

Green bioanalytical method for bilastine and cetirizine in plasma using deep eutectic solvent-assisted extraction and quality by design optimization and its application to pharmacokinetic study.

Valapa Anusha^{1*}, Dr. Rajkumari Thagele¹ and Venkata Suresh P²

¹Department of Pharmacy, Career point university, Alaniya, Kota, Rajasthan 325003

²Department of pharmaceutical analysis, Chalapathi Institute of Pharmaceutical Sciences, Lam, Guntur, Andhra Pradesh 522034

*Correspondence Author: anushavalapa1209@gmail.com

Received: 14th June, 2026; Revised: 20th July 2026; Accepted: 20th August, 2026; Available Online: 05th September 2026

ABSTRACT

Background: Sustainable bioanalytical method development requires consideration of analytical performance, robustness, sample-preparation efficiency, and environmental impact. This study developed an Analytical Quality by Design (AQbD)-guided RP-HPLC method for simultaneous determination of bilastine and cetirizine in rat plasma using loratadine as internal standard and deep eutectic solvent (DES)-assisted sample preparation.

Methods: Method variables were identified through preliminary experimentation, Ishikawa analysis, and Failure Mode and Effects Analysis (FMEA). Seven chromatographic variables were screened using a Taguchi design, followed by Central Composite Design (CCD) optimization of critical factors. A choline chloride–propylene glycol DES was used for plasma extraction. Separation was achieved on a Reprosil XR 120 C18 column using 10 mM KH₂PO₄ (pH 3.0 ± 0.1): acetonitrile (55:45, v/v) at 1.0 mL min⁻¹ with detection at 260 nm.

Results: Acetonitrile proportion, buffer pH, and flow rate were identified as critical method parameters. The method was linear over 10–400 ng mL⁻¹, with R² values of 0.9984 and 0.9994 for BIL and CET, respectively, and a validated LLOQ of 10 ng mL⁻¹ for both analytes. Validation demonstrated acceptable precision, accuracy, recovery, matrix effect, robustness, and stability. Environmental and practical performance was assessed using complementary metrics. A preclinical pharmacokinetic assessment further demonstrated the potential applicability of the method for concentration–time analysis following bilastine (BIL) and cetirizine (CET) co-administration.

Conclusion: The integrated deep eutectic solvent-assisted extraction and quality by design approach provided a robust, sustainability-oriented RP-HPLC bioanalytical method for simultaneous estimation of bilastine and cetirizine in plasma samples and preclinical pharmacokinetic application.

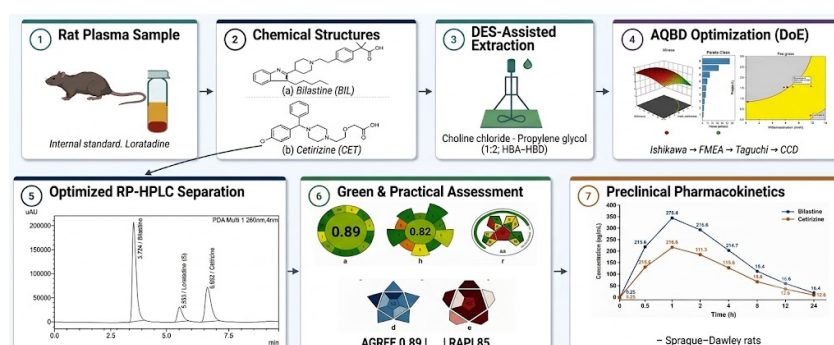
Keywords: Analytical Quality by Design; Bilastine; Cetirizine; Deep eutectic solvent; Green analytical chemistry; RP-HPLC; DoE; Pre-clinical.

How to cite this article: Anusha V, Thagele R, Suresh PV, Green bioanalytical method for bilastine and cetirizine in plasma using deep eutectic solvent-assisted extraction and quality by design optimization and its application to pharmacokinetic study.. Int J Drug Deliv Technol. 2026;16(7): 1363-1379; DOI: 10.25258/ijddt.16.7.147

Source of support: Nil.

Conflict of interest: None

GRAPHICAL ABSTRACT



*Author for Correspondence: anushavalapa1209@gmail.com

INTRODUCTION

Histamine H₁-receptor antagonists are widely used in the management of allergic rhinitis, rhinoconjunctivitis, and urticaria. Bilastine (BIL) and cetirizine (CET) are second-generation antihistamines with predominantly peripheral H₁-receptor activity. BIL is structurally distinct from CET, which belongs to the piperazine class, and these differences in molecular and physicochemical

characteristics are relevant to their analytical behavior. The structural features of BIL and CET are shown in Fig. 1. From a bioanalytical perspective, differences in physicochemical properties, together with the complexity of plasma matrices and the relatively low analyte concentrations encountered in biological samples, can complicate simultaneous extraction, chromatographic separation, and quantitative determination [3,7,9].

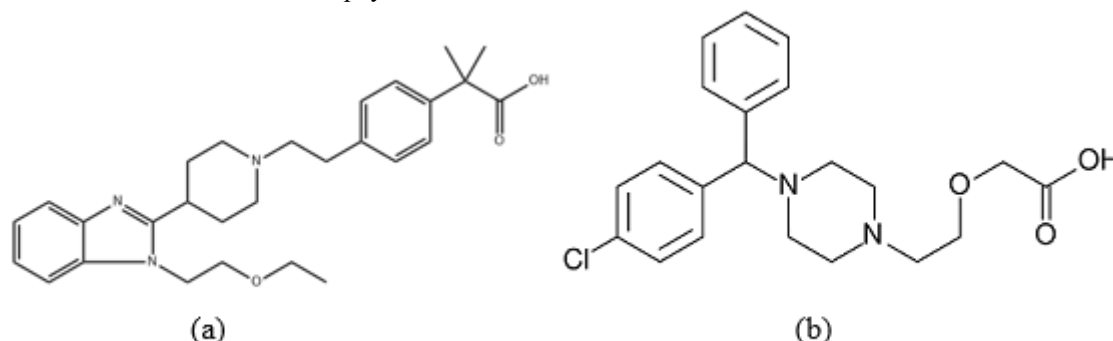


Fig.1. Chemical structures (a) Bilastine (b) Cetirizine.

A range of analytical methods has been reported for BIL, encompassing chromatographic, spectroscopic, and bioanalytical techniques. Padte *et al.* developed an RP-HPLC method for BIL in bulk drug and pharmaceutical formulations [1]. Biagi *et al.* employed near-infrared spectroscopy for in-line quantitative determination of BIL during pharmaceutical powder blending [8], whereas Radwan *et al.* reported a sensitive spectrofluorimetric procedure for BIL in pharmaceutical preparations and biological fluids [9]. These studies demonstrate the availability of relatively simple analytical approaches for BIL determination, but they were not designed for simultaneous BIL–CET quantification in plasma.

More recent BIL studies have extended method development toward experimental optimization, biological matrices, and environmental considerations. Roshdy *et al.* developed a green QbD-assisted RP-HPLC method for simultaneous determination of BIL and montelukast using factorial experimental optimization [2]. Ngo and Pham reported an LC–MS/MS method for quantification of BIL in rat plasma [3], while Ahmed *et al.* developed a sensitive spectrofluorimetric method applicable to pharmaceutical preparations and spiked rat plasma [4]. Although these studies broadened the analytical scope of BIL determination, they did not combine BIL with CET in a plasma assay incorporating DES-assisted sample preparation and sequential risk-based chromatographic optimization.

Comparable developments have been reported for CET. Abd-AlGhafar *et al.* employed a 2³ factorial design for RP-HPLC–UV determination of CET and azelastine, illustrating the usefulness of experimental design in chromatographic optimization [5]. Kormány *et al.* applied design-space concepts to CET impurity profiling and the systematic selection of alternative chromatographic

columns [6]. More recently, Alqahtani *et al.* used CCD optimization together with green chemistry assessment for spectrofluorimetric CET determination [10]. For biological matrices, Khattak *et al.* developed a rapid and cost-effective RP-HPLC/UV method for CET/levocetirizine determination in rat plasma [7]. Despite these advances, the reported methods did not integrate simultaneous BIL–CET plasma determination with DES-assisted extraction and sequential risk assessment, factor screening, and response-surface optimization.

The limitations identified in the available analytical literature provided the basis for adopting an Analytical Quality by Design (AQbD) strategy in the present work. Rather than optimizing chromatographic conditions through isolated one-factor-at-a-time experiments, potential sources of variability were systematically identified and prioritized. Preliminary experimentation and Ishikawa analysis were followed by Failure Mode and Effects Analysis (FMEA) for risk prioritization. Seven chromatographic variables were subsequently screened using a Taguchi design, and the variables identified as critical were subjected to Central Composite Design (CCD) optimization. Resolution, tailing factor, and theoretical plate count were selected as critical chromatographic responses. This sequential FMEA–Taguchi–CCD strategy was adopted to separate risk assessment and factor screening from response-surface optimization, thereby improving experimental efficiency and understanding of the variables governing chromatographic performance.

Sample preparation was considered concurrently with chromatographic development because extraction efficiency and matrix removal directly influence the reliability of plasma bioanalysis. Deep eutectic solvents

(DESs), generally formed through interactions between hydrogen-bond acceptors and hydrogen-bond donors, have attracted considerable interest as tunable extraction media in analytical chemistry [11,12]. Their physicochemical characteristics can be modified through component selection, molar ratio, and water content, facilitating their application in liquid-phase microextraction and related sample-preparation procedures [13,14].

In the present work, a choline chloride–propylene glycol DES was investigated as an extraction-assisting medium in conjunction with acetonitrile-mediated protein precipitation. DES composition and volume, water content, acetonitrile volume, vortexing, sonication, centrifugation, and plasma volume were evaluated during sample-preparation optimization.

Environmental performance was also considered during method development. AGREE provides a quantitative assessment based on the principles of Green Analytical Chemistry [15], while ComplexGAPI considers the environmental implications of different stages of the analytical workflow [16]. Because individual greenness metrics differ in their scope and assessment criteria, the use of complementary tools provides a broader perspective on the environmental characteristics of an analytical procedure [18]. The Blue Applicability Grade Index (BAGI) was additionally used to examine the practical applicability of the method for routine laboratory analysis [17]. Together, these assessment approaches allow environmental performance and operational practicality to be considered alongside conventional analytical performance.

As the proposed procedure is intended for quantitative determination of BIL and CET in plasma, bioanalytical validation was performed with reference to the U.S. FDA/ICH M10 Bioanalytical Method Validation and Study Sample Analysis guidance [19]. The validation framework was applied to establish the suitability of the method for quantitative bioanalysis through evaluation of relevant performance characteristics, including selectivity, calibration performance, sensitivity, accuracy, precision, recovery, matrix effect, dilution integrity, robustness, and stability.

Accordingly, the present study aimed to develop and validate an RP-HPLC method for the simultaneous determination of BIL and CET in rat plasma, integrating DES-assisted sample preparation with a sequential FMEA–Taguchi–CCD AQbD strategy. Environmental sustainability and practical applicability were further evaluated using complementary assessment metrics, and the potential bioanalytical utility of the method was explored through a preclinical pharmacokinetic application of BIL and CET following oral co-administration. The study therefore integrates simultaneous plasma analysis, DES-assisted extraction, risk-based method development, DoE-driven optimization, bioanalytical validation, multidimensional sustainability assessment, and

pharmacokinetic application within a single RP-HPLC workflow.

MATERIALS AND METHODS

Chemicals, reagents and materials

Bilastine (BIL), cetirizine (CET) and loratadine (LOR) (internal standard) reference standard was obtained from Pharmallities (Hyderabad, India). While HCl, HPLC-grade acetonitrile and methanol, KH_2PO_4 , orthophosphoric acid, choline chloride, propylene glycol, and ultrapure water were of analytical/HPLC grade procured from Merck, India. Blank rat plasma was obtained from Dr PathLabs (Hyderabad, India).

Equipment and instrumentation

Chromatographic analysis was performed using a Shimadzu 2030 LC HPLC system with PDA detection. A Shimadzu ATX324R analytical balance, Metrohm 913 pH meter, REMI 101R refrigerated centrifuge, REMI C22 vortex mixer, PAL Life Sciences bath sonicator, Eppendorf micropipettes, deep freezer, and Class A volumetric glassware were used for sample preparation and analysis.

Software and green analytical tools

Experimental design and method optimization were performed using Design-Expert® version 13 (Stat-Ease Inc., Minneapolis, MN, USA). The environmental sustainability and practical applicability of the developed method were assessed using complementary analytical metrics, including AGREE, AGREEprep, MoGAPI, BAGI and RAPI.

Preparation of primary standard stock solutions

Individual stock solutions of Bilastine (BIL), Cetirizine HCl (CET), and Loratadine (internal standard) were prepared separately in methanol. Accurately weighed **10.00 mg** of each reference standard was transferred into a separate 10 mL volumetric flask. Approximately 7 mL of methanol was added, and each solution was sonicated until completely dissolved. The volume was then made up to the mark with methanol and mixed thoroughly. The resulting stock solutions contained 1.0 mg/mL (1000 $\mu\text{g/mL}$) of the respective standards.

Preparation of plasma calibration and quality control standards

Transfer approximately 990 μL blank rat plasma into appropriately labelled tubes and add 10 μL of the respective mixed calibration working solution. The resulting plasma standards contain Bilastine and Cetirizine at 10, 20, 40, 60, 80, 100, 200 and 400 ng/mL. Add the internal standard at a concentration of 200 ng/mL to all calibration standards, QC samples (LLOQ-10, LQC- 30, MQC-200 and HQC-320ng/mL).

Preparation of the Deep Eutectic Solvent

Accurately weigh choline chloride and propylene glycol in the required 1:2 molar ratio. Transfer both components into a clean glass container. Heat at approximately 60–70°C under continuous stirring. Continue mixing until a

clear, homogeneous liquid is formed. Allow the DES to cool to room temperature. Store in a tightly closed container protected from moisture. A small proportion of water, for example 10–20%, may subsequently be investigated during optimization to decrease viscosity.

DES-assisted extraction from rat plasma

For each sample; Transfer 200 μ L of spiked rat plasma into a 1.5 or 2.0mL polypropylene centrifuge tube. Add 200ng/mL of Loratadine internal-standard working solution. Vortex for approximately 20–30 seconds. Add approximately 100 μ L ChCl:propylene glycol DES (1:2). Vortex vigorously for approximately 1 minute. Add approximately 500 μ L cold acetonitrile to facilitate plasma-protein precipitation. Vortex for approximately 2 minutes. Sonicate for approximately 5 minutes to enhance analyte transfer and sample homogenization. Centrifuge at approximately 10,000–12,000 $\times g$ for 10 minutes. Carefully collect the clear supernatant. Dilute an appropriate portion of the supernatant with mobile phase if necessary to minimize solvent-strength and DES-related chromatographic effects. Transfer into an HPLC vial. Inject 10 μ L into the chromatographic system.

Method optimization by AQbD and DOE approach

Extraction parameters influencing recovery of BIL and CET from rat plasma samples were systematically optimized. Key variables included pH extraction solvent volume and sonication time are the key variables. By using a Design of Experiments (DOE) optimization was performed to achieve maximum extraction efficiency while minimizing solvent use.

Initial Experimental Investigation and Failure Mode and Effects Analysis

Preliminary chromatographic experiments were conducted to identify suitable analytical conditions and potential method risk parameters affecting the simultaneous

determination of Bilastine and Cetirizine using Loratadine as an internal standard. Initial trials involved evaluation of the stationary phase, aqueous buffer, organic modifier, mobile-phase composition, pH, flow rate, and other chromatographic variables to achieve adequate resolution, symmetrical peak shape, and satisfactory column efficiency. A C18 stationary phase in combination with potassium dihydrogen phosphate buffer and acetonitrile was found suitable for chromatographic separation. The detection wavelength was also evaluated during preliminary method development based on the UV absorption characteristics of Bilastine, Cetirizine, and Loratadine. Considering the simultaneous response of all three components, 260 nm was selected as the optimized detection wavelength for further chromatographic studies. The optimized method subsequently employed 10 mM KH_2PO_4 buffer at pH 3.0 ± 0.1 with acetonitrile (55:45, v/v), at a flow rate of 1.0 mL/min.

Following preliminary experimentation, potential sources of method variability were identified through brainstorming and categorized under the major domains of Measurement, Method, Manpower, Materials, Machines, and Environment. These included mobile-phase composition, buffer pH, buffer concentration, flow rate, column temperature, injection volume, detection wavelength, reagent and column quality, analyst-related factors, instrument performance, and environmental conditions. The identified variables were systematically organized using an Ishikawa (fishbone) diagram (Fig. 2) to visualize their potential influence on the critical chromatographic responses, namely resolution, tailing factor, and theoretical plate count. The identified risk parameters were subsequently subjected to Failure Mode and Effects Analysis (FMEA) for quantitative risk prioritization and selection of variables for further experimental screening.

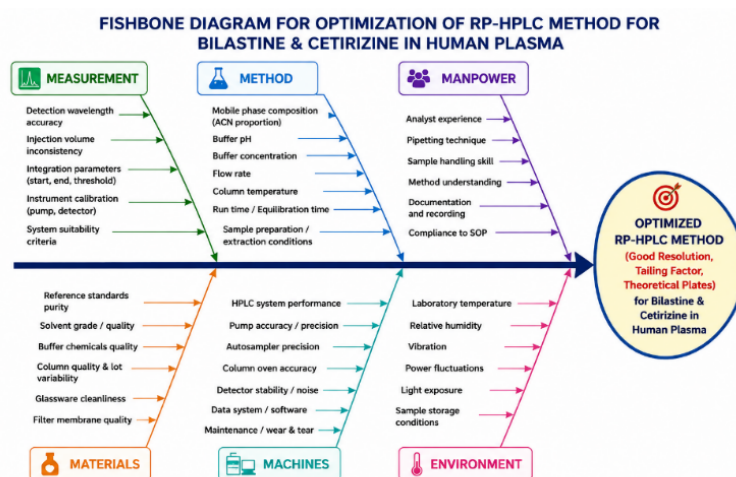


Fig.2. Ishikawa (fishbone) diagram illustrating potential factors influencing the RP-HPLC method.

Failure Mode and Effects Analysis (FMEA)

FMEA was employed to quantitatively prioritize the identified method risk parameters according to their potential influence on chromatographic performance. Each

failure mode was assigned scores for severity (S), occurrence (O), and detectability (D), and the corresponding Risk Priority Number (RPN) was calculated as:

$$RPN = S \times O \times D$$

Higher RPN values indicated variables requiring greater experimental attention, whereas variables with lower RPN values were considered to present comparatively lower risk to method performance. The resulting RPN-versus-failure-mode plot (Fig. 3) facilitated visual ranking of the

investigated variables. Buffer pH, organic modifier composition, flow rate, column temperature, buffer concentration, and injection volume were among the variables identified as potentially important during risk assessment and were therefore considered for subsequent experimental screening.

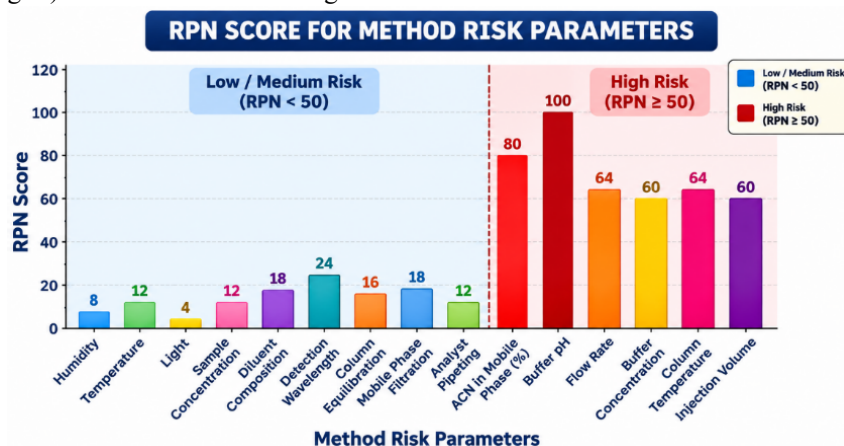


Fig. 3. FMEA-based RPN scores for method risk parameters, distinguishing low/medium-risk (RPN < 50) and high-risk (RPN ≥ 50) factors.

DoE-Based Taguchi Screening of Potential Method Risk Parameters

Following FMEA-based risk prioritization, the potentially influential chromatographic variables were subjected to Design of Experiments (DoE)-based Taguchi screening to experimentally determine their relative effects on the critical chromatographic responses. The selected method parameters were investigated at predefined low (−1) and high (+1) levels, and the experimental design was generated using Design-Expert software. The critical analytical responses monitored during screening were resolution, tailing factor, and theoretical plate count. The experimental runs proposed by the Taguchi design were performed under the specified chromatographic conditions, and the experimentally obtained responses were entered into the design matrix. The influence of individual factors was evaluated using analysis of variance (ANOVA), main-effect analysis, and Pareto charts. The Pareto analysis demonstrated that the magnitude and direction of factor effects differed among the chromatographic responses. Buffer pH showed the strongest overall influence, while flow rate and acetonitrile content of the mobile phase also produced substantial effects, particularly on resolution and column efficiency. Detection wavelength showed an appreciable influence on tailing factor, whereas the remaining investigated variables exhibited comparatively smaller effects.

Response-surface modelling and identification of the Method Operable Design Region by Central Composite Design

Following risk assessment and Taguchi screening, acetonitrile content in the mobile phase (A), buffer pH (B), and flow rate (C) were identified as the critical method parameters (CMPs) exerting the greatest influence on chromatographic performance. Resolution (Rs), tailing

factor, and theoretical plate count were selected as critical analytical responses representing separation efficiency, peak symmetry, and column efficiency, respectively. The selected CMPs were subsequently subjected to response-surface optimization using a Central Composite Design (CCD) in Design-Expert software version 13 (Stat-Ease Inc., Minneapolis, MN, USA). The CCD was selected to evaluate the linear, two-factor interaction, and quadratic effects of the three CMPs while minimizing the experimental burden associated with conventional univariate optimization.

A three-factor CCD comprising **20 experimental runs**, including factorial, axial, and replicated centre-point experiments, was employed. ACN percentage, buffer pH, and flow rate were varied systematically within the predefined experimental domain, while other chromatographic conditions were maintained constant. The experimentally observed values of Rs, tailing factor, and theoretical plates were entered into the design matrix and fitted to second-order polynomial models. Model adequacy was assessed by analysis of variance (ANOVA), model *F*-value, associated *p*-values, lack-of-fit, coefficient of determination (*R*²), adjusted *R*², predicted *R*², coefficient of variation (CV), and adequate precision. Three-dimensional response-surface and contour plots were generated to visualize the individual and interactive effects of the CMPs. The Method Operable Design Region (MODR) was subsequently identified by simultaneous consideration of the predefined chromatographic objectives, particularly adequate resolution, acceptable peak symmetry, and high column efficiency.

Control Strategy and Optimized Chromatographic Conditions

The control strategy was established based on the AQbD-defined operating region to ensure consistent chromatographic performance. Separation was achieved on a Reprosil XR 120 C18 column (250 × 4.6 mm, 5 µm) using 10 mM KH₂PO₄ buffer (pH 3.0 ± 0.1):acetonitrile (55:45, v/v) under isocratic conditions at 1.0 mL min⁻¹, with PDA detection at 260 nm, an injection volume of 10 µL, and column temperature of 30°C. Under the optimized conditions, Bilastine, Loratadine (IS), and Cetirizine eluted at 3.739, 5.593, and 6.751 min, respectively, confirming well-defined sequential elution within the chromatographic run. The corresponding tailing factors were 1.232, 1.351, and 1.321, respectively. Method performance was controlled by maintaining resolution at $R_s \geq 2.0$, tailing factor <2.0, theoretical plates >2000, and retention-time precision ≤1% RSD.

DES-assisted extraction from rat plasma extraction procedure

For each sample; Transfer 200 µL of spiked rat plasma into a 1.5 or 2.0 mL polypropylene centrifuge tube. Add 200ng/mL of Loratadine internal-standard working solution. Vortex for approximately 20–30 seconds. Add approximately 100 µL ChCl:propylene glycol DES (1:2). Vortex vigorously for approximately 1 minute. Add approximately 500–600 µL cold acetonitrile to facilitate plasma-protein precipitation. Vortex for approximately 2 minutes. Sonicate for approximately 5 minutes to enhance analyte transfer and sample homogenization. Centrifuge at approximately 10,000–12,000 ×g for 10 minutes. Carefully collect the clear supernatant. Dilute an appropriate portion of the supernatant with mobile phase if necessary to minimize solvent-strength and DES-related chromatographic effects. Transfer into an HPLC vial. Inject 10 µL into the chromatographic system.

Method Validation

The developed bioanalytical RP-HPLC method for the simultaneous determination of Bilastine (BIL) and Cetirizine (CET) in rat plasma using Loratadine as the internal standard (IS) was validated with respect to selectivity and specificity, sensitivity, linearity, precision and accuracy, matrix effect, extraction recovery, robustness, dilution integrity, and different stability parameters. The calibration range for both analytes was 10.00–400.00 ng/mL. Quality-control samples were prepared at LLOQ QC (10.00 ng/mL), LQC (30.00 ng/mL), MQC (200.00 ng/mL), and HQC (320.00 ng/mL). Six replicate determinations were employed, where applicable, to assess the precision and reliability of the developed method.

Selectivity and Specificity

The selectivity and specificity of the developed method were evaluated using different lots of blank rat plasma to investigate possible interference from endogenous plasma components at the retention times of Bilastine, Cetirizine, and Loratadine (IS). Blank plasma samples were processed using the proposed extraction procedure and chromatographed under the optimized conditions. The chromatograms of blank plasma were compared with those

of the corresponding LLOQ samples and internal-standard-spiked samples. The method was considered selective when no significant endogenous or interfering peaks were observed at the retention times of the analytes and IS. The response of any interfering component at the analyte retention time was evaluated relative to the response obtained at the LLOQ.

Sensitivity and Lower Limit of Quantification

The sensitivity of the developed method was assessed at the lower limit of quantification (LLOQ) of 10.00 ng/mL for both Bilastine and Cetirizine. Six independently prepared LLOQ QC samples were extracted and analyzed under the optimized chromatographic conditions. The mean measured concentration, standard deviation, coefficient of variation (%CV), and percentage nominal concentration were determined. The LLOQ represented the lowest concentration of the calibration range that could be quantified with acceptable precision and accuracy. Signal-to-noise characteristics may additionally be assessed from the chromatographic response where required.

Linearity and Calibration Curve

The linearity of the method was evaluated over the concentration range of 10.00–400.00 ng/mL for both analytes. Calibration standards were prepared in screened blank rat plasma at concentrations of 10.00, 20.00, 40.00, 60.00, 80.00, 100.00, 200.00, and 400.00 ng/mL. Each calibration sample contained a constant concentration of Loratadine as the internal standard and was subjected to the same extraction procedure as the study samples. Calibration curves were constructed by plotting the peak-area ratio of analyte to internal standard on the y-axis against the corresponding nominal plasma concentration on the x-axis. The regression equation and coefficient of determination (R^2) were used to assess the linear relationship between concentration and chromatographic response.

Precision and Accuracy

Within-batch and between-batch precision and accuracy were evaluated using QC samples at four concentration levels: LLOQ QC (10.00 ng/mL), LQC (30.00 ng/mL), MQC (200.00 ng/mL), and HQC (320.00 ng/mL) for both Bilastine and Cetirizine. Six replicate samples were analyzed at each QC level. Intra-day precision and accuracy were evaluated within the same analytical run, whereas inter-day performance was evaluated on different analytical days. Precision was expressed as the coefficient of variation (%CV or %RSD), while accuracy was expressed as the percentage of the measured concentration relative to the corresponding nominal concentration.

Matrix Effect

The influence of endogenous plasma components on the determination of Bilastine and Cetirizine was assessed using different lots of blank rat plasma. Plasma samples from six independent matrix lots were processed and subsequently evaluated at LQC and HQC levels. Three determinations were considered for each plasma lot, and

the mean concentration obtained for each batch was calculated. The overall mean, standard deviation, %CV, and percentage nominal concentration were determined at both QC levels to assess variability attributable to the biological matrix. The consistency of the matrix factor across different plasma lots was used to assess the reproducibility of analyte determination in the presence of plasma components.

Extraction Recovery

Extraction recovery was evaluated at LQC (30.00 ng/mL), MQC (200.00 ng/mL), and HQC (320.00 ng/mL) for both analytes. Six replicates were prepared at each concentration level. The concentrations obtained from extracted QC samples were compared with the corresponding unextracted QC samples prepared at equivalent concentrations.

Dilution Integrity

Dilution integrity was evaluated to demonstrate that samples containing concentrations above the upper calibration limit could be diluted accurately using blank plasma without affecting analytical performance. The high-concentration samples were subjected to two-fold and four-fold dilution, corresponding to nominal diluted concentrations of 200.00 and 100.00 ng/mL, respectively, when the high calibration concentration of 400.00 ng/mL was considered. Six replicate samples were analyzed at each dilution level, and the mean concentration, SD, %CV, and percentage nominal concentration were calculated.

Robustness

The robustness of the developed chromatographic procedure was evaluated by introducing small deliberate variations around the optimized chromatographic conditions. The optimized method employed 10 mM KH_2PO_4 buffer (pH 3.0 ± 0.1) and acetonitrile in a 55:45 (v/v) ratio at a flow rate of 1.0 mL/min with detection at 260 nm. The influence of deliberate changes in flow rate, mobile-phase composition, and detection wavelength was investigated. Six replicate determinations were performed under each condition, and changes in mean analytical response, accuracy, and %RSD were evaluated relative to the actual optimized condition. The percentage difference between the modified and optimized conditions was also calculated to demonstrate that small deliberate variations did not substantially affect method performance.

Stability Studies

Stability studies were performed at LQC (30.00 ng/mL) and HQC (320.00 ng/mL) using six replicate samples at each level. For each stability experiment, the concentrations obtained from stability samples were compared with those obtained from freshly prepared comparison samples. **Bench-Top Stability**

Bench-top stability was evaluated to determine the stability of Bilastine and Cetirizine in plasma during routine sample handling at ambient laboratory temperature. Six replicates each of LQC and HQC samples were maintained at room temperature for the predefined

bench-top stability period and subsequently processed and analyzed. The results were compared with freshly prepared LQC and HQC samples, and the percentage stability was calculated.

Freeze–Thaw Stability

Freeze–thaw stability was evaluated using six replicate LQC and HQC samples subjected to repeated freezing and thawing cycles. Samples were frozen at the designated storage temperature and subsequently allowed to thaw completely at room temperature. After completion of the predefined freeze–thaw cycles, samples were processed and analyzed together with freshly prepared comparison QC samples. The percentage stability at both QC levels was determined from the respective mean concentrations.

Autosampler Stability

Autosampler stability was assessed to establish the stability of the processed plasma extracts while awaiting chromatographic analysis. Six replicate LQC and HQC samples were processed and maintained in the autosampler under the defined temperature conditions for the specified period. Following storage, the samples were reinjected and their concentrations were compared with those of freshly prepared comparison samples. Mean concentration, SD, %CV, percentage nominal concentration, and percentage stability were determined.

Re-Injection Stability

Re-injection stability was evaluated to determine whether previously processed samples could be reliably reanalyzed following an interruption or delay in chromatographic analysis. Six replicate LQC and HQC samples were analyzed initially and subsequently reinjected after the predefined storage interval. The mean concentrations obtained following re-injection were compared with the corresponding initial/fresh sample concentrations, and percentage stability was calculated.

Long-Term Storage Stability

Long-term stability was evaluated to establish the stability of Bilastine and Cetirizine in plasma during prolonged frozen storage. Six replicate LQC and HQC samples were stored under the predefined long-term storage conditions. At the end of the specified storage period, the samples were thawed, processed, and analyzed together with freshly prepared comparison QC samples. Long-term stability was determined by comparing the mean measured concentrations of stored and fresh samples.

Stock-Solution Stability

Stock-solution stability was assessed for Bilastine, Cetirizine, and Loratadine (IS) by comparing the analytical responses obtained from stored stock solutions with those obtained from freshly prepared comparison stock solutions. Six replicate determinations were performed for each compound.

Refrigerated Stock-Solution Stability

Refrigerated stock-solution stability was evaluated by storing the standard stock solutions of Bilastine, Cetirizine, and Loratadine under refrigerated conditions

for the predefined storage period. At the end of the storage period, six replicate responses from each stored stock solution were compared with those obtained from freshly prepared comparison stock solutions. The percentage stability was calculated from the ratio of the mean response of the refrigerated stock solution to the mean response of the freshly prepared stock solution.

Animals

Healthy Sprague–Dawley rats weighing approximately 250–350 g were used for the preclinical pharmacokinetic study. The animals were maintained under standard laboratory conditions with free access to food and water and were acclimatized before experimentation. Twelve animals were randomly divided into two groups ($n = 6$ per group) for administration of the test treatments. All animal procedures were performed following approval from the Institutional Animal Ethics Committee (IAEC) and in accordance with applicable CPCSEA guidelines.

In vivo pharmacokinetic study

The developed bioanalytical method was applied to determine the plasma concentration–time profiles of Bilastine (BIL) and Cetirizine (CET) following oral administration to Sprague–Dawley rats. A pharmacokinetic study was designed in healthy Sprague–Dawley rats weighing 250–350 g. The animals were received an oral combination of **Bilastine (20 mg/kg) and Cetirizine (5 mg/kg)**. Blood samples were collected at predetermined intervals (0.5, 1, 2, 4, 8, 12, and 24 h) and centrifuged to separate plasma. Plasma samples were processed using the optimized deep eutectic solvent (DES)-assisted extraction procedure, with loratadine as the internal standard, and analyzed using the validated RP-HPLC method. Plasma concentrations were quantified using the validated calibration curve, and pharmacokinetic parameters, including C_{max} , T_{max} , AUC_{0-t} , $AUC_{0-\infty}$, and $t_{1/2}$, were subsequently determined.

RESULTS AND DISCUSSION

Analytical Target Profile (ATP) and Critical Quality Attributes (CQAs)

The Analytical Target Profile (ATP) was established with the objective of developing a selective, robust, and efficient RP-HPLC method for the simultaneous determination of Bilastine (BIL) and Cetirizine (CET) in rat plasma using Loratadine as the internal standard. Chromatographic performance was primarily assessed through resolution, tailing factor, and theoretical plate count, which were considered the critical quality attributes (CQAs) of the method. The desired chromatographic performance included adequate separation of the analytes and internal standard, acceptable peak symmetry, and sufficient column efficiency. Accordingly, the method targeted a critical-pair resolution of ≥ 2.0 , a tailing factor below 2.0 with approximately 0.8–1.5 preferred, and theoretical plates greater than 2000. These CQAs were subsequently used as responses during experimental screening and optimization.

Preliminary Trials for Identification of Failure Modes

Preliminary chromatographic trials were performed to identify suitable analytical conditions and potential variables capable of influencing the selected CQAs. Different stationary phases, aqueous and organic mobile-phase components, buffer pH, buffer concentration, flow rate, column temperature, injection volume, and detection wavelength were considered. A C18 stationary phase with potassium dihydrogen phosphate buffer and acetonitrile provided satisfactory chromatographic separation. Based on the UV absorption characteristics and simultaneous analytical response of Bilastine, Cetirizine, and Loratadine, 260 nm was selected as the working detection wavelength. The preliminary experiments provided the basis for identifying potential sources of analytical variability.

The identified risk parameters were categorized under the major domains of Materials, Methods, Machines, Manpower, Measurement, and Environment and represented using an Ishikawa (fishbone) diagram. This systematic risk-identification approach enabled visualization of the possible causes of variation in resolution, peak symmetry, and chromatographic efficiency and provided the foundation for subsequent FMEA-based risk assessment.

Failure Mode and Effects Analysis (FMEA)

Failure Mode and Effects Analysis was employed to quantitatively prioritize the potential method risk parameters identified during preliminary experimentation and Ishikawa analysis. Direct experimental evaluation of every potential source of variability would require a large number of experimental runs; therefore, the Risk Priority Number (RPN) approach was used to identify variables requiring further investigation. Each potential failure mode was assigned scores for severity (S), occurrence (O), and detectability (D). Higher RPN values indicated greater potential risk to chromatographic performance. The calculated RPN values were plotted against the corresponding method risk parameters to facilitate their comparative assessment. The risk analysis indicated that buffer pH and acetonitrile content were among the highest-risk chromatographic variables, while flow rate, buffer concentration, column temperature, and injection volume also warranted experimental investigation. Detection wavelength was additionally retained during screening because of its possible influence on analytical response and peak characteristics. Consequently, seven chromatographic parameters were carried forward for experimental screening rather than attempting to investigate all potential failure modes identified in the Ishikawa analysis.

Screening of Potential Method Risk Parameters by Taguchi Design

A Taguchi experimental design was employed to screen the seven selected method parameters: acetonitrile content in the mobile phase (A), buffer pH (B), flow rate (C), buffer concentration (D), column temperature (E), injection volume (F), and detection wavelength (G). Each factor was investigated at two levels, and an eight-run

experimental matrix was generated. The three CQAs selected as responses were resolution (R1), tailing factor (R2), and theoretical plate count (R3).

The experimental matrix covered 42–48% acetonitrile, pH 2.7–3.3, flow rate 0.9–1.1 mL/min, buffer concentration 5–15 mM, column temperature 25–35°C, injection volume 5–15 μ L, and detection wavelength 230–270 nm. Across the eight experimental runs, resolution varied from 1.72 to 2.56, tailing factor from 1.16 to 1.43, and theoretical plate count from 7130 to 9720, demonstrating that deliberate changes in the chromatographic factors produced measurable differences in method performance.

Among the experimental conditions, Run 4 produced the highest resolution (2.56) and theoretical plate count (9720) together with the lowest tailing factor (1.16). In contrast, Run 5 produced comparatively poorer chromatographic performance, with a resolution of 1.72, tailing factor of 1.43, and theoretical plate count of 7130. The observed

variability across the Taguchi runs confirmed that the selected factors did not contribute equally to chromatographic performance and justified further statistical screening.

Pareto Analysis and Selection of Critical Method Parameters

The Taguchi responses (Fig.4) were subsequently evaluated using statistical effect analysis and Pareto charts. Buffer pH (B) showed the greatest overall influence on the chromatographic responses, particularly resolution, tailing factor, and theoretical plate count. Flow rate (C) also demonstrated a pronounced influence, while acetonitrile content in the mobile phase (A) produced an appreciable effect on resolution and column efficiency. Detection wavelength showed an observable influence particularly on the tailing response, whereas buffer concentration, column temperature, and injection volume produced comparatively smaller overall effects.

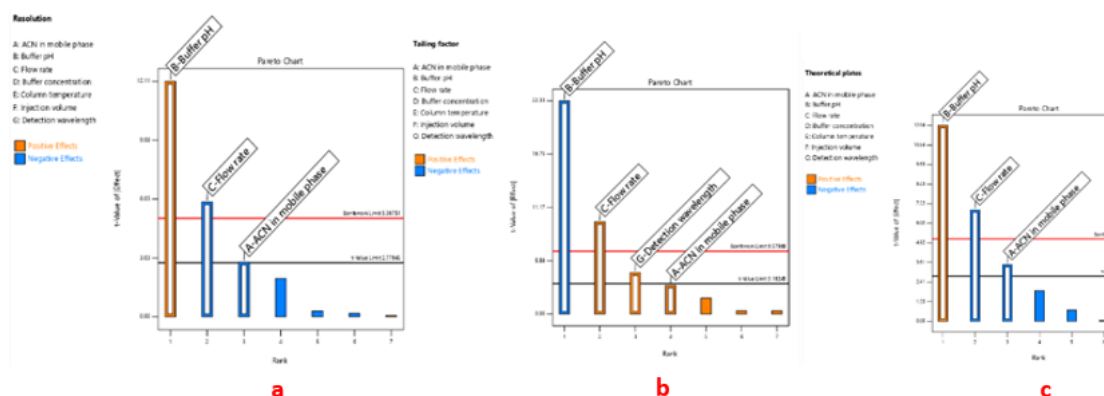


Fig. 4. Pareto charts showing the standardized effects of screened method parameters on (a) Resolution, (b) Tailing factor, and (c) Theoretical plate count following Taguchi screening.

Considering the combined influence of the investigated variables across all three CQAs, acetonitrile content, buffer pH, and flow rate were identified as the principal critical method parameters. These three variables were therefore selected for subsequent optimization using a Central Composite Design (CCD). The CCD was employed to investigate their linear, interaction, and quadratic effects and to define an experimental design space capable of simultaneously providing adequate resolution, acceptable peak symmetry, and high chromatographic efficiency.

Risk Assessment by FMEA and Selection of Variables for DoE

A systematic Failure Mode and Effects Analysis (FMEA) was performed to identify chromatographic and operational variables that could potentially influence method performance. The risk priority number (RPN), calculated from severity, occurrence, and detectability scores, provided a quantitative basis for ranking the variables. As shown in the FMEA table and corresponding RPN plot, buffer pH exhibited the highest risk (RPN = 100), followed by acetonitrile content in the mobile phase (RPN = 80). Flow rate and column temperature each

showed an RPN of 64, while buffer concentration and injection volume showed an RPN of 60. These parameters were therefore categorized as high-risk variables. In comparison, detection wavelength showed a moderate risk (RPN = 24), whereas diluent composition, mobile-phase filtration, column equilibration, room temperature, sample concentration, analyst/pipetting, humidity, and light exposure showed comparatively low RPN scores.

The RPN score graph further illustrates the clear separation between high- and low-risk variables, supporting a risk-based reduction in the number of factors requiring experimental investigation. Rather than directly subjecting all identified variables to response-surface optimization, the high-risk chromatographic factors will be taken forward for Taguchi screening design. This screening step will experimentally assess their relative influence on critical chromatographic responses and eliminate factors having negligible effects. The most influential factors identified through Taguchi screening will subsequently be selected as critical method parameters (CMPs) and investigated using a Central Composite Design (CCD). CCD will enable evaluation of linear, interaction, and quadratic effects and facilitate

construction of response-surface models for defining the optimal chromatographic region. Thus, the sequential FMEA → Taguchi screening → CCD optimization strategy provides a structured risk- and experiment-based approach for progressing from initial variable identification to final method optimization while minimizing unnecessary experimental runs.

Response-surface analysis and statistical optimization

The CCD results (Fig.5) demonstrated that chromatographic performance was governed by significant linear, interaction, and quadratic effects of CAN (acetonitrile) percentage, buffer pH, and flow rate. All three response models were statistically significant ($p < 0.0001$), whereas lack-of-fit was non-significant for resolution ($p = 0.1635$), tailing factor ($p = 0.2614$), and theoretical plate count ($p = 0.4351$). These findings indicate that the quadratic models adequately represented the experimental domain and that the residual variation was predominantly attributable to random experimental error rather than systematic model inadequacy.

Effect of critical method parameters on resolution

The quadratic model for resolution was highly significant, with an F-value of 556.51 ($p < 0.0001$). All three principal factors significantly influenced resolution; among them, buffer pH showed the strongest effect ($F = 1550.71$), followed by ACN percentage ($F = 1030.34$) and flow rate ($F = 872.81$). The AB, AC, and BC interaction terms and all three quadratic terms were also statistically significant ($p < 0.0001$), demonstrating that resolution was controlled by both individual factor effects and interactions within the investigated design space.

The fitted model showed excellent explanatory and predictive performance ($R^2 = 0.9980$, adjusted $R^2 = 0.9962$ and predicted $R^2 = 0.9883$), with a low CV of 0.9732%. The adequate precision value of 79.77, substantially exceeding the generally acceptable signal-to-noise ratio of 4, further demonstrated a strong model signal. The coded polynomial equation was:

$$\text{Resolution} = 2.61 - 0.19A + 0.24B - 0.18C + 0.11AB - 0.0803AC + 0.08BC - 0.12A^2 - 0.14B^2 - 0.10C^2.$$

Effect of critical method parameters on tailing factor

The quadratic model describing peak tailing was also highly significant ($F = 326.86$, $p < 0.0001$). Among the linear factors, flow rate produced the greatest statistical influence ($F = 837.54$), followed by buffer pH ($F = 727.23$) and ACN percentage ($F = 524.97$). The AB and AC interactions were significant at $p < 0.0001$, while BC

remained significant at $p = 0.0003$. Significant quadratic effects for A^2 , B^2 , and C^2 further confirmed curvature in the tailing response.

Model fitting was excellent, with $R^2 = 0.9966$, adjusted $R^2 = 0.9936$ and predicted $R^2 = 0.9813$. The low CV (0.6844%) and high adequate precision (60.326) indicate good reproducibility and a strong signal across the experimental domain. The resulting coded equation was:

$$\text{Tailing factor} = 1.15 + 0.05A - 0.06B + 0.06C + 0.02AB + 0.01AC - 0.01BC + 0.04A^2 + 0.03B^2 + 0.03C^2.$$

Effect of critical method parameters on theoretical plate count

The theoretical plate response also fitted a significant quadratic model ($F = 202.69$, $p < 0.0001$). Flow rate was the dominant linear factor ($F = 615.01$), followed by buffer pH ($F = 389.89$) and ACN percentage ($F = 316.61$). The AB interaction was particularly pronounced ($F = 77.73$, $p < 0.0001$), while AC ($p = 0.0055$) and BC ($p = 0.0009$) were also statistically significant. All quadratic terms were significant, confirming a nonlinear relationship between the CMPs and chromatographic efficiency.

The model exhibited $R^2 = 0.9945$, adjusted $R^2 = 0.9896$ and predicted $R^2 = 0.9734$, with a CV of only 1.24% and adequate precision of 51.32. The non-significant lack-of-fit ($p = 0.4351$) further supports the suitability of the quadratic model for navigating the experimental region. The resulting coding equation was: Theoretical Plates = $9806.38 - 54A + 602.10B - 756.21C + 351.25AB - 140.5AC + 184.5BC - 366.12A^2 - 429.23B^2 - 303.54C^2$.

Response-surface interpretation and MODR

The three-dimensional (Fig.5) response surface plots illustrate the combined effects of acetonitrile concentration (A) and buffer pH (B) on the critical chromatographic responses. In (a), resolution showed a curved response, indicating that both factors and their interaction influenced analyte separation, with better resolution obtained around the central experimental region. In (b), the tailing factor increased away from the optimum region, supporting the selection of intermediate conditions for improved peak symmetry. In (c), the theoretical plate count exhibited a nonlinear response, with higher column efficiency observed near the central region.

Collectively, the response surfaces supported the selection of approximately 45% acetonitrile and pH 3.0 as optimum conditions, providing a suitable balance of resolution, peak symmetry, and column efficiency.

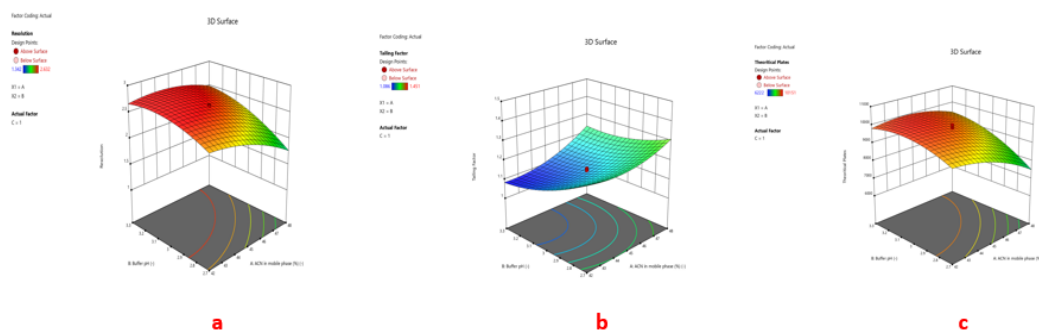


Fig. 5. Three-dimensional response surface plots showing the effects of acetonitrile concentration (A) and buffer pH (B) on (a) resolution, (b) tailing factor, and (c) theoretical plate count.

The overlay plot defines the method operable design region (MODR) based on acetonitrile content and buffer pH. The yellow region (Fig.6) represents conditions satisfying the predefined chromatographic criteria,

whereas the grey region indicates unacceptable conditions. The selected optimum (45% acetonitrile, pH 3.0) lies within the MODR, supporting the robustness of the optimized method.

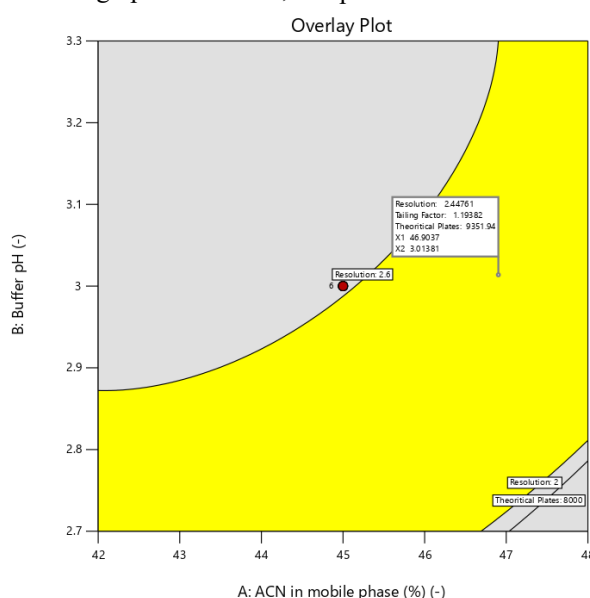


Fig. 6. Overlay plot showing the method operable design region (MODR) for acetonitrile content and buffer pH, with the selected optimum at 45% acetonitrile and pH 3.0.

METHOD DEVELOPMENT

Optimization of DES-Assisted Extraction Parameters

The DES-assisted extraction procedure was systematically optimized to achieve efficient recovery of Bilastine (BIL) and Cetirizine (CET) from spiked rat plasma.

The investigated parameters (Fig.7) included DES composition and volume, water content, acetonitrile volume, vortex mixing time, sonication time, centrifugation force, and plasma volume. Extraction recovery of both analytes was considered the primary criterion, together with sample clarity, ease of handling, and compatibility of the resulting extract with the RP-HPLC system. The ChCl:propylene glycol molar ratio significantly influenced extraction performance. Among the ratios evaluated (1:1, 1:2, and 1:3), the 1:2 ratio provided the highest recoveries of 96.73% for BIL and 97.12% for CET, compared with 91.84 and 90.96% at 1:1

and 94.18 and 93.67% at 1:3, respectively. This improvement may be attributed to a favourable balance between solvent polarity, hydrogen-bonding interactions, and viscosity; therefore, the 1:2 ratio was selected.

Optimization of the DES volume showed that increasing the volume from 50 to 100 μ L improved BIL and CET recoveries from 92.46 and 91.72% to 96.58 and 97.03%, respectively. A further increase to 150 μ L reduced recovery, indicating that excess DES offered no additional extraction advantage. Similarly, incorporation of 10% water into the DES improved recoveries to 96.41% for BIL and 96.86% for CET, compared with approximately 92% without water. Increasing the water content to 20% resulted in a moderate decline in recovery, suggesting that 10% water provided an appropriate balance between reduced viscosity and preservation of DES extraction characteristics.

The volume of acetonitrile, used primarily for plasma protein precipitation, was also optimized. The highest recoveries were obtained with 500 μ L, reaching 96.92% for BIL and 97.28% for CET. Lower recovery was observed with 400 μ L, whereas increasing the volume to 600 μ L provided no further improvement and would increase solvent consumption and sample dilution. Thus, 500 μ L was selected for subsequent extraction.

Extraction efficiency was further influenced by sample-processing conditions. Increasing the vortex time from 1 to 2 min improved recoveries to 96.64% for BIL and 97.06% for CET, while extending mixing to 3 min produced no additional benefit. Likewise, 5 min sonication provided maximum recoveries of 96.77 and 97.19%, respectively, whereas prolonged sonication to 10 min resulted in a slight decrease. These findings supported the selection of 2 min vortexing followed by 5 min sonication.

Centrifugation at 10,000 $\times$ g provided efficient sample clarification and recoveries of 96.55% for BIL and 96.94% for CET. Increasing the centrifugal force to 14,000 $\times$ g did not appreciably improve extraction.

Plasma volume was finally optimized between 100 and 300 μ L, with 200 μ L plasma providing the highest recoveries (96.81% for BIL and 97.16% for CET), while the larger volume resulted in reduced recovery, probably because of the increased matrix burden.

Overall, the optimized extraction conditions comprised 200 μ L plasma, 100 μ L ChCl:propylene glycol DES (1:2) containing 10% water, 500 μ L acetonitrile, 2 min vortexing, 5 min sonication, and centrifugation at 10,000 $\times$ g. The selected conditions consistently provided approximately 96–97% recovery for both analytes, supporting their suitability for subsequent bioanalytical evaluation.

Based on this optimization, the proposed final conditions are ChCl:propylene glycol 1:2 molar ratio, 100 μ L DES containing 10% water, 200 μ L plasma, 500 μ L acetonitrile, 2 minutes vortexing, 5 minutes sonication and centrifugation at 10,000 $\times$ g. These conditions should then be experimentally verified using extraction recovery, precision, matrix effect and chromatographic performance before being designated as the final validated extraction procedure.

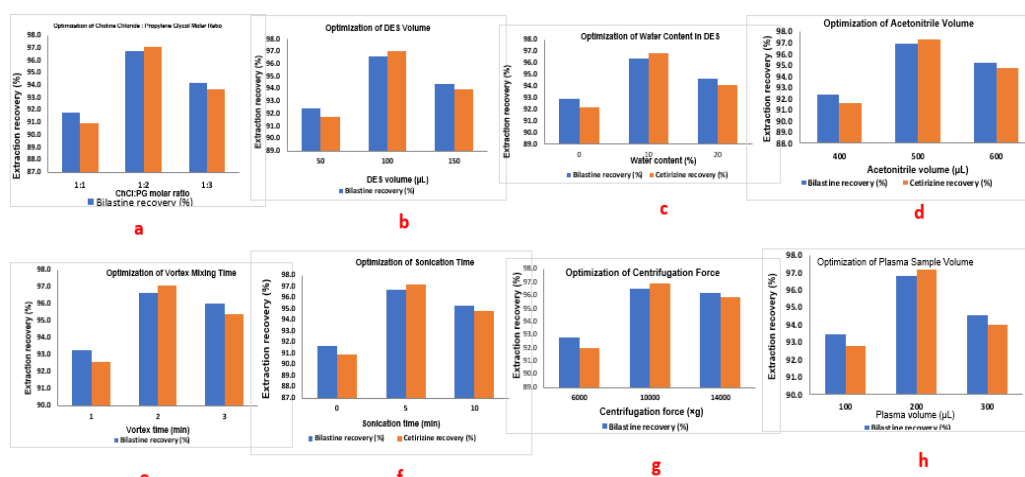


Fig. 7. Optimization of DES-assisted plasma extraction parameters for Bilastine and Cetirizine: (a) DES molar ratio, (b) DES volume, (c) water content, (d) acetonitrile volume, (e) vortex time, (f) sonication time, (g) centrifugation force, and (h) plasma volume.

Optimization of Chromatographic Conditions

Preliminary chromatographic trials were performed using various columns and mobile-phase compositions to achieve satisfactory simultaneous separation of Bilastine (BIL), Cetirizine (CET), and Loratadine (IS). Different combinations of aqueous buffers and organic modifiers, along with variations in pH, flow rate, and detection wavelength, were evaluated. Among the conditions examined, a C18 stationary phase with KH_2PO_4 buffer–acetonitrile provided better peak separation and symmetry, with 260 nm selected for detection.

Subsequently, AQbD-based risk assessment and screening identified acetonitrile content, buffer pH, and flow rate as critical variables, which were optimized using CCD. The final conditions (Fig.8) comprised 10 mM KH_2PO_4 (pH 3.0 ± 0.1):acetonitrile (55:45, v/v) at 1.0 mL min⁻¹. Under these conditions, BIL, Loratadine, and CET eluted at 3.739, 5.593, and 6.751 min, respectively, with satisfactory peak symmetry and resolution, supporting further bioanalytical validation.

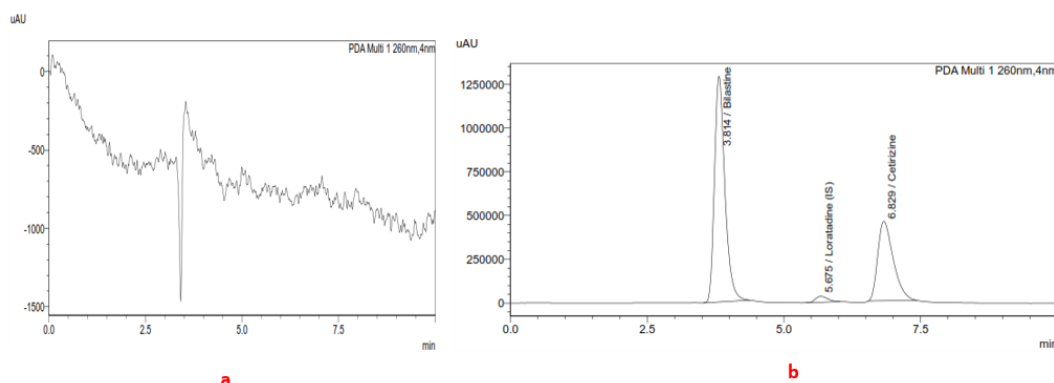


Fig. 8. Representative chromatograms: **(a)** blank and **(b)** optimized standard chromatogram of Bilastine, Loratadine (IS), and Cetirizine.

Method validation

The method showed (Table.1) adequate selectivity, with no significant endogenous interference at the retention regions of BIL, CET, and Loratadine (IS). The validated LLOQ was 10 ng/mL for both analytes, with accuracies of 97.08% (BIL) and 97.13% (CET) and %CV values of 1.28% and 1.58%, respectively, confirming adequate sensitivity and reproducibility. The calculated LOD/LOQ values were 0.59/1.80 ng/mL for BIL and 0.53/1.78 ng/mL for CET. However, 10 ng/mL was retained as the validated LLOQ, representing the lowest experimentally validated calibration level.

Linear responses were obtained over 10–400 ng/mL, with R^2 values of 0.9984 for BIL and 0.9994 for CET. Across the calibration range, accuracy was 95.84–97.43% for BIL and 95.68–97.36% for CET, with %RSD $\leq 0.603\%$, demonstrating consistent quantitative performance. At LLOQ, LQC, MQC, and HQC levels, intra- and inter-day accuracies ranged from 95.73–97.38% for BIL and 95.68–97.54% for CET, with %RSD $\leq 1.62\%$. These results demonstrated satisfactory repeatability and intermediate precision.

BIL recoveries were 94.99, 96.19, and 96.37%, while CET recoveries were 95.84, 96.39, and 95.40% at LQC, MQC, and HQC, respectively. The low variability (%CV $< 0.86\%$) indicated consistent extraction efficiency across QC levels. Apparent matrix factors ranged from 0.9663–0.9672 for BIL and 0.9667–0.9687 for CET, with %CV $\leq 0.331\%$, indicating minimal matrix-related variability. Two- and four-fold diluted samples showed accuracies of 96.66–97.18% for BIL and 96.58–96.79% for CET, with %CV $\leq 0.65\%$, confirming reliable quantification following sample dilution. Deliberate variations in flow rate, mobile-phase composition, and detection wavelength produced only minor changes in analytical response, with %RSD $< 0.65\%$, demonstrating satisfactory method robustness. BIL and CET remained stable under autosampler, freeze–thaw, bench-top, long-term storage, and re-injection conditions, with measured stability ranging from approximately 99.08–99.63% and consistently low variability. After 7 days of refrigerated storage, BIL, CET, and Loratadine retained 99.43%, 99.34%, and 99.48%, respectively, relative to freshly prepared solutions, indicating satisfactory stock-solution stability.

Table. 1. Validation performance characteristics of the developed bioanalytical RP-HPLC method for Bilastine in rat plasma.

Validation parameter	Level/Condition	Bilastine (BIL)	Cetirizine (CET)
Linearity	Range (ng/mL)	10–400	10–400
	Measured range (ng/mL)	9.637–388.840	9.573–385.560
	%RSD	0.302–0.603	0.302–0.603
	%Accuracy	95.84–97.43	95.68–97.36
	R^2	0.9984	0.9994
Within-run precision *(MMC, %RSD, %AC)	LLOQ (10 ng/mL)	9.573; 1.412%; 95.728%	9.657; 1.329%; 96.57%
	LQC (30 ng/mL)	28.926; 1.18%; 96.42%	28.782; 1.09%; 95.94%
	MQC (200 ng/mL)	194.32; 0.84%; 97.16%	194.86; 0.77%; 97.43%
	HQC (320 ng/mL)	309.792; 0.63%; 96.81%	308.096; 0.58%; 96.28%
Between-run precision	LLOQ	9.611; 1.561%;	9.568; 1.62%;

Validation parameter	Level/Condition	Bilastine (BIL)	Cetirizine (CET)
*(MMC, %RSD, %AC)		96.11%	95.68%
	LQC	29.214; 1.27%; 97.38%	29.136; 1.21%; 97.12%
	MQC	191.78; 0.92%; 95.89%	192.72; 0.88%; 96.36%
	HQC	311.072; 0.71%; 97.21%	312.128; 0.69%; 97.54%
Recovery *(%RC, %RSD)	LQC	94.985%; 0.838%	95.835%; 0.859% RSD
	MQC	96.187%; 0.802%	96.389%; 0.819% RSD
	HQC	96.370%; 0.763%	95.399%; 0.783% RSD
Matrix factor *(MF, %RSD)	LQC	0.9663; 0.27	0.9667; 0.33
	HQC	0.9672; 0.153	0.9687; 0.15
Autosampler stability	LQC / HQC	99.546 / 99.630%	99.575 / 99.566%
Freeze–thaw stability	LQC / HQC	99.268 / 99.421%	99.409 / 99.404%
Bench-top stability	LQC / HQC	99.492 / 99.464%	99.423 / 99.399%
Long-term stability	LQC / HQC	99.084 / 99.205%	99.120 / 99.166%

*MMC: Mean measured concentration; %RSD: Relative standard deviation; %AC: %Accuracy; MF: matrix factor.

Preclinical Pharmacokinetic Application

The applicability of the developed bioanalytical method was further demonstrated using a plasma concentration–time profile of Bilastine and Cetirizine following oral co-administration in rats (Fig.9, Table.2). Both analytes showed a rapid increase in plasma concentration, reaching C_{max} at 1 h, with maximum concentrations of 346.80 ng/mL for Bilastine and 216.70 ng/mL for Cetirizine. Thereafter, plasma concentrations declined progressively up to 24 h, with terminal half-lives of 6.39 and 6.53 h, respectively.

The estimated AUC_{0–24} values were 2480.45 and 1587.88 ng·h/mL, while AUC_{0–∞} values were 2654.57 and

1706.60 ng·h/mL for Bilastine and Cetirizine, respectively, indicating greater systemic exposure for Bilastine.

The estimated MRT_{0–∞} values were comparable (8.17 and 8.35 h), whereas the apparent oral clearance (CL/F) and volume of distribution (Vd/F) were 7.53 L/h/kg and 69.4 L/kg for Bilastine and 2.93 L/h/kg and 27.6 L/kg for Cetirizine, respectively. Importantly, all quantifiable concentrations remained within the validated analytical range of 10–400 ng/mL, supporting the potential applicability of the developed method for pharmacokinetic sample analysis.

Table. 2. Pharmacokinetic parameters of Bilastine and Cetirizine following oral co-administration Sprague–Dawley in rats.

Pharmacokinetic parameter	Bilastine	Cetirizine
C _{max} (ng/mL)	346.80	216.70
T _{max} (h)	1.00	1.00
AUC _{0–24} (ng·h/mL)	2480.45	1587.88
AUC _{0–∞} (ng·h/mL)	2654.57	1706.60
t _{1/2} (h)	6.39	6.53
MRT _{0–∞} (h)	8.17	8.35
CL/F (L/h/kg)	7.53	2.93
Vd/F (L/kg)	69.4	27.6

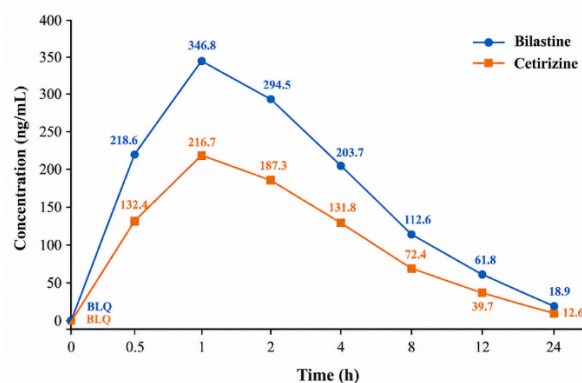


Fig.9. Plasma concentration–time profiles of Bilastine and Cetirizine following oral co-administration in Sprague–Dawley rats.

Assessment of Greenness and Analytical Performance

The sustainability, practicality, and analytical performance of the developed method was evaluated using complementary assessment metrics. The method achieved an AGREE score of 0.89, indicating high compliance with Green Analytical Chemistry principles, while an AGREEprep score of 0.82 demonstrated a favorable environmental profile of the plasma sample-preparation procedure. The MoGAPI score of 83 further supported the

overall greenness of the analytical workflow. Practical applicability assessed by BAGI yielded a score of 81, indicating good suitability for routine bioanalysis. Furthermore, the RAPI score of 85 demonstrated strong analytical performance. Collectively, these assessments indicate that the developed method provides a favorable balance between environmental sustainability, practical applicability, and analytical performance (Fig. 10a–e).

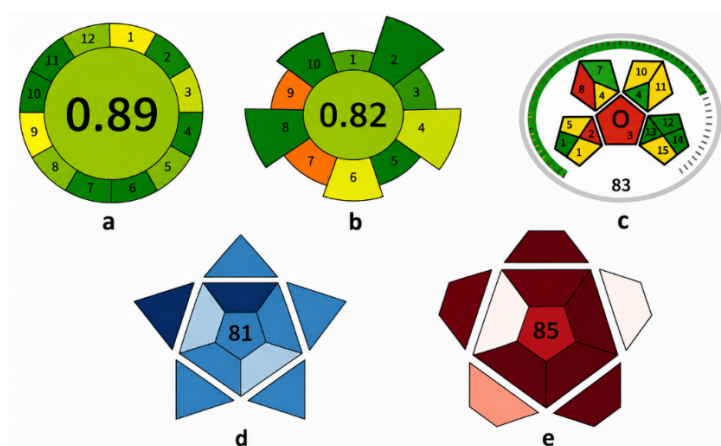


Fig. 10. Assessment of the developed bioanalytical RP-HPLC method using (a) AGREE (0.89), (b) AGREEprep (0.82), (c) MoGAPI (83), (d) BAGI (81), and (e) RAPI (85) metrics.

CONCLUSION

A robust RP-HPLC method was developed for simultaneous determination of bilastine and cetirizine in rat plasma by integrating DES-assisted extraction with sequential FMEA–Taguchi–CCD optimization. The method demonstrated satisfactory chromatographic performance and reliable validation across 10–400 ng/mL. The complementary greenness and applicability metrics supported its environmental and practical performance. Furthermore, preclinical pharmacokinetic assessment following oral co-administration demonstrated its applicability to concentration–time analysis, with both analytes remaining within the validated analytical range. Overall, the proposed approach offers a practical, sustainability-oriented bioanalytical strategy suitable for

simultaneous BIL–CET determination and pharmacokinetic applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are thankful to the Chalapathi Drug Testing Laboratory and Chalapathi Institute of Pharmaceutical Sciences for providing the research facilities.

REFERENCES

1. Padte CK, Batwal PR, Patil MS, Jain AS. Development and validation of analytical method for estimation of bilastine in bulk and pharmaceutical (tablet) dosage form. *Journal of the Indian Chemical*

- Society*. 2021;98(11):100172. doi:10.1016/j.jics.2021.100172.
2. Roshdy A, Abdel Salam R, Hadad G, Belal F, Elmansi H. Green quality by design HPLC approach for the simultaneous determination of Bilastine and Montelukast. *BMC Chemistry*. 2023;17:43. doi:10.1186/s13065-023-00953-y.
3. Ngo QT, Pham TH. Development and validation of a LC-MS/MS method for quantification of bilastine in rat plasma. *Journal of Pharmaceutical and Cosmetic Control*. 2024;22(1):15–20.
4. Ahmed AS, Badr El-Din KM, Khorshed AA, Derayea SM, Oraby M. Validation of a green and sensitive spectrofluorimetric method for determination of Bilastine and its application to pharmaceutical preparations, content uniformity test, and spiked rat plasma. *BMC Chemistry*. 2025;19:258. doi:10.1186/s13065-025-01622-y.
5. Abd-AlGhafar WN, Aly FA, Sheribah ZA, Saad S. Factorial design-assisted reverse phase HPLC–UV approach for the concurrent estimation of cetirizine and azelastine in aqueous humor. *Scientific Reports*. 2022;12:22435. doi:10.1038/s41598-022-26774-y.
6. Kormány R, Soós B, Horváth K. Updating the European Pharmacopoeia impurity profiling method for cetirizine and suggesting alternative column, using design space comparison. *Journal of Pharmaceutical and Biomedical Analysis*. 2024;237:115776. doi:10.1016/j.jpba.2023.115776.
7. Khattak M, Iqbal Z, Jan A, Ali N, Nasir F, Shahbaz N. Development and validation of rapid and cost-effective RP-HPLC/UV method for determination of cetirizine/levocetirizine in blood plasma of smokers and non-smokers: a two cohort comparative study. *Journal of Chromatographic Science*. 2025;63(4):bmae058. doi:10.1093/chromsci/bmae058.
8. Biagi D, Nencioni P, Valleri M, Calamassi N, Mura P. Development of a Near Infrared Spectroscopy method for the in-line quantitative bilastine drug determination during pharmaceutical powders blending. *Journal of Pharmaceutical and Biomedical Analysis*. 2021;204:114277. doi:10.1016/j.jpba.2021.114277.
9. Radwan AS, Elkhoudary MM, Hadad GM, Belal F, Salim MM. A highly sensitive spectrofluorimetric method for the determination of bilastine in its pharmaceutical preparations and biological fluids. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. 2022;276:121246. doi:10.1016/j.saa.2022.121246.
10. Alqahtani T, Alqahtani A, Almrasy AA. Spectrofluorimetric cetirizine analysis using erythrosin B static quenching with mechanistic studies, central composite design optimization, and green chemistry evaluation. *Journal of Photochemistry and Photobiology A: Chemistry*. 2026;471:116733. doi:10.1016/j.jphotochem.2025.116733.
11. Santana-Mayor Á, Rodríguez-Ramos R, Herrera-Herrera AV, Socas-Rodríguez B, Rodríguez-Delgado MÁ. Deep eutectic solvents. The new generation of green solvents in analytical chemistry. *TrAC Trends in Analytical Chemistry*. 2021;134:116108. doi:10.1016/j.trac.2020.116108.
12. Hansen BB, Spittle S, Chen B, Poe D, Zhang Y, Klein JM, et al. Deep eutectic solvents: a review of fundamentals and applications. *Chemical Reviews*. 2021;121(3):1232–1285. doi:10.1021/acs.chemrev.0c00385.
13. Santos LB, Assis RS, Barreto JA, Bezerra MA, Novaes CG. Deep eutectic solvents in liquid-phase microextraction: Contribution to green chemistry. *TrAC Trends in Analytical Chemistry*. 2022;146:116478. doi:10.1016/j.trac.2021.116478.
14. Tang W, An Y, Row KH. Emerging applications of (micro)extraction phase from hydrophilic to hydrophobic deep eutectic solvents: opportunities and trends. *TrAC Trends in Analytical Chemistry*. 2021;136:116187. doi:10.1016/j.trac.2021.116187.
15. Pena-Pereira F, Wojnowski W, Tobiszewski M. AGREE—Analytical GREEnness Metric Approach and Software. *Analytical Chemistry*. 2020;92(14):10076–10082. doi:10.1021/acs.analchem.0c01887.
16. Płotka-Wasyłka J, Wojnowski W. Complementary green analytical procedure index (ComplexGAPI) and software. *Green Chemistry*. 2021;23:8657–8665. doi:10.1039/D1GC02318G.
17. Manousi N, Wojnowski W, Płotka-Wasyłka J, Samanidou V. Blue applicability grade index (BAGI) and software: a new tool for the evaluation of method practicality. *Green Chemistry*. 2023;25:7598–7604. doi:10.1039/D3GC02347H.
18. Sajid M, Płotka-Wasyłka J. Green analytical chemistry metrics: a review. *Talanta*. 2022;238:123046. doi:10.1016/j.talanta.2021.123046.
19. U.S. Food and Drug Administration. M10 Bioanalytical Method Validation and Study Sample Analysis: Guidance for Industry. U.S. Department of Health and Human Services, Food and Drug Administration, CDER and CBER; November 2022. This is confirmed directly by the FDA.
20. Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Compendium of CPCSEA 2018. Animal Welfare Division, Ministry of Environment, Forest and

Green bioanalytical method for bilastine and cetirizine in plasma using deep eutectic solvent-assisted extraction and quality by design optimization and its application to pharmacokinetic study.

Climate Change, Government of India, New Delhi;
2018. The current official regulator is now CCSEA,

whose website continues to host the CPCSEA
Compendium.