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Yurii Ihnatsevych

I. Horbachevsky Ternopil National Medical University

Marjan Piponski

Galafarm

Oleksandra Oleshchuk

I. Horbachevsky Ternopil National Medical University

Mamdouh R. Rezk

Cairo University

Marta Sulyma

Danylo Halytsky Lviv National Medical University

Andrii Sverstyuk

I. Horbachevsky Ternopil National Medical University

Mariana Horyn

I. Horbachevsky Ternopil National Medical University

Liubomyr Kryskiw

I. Horbachevsky Ternopil National Medical University

Liliya Logoyda

`llogoyda@tdmu.edu.ua`

I. Horbachevsky Ternopil National Medical University

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A Chaotropic Agent-Assisted, AQbD-Guided RP-HPLC Method for the Simultaneous Determination of Rosuvastatin, Atorvastatin, and Ezetimibe: A Rapid, Robust, and Green Approach

¹Yurii Ihnatsevykh, ²Marjan Piponski, ³Oleksandra Oleshchuk, ⁴Mamdouh R. Rezk, ⁵Marta Sulyma, ⁶Andrii Sverstyuk, ¹Mariana Horyn, ¹Liubomyr Kryskiw, ^{1*}Liliya Logoyda

¹Department of Pharmaceutical Chemistry, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

²Quality Control Sector, Galafarm Skopje, st.51 No23, 1000 Skopje, North Macedonia

³Department of Pharmacology with Clinical Pharmacology, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

⁴Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt

⁵Department of General, Medical and Toxicological Chemistry, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

⁶Department of Medical Informatics, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

* Correspondence: Liliya Logoyda

logojda@tdmu.edu.ua

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Abstract

The simultaneous determination of rosuvastatin (ROS), atorvastatin (ATOR), and ezetimibe (EZET) in pharmaceutical formulations is challenging due to their distinct physicochemical properties. This study details the development and validation of a rapid, robust, and stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method, guided by Analytical Quality by Design (AQbD), with a focus on method robustness and environmental sustainability. Critical method parameters (CMPs)—potassium hexafluorophosphate (KPF₆) concentration, column temperature, and acetonitrile (ACN) fraction—were systematically optimized using a Box-Behnken design (BBD) and response surface methodology (RSM). The optimized conditions comprised a 0.2 mM KPF₆ aqueous buffer, a column temperature of 48 °C, and a 60% ACN mobile phase. Under these conditions, efficient separation of ROS, ATOR, and EZET was achieved, with a retention time of 2.29 min for the most lipophilic analyte, ATOR. The method demonstrated excellent linearity ($r^2 \geq 0.996$) over 12.0–50.0 µg/mL, high accuracy (98–102% recovery), and precision (%RSD < 2%). Greenness and sustainability were quantitatively confirmed using AGREE (0.61), MoGAPI (75), AGSA (63.89), CACI (70), CaFRI (72), and BAGI (80.0) metrics. This AQbD-guided RP-HPLC method is rapid, reliable, robust, and environmentally favorable, making it highly suitable for routine quality control of pharmaceutical formulations containing ROS, ATOR, and EZET.

1. INTRODUCTION

Cardiovascular diseases (CVDs) remain the leading cause of mortality and disability worldwide, resulting in a substantial medical, social, and economic burden. According to the *Global Burden of Disease Study 2023*, CVDs accounted for approximately 19.2 million deaths in 2023, while their global burden amounted to 437 million disability-adjusted life years (DALYs) [1]. Projections regarding the prevalence and consequences of CVDs over the coming decades remain unfavorable. According to scientific estimates, the global burden of cardiovascular diseases is expected to increase substantially by 2050 [2]. Elevated levels of low-density lipoprotein cholesterol (LDL-C) represent one of the key modifiable risk factors for the development of atherosclerotic cardiovascular diseases. Therefore, the effective reduction of LDL-C levels is an essential component of both primary and secondary prevention of CVDs and constitutes one of the priority areas of modern pharmacotherapy. The use of combination lipid-lowering therapy, particularly the combination of statins—rosuvastatin (ROS) or atorvastatin (ATOR)—with ezetimibe (EZET), is becoming increasingly important. Owing to their different yet complementary mechanisms of action, these medicinal products provide a more pronounced reduction in LDL-C levels compared with statin monotherapy. The use of such combinations makes it possible to achieve target lipid profile parameters while using lower statin doses, potentially improving treatment tolerability and enhancing patient adherence to therapy. The clinical efficacy and appropriateness of combining ROS or ATOR with EZET have been confirmed by the results of recent clinical studies and meta-analyses [3–6]. At the same time, the quality and safety of combination medicinal products and individualized treatment regimens largely depend on the availability of reliable, rapid, and accurate methods for their pharmaceutical analysis. The simultaneous chromatographic determination of ROS, ATOR and EZET within a single analytical procedure represents a considerable challenge for reversed-phase high-performance liquid chromatography (RP-HPLC). The main analytical challenge arises from substantial differences in the physicochemical properties of these molecules, including their high lipophilicity, distinct ionization behavior, and propensity for secondary electrostatic interactions with the residual silanol groups of the stationary phase. These factors often result in peak tailing and inadequate resolution of critical pairs of compounds. An analysis of the current literature indicates that most existing official and pharmacopoeial methods have been developed only for single-component products or binary mixtures, such as ROS/EZET or ATOR/EZET [7–9]. The limited number of attempts to simultaneously determine a broader range of lipid-lowering agents often relies on complex gradient elution programs, prolonged analysis times, or the use of toxic organic solvents, which is inconsistent with the principles of green chemistry [10–13]. Moreover, most of these methods have been developed using the traditional empirical “one factor at a time” (OFAT) approach. This approach focuses on optimizing only one parameter while keeping all other factors constant; therefore, it is inherently incapable of identifying multifactorial interactions among chromatographic variables and does not ensure the long-term robustness of the analytical method under minor variations in analytical conditions [14]. A modern alternative, recommended by leading regulatory authorities, including the International Council for Harmonisation (ICH), the U.S. Food and Drug Administration (FDA), and the European Medicines Agency (EMA), is the concept of Analytical Quality by Design (AQbD) [15–17]. This concept involves a systematic approach that begins with clearly defined analytical objectives and is based on risk management and the application of Design of Experiments (DoE). The use of DoE, particularly the Box–Behnken design (BBD), makes it possible to mathematically characterize the multidimensional design space, thereby ensuring a high degree of method robustness. This study aimed to develop and validate a rapid, sensitive, and eco-friendly RP-HPLC method for simultaneous quantitation of ROS, ATOR, and EZET using an AQbD approach. To address peak asymmetry and optimize separation, the synergistic effects of the chaotropic agent potassium hexafluorophosphate (KPF₆), column temperature, and acetonitrile (ACN) fraction were systematically investigated for the first time in this context.

2. MATERIALS AND METHODS

2.1 INSTRUMENTALS AND CHEMICALS

RP-HPLC analyses were carried out using a Shimadzu LC-2050 C 3D system equipped with a diode-array detector (DAD). Chromatographic data were integrated using LabSolutions software. An ultrasonic bath (Elmasonic Easy 40 H, Germany) was employed for mobile-phase degassing and sample dissolution. Accurate weighing of reagents and samples was performed using RADWAG AS 200/C analytical balances. Used chromatographic columns: Luna C18 (100 × 4.6 mm 3 µm) purchased from Phenomenex and Zorbax SB C18 (150 × 4.6 mm 3.5 µm) purchased from Agilent Technologies. ROS, ATOR and EZET (purity ≥ 98% (HPLC)) were supplied from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). ROS, ATOR and EZET tablets were purchased from a local pharmacy.

HPLC-grade ACN, methanol (MeOH) and water (≥ 99.9% purity) were acquired from Honeywell, Riedel-de Haen (Germany). Potassium hexafluorophosphate (KPF₆), potassium dihydrogen phosphate (KH₂PO₄), and formic acid (HPLC grade) used for buffer preparation were purchased from Honeywell Fluka. A filter regenerated cellulose (RC) The test solutions were filtered through a 0.45 µm membrane filter (Phoenix, Germany). All reagents and chemicals used throughout the study were of analytical grade.

2.2 SAMPLE PREPARATION

2.2.1 Preparation of Stock Standard Solution

A stock standard solution containing ROS, ATOR and EZET was prepared by accurately weighing 25 mg of each analyte into a 25 mL volumetric flask. Approximately 15 mL of MeOH was added, and the mixture was sonicated until complete dissolution of all components. The solution was then diluted to the mark with MeOH and mixed thoroughly.

2.2.2 Preparation of working standard solutions

A mixed stock solution containing 1 mg/mL of ROS, ATOR, and EZET was prepared by accurately weighing 25 mg of each reference standard into a 25 mL volumetric flask, dissolving in ~15 mL of MeOH by sonication, and diluting to volume with MeOH. Working standard solutions were prepared by appropriate dilution of this stock solution.

2.3 CHROMATOGRAPHIC CONDITIONS

Chromatographic separation was carried out under isocratic elution using a Luna C18 column (100 × 4.6 mm, 3 µm) at 48 ± 2 °C. The mobile phase comprised ACN and an aqueous buffer containing 0.1% KH₂PO₄, 0.1% HClO₄, and 0.2% KPF₆ mixed in a 60:40 (v/v) ratio. Before analysis, the mobile phase was degassed by ultrasonic treatment. The chromatographic system operated at a flow rate of 1.0 mL/min, with an injection volume of 3 µL. Detection was performed using a UV detector at 240 nm, and the total chromatographic run time was 4.0 min. Prior to sample analysis, the column was conditioned with the mobile phase for 30 min to ensure a stable baseline.

3. RESULTS AND DISCUSSION

3.1 PRELIMINARY STUDIES AND SELECTION OF CRITICAL METHOD PARAMETERS

The simultaneous chromatographic quantitative determination of highly lipophilic and structurally complex multicomponent pharmaceutical products represents a significant analytical challenge. Conventional analytical methods, HPLC, often require separate analytical procedures for each analyte, resulting in increased analysis time and excessive consumption of solvents and materials. Furthermore, differences in the chemical properties of the analytes, including polarity and solubility, complicate their separation and identification and require careful optimization of the mobile-phase

composition [18]. To establish a reliable analytical profile, a comprehensive preliminary screening of the chromatographic conditions was performed. The incorporation of appropriate solvents, buffers, and additives into the mobile phase is critically important for controlling retention behavior in reversed-phase liquid chromatography [19]. Therefore, the concentration of the chaotropic salt KPF₆ was selected as the first critical method parameter (CMP). The addition of such modifiers to the mobile phase represents a fundamental concept in modern liquid chromatography for controlling secondary interactions between analytes and the stationary phase, which commonly lead to peak asymmetry [20]. Preliminary experiments demonstrated that variations in the KPF₆ concentration within a specific analytical range significantly affected the overall column efficiency. Column temperature was identified as the second critical method parameter. Temperature is a key variable in high-performance liquid chromatography, governing the thermodynamics of separation and directly affecting mobile-phase viscosity and the mass-transfer kinetics of analytes within the chromatographic column [21]. Operating at optimally elevated temperatures can substantially improve peak separation and enhance overall chromatographic system efficiency. The volume fraction of the organic modifier, ACN, was evaluated as the third critical method parameter. The use of aqueous–ACN mobile phases significantly affects the retention, selectivity, and ionization behavior of compounds in HPLC [22]. According to retention modeling principles, the proportion of the organic solvent is a major factor controlling the overall analysis time. Because the physicochemical interactions among the chaotropic salt, column temperature, and organic modifier are highly complex, the conventional empirical “one factor at a time” (OFAT) optimization approach is insufficient. The application of Quality by Design (QbD) principles represents a modern systematic approach recommended by regulatory authorities, particularly within the ICH Q8–Q10 guidelines, for developing robust analytical methods capable of withstanding variations in experimental conditions [15]. The integration of Design of Experiments (DoE) methodology is essential for systematic pharmaceutical development and the comprehensive evaluation of multifactorial interactions [23]. A multivariate experimental design approach was therefore employed to efficiently map the design space and provide greater regulatory flexibility [24]. Preliminary experiments are presented in **Figure 1S-13S**.

3.2 STATISTICAL ANALYSIS OF THE BOX-BEHNKEN DESIGN AND DEVELOPMENT OF COMPREHENSIVE MATHEMATICAL MODELS

To establish a mathematically justified relationship between the identified critical method parameters (CMPs) and critical analytical attributes (CAAs), an experimental design based on the Box–Behnken design (BBD) was implemented (**Table 1**). The experimental matrix consisted of 15 experimental runs performed in a randomized order to minimize the influence of uncontrolled external factors. The design included 12 factorial points located at the midpoints of the edges of the three-dimensional factor space and three replicate center points. The inclusion of center points is an essential component of response surface methodology, as they enable an independent estimation of pure experimental error and allow the overall stability of the chromatographic system to be assessed throughout the analytical sequence [23, 25].

Table 1. Box–Behnken Design (BBD) Experimental Matrix and the Obtained Chromatographic System Responses

StdOrder	RunOrder	PtType	Blocks	cKPF ₆ , mM	t, °C	ACN, %	NTP	Rs	Tf	Rt
5	1	2	1	0,2	45	50	16819	10,803	1,068	7,43
8	2	2	1	0,5	45	60	17952	16,388	1,087	10,41
11	3	2	1	0,35	42	60	13772	5,297	1,203	3,85
14	4	0	1	0,35	45	55	13657	7,508	1,134	4,95
10	5	2	1	0,35	48	50	10926	9,181	1,127	6,83

1	6	2	1	0,2	42	55	12155	6,809	1,154	4,97
4	7	2	1	0,5	48	55	20693	12,474	1,059	14,33
2	8	2	1	0,5	42	55	13285	7,473	1,146	5,02
12	9	2	1	0,35	48	60	10515	4,996	1,21	3,57
15	10	0	1	0,35	45	55	13657	7,508	1,134	4,95
7	11	2	1	0,2	45	60	14059	5,444	1,205	3,8
13	12	0	1	0,35	45	55	13657	7,508	1,134	4,95
3	13	2	1	0,2	48	55	13802	7,484	1,135	4,9
6	14	2	1	0,5	45	50	417	1,137	2,000	12,11
9	15	2	1	0,35	42	50	16527	10,725	1,063	7,5

Full quadratic polynomial models were applied to identify and characterize the relationships among the investigated variables. The use of these models makes it possible to capture the multidimensional complexity of the chromatographic system, which is critically important within QbD framework. Their mathematical structure incorporates linear main effects, two-factor interaction effects, and nonlinear quadratic curvature. This approach enables an accurate mathematical representation of the complex physicochemical interactions among the chaotropic salt concentration, column temperature, and volume fraction of the organic modifier [26]. The statistical reliability and predictive performance of the obtained quadratic models were thoroughly evaluated using analysis of variance (ANOVA). The regression models developed for the most important chromatographic responses, including the overall analysis time (R_t) and the resolution of the critical peak pair (R_s), demonstrated high statistical significance, with overall p-values below 0.05 (**Figure 1**). These findings indicate that the developed equations adequately describe the underlying mass-transfer processes occurring within the chromatographic column and that the observed relationships are unlikely to be attributable to experimental noise [27].

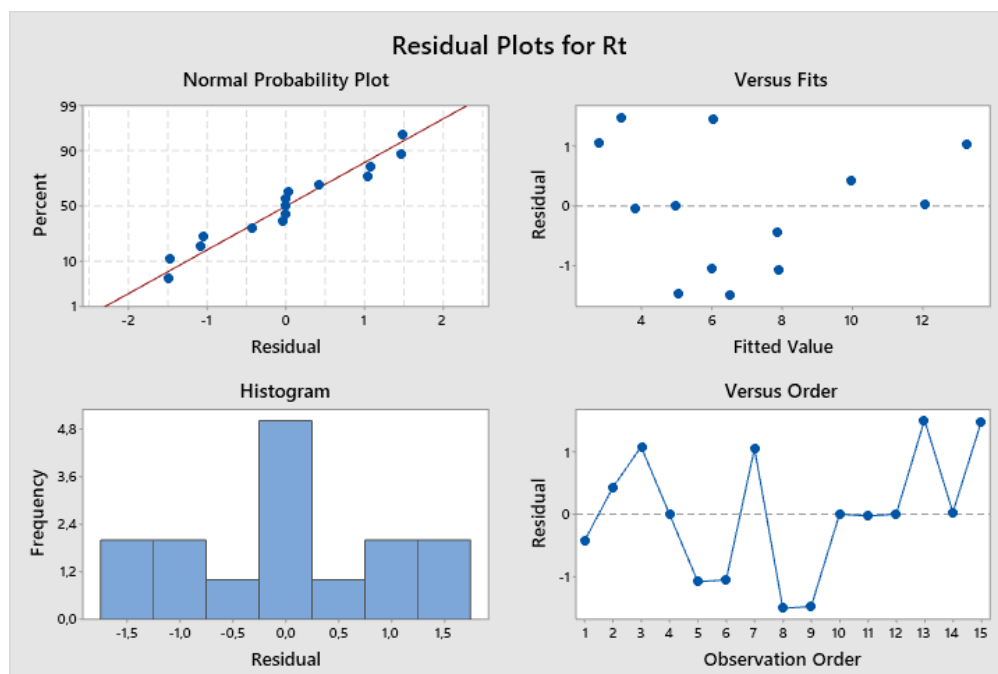


Figure 1. Residual plots for the retention time of ATOR (R_t), confirming the normal distribution of experimental errors and the overall adequacy of the developed regression model

The coefficient of determination (R^2) was considered a key indicator of model reliability and predictive capability. The full quadratic model developed for retention time (R_t) achieved a high R^2 value of 87.99%, indicating that the model explained nearly 88% of the total variability in analysis time. The model developed for chromatographic resolution (R_s) yielded an R^2 value of 65.39%. For

highly complex multicomponent pharmaceutical separations involving structurally related compounds, including highly lipophilic statins and ezetimibe, and incorporating nonlinear effects and chaotropic agents, these coefficients of determination indicate satisfactory model performance and support the adequacy of the experimental design. Furthermore, the lack-of-fit test for both models was statistically insignificant relative to the pure error ($p > 0.05$), demonstrating that the polynomial equations adequately described the design space without evidence of systematic model deviation [28].

3.3. EFFECTS OF THE FACTORS ON THE CRITICAL ANALYTICAL ATTRIBUTES OF ATOR: RESPONSE SURFACE AND PARETO CHART ANALYSIS

To gain a deeper understanding of multifactorial interactions and visualize the effects of the independent chromatographic parameters on the critical analytical attributes (CAAs) of the target analyte, ATOR, standardized Pareto charts and three-dimensional (3D) response surface plots were generated (**Figure 2**). Since the optimization at this stage of method development was specifically focused on atorvastatin, which is the most lipophilic component, determines the overall analysis time, and is prone to peak asymmetry, all mathematical models were interpreted in relation to its chromatographic behavior.

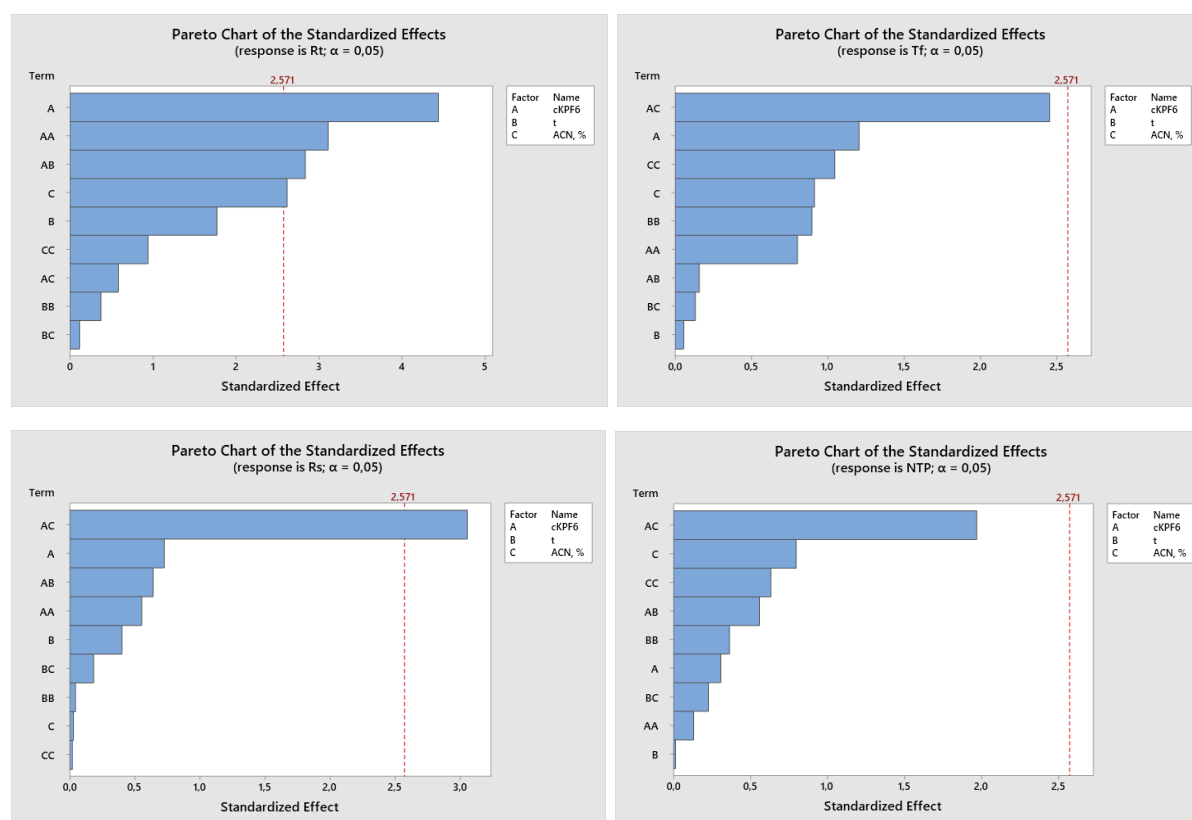


Figure 2. Standardized Pareto charts for evaluating the statistical significance of the effects of chromatographic factors on the critical analytical attributes of ATOR: (a) retention time (R_t); (b) peak asymmetry factor (T_x); (c) resolution of the critical peak pair (R_s); and (d) column efficiency (NTP). The vertical reference line indicates the critical t -value (2.571) at a significance level of $\alpha = 0.05$

Analysis of the standardized Pareto charts enabled the graphical ranking of all linear, quadratic, and interaction effects according to their statistical significance relative to the critical t -value threshold. According to the obtained charts, the volume fraction of the organic modifier, ACN (X_3), exhibited the most pronounced and dominant effect on the overall R_t of ATOR. An increase in ACN concentration resulted in a sharp exponential decrease in R_t , which is fully consistent with classical thermodynamic models of retention in reversed-phase chromatography [20]. At the same time, the Pareto charts revealed statistically significant interaction effects among the investigated parameters that could not have been identified using the conventional empirical “one factor at a time” (OFAT)

approach [24]. The mathematical relationships between the investigated critical analytical attributes of the method and the actual values of the independent chromatographic factors were described by the calculated second-order polynomial regression equations expressed in uncoded units:

$$\mathbf{NTP} = 291912 - 530783 \cdot c(\text{KPF}_6) - 13011 \cdot t + 3631 \cdot \text{ACN} + 15633 \cdot c(\text{KPF}_6)^2 + 108 \cdot t^2 - 68 \cdot \text{ACN}^2 + 3201 \cdot c(\text{KPF}_6) \cdot t + 6765 \cdot c(\text{KPF}_6) \cdot \text{ACN} + 39 \cdot t \cdot \text{ACN}$$

$$\mathbf{R_s} = 238 - 510 \cdot c(\text{KPF}_6) - 2,6 \cdot t - 3,16 \cdot \text{ACN} + 43,2 \cdot c(\text{KPF}_6)^2 + 0,009 \cdot t^2 - 0,0015 \cdot \text{ACN}^2 + 2,40 \cdot c(\text{KPF}_6) \cdot t + 6,87 \cdot c(\text{KPF}_6) \cdot \text{ACN} + 0,021 \cdot t \cdot \text{ACN}$$

$$\mathbf{Tf} = -15,8 + 18,8 \cdot c(\text{KPF}_6) + 1,06 \cdot t - 0,362 \cdot \text{ACN} + 3,97 \cdot c(\text{KPF}_6)^2 - 0,0111 \cdot t^2 + 0,00467 \cdot \text{ACN}^2 - 0,038 \cdot c(\text{KPF}_6) \cdot t - 0,350 \cdot c(\text{KPF}_6) \cdot \text{ACN} - 0,00095 \cdot t \cdot \text{ACN}$$

$$\mathbf{Rt} = 151 - 336 \cdot c(\text{KPF}_6) + 1,39 \cdot t - 4,39 \cdot \text{ACN} + 119,0 \cdot c(\text{KPF}_6)^2 - 0,0358 \cdot t^2 + 0,0324 \cdot \text{ACN}^2 + 5,21 \cdot c(\text{KPF}_6) \cdot t + 0,64 \cdot c(\text{KPF}_6) \cdot \text{ACN} + 0,0065 \cdot t \cdot \text{ACN}$$

To provide a deeper understanding of the multifactorial interactions, the developed models were comprehensively visualized using three-dimensional response surface plots (**Figure 3**).

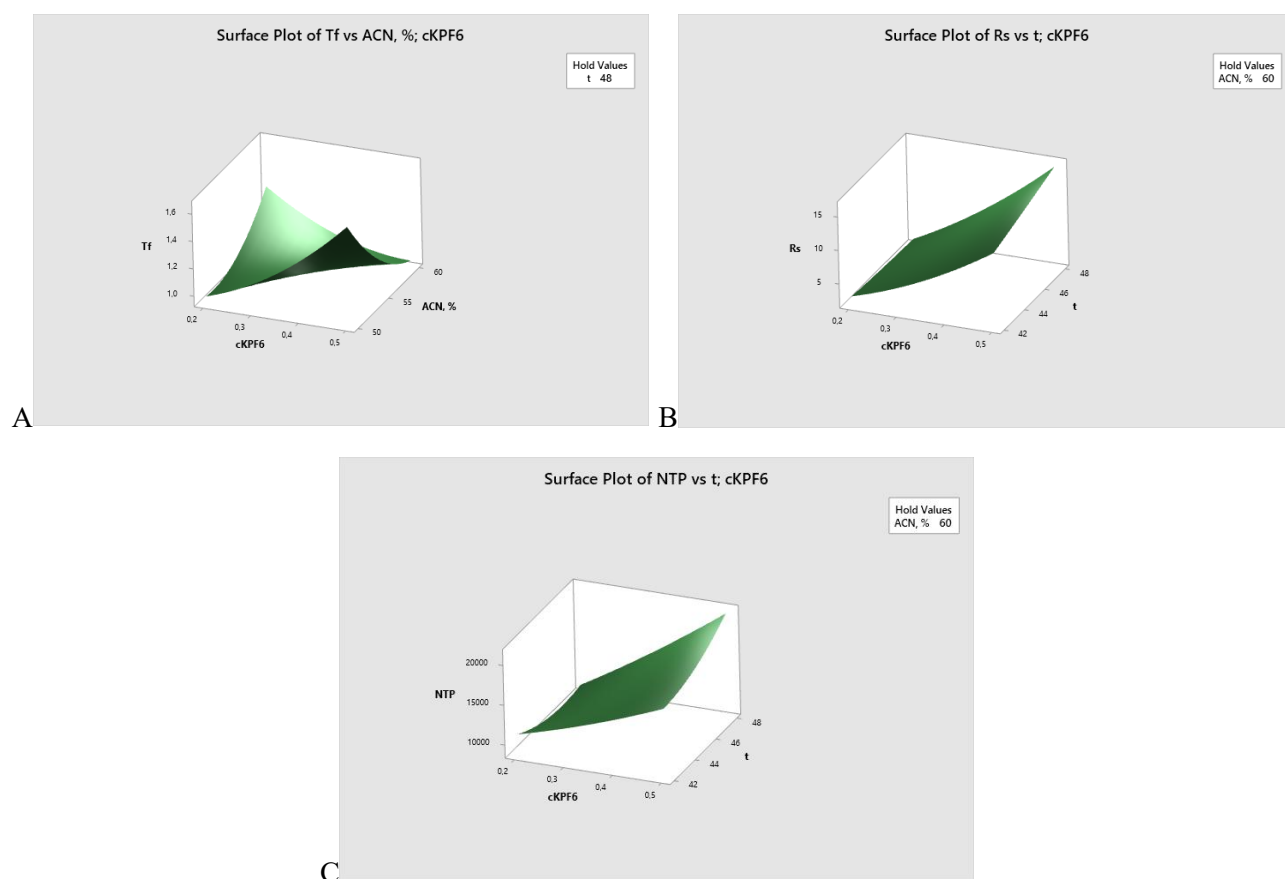


Figure 3. Three-dimensional response surface plots illustrating the synergistic effects and nonlinear curvature of the interactions among the concentration of the chaotropic salt [$c(\text{KPF}_6)$], t , and the content of the organic modifier (ACN) on: (A) the peak asymmetry factor (Tf); (B) the resolution of the critical peak pair (R_s); and (C) column efficiency (NTP).

When considering the effect on chromatographic resolution (R_s), the three-dimensional response surface plots revealed a complex synergistic interaction between column temperature (X_2) and the percentage of the organic modifier (X_3). ATOR is a structurally bulky molecule. Increasing the column temperature reduces the viscosity of the mobile phase and substantially improves intraparticle mass-transfer kinetics, thereby promoting the narrowing of the analyte's chromatographic band [21]. However, the 3D plots clearly demonstrate that this beneficial temperature effect is fully achieved

only at a balanced proportion of the organic modifier. Excessive elution strength resulting from a high ACN content at elevated temperatures leads to excessive chromatographic compression, which critically impairs the ability of the system to separate ATOR from co-eluting impurities. The most pronounced physicochemical relationships were observed when analyzing the response surfaces for the column efficiency (NTP) and peak asymmetry factor (T_x) of ATOR. In this case, the concentration of the chaotropic salt KPF_6 (X_1) played a decisive role. The response surfaces for these parameters exhibited pronounced quadratic curvature. The molecular structure of ATOR contains polar functional groups capable of undergoing undesirable secondary electrostatic interactions with residual ionized silanol groups on the stationary phase, which are traditionally considered a major cause of pronounced peak tailing [19]. As demonstrated by the contour plots, the gradual addition of KPF_6 to the mobile phase resulted in a substantial increase in the number of theoretical plates (NTP) and brought the peak asymmetry factor (T_f) closer to the ideal value of 1.0. The chaotropic PF_6^- anion effectively masks the active sites of the stationary-phase surface. However, the presence of a distinct optimum on the 3D response surface, as indicated by the curvature of the plot, demonstrates that an uncontrolled increase in salt concentration does not provide further improvement. On the contrary, an excessive concentration of KPF_6 may induce competitive interactions at the sorbent surface or cause localized changes in mobile-phase viscosity, thereby slightly reducing the overall efficiency of the chromatographic system [23]. Mapping this design space enabled reliable visualization of the boundaries within which the chromatographic behavior of ATOR remained stable.

3.4. MULTIVARIATE OPTIMIZATION AND ESTABLISHMENT OF THE DESIGN SPACE

The final and most important stage in analytical method development within the AQbD framework is the simultaneous compromise optimization of all investigated critical analytical attributes (CAAs). In chromatographic practice, the individual optima of different parameters often conflict with one another. For example, a substantial reduction in analysis time to improve method throughput requires an increase in the elution strength of the mobile phase, typically achieved by increasing the ACN content. However, this may lead to a critical decrease in the resolution (R_s) of closely eluting peaks and a reduction in overall column efficiency. To address this analytical challenge and identify a single, mathematically justified operating point, a multivariate optimization approach based on the Derringer composite desirability function was applied.

The mathematical algorithm involved transforming each experimental response (y_i) into an individual desirability function (d_i), with values ranging from 0, representing a completely unacceptable result, to 1, representing an ideal response [29]. During the optimization procedure in the Minitab environment, stringent limits were established for the analytical target profile (ATP), with the aim of developing a rapid and robust analytical method. The main optimization priorities were defined as follows: minimization of the retention time of the most lipophilic component, ATOR ($R_t \leq 2.5$ min); achievement of reliable baseline separation for all critical peak pairs ($R_s \geq 2.0$); maximization of column efficiency, expressed as the number of theoretical plates (NTP); and optimization of peak shape by bringing the peak asymmetry factor close to the ideal value of unity ($T_f \approx 1.0$).

By calculating the geometric mean of all individual desirability functions, the software generated a composite desirability model for the chromatographic system (D). The maximum value of this function ($D = 0.803$) clearly defined the final coordinates of the optimal chromatographic conditions within the investigated factor space, corresponding to the Method Operational Design Region (MODR): concentration of the chaotropic agent KPF_6 in the aqueous phase: 0.2%; column oven temperature: 48°C; volume fraction of ACN in the mobile phase: 60%. (**Figure 4**).

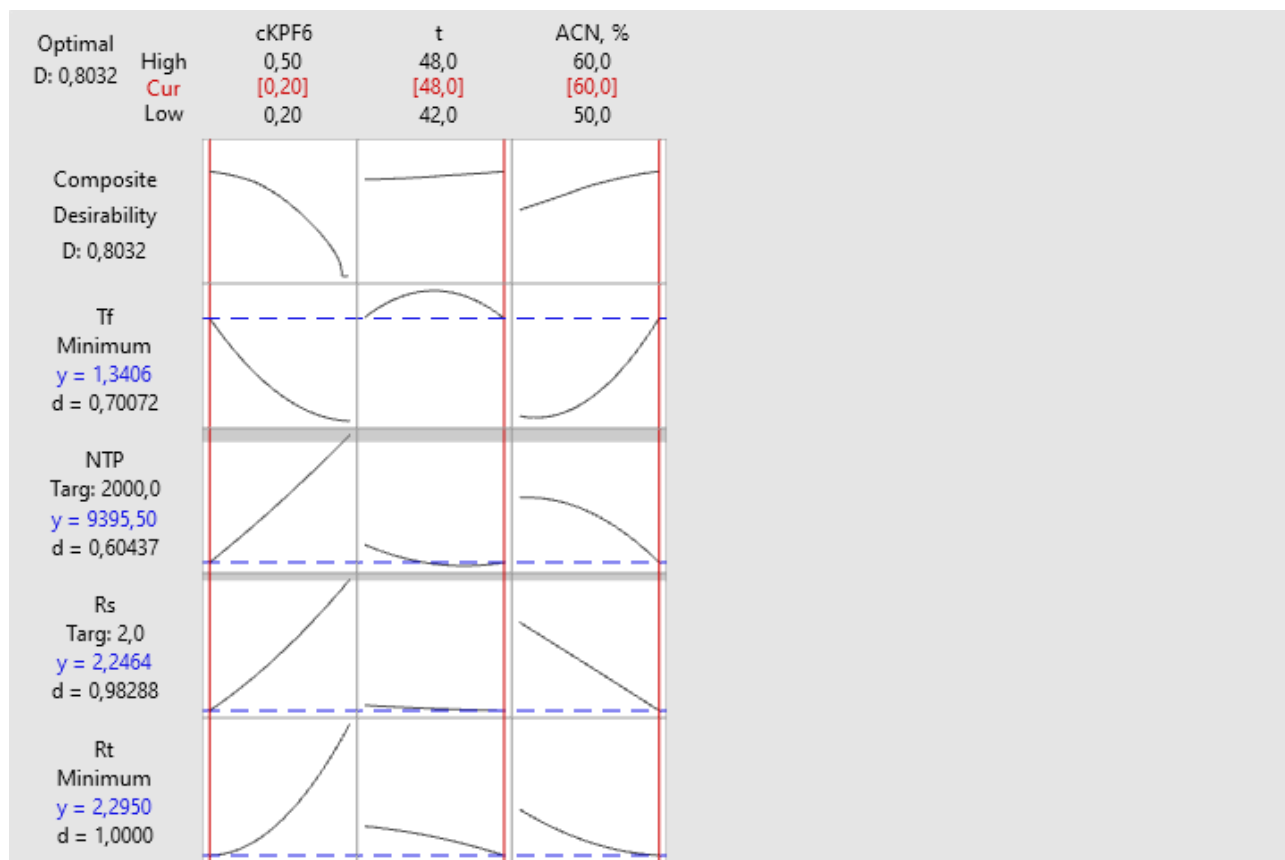


Figure 4. Multivariate compromise optimization of the analytical target profile using the Derringer composite desirability function ($D = 0.8032$)

The physicochemical rationale underlying the identified optimum is based on the synergistic interaction of the selected factors. The high ACN content (60%) provided rapid elution of the lipophilic molecules, resulting in a predicted retention time for ATOR (R_t) of only 2.29 min. To prevent peak coalescence and peak tailing, which may occur under conditions of rapid analyte migration, the system employed an elevated column temperature (48 °C) to enhance mass transfer, together with the chaotropic salt KPF₆. A salt concentration of 0.2% was found to represent an optimal compromise: it was sufficient to effectively shield the residual silanol groups of the stationary phase, thereby maintaining the peak asymmetry factor (T_f) at a favorable value of 1.34, while minimizing the risk of salt precipitation in the chromatographic system when mixed with a high proportion of the organic solvent [30]. The model also predicted adequate separation of the chromatographic zones ($R_s = 2.25$) and high column efficiency ($NTP = 9395$). To graphically confirm the optimization results, the response contour plots were overlaid, enabling clear mapping of the multidimensional design space [31]. The resulting visualized “robustness window” demonstrated that the method could be reliably applied under routine laboratory conditions: variations in the salt concentration within ± 0.05 mM or in column temperature within ± 1.5 °C did not result in deterioration of the analytical performance. Final experimental verification was performed by conducting confirmatory runs under the calculated optimal conditions. The experimental chromatograms showed close agreement with the theoretical predictions generated by the optimization software, with deviations for all CAAs not exceeding 2.5%. These results confirmed the reliability of the regression models and supported progression to the next major stage of the study—the analytical method validation.

3.5. VALIDATION OF THE PROPOSED METHOD

To confirm the practical applicability, operational reliability, and reproducibility of the optimized RP-HPLC method, a comprehensive metrological validation was performed. This process is an essential stage of pharmaceutical development, as it ensures the accuracy of the obtained analytical results and demonstrates that the analytical system is robust against random variations. The key

validation characteristics were experimentally evaluated in accordance with the fundamental principles and recommendations of ICH Q2(R2) guideline. Since the optimal chromatographic conditions were established using DoE-based modeling, all validation experiments were performed directly at the identified compromise operating point: the concentration of the chaotropic agent KPF₆ in the aqueous phase was 0.2, the column oven temperature was 48 °C, and the volume fraction of acetonitrile in the mobile phase was 60%.

The ability of the analytical procedure to produce results that are directly proportional to the concentrations of the active substances in the sample was evaluated using a series of multicomponent model solutions. For the construction of calibration curves, five concentration levels within the range of 10.0–50.0 µg/mL were experimentally investigated for each of the three analytes: ROS, ATOR and EZET. This analytical range fully covered the required working concentrations and enabled reliable control of the active substance content. The obtained experimental datasets were mathematically processed using the least-squares linear regression method, in which the nominal concentration of each substance was used as the independent variable and the corresponding chromatographic peak area was used as the dependent variable. Regression and validation parameters of the proposed HPLC method are presented in **Table 2**. Analysis of the obtained first-order polynomial regression equations demonstrated the high quality and adequacy of the developed mathematical models. The calculated coefficients of determination (r^2) for ROS, ATOR and EZET exceeded the acceptance criterion of 0.996. From the perspective of the physicochemical principles of liquid chromatography, such high linearity confirms that, throughout the selected concentration range, mass transfer between the mobile phase and the alkyl chains of the stationary phase remained stable and that the detector operated within its linear response range without signal saturation effects.

Table 2. Regression and validation parameters of the proposed HPLC method

Parameters	ROS	EZET	ATOR
Linearity range (µg/mL)	12.0–50.0	12.0–50.0	12.0–50.0
Regression equation ($A = bC + a$)	$A = 20891.74C + 64252.20$	$A = 21371.91C + 56342.70$	$A = 2204.68C + 11710.40$
Slope (b)	20891.74	21371.91	22204.68
Intercept (a)	64252.20	56342.70	11710.40
Coefficient of determination (r^2)	0.9964	0.9961	0.9968
Standard deviation of the intercept (S_a)	24149.85	25492.24	24024.10
Limit of detection (LOD) (µg/mL)	3.81	3.94	3.57
Limit of quantification (LOQ) (µg/mL)	11.56	11.93	10.82
Accuracy(%R)	99.53±0.54	99.07±0.35	100.32±0.49
Precision (%RSD), Repeatability	0.712	0.904	0.698
Precision (%RSD), Intermediate precision	0.694	0.854	0.984

The accuracy of the method was evaluated by analyzing laboratory-prepared mixtures containing known concentrations of the three pharmaceuticals at three concentration levels, with each level

analyzed in triplicate. The mean recoveries ranged from 99.07 to 100.32%, with low standard deviations (**Table 2**), confirming the accuracy of the proposed method. Repeatability was assessed by analyzing three concentration levels of each pharmaceutical in triplicate within a single day. The obtained RSD values were below 1.0% for all analytes (**Table 2**), indicating excellent repeatability. Intermediate precision was evaluated by analyzing the same three concentration levels in triplicate over three consecutive days. As shown in **Table 2**, the RSD values for all pharmaceuticals were below 1.0%, demonstrating the high intermediate precision of the method. The robustness of the proposed method was assessed as part of the method development process using a risk-based QbD approach.

3.5. ASSESSMENT OF THE ENVIRONMENTAL SUSTAINABILITY OF THE ANALYTICAL METHOD

The developed analytical method was comprehensively evaluated using several complementary metrics of green, sustainable, and practical analytical chemistry. The obtained scores were AGREE = 0.61, MoGAPI = 75, AGSA = 63.89, CACI = 70, CaFRI = 72, and BAGI = 80.0 (**Figure 5**) [32-37]. The AGREE score of 0.61 indicates a favorable overall greenness profile of the analytical procedure and demonstrates good compliance with the principles of Green Analytical Chemistry. The MoGAPI score of 75 confirms a high level of greenness of the developed procedure. This result reflects the favorable characteristics of the method in terms of sample preparation, reagent and solvent consumption, analytical procedure, and waste generation. The AGSA score of 63.89 further supports the environmentally favorable profile of the method, indicating good implementation of the principles of Green Analytical Chemistry. The CACI score of 70 demonstrates good practical applicability of the analytical procedure, considering factors associated with its operational characteristics and suitability for routine analysis. The CaFRI value of 72 indicates a favorable carbon-footprint profile and suggests that the developed procedure has a relatively low environmental burden associated with energy consumption, solvent and reagent use, and waste generation. The highest score was obtained for BAGI (80.0), indicating a high level of practical applicability of the developed method. This result confirms that the procedure combines environmental sustainability with favorable analytical and operational characteristics. Overall, the obtained results demonstrate that the developed analytical method provides a balanced combination of greenness, sustainability, and practical applicability. The relatively high scores obtained with all evaluated metrics confirm its potential as an environmentally friendly and practically suitable procedure for routine pharmaceutical analysis.

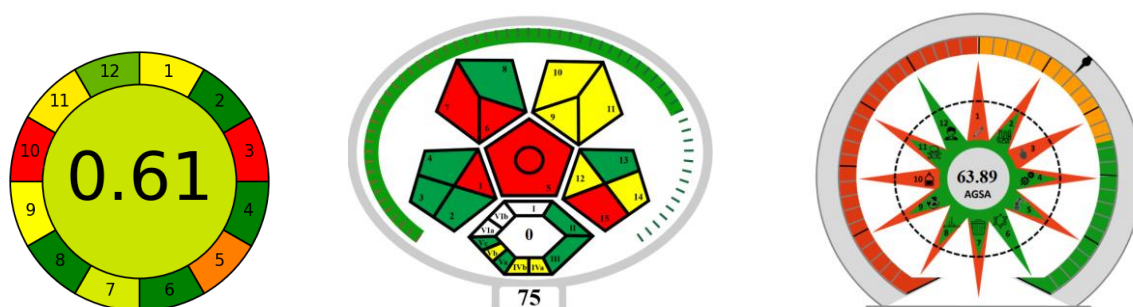




Figure 5. Metric assessment of the proposed RP-HPLC method (AGREE, MoGAPI, AGSA, CACI, CaFRI, BAgI)

3.6. CORRELATION ANALYSIS OF EXPERIMENTAL FACTORS AND CHROMATOGRAPHIC RESPONSES OF MULTIPLE ANALYTES

For the first analyte (ROS), a strong positive correlation was observed between NTP and R_t ($r = 0.876$), indicating a coordinated change in column efficiency and R_t under the investigated conditions. In addition, a strong negative correlation was observed between T_f of the first analyte and R_s of EZET ($r = -0.924$). R_t of the first analyte was also positively correlated with R_t of EZET ($r = 0.843$) and negatively correlated with its T_f ($r = -0.727$). Several strong correlations were identified between the chromatographic characteristics of EZET and ATOR. The strongest relationship was observed between T_f of the two analytes, with a correlation coefficient of $r = 0.999$. A very strong positive correlation was also observed between R_t of EZET and ATOR ($r = 0.961$). In addition, the resolution of EZET was strongly positively correlated with R_t of ATOR ($r = 0.913$). These findings indicate a close relationship between the chromatographic behavior of these two analytes under the investigated conditions. Statistically significant relationships were also identified between the first analyte and ATOR. In particular, a strong negative correlation was observed between T_f of the first analyte and R_t of ATOR ($r = -0.857$), whereas a positive correlation was observed between the R_t of these analytes ($r = 0.704$). Among the investigated chromatographic conditions, ACN content demonstrated a moderate negative correlation with R_t of the first analyte ($r = -0.509$) and EZET ($r = -0.420$). The concentration of KPF_6 was positively correlated with R_t of EZET ($r = 0.585$) and ATOR ($r = 0.606$), whereas an inverse relationship was observed between KPF_6 concentration and T_f of the first analyte ($r = -0.617$) (Table 1S, 2S, Figure 14S-28S).

Overall, the correlation analysis demonstrated pronounced relationships among the chromatographic characteristics of the three investigated analytes, particularly between EZET and ATOR. However, the observed correlations should be interpreted as associations between chromatographic responses rather than as direct evidence that one analyte directly affects another. Further regression analysis and dedicated selectivity/interference studies would therefore be required to confirm whether changes in the concentration of one analyte have a statistically significant effect on the quantitative determination of another analyte.

4. CONCLUSION

In this study, a novel, rapid, and robust RP-HPLC method was successfully developed and validated for the simultaneous determination of ROS, ATOR, and EZET using an AQbD-guided approach. The systematic optimization of CMPs via a Box-Behnken design enabled the clear definition of a Method Operational Design Region (MODR) at 0.2 mM KPF_6 , 48 °C, and 60% ACN. This yielded a highly efficient separation with an ATOR retention time of just 2.29 min. The method demonstrated

excellent linearity ($r^2 \geq 0.996$), accuracy (98–102%), and precision (%RSD < 2%) over the analytical range of 12.0–50.0 $\mu\text{g/mL}$. Its environmental sustainability was confirmed by multiple greenness assessment tools. The method's robustness, speed, and eco-friendliness make it a superior alternative to conventional methods and a valuable tool for routine quality control of combined lipid-lowering pharmaceutical products.

Conflict of interest

The authors confirm that there is no possible conflict of interest in the manuscript.

Data availability

All data generated or analysed during this study are included in this published article.

5. Declaration

1. Ethical approval and consent to participate

Not applicable

2. Consent for publication

Not applicable

3. Ethical Guidelines

Guidelines according to BMC Chemistry

4. Availability of data and materials

All data generated or analysed during this study are included in this published article.

5. Funding

Non-funding

6. Competing interest

The authors declare that they have no competing interests

7. Author's Contribution

Yurii Ihnatsevyh Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, **Liliya Logoyda**: Supervision, Conceptualization, Writing – review and editing, **Marjan Piponski**, **Oleksandra Oleshchuk**, **Mamdouh R. Rezk**, **Marta Sulyma**, **Andrii Sverstyuk**, **Mariana Horyn**, **Liubomyr Kryskiw**: Validation, Formal analysis, Visualization, Writing – review and editing.

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