

Factor	Coded values for a given factor	Levels	
		-1	+1
Run time (min)	A	8	12
Flow rate (mL/min)	B	0.6	1.0
Column temperature (°C)	C	20	30

2.9. Assessing experimental results and selection of the ultimate method condition

The method conditions were assessed using the CCD methods. Variables including tailing factor, theoretical plates, and retention time were assessed in the initial stage. This approach discovered different chromatographic conditions for abiraterone acetate and prednisolone. Proven acceptable limits were created within robust zones where deliberate alterations in method parameters did not affect method quality, assuring dependability throughout validation testing [19]. The most suitable chromatographic conditions were optimized with design expert tools.

2.10. Risk assessment

An analytical technique's suitability for its intended use is determined through validation. Using the ICH Q2 (R1) guidelines, the developed HPLC method for quantifying prednisolone and abiraterone acetate was verified [20].

2.11. Analytical method validation

The purpose of validating an analytical technique is to ensure that it works effectively for its intended use. In this case, the HPLC method developed to measure abiraterone acetate and prednisolone was carefully tested and validated following the ICH Q2 (R1) guidelines to confirm its accuracy, reliability, and suitability [21].

2.11.1. Linearity

In order to assess linearity, the standard stock solution was diluted to produce aliquots with final concentrations of PDS ranging from 0.5 to 30 µg/mL and ABTA ranging from 50 to 300 µg/mL. 10 µL of each combination was put into the column. The regression equation and coefficient of determination values for the medications were computed, and a calibration curve was made by plotting peak area versus drug concentration.

2.11.2. Precision

Precision refers to the method's capacity to consistently analyze multiple replicates under varying conditions. To determine the precision, samples of both analytes (ABTA and PDS) underwent interday and intraday quality control (QC) analysis. The acceptance criterion for %RSD was set at ≤2%.

2.11.3. Accuracy

Recovery studies on a laboratory-prepared formulation at three levels (80%, 100%, and 120%) of the standard solution were used to evaluate the accuracy of the approach. The percentage recovery of ABTA and PDS was calculated, and acceptance criteria of 98% to 102% based on ICH guidelines were established.

2.11.4. LOD and LOQ

The lowest quantity of a material that can be consistently detected, even if it cannot be quantified accurately, is known as the limit of detection (LOD). However, the lowest concentration that can be precisely determined is known as the limit of quantitation (LOQ). To calculate the LOD, researchers identify the lowest concentration that can be clearly distinguished from a blank sample, ensuring the substance can be detected. Both LOD and LOQ were determined following ICH guidelines using these formulas:

$$LOD = 3.3 \times \sigma / SD$$

$$LOQ = 10 \times \sigma / SD$$

Here, σ represents the standard deviation of the y-intercept of the regression line, and SD is the slope of the calibration curve.

2.11.5. Robustness

Robustness was measured using the impact of small chromatographic changes on peak area, retention time (RT), and theoretical plates. Samples were subjected to analysis at different column oven temperatures (20°C and 30°C), flow rates (0.6 and 1.0 mL/min), and detection wavelengths (258 and 262 nm). The effects of various technique adjustments were investigated, and the findings were used to establish the method's stability under minor changes.

2.12. System suitability

To examine system performance, six duplicate samples of the ABTA and PDS solutions were injected. The parameters tested were retention time, column efficiency, tailing factor, theoretical plates, and peak area. These parameters were utilized to guarantee that the system was reliable and consistent throughout the analysis.

Table 2. Optimization of parameters for analysis of abiraterone acetate and prednisolone

Sr. No.	Factor-1 Run time	Factor-2 Flow rate	Factor-3 Column temperature	Response-1 Retention time of abiraterone acetate	Response-2 Retention time of prednisolone	Response-3 NTP of abiraterone acetate	Response-4 NTP of prednisolone	Response-5 The tailing factor of abiraterone acetate	Response-6 The tailing factor of prednisolone
1	10	0.80	16.59	5.403	3.37	9454	8352	1.271	1.2
2	8.00	0.60	20.00	7.413	4.512	11237	9705	1.242	1.171
3	8.00	1.00	20.00	4.44	2.72	8693	6885	1.271	1.109
4	10.00	0.80	33.41	5.5	3.383	8985	8333	1.305	1.212
5	10.00	0.80	25.00	5.52	3.347	8211	8475	1.271	1.157
6	12.00	0.60	20.00	7.147	4.48	8874	9838	1.32	1.185
7	8.00	0.60	30.00	7.163	4.483	9842	10073	1.315	1.151
8	12.00	0.60	30.00	6.85	4.437	8865	9883	1.311	1.161
9	10.00	0.80	25.00	5.15	3.333	7937	8530	1.346	1.179
10	10.00	0.46	25.00	9.123	5.797	9852	10787	1.246	1.165
11	10.00	1.14	25.00	3.76	2.363	7066	6605	1.273	1.131
12	12.00	1.00	30.00	4.38	2.703	6973	7202	1.294	1.15
13	10.00	0.80	25.00	5.323	3.353	6759	8377	1.314	1.187
14	10.00	0.80	25.00	5.413	3.363	6762	8376	1.29	1.142
15	6.64	0.80	25.00	5.403	3.36	6970	8574	1.259	1.158
16	10.00	0.80	25.00	5.413	3.363	6727	8302	1.267	1.189
17	10.00	0.80	25.00	5.423	3.363	6602	8294	1.303	1.161
18	8.00	1.00	30.00	5.363	2.697	6522	7265	1.26	1.14
19	13.36	0.80	25.00	5.343	3.35	5972	8324	1.341	1.175
20	12.00	1.00	20.00	4.447	2.703	5826	7211	1.297	1.148

2.13. Assay

Before being pulverized, twenty pills were individually weighed to determine their average weight. The powdered tablet, which contained 100 mg of ABTA and 1 mg of PDS, was dumped into a 100 mL volumetric flask. After adding 20 milliliters of mobile phase, the mixture was sonicated for five minutes at a controlled temperature to dissolve the powder. The same solvent was used to get the volume up to the mark. One milliliter of this solution was pipetted into a ten-milliliter volumetric flask, diluted with the appropriate amount of mobile phase, then mixed properly and filtered using a membrane filter with a pore size of 0.45 µm. A final concentration of 200 µg/mL of ABTA and 2 µg/mL of PDS, which falls within the linearity range, was obtained by further dilutions using methanol. The drug content in each tablet and the bulk drug was determined by utilizing the standard calibration curve.

2.14. Green assessment

The growing demand for adopting sustainable processes to meet the Green Analytical Chemistry (GAC) criteria presents a significant challenge for the pharmaceutical sector. One of the most widely used techniques in several stages of pharmaceutical analysis, high-performance liquid chromatography (HPLC), generates a significant amount of organic hazardous waste. Consequently, it is now crucial to use GAC concepts in pharmaceutical analysis. Four key areas may be used to summarize these principles: (1) decreasing or eliminating the use of reagents in analytical processes; (2) conserving energy; (3) appropriately handling analytical waste; and (4) improving operator safety [22].

One of the green evaluation tools used to evaluate these processes is the High-Performance Liquid Chromatography-Environmental Assessment Tool (HPLC-EAT), which is simple and easy to use, and the software is freely available, making it an accessible option for evaluating the environmental impact of analytical methods. HPLC-EAT summarizes the safety, health, and environmental effects of all solvents used in the chromatography process and assigns a final score that indicates the method's overall "greenness" based on the type and quantity of solvents used: the lower the score, the less of an impact on the environment and human health.

Additionally, the Green Analytical Procedure Index, or GAPI, is an experimental tool that evaluates the whole greenness of analytical methods from sample collection to the end output. GAPI measures and quantifies the environmental impact of each process step using a specific symbol made up of five pentagrams. A color-coded scheme that goes from green (low impact) to yellow (medium effect) to red (high impact) is used to illustrate the impact. The influences of the environment at every level are so clearly shown. This all-encompassing strategy guarantees that each analytical procedure's environmental impact is thoroughly assessed and optimized [23].

The Blue Analytical Greenness Index (BAGI) tool is an additional tool to the GAPI, ComplexGAPI, AGREE, and AGREEprep green evaluation tools. In contrast to its green counterparts, BAGI emphasizes the practical elements of analytical procedures while concentrating on the "blue" concepts of White Analytical Chemistry (WAC). The type of analysis, the number of analytes that are determined simultaneously, the number of samples that can be analyzed in an hour, the type of reagents and materials used in the analytical method, the necessary instrumentation, the number of samples that can be treated simultaneously, the need for preconcentration, the degree of automation, the type of sample preparation, and the amount of sample are among the ten essential characteristics that are evaluated by this tool. A pictogram and a score that represent the method's usefulness and applicability are produced using these criteria [24].

3. Results

During the initial phase of method development, using a methanol-water mixture as the mobile phase failed to produce a detectable peak. Subsequent trials with acetonitrile and water in a 50:50 v/v ratio also showed no peak. Switching to a methanol-acetonitrile mixture at a 40:60 v/v ratio resulted in asymmetric peaks. Further adjustments to improve peak shape and symmetry led to the optimized mobile phase composition of methanol to acetonitrile in a 10:90 v/v ratio. The central composite design was then employed to optimize various parameters within the design space for better performance.

3.1. HPLC method development by QbD approach

3.1.1. Quality Target Product Profile (QTPP)

Retention duration, theoretical plate count, and tailing factor were the chosen Quality Target Product Profile (QTPP) parameters for maximizing HPLC chromatographic conditions for both medications.

3.1.2. Critical quality attributes

The Critical Quality Attributes (CQA), like run duration, flow rate, and column temperature, were determined to be crucial for guaranteeing the effectiveness and dependability of the method being used.

3.1.3. Factorial design

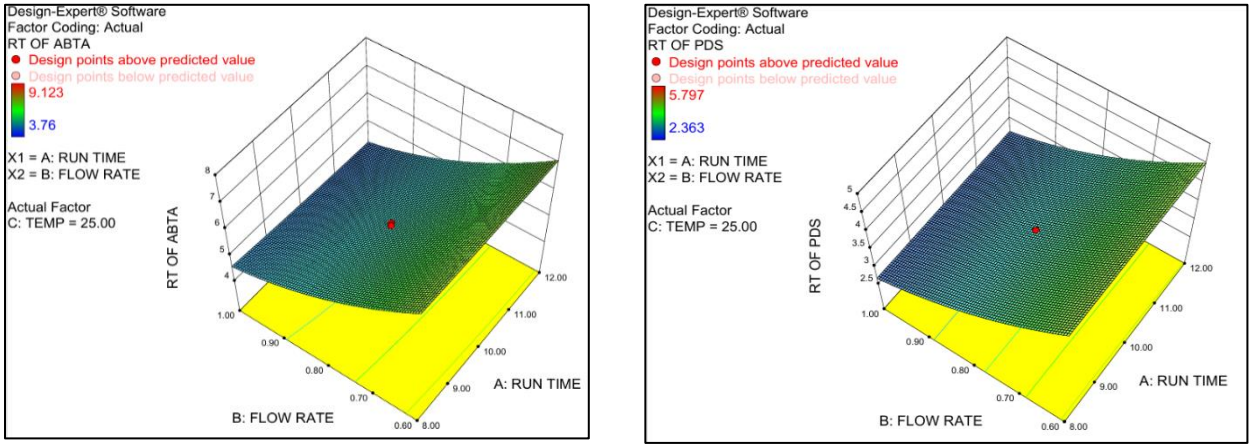
The suggested HPLC technique was developed using the central composite design (CCD). As seen in Table 2, this method made it easier to optimize several parameters.

3.1.4. Design space

The response surface study type, CCD, was employed for 20 runs. The suggested CCD experimental design was used, and run time, flow rate, and column temperature were measured against six responses: retention time of ABTA and PDS. NTP of ABTA and PDS, and tailing factor of ABTA and PDS. The findings were summarised.

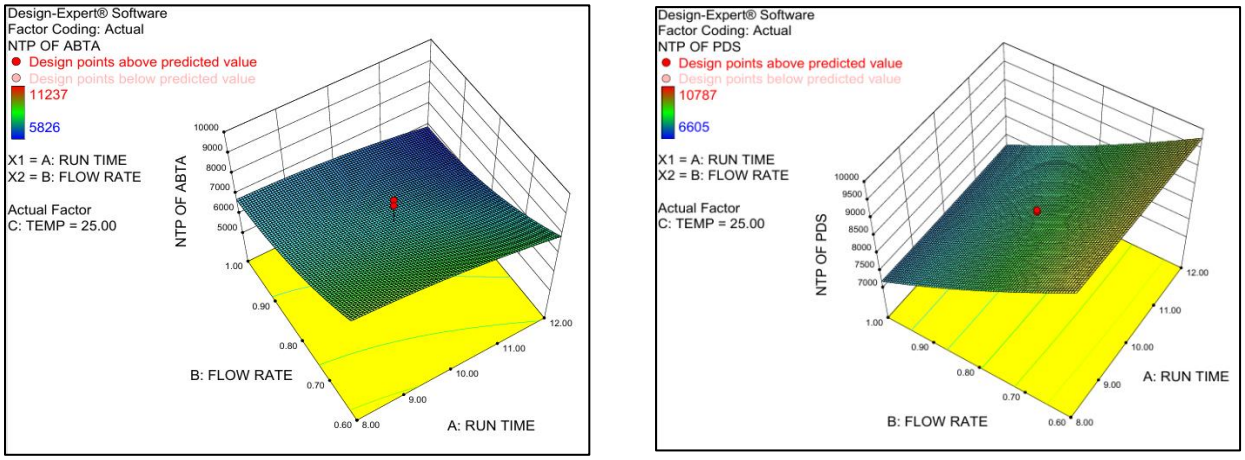
From Fig. 3(A) and the equation for the retention time of abiraterone acetate (for actual values) = +19.05175 + 0.23526*A - 26.21653*B - 0.11024*C - 0.12406*A*B -0.012963*A*C + 0.17538*B*C + $0.00637149*A^2$ + $10.08144*B^2$ + $0.00212960*C^2$, it was concluded that as the run time (A) goes up, the retention time of abiraterone acetate increases. This is supported by a positive coefficient of +0.23526. On the other side, with the flow rate (B) going down, which is indicated by a negative coefficient of -26.21653, the retention time also rises. Lastly, when the column temperature (C) decreases, as shown by a negative coefficient of -0.11024, this, too, leads to a longer retention time for abiraterone acetate.

From Fig. 3(B) and equation for retention time of prednisolone (for actual values) = +11.50494 - 0.000819608*A -15.15355*B - 0.013252*C + 0.022500*A*B + 0.000175000*A*C + 0.006750000*B*C - $0.00128063*A^2$ + $6.28009*B^2$ + $0.00009915500*C^2$, it was concluded that as ß1 negative coefficient (-0.000819608) indicates that as run time (A) decreases, ß2 negative coefficient (-15.15355) indicates that as the flow rate (B) decreases, and the ß3 negative coefficient (-0013252) indicates that as the column temperature (C) decreases, the value of the retention time of prednisolone increases.



(A) (B)

Fig. 3. 3D surface plot for the effect of a combination of factors on (A) retention time of abiraterone acetate and (B) retention time of prednisolone by using a central composite design



(A) (B)

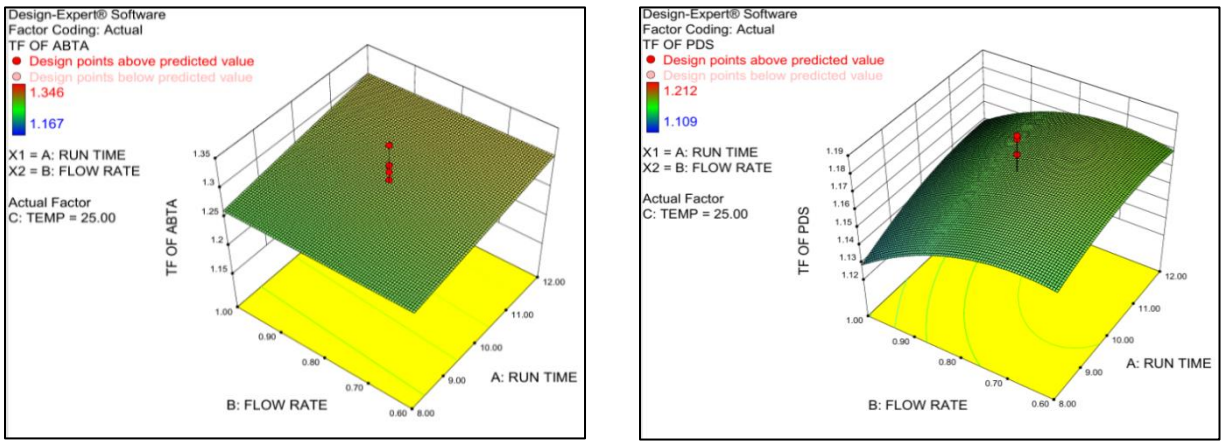
Fig. 4. A 3D surface plot depicting the effect of a combination of factors on (A) theoretical plates of abiraterone acetate and (B) theoretical plates of prednisolone by utilizing a central composite design

From Fig. 4(A) and equation for theoretical plates of abiraterone acetate (for actual values) = +56358.55600 - 1008.38454*A - 30142.10068*B - 2230.67048*C + 288.75000*A*B + 58.80000*A*C + 47.50000*B*C - 48.24011*A^2 + 12747.59251*B^2 + 31.15124*C^2, it was deduced that as ß1 negative coefficient (1008.38454) shows that when run time (A) is decreased, ß2 negative coefficient (-30142.10068) suggests that when flow rate (B) is decreased, and ß3 negative coefficient (-2230.67048) illustrates that when column temperature (C) is decreased, then the value of theoretical plates of abiraterone acetate was increased.

Looking at Fig. 4(B) and the equation related to the theoretical plates of prednisolone (based on actual values) = +13785.12681 + 28.52600*A - 11789.68032*B + 137.07130*C + 100.000*A*B - 8.90000*A*C - 5.25000*B*C + 5.30613*A2 + 2713.80512*B2 - 0.65716*C2, the results showed that when the run time (A) goes up, represented by a ß1 positive coefficient of +28.52611, the theoretical plates of prednisolone increase. On the other hand, a decrease in flow rate (B), indicated by a ß2 negative coefficient of -11789.68032, also leads to the rise in these theoretical plates. Additionally, as the column temperature (C) rises, with a ß3 positive coefficient of +137.07130, the value of the theoretical plates continues to grow.

From Fig. 5(A) and equation for the tailing factor of abiraterone acetate (for actual values) = +1.15704 +0.009954960*A -0.00753892*B +0.001569630*C, it was found that the value of tailing factor of abiraterone acetate was increased as ß1 positive coefficient (+0.009954960) indicates that as the run time (A) increases, ß2 negative coefficient (-0.00753892) suggest that as the flow rate (B) decreases and ß3 positive coefficient (+0.001569630) indicates that as the column temperature (C) increases.

From Fig. 5(B) and equation for the tailing factor of prednisolone (for actual values) = +1.21079 + 0.0355990*A +0.10011*B - 0.020248*C + 0.007812500*A*B- 0.000412500*A*C + 0.009625000*B*C - 0.00139042*A^2 - 0.30256*B^2 + 0.0003361470*C^2, where ß1 positive coefficient (+0.035590) means that increasing run time (A) increases the value of tailing factor of prednisolone, ß2 positive coefficient (+0.10011) indicates that as flow rate (B) increases the value of tailing factor of prednisolone increases, and ß3 negative coefficient (-0.020248) indicates that on decreasing column temperature (C) the value of tailing factor of prednisolone was increased.



(A) (B)

Fig. 5. A 3D surface plot for the effect of a combination of factors on (A) the tailing factor of abiraterone acetate and (B) the tailing factor of prednisolone by using a central composite design

Table 3. Optimized Chromatographic Conditions

Mobile phase	Methanol: Acetonitrile (90:10)
Flow rate	0.8 mL/min
Column	BDS Hypersil C18 column (250 mm × 4.6 mm) having 5.0 µm particle size
Diluent	Methanol
Detection wavelength	260 nm
Column temperature	25ºC
Injection volume	10 µL
Run time	8 minutes

Table 4. Optimized solution for method development

Run time (min)	Flow rate (mL/min)	Temp.	Retention time of abiraterone acetate	Retention time of prednisolone	NTP of abiraterone acetate	NTP of prednisolone	The tailing factor of abiraterone acetate	The tailing factor of prednisolone
8	0.8	25ºC	5.230	3.3357	11608	8660	1.424	1.172

3.1.5. Optimized condition obtained

Numerical optimization was used to determine the best approach by "trading off" different CAAs to maximize theoretical plates and minimize retention time, resulting in a desirability function of 1. The optimized conditions revealed that the process lasts for 8 minutes, with a flow rate set at 0.8 mL per minute, and the temperature of the column is maintained at 25ºC, resulting in the desirability of 1.0 and all CAAs within the intended limits (Table 4). Table 3 shows the optimized run time, flow rate, and column temperature parameters, whereas Fig. 6 shows the chromatogram of the optimized method.

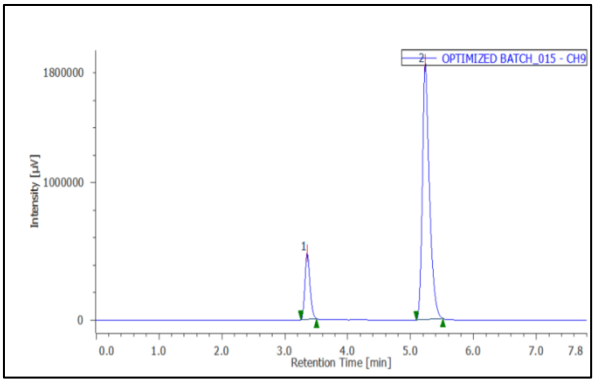


Fig. 6. Chromatogram of optimized method

Table 4. Optimized solution for method development

Run time (min)	Flow rate (mL/min)	Temp.	Retention time of abiraterone acetate	Retention time of prednisolone	NTP of abiraterone acetate	NTP of prednisolone	The tailing factor of abiraterone acetate	The tailing factor of prednisolone
8	0.8	25ºC	5.230	3.3357	11608	8660	1.424	1.172

3.2. Method validation

3.2.1. System suitability

The system suitability test was performed on a representative chromatogram to evaluate different parameters such as retention time, which was 5.273 min for ABTA and 3.363 min for PDS. The theoretical plates for ABTA have been estimated to be 10746 and for PDS 8217, whereas the tailing factor for ABTA was 1.170 and for PDS 1.197. Fig. 7 depicts a 3D surface plot of desirability for the optimized method.

3.2.2. Linearity

The calibration curves for PDS (0.5-3 µg/mL) (Fig. 8A) and ABTA (50-300 µg/mL) (Fig. 8B) were created. Using linear least squares regression, the peak area versus concentration data were examined. The regression equation for ABTA and PDS was y = 14730x + 121326 and y = 256275x + 37087, respectively, with regression coefficients of 0.9994 and 0.9997 (Table 5).

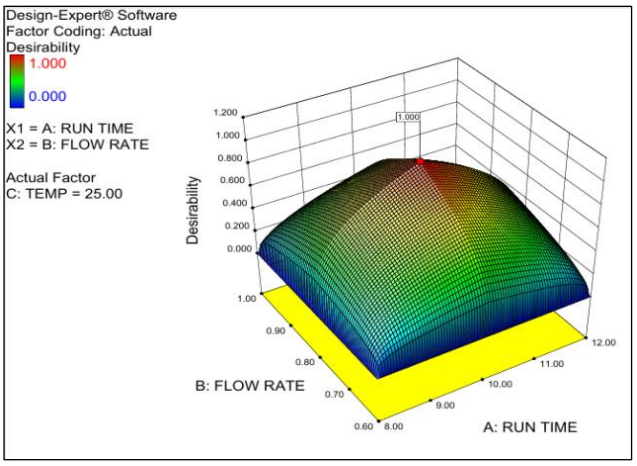
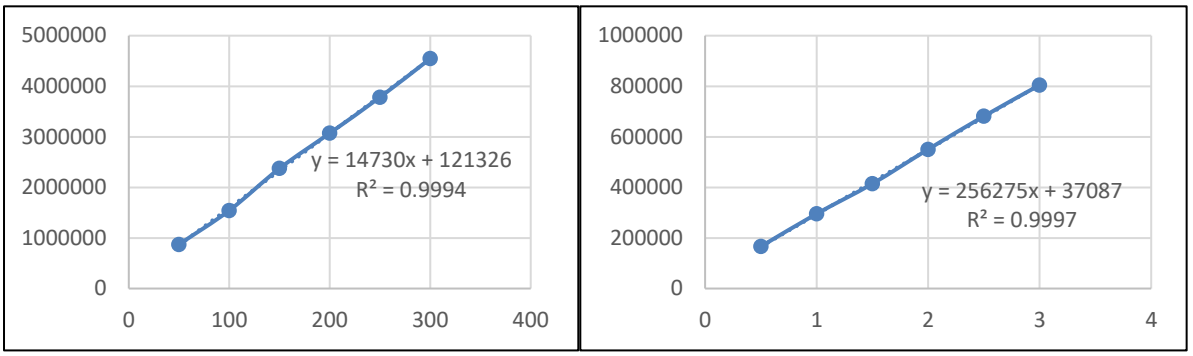


Fig. 7. 3D surface plot of desirability for optimized method



(A) (B)

Fig. 8. Linearity graph of (A) abiraterone acetate and (B) prednisolone

Table 5. Linearity of abiraterone acetate and prednisolone standard curves

Sr no.	Abiraterone acetate		Prednisolone	
	Concentration	Peak area	Concentration	Peak area
1	50	873811	0.5	166469
2	100	1543942	1.0	295722
3	150	2374943	1.5	414443
4	200	3072470	2.0	550544
5	250	3783160	2.5	681461
6	300	4546346	3.0	804767

3.2.3. Precision

The method's accuracy was evaluated by conducting interday and intraday analyses for both analytes. Six replicate measurements were conducted for each drug at concentrations of 1.5 µg/mL for ABTA and 150 µg/mL for PDS. It was discovered that both drugs had a %RSD (relative standard deviation) of less than 2% for peak area, NTP, tailing factor, and retention time (Table 6 and Table 7). This suggests that the optimized approach is precise because there is little variation in the measurements.

3.2.4. Accuracy

The optimized method's accuracy was assessed by measuring the % recovery of both ABTA and PDS at three levels: 80%, 100%, and 120% of the standard concentration. Both analytes had percentage recovery values ranging from 98-102% (Table 8 and Table 9). According to the ICH criteria, the findings show that the procedure is accurate as the recoveries are within acceptable ranges.

3.2.5. Robustness

In order to demonstrate the HPLC technique's robustness, samples were evaluated under purposefully altered chromatographic circumstances. The detection wavelength and column temperature were adjusted by ± 2% and ± 10°C, respectively, while the mobile phase flow rate was altered from 0.8 mL/min to 0.6 mL/min and 1.0 mL/min. The effects on retention time and peak parameters were investigated (Table 10 and Table 11).

Table 6. Intraday and Interday precision data for abiraterone acetate

r.no.	Concentration (ug/mL)	Intraday				Interday			
		Peak area	Theoretical plates	Tailing factor	Retention time	Peak area	Theoretical plate	Tailing factor	Retention time
1	150	2361174	10646	1.178	5.28	2338037	8540	1.313	5.277
2	150	2368803	10630	1.173	5.28	2398282	8548	1.361	5.283
3	150	2323127	10667	1.17	5.28	2356400	8504	1.328	5.277
4	150	2412322	10607	1.187	5.28	2361923	8296	1.351	5.287
5	150	2369800	10712	1.179	5.28	2324328	8319	1.311	5.287
6	150	2376013	10577	1.193	5.277	2340635	8340	1.333	5.287
Mean		2368539.83	10639.83	1.18	5.28	2353267.50	8424.50	1.33	5.28
Standard deviation		28604.07	47.13	0.01	0.01	25846.00	118.06	0.02	0.01
% RSD		1.21	0.44	0.73	0.02	1.10	1.40	1.51	0.09

Table 7. Intraday and Interday precision data for prednisolone

Sr no.	Concentration (ug/mL)	Intraday				Interday			
		Peak area	Theoretical plates	The Tailing factor	Retention time	Peak area	Theoretical plate	The Tailing factor	Retention time
1	1.5	424930	8233	1.179	3.367	424847	8116	1.22	3.36
2	1.5	424455	8277	1.19	3.363	416354	8216	1.262	3.363
3	1.5	4223875	8242	1.22	3.363	413426	8253	1.214	3.36
4	1.5	423873	8365	1.166	3.363	424083	8257	1.208	3.363
5	1.5	423641	8306	1.19	3.363	411582	8216	1.214	3.36
6	1.5	429811	8327	1.198	3.367	422259	8287	1.26	3.363
Mean		423430.83	8300	1.19	3.36	418758.50	8224.17	1.23	3.36
Standard deviation		1835.02	42.83	0.02	0.01	5716.52	59.48	0.02	0.01
% RSD		0.43	0.52	1.53	0.06	1.37	0.72	1.91	0.05

Table 8. Accuracy data for Abiraterone acetate

Level	Tablet powder solution added(ug/mL)	Standard stock solution added(ug/mL)	Total amount (ug/mL)	Peak area	Calculated concentration	%Recovery	Standard deviation	%RSD
80%	100	80	180	2771039	179.88	99.35%	0.94	0.52
	100	80	180	2751324	178.54			
	100	80	180	2744211	178.06			
100%	100	100	200	3068937	200.10	99.70%	0.60	0.30
	100	100	200	3053407	199.05			
	100	100	200	3053483	199.06			
120%	100	120	220	3347432	219.01	100.10%	1.49	0.67
	100	120	220	3358157	219.74			
	100	120	220	3389734	221.88			

Table 9. Accuracy data for prednisolone

Level	Tablet powder solution added(ug/mL)	Standard stock solution added(ug/mL)	Total amount (ug/mL)	Peak area	Calculated concentration	%Recovery	Standard deviation	%RSD
80%	1	0.8	1.8	490678	17.69	99.59%	0.22	1.24
	1	0.8	1.8	502134	18.14			
	1	0.8	1.8	496633	17.93			
100%	1	1	2	546050	19.86	100.19%	0.16	0.80
	1	1	2	551656	20.07			
	1	1	2	554149	20.17			
120%	1	1.2	2.2	606762	22.22	99.47%	0.30	1
	1	1.2	2.2	591832	21.64			
	1	1.2	2.2	595034	21.77			

3.2.6. LOD and LOQ

For PDS, the limit of detection was 0.12 ug/mL, whereas for ABTA, it was 18.47 ug/mL. PDS had a limit of quantification of 0.37 ug/mL, while ABTA had a limit of quantification of 55.97 ug/mL.

3.2.7. Assay

When the test was done on tablets, the optimized chromatogram of ABTA and PDS revealed a retention time of 5.297 min for abiraterone acetate and 3.363 min for prednisolone. The % purity of ABTA was discovered to be 99.11%, and PDS was discovered to be 99.16% for tablet label claims. The assay findings showed that the technique could identify medications even when there were excipients present in tablet powder.

Table 10. Robustness study of abiraterone acetate

Flow rate	Flow minus (0.6 mL/min)			Flow plus (1.0 mL/min)		
Sr no.	Peak area	Retention time	Theoretical plates	Peak area	Retention time	Theoretical plates
1	3177672	6.92	8718	1906655	4.31	7367
2	3135488	6.92	8782	1916125	4.31	7178
3	3163112	6.91	8879	1914292	4.32	7190
Mean	3158757	6.92	8793	1912357	4.31	7245
Standard deviation	21426.50	0.01	81.06	5022.69	0.01	105.83
%RSD	0.68	0.08	0.92	0.26	0.13	1.46
Temperature	Temperature minus(20°C)			Temperature plus(30°C)		
Sr no.	Peak area	Retention time	Theoretical plate	Peak area	Retention time	Theoretical plates
1	2370669	5.52	7424	2415362	5.19	7168
2	2436231	5.52	7323	2409754	5.19	7176
3	2403975	5.53	7248	2406362	5.11	7139
Mean	2403625	5.52	7332	2410493	5.16	7161
Standard deviation	32782.40	0.01	88.32	4545.24	0.05	19.47
%RSD	1.36	0.10	1.20	0.19	0.89	0.27
Wavelength	Wavelength minus (258 nm)			Wavelength plus(262nm)		
Sr no.	Peak area	Retention time	Theoretical plate	Peak area	Retention time	Theoretical plate
1	1892989	4.32	7047	2150056	4.33	6887
2	1931950	4.32	6983	2135002	4.32	6795
3	1931942	4.33	6863	2159653	4.00	6847
Mean	1918960	4.32	6964	2148237	4.21	6843
Standard deviation	22491.83	0.01	93.41	12425.76	0.07	46.13
%RSD	1.17	0.13	1.34	0.58	1.60	0.67

Table 11. Robustness study of prednisolone

Flow rate	Flow minus (0.6 mL/min)			Flow plus (1.0 mL/min)		
Sr no.	Peak area	Retention time	Theoretical plates	Peak area	Retention time	Theoretical plates
1	588002	4.45	9591	347493	2.70	6476
2	590207	4.45	9573	343962	2.71	6442
3	594111	4.46	9641	349113	2.70	6437
Mean	590773	4.45	9602	346856	2.70	6452
Standard deviation	3093.63	0.01	35.23	2633.92	0.01	21.22
%RSD	0.52	0.13	0.37	0.76	0.21	0.33
Temperature	Temperature minus(20°C)			Temperature plus(30°C)		
Sr no.	Peak area	Retention time	Theoretical plate	Peak area	Retention time	Theoretical plates
1	432667	3.387	7974	432243	3.347	8024
2	436514	3.386	7932	435297	3.347	7994
3	442087	3.387	7815	438461	3.337	7903
Mean	437089	3.387	7907	435334	3.347	7974
Standard deviation	4736.28	0.00	82.40	3109.16	0.01	63.01
%RSD	1.08	0.02	1.04	0.71	0.17	0.79
Wavelength	Wavelength minus (258 nm)			Wavelength plus(262nm)		
Sr no.	Peak area	Retention time	Theoretical plate	Peak area	Retention time	Theoretical plate
1	343813	2.703	6555	382279	2.703	6475
2	346983	2.704	6529	376671	2.703	6469
3	349448	2.707	6498	389832	3.000	6423
Mean	346748	2.704	6527	382927	2.802	6465
Standard deviation	2824.84	0.00	28.54	6604.41	0.01	37.58
%RSD	0.81	0.08	0.44	1.72	0.28	0.58

3.3. Greenness assessment

The green analytical method's index assesses the feasibility of the various processes involved in the specified method. It takes into account sample preparation, handling, chemicals consumed, and instrumentation. The assessment uses a color-coding system, where a little environmental effect is denoted by green, a moderate impact by yellow, and a large impact by red. The HPLC technology employed demonstrated environmentally friendly attributes, with all parameters tested falling within the green zones, as illustrated in Fig. 9 [25,26]. Furthermore, the AGREE tool was utilized to determine the environmental friendliness profile of the analytical procedures using numerical values. The result obtained was 0.83, as shown in Fig. 10, confirming the outstanding green attributes of the developed HPLC method. Based on the concepts of Green Analytical Chemistry (GAC), the evaluation parameter is a 0–1 scale. Scores closer to 0 signify that the method poses a greater environmental risk and is more hazardous, while scores closer to 1 indicate a greener method with a minimal impact on both the system and the analyst [27].

The Blue Applicability Grade Index (BAGI) assesses the feasibility of 10 key components of an analytical process. Fig. 11 displays the outcomes of the suggested approach carried out using BAGI. The evaluated approach received a high score of 82.5, over 60, demonstrating its applicability [27,28].

The obtained HPLC-EAT scores depicted in Fig. 12 for the proposed method were found to be a 152.046 safety impact, a 24.058 health impact, and an environmental impact of 25.344. These scores offer an overview of the proposed HPLC method's safety, health, and environmental effect suggesting that the method used is more favorable and has a lesser environmental footprint.

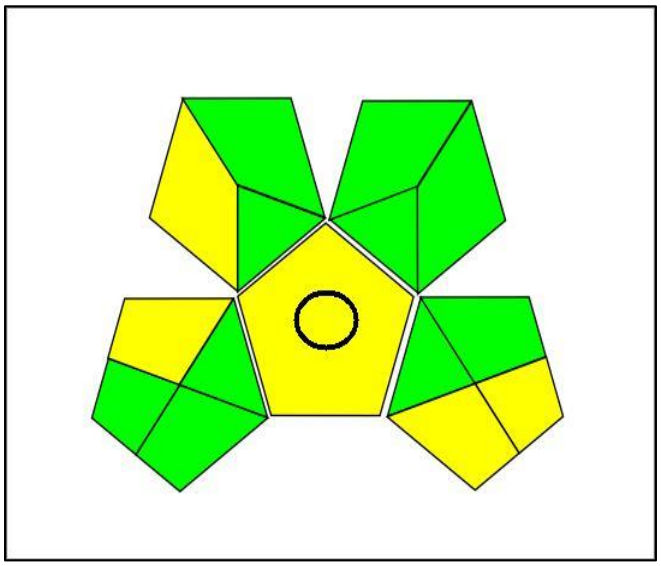


Fig. 9. Greenness evaluation by GAPI.

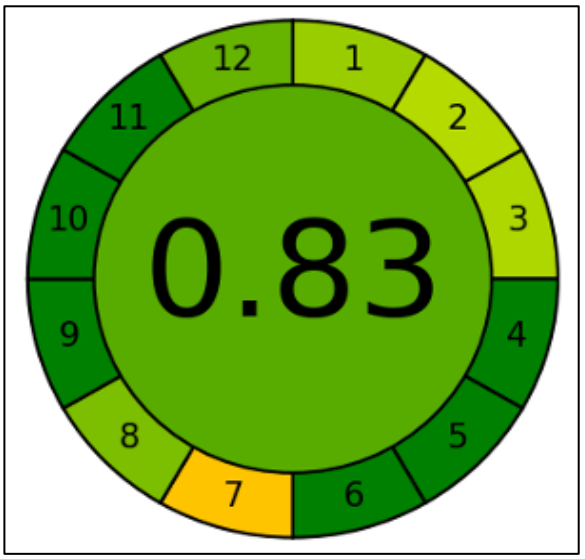


Fig. 10. Greenness evaluation by AGREE

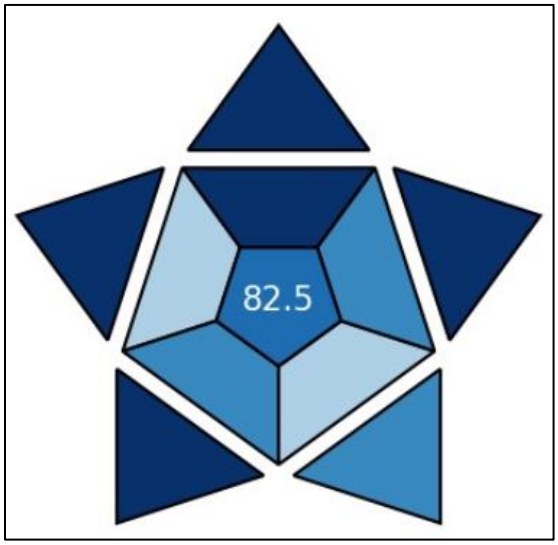


Fig. 11. Greenness evaluation by BAGI

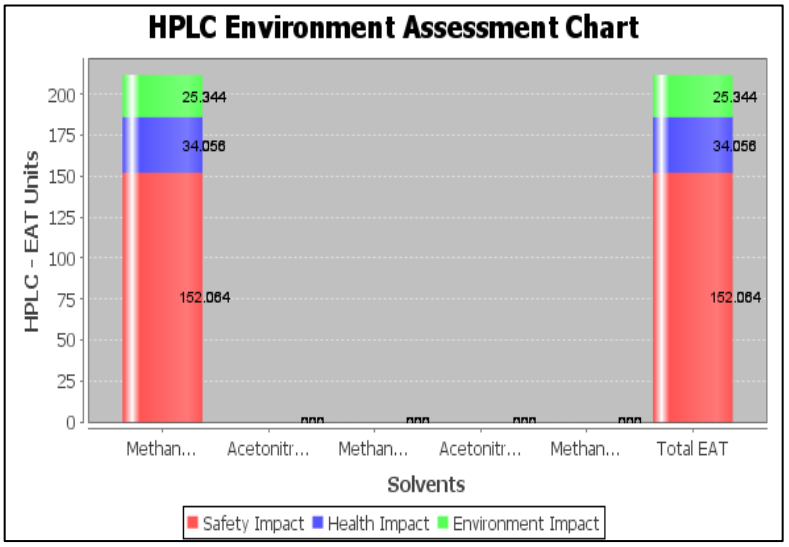


Fig. 12. Greenness evaluation by HPLC-EAT

4. Conclusion

The current study successfully used a Quality by Design (QbD) methodology to design and verify a new RP-HPLC technique for the simultaneous measurement of prednisolone (PDS) and abiraterone acetate (ABTA). By using the Central Composite Design (CCD), it was possible to optimize crucial parameters, including run duration, flow rate, and column temperature, resulting in a robust and effective separation in just eight minutes. The method demonstrated excellent resolution, minimal peak tailing, and high reproducibility. Validation results confirmed compliance with ICH guidelines, ensuring precision, accuracy, linearity, and robustness. The integration of QbD principles allowed a systematic risk assessment and ensured the versatility of the approach for regular pharmaceutical quality control. Additionally, green analytical chemistry principles were incorporated to enhance sustainability. Evaluations using GAPI, AGREE, HPLC-EAT, and BAGI tools highlighted the method's eco-friendly profile, with reduced solvent consumption and minimal environmental impact. This integration of QbD and green principles supports both analytical performance and environmental stewardship, making the method a valuable tool for sustainable pharmaceutical practices.

Author Contributions: Conceptualization: P.J.; Data curation: P.J., B.M., P.G.; Formal analysis: P.J., P.C.; Investigation: R.J., P.J.; Methodology: P.J., B.M., P.G.; Project administration: P.J., P.C., A.P., P.G.; Resources: P.J., P.C., B.M.; Supervision: A.J., P.J.; Validation: A.J., R.J., P.J.; Visualization: P.J., B.M.; Writing–original draft: P.J., P.C., P.G.; Writing–review & editing: P.J., B.M.

Funding: None to declare.

Acknowledgments: I would like to express my sincere gratitude to our principal, Dr. Ashish Jain, for providing the necessary resources and support to carry out this research. Your encouragement has been instrumental in my academic journey. I am deeply indebted to my guide, Dr. Reshma Jadhav, for their expert guidance, invaluable insights, and continuous support throughout the project. Your mentorship has greatly contributed to the success of this research. I also wish to sincerely thank Dr. Bhushan Rane, PG Incharge, for his valuable cooperation and guidance, which greatly helped in the successful completion of this work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jena, B. R.; Panda, S. P.; Kulandaivelu, U.; Alavala, R. R.; Rao, G. S. N. K.; Swain, S.; Pattnaik, G.; Ghose, D. AQbD Driven Development of an RP-HPLC Method for the Quantitation of Abiraterone Acetate for Its Pharmaceutical Formulations in the Presence of Degradants. *Turk. J. Pharm. Sci.* **2021**, *18* (6), 718–729. DOI: 10.4274/tjps.galenos.2021.74150
2. Bashar, T.; Apu, M. N. H.; Mostaid, M. S.; Islam, M. S.; Hasnat, A. Pharmacokinetics and Bioavailability Study of a Prednisolone Tablet as a Single Oral Dose in Bangladeshi Healthy Volunteers. *Dose Response* **2018**, *16* (3), Art. No: 1559325818783932. DOI: 10.1177/1559325818783932
3. Attard, G.; Murphy, L.; Clarke, N. W.; Cross, W.; Jones, R. J.; Parker, C. C.; Gillessen, S.; Cook, A.; Brawley, C.; Amos, C. L.; Atako, N.; Pugh, C.; Buckner, M.; Chowdhury, S.; Malik, Z.; Russell, J. M.; Gilson, C.; Rush, H.; Bowen, J.; Lydon, A.; Pedley, I.; O'Sullivan, J. M.; Birtle, A.; Gale, J.; Srihari, N.; Thomas, C.; Tanguay, J.; Wagstaff, J.; Das, P.; Gray, E.; Alzoueb, M.; Parikh, O.; Robinson, A.; Syndikus, I.; Wylie, J.; Zarkar, A.; Thalmann, G.; de Bono, J. S.; Dearnaley, D. P.; Mason, M. D.; Gilbert, D.; Langley, R. E.; Millman, R.; Matheson, D.; Sydes, M. R.; Brown, L. C.; Parmar, M. K. B.; James, N. D.; Systemic Therapy in Advancing or Metastatic Prostate cancer: Evaluation of Drug Efficacy (STAMPEDE) investigators. Abiraterone Acetate and Prednisolone with or without Enzalutamide for High-Risk Non-Metastatic Prostate Cancer: A Meta-Analysis of Primary Results from Two Randomised Controlled Phase 3 Trials of the STAMPEDE Platform Protocol. *Lancet* **2022**, *399*(*10323*), 447–460. DOI: 10.1016/S0140-6736(21)02437-5
4. Chandra Reddy, B. J.; Sarada, N. C. Development and Validation of a Novel RP-HPLC Method for Stability-Indicating Assay of Abiraterone Acetate. *J. Liq. Chromatogr. Relat. Technol.* **2016**, *39* (7), 354–363. DOI: 10.1080/10826076.2016.1163500
5. Beg, S.; Malik, A. K.; Afzal, O.; Altamimi, A. S. A.; Kazmi, I.; Al-Abbasi, F. A.; Almalki, W. H.; Barkat, M. A.; Kawish, S. M.; Pradhan, D. P.; Rahman, M. Systematic Development and Validation of a RP-HPLC Method for Estimation of Abiraterone Acetate and Its Degradation Products. *J. Chromatogr. Sci.* **2021**, *59* (1), 79–87. DOI: 10.1093/chromsci/bmaa080
6. Sankar, P. R.; Eswarudu, M. M.; Siva, P.; Viswanath, A. Optimized and Validated RP-HPLC Method for Quantification of Abıraterone Acetate (an Antı-Prostate Cancer Drug) in Pharmaceutıcal Dosage Form. *High Technol. Lett.* **2021**, *27* (*7*), 21-32.
7. Ashok, R.; Prakash, P. P.; Selvan, T. Development and Validation of Analytical Method for Estimation of Prednisolone in Bulk and Tablets Using UV-Visible Spectroscopy. *Int. J. Pharm. Pharm. Sci.* **2011**, *3* (4), 184–186.
8. Optimization and Validation of Spectrophotometric Methods for the Determination of Prednisolone in Chitosan Nanoparticles. *J. Hunan Univ. Nat. Sci.* **2024**, *51* (*4*), 83-91. DOI: 10.55463/issn.1674-2974.51.4.10.
9. Ghosh, S.; Kumar, D. S.; Sahu, A.; Banji, D.; Harani, A.; Chaitanya, C. K.; Kalapana, M. Development and Validation for Prednisolone in Tablet Dosage Form by Reverse Phase-HPLC. *Asian J. Chem.* **2011**, *23* (*11*), 5092–5094.
10. Razzaq, S. N.; Khan, I. U.; Mariam, I.; Razzaq, S. S. Stability Indicating HPLC Method for the Simultaneous Determination of Moxifloxacin and Prednisolone in Pharmaceutical Formulations. *Chem. Cent. J.* **2012**,

6 (1), Art. No: 94. DOI: 10.1186/1752-153X-6-94

11. S. Chitlange, S.; K. Chaturvedi, K.; B. Wankhede, S. Development and Validation of Spectrophotometric and HPLC Method for the Simultaneous Estimation of Salbutamol Sulphate and Prednisolone in Tablet Dosage Form. *J. Anal. Bioanal. Tech.* **2011**, *2*, Art. No: 19. DOI: 10.4172/2155-9872.1000117

12. Potnuri, N. R.; Rao, D. G.; Prasad, R. Y. A Novel Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Moxifloxacin and Prednisolone in Bulk and Their Combined Dosage Form. *Int. J. Pharm. Sci. Res.* **2015**, *6* (5), 1965-1973. DOI: 10.13040/IJPSR.0975-8232.6(5).1965-73

13. Solanki, T. D.; Patel, S. K.; Patel, B. K. Development and Validation of Stability Indicating Method for Simultaneous Estimation of Ofloxacin and Prednisolone in Pharmaceutical Dosage Form. *Pharm. Biol. Eval.* **2016**, *3*, 256–263.

14. Saad, F.; Efstathiou, E.; Attard, G.; Flaig, T. W.; Franke, F.; Goodman, O. B., Jr; Oudard, S.; Steuber, T.; Suzuki, H.; Wu, D.; Yeruva, K.; De Porre, P.; Brookman-May, S.; Li, S.; Li, J.; Thomas, S.; Bevans, K. B.; Mundle, S. D.; McCarthy, S. A.; Rathkopf, D. E.; ACIS Investigators. Apalutamide plus Abiraterone Acetate and Prednisone versus Placebo plus Abiraterone and Prednisone in Metastatic, Castration-Resistant Prostate Cancer (ACIS): A Randomised, Placebo-Controlled, Double-Blind, Multinational, Phase 3 Study. *Lancet Oncol.* **2021**, *22* (11), 1541–1559. DOI: 10.1016/S1470-2045(21)00402-2

15. Dewani, A. P.; Vekariya, H. J. Optimization of High-Performance Liquid Chromatographic Method Using Central Composite Design for the Simultaneous Estimation of Ciprofloxacin and Loteprednol. *J. Chromatogr. Sci.* **2023**, *61* (3), 279–287. DOI: 10.1093/chromsci/bmac016

16. Kumar, N.; D, S. Analytical Method Development by Using QbD - an Emerging Approach for Robust Analytical Method Development. *J. Pharm. Sci. Res.* **2020**, *12(10),* 1298–1305.

17. Holm, P.; Allesø, M.; Bryder, M. C.; Holm, R. Q8(R2): Pharmaceutical Development. In *ICH Quality Guidelines*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, **2017**; pp 535–577.

18. Shamim, A.; Ansari, M. J.; Aodah, A.; Iqbal, M.; Aqil, M.; Mirza, M. A.; Iqbal, Z.; Ali, A. QbD-Engineered Development and Validation of a RP-HPLC Method for Simultaneous Estimation of Rutin and Ciprofloxacin HCl in Bilosomal Nanoformulation. *ACS Omega* **2023**, *8* (24), 21618–21627. DOI: 10.1021/acsomega.3c00956

19. Patel, K. Y.; Dedania, Z. R.; Dedania, R. R.; Patel, U. QbD Approach to HPLC Method Development and Validation of Ceftriaxone Sodium. *Futur. J. Pharm. Sci.* **2021**, *7*, Art. No: 141. DOI: 10.1186/s43094-021-00286-4

20. The Application of Quality by Design to Analytical Methods. *Pharmaceutical Technology* **2007**, *31(10).* https://www.pharmtech.com/view/application-quality-design-analytical-methods (Accessed on 24-11-2024).

21. Borman, P.; Elder, D. Q2(R1) Validation of Analytical Procedures: Text and Methodology. In *ICH Quality Guidelines*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, **2017**; pp 127–166.

22. Nakov, N.; Acevska, J.; Brezovska, K.; Kavrakovski, Z.; Dimitrovska, A.. Green Strategies toward Eco-Friendly HPLC Methods in Pharma Analysis. High Performance Liquid Chromatography - Recent Advances and Applications **2023**. DOI: 10.5772/intechopen.110035

23. Płotka-Wasylka, J. A New Tool for the Evaluation of the Analytical Procedure: Green Analytical Procedure Index. *Talanta* **2018**, *181*, 204–209. DOI: 10.1016/j.talanta.2018.01.013

24. Manousi, N.; Wojnowski, W.; Płotka-Wasylka, J.; Samanidou, V. Blue Applicability Grade Index (BAGI) and Software: A New Tool for the Evaluation of Method Practicality. *Green Chem.* **2023**, *25 (19),* 7598 –7604. DOI: 10.1039/d3gc02347h.

25. Fares, M. Y.; Hegazy, M. A.; El-Sayed, G. M.; Abdelrahman, M. M.; Abdelwahab, N. S. Quality by Design Approach for Green HPLC Method Development for Simultaneous Analysis of Two Thalassemia Drugs in Biological Fluid with Pharmacokinetic Study. *RSC Adv.* **2022**, *12 (22),* 13896–13916. DOI: 10.1039/d2ra00966h

26. Imam, M. S.; Batubara, A. S.; Gamal, M.; Abdelazim, A. H.; Almrasy, A. A.; Ramzy, S. Adjusted Green HPLC Determination of Nirmatrelvir and Ritonavir in the New FDA Approved Co-Packaged Pharmaceutical Dosage Using Supported Computational Calculations. *Sci. Rep.* **2023**, *13 (1),* Art. No: 137. DOI: 10.1038/s41598-022-26944-y

27. Pena-Pereira, F.; Wojnowski, W.; Tobiszewski, M. AGREE-Analytical GREEnness Metric Approach and Software. *Anal. Chem.* **2020**, *92 (14),* 10076–10082. DOI: 10.1021/acs.analchem.0c01887

28. Wejnerowska, G.; Narloch, I. A. Comprehensive Assessment of Sample Preparation Methods for the Determination of UV Filters in Water by Gas Chromatography–Mass Spectrometry: Greenness, Blueness, and Whiteness Quantification Using the AGREEprep, BAGI, and RGB 12 Tools. *Applied Sciences* **2024**, *14(17),* 7690–7690. DOI: 10.3390/app14177690