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A quality by design approach with comprehensive green analytical chemistry assessment: Development, validation, and application of a high-performance liquid chromatographic method for quantifying meropenem trihydrate in nanosponges and marketed formulations

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ABSTRACT

Meropenem trihydrate (MPN), pivotal in antimicrobial therapeutics, necessitates accurate analytical methods for its quantification across pharmaceutical formulations. The research aimed to develop a Quality by Design (QbD)-driven high-performance liquid chromatography (HPLC) method and validate it for the quantification of MPN in traditional and novel formulations, with a focus on environmental sustainability. The study employed a QbD approach to develop an HPLC method, ensuring its robustness and adaptability. The method's universality was evaluated in both traditional powders for injection formulation and a novel beta-cyclodextrin nanosponges formulation. Rigorous validation was conducted per the International Conference on Harmonisation Q2 (R1) guidelines, including extensive stability and degradation studies to ascertain the method's tenacity under multifarious conditions. The QbD-driven HPLC method showcased impeccable precision and accuracy, with a recovery rate of 99% for the marketed product and an encapsulation efficiency of 88.7% for nanosponges. Furthermore, seven different green analytical chemistry tools were used, and they indicated a significant reduction in environmental impact compared to pre-existing methodologies. In conclusion, our QbD-driven HPLC method for MPN quantification melds technical prowess with environmental responsibility, signifying a noteworthy stride in pharmaceutical research. The method's high precision and stability assessment provide clinicians with a reliable tool for ensuring accurate dosing in critically ill patients, ultimately enhancing therapeutic efficacy and reducing treatment failure risks. Furthermore, the method supports sustainable drug analysis, minimizing ecological hazards associated with pharmaceutical waste. The method's adaptability and greenness set a benchmark for future analytical methodologies, emphasizing analytical rigor, and ecological conscientiousness.

INTRODUCTION

The global healthcare landscape grapples with chronic bacterial infections, resulting in prolonged patient suffering and inflated healthcare expenses. These infections profoundly impact patients' quality of life and necessitate effective treatment strategies [1,2]. In 2017 and 2019, the Indian Council of Medical Research reported several pathogens that were found to be responsible for such maladies. These findings have been corroborated by the World Health Organization and the Center for Disease Control and Prevention, signifying the escalating necessity to address this global health threat [2–5].

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Among the arsenal of antibiotics utilized to combat chronic bacterial infections, meropenem trihydrate (MPN) is a broad-spectrum and last-resort antibiotic option [6]. Its effectiveness against a vast range of bacteria designates it as an invaluable therapeutic agent for managing severe infections. Currently, MPN is marketed as an intravenous formulation with various doses in the treatment regimen. However, the quest for optimization has driven research toward nanoformulations such as meropenem-loaded solid lipid nanoparticles [7], liposomes [8], and mesoporous silica nanoparticles [6]. These innovations promise enhanced drug delivery and heightened treatment efficacy [9,10].

The accurate and precise quantification of MPN in dosage forms is paramount for elucidating its dose-dependent therapeutic action. Various analytical methods have been reported for MPN quantification, including ultraviolet (UV) spectrometry [11], high-performance liquid chromatography-ultraviolet spectrometry (HPLC-UV) [11–15], and liquid chromatography-mass spectrometry (LC-MS) [15,16]. Despite the availability of these methodologies, high-performance liquid chromatography (HPLC) with UV detection is often the preferred choice for quantifying drugs in pharmaceutical formulations, credited for its rapidness, robustness, reliability, selectivity, and sensitivity, which surpass traditional UV techniques [17–19]. Additionally, the lower cost and simpler instrumentation of HPLC-UV compared to LC-MS make it more accessible for a wide range of analytical laboratories. Therefore, we used the HPLC-UV method in the current investigation to quantify MPN [11,12]. However, a review of the literature reveals significant limitations within reported methods for estimating MPN. These shortcomings include poor sensitivity [14,20,21], excessive use of organic solvents that are not environmentally friendly and lead to high analysis costs [14,20–22], long run times that render the process time-intensive [11,23–25], complex method of elution [26–28], complicated mobile phase preparations [29], the complex and costly use of detectors such as mass spectrometry [26–28], incomplete method validation [14,20–28,30–33], and a notable absence of a reliable analytical HPLC method for the estimation of MPN. The reported analytical methods are lacking in providing comprehensive details of the impact of various factors as well as the interaction effect of factors on responses [11,14,20–30,31–33]. Additionally, there is a dearth of methods for quantifying the MPN in the marketed formulation and novel nanoformulation (nanosponges). Hence, the pressing need remains to develop a robust and validated HPLC-UV analytical method to quantify MPN in pharmaceutical formulations. The detailed drawbacks of existing methods are summarized in Table 1 below, highlighting the novelty and necessity of this study to address the gaps in current research and methodology.

Systematic evaluation of factors affecting analytical method performance is essential but challenging. This study employs a quality by design (QbD) approach to method development, focusing on identifying and controlling critical factors to ensure robustness, reliability, accuracy, and precision. The QbD approach also minimizes variability and enhancing method performance [34–37]. The objective is to develop an HPLC-UV method for quantifying MPN in pharmaceutical formulations using this systematic framework.

This study also examines the stability and degradation behavior of MPN under stress conditions to identify degradation pathways and stability concerns, ensuring the safety and efficacy of pharmaceutical formulations. Additionally, it addresses gaps in the literature by analyzing MPN quantification in marketed formulations and innovative nanosponges.

Growing awareness of environmental impacts from chemicals underscores the need for sustainable practices aligned with the United Nations' Sustainable Development Goals (SDGs), particularly SDG 12: "Responsible Consumption and Production". These efforts also support SDG 3: "Good Health and Well-being" and SDG 14: "Life Below Water" by minimizing harmful chemical effects, and driving eco-friendly analytical methodologies [38,39].

Green analytical chemistry (GAC) promotes sustainable analytical practices that balance accuracy, robustness, and environmental stewardship [40,41]. This paradigm shift prioritizes energy efficiency, waste reduction, and minimal environmental impact in liquid chromatography, aligning with SDG 12 and related goals. Advanced greenness evaluative tools, from Analytical Eco-Scale (AES) to the analytical greenness (AGREE) calculator, provide comprehensive assessments of analytical methods, supporting a synergistic evaluation approach [42,43]. Our developed method was compared against the existing analytical methods ("reported method with most citations in Scopus" [11] and the "latest reported method available in Scopus" [14]). These methods were chosen due to their widespread acclaim and the most up-to-date information available in the scientific community. By juxtaposing our developed method with these highly regarded counterparts, we aimed to demonstrate its sustainable essence and competitive prowess within existing analytical approaches.

This research advances analytical methodologies for MPN quantification, providing insights into its stability and degradation behavior. Our greenness assessment highlights the method's minimal environmental impact, achieved through advanced tools, and sustainable practices. These efforts contribute to developing MPN-based formulations for chronic bacterial infections while promoting eco-friendly analytical research.

MATERIALS

MPN (purity ≥98%) was procured from Sigma Aldrich. The buffer salts utilized in this research, including ammonium acetate (purity ≥97%), ammonium formate (purity 97%), and sodium acetate (purity ≥99%), were procured from Merck Life Science Pvt. Ltd. (Mumbai, India). Potassium dihydrogen phosphate (purity, min. 99%) was from Himedia Labs Pvt. Ltd. (Mumbai, India), and sodium hydroxide pellets (purity, min. 97%) were from Finar Ltd. (Gujarat, India). The HPLC-grade solvents of acetonitrile (purity, min 99.9%), hydrochloric acid (37%), hydrogen peroxide (30%), glacial acetic acid (100%), and orthophosphoric acid (purity, min 85%) were procured from Merck Life Science Pvt. Ltd. (Mumbai, India). Methanol (purity, min. 99.80%), triethylamine LR (purity, min. 99.00%), and formic acid (purity, 85%) were purchased from Finar Ltd. (Gujarat, India). Beta-cyclodextrin (β-CD) was procured as *ex-gratis*

Table 1. Comparison of the proposed analytical method with reported methods.

Reported methods	Drawbacks of the reported method	Advantages of the proposed method over reported methods	Reference of the reported method
HPLC-UV	✓ DL and QL aren't specified ✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied stability and degradation studies in the method's development	✓ Development under a QbD approach, a novel strategy in contrast to previous methods, systematically addressed variability and risk. ✓ Complete and thorough validation of the optimized method ✓ Comprehensive stability studies and forced degradation studies have been performed with various stressor agents, demonstrated the specificity of the method ✓ Increased sensitivity ✓ Accurate and robust method ✓ Simplicity and adherence to green principles characterized the method as both eco-friendly and user-friendly. ✓ Reduction of heavy capital and operating costs enhanced cost-effectiveness. ✓ Comprehensive green analysis affirmed the method's environmental sustainability.	[30]
HPLC-UV	✓ DL and QL aren't specified ✓ Complicated mobile phase composition ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability and degradation studies in the method's development ✓ High injection volume		[29]
HPLC-MS	✓ Complex gradient elution ✓ Expensive MS detector ✓ Incomplete method validation ✓ Increased capital and operating cost ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability and degradation studies in the method's development		[26–28]
HPLC-UV	✓ Longer run time ✓ Increased capital and operating cost ✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied stability and degradation studies in the method's development ✓ High injection volume		[23, 25]
HPLC-UV	✓ Longer run time ✓ Increased capital and operating cost ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability and degradation studies in the method's development ✓ High injection volume		[11]

(continued)

Reported methods	Drawbacks of the reported method	Advantages of the proposed method over reported methods	Reference of the reported method
HPLC-UV	✓ Poor sensitivity ✓ Incomplete method validation ✓ High organic phase ratio ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability in the method's evelopment ✓ High injection volume		[20]
HPLC-UV	✓ Poor sensitivity ✓ High organic phase ratio ✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied stability and degradation studies in the method's development ✓ High injection volume		[14, 21]
HPLC-UV	✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability and degradation studies in the method's development ✓ High injection volume		[31]
HPLC-UV	✓ High organic phase ratio ✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability and degradation studies in the method's development		[22]
HPLC-UV	✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability and degradation studies in the method's development		[32]
HPLC-UV	✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied stability and degradation studies in the method's development ✓ High injection volume.		[33]
HPLC-UV	✓ Longer run time ✓ Increased capital and operating cost ✓ Incomplete method validation ✓ Failure to identify and manage potential sources of variability and risk ✓ Lack of applied mobile phase stability and degradation studies in the method's development ✓ High injection volume		[24]

from Ashland, India, and diphenyl carbonate (DPC) was purchased from Spectrochem, Pvt. Ltd. (Mumbai, India) for nanosponges preparation. Ferric chloride (purity ≥99%) and acetone (purity, 99% min) were procured from Merck Life Science Pvt. Ltd. (Mumbai, India).

INSTRUMENTATION

The present research study employed the LC-2010C HT, a HPLC instrument manufactured by Shimadzu, Japan. This advanced device is equipped with a dual-wavelength UV detector, a column oven, and an autosampler, all provided by Shimadzu. To record and analyze the chromatographic data, LC Solution software version 5.57 was utilized. The various analytical columns used are Kinetex C_{18} (250 mm*4.6 mm*5 µ) column from Phenomenex, USA; Hyperclone C_{18} (250 mm*4.6 mm*5 µ) column from Phenomenex; Chromasol Jade C_{18} (250 mm*4.6 mm*5 µ) column from Intek; and Chromasol Onyx C_{18} (250 mm*4.6 mm*5 µ) column from Intek.

The mobile phase was filtered through a 0.22-micron cellulose nitrate membrane using a Millipore glass vacuum filtration unit and sonicated for 10 minutes in a GT Sonic Ultrasonic Cleaner (Servewell Instruments, India). pH measurements were performed with a Systronics Micro-Controller-based pH system 361, ensuring precise control.

METHOD DEVELOPMENT

QbD approach

The study adopted the Analytical QbD framework to develop a robust and reproducible HPLC-UV method for estimating MPN in marketed and nanosponges formulations, aligning with International Conference on Harmonisation (ICH) Q2(R1), Q8(R2), and Q14 guidelines [44–46].

Defining the analytical target profile (ATP) and determining critical performance attributes (CPAs)

The ATP was defined to ensure method performance. CPAs were identified based on prior knowledge, literature review, and preliminary trials [35].

Risk assessment

A systematic risk assessment was performed to evaluate the impact of Critical Method Attributes (CMAs) and Critical Procedure Parameters (CPPs) on CPA. This risk assessment was conducted using an Ishikawa Fishbone Diagram (Fig. 1) to identify potential risks associated with method and procedure attributes. Preliminary trials conducted using a one-factor-at-a-time (OFAT) design, allowed for the identification of critical factors affecting method performance [47].

Preparation of the mobile phase and standard solutions

The mobile phase was prepared by dissolving HPLC-grade ammonium acetate in 1,000 ml of MilliQ water and adjusting to the desired pH of 5.1. A 1,000 µg/ml standard stock solution of MPN was prepared by dissolving 10 mg of MPN in the mobile phase, with subsequent dilutions to achieve the required concentrations.

Design of experiments (DoEs)

DoE principles were applied to optimize CMAs and CPPs. Screening and optimization experiments were conducted to evaluate the effect of independent factors on dependent variables using Design-Expert software (v9.0.5.1, Stat-Ease Inc., USA).

Screening of independent factors using fractional factorial design

A two-level fractional factorial design ($2V^{5-1}$) with three center points and triplicates was employed to screen five independent factors (Table 2) based on their main and interaction effects. The design suggested 51 experiments, enabling efficient factor prioritization. Factor levels were chosen based on literature and preliminary studies [48].

Optimization of factors using Box-Behnken design

Following the screening phase, the three most significant factors were optimized using the Box-Behnken design, an economical response surface methodology suitable for optimizing three independent factors. This design estimates the main and second-order interaction effects of input factors on responses with minimal experimentation. The software suggested 12 experiments, and the optimum levels of the factors were identified through these trials.

Development and verification of the method operable design region (MODR)

The MODR defines a flexible multidimensional space where the interaction of independent factors ensures minimal variability [35]. MODR was established through numerical optimization, with specified limits for all individual responses.

Numerical optimization identified the optimal solution with the highest desirability score. The MODR was further validated through three confirmatory experimental runs, and the results were consistent with model-predicted values.

VALIDATION OF THE OPTIMIZED HPLC METHOD

Validation was conducted according to ICH Q2(R1) guidelines to assess the method's performance for its intended use [49].

Specificity

Specificity was evaluated by injecting replicates of blank (diluent), MPN standard (5 µg/ml), marketed formulation (equivalent to 10 µg/ml of MPN), placebo, and novel nanosponges formulation (equivalent to 10 µg/ml of MPN). Chromatograms were analyzed for potential interference from excipients or diluents at MPN's retention time (Rt).

Linearity

The method's linearity was tested using MPN concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, and 12.5 µg/ml. Each concentration was injected in quintuplicate, and a linear regression graph was plotted between the concentration (µg/ml) and peak area (mV-min). The regression equation's slope and intercept were calculated [50].

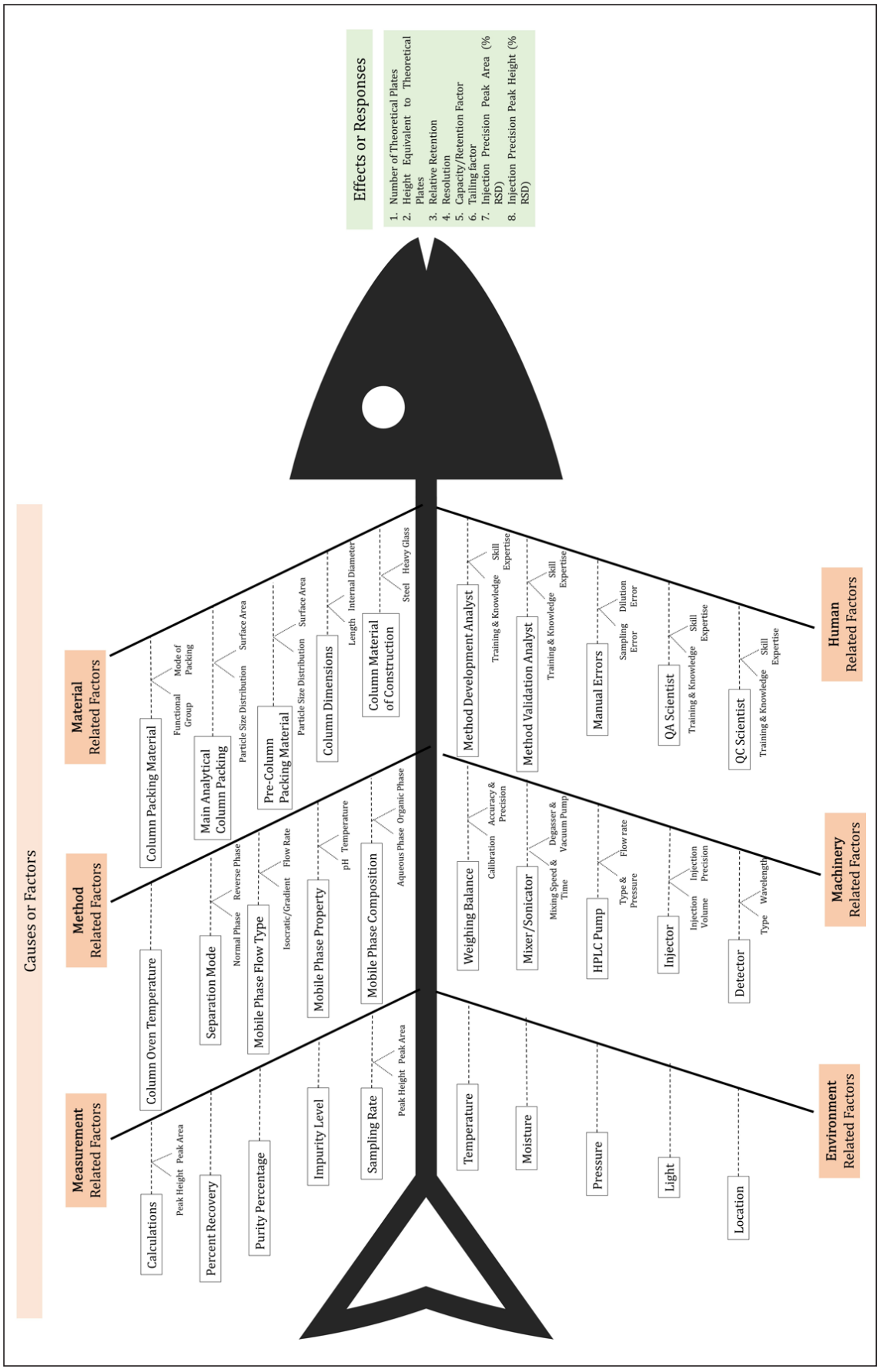


Figure 1. Identification of risk factors by the Ishikawa Fishbone diagram.

Table 2. Independent factors with its level, center points, and their dependent factors in $2V^{(5-1)}$ fractional factorial design.

Factors	Level of factor		Center points
Independent factors	**Low (-1)**	**High (-1)**	**Medium (0)**
A: pH of the aqueous buffer	4.6	5.6	5.1
B: Flow rate (ml/minute) of the mobile phase	0.6	1.2	0.9
C: Acetonitrile ratio in the mobile phase (%)	6	10	8
D: Strength of the aqueous buffer (mM)	5	15	10
E: Injection volume (µl)	5	15	10
Dependent factors			
Y1: R_t (minute)			
Y2: Peak area (mV-min)			
Y3: Number of theoretical plates (NTP)			
Y4: Tailing factor (T_f5%)			
Y5: Tailing factor (T_f10%)			

Accuracy and precision

Accuracy was assessed by injecting three concentrations (4, 5, and 6 µg/ml) in sextuplicate. It was evaluated by analyzing these concentrations, and the mean percentage recovery was calculated for each concentration [50].

Precision was assessed using four quality control (QC) concentrations: quantitation limit (QL), lower QC (3× QL), middle QC (average of lower and higher QC), and higher QC (70% of the highest linearity concentration). Intra-day precision was evaluated by analyzing sextuplicates of the QC concentrations at two different time points within the same day (09:00 and 21:00). Inter-day precision was assessed by performing duplicate injections of the QC concentrations over three consecutive days. For both intra-day and inter-day analyses, the peak area and percentage relative standard deviation (%RSD) were calculated to determine precision.

Statistical comparison with a reported method to validate our method's performance, we compared it with the previously reported method by Mendez *et al.* [11] using two statistical tests [11].

Comparison of method precision (F-test)

The *F*-test was used to compare how precise both methods are by comparing their variations in measurements. This tcresults than the other.

Comparison of method accuracy (t-test)

Student's *t*-test was used to compare the accuracy between both methods by comparing their mean recovery values. This helps determine if both methods give similar results when measuring the same thing. Both statistical tests were performed at a 95% confidence level. If the calculated values were less than the critical values from standard statistical tables, it would indicate no significant difference between the methods.

Sensitivity

The detection limit (DL) and QL were calculated using the following formulas:

$$DL = 3.3 * \frac{\sigma}{S}$$

$$QL = 10 * \frac{\sigma}{S}$$

where σ = the residual standard deviation of the regression line

S = slope of the regression line.

Robustness

Robustness was evaluated by analyzing the impact of minor variations in experimental conditions (Table 3). An MPN concentration of 5 µg/ml was injected thrice under altered conditions, and parameters such as peak area, R_t, T_f5%, T_f10%, and NTP were monitored for %RSD.

System suitability

System suitability was confirmed by injecting 5 µg/ml of MPN in sextuplicate. Parameters such as peak area, R_t, T_f5%, T_f10%, and NTP were calculated along with %RSD to ensure the system met the required criteria.

BENCHTOP STABILITY STUDIES OF THE MPN SOLUTION

The stability and degradation studies for MPN were conducted to evaluate its behavior under various conditions. Benchtop stability of the MPN solution was assessed using two concentrations, 4.59 µg/ml (lower QC) and 8.75 µg/ml (higher QC). Fresh solutions were prepared and stored at ambient temperature over three days, labeled D1, D2, and D3. On Day 3, all solutions were analyzed in triplicate. The mean peak areas of D1 (48 hours old) and D2 (24 hours old) were compared to D3 (freshly prepared) to calculate the percentage stability and similarity indices.

$$\text{Similarity index for 48 hours stability} = \frac{\text{Mean peak area of D1} \times \text{Amount of D3}}{\text{Mean peak area of D3} \times \text{Amount of D1}}$$

$$\text{Similarity index for 24 hours stability} = \frac{\text{Mean peak area of D2} \times \text{Amount of D3}}{\text{Mean peak area of D3} \times \text{Amount of D2}}.$$

AUTOSAMPLER POST-OPERATIVE STABILITY STUDIES OF MPN SOLUTION

Autosampler post-operative stability studies were conducted to determine the integrity of MPN stored at 4°C

Table 3. Different conditions for robustness studies.

Condition	Lower limit	Higher limit
Acetonitrile ratio in the mobile phase (%)	9	11
Column oven temperature (°C)	23	27
Wavelength (nm)	295	299
Flow rate (ml/minute)	0.67	0.83
Injection volume (µl)	9	11

in autosampler vials for 24 hours. The lower and higher QC concentrations were analyzed in triplicate and compared to freshly prepared samples. Stability percentages and similarity indices were calculated based on peak area comparisons [51].

Similarity index for autosampler postoperative stability

$$= \frac{\text{Mean peak area of 24 hours autosampler stored sample} \times \text{Amount of fresh sample}}{\text{Mean peak area of fresh sample} \times \text{Amount of autosampler stored sample}}$$

BENCHTOP STABILITY STUDIES OF THE MOBILE PHASE

The benchtop stability of the mobile phase was assessed by preparing and storing it at ambient temperature for three consecutive days (labeled M1, M2, and M3). On Day 3, a 5 µg/ml MPN solution was analyzed using all three mobile phases in triplicate, with M1 (48 hours old) and M2 (24 hours old) compared to M3 (freshly prepared).

Similarity index for 48 hours stability

$$= \frac{\text{Mean peak area of MPN in M1} \times \text{Amount of MPN}}{\text{Mean peak area of MPN in M3} \times \text{Amount of MPN}}$$

Similarity index for 24 hours stability

$$= \frac{\text{Mean peak area of MPN in M2} \times \text{Amount of MPN}}{\text{Mean peak area of MPN in M3} \times \text{Amount of MPN}}$$

DEGRADATION STUDIES

Degradation studies examined MPN under stress conditions, including acid, alkali, oxidative, and photolytic exposures. Acid-induced degradation was conducted under real-time and accelerated conditions by treating MPN (5 µg/ml) with 0.1 N and 1 N HCl. Samples were stored at ambient temperature for 24 hours or at 60°C for 6 hours, then neutralized with NaOH and analyzed. Alkali-induced degradation was performed similarly, using 0.1 N and 1 N NaOH for treatment and subsequent neutralization with HCl. Oxidative degradation involved treating MPN with 3% H_2O_2, either stored at ambient temperature for 24 hours or at 60°C for 6 hours, followed by analysis. Photolytic degradation was evaluated by exposing MPN to UV light (direct sunlight) for 24 hours, and the resulting degradation products were analyzed. This comprehensive assessment of MPN's stability and degradation under varying conditions provides insights into its robustness and suitability for analytical applications [51].

APPLICATION OF THE DEVELOPED ANALYTICAL METHOD FOR QUANTIFICATION OF MPN IN MARKETED PRODUCT AND NOVEL NANOFORMULATIONS

The developed HPLC method is considered a valuable tool only after its practical application to quantify MPN in dosage forms. MPN was analyzed in the marketed powder for injection formulation (Merosure 125 mg, Alkem Laboratories, India) and the novel β-CD nanosponges formulation to test the practical feasibility of the method.

Quantification of MPN in the marketed formulation

The validated method was used to analyze and quantify the MPN in the marketed powder for injection formulation. The marketed formulation's standard stock solution was prepared by accurately weighing 5.45 mg (equivalent to 5 mg of MPN), transferring it to a 10 ml volumetric flask, and filling the volume with the mobile phase at a final concentration of 500 µg/ml. Further dilutions were done with the mobile phase to obtain the desired concentration (10 µg/ml). The validated method was used to analyze the desired concentration, and the R_t and peak area were measured. The amount of MPN in the desired concentration was calculated by comparing the peak area [35].

Quantification of MPN in β-CD-nanosponges

Preparation of β-CD cross-linked nanosponges

β-CD nanosponges were prepared using the melt method, involving a cross-linking reaction between β-CD and DPC in a 1:4 weight ratio. The homogenously triturated mixture was reacted for 5 hours at 100°C and left overnight at ambient temperature. The resulting cross-linked nanosponges were purified via Soxhlet extraction using acetone, and the phenol by-product was removed. The purified nanosponges were dried at 50°C and tested with 1% ferric chloride solution to ensure phenol-free purity, resulting in a pure white powder stored at 25°C for future use [52].

Preparation of MPN encapsulated β-CD-nanosponges

MPN encapsulation into nanosponges was achieved using a solvent immersion technique. A 1:1 ratio of MPN and nanosponges was used, where the nanosponges were dispersed in water and sonicated with a probe sonicator (20% amplitude for 10 minutes with 2-second breaks every 10 seconds). MPN was then added to the dispersion, stirred for 6 hours, and centrifuged at 2,500 rpm for 10 minutes. The colloidal supernatant containing drug-encapsulated nanosponges was collected, freeze-dried, and stored for further analysis [52].

Encapsulation efficiency

To determine encapsulation efficiency, MPN-encapsulated nanosponges were weighed and dispersed in methanol, followed by sonication to break the nanosponges. The sonicated mixture was centrifuged, and the supernatant was diluted with the mobile phase for HPLC analysis. The Rt and peak areas were recorded, and the amount of MPN was calculated by comparing the peak areas with the standard. Encapsulation efficiency was calculated using the below-mentioned formulas [52].

$$\text{Encapsulation efficiency} = \frac{\text{Entrapped amount of drug} \times 100}{\text{Total amount of drug added}}$$

STATISTICAL ANALYSIS

All the data obtained from the experiments were subjected to statistical analysis. Results are presented as mean

values and their respective % RSD. Statistical significance was determined at a 95% confidence level.

RESULT AND DISCUSSION

Defining an ATP and determining CPAs

The ATP was established to ensure a robust and reliable HPLC-UV method for quantifying MPN, addressing systematic and inherent variability while ensuring system suitability. As part of the QbD approach, CPAs were identified to significantly impact the method's performance. They are (a) Rt (min), (b) peak area (mV-min), (c) tailing factor, and (d) number of theoretical plates.

Risk assessment

A risk assessment was conducted using an Ishikawa fishbone diagram to identify the CMAs and CPPs that could influence the analytical method. Preliminary trials (Table 4), based on an OFAT design, were conducted, and the variables such as buffer pH, flow rate, acetonitrile ratio in the mobile phase, buffer strength, and injection volume were identified as critical based on their significant impact on method performance.

Selection of the mobile phase

Acetonitrile was chosen as the organic mobile phase because it has a higher vapor pressure than methanol and a lower internal pressure in the column, leading to better efficiency and column lifetime. The aqueous mobile phase composition was optimized by evaluating the buffer type, pH, and concentration. Considering MPN's pKa values (2.9 and 7.4), a buffer pH of 5.1 was selected to maintain the drug in a consistent ionized or unionized state without compromising the silica column integrity.

Four buffer systems (ammonium acetate, ammonium formate, sodium acetate, and potassium dihydrogen phosphate) were screened at a concentration of 10 mM with a mobile phase ratio of 92:08 (aqueous:organic). Ammonium acetate demonstrated superior performance, yielding a peak area of 87,141.33 ± 2.08 mV-min and 11,168.70 ± 9.71 theoretical plates, compared to sodium acetate with 82,345.33 ± 123.50 mV-min and 11,158.30 ± 18.00 theoretical plates.

Selection of the stationary phase

In continuation of the OFAT design, various stationary phases, including the Phenomenex Kinetex C_{18} (250 mm*4.6 mm*5 µ) column, Phenomenex Hyperclone C_{18} (250 mm*4.6 mm*5 µ) column, Intek Chromasol Jade C_{18} (250 mm*4.6 mm*5 µ) column, and Intek Chromasol Onyx C_{18} (250 mm*4.6 mm*5 µ) column, were screened.

Acetonitrile and 10 mM ammonium acetate in a ratio of 8:92 were used in the preliminary trials for the stationary phase selection. An MPN concentration of 5 µg/ml was prepared and analyzed in the four stationary phases.

Among the four stationary phases, the Phenomenex Kinetex C_{18} column significantly showed consistent results with a better peak area, and therefore, it was selected as the ideal stationary phase for the method development.

Design of experiments

Screening of independent factors using fractional factorial design

Screening of 5 independent factors [Factor A: pH of the buffer; Factor B: flow rate; Factor C: acetonitrile ratio in the mobile phase; Factor D: strength of the buffer; and Factor E: injection volume] was carried out using a fractional factorial design. Dependent responses were measured for all experimental runs, as tabulated in Table 5, and the relationship between factors and their respective responses was analyzed

Table 4. Preliminary trial for the identification of risk factors.

Aqueous buffer	Strength of the aqueous buffer (mM/l)	pH of the aqueous buffer	Mobile phase ratio (acetonitrile: aqueous buffer)	Flow rate of mobile phase (ml/ minute)	R_t (min)	Peak area (mV-min)	NTP	T_f 5%	T_f 10%
Ammonium acetate	10	5.1	08:92	0.9	5.471 ± 0.0005	87,141.33 ± 2.08	11,168.70 ± 9.71	1.474 ± 0.004	1.438 ± 0.003
Sodium acetate	10	5.1	08:92	0.9	5.473 ± 0.002	82,345.33 ± 123.50	11,158.30 ± 18.00	1.470 ± 0.001	1.436 ± 0.003
Potassium dihydrogen phosphate	10	5.1	08:92	0.9	4.906 ± 0.007	73,459.00 ± 93.66	10,285.70 ± 315.52	1.445 ± 0.001	1.412 ± 0.002
Ammonium formate	10	5.1	08:92	0.9	6.714 ± 0.009	72,638.00 ± 16.09	10,408.30 ± 73.55	1.458 ± 0.0005	1.424 ± 0.0005
Ammonium acetate	20	5.1	08:92	0.9	5.476 ± 0.001	87,490.66 ± 329.16	11,154.70 ± 32.03	1.467 ± 0.004	1.432 ± 0.002
Sodium acetate	20	5.1	08:92	0.9	5.460 ± 0.021	82,610.67 ± 153.13	11,124.70 ± 35.79	1.443 ± 0.003	1.411 ± 0.001
Ammonium acetate with 0.1% triethylamine	10	5.1	08:92	0.9	5.471 ± 0.002	87,456.33 ± 87.32	11,147.30 ± 15.04	1.471 ± 0.003	1.437 ± 0.0005

Table 5. Experimental runs and their results based on fractional factorial design.

Run	A: pH of the buffer	B: Flow rate (ml/minute)	C: Acetonitrile ratio in the mobile phase (%)	D: Strength of buffer (mM)	E: Injection volume (µl)	Rt (minute)	Peak Area (mV-minute)	NTP	T_f5 %	T_f10 %
1	4.6	0.6	10	5	5	6.412	52,862	14,896.853	1.314	1.263
2	5.6	0.6	6	5	5	5.944	53,141	3,045.556	1.056	1.044
3	5.1	0.9	8	10	10	6.077	71,310	15,079.129	1.259	1.223
4	4.6	1.2	6	5	5	7.411	26,596	16,110.535	1.091	1.08
5	4.6	1.2	10	5	15	3.256	79,072	9,861.406	1.345	1.3
6	4.6	0.6	10	5	5	6.422	52,854	14,544.198	1.306	1.26
7	5.6	0.6	10	15	5	6.497	52,784	14,508.543	1.334	1.283
8	4.6	0.6	10	5	5	6.445	52,572	14,820.499	1.302	1.257
9	4.6	0.6	6	15	5	4.41	54,564	1,902.916	1.088	1.095
10	4.6	1.2	6	15	15	7.404	78,735	16,690.671	1.112	1.1
11	4.6	0.6	10	15	15	6.448	156,687	16,002.612	1.323	1.271
12	5.1	0.9	8	10	10	6.089	70,591	15,367.166	1.248	1.211
13	4.6	0.6	6	15	5	4.411	54,527	1,887.449	1.094	1.099
14	5.6	0.6	10	5	15	6.66	161,469	18,369.087	1.285	1.237
15	5.6	1.2	6	5	15	8.452	83,627	17,161.88	1.081	1.071
16	4.6	0.6	10	15	15	6.437	156,442	15,676.875	1.32	1.273
17	5.6	0.6	6	15	15	5.036	162,469	2,706.772	0.928	0.902
18	5.6	0.6	6	5	5	5.928	53,686	2,869.054	1.051	1.037
19	4.6	0.6	10	15	15	6.456	156,846	15,683.331	1.32	1.27
20	5.1	0.9	8	10	10	6.054	70,815	15,089.201	1.25	1.215
21	4.6	1.2	10	15	5	3.26	26,243	8,534.627	1.4	1.345
22	4.6	1.2	10	15	5	3.255	26,150	8,400.828	1.384	1.344
23	5.6	0.6	10	15	5	6.522	52,873	14,606.342	1.331	1.282
24	5.6	0.6	10	5	15	6.705	161,259	18,266.342	1.291	1.239
25	5.6	0.6	6	5	5	5.96	52,595	3,222.058	1.061	1.05
26	4.6	1.2	6	5	5	7.412	26,407	16,382.453	1.091	1.083
27	4.6	1.2	10	15	5	3.263	26,205	8,364.163	1.395	1.352
28	5.6	1.2	6	15	5	7.735	26,891	16,276.155	1.11	1.098
29	5.6	1.2	6	5	15	8.405	83,992	17,799.513	1.09	1.08
30	5.6	1.2	10	5	5	3.607	28,741	9,533.336	1.355	1.309
31	5.6	1.2	6	15	5	7.85	26,700	17,059.601	1.109	1.095
32	4.6	1.2	6	15	15	7.381	78,529	16,979.997	1.118	1.104
33	5.6	1.2	10	5	5	3.573	28,447	9,575.347	1.359	1.319
34	5.6	1.2	10	15	15	3.385	81,141	10,305.281	1.358	1.32
35	5.6	1.2	10	15	15	3.384	80,948	10,376.893	1.376	1.331
36	4.6	0.6	6	5	15	1.543	157,155	254.958	1.06	1.05
37	5.6	1.2	6	15	5	7.789	26,634	17,156.649	1.112	1.098
38	4.6	1.2	6	15	15	7.387	78,536	16,778.151	1.115	1.103
39	4.6	1.2	10	5	15	3.26	78,598	8,433.206	1.393	1.347
40	5.6	0.6	6	15	15	5.012	162,815	2,576.624	0.901	0.872
41	5.6	1.2	6	5	15	8.331	83,018	18,620.729	1.092	1.081
42	4.6	1.2	6	5	5	7.383	26,279	15,835.687	1.095	1.084

(continued)

Run	A: pH of the buffer	B: Flow rate (ml/minute)	C: Acetonitrile ratio in the mobile phase (%)	D: Strength of buffer (mM)	E: Injection volume (µl)	Rt (minute)	Peak Area (mV-minute)	NTP	T_f5 %	T_f10 %
43	5.6	0.6	10	5	15	6.725	161,293	18,054.803	1.281	1.229
44	4.6	0.6	6	5	15	1.545	156,874	256.608	1.058	1.048
45	4.6	0.6	6	15	5	4.409	54,600	1,918.383	1.081	1.09
46	5.6	1.2	10	5	5	3.594	28,542	9,832.504	1.351	1.308
47	5.6	1.2	10	15	15	3.407	80,745	9,955.54	1.366	1.321
48	5.6	0.6	6	15	15	5.06	162,122	2,836.92	0.954	0.931
49	5.6	0.6	10	15	5	6.532	530,56	14,331.897	1.345	1.292
50	4.6	1.2	10	5	15	3.258	78,835	9,147.306	1.369	1.324
51	4.6	0.6	6	5	15	1.54	157,435	253.307	1.062	1.051

using graphical effects analysis, specifically Pareto charts (Fig. 2).

The Pareto charts revealed that factors A, B, and C had *t*-values exceeding their Bonferroni limits, confirming their significance. Conversely, factors D and E were deemed non-significant due to their *t*-values falling below the Bonferroni limits.

Subsequently, numerical optimization was performed to determine the ideal values for the non-significant factors (buffer strength and injection volume) to achieve the desired responses. Among the potential solutions offered by the software, the solution with the highest desirability (desirability = 1) was chosen. As per the numerical optimization, the ideal injection volume was 10 µl, and the ideal buffer strength was 10 mM.

The factors A, B, and C were found significant and subjected to optimization using the Box-Behnken design.

Optimization of factors using Box-Behnken design

A 2-level Box-Behnken design with their center points was performed for three independent factors [A: pH of the buffer, B: flow rate, and C: acetonitrile ratio in the mobile phase]. The software recommended 12 experimental runs were performed, and the results are tabulated in Table 6. The effect of individual independent factors on responses was analyzed using a quadratic regression model through analysis of variance (ANOVA). The results of the ANOVA test for all the independent factors from the quadratic model represent the main effects and interaction effects of factors on responses, and the results are tabulated in Table 7. The impact of individual factors on responses was briefly discussed in a further section.

Analysis of the effect of individual independent factors on the Rt

The quadratic model and ANOVA results led to the following coded equation for Rt (Y1):

$$(Y1 = +5.47 + 0.4524A + 0.0919B - 0.5198C - 0.2200AB - 0.3518AC - 1.67BC). \quad (1)$$

This equation, alongside Figure 3(R1), suggests that the buffer pH and flow rate positively affect Rt, while the acetonitrile ratio in the mobile phase negatively impacts it. At pH 4.6, MPN becomes more polar, and as the pH increases, its lipophilicity rises, enhancing retention. Increasing the acetonitrile ratio in the mobile phase reduces Rt due to decreased polarity. The flow rate also influences Rt, with a higher flow rate resulting in a slight increase in retention due to the interaction with acetonitrile concentration.

Analysis of the effect of individual independent factors on the peak area

The coded equation for peak area (Y2) is:

$$Y2 = +80145.83 + 886.62A - 26332.38B - 220.25C + 374.00AB + 308.75AC + 424.75BC \quad (2)$$

Here, buffer pH positively impacts peak area, while flow rate and acetonitrile ratio negatively affect it. Increasing the buffer pH enhances the degree of ionization, slightly increasing the peak area. Higher flow rates reduce peak area due to decreased analyte Rt, while an increased acetonitrile ratio causes a negligible decrease in peak area due to changes in ionization (Fig. 3(R2)).

Analysis of the effect of individual independent factors on the number of theoretical plates

The coded equation for the number of theoretical plates (Y3) is:

$$Y3 = +11014.08 + 613.00A + 2118.38B + 1572.88C - 107.50AB - 57.00AC - 5345.25BC \quad (3)$$

Flow rate, buffer pH, and acetonitrile ratio all positively affect theoretical plates. Contrary to theoretical understanding, increasing the flow rate resulted in a higher number of theoretical plates due to interactions with other factors. Increased pH enhances ionization, improving elution efficiency, and an increased acetonitrile ratio improves elution efficiency, increasing the number of theoretical plates (Fig. 3(R3)).

Analysis of the effect of individual independent factors on the tailing factor 5% and the tailing factor 10%

Equations 4 and 5 are coded equations for the tailing factor 5% (Y4) and the tailing factor 10% (Y5), respectively.

$$Y4 = + 1.20 - 0.0138c + 0.0317B + 0.1373C \quad (4)$$

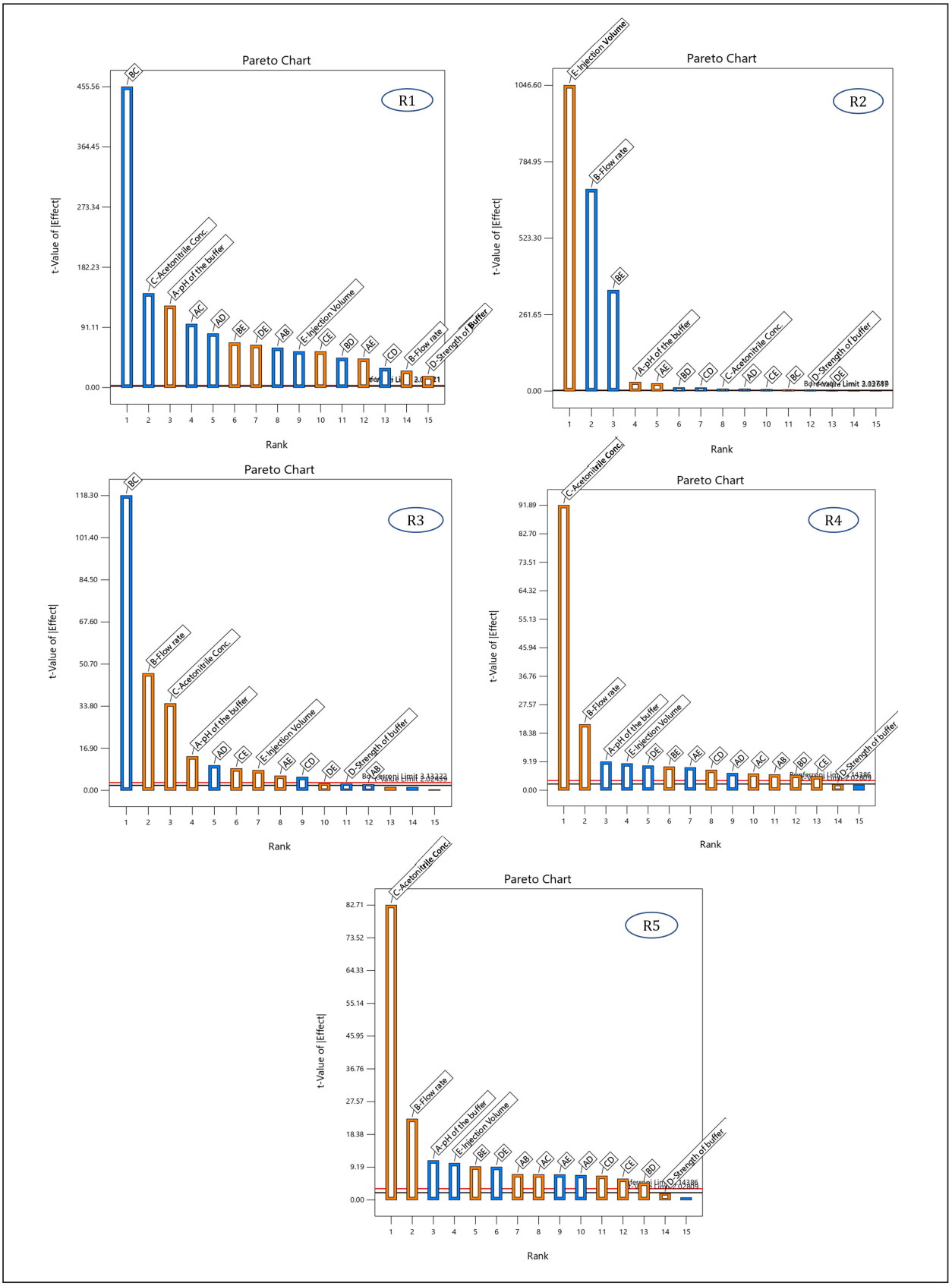


Figure 2. Pareto chart illustrating the effect of independent variables [(A) pH of the buffer, (B) flow rate, (C) acetonitrile ratio in the mobile phase, (D) strength of the buffer, and (E) injection volume] on respective responses [(R1) Rt of MPN, (R2) peak area of MPN, (R3) number of theoretical plates, (R4) tailing factor 5%, and (R5) tailing factor 10%].

Table 6. Experimental runs and their results based on Box-Behnken design.

Run	A: pH of the buffer	B: Flow rate (ml/minute)	C: Acetonitrile ratio in the mobile phase (%)	R_t (minute)	Peak area (mV-min)	NTP	T_f5%	T_f10%
1	5.1	1.2	10	3.374	54,018	9,360	1.37	1.326
2	5.6	0.6	8	6.048	106,991	9616	1.151	1.116
3	5.1	0.6	6	4.23	107,123	1,,978	1.032	1.022
4	5.1	1.2	6	7.744	53,609	16,905	1.101	1.089
5	4.6	0.9	10	4.847	78,730	12,031	1.347	1.3
6	4.6	0.9	6	5.183	79,788	8,771	1.088	1.082
7	4.6	0.6	8	4.703	105,966	8,175	1.193	1.168
8	4.6	1.2	8	5.327	52,553	12,627	1.242	1.213
9	5.6	1.2	8	5.792	55,074	13,638	1.229	1.202
10	5.6	0.9	6	6.791	80,944	10,111	1.045	1.029
11	5.6	0.9	10	5.048	81,121	13,143	1.335	1.289
12	5.1	0.6	10	6.521	105,833	15,814	1.312	1.262

Table 7. Results of the ANOVA test for all the independent factors from the quadratic model.

Responses	R_t of MPN		Peak area of MPN		NTP		T_f5%		T_f10%	
F-value	31,290,000		11,110,000,000		346,100,000		60.04		60.04	
p-value	**Model**	< 0.0001	**Model**	< 0.0001	**Model**	< 0.0001	**Model**	< 0.0001	**Model**	< 0.0001
	A	< 0.0001	A	< 0.0001	A	< 0.0001	A	0.001	A	< 0.0001
	B	< 0.0001	B	< 0.0001	B	< 0.0001	B	< 0.0001	B	< 0.0001
	C	< 0.0001	C	< 0.0001	C	< 0.0001	C	< 0.0001	C	< 0.0001
	AB	< 0.0001	AB	< 0.0001	AB	< 0.0001			AB	< 0.0001
	AC	< 0.0001	AC	< 0.0001	AC	< 0.0001			AC	< 0.0001
	BC	< 0.0001	BC	< 0.0001	BC	< 0.0001			BC	0.0035
R^2	1		1		1		0.997		1	
Adjusted R^2	1		1		1		0.9959		1	
Predicted R^2	1		1		1		0.9933		1	
Adequate precision	19,820.457		247,500		67,703.6422		75.5201		1,379.9483	
%CV	0.0053		0.0004		0.0026		0.644		0.0246	

$$Y5 = +1.17 - 0.0159A + 0.0327B + 0.1194C + 0.0103AB + 0.0105AC - 0.0007BC.$$

Flow rate and acetonitrile ratio increase the tailing factor, as higher flow rates reduce analyte-column interaction time, causing band broadening and peak tailing. Increased acetonitrile reduces polarity, resulting in broader peaks and higher tailing factors. Buffer pH, conversely, improves peak symmetry by altering the ionization state, and reducing tailing (Fig. 3 (R4) and (R5)).

Development and verification of method operable design space

After using a regression model to analyze the factors, numerical optimization was used to develop the design space. The software generates 47 prospective solutions, of which the optimized solution was chosen (Fig. 4.) based on the highest desirability value (0.807). Conclusively, DOE helped in a complete understanding of the method, allowing for improved method development and subsequent method transfer with minimum systematic and inherent random variability. Based on the analysis of the Box-Behnken design, the optimal parameters were determined and tabulated in Table 8 for the developed method. Three sets of experimental runs were conducted as per optimized analytical conditions. The observed experimental results matched the model-predicted results, as tabulated in Table 9. The table provides a side-by-side view of predicted outcomes against actual observations, assessed at a two-sided 95% confidence interval (CI) and a 99% population interval.

Validation of the optimized HPLC method

The optimized HPLC method was validated by performing a rigorous set of experiments recommended by ICH Q2(R1) guidelines that assessed the method's performance

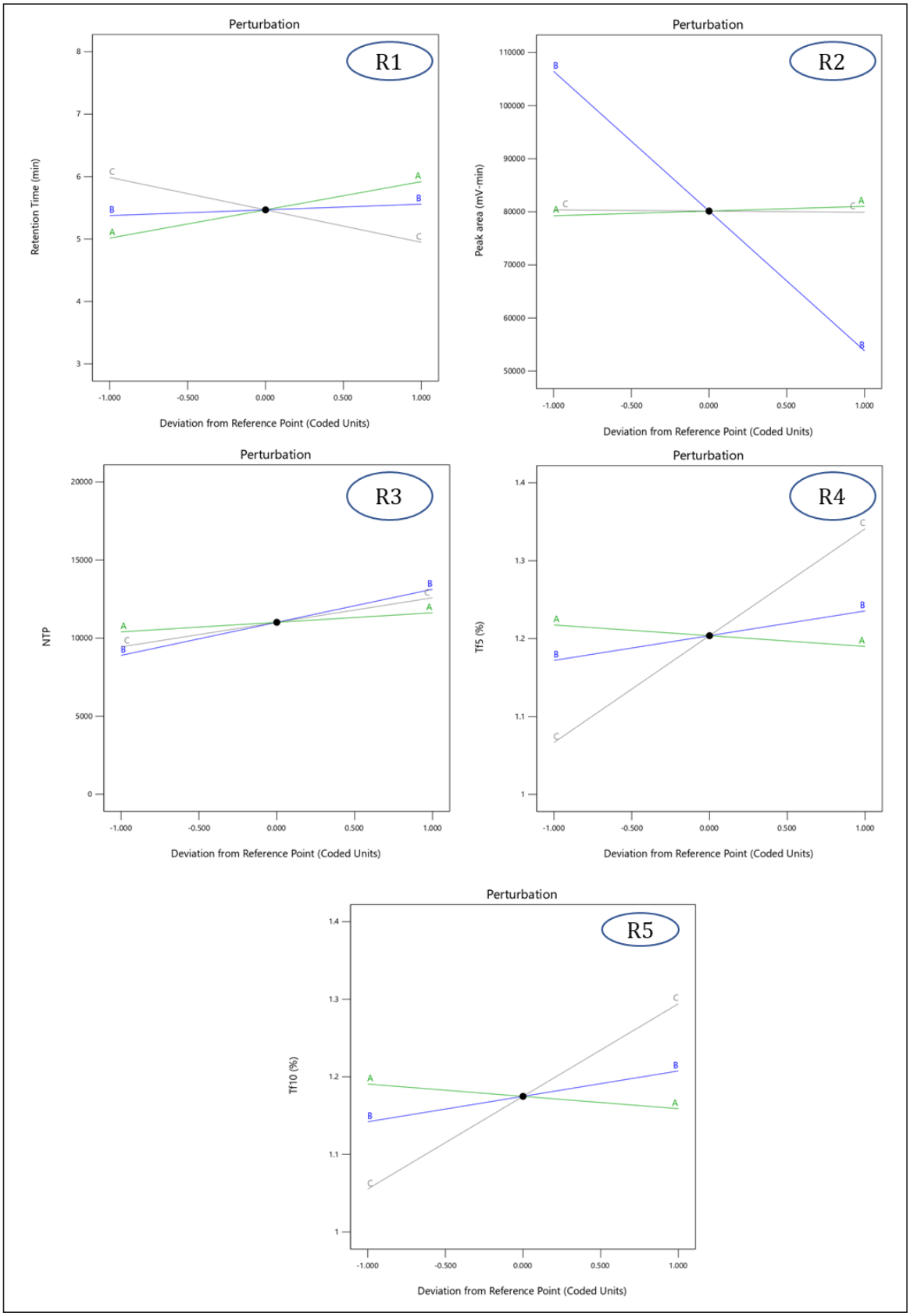


Figure 3. Perturbation plots demonstrating the effect of independent variables [(A) pH of the buffer, (B) flow rate, and (C) acetonitrile ratio in the mobile phase] on individual responses [(R1) Rt of MPN, (R2) peak area for MPN, (R3) number of theoretical plates, (R4) tailing factor 5%, and (R5) tailing factor 10%].

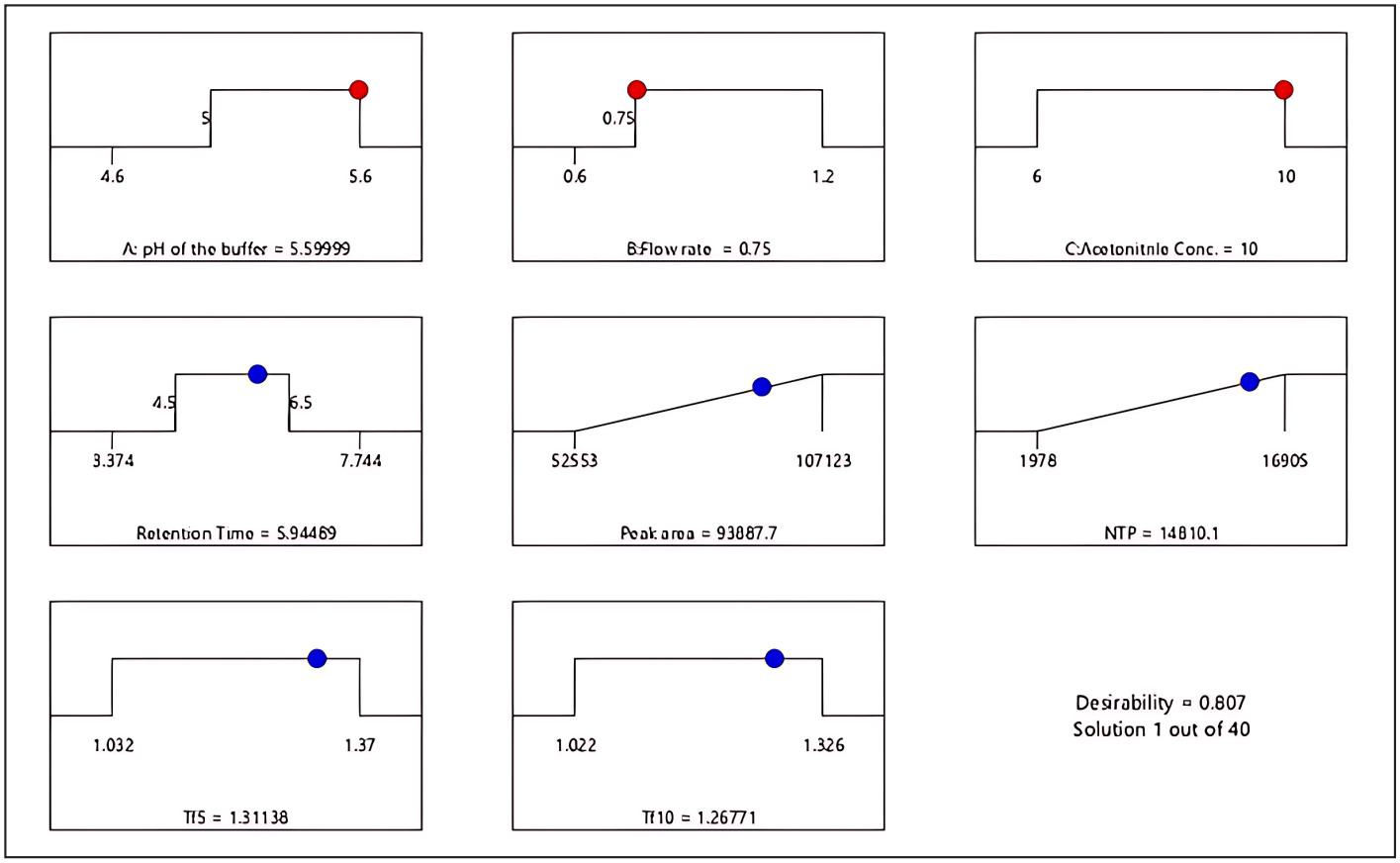


Figure 4. Numerical optimization indicates an optimized solution with the highest desirability value.

Table 8. Optimal chromatographic conditions.

Stationary phase	Kinetex C_{18} (250 mm*4.6 mm*5 µ)
Concentration of ammonium acetate buffer (mmol/l)	10
pH of buffer	5.6
Acetonitrile: buffer ratio	10:90
Injection volume (µl)	10
Wavelength (nm)	297
Flow rate (ml/minute)	0.75
Column oven temperature (°C)	25

under various conditions. The results of all the validation parameters are listed in Table 10.

Specificity

The method represented a distinctive peak for MPN at a Rt of 5.93 minutes, as illustrated in Figure 5. No interferences were observed at the Rt of the MPN from the blank and placebo of the novel formulation. The developed method eluted the MPN from the marketed formulation and the novel nanosponges formulation at a Rt of 5.91 and 5.89 minutes, respectively. Hence, the optimized method was demonstrated to be highly specific in determining MPN.

Linearity

MPN were analyzed in quintuplicate for a concentration range of 0.1 to 12.5 µg/ml. Individual linear plots were constructed for each trial, and the mean of all trials was calculated. According to the plotted linear regression graph, the linear regression equation for the mean value was y = 18155x+1145. The coefficient of determination (R^2) was found to be 0.9991. Out of five linear regression plots, no two consecutive R^2 values were less than 0.999. Therefore, the optimized method was significantly linear in the MPN concentration range and subjected to further experimentation.

Accuracy and precision

Three different MPN concentrations were injected in sextuplicate, and the mean percentage recovery of the concentrations 4, 5, and 6 µg/ml were 98.39%, 101.61%, and 100.98%, respectively. The accuracy results showed that the mean recovery percentage of analysis of MPN was well within the range of 85%–115%. Thus, the optimized method demonstrates suitability for the accurate quantification of MPN.

Four QC concentrations of MPN were injected in duplicate, and in the intraday analysis, the mean peak areas of the concentrations of 1.53, 4.59, 8.75, and 6.67 µg/ml were 28,764.17, 86,155.33, 125,341.00, and 164,384.5 mV-min, respectively. In the inter-day analysis, the same four QC concentrations of MPN were injected in duplicate; the mean

Table 9. Predicted and actual point prediction at two-sided 95% CI.

Analysis	Predicted mean	Predicted median	Observed	Standard deviation	SE mean	95% CI low for mean	95% CI high for mean
Rt (minute)	5.944	5.944	5.938	0.000288675	0.000248255	5.94425	5.94553
Peak area (mV-min)	93,887.7	93,887.7	93895	0.288675	0.248255	93,887.1	93,888.3
NTP	14,810.1	14,810.1	14813	0.288675	0.248255	14,809.5	14,810.8
T_f5%	1.311	1.311	1.312	0.00775202	0.0046807	1.30058	1.32217
T_f10%	1.267	1.267	1.268	0.000288675	0.000248255	1.26707	1.26835

peak areas of the concentrations of 1.53, 4.59, 8.75, and 6.67 µg/ml were 28,756.33, 86,175.5, 125,464.2, and 164,477.5 mV-min, respectively. The precision results showed that the % RSD of the peak area for all the concentrations in intraday and interday analyses was less than 2%. Thus, the optimized method demonstrates suitability for the precise quantification of MPN. The optimized technique has exhibited a high degree of suitability for the precise quantification of MPN.

The analytical performance of our developed method was statistically compared with the previously reported method by Mendez *et al.* [11] The precision comparison using *F*-test revealed that our method demonstrates significantly better precision, with %RSD values ranging from 0.041% to 0.167% compared to their reported values of 0.78% (intra-day) and 0.85% (inter-day). The calculated *F*-values were lower than the critical *F*-value at a 95% confidence level, confirming the superior precision of our method. In terms of accuracy, our method showed recovery values ranging from 98.39% to 101.61%, which was comparable to their reported range of 99.11%–100.10%. The Student's *t*-test performed on the recovery data showed no significant difference between the two methods at a 95% confidence level, indicating that both methods are equally accurate. These statistical comparisons demonstrate that our method maintains equivalent accuracy while offering improved precision compared to the previously reported method.

Sensitivity

The DL was calculated based on the residual standard deviation of the regression line and its slope. The DL and QL were determined and found to be 0.51 and 1.53 µg/ml, respectively.

Robustness

An MPN concentration of 5 µg/ml was injected in triplicate, and the impact of small variations in the operating conditions, like the acetonitrile ratio in the mobile phase, column oven temperature, wavelength, flow rate, and injection volume, was analyzed. For all the varied conditions, the mean %RSD of peak area, Rt, number of theoretical plates, tailing factor 5%, and tailing factor 10% were less than 2. The optimized methodology has been demonstrated to have a high degree of robustness in obtaining precise and accurate quantifications of MPN in response to variations in operating conditions.

System suitability

An MPN concentration of 5 µg/ml was injected in sextuplicate and analyzed for mean Rt, peak area, number of theoretical plates, tailing factor 5%, and tailing factor 10%. The consistent results from sextuplicate trials ensure the suitability of the analytical system for the intended application of quantification of MPN in dosage forms.

Benchtop stability studies of MPN solution

The benchtop stability study was carried out to assess the stability of MPN in solution under ambient temperature conditions, and the results are depicted in Table 11. The analysis of lower and higher QC MPN concentrations (4.59 and 8.75 µg/ml, respectively) over time revealed that the stability of MPN was significantly affected by storage duration at ambient temperature. Results showed that the Rt of MPN remained the same (5.942 minutes), and the MPN concentration of 4.59 and 8.75 µg/ml decreased to 91.31% and 92.51% of the initial concentration after 24 hours, respectively, and to 77.38% and 78.91% after 48 hours, respectively. This implies that the MPN undergoes hydrolysis, which gradually converts the active form of the drug into the inactive form. The similarity index for the 48 and 24 hours stability was determined and tabulated in Table 11.

Autosampler post-operative stability studies of MPN solution

Autosampler post-operative stability studies were conducted to assess the stability of MPN in solution form, stored in an autosampler at 4°C for 24 hours. Over time, the analysis of lower and higher QC MPN concentrations (4.59 and 8.75 µg/ml) revealed that storage duration at 4°C negligibly influenced the MPN stability. The results indicated that the Rt of MPN remained the same (5.937 minutes), and the MPN concentration of 4.59 and 8.75 µg/ml was stable and remained at 99.83% and 98.75% of the initial concentration after 24 hours at 4°C, respectively, as represented in Table 11. This implies that the rate of hydrolysis was significantly reduced at lower temperatures (refrigerated conditions).

Benchtop stability studies of the mobile phase

Benchtop stability studies were performed on the mobile phase to evaluate its stability at ambient temperature over a specific period and its ability to elute MPN. The analysis of the MPN concentration of 5 µg/ml over time showed that the stability of the mobile phase had a minimal impact on the concentration of MPN, and the Rt of MPN remained the same (5.940 minutes). The results indicated that the MPN concentration decreased to 96.62% and 97.03% of the initial concentration after 48 and 24 hours, respectively, as represented

Table 10. Results of all the validation parameters.

Specificity											
Analyte			**Mean Rt (minute)**								
Bulk MPN			5.930 ± 0.00								
MPN marketed formulation			5.911 ± 0.02								
MPN in novel nanosponges formulation			5.892 ± 0.01								
Linearity											
Concentration range			**Mean regression equation**					**Mean coefficient of determination**			
0.1–12.5 µg/ml			*y* = 18,155x + 1,145					0.9991			
Accuracy											
Three standard concentrations from the linearity range (µg/ml)			**Mean peak area (mV-min)**			**Calculated concentration (µg/ml)**		**Mean percentage recovery (%)**			
4			72,594.33 ± 164.87			3.94 ± 0.01		98.39 ± 0.23			
5			93,380.50 ± 232.38			5.08 ± 0.01		101.61 ± 0.26			
6			111,141.16 ± 905.97			6.06 ± 0.05		100.98 ± 0.83			
Sensitivity											
Residual standard deviation of the regression line (σ)			2,782.37								
Slope of the regression line			18,155.49								
DL			0.51 µg/ml								
QL			1.53 µg/ml								
Precision											
			Intra-day precision					**Inter-day precision**			
QC solutions			**Mean peak area (mV-min)**		**%RSD**			**Mean peak area (mV-min)**		**% RSD**	
QL			28,764.17 ± 23.69		0.082			28,756.33 ± 12.99		0.045	
LQC			86,155.33 ± 43.57		0.050			86,175.50 ± 36.17		0.041	
MQC			125,341.00 ± 79.49		0.063			125,464.20 ± 99.34		0.079	
HQC			164,384.50 ± 155.41		0.094			164,477.50 ± 275.56		0.167	
Robustness											
Chromatographic conditions			**Mean Rt (minute)**	**% RSD**	**Mean peak area (mV-min)**	**% RSD**	**Mean NTP**	**% RSD**	**Mean T_f 5%**	**% RSD**	**Mean T_f 10%**	**% RSD**
Ratio of acetonitrile in mobile phase (%)	Lower limit	9	6.103 ± 0.001	0.016	94,067.67 ± 58.60	0.062	13,329.12 ± 85.82	0.644	1.30 ± 0.002	0.178	1.25 ± 0.001	0.080
	Higher limit	11	5.771 ± 0.005	0.087	93,553.00 ± 45.57	0.049	15,042.23 ± 31.20	0.207	1.33 ± .002	0.156	1.29 ± 0.001	0.090
Injection volume (µl)	Higher limit	11	5.976 ± 0.02	0.263	103,242.67 ± 3.79	0.004	14,553.19 ± 36.77	0.253	1.32 ± 0.001	0.076	1.23 ± 0.001	0.094
	Lower limit	9	5.973 ± 0.02	0.407	84,434.33 ± 11.06	0.013	15,183.08 ± 6.12	0.040	1.31 ± 0.005	0.446	1.26 ± 0.007	0.601
Temperature (°C)	Higher limit	27	5.923 ± 0.02	0.391	93,478.67 ± 263.07	0.281	14,151.01 ± 34.48	0.244	1.30 ± 0.006	0.466	1.26 ± 0.0005	0.046
	Lower limit	23	5.965 ± 0.03	0.514	93,414.67 ± 224.16	0.240	14,777.23 ± 70.56	0.477	1.31 ± 0.001	0.088	1.26 ± 0.001	0.137

(continued)

Chromatographic conditions			Mean Rt (minute)	% RSD	Mean peak area (mV-min)	% RSD	Mean NTP	% RSD	Mean T_f 5%	% RSD	Mean T_f 10%	% RSD
Detection wavelength (nm)	Higher limit	299	5.976 ± 0.02	0.263	93,409.33 ± 251.62	0.269	14,653.19 ± 36.77	0.251	1.32 ± 0.001	0.076	1.24 ± 0.001	0.094
	Lower limit	295	5.973 ± 0.02	0.407	93,567.00 ± 246.60	0.264	14,769.75 ± 7.88	0.189	1.31 ± 0.005	0.446	1.26 ± 0.007	0.601
Flow rate (ml/minute)	Higher limit	0.83	5.783 ± 0.002	0.046	91,370.33 ± 17.04	0.019	15229.12 ± 24.83	0.163	1.33 ± 0.001	0.075	1.36 ± 0.01	0.920
	Lower limit	0.67	6.114 ± 0.003	0.049	96,456.67 ± 26.27	0.027	14,405.57 ± .75	0.019	1.30 ± 0.001	0.077	1.24 ± 0.001	0.046

System suitability

Parameters	Mean response	%RSD
R_t (minute)	5.932 ± 0.01	0.238
Mean peak area (mV-min)	93,838.80 ± 29.65	0.031
NTP	14,831.14 ± 26.70	0.180
Mean T_f 5%	1.32 ± 0.002	0.152
Mean T_f 10%	1.26 ± 0.002	0.205

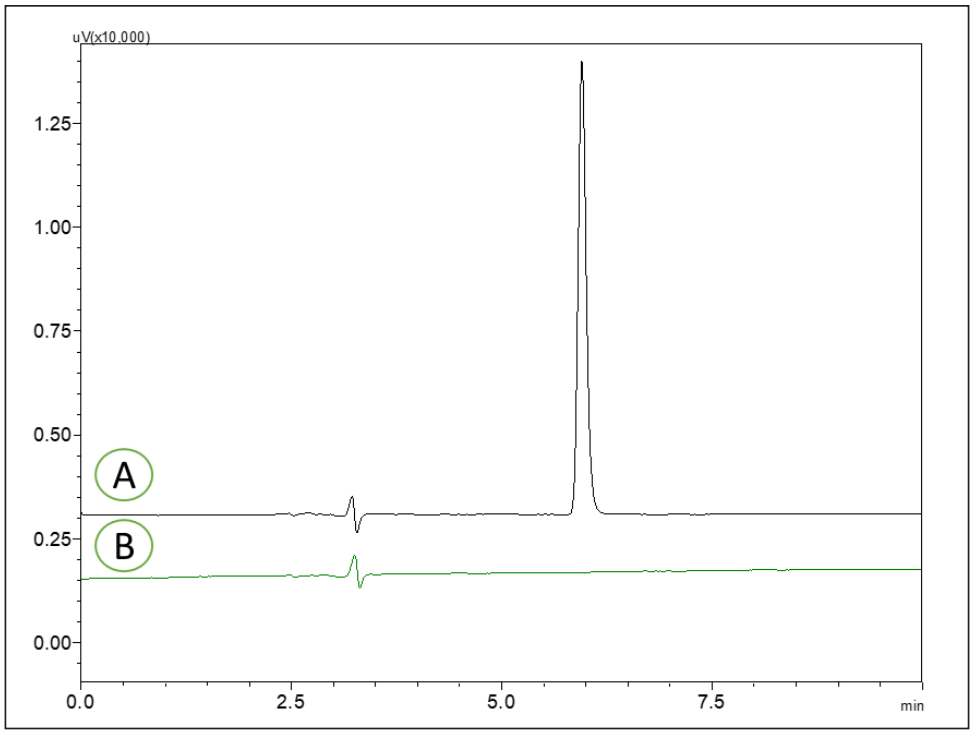


Figure 5. HPLC chromatograms of MPN reference standard 5 µg/ml (A) and HPLC chromatograms of blank (B).

in Table 11. This demonstrates that the mobile phase was stable for 48 hours and can elute MPN effectively even after 48 hours.

Acid—induced degradation studies

Real-Time Conditions: MPN was subjected to 0.1 N and 1 N HCl at ambient temperature for 24 hours to evaluate its stability under acidic conditions. The analysis showed significant degradation, with MPN concentrations reducing by 82.23% and 89.16%, respectively. Degradation peaks were observed near the solvent front (1–3 minutes), indicating acid-catalyzed protonation of the MPN molecule. This reaction heightened molecular reactivity, leading to hydrolysis and the formation of inactive degradation products.

Accelerated Conditions: MPN solutions were exposed to 0.1 N and 1 N HCl at 60°C to simulate accelerated degradation. Substantial degradation was noted, with the primary peak showing splitting and additional peaks near the solvent front. The degradation process was attributed to combined acid-

Table 11. Results of stability studies.

Benchtop stability studies of MPN solution										
Amount of MPN (µg)	**Mean Rt of D3 (minute)**	**Mean peak area of D3 (mV-min)**	**Mean Rt of D1 (minute)**	**Mean peak area of D1 (mV-min)**	**Similarity index for 48 hours stability**	**% Stability of MPN after 48 hours**	**Mean Rt of D2 (minute)**	**Mean peak area of D2 (mV-min)**	**Similarity index for 24 hours stability**	**%Stability of MPN after 24 hours**
4.59	5.949 ± 0.007	86,249.37 ± 64.12	5.942 ± 0.004	65,624.24 ± 70.12	0.761 ± 0.00025	77.38 ± 0.08	5.936 ± 0.001	77,233.88 ± 64.38	0.896 ± 0.00008	91.31 ± 0.08
8.75	5.939 ± 0.01	164,796.39 ± 50.26	5.944 ± 0.02	126,497.25 ± 100.20	0.768 ± 0.00037	78.91 ± 0.06	5.944 ± 0.02	148,103.15 ± 98.12	0.899 ± 0.00032	92.51 ± 0.06

Autosampler post-operative stability studies					
Amount of MPN (µg)	**Mean Rt of fresh sample (min)**	**Mean peak area of fresh sample (mV-min)**	**Mean Rt of 24 hours autosampler stored sample (min)**	**Mean peak area of 24 hours autosampler stored sample (mV-min)**	**Similarity index for autosampler postoperative stability**	**% Stability of MPN after 24 hours autosampler storage**
4.59	5.940 ± 0.03	86,275.10 ± 25.33	5.942 ± 0.007	84,335.85 ± 54.01	0.978 ± 0.00034	99.83 ± 0.07
8.75	5.935 ± 0.001	164,796.39 ± 101.25	5.931 ± 0.01	158,015.99 ± 165.29	0.959 ± 0.00041	98.75 ± 0.10

Benchtop stability studies of mobile phase										
Amount of MPN (µg)	**Mean Rt of M3 (minute)**	**Mean peak area of MPN in M3 (mV-min)**	**Mean Rt of M1 (minute)**	**Mean peak area of MPN in M1 (mV-min)**	**Similarity index of MPN in 48 hours old mobile phase with fresh mobile phase**	**%Stability of MPN in 48 hours old mobile phase**	**Mean Rt of M2 (minute)**	**Mean peak area of M2 (mV-min)**	**Similarity index of MPN in 24 hours old mobile phase with fresh mobile phase**	**%Stability of MPN in 24 hours old mobile phase**
5	5.941 ± 0.004	93,455.82 ± 63.13	5.938 ± 0.05	88,854.25 ± 45.21	0.951 ± 0.00016	96.62 ± 0.05	5.941 ± 0.04	89,226.35 ± 48.57	1.00 ± 0.00004	97.03 ± 0.05

catalyzed hydrolysis and thermal effects, emphasizing the need for controlled storage conditions to maintain drug stability.

Alkali—induced degradation studies

Real-Time Conditions: Stability studies involved exposing MPN to 0.1 N and 1 N NaOH at ambient temperature. After 24 hours, degradation levels reached 75.09% and 91.19%, respectively. The basic environment deprotonated the MPN molecule, promoting beta-lactam ring cleavage and subsequent degradation.

Accelerated Conditions: MPN solutions in 0.1 N and 1 N NaOH were heated to 60°C. Significant degradation was observed, characterized by the splitting of the main peak and additional degradation peaks near the solvent front. The combined effects of base-catalyzed hydrolysis and thermal degradation led to the formation of less active products, indicating the susceptibility of MPN to alkaline and high-temperature conditions.

Oxidative degradation studies

Real-Time Conditions: MPN stability was evaluated by exposing solutions to 3% hydrogen peroxide (H_2O_2) at ambient temperature for 24 hours. Results indicated 30% degradation, likely due to oxidation of the sulfur atom in the thiazolidine ring or cleavage of the beta-lactam ring.

Accelerated Conditions: To simulate oxidative stress, MPN solutions were treated with 3% H_2O_2 and heated to 60°C. Accelerated degradation was evident, with peak splitting and new degradation peaks near the solvent front. Free radical formation and elevated temperatures exacerbated bond cleavage and molecular destabilization.

Photolytic degradation studies

Photolytic stability was assessed by exposing MPN to UV light and direct sunlight for 24 hours. The concentration of MPN decreased by 20%, driven by photodegradation. UV-induced bond cleavage formed unstable degradation products, with peaks observed near the solvent front. This study underscores MPN's sensitivity to light exposure and highlights the importance of protecting the drug from UV and sunlight.

Application of the developed analytical method for quantification of MPN in marketed products and novel nanoformulations

The validated HPLC method was successfully applied for the quantification of MPN in a marketed powder for injection formulation (Merosure 125 mg, Alkem Laboratories, India) and a novel β-CD nanosponges formulation, demonstrating its feasibility for real-world applications.

Quantification of MPN in the marketed formulation

A stock solution of 10 µg/ml of MPN prepared from the marketed product was analyzed using the developed method. The results showed consistent Rt and peak area, with a recovery rate of 99%, highlighting the method's high accuracy and precision.

Quantification of MPN in β-CD-nanosponges

A stock solution of 10 µg/ml of MPN prepared from the marketed product was analyzed using the developed method. The results showed consistent Rt and peak area, with a recovery rate of 99%, highlighting the method's high accuracy and precision.

THE GREENNESS OF THE ANALYTICAL METHOD

The principles of GAC focus on reducing or eliminating the detrimental impact of chemicals employed in analytical methodologies, conserving energy, and curtailing waste generation, all while maintaining the method's analytical efficacy. The green credential of an analytical method is ascertained using multifaceted GAC benchmarks. A myriad of green assessment tools, such as the AES, the HPLC-Eco-Scale Analytical Tool (HPLC-EAT), the Analytical Method Volume Intensity (AMVI), the Complementary Green Analytical Procedure Index (ComplexGAPI), the Analytical Method Greenness Score (AMGS), and both AGREE calculators [AGREE and analytical greenness preparation (AGREEprep)], have been harnessed to holistically evaluate every facet of the analytical procedure [53, 54]. Our proposed method was compared against the "reported method with most citations in Scopus" [11] and the "latest reported method available in Scopus" [14]. These comparisons aimed to showcase the green credentials of the developed method in relation to other existing analytical approaches [43].

Assessment of greenness using the AES tool

The AES is a semi-quantitative environmental scorecard that evaluates analytical processes using a penalty point system. It considers the quantity and hazards of chemicals, energy consumption, occupational hazards, and waste generation [42, 55–57].

The AES rating is derived by deducting the cumulative penalty points from the ideal score (100). Thus, a score closer to 100 indicates a greener method [58, 59].

$$AES = 100 - \text{Total penalty points}$$

For the developed method, AES calculations show a score of 89, making it the most eco-friendly compared to the reported methods, which scored 87 and 85, respectively. Acetonitrile was used in all methods, with each earning penalty points based on its quantity, hazard rating, and hazard pictograms. The developed method used less than 10 ml of acetonitrile, resulting in 4 penalty points, while the other methods had higher penalty points due to methanol's increased hazard pictograms. Waste generation was also lower for the developed method, contributing fewer penalty points (three compared to five for others). All methods used HPLC with similar energy consumption, earning one penalty point for energy. Waste treatment was not applied to any method, resulting in an additional three penalty points.

Table 12 shows the AES scores for all three methods, highlighting the developed method's superior environmental performance.

Assessment of greenness using the HPLC-EAT tool

The HPLC-EAT evaluates the greenness of chromatographic methods by considering the safety, health, and environmental impacts of solvents. The tool is freely available at: http://pubs.rsc.org/en/Content/ArticleLanding/2011/GC/c0gc00667j. The score is calculated based on solvent mass and its safety, health, and environmental characteristics using the equation:

Table 12. AES scoring.

AES parameters	Developed method		The reported method with the most citation in Scopus		The latest reported method in scopus	
		Penalty points		Penalty points		Penalty points
Reagents	Acetonitrile		Acetonitrile		Methanol	
Amount	<10ml	1	<10ml	1	<10ml	1
Hazard Scale	Danger	2	Danger	2	Danger	2
No. of hazard pictograms	Two	2	Two	2	Three	3
Sub total (Amount*Hazard Scale*No. of hazard pictograms)		4		4		6
Reagents	Ammonium acetate buffer		Phosphate buffer		TRIS buffer	
Amount	10–100 ml	2	10–100 ml	2	10–100 ml	2
Hazard Scale	None	0	None	0	None	0
No. of hazard pictograms	None	0	None	0	None	0
Sub total (Amount*Hazard Scale*No. of hazard pictograms)		0		0		0
Total penalty points from reagents (Sum of sub-totals)		4		4		6
Energy consumption	≤ 1.5 kWh	1	≤ 1.5 kWh	1	≤ 1.5 kWh	1
Occupational hazard	Hermetized	0	Hermetized	0	Hermetized	0
Waste generated	1-10ml	3	>10ml	5	>10ml	5
Waste treatment	None	3	None	3	None	3
Total penalty points		**11**		**13**		**15**
AES score		**89**		**87**		**85**

There was no major significance for the bold text; it was bolded as it represented the total penalty points and AES score.

Table 13. AMVI scoring.

AMVI parameters	Developed method	The reported method with the most citations in scopus
Sample preparation solvent		
The volume of solvent required to prepare the standard stock solution (ml)	10	100
The volume of solvent required to prepare the samples (ml)	22	40
Total (ml)	**32**	**140**
HPLC solvent		
Flow rate (ml/minute)	0.75	1
Total runtime (minute)	10	15
Number of injections for 1 full analysis (including blanks and sample injections)	6	6
Number of potential analytes	1	1
Total (ml)	= (0.75*10*6) **= 45**	= (1*15*6) **= 90**
Total solvent consumption (ml)	= 32 + 45 **= 77**	= 90 + 140 **= 230**
AMVI	= 77/1 **= 77**	= 230/1 **=230**

$$HPLC{-}EAT = S_1m_1 + H_1m_1 + E_1m_1 + S_2m_2 + H_2m_2 + E_2m_2 + \ldots\ldots S_nm_n + H_nm_n + E_nm_n$$

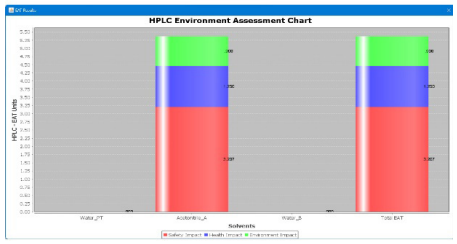
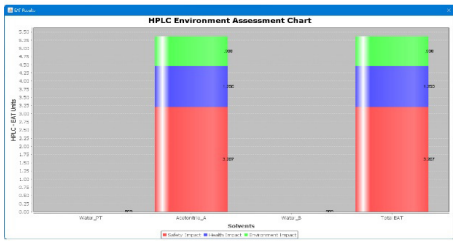
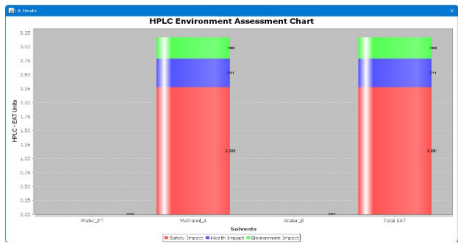
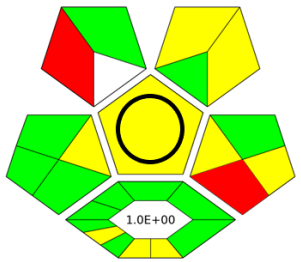
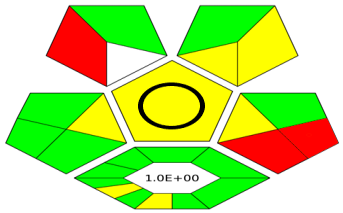
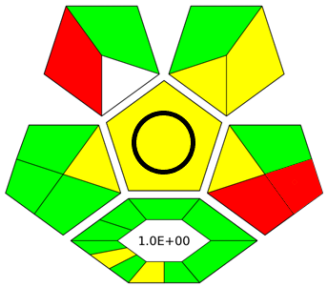
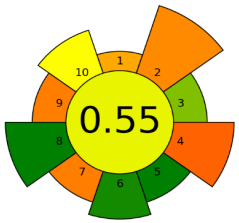
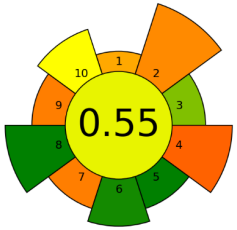
Here, *S*, *H*, and *E* represent safety, health, and environmental concerns, and m is the solvent mass used. A lower score indicates a greener method [53, 60].

We used this tool to assess the developed and existing methods, resulting in scores of 1.59, 4.81, and 6.32, respectively, showing that our method is more environmentally friendly (Table 14).

Assessment of greenness using the AMVI tool

The AMVI tool is a standardized metric designed for pharmaceutical laboratories to evaluate the environmental impact of analytical methods. It calculates total solvent consumption, including solvents used for sample preparation and during

Table 14. Results of various greenness assessments.

Greenness assessment tools	Developed method	The reported method with the most citations in scopus	The latest reported method available in scopus
AES	89	87	85
HPLC-EAT			
AMVI	77	230	----
ComplexGAPI			
AMGS	218.84	284.03	---
AGREE			
AGREEprep			---

HPLC analysis, and accounts for the number of samples and chromatographic peaks analyzed [53, 61]. The formula is:

$$\text{AMVI} = \frac{\text{Total solvent consumption}}{\text{Number of chromatographic peaks of interest}}$$

A lower AMVI value indicates a greener method. We calculated AMVI scores for the developed method and the most cited method, yielding scores of 77 and 230, respectively. Our method proved more environmentally friendly (Table 13).

Assessment of greenness using the ComplexGAPI tool

The Green Analytical Procedure Index (GAPI) provides a semi-quantitative environmental assessment using a pentagonal design divided into 15 segments representing various aspects of the analytical process (Table 14). GAPI uses a color-coding system—green, yellow, and red—to denote low, medium, and high environmental impacts. Despite its detailed analysis, GAPI's complexity and limitations in evaluating all elements accurately prompted the development of ComplexGAPI, which includes a hexagonal component to emphasize pre-analysis activities. ComplexGAPI provides a free software application mostwiedzy.pl/complexgapi that simplifies the evaluation by directly inputting method parameters and generating a pictogram. It incorporates GAPI parameters alongside additional considerations, such as yield/selectivity, reagents and solvents, instrumentation, and the E-factor. The pictogram includes a central circle, indicating a quantitative technique, while the E-factor measures waste generation [62, 63].

The ComplexGAPI analysis showed that the developed method had an eco-friendly profile with 9 green, 13 yellow, and 2 red segments. This was compared to the most cited method (9 green, 12 yellow, and 3 red) and the latest method (7 green, 14 yellow, and 3 red). The developed method used less than 10 ml of solvent per sample, produced less waste (1–10 ml), and had a lower occupational risk due to hermetic sealing, contributing to its higher green rating. All methods achieved yields above 89%, with processing at room temperature in less than 1 hour, earning green scores for these parameters. The developed method met 5–6 green economy rules, indicating its superior environmental compatibility for HPLC quantification of MPN [64].

Assessment of greenness using the AMGS tool

The AMGS is a quantitative tool for assessing the environmental footprint of analytical methods. It considers factors such as instrument energy consumption, solvents' cumulative energy demand, and waste generation based on Environment, Health, and Safety parameters. The AMGS program, which is freely available online, provides a comprehensive evaluation of a method's environmental sustainability. The program is accessible at no cost at www.acsgcipr.org/amgs [53, 64].

For our developed method, the AMGS revealed a greener profile, with a score of 218.84, compared to the most-cited method's score of 284.03. A lower score indicates a greener method, confirming our developed method's superior environmental sustainability.

Assessment of greenness using the analytical greenness calculator (AGREE) tool

The AGREE tool is a comprehensive, user-friendly tool that aligns with the 12 principles of GAC, offering a more flexible and interpretable analysis compared to previous tools. It assigns a score from 0 to 1 to each criterion, indicating the degree of adherence to GAC principles. The final score, represented on a clock face, suggests the method's overall eco-friendliness, with a score above 0.6 indicating a green method [41]. This open-source program is available at https://mostwiedzy.pl/AGREE [53].

The AGREE analysis of the developed and compared methods revealed that all three methods are considered green. The developed method scored 0.65, slightly higher than the compared methods (0.64 and 0.62). All methods utilized LC-based analysis, with solvent volumes and waste generation similar across the methods. While the results were not significantly different, our method had a slight advantage in terms of greenness.

Assessment of greenness using the AGREEprep sample calculator tool

The AGREEprep tool, an extension of the AGREE framework, focuses on evaluating the environmental impact of sample preparation steps in analytical methods. Based on the green sample preparation principles, AGREEprep assigns a subscore (0–1) for each principle, offering a detailed environmental assessment. A pictogram is generated to reflect the method's overall environmental footprint and hazardous content. The tool is freely accessible via https://mostwiedzy.pl/AGREEprep [53].

We used AGREEprep to assess the environmental footprint of the developed method and the most-cited method in Scopus. Both methods utilize online sample preparation, and over 75% of the solvents used are sustainable or renewable water-based buffers. Parameters like waste production, sample size, and throughput were similar across both methods. The energy consumption for both methods was less than 1.5 kWh. Acetonitrile, a hazardous solvent used in both methods, was also considered. AGREEprep gave a score of 0.57 for the developed method and 0.55 for the most-cited method, indicating a slightly greener profile for the developed method.

Indeed, the utilization of seven distinct greenness assessment tools allowed us to comprehensively evaluate various facets of our developed HPLC method. By employing this array of tools, each with its own unique focus, we ensured a thorough exploration of diverse environmental parameters. The unanimous outcomes from these tools consistently favoring our method over the existing ones affirm its overall superiority in terms of ecological friendliness from multiple angles.

CLINICAL AND PATIENT IMPLICATIONS

The developed HPLC-UV method for MPN quantification ensures accurate dosing, crucial for preventing treatment failure and antimicrobial resistance. Its high precision and robustness make it a reliable tool for QC in pharmaceutical formulations, supporting effective patient therapy. The stability and degradation studies provide essential data for optimizing

storage conditions, preserving drug efficacy, and ensuring safe clinical use. Additionally, the method's application to nanosponges highlights its potential to enhance drug stability and controlled release, which could improve patient compliance and therapeutic outcomes. By integrating GAC principles, this method reduces solvent consumption and environmental impact, aligning with sustainable pharmaceutical practices. Overall, this study offers a clinically relevant, reliable, and eco-friendly analytical approach to support effective antibiotic therapy.

CONCLUSION

In a world increasingly aligned with the United Nations' SDGs, the fusion of pharmaceutical research and environmental stewardship gains paramount importance. This study vividly embodies this synergy by introducing an HPLC method for quantifying MPN. Conceived through the meticulous Quality-by-Design approach, our method validates the aspects of precision, reliability, and adaptability, as evidenced by its proficient application across both the marketed formulation and the novel nanosponge formulation. Validations as per the stringent ICH Q2 (R1) guidelines cement the method's credibility, while its demonstrable resilience in exhaustive stability and degradation evaluations ensures its broader applicability in diverse pharmaceutical scenarios. However, our study's commitment to environmental prudence shines brightly beyond these technical accolades. The embrace of GAC and a rigorous assessment of the method's ecological footprint heralds a new era in pharmaceutical research, where technical excellence seamlessly combined with environmental consciousness. Further, our method's marked reduction in environmental impact, when juxtaposed against prevailing methodologies in literature, establishes its pioneering status in analytical competence and sustainability. As the pharmaceutical landscape evolves, our research signals a pressing call to the broader scientific community: the path ahead should exemplify technical prowess and be deeply rooted in ecological responsibility. Our work, thus, stands as a beacon, pointing towards a future where pharmaceutical innovation and planetary well-being walk hand in hand.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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