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Original Article

Designing and Optimisation of QbD Guided Green NADES Based RP-HPLC Method for Stability-Indicating Analysis of Cyclophosphamide: An Agree Assessment

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ABSTRACT

Background: Innovative green chromatographic techniques have been established to satisfy the increasing need for ecologically sustainable analytical procedures without sacrificing analytical performance. **Objectives:** Herein, an Analytical Quality-by-Design (AQbD) framework was adopted to develop and assess a novel NADES-based RP-HPLC method for the detection of cyclophosphamide in bulk medication and in house capsule formulations. **Materials and Methods:** The previous analytical methods reported for cyclophosphamide estimation utilise hazardous chemical solvents and lack systematic optimization, hence there is a need for development of more environmentally friendly and resilient alternatives. **Results:** Herein, a factorial design of experiments discovered major chromatographic factors and their interactions during technique optimization. Using a phosphate buffer (pH 7.2) as mobile phase A and a methanol-NADES-water combination as mobile phase B, the optimized approach separated cyclophosphamide at 2.5 min. Excellent chromatographic performance was achieved with a resolution of ≥1.8, tailing factor of ≤1.1, and theoretical plate counts of ~2500. Validation using ICH Q2(R1) revealed linearity (r^2=0.999), high sensitivity (LOD 0.3 µg mL^{-1}; LOQ 1.0 µg mL^{-1}), accuracy (98-102% recovery), precision (%RSD <2%), and resilience. Forced degradation tests showed that the approach indicates stability, with unique degradation products only under acidic and oxidative stress. Application to bulk medication and in house capsule formulations provided assay values near to label claim with impurities below 0.1%. The use of NADES resulted in a partial replacement of the traditional mobile phase with organic solvent, which has an environmental footprint. In addition, a high score of 0.88 on the AGREE greenness metric indicated environmentally friendly and possesses an excellent sustainable profile. **Conclusion:** The proposed system for routine pharmaceutical quality control is robust, green, and AQbD-compliant.

Keywords: AGREE Greenness Assessment, Analytical Quality by Design (AQbD), Cyclophosphamide, Green RP-HPLC, Natural Deep Eutectic Solvents (NADES), Stability-Indicating Method.

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INTRODUCTION

Cyclophosphamide is a chemically produced oxazaphosphorine alkylating agent that has been utilized for over sixty years in the management of various malignant and immune-mediated illnesses. It is given as an inactive prodrug that is changed into 4-hydroxycyclophosphamide by the liver's cytochrome P450 system. This new substance is in balance with its ring-open aldophosphamide. After β-elimination, phosphoramide mustard, the final DNA-cross-linking metabolite that causes the cytotoxic impact, and acrolein, which is thought to cause urotoxicity, are formed.[1] Cyclophosphamide is used in several chemotherapy regimens for breast and ovarian cancers, lymphomas, leukemias, multiple myeloma, and neuroblastoma. It is also used as an immunosuppressant for autoimmune disorders, vasculitis, and nephrotic syndrome.

In the past, compendial analysis of cyclophosphamide was performed using Thin Layer Chromatography (TLC) techniques. These techniques provided only a semi-quantitative assessment of related substances and lacked sensitivity for low level contaminants. In order to circumvent these constraints, a number of liquid chromatographic techniques for assay and impurity profiling have been developed. In comparison to TLC, ion chromatographic procedures that make use of cation exchange columns and conductivity-UV detection have made it possible

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to simultaneously determine cyclophosphamide and process impurities like propanolamine with a higher degree of precision.[2] However, these procedures require specialized instrumentation and have run times that can last for up to fifty min. More recently, the reported method provides acceptable sensitivity and validation performance for cyclophosphamide estimation, employing C18 columns and mixed aqueous-organic mobile phases have been proposed, including a stability-indicating RP-HPLC method on an X-Bridge C18 column. However, it relies on a complex mobile phase containing acetonitrile and lacks systematic AQbD-based optimization and greenness evaluation.[3]

In parallel, reported HTLC-ESI-MS/MS method enables rapid and highly selective simultaneous quantification of cyclophosphamide and its active metabolite CEPM in human plasma with excellent precision and accuracy, supporting its application in clinical pharmacokinetic studies.[4] This method, on the other hand, requires special LC-MS/MS equipment and online extraction systems. There is more cost required in fact to run and its is not feasible for the environment or for regular quality control labs. In addition, many of the previously published chromatographic methods require large quantities of acetonitrile and other harmful organic solvents which disobey the principles of green analytical chemistry. More sustainable methods of therapeutic monitoring and quality control are needed, as shown by a recent multi-tool greenness assessment of anticancer drug tests, which revealed that traditional HPLC and LC-MS techniques frequently score low on eco scale and AGREE metrics.[5] Additionally, there are very few cyclophosphamide techniques that have been developed using the Quality by Design (AQbD) framework, which officially defines design space, robustness, and lifecycle management.

Natural Deep Eutectic Solvents (NADES) are a viable ecological alternative to traditional organic solvents since they are biodegradable, have tunable physicochemical features, are not very harmful, and are easy to make.[6] Despite their growing use in extraction and sample preparation, NADES are still not widely used as mobile phase components in chromatographic techniques. In RP-HPLC method, use of NADES can definitely enhance sustainability without lingering the effectiveness of chromatography.[7] Herein, an Analytical Quality by Design (AQbD) method has been proposed for optimisation of mobile phase and other parameters. In accordance with the regulatory guidelines, this method offers robust and reproducible analytical procedure and additionally provide the AGREE metric which make it easy to empirically assess environmental sustainability all through analytical processes.

This method presents a green, NADES-based RP-HPLC method for estimation of cyclophosphamide encompassing AQbD-guided approach. This methodology has been obeying the ICH Q2(R1) guidelines, with inclusion of forced degradation studies. Moreover, the method supports AGREE assessment for RP-HPLC work with score of 0.88, governing good greenness methodology. This work can be considered as a robust and eco-friendly alternative to conventional pharmaceutical quality management.

MATERIALS AND METHODS

Chemicals and reagents

The cyclophosphamide reference standard was procured from a reliable supplier and utilized without any additional purification. In house capsule formulation (50 mg) was prepared in laboratory. Analytical grade choline chloride, ethylene glycol, and D-(+)-glucose were utilized in the preparation of NADES. Lactose monohydrate, magnesium stearate and standard chemical suppliers were utilized to acquire potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide, orthophosphoric acid, methanol (HPLC grade), and hydrogen peroxide (30-35%). Analytical reagent grade hydrochloric acid and sodium hydroxide were employed in stress studies. The Milli-Q system was employed to purify the water that was utilized throughout the study.

Instruments and chromatographic system

A modular HPLC system with a quaternary pump, an on-line degasser, a manual injector, a column oven, and a UV or Photodiode Array (PDA) detector controlled by chromatography data software was used for chromatographic analysis. Separation was achieved on an X Bridge C18 column (250 × 4.6 mm, 3.5 µm) fitted with a compatible guard column. The column temperature stayed at 25°C, and samples were added using a manual Rheodyne injector with a set injection volume of 20 µL.

Preparation of NADES

We looked into two Natural Deep Eutectic Solvents (NADES) as green organic modifiers: Choline chloride: ethylene glycol (ChCl: EG, 1:2 molar ratio) and choline chloride: glucose (ChCl: Glc, 1:5 molar ratio). For each NADES, the necessary molar quantities of choline chloride and the respective hydrogen-bond donor were measured into a round-bottom flask and heated to 80-90°C with continuous magnetic stirring until a clear, homogeneous liquid was achieved.[8] Then this solution was allowed to cool at room temperature followed by their sealing in amber-colored vials. During the process, at each stage, the NADES solution was tested critically for crystallization and phase separation. Finally, we diluted the NADES solutions using water at 25°C and ensured they have optimum viscosity, density, and pH.[7]

Laboratory-prepared capsule formulation

The applicability of the proposed RP-HPLC method was demonstrated by the formulation of the in-house prepared capsules of cyclophosphamide as a finished dosage form. To prepare hard gelatin capsules, cyclophosphamide was accurately weighed, mixed with other commonly used excipients for pharmaceutical formulations, lactose monohydrate as a diluent,

and magnesium stearate as a lubricant, to form a uniform powder blend for each capsule. Hard gelatin capsules were filled by hand with the blend using a capsule-filling machine. The prepared capsules were kept at room temperature in airtight containers until they were analysed.

Mobile phase and diluent

We utilised DoE software for optimisation of final chromatographic conditions such as peak symmetry, column efficiency, good resolution and optimum runtime. Herein, 25 mM potassium dihydrogen phosphate buffer solution (pH 7.2) was used as mobile phase A, which was filtered via a 0.45 µm membrane filter, and degassed ultrasonically. In mobile phase B, methanol (35%, v/v) and a natural deep eutectic solvent (NADES, 30%, v/v), was used with remaining proportion of water. The selected NADES system was choline chloride-ethylene glycol (ChCl: EG). Now, at t=0 min, 35% mobile phase B was used to perform chromatographic separation at an optimized flow rate of 0.55 mL min^{-1}. The optimised diluent was methanol-water mixture (50:50, *v/v*) to ensure complete dissolution of cyclophosphamide and compatibility with mobile phases, to avoid precipitation or phase separation [8]. These optimized conditions were subsequently employed for method validation and routine analysis.

Chromatographic conditions

Based on Design of Experiments-guided numerical optimization, chromatographic separation was performed using a gradient elution program with mobile phase A consisting of 25 mM potassium dihydrogen phosphate buffer adjusted to pH 7.2 and mobile phase B composed of methanol (35%, v/v) and a natural deep eutectic solvent (NADES, 30%, v/v), with water constituting the remaining proportion.[9] While the column temperature was kept at 25°C, the chromatographic run was started with 35% mobile phase B at time t=0 min. The flow rate was optimized to be 0.55 mL per minute, and the column temperature remains constant. Spectra were acquired spanning the wavelength range of 190-400 nm for conducting Photodiode Array (PDA) Analysis. This was done in order to enable peak purity assessment. The detection was carried out at a wavelength of 200 nm. After establishing these optimal chromatographic settings, they were later utilized for all of the tests that were conducted to validate the procedure and analyze samples.

Preparation of standard solutions

A primary stock solution of cyclophosphamide was prepared by accurately weighing about 50 mg of the reference standard into a 50 mL volumetric flask, dissolving in approximately 30 mL of methanol: water (50:50, v/v), sonicating for 5 min, cooling to room temperature, and diluting to volume with the same solvent to obtain a concentration of 1.0 mg/mL. Working standard solutions for system suitability and calibration were obtained by appropriate dilution of the stock solution with diluent to cover the intended concentration range (e.g., LOQ to 150% of the specification level). A cyclophosphamide standard solution was used for system suitability and QbD experiments.[10]

Preparation of sample solutions

For bulk drug analysis, an accurately weighed portion of cyclophosphamide API equivalent to about 50 mg was transferred into a 50 mL volumetric flask, dissolved in 30 mL of methanol: water (50:50, v/v) with sonication, if necessary, cooled to room temperature, and diluted to volume with the same diluent. After cooling, we diluted it to volume with diluent, and finally, we filtered it using a 0.22 µm membrane filter. As needed, more dilution was carried out using diluent in order to achieve the desired concentration.[9]

QbD-based method development and optimization

The Critical Method Parameters (CMPs) were selected based on the preliminary development experiments, literature data on the chromatographic analysis of cyclophosphamide in reverse phase HPLC (RP-HPLC) and from prior knowledge of RP-HPLC method development.[11] In the present work, the different factors such as buffer pH (A), methanol (B) content in the mobile phase, NADES content in the mobile phase (C), initial %B at t=0 (D), and flow rate (E) were chosen because these parameters are known to have a significant effect on chromatographic performance parameters such as resolution, tailing factor, theoretical plates, and run time (Table S1). For each parameter, the ranges of the independent variables were determined in preliminary screening studies, with the levels chosen to cover the area within which measurable changes in chromatographic performance were seen but did not adversely affect system suitability. These ranges were then explored on a factorial Design of Experiments (DoE) to establish the analytical design space and to find robust operating conditions.[12]

Resolution values in the trial ranged from 1.35 to 1.90, which means that separation was good under most conditions. Higher resolution was usually seen at lower flow rates and medium amounts of methanol and NADES.[13] At an ideal pH and with a well-balanced organic modifier composition, the tailing factor ranged from 1.00 to1.48, showing satisfactory peak symmetry. The range of theoretical plates representing column efficiency was between 1820 to 2500. As the pH was tuned to be between 7.0 and 7.2, flow rates were reduced, and the initial % B was adequate, the plate counts increased, indicating better mass transfer and column performance. Analysis times were lower for samples with higher organic content and those with optimal flow conditions, and the run times ranged from 17.5 to 23.2 min. The optimized and centre point experimental runs showed a good balance in all responses, proving that the chosen chromatographic settings give the best compromise in terms of analysis time, resolution, peak symmetry, and efficiency.[14] The results show that the DoE

methodology was successful in finding stable operating conditions for the green RP-HPLC technology that was developed.

Even though the optimized method resulted in theoretical-plates of around 2500, the selected chromatographic conditions were the best balance of the chromatographic efficiency, green solvent usage, analysis time and the overall robustness of the method, achieved through the AQbD optimization. During preliminary testing further improvement of columns' efficiency was considered, but the resulting changes in mobile phase composition and gradient conditions either increased the analysis time or resulted in less desirable peak symmetry and environmental impact. Since the method fulfilled all predefined system suitability criteria, including adequate resolution and acceptable peak symmetry, the selected operating conditions were considered appropriate for routine pharmaceutical analysis.[15,16]

Forced degradation studies

Forced degradation studies were conducted on cyclophosphamide under a variety of stress conditions, with a particular emphasis placed on two conditions that are known to produce unknown impurity peaks.[17] The purpose of these studies was to demonstrate the capability of the green method to indicate stability and to evaluate the behavior of impurities under stress. For each of the stress conditions, a solution or suspension of cyclophosphamide (equivalent to approximately 1 mg/mL) was subjected to the following: acidic hydrolysis with 0.1 M hydrochloric acid at elevated temperature, basic hydrolysis with 0.1 M sodium hydroxide, oxidative degradation with 3% hydrogen peroxide, and thermal stress as appropriate.[18] Herein, at predefined time points, aliquots were withdrawn, neutralized where necessary, diluted with diluent to the working concentration (25-50 µg/mL), and analysed under the optimized chromatographic conditions. Chromatograms were examined for formation of known and unknown degradant peaks, and their separation from the cyclophosphamide peak was verified. For key stressed samples, PDA data was used to assess peak purity of the cyclophosphamide peak. The two stress conditions that produced prominent unknown impurity peaks were characterized in detail, and the relative retention times and behavior of these unknown peaks were documented.[19]

Methods validation

The green RP-HPLC method developed with NADES-modified mobile phase was validated in accordance with ICH Q2 (R1) for the determination of cyclophosphamide.[20] Validation included assessment of specificity (including placebo and stressed samples), linearity and range, detection and quantitation limits, accuracy, precision, robustness, (pH, methanol fraction, NADES fraction, flow rate and column temperature), and system suitability. Detailed procedures and acceptance criteria followed standard chromatographic validation practice and were aligned with quantitative testing requirements.

Statistical Analysis

Design of Experiments (DoE) and statistical analysis were performed using Design Expert® software, version 13 (Stat-Ease, Inc., Minneapolis, MN, USA). A two-level factorial design was used to evaluate the effects of selected factors on the responses. Analysis of Variance (ANOVA) was applied to assess the statistical significance of the model terms and curvature using Type III - Partial sum of squares. Factor coding was in coded units. Model adequacy was evaluated based on F-values, *p*-values, and lack-of-fit test.

RESULTS AND DISCUSSION

QbD-Based Method Optimization

Natural Deep Eutectic Solvents (Nades) Characterization

The physicochemical properties of the prepared natural deep eutectic solvents, choline chloride-ethylene glycol (ChCl: EG, 1:2) and choline chloride-glucose (ChCl: Glc, 1:5), were systematically evaluated to support their application in the developed green RP-HPLC method (Figure 1). Table 1 shows that the NADES ChCl: EG had greater mass transfer, less backpressure, and higher miscibility with organic solvents than ChCl: Glc, thanks to its lower viscosity and density. These qualities are especially helpful for chromatography. The near-neutral pH values of the two NADES systems showed that the analyte was stable and worked well with the phosphate buffer-based mobile phase.[8]

Between the two NADES systems evaluated, ChCl: EG (1:2) was selected because it provided a better overall chromatographic balance, including improved peak shape, acceptable retention, and better compatibility with UV detection. The selection was based not only on viscosity and miscibility, but also on polarity/solvation characteristics and the expected chromatographic efficiency of the mobile phase, consistent with published reports

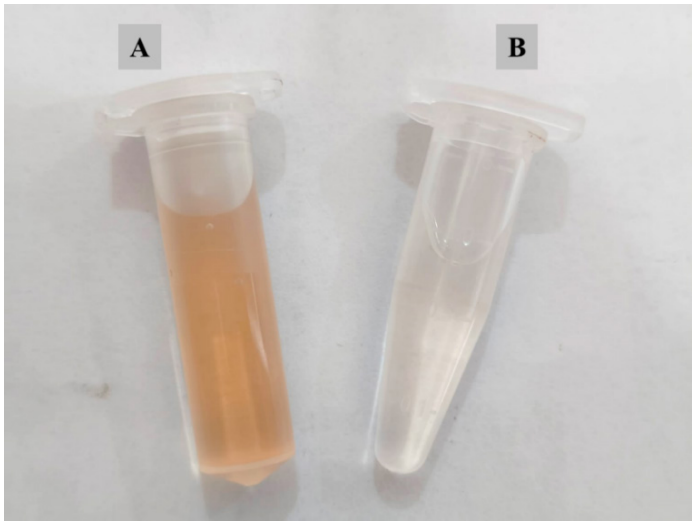


Figure 1: Choline chloride-glucose (ChCl: Glc, 1:5) (A) as a viscous, light amber liquid and choline chloride-ethylene glycol (EG, 1:2) (B) as a transparent, colorless, free-flowing liquid.

Table 1: Physicochemical properties of synthesized NADES (mean±SD, *n*=3).

NADES system	Molar ratio	Density (g mL^{-1})	Viscosity (mPa·s, 25°C)	pH
ChCl: EG	1:2	1.12±0.01	38.6±1.4	6.8±0.1
ChCl: Glc	1:5	1.29±0.02	186.4±5.8	6.5±0.2

Table 2: Linearity of cyclophosphamide (LOQ-150%).

Concentration level	Concentration (µg mL^{-1})	Mean peak area±SD (*n*=3)
LOQ	1.0	12548±184
25%	5.0	62145±512
50%	10.0	124380±905
75%	15.0	186925±1248
100%	20.0	249870±1825
125%	25.0	311450±2056
150%	30.0	374120±2685

Regression equation: y=12460x + 85, Correlation coefficient (r^2): 0.999.

on NADES use in HPLC.[9,22] Both NADES did not crystallize, separate into different phases, or change in appearance after two weeks of being stored at room temperature. This shows that they are physically stable and can be used in ordinary chromatography.

QbD Method Development and Optimization

We used Analysis of Variance (ANOVA) to see if the chosen factorial model was good enough for resolution and to see if it was statistically significant. The results are shown in Table S2. The model had low variability, as shown by the small model sum of squares compared to the total variation. This means that the experimental factors chosen correctly to describe the reaction in the domain that was studied. The curve term had a *p*-value of 0.4690 (F=0.5486), which means it was not statistically significant. This confirmed that a linear factorial model was enough to explain the connection between the factors that were studied and resolution. The residual mean square value of 0.0295 shows that there is not much variation that cannot be explained, which supports the idea that the testing data is reliable. The lack-of-fit test also showed that there was no statistically significant difference (p=0.7278), which means that the predicted values and experimental values were very close to each other. This means that the model fits the data well and there is no systematic mistake. The relatively low input from pure error adds to the evidence that the experiments can be repeated.[23] As a whole, the ANOVA results demonstrate that the selected factorial model is statistically sound and may be utilized to characterize and enhance chromatographic resolution in the investigated green RP-HPLC circumstances. This gives it more credibility as a way to improve processes and get strong analytical results.

Figures S1A-S1D show the results of a statistical experiment that used chromatographic resolution as the response variable to refine and validate the ecologically sustainable RP-HPLC technology. The main factors affecting resolution, according to Pareto analysis (Figure S1A), were the mobile phase composition and flow rate. As per the results, it conformed the optimum solvent percentage necessary for eco-friendly environment and proper peak separation. As per the ICH Q2 (R1) guidelines, the proposed model was gotten to be accurate and reliable to predict resolution in close alignment of regression line. In addition, the comparison of projected and experimental data ensured the data alignment as depicted in Figure S1B.

As per Figure S1C, which demonstrated a large green design space along with experimental data producing acceptable resolution values, the method was said to be durable and operational. At the same time, a three-dimensional response surface plot, illustrated the combined and interactive effects of key chromatographic variables on resolution (Figure S1D). With this, the method is supposed to be adequate and predictable with smooth surface profile and minimal variation between experimental and predicted values. Therefore, the RP-HPLC method was supposed to be suitable for estimation of cyclophosphamide in pharmaceutical formulations, ensuring that optimization constructed stable and trustworthy results under eco-friendly instances.[24]

To obtain the optimal chromatographic parameters keeping resolution as a response parameter, a desirability function was studied. The desirability plot (Figure S2) shows that the best conditions meet all the analytical goals in the experimental design space, with a value of 1.000 being the most desirable. As shown in the prediction plot, the expected response value of 1.66 shows that the conditions give good and reliable chromatographic performance.

The tailing factor factorial model worked well, as shown by ANOVA (Table S3). It was found that the curvature effect was not statistically significant (p=0.4794), which means that a linear factorial model correctly described the relationship between chromatographic factors and peak tailing in the experimental range. The test for lack of fit was not significant (p=0.6864), which means the model is good and the predictions are very close to what happened. The small pure error and low residual mean square value (0.0260) show that the trial data can be used repeatedly. The ANOVA test shows that the factorial model is statistically sound and can be used to change the tailing factor and make sure that the peaks are symmetrical in green RP-HPLC conditions.[25]

The RP-HPLC method for tailing factor was statistically improved using a factorial design, as shown in Figures S3A-S3D. A Pareto chart, Figure S3A, shows the most important factors

that affect peak tailing in the set of variables that were tested. The composition of the mobile phase and the flow rate were the most important factors. Other factors did not have as much of an impact. The expected versus experimental plot in Figure S3B showed that the model we chose was good enough to make correct predictions. The contour plot in Figure S3C showed that the method was reliable because it showed a large design space where the right tailing factor values were always found. Figure S3D, a three-dimensional response surface plot, further showed how important variables worked together to affect peak symmetry, and there was very little discrepancy between the experimental and anticipated results.[26] In short, the data reveal that the method is strong, well optimized, and good for frequent pharmaceutical analysis in an RP-HPLC setting that is good for the environment.

A wide optimal region was found by the tailing factor design space analysis, where satisfactory peak symmetry was constantly maintained. Figure S4 shows the contour plot, which shows that the optimized chromatographic conditions had a predicted tailing factor of 1.22, which means that the system was suitable and the peaks were nearly perfectly symmetrical. Aside from proving the method's durability, the fact that the design points cluster within the acceptable zone and the uniform green region show that small changes in chromatographic settings have no big impact on peak shape. These findings provide more evidence that the improved RP-HPLC settings are reliable for conventional pharmaceutical analysis in an environmentally conscious setting.[24]

This factorial model for theoretical plate number-a measure of the column's efficacy—was determined to be adequate by the analysis of variance. Table S4 shows that the investigated chromatographic variables' effects on theoretical plates were well described by a linear factorial model, as the curvature effect was not statistically significant (F=1.01, p=0.3297). Since the experimental and projected values were so near to one another, indicating that the model was suitable, the lack-of-fit test also yielded no significant results (p=0.7069). Both the low pure error contribution and the residual mean square value of 46996.69 indicate that the experiment can be repeated with satisfactory results. An Analysis of Variance (ANOVA) shows that the selected factorial model is appropriate for increasing the theoretical plate number, which ensures constant chromatographic efficiency using the standard green RP-HPLC parameters, and is also statistically sound.[27]

Figures S5A-S5D show the results of a factorial design optimization of the created RP-HPLC process utilizing theoretical plate number as the response parameter. Figure S5A, a Pareto chart, showed that, within the range of variables examined, mobile phase composition and flow rate had the greatest impact on column efficiency, with other variables showing relatively smaller effects. Confirming the appropriateness and predictive capabilities of the selected model, the anticipated vs experimental plot Figure S5B showed good agreement between observed and model-predicted values. Figure S5C contour plot showed a large design space with consistently high theoretical plate values, which meant the approach, was more resilient and the columns were more efficient. A smooth response surface and low divergence between predicted and experimental points were further demonstrated in the three-dimensional response surface plot, Figure S5D, which showed the combined influence of major chromatographic factors on theoretical plate number. When run under ideal, eco-friendly RP-HPLC settings, the results show that the new approach consistently produces good chromatographic efficiency.[28]

The design space study for theoretical plate number showed a wide optimal region with good column efficiency. The contour map shows that the expected theoretical plate value under optimum chromatographic conditions was 2103.13, demonstrating good column performance and analyte separation (Figure S6). The

Table 3: LOD and LOQ of cyclophosphamide (S/N method).

Parameter	Concentration (µg mL^{-1})	Signal-to-noise ratio
LOD	0.3	≈ 3
LOQ	1.0	≈ 10

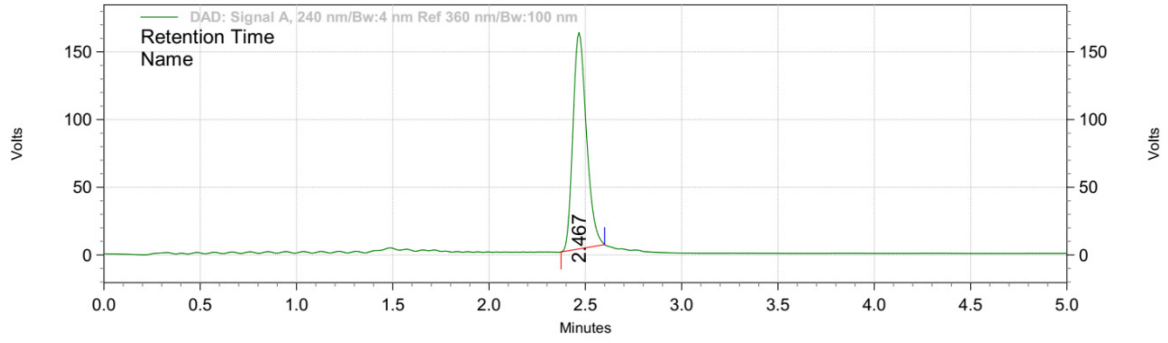


Figure 2: Optimised RP-HPLC chromatogram displaying a sharp peak at 2.467 min of cyclophosphamide.

Table 4: Accuracy (Recovery studies, *n*=3).

Spiking level	Amount added (µg mL^{-1})	Amount found (µg mL^{-1})±SD	% Recovery
98%	19.6	19.52±0.18	99.6
100%	20.0	20.08±0.15	100.4
102%	20.4	20.32±0.21	99.6

Table 5: Precision of the method.

(A) Repeatability (intra-day, *n*=6)		
Parameter	**Mean assay (%)**	**%RSD**
Assay	99.8	0.84
(B) Intermediate precision (inter-day, ***n***=6)		
Day	Mean assay (%) ±SD	%RSD
Day 1	99.7±0.92	0.92
Day 2	100.1±0.88	0.88

uniform green region and distribution of design points inside the acceptable zone show that the approach is robust and that tiny chromatographic parameter changes do not impair column efficiency.

The suggested factorial model for run time optimization was validated by ANOVA. The curvature impact was not statistically significant (F=0.5740, p=0.4590), showing that a linear factorial model properly characterized the effect of the examined chromatographic variables on experimental domain run time (Table S5). Herein, the lack-of-fit test was found to be non-significant (p=0.0921), encouraging model suitability and strong agreement between experimental and projected values. The low pure error contribution indicates good experimental repeatability. ANOVA study shows that the suggested factorial model is statistically valid and suitable for optimizing run time, providing efficient and reliable chromatographic performance under green RP-HPLC circumstances.[29]

The created RP-HPLC method was made better using a factorial Design of Experiments method, with run time as the response parameter, as shown in Figures S7A-D. The Pareto chart Figure S7A showed that the makeup of the mobile phase and the flow rate had the most significant effects on run time. Other variables had much smaller effects in the area that was being studied. The predicted versus experimental plot Figure S7B showed that the measured and model-predicted run time values were very close to each other. This proved that the chosen model was accurate and could make accurate predictions. The contour plot in Figure S7(C) demonstrated a substantial design space in which the run time values were consistent within a reasonable range of approximately 17.5 to 23.2 min. It confirmed that the method is robust to be adopted in many circumstances. As per Figure S7(D), important chromatographic parameters influencing run time were studied, and it was found that there is minimal difference between the expected and experimental results. Under ideal chromatographic conditions, and to maintain overall performance and efficiency of the method, run duration of 19.2 min was taken into consideration. This conforms that proposed RP HPLC method is sufficient to estimate drug in in environmentally friendly RP-HPLC surroundings.[25] Additionally, larger runtime design space provides a huge operating range where efficient chromatographic separation was constantly accomplished. As per software the expected run time under progressed chromatographic conditions was 19.175 min as depicted in contour map (Figure S8). In addition, it can be concluded that analysis ran for an acceptable extent of time without negotiating chromatographic efficiency. As per the Figures, method's strength is shown by the evenly distributed colours and the presence of design points that fall inside the permitted range. Therefore, these results show that the run time is unaffected by even small changes to the chromatographic parameters. These findings show that the adjusted RP-HPLC parameters are suitable for routine pharmaceutical analysis, which is in line with the principles of green analytical chemistry.

Chromatographic Method Optimization

Chromatographic Conditions

Herein, DoE-guided numerical optimization method was utilised to analyse final chromatographic conditions to suit all of the critical analytical performance requirements at the same time. The optimized RP-HPLC method consisted of a phosphate buffer (pH 7.2) for mobile phase A, methanol and a natural deep eutectic solvent (NADES, 30% v/v), and water for the rest of the mobile phase B. The optimum flow rate for the chromatographic operation was chosen as 0.55 mL min^{-1}, beginning with 35% mobile phase B at t=0 min. All the conditions were setup to obtain optimal equilibrium among resolution, column efficiency, peak symmetry, and runtime obeying green principles. Finally, the established optimum conditions were successively employed for method validation and routine sample analysis.[28]

Under ideal RP-HPLC conditions, the representative cyclophosphamide chromatogram showed a distinct, symmetrical peak with a retention period of 2.467 min (Figure 2). The optimization of the chromatographic conditions was done under the following conditions: all representative chromatograms in this study were recorded at the optimized chromatographic conditions. The observed minor differences in retention time of cyclophosphamide between standard samples and assay/stressed samples were not considered to be the result of any changes in the chromatographic conditions, but rather due to differences in the matrix and the stress-treatment conditions. These variations were

in the normal range for gradient RP-HPLC analysis and did not affect the identification of the analytes nor the chromatographic performance.

As indicated by early and steady elution, the improved mobile phase composition and flow rate enable fast analysis without compromising chromatographic performance. A constant baseline, low noise, and no interference peaks surrounding the analyte demonstrated the method's specificity and selectivity. The high-anticipated plate values determined during process optimization, which align with the symmetrical shape and low peak breadth, indicate minimum peak tailing and efficient mass transfer. The short retention time of high-throughput analysis cuts down on the amount of solvent used and makes the analysis process more efficient.[30] Finally, the method validation and routine pharmaceutical analysis are both supported by the representative chromatogram, to ensure the durability and reproducibility of the enhanced chromatographic conditions. A dedicated column-lifetime study under repeated NADES exposure was not performed in this work. However, the method was operated with filtered mobile phases and samples, a guard column was used, and backpressure and system suitability were monitored throughout analysis. These precautions are consistent with standard HPLC column-care recommendations and reduce the likelihood of rapid stationary-phase deterioration.

ICH Q2(R1) Validation Results

Herein, the proposed method was validated to confirm its applicability for quantitative analysis as per guidelines set up by the ICH Q2(R1). We performed the parameters such as linearity, accuracy, precision, robustness, specificity, and sensitivity. The results are shown in Tables 2-10.

Specificity

A method is said to be specific when it achieved the by getting evaluation of blank and control solutions performed. The analysis revealed that there were no interfering peaks during the retention time of cyclophosphamide, which indicated that there was no matrix interference. Furthermore, peak purity assessment of stressed samples employing PDA detection demonstrated that degradation products did not co-elute with the analyte.[31] This was demonstrated by the fact that the analyte peak was spectrally uniform. In light of these findings, the specificity and stability-indicating capabilities of the approach have been validated.

Linearity

Over the concentration range from LOQ to 150% of the target concentration, the technique was linear. The calibration curve revealed a linear correlation (r^2) of 0.999 between peak area and concentration (Table 2). The approach is suitable for accurate quantitative analysis across the working range.

Limit of Detection and Limit of Quantification

A sufficient level of technique sensitivity was established by the LOD and LOQ, which were obtained by employing the Signal-to-Noise (S/N) approach (Table 3). Confirmation that the method is sensitive enough for routine analysis and trace-level quantification is provided by the low limits of detection and limits of quantification values.[32]

Accuracy

Using recovery trials at three different concentration levels 98%, 100%, and 102%-each of which was assessed in triplicate, the accuracy of the results was determined. The fact that the procedure is accurate and does not exhibit any systematic bias is demonstrated by the fact that the mean percentage recoveries ranged from 98 to 102% over an acceptable range of variability (Table 4).[33]

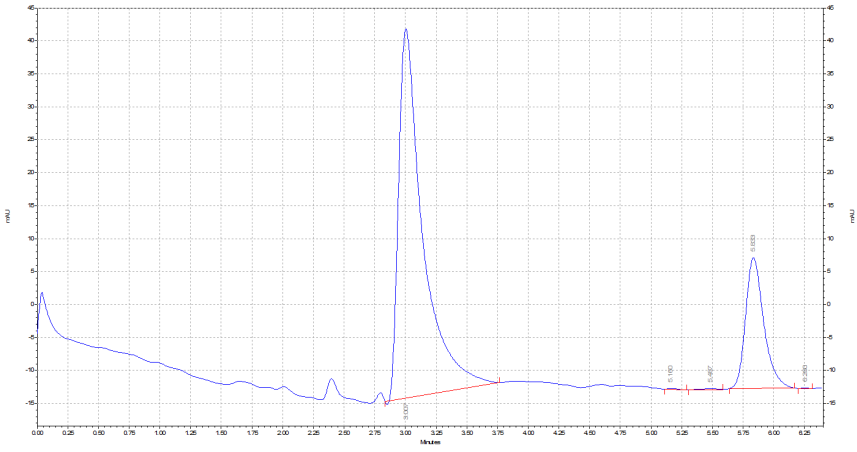


Figure 3: RP-HPLC chromatogram of cyclophosphamide under acidic stress conditions displaying degradation product peak, and parent peak: well, resolved.

Table 6: Robustness study (±10% deliberate variations).

Parameter varied	Condition	Resolution	Tailing factor	Run time (min)
Flow rate	0.50 mL min^{-1}	1.82	1.07	20.8
	0.60 mL min^{-1}	1.75	1.10	18.2
Methanol %	−10%	1.78	1.09	20.1
	+10%	1.80	1.06	18.6
pH	7.0	1.76	1.11	19.5
	7.4	1.83	1.05	18.9

All parameters remained within acceptance limits.

Table 7: Validation summary as per ICH Q2(R1).

Validation parameter	Acceptance criteria	Result	Compliance
Specificity	No interference	No interference	Pass
Linearity	$r^2 \geq 0.999$	0.999	Pass
LOD	S/N ≈ 3	0.3 µg mL^{-1}	Pass
LOQ	S/N ≈ 10	1.0 µg mL^{-1}	Pass
Accuracy	98-102%	99.6-100.4%	Pass
Precision	%RSD ≤ 2%	<1%	Pass
Robustness	No significant change	Stable	Pass

Precision

Method repeatability and intermediate precision were tested. All measurements had Relative Standard Deviation (%RSD) values below 2% (Table 5), confirming ICH-compliant procedure repeatability and reproducibility.

Robustness

In this method, deliberately ±10% variations in selected chromatographic parameters were performed. As a result, no significant effect on system suitability or assay performance was observed (Table 6), confirming the method is found to be robust.[34]

Validation Summary

Table 7 summarises results of method validation ensuring all evaluated parameters met the acceptance standards mentioned by ICH Q2(R1). In all, the proposed method was found to be linear, accurate, precise, robust, specific and sensitive. This enables analyst to perform estimation of drug cyclophosphamide for routine pharmaceutical analysis or quality control testing.

Forced Degradation Studies

Forced degradation studies were conducted to establish the stability-indicating capability of the developed green RP-HPLC method and to evaluate the behavior of cyclophosphamide and its impurities under stress conditions. Cyclophosphamide was subjected to acidic, basic, oxidative, and thermal stress, and the resulting samples were analysed under the optimized chromatographic conditions.[35] The extent of degradation and the formation of degradant peaks varied depending on the applied stress. Representative chromatograms are displayed over a shorter time window (up to 6.5 min) to clearly illustrate the separation and peak purity of cyclophosphamide and its major degradation products. However, all stressed samples were chromatographed over the full 18-19 min run time, during which no additional late-eluting peaks were observed beyond those discussed, confirming method selectivity and stability-indicating capability.

Confirming that cyclophosphamide is susceptible to acid-mediated transformation, the acidic stress chromatogram showed that the medication degraded significantly under acidic hydrolysis conditions. In contrast to the unstressed sample, the chromatogram displayed an extra peak at around 5.833 minutes of retention time after being exposed to 0.1 M hydrochloric acid at increased temperature.[10]

This peak is indicative of an unidentified degradation product that is produced in acidic conditions. Herein, Figure 3 illustrates that the cyclophosphamide peak was identified at a retention time of approximately 3.007 min, with a high degree of peak symmetry and intensity, suggesting partial but not complete degradation. Prominently, the degradant peak (Retention time: 5.883) was well resolved from the main drug peak, with clear baseline separation, demonstrating the selectivity of the developed RP-HPLC method. This proved that the approach could accurately indicate stability. The presence of a distinct degradation peak only under acidic conditions further supports the specificity of the method in differentiating cyclophosphamide from its degradation products.

In a similar manner, the oxidative stress chromatogram of cyclophosphamide displayed a obvious deterioration under oxidative environment, which in turn confirmed the drug's

vulnerability towards oxidation. An extra deterioration peaks at around 3.98 min and 1.627 min were visible in the chromatogram after treatment with 3% hydrogen peroxide; these peaks were not present in the unstressed sample. This signal represents a different degradation mechanism than acidic hydrolysis and is associated with an unidentified oxidative degradant.[36] The Figure 4 depicts the cyclophosphamide peak at 2.41 min, which has good peak symmetry and intensity, demonstrating partial degradation but not total loss. The oxidative degradant peak was well separated from the cyclophosphamide peak, demonstrating that RP-HPLC can resolve degradation products.[37] Oxidation is one of cyclophosphamide's two main stress mechanisms, as other stressors did not degrade it.

Basic hydrolysis and heat stress conditions, on the other hand, caused little or no degradation, and the chromatograms did not show any new peaks. The cyclophosphamide peak exhibited a constant retention period and peak shape, demonstrating the stability of the drug ingredient under basic and thermal stress within the studied parameters. Using PDA detection on the stressed samples that made degradants, peak purity analysis showed that the cyclophosphamide peak was spectrally uniform, with purity angles below purity thresholds. This showed that there were no co-eluting contaminants. The distinct separation of cyclophosphamide from degradation products under the two stress conditions, which yield unknown contaminants, validates that the established RP-HPLC approach is indicative of stability.[38] Overall, these findings indicate that the method can precisely quantify cyclophosphamide despite the presence of its metabolites. This renders it advantageous for stability assessments and routine quality control.

Table 8: Assay results of cyclophosphamide bulk drug (*n*=6).

Sl. No.	Assay (% of label claim)
1.	99.6
2.	100.2
3.	99.8
4.	100.1
5.	99.9
6.	100.0

Mean±SD: 99.93±0.22. %RSD: 0.22. Total impurities: <0.1%.

Bulk Drug Analysis

Herein, the verified RP-HPLC method's applicability was confirmed through the analysis of six cyclophosphamide bulk drug samples. The mean assay was 99.93±0.22% (% RSD=0.22), indicating exceptional precision and accuracy (Table 8). The assay findings varied from 99.6% to 100.2% of the labelled claim. The absence of any extraneous peaks indicating related compounds or breakdown products proved the bulk drug's high purity. The total impurity level was less than 0.1 percent. The results demonstrate that the approach is suitable for routine quality checks on cyclophosphamide bulk material.[18]

Table 9: Assay and content uniformity of cyclophosphamide in-house capsule formulation, Content uniformity (*n*=10).

In-house capsule No.	Assay (% of label claim)
1	99.2
2	100.5
3	101.1
4	99.8
5	100.3
6	99.6
7	100.9
8	99.7
9	100.1
10	100.4

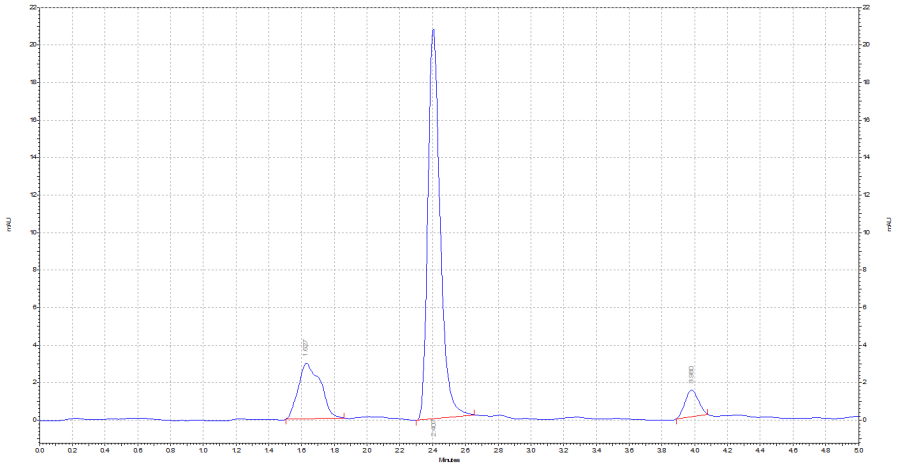


Figure 4: The RP-HPLC chromatogram of cyclophosphamide under oxidative stress conditions exhibiting degradation product peak.

Table 10: Comparison of the proposed RP-HPLC method with reported analytical methods for cyclophosphamide.

Parameter	Proposed Method	Ion Chromatography[41]	RP-HPLC[3]	USP Monograph[42]	Ion Chromatography[43]
Analytical technique	RP-HPLC (NADES-based)	Ion Chromatography	RP-HPLC	TLC	Ion Chromatography
Column	X-Bridge C18	IonPac SCS-1 (2 × 250 mm)	X-Bridge C18	-	IonPac SCS-1
Mobile phase	Phosphate buffer pH 7.2 + MeOH: NADES	0.2 mL H_3PO_4 in 1 L H_2O	Phosphate buffer pH 7.0 + H_2O: MeOH: ACN	Chloroform: MeOH	0.2 mL H_3PO_4 in 1 L H_2O
Organic solvent	NADES (green)	None	Acetonitrile (toxic)	Chloroform	None
Flow rate (mL min^{-1})	0.55	0.25	0.50	-	0.25
Run time (min)	~18-19	~50	~25	-	~50
Detection	UV (200 nm)	Conductivity + UV (200 nm)	UV (200 nm)	UV	Conductivity + UV
Injection volume (µL)	20	25	Not specified	-	25
LOD (µg mL^{-1})	0.3	3 (Imp A, B, D)	Not reported	-	3
LOQ (µg mL^{-1})	1.0	10 (Imp A, B, D)	Not reported	-	10
Linearity range	LOQ-150% (1-30 µg mL^{-1})	10-50 µg mL^{-1}	LOQ-150%	-	10-50 µg mL^{-1}
Correlation coefficient (r^2)	0.999	0.9995-0.9997	0.9987-0.9999	-	0.9995
Accuracy (% recovery)	98-102%	90-110%	Reported acceptable	-	90-110%
Precision (%RSD)	<2.0	1.5-2.6	Acceptable	-	1.5-2.6
Critical resolution	≥1.8 (optimized)	8.2 (Imp D-propanolamine)	Reported good	-	8.2
Forced degradation	Yes (comprehensive)	Not reported	Yes	No	Not reported
QbD approach	Yes	No	No	No	No
Greenness profile	Excellent	Good (no organic solvent)	Poor (ACN-based)	Poor	Good
Instrument requirement	Standard HPLC	Specialized IC	Standard HPLC	TLC scanner	Specialized IC
Application	API + in-house capsules	API, powder, in-house capsules	In-house capsules only	API	API, powder

In house capsule Formulation Analysis

The optimized approach effectively analysed cyclophosphamide in-house capsule formulations. Ten laboratory-prepared capsule units were randomly selected and individually analysed to evaluate the applicability of the developed method for finished dosage forms. Capsule contents were accurately weighed, dissolved in the diluent, sonicated, filtered through a 0.45 µm membrane filter, and diluted to the required concentration prior to chromatographic analysis. The assay study was performed and the results are between 99.2% and 101.1%, and an average of

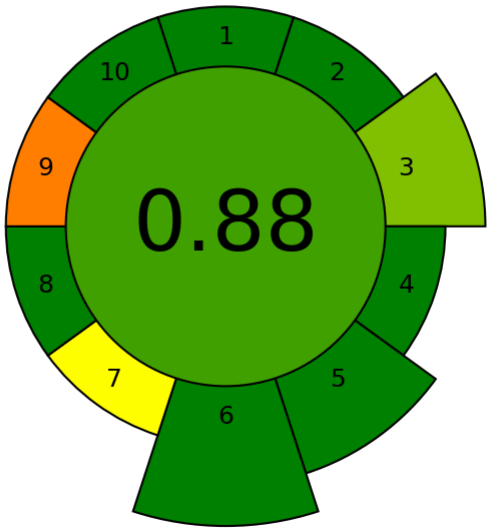


Figure 5: AGREE score (0.88) of the developed NADES-based RP-HPLC method.

100.16±0.63%. The Table 9 shows that the acceptable value was within the pharmacopeia's range. After 10, 20, and 30 minutes, the mixture released the drug at rates of 62.4±2.1%, 82.7±1.8%, and 96.3±1.4%. This achieved ≥80% release within 30 min. The method is specific for evaluating finished pharmaceutical goods because excipients did not interfere with the chromatograms.[23] In all, the method proved reliable and robust for both bulk drug and in-house capsule formulation analysis, supporting its routine application in pharmaceutical quality control.

Mean±SD: 100.16±0.63 Acceptance Value (AV): Within pharmacopeial limits.

Comparison with Reported Methods

Table 10 herein compares the developed method with established analytical techniques. In comparison to other literature approaches, the proposed RP-HPLC technique employs eco-friendly solvents, minimizes organic solvent usage, maintains an acceptable runtime, and adheres to Analytical Quality-by-Design (AQbD) principles. The method's environmentally friendly and sustainable analytical characteristics are improved by its comparable or superior sensitivity, precision, and accuracy when utilizing a Natural Deep Eutectic Solvent (NADES). This method is optimal for reviewing of routine bulk drugs and pharmaceutical formulations.

Greenness Assessment

We carefully checked if the new RP-HPLC process was safe for the environment by using the AGREE metric, solvent consumption analysis, and the Analytical Eco-Scale method. Figure 5 shows that the suggested NADES-based method got an excellent AGREE score of 0.88, which means that it followed the twelve principles of green analytical chemistry[39] very well. Compared to traditional RP-HPLC methods that use acetonitrile, this method had a better environmental profile. This procedure did not utilise any toxic or harmful solvents and used only natural deep eutectic solvents, which are degraded naturally. The incorporation of NADES partially substituted the conventional organic solvent content of the mobile phase, thereby improving the environmental sustainability of the chromatographic method. In addition, reduced solvent waste and environmental impact were achieved through a combination of a lower flow rate, a shorter effective analytical window, and the partial replacement of organic solvent with NADES. Thanks to its minimal reagent toxicity, waste generation, and energy usage penalty points, the approach was rated "Excellent/Green" in the Analytical Eco-Scale evaluation. The AGREE, solvent usage, and Eco-Scale assessments show that the proposed RP-HPLC method is greener than standard chromatographic methods while preserving analytical performance.[5] Green chemistry principles are included without affecting accuracy, robustness, or stability-indicating capabilities.

CONCLUSION

Utilizing a NADES-based mobile phase and an AQbD-driven optimization approach, an RP-HPLC technique that is environmentally friendly, robust, and stability indicating was effectively developed and validated for the quantification of cyclophosphamide in bulk drug and in-house capsule formulations. The identification of chromatographic settings that yield a resolution of at least 1.8, a tailing factor not exceeding 1.1, theoretical plates approaching 2500, and a total run time of approximately 18-19 min, with the analyte eluting at approximately 2.5 min, was achieved through Design of Experiments (DoE)-guided numerical optimization. Method validation per ICH Q2 (R1) shown exceptional linearity in the LOQ-150% range (r^2=0.999), elevated sensitivity (LOD 0.3 µg mL^{-1}; LOQ 1.0 µg mL^{-1}), reasonable accuracy (98-102% recovery), and precision with % RSD under 2%. The method's stability was confirmed in forced degradation tests, which allowed for the observation of separate breakdown products under acidic and oxidative stress conditions alone. The cyclophosphamide peak showed spectrum uniformity, according to the PDA peak purity analysis. Impurity levels were less than 0.1% and assay values were very close to the label claim for both bulk drug (99.6-100.2% accuracy) and in-house capsule formulations (mean 100.16% accuracy). The proposed method reduced the dependence on conventional hazardous organic solvents by partially replacing them with a biodegradable natural deep eutectic solvent while maintaining satisfactory chromatographic performance. This method received a high environmental sustainability score of 0.88 from the AGREE tool. Its "Excellent/Green" Eco-Scale rating attests to its eco-friendliness. If NADES works well in RP-HPLC mobile

phases, it could pave the way for using this method with other pharmaceutical chemicals and anticancer drugs, even those in biological matrices.

ABBREVIATIONS

AQbD: Analytical Quality by Design; **RP-HPLC:** Reverse Phase High-Performance Liquid Chromatography; **NADES:** Natural Deep Eutectic Solvents; **DoE:** Design of Experiments; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **RSD:** Relative Standard Deviation; **PDA:** Photodiode Array; **UV:** Ultraviolet; **ANOVA:** Analysis of Variance; **API:** Active Pharmaceutical Ingredient; **EG:** Ethylene Glycol; **Glc:** Glucose; **ChCl:** Choline Chloride; **ChCl: EG:** Choline Chloride-Ethylene Glycol; **ChCl-Glc:** Choline Chloride-Glucose; **KH_2PO_4:** Potassium Dihydrogen Phosphate; **NaOH:** Sodium Hydroxide; **HCl:** Hydrochloric Acid; **H_2O_2:** Hydrogen Peroxide; **HPLC:** High-Performance Liquid Chromatography; **HTLC-ESI-MS/MS:** High-Throughput Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry; **LC-MS/MS:** Liquid Chromatography-Tandem Mass Spectrometry; **TLC:** Thin Layer Chromatography; **IC:** Ion Chromatography; **MeOH:** Methanol; **ACN:** Acetonitrile; **v/v:** Volume per Volume; **w/v:** Weight per Volume; **min:** Minutes; **µL:** Microlitre; **µg:** Microgram; **mL:** Millilitre; **mg:** Milligram; **nm:** Nanometre**; °C:** Degree Celsius; **S/N:** Signal-to-Noise Ratio; SD**:** Standard Deviation; **r^2:** Correlation Coefficient; **AV:** Acceptance Value; **RT:** Retention Time.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

All research and writing have been equally contributed by both authors.

SUMMARY

An AQbD-guided green RP-HPLC method using NADES was developed for cyclophosphamide analysis in bulk and in-house capsule formulations. Factorial design optimized chromatographic conditions, achieving rapid separation at 2.5 min with excellent resolution, peak symmetry, and efficiency. The method demonstrated strong validation performance with high linearity (r^2=0.999), sensitivity, accuracy, and precision as per ICH guidelines. Stability-indicating capability was confirmed through forced degradation studies. Application to pharmaceutical samples showed assay values near label claim with minimal impurities. Greenness assessment (AGREE score 0.88) indicated a significant reduction in hazardous solvent use, confirming the method as robust, eco-friendly, and suitable for routine quality control.

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Table S1: Experimental design matrix showing factorial combinations of chromatographic variables and their effects on resolution, tailing factor, theoretical plates, and run time.

		Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Response 1	Response 2	Response 3	Response 4
Std	Run	A:Buffer pH	B:% Methanol in mobile phase B	C:% NADES in mobile phase B	D:Initial %B at t=0	E:Flow rate	Resolution	Tailing Factor	Theoretical Plates	Run Time
			%	%	%	mL/min				min
4	1	7.2	45	30	25	0.55	1.45	1.45	1850	22.1
14	2	7.2	35	50	35	0.45	1.42	1.38	1920	18.8
1	3	6.8	35	30	25	0.55	1.68	1.12	2150	19.2
2	4	7.2	35	30	25	0.45	1.65	1.15	2080	17.9
8	5	7.2	45	50	25	0.45	1.52	1.28	1980	19.5
3	6	6.8	45	30	25	0.45	1.48	1.32	1950	18.2
15	7	6.8	45	50	35	0.45	1.72	1.08	2280	17.5
6	8	7.2	35	50	25	0.55	1.7	1.1	2250	18
5	9	6.8	35	50	25	0.45	1.38	1.48	1820	23.2
10	10	7.2	35	30	35	0.55	1.35	1.42	1880	19.8
9	11	6.8	35	30	35	0.45	1.62	1.18	2120	20.1
13	12	6.8	35	50	35	0.55	1.6	1.2	2050	18.5
7	13	6.8	45	50	25	0.55	1.5	1.3	2020	20.5
18	14	7	40	40	30	0.5	1.46	1.35	1970	19
11	15	6.8	45	30	35	0.55	1.75	1.05	2320	17.8
19	16	7	40	40	30	0.5	1.73	1.07	2300	18.3
17	17	7	40	40	30	0.5	1.85	1.02	2450	18
16	18	7.2	45	50	35	0.55	1.88	1.01	2480	17.9
12	19	7.2	45	30	35	0.45	1.9	1	2500	17.8

Table S2: ANOVA for selected factorial model Response 1: Resolution .

Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.0000	0				
Curvature	0.0162	1	0.0162	0.5486	0.4690	
Residual	0.5010	17	0.0295			
Lack of Fit	0.4212	15	0.0281	0.7038	0.7278	not significant
Pure Error	0.0798	2	0.0399			
Cor Total	0.5172	18				

Factor coding is **Coded**. Sum of squares is **Type III – Partial**.

Table S3: ANOVA for selected factorial model. Response 2: Tailing Factor.

Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.0000	0				
Curvature	0.0136	1	0.0136	0.5229	0.4794	
Residual	0.4417	17	0.0260			
Lack of Fit	0.3784	15	0.0252	0.7975	0.6864	not significant
Pure Error	0.0633	2	0.0316			
Cor Total	0.4553	18				

Factor coding is Coded. Sum of squares is Type III – Partial.

Table S4: ANOVA for selected factorial model. Response 3: Theoretical Plates.

Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.0000	0				
Curvature	47329.93	1	47329.93	1.01	0.3297	
Residual	7.989E+05	17	46996.69			
Lack of Fit	6.783E+05	15	45222.92	0.7500	0.7069	not significant
Pure Error	1.206E+05	2	60300.00			
Cor Total	8.463E+05	18				

Factor coding is Coded. Sum of squares is Type III – Partial.

Table S5: ANOVA for selected factorial model. Response 4: Run Time.

Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.0000	0				
Curvature	1.39	1	1.39	0.5740	0.4590	
Residual	41.16	17	2.42			
Lack of Fit	40.63	15	2.71	10.29	0.0921	not significant
Pure Error	0.5267	2	0.2633			
Cor Total	42.55	18				

Factor coding is **Coded**. Sum of squares is **Type III – Partial.**

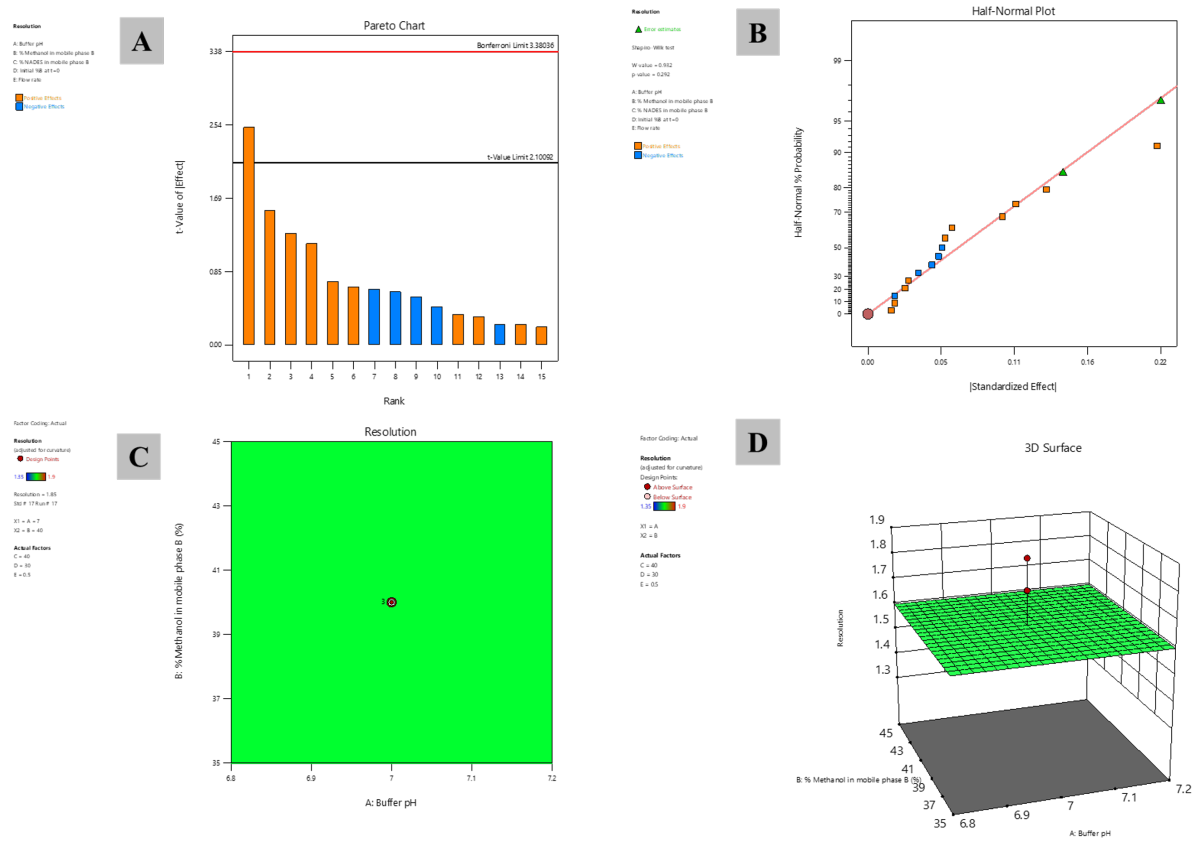


Figure S1: (A) Pareto chart, (B) predicted versus experimental plot, (C) contour plot, and (D) response surface plot for optimization of chromatographic resolution.

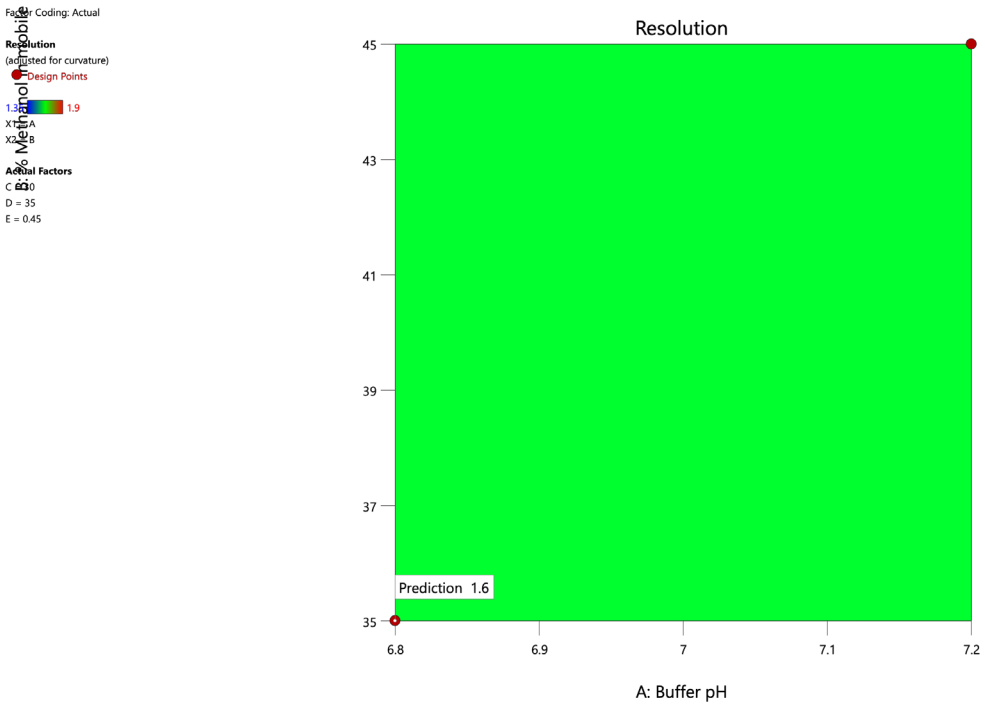


Figure S2: Design space contour plot showing the optimal region for tailing factor with a predicted value of 1.6.

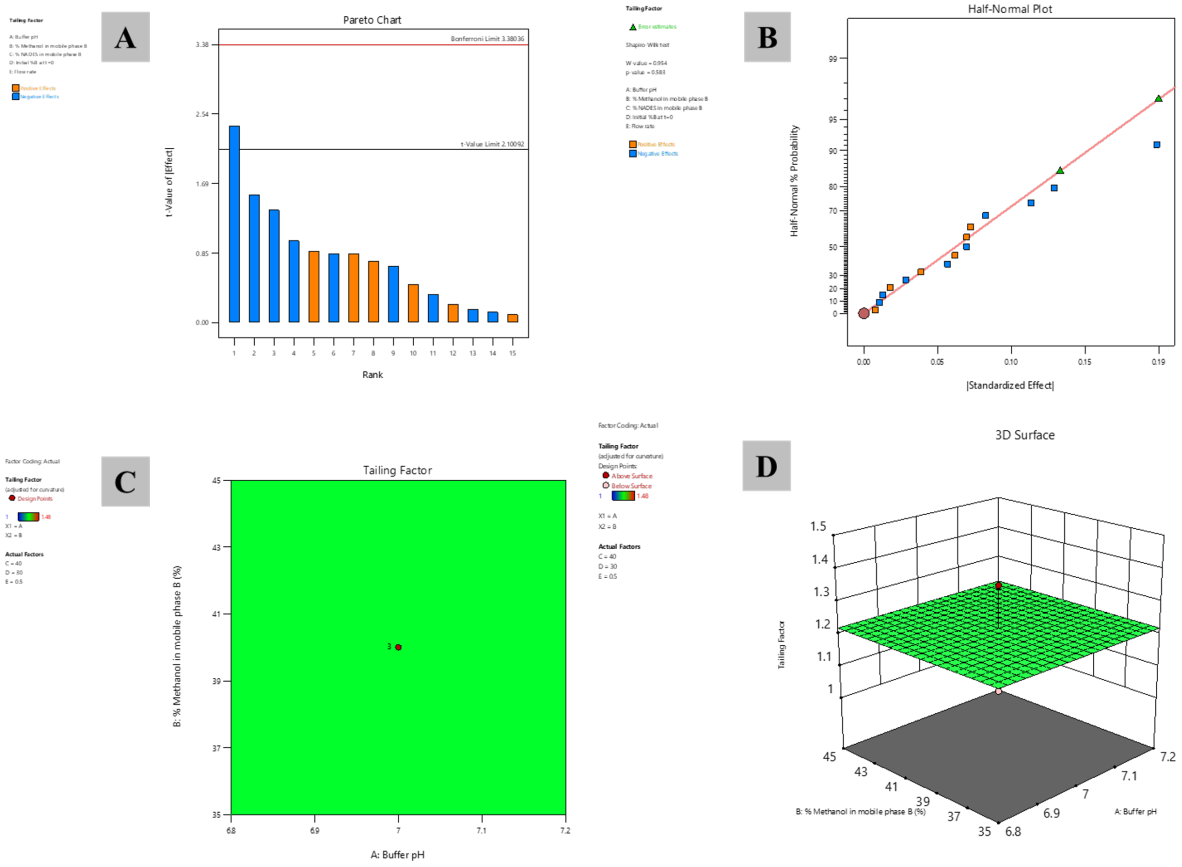


Figure S3: (A) Pareto chart, (B) predicted versus experimental plot, (C) contour plot, and (D) response surface plot for optimization of tailing factor.

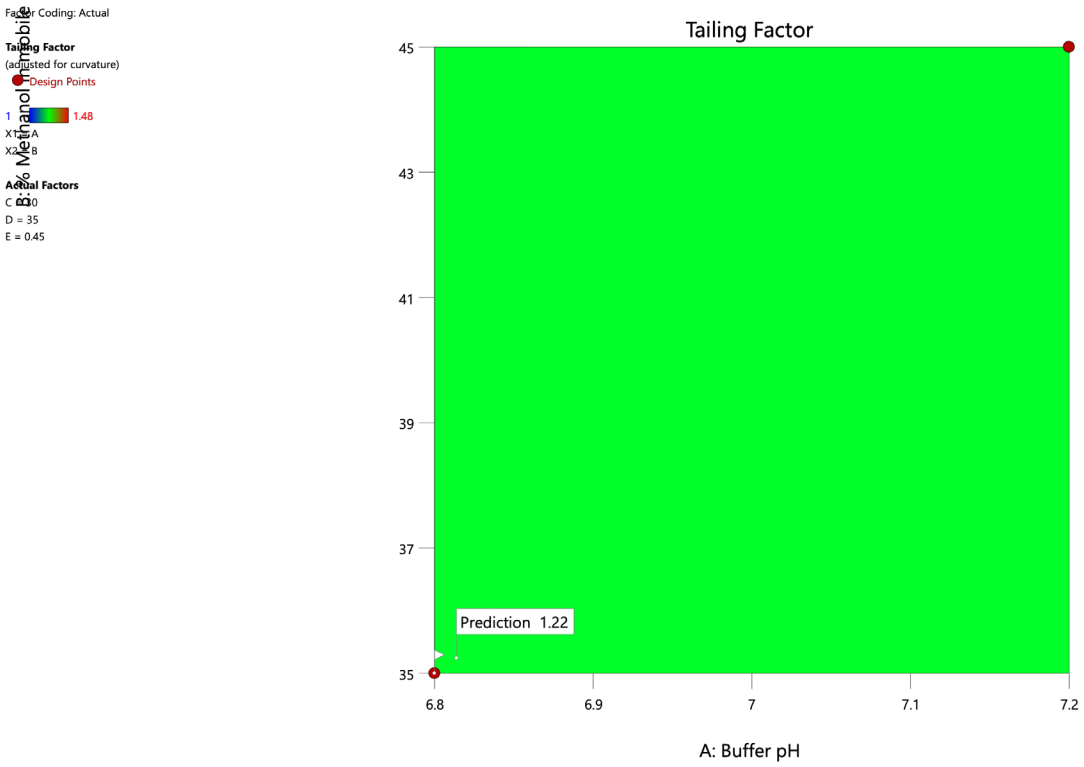


Figure S4: Design space contour plot showing the optimal region for tailing factor with a predicted value of 1.22.

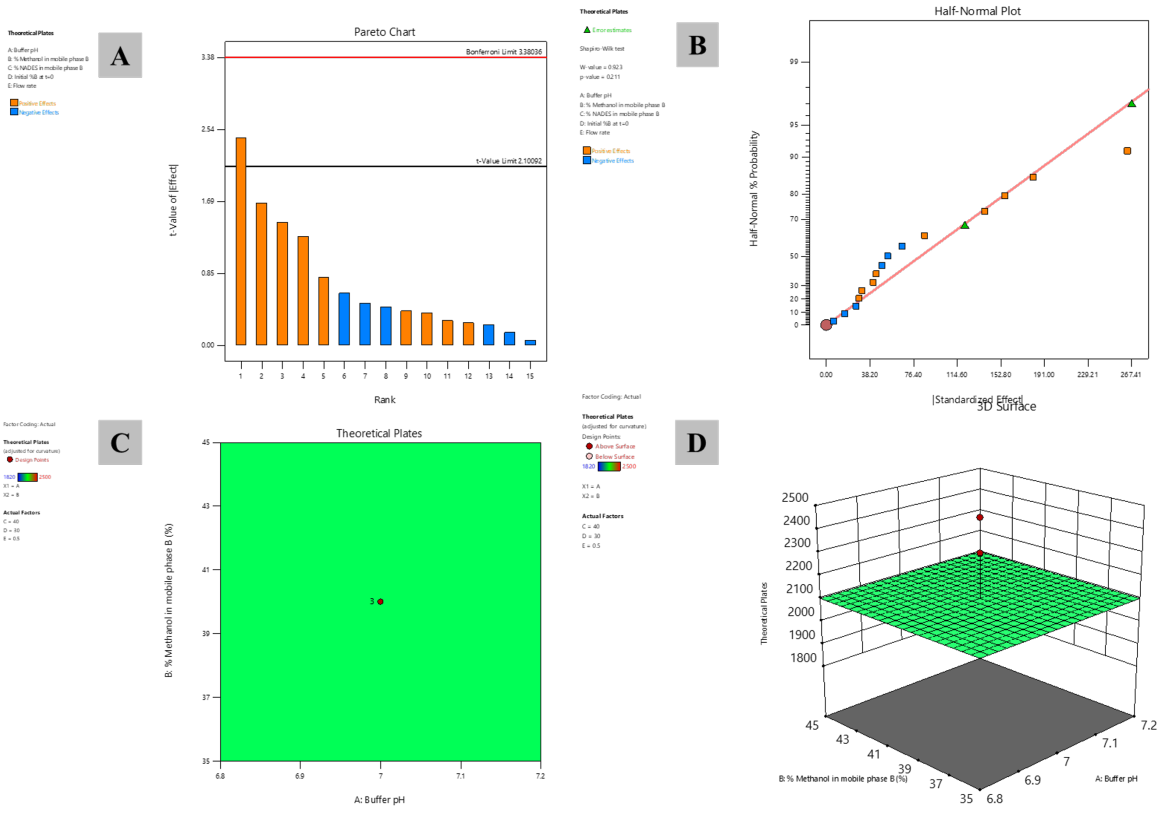


Figure S5: Pareto chart (A), predicted versus experimental plot (B), contour plot (C), and response surface plot (D) illustrating optimization of theoretical plate number.

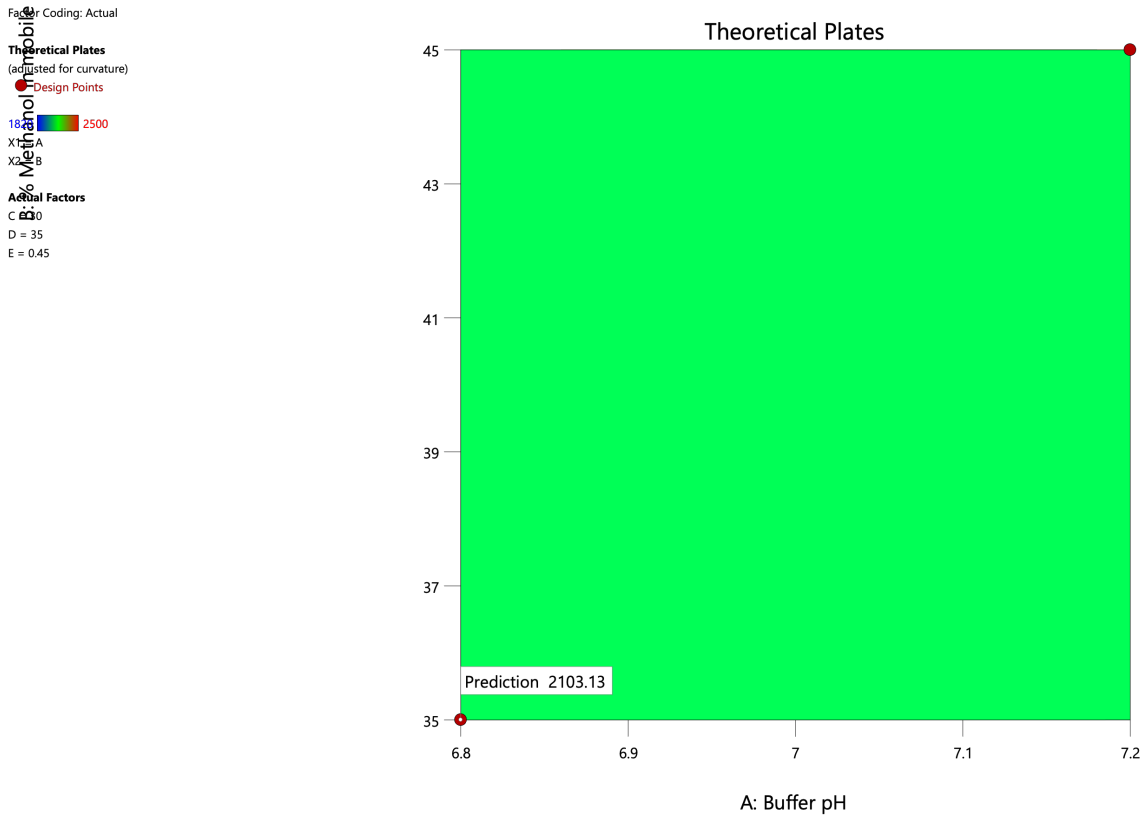


Figure S6: Design space contour plot showing the optimal region for theoretical plates with a predicted value of 2103.13.

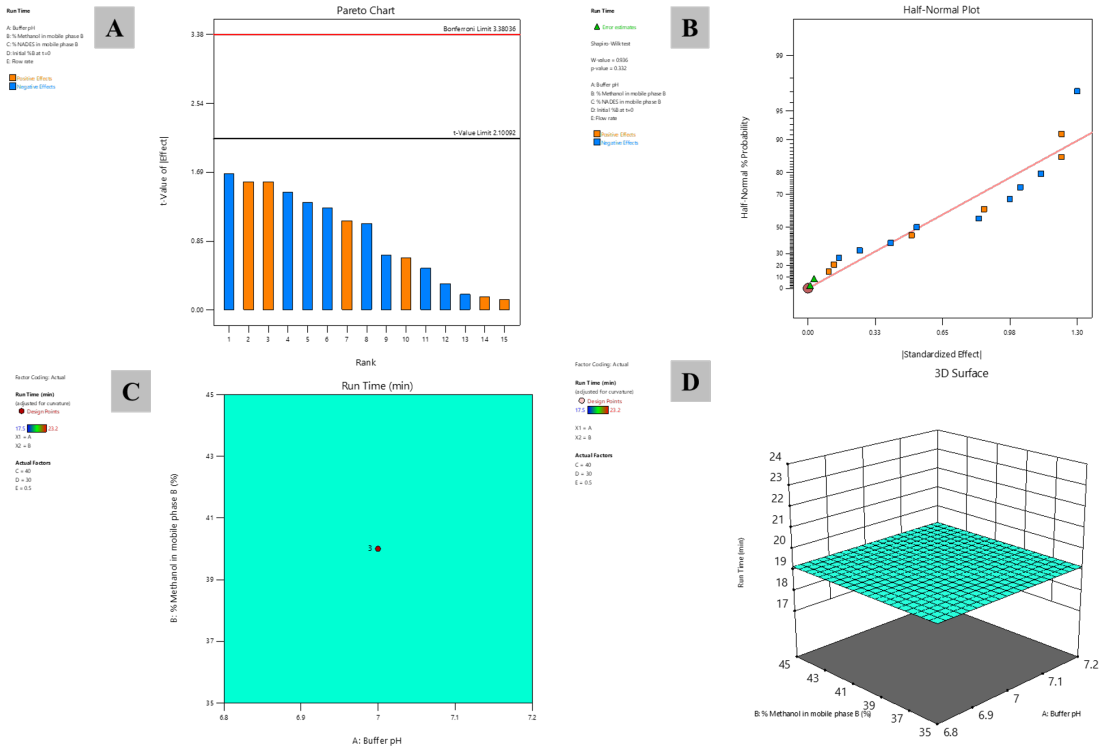


Figure S7: Pareto chart (A), predicted versus experimental plot (B), contour plot (C), and response surface plot (D) illustrating factorial design-based optimization of chromatographic run time.

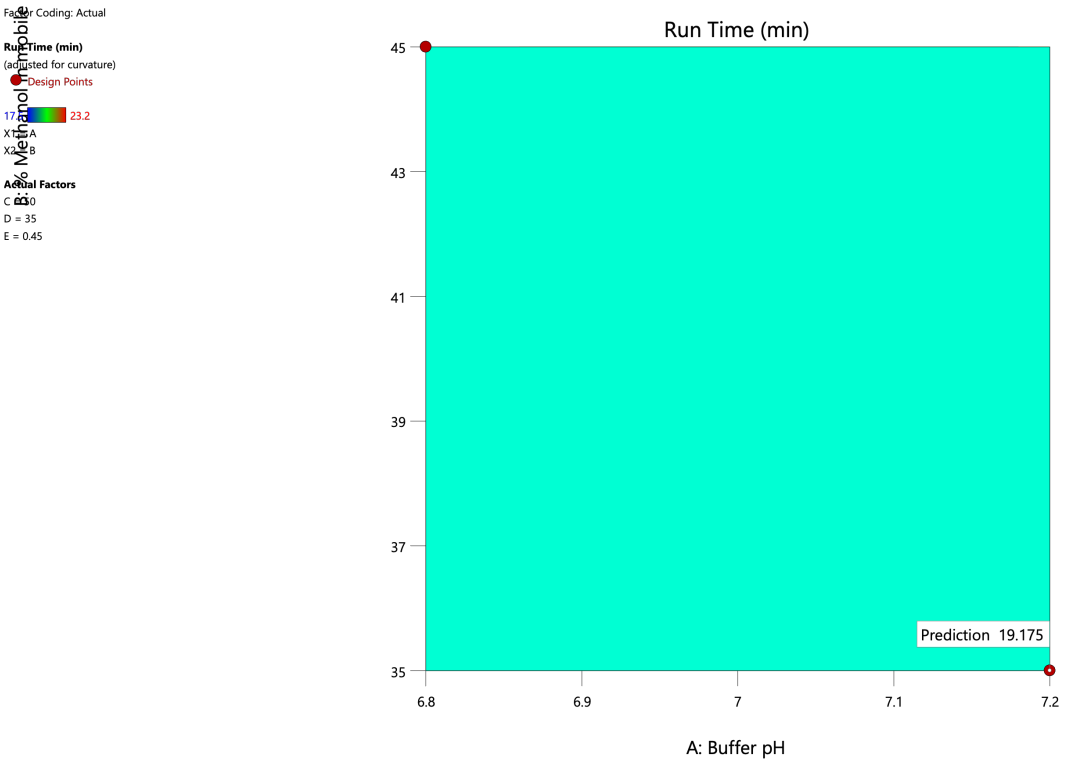


Figure S8: Design space contour plot showing the optimal region for run time with a predicted value of 19.17 min.