

QBD ENABLED DEVELOPMENT AND VALIDATION OF A GREEN REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS QUANTIFICATION OF SELEGILINE AND BRAHMI (*BACOPA MONNIERI*) EXTRACT

RAKESH KUMAR^{ID}, RAJESH KUMAR^{*ID}, SUMANT SAINI^{ID}, VIJAY MISHRA^{ID}

School of Pharmaceutical Sciences, Lovely Professional University, Phagwara (Punjab)-144411, India

*Corresponding author: Rajesh Kumar; *Email: rajksach09@gmail.com

Received: 01 Feb 2026, Revised and Accepted: 02 May 2026

ABSTRACT

Objective: This study aimed to develop and validate a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous quantification of Selegiline and Brahmi (*Bacopa monnieri*) extract.

Methods: The chromatographic method was optimized using the quality by design approach. Initially, risk assessment was carried out. From this, plausible factors were selected and screened using a screening design. Following this, the critical variables were optimized, and the design space thus obtained was verified using monte carlo simulations. Chromatographic separation was achieved using a C-18 column with a mobile phase consisting of acetonitrile and 0.72% anhydrous sodium sulphate (31.5:68.5 v/v) at a flow rate of 1 ml/min, with detection wavelength optimised at 205 nm. The method was validated in accordance with ICH Q2(R2) guidelines, assessing linearity, accuracy, precision, limit of detection, and limit of quantification.

Results: The developed method was linear over the concentration ranges of 2-10 µg/ml for Selegiline and 5-25 µg/ml for brahmi extract (BE). The method demonstrated excellent linearity for both drugs, with R values exceeding 0.99. Accuracy and precision values fell within acceptable ranges, indicating strong repeatability. In the system suitability test, the theoretical plates were found to be greater than 2000, and the tailing factor was less than 2. LOD and LOQ for Selegiline and BE were 0.86, 2.35, 0.71, and 2.35 µg/ml, respectively. The method was evaluated for its environmental impact using its green score.

Conclusion: The validated RP-HPLC method was robust and reliable for the simultaneous quantification of Selegiline and Brahmi extract, making it suitable for quality control applications in pharmaceutical formulations.

Keywords: Selegiline, *Bacopa monnieri*, Bacoside A, RP-HPLC, Validation, ICH guidelines

© 2026 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<https://creativecommons.org/licenses/by/4.0/>) DOI: <https://dx.doi.org/10.22159/ijap.2026v18i4.58355> Journal homepage: <https://innovareacademics.in/journals/index.php/ijap>

INTRODUCTION

Selegiline (Sel) is chemically (2R)-N-methyl-1-phenyl-N-prop-2-ynylpropan-2-amine, known as L-deprenyl. It is a white powder with a molecular weight of 187.28 g/mol and a log P value of 2.7. In addition to this, it is a substituted phenethylamine and amphetamine, a methamphetamine derivative, and the pure levorotatory enantiomer of deprenyl, a drug mainly used to treat Parkinson's disease (PD) and severe depressive disorders [1]. Additionally, it is used as a selective monoamine oxidase B (MAO-B) inhibitor, which helps elevate levels of certain neurotransmitters in the brain, primarily dopamine. That helps enhance motor function and reduce PD symptoms.

Furthermore, it is recommended to frequently complement the effects of other anti-Parkinson's drugs, such as levodopa, and to assist in minimizing motor fluctuations. Sometimes, it is used to treat depression, especially when other antidepressants are ineffective. The United States Food and Drug Administration (USFDA) has approved Sel, an MAO inhibitor, as an additional treatment for individuals with PD and major depressive disorder. The MAO enzymes catabolize neurotransmitters such as norepinephrine, serotonin, and dopamine. Most importantly, blocking the action of these enzymes inhibits the reuptake of neurotransmitters in the central nervous system, thereby increasing the levels of physiologically active monoamines at the synaptic cleft. Despite these potential benefits, its low solubility and oral bioavailability limit its use as a BCS Class II compound. Additionally, it has side effects and undergoes high first-pass metabolism, which prevents it from crossing the blood-brain barrier (BBB) and results in low bioavailability, suggesting the need for an alternative drug-delivery approach. This has led to recent advances in drug delivery systems that deliver the drug directly to the brain, either by crossing the BBB via the nasal route of administration [2]. Similarly, *Bacopa monnieri* is a plant commonly used in Ayurvedic medicine to

improve memory, alleviate sleeplessness, treat epilepsy, and serve as an anxiolytic. It contains alkaloid brahmine, nicotine, herpestine, bacosides A and B, saponins A, B, and C, triterpenoid saponins, stigmastanol, β -sitosterol, betulinic acid, D-mannitol, stigmastanol, α -alanine, aspartic acid, glutamic acid, serine, and pseudojubenin glycoside. Numerous clinical research studies have demonstrated that *Bacopa* enhances verbal learning, improves delayed word recall, facilitates memory acquisition, and reduces anxiety. Bacoside A is the main active constituent of *B. monnieri*, which is responsible for its being regarded as a brain tonic. Its principal constituents include the saponin bacoside A3, bacoside II, bacosaponin C, and jubenin isomer of bacosaponin C [3]. It has been referred to as a relaxing cognitive enhancer. The triterpenoid saponins are thought to be responsible for most of the herb's pharmacological effects. *Bacopa* has a high therapeutic index and is typically well tolerated [4].

This combination offers a comprehensive approach for brain health by pairing Selegiline's ability to inhibit dopamine breakdown with *Bacopa*'s capacity to protect the neurons that produce it, resulting in sustained dopamine persistence. They offer dual-action neuroprotection by shielding the brain from the oxidative stress of metabolism and environmental free radicals, while simultaneously creating a cognitive synergy where Selegiline enhances mental drive and *Bacopa* strengthens memory through the cholinergic system [5, 6]. *Bacopa* further acts as an adaptogenic "buffer" to smooth out any jitteriness or over-stimulation caused by the MAO-B inhibitor, providing a balanced state of calm focus. In the context of Parkinson's management, this pairing uses Selegiline for primary motor symptom control and *Bacopa* as an experimental adjunct to address non-motor issues like brain fog and anxiety. Ultimately, this combination aims to improve the brain's structural "wiring" by combining Selegiline's protective effects with *Bacopa*'s potential to promote the growth of new nerve endings.

The simultaneous analysis comes with various challenges. The first is regarding the wavelength maxima. The value is around 200 nm, which is known to be too much interfered with by the fingerprint regions of various mobile phases and solvents used. Secondly, there is a vast difference in polarity. Selegiline is non-polar in nature. However, brahmi extract is polar, as several sugar moieties are attached to the bacoside molecule present in it. Alongside selegiline, which also owes to a high number of amino groups, it has high tailing. All of these make it quite arduous to simultaneously analyze both molecules qualitatively and quantitatively.

Looking into the potential of both these drugs, this combination has been proposed for alleviating neurodegeneration in PD. The present study aims to develop and validate a reverse-phase HPLC method for the simultaneous estimation of both drugs as an essential part of a developed combination formulation [7]. The current study aims to develop a simple, precise, and robust simultaneous method for quantifying Sel and Brahmi extract (BE) in bulk form. Additionally, this method can be used to quantify this combination of drugs in nanoformulations (fig. 1) [8, 9].

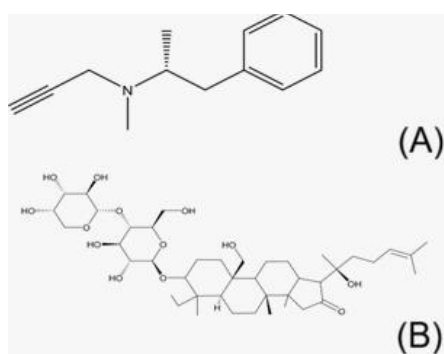


Fig. 1: Chemical structures of (A) Selegiline, and (B) Bacoside A

MATERIALS AND METHODS

Materials

Selegiline was obtained as an *ex gratia* gift sample from M/S Embio Laboratories Ltd., Mumbai, Maharashtra. Brahmi extract and Bacoside A marker (96.9 % pure) were purchased from Natural Remedies, Bangalore. The solvents, namely acetonitrile, methanol (procured from Rankem Chemicals, India), and sulphuric acid (procured from Loba Chemie Pvt., India), were of analytical grade (AR grade). HPLC-grade water (Sigma Aldrich, Maharashtra, India) was used throughout the study.

Instrumentation

Ultrafast liquid chromatography system consisting of a binary pump (LC-20 AD; Prominence, Shimadzu, Japan), a degasser unit (DGU-20A5), photodiode array detector (SPDM20A; Shimadzu, Japan), Rheodyne injector with 20 μ l** sample injector loop, and Nucleodur C18 column with dimension 250 x 4.6 mm (5 mm) was used for development of the analytical method. Empower 3 software was used to record and analyze the data.

Analytical method development

Determination of isosbestic point

For the simultaneous estimation of the two molecules, the isosbestic point was first ascertained. A series of solutions was prepared in which the total concentration of both drugs remained constant, while their relative concentrations varied. The ultraviolet-visible (UV-Vis) absorption spectra of each solution were recorded across a suitable wavelength range. All the recorded spectra were then plotted on the same graph, with absorbance on the y-axis and wavelength on the x-axis. The isosbestic point(s) were identified as the specific wavelength(s) at which the absorption spectra intersected. At this wavelength, the molar absorptivities of the two interconverting species were the same, resulting in a constant absorbance despite changes in their relative concentrations. A sharp isosbestic point was often considered evidence that only two spectroscopically distinct species interconverted without the formation of significant intermediates. The said intersection was named as an isosbestic point and employed for the simultaneous estimation of two drugs [10].

Selection of chromatographic conditions

Various initial trials were conducted to select suitable chromatographic conditions for the simultaneous estimation of Sel and BE. A wide range of mobile phases and flow rates was tested. The trials discussed in this section are summarized in table 1.

Preparation of standard stock solution

Sel (10 mg) was dissolved in methanol in a standard volumetric flask (10 ml), and the volume was adjusted to 10 ml to get the final concentration of 1000 μ g/ml. Aliquot (1 ml) of this solution was withdrawn and further diluted to 10 ml to obtain a concentration of 100 μ g/ml. Afterwards, 1 ml of this solution was further diluted up to 10 ml to get a concentration of 10 μ g/ml [11]. Similarly, 10 mg of BE (containing 20 % of bacoside A) was dissolved in a few ml of methanol in a standard volumetric flask (10 ml), and the volume was adjusted to 10 ml to get the final concentration of 1000 μ g/ml. As mentioned above, the stock solution was diluted to 25 μ g/ml [12]. Further dilutions were made to have a concentration of BE in a range of 5-25 μ g/ml.

Table 1: Various trial compositions employed during analytical method development

Trials	Flow rate (ml/min)	Mobile phase composition	RT	TF	PC	Resolution
Trial 1	1.0	ACN 90: 10 0.1% OPA	4.5 min	≥ 2	≥ 2000	No sharp, well-eluted peaks were there; broad and tailing peaks were observed
Trial 2	1.0	Methanol 90: 10 0.1% OPA	Not clear			Peaks were not well defined; peaks had excessive broadening and poor efficiency
Trial 3	0.8	Methanol 90: 10 0.2% OPA	8.5			RT was increased but peaks were asymmetric and had poor resolution
Trial 4	1.0	ACN 80: 20 0.1% OPA	4.34 min			There was a partial separation, however, there was an inadequate resolution between the peaks
Trial 5	1.0	ACN 70: 30 0.1% OPA	Not clear			There was poor selectivity and peak separation
Trial 6	0.8	ACN 50: 50 0.2% OPA	Not clear			Broad peaks were observed
Trial 7	1.0	ACN 30: 70 0.2% OPA	Not clear			A very poor peak shape with peak merging was observed
Trial 8	1.0	ACN: Buffer (0.72% anhydrous sodium sulphate) (31.5:68.5)	4.07, 20.682 min	Sel-256495 BE 16280	Sel-7001 $\pm$ 72 BE-3254 $\pm$ 59	Excellent resolution (30.76) with sharp and well-defined peaks was observed

For each confirmation, results were evaluated based on the tailing factor, peak area, and the various trial compositions used during method development. ACN = Acetonitrile, OPA= orthophosphoric acid

Systematic method development using quality by design principles

To develop a robust analytical method, a systematic approach centered on analytical quality by design (QbD) principles was employed. The study involved applying QbD principles and designing an experimental approach to account for the various factors impacting chromatographic output, thereby optimizing and achieving maximum efficacy.

Defining target profile and corresponding critical analytical attributes (CAAs)

Before designing the new analytical method using QbD principles, we first defined the key requirements for the method in an Analytical Target Profile (ATP). These targets, guided by industry standards, are listed in table 2 for the chromatographic analysis. Based on a thorough review of existing literature and team discussion, we selected the following as Critical Analytical Attributes (CAAs): peak area, retention time, tailing factor, and theoretical plate count.

Risk assessment studies

To perform risk assessment studies for the LC method, an Ishikawa fish-bone diagram was drawn using Minitab 17 software (M/S Minitab Inc., Philadelphia, USA) to establish a cause-and-effect relationship among material attributes (MAs) and process parameters (PPs) and to identify the likely impact on critical attributes of the analysis (CAAs). Failure Mode and Effects Analysis (FMEA) was performed to identify high-risk factors with a critical impact on the CQAs. Rank-order scores, ranging from 1 to 10 each, were assigned to the CMAs for each of the parameters, viz. Severity (S), occurrence (O), and detectability (D) are based on prior knowledge and experience through extensive brainstorming exercises to calculate the risk priority.

$$\text{RPN} = \text{Severity (S)} \times \text{Occurrence (O)} \times \text{Detectability (D)}$$

The value of RPN score can vary from 1 to 1000. A value up to 300 is considered low risk, 301-700 is considered medium risk, and 701-1000 is regarded as high risk. The factors associated with medium and high risk were examined in further factor screening studies.

Table 2: Analytical target profile (ATP) postulated for liquid chromatographic analysis of Sel and BE

S. No.	ATP	Objective(s)	Explanation
1.	Sample	Sel/BE	Development of an analytical method and estimation of active molecules in pharmaceutical dosage forms
2.	Type of method	Reversed-phase	The nonpolar stationary phase tends to provide improved retention of molecules.
3.	Instrument requirement	Auto sampler with quaternary HPLC pump	A quaternary pump provides a higher resolution
4.	Sample nature	Liquid	The analyte must be in the liquid phase to ensure complete miscibility
5.	Standard preparation	Dilution in a linear method	Standard dilution of bioactive/drug solution to ensure its proper elution
6.	Sample preparation	Manual handling	The sample preparation is carried out manually. Followed by mixing in the mobile phase
7.	Method application	Estimation of Sel/BE	The method is applicable for detecting FA in pharmaceutical dosage forms as well as in biological matrices.

Table 3A and 3B: A model matrix, which contains the chosen CMPs at their low (-1), medium (0), and high (+1) levels, resulting in a total of 13 experimental runs, including five replicated central points (0, 0). A drug solution of Sel and BE was used throughout the studies to investigate the influence of X1 and X2 on the selected CAAs, viz., PA, RT, TF, and PC

Table 3A

Run	Mobile phase ratio	Flow rate
1	0	0
2	+1	-1
3	-1	0
4	0	0
5	0	0
6	-1	+1
7	-1	-1
8	0	+1
9	0	0
10	0	0
11	+1	+1
12	0	-1
13	+1	0
Factor descriptions along with their coded and actual levels		
Critical Method Parameters	Low (-1)	Intermediate (0)
Mobile phase ratio (ACN)	25	42.5
Flow rate (ml. min ⁻¹)	0.8	1.0
		High (+1)
		60
		1.2

Table 3B Optimization model runs with the corresponding values

Run no.	Mobile phase ratio (%)	Flow rate (ml min ⁻¹)	RT BE (min)	RT Sel (min)	PA BE (AU)	PA Sel (AU)	TF BE	TF Sel	PC Sel	PC BE
1	25	0.8	26.2	11.7	1229319	702916	1.30	1.32	7628	3513
2	25	1.0	25.5	11.0	1310946	432832	1.25	1.22	7927	3785
3	25	1.2	24.8	10.3	1269996	241989	1.35	1.85	7777	3667
4	42	0.8	21.5	7.0	1502865	836232	1.15	1.13	7717	4488
5	42	1.0	20.8	6.3	1639365	288768	1.05	1.08	8533	5013
6	42	1.2	20.2	5.7	1584765	260905	1.10	1.30	7696	3885
7	60	0.8	19.0	4.5	1365546	838320	1.20	1.22	6905	4017
8	60	1.0	18.5	4.0	1475292	670101	1.15	1.12	7295	4388
9	60	1.2	18.0	3.5	1420692	474905	1.25	1.75	7107	4213
10	42	0.8	21.5	7.0	1502865	587279	1.15	1.13	7396	4485
11	42	0.8	21.5	7.0	1502319	581493	1.15	1.18	7406	4517
12	42	0.8	21.5	7.0	1502046	826986	1.15	1.20	7393	4238
13	42	0.8	21.5	7.0	1502046	445031	1.15	1.17	7405	4513

In this table RT stands for Retention Time, PA= Peak area, TF=Tailing Factor, PC= Plate count

Factor optimization studies

Two Critical Method Parameters (CMP), the mobile phase ratio (X1) and flow rate (X2), were optimized using a Face-Centered Central Composite Design (FCCD) in design-expert 11. These factors were selected for optimization after a risk assessment and screening exercise showed they had a statistically significant impact. Tables 3A and 3B present the model matrix, which includes the chosen CMPs at their low (-1), medium (0), and high (+1) levels, yielding a total of 13 experimental runs, including five replicated central points (0, 0). A drug solution of Sel and BE was employed throughout the studies to investigate the influence of X1 and X2 on the selected CAAs, viz., PA, RT, TF, and PC (table 3A and 3B).

Optimization, data analysis, and model validation

The data were fit to a second-order polynomial (quadratic) using the Multiple Linear Regression Analysis (MLRA) feature in Design-Expert® 13 to establish the relationship between factors and responses. This analysis was evaluated through analysis of variance (ANOVA) and the coefficient of correlation (r) to assess the adequacy of the model [13]. Additionally, the lack of fit and factor response relationship was investigated. Following this, quadratic model fitting was performed to encompass both main and interaction effects, and the results were graphically visualized using

3D response surface plots and 2D contour plots.

Briefly, a Central Composite Design was used for two factors, each varying at three levels: low (-1), medium (0), and high (+1). The results were analyzed. The veracity of the model was first established by fitting the model into a quadratic model using the following equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2$$

Y is the response, β_0 is the intercept, β_1 and β_2 are the coefficients of linear terms X_1 and X_2 , respectively. β_3 is the coefficient of factor interaction, β_4 and β_5 are coefficients of the corresponding quadratic terms. The results were analyzed by computing the coefficient of correlation (r) and the value of type-1 error (p-value)

Determination of optimum chromatographic solution

A mathematical optimization tool was used to determine the optimal chromatographic conditions for various CAAs, minimizing TF and RT and maximizing PA and PC. Additionally, the desirability function was adjusted to maximize its value, aiming for a value as close to unity as possible. Following that, a graphical method was employed to optimize, allowing identification and visualization of the best chromatographic solution within the defined design space [14] (table 4).

Table 4: Ranges, goals, and criteria for various factors and responses during optimization studies, along with weightness and importance gradings

Name	Goal	Lower limit		Upper limit	Lower weight	Upper weight	Importance			
MPR	is in range	25		60	1	1	3			
Flow rate	is in range	0.8		1.2	1	1	3			
RT BE	minimize	18		26.2	1	0.2	3			
RT Sel	minimize	3.5		11.7	1	0.2	3			
PA Sel	maximize	1.22932		1.63937	0.3	1	3			
PA BE	maximize	241989		838320	0.3	1	3			
TF BE	minimize	1.05		1.35	1	0.4	3			
TF Sel	minimize	1.08		1.85	1	0.4	3			
PC Sel	maximize	6905		8533	0.5	1	5			
PC BE	maximize	3513		5013	0.5	1	5			
Overall optimal conditions and desirability value										
MPR	Flow rate	RT BE	RT Sel	PA Sel	Pa BE	TF BE	TF Sel	Pc Sel	Pc BE	Desirability
46.9	0.97	20.0	5.5	1625042	431375	1.06	0.987443	8030.31	4812.45	0.908

Where PA= Peak Area, RT= Retention time, TF= Tailing Factor, PC= Plate count, Sel= Selegiline, BE=Brahmi Extract, MPR= Matrix-Phase Ratio

Determination of multicollinearity

The degree of multicollinearity among the selected factors for optimization was evaluated by calculating the Variance Inflation Factor (VIF) and the correlation coefficients among the variables through a matrix plot [15].

MODR verification using Monte Carlo simulations

The defined MODR was examined for model uncertainty and validated against the probabilistic design space derived through Monte Carlo simulations. The simulation model was developed using the polynomial equation obtained from the optimization analyses [16]. For each investigated CAA, the model was simulated 50,000 times, assuming a normal distribution for all factors and CAAs, using Minitab 17 software (Minitab LLC, USA).

Partial least squares (PLS) regression

Following the factor optimization studies (e. g., using Response Surface Methodology), Partial Least Squares (PLS) regression was performed in Minitab. This was done to gain a more profound, multivariate understanding of the complex relationships between the experimental factors and multiple chromatographic responses. While optimization studies identify optimal settings for individual or combined responses, PLS provides insights into how factors jointly influence all responses simultaneously, especially when responses are correlated or predictors exhibit multicollinearity, which traditional regression methods might struggle with. This enables a more comprehensive characterization of the LC method's

robustness and performance across the entire design space. The CCD experimental data, comprising factor settings (pH, Flow Rate, and Organic Modifier %) and corresponding responses (i. e., Peak Area, RT, TF, and Plate Count), were loaded. The PLS model was fitted. Relevant results and plots (Summary, VIP, Coefficients, Score, Loading, and Residual plots) were selected for interpretation. The outputs were then analyzed.

Method validation

After optimizing the operating conditions, the developed analytical method was validated in accordance with the International Conference on Harmonization (ICH) Q2(R2) guidelines for several parameters, including linearity, accuracy, precision, robustness, and system suitability [17].

Linearity and range

The linearity range of the analytical method was validated by assessing the range and patterns of residuals obtained from least-squares linear regression. The linearity of the HPLC method was determined by analyzing the prepared dilutions of BE in the concentration range of 5-25 µg/ml, and Sel in the range of 2-10 µg/ml, respectively, plotting the area under the peak (AUP) against the drug concentration (n=6). The calibration and corresponding residual plots were constructed using a spreadsheet package (Microsoft Excel, Microsoft Inc., Washington, USA). The calibration plot and the corresponding residual plot were constructed using a spreadsheet package (Microsoft Excel, Microsoft Inc., Washington, USA) [18].

Detection and quantification limits

The lowest detectable (i. e., DL) and quantifiable limits (i. e., QL) were calculated as per the formula given in eq. 1 and 2 respectively [19]. The value of constant (k) is 3.3 and 10, respectively.

$$DL = 3.3 \times \frac{\sigma}{S} \dots\dots \text{Eq. 1.}$$

$$QL = 10 \times \frac{\sigma}{S} \dots\dots \text{Eq. 2.}$$

Where σ stands for standard deviation in the response of the lowest concentration, and S refers to the slope of the calibration plot.

System suitability

System suitability of the developed analytical method was evaluated by subjecting hexaplicate injections, each with a fixed concentration. Subsequently, the values of capacity factor (k), Tailing Factor, Plate Count, asymmetry factor (As), and injection repeatability (% RSD) were determined [20].

Accuracy

The average percentage recovery, along with % Relative Standard Deviation (RSD), for the three quality test samples, i. e., LQC, MQC, and HQC, of BE and Sel was estimated to verify whether the accuracy of the developed analytical technique lies within acceptable limits [21].

$$\text{Recovery} = \frac{\text{Drug recovered}}{\text{Drug added}} \times 100 \dots\dots \text{Eq. 3.}$$

Precision

The precision of the developed HPLC method was also estimated at three drug concentration levels (LQC, MQC, and HQC) on the same day (i. e., intra-day precision) and the next day (i. e., inter-day precision). The mean percentage recovery and percentage relative standard deviation (RSD) values were also calculated using Equation 4 to ensure that the method precision lies within the specified limits.

$$RSD = \frac{\sigma}{\bar{x}} \times 100 \dots\dots \text{Eq. 4.}$$

Where RSD is the relative standard deviation, σ is the standard deviation, and $\bar{x}$ is the mean value

Robustness

The robustness of the method was evaluated by making minute changes in the experimental conditions such as by changing the flow rate of the mobile phase (0.8, 1, and 1.2 ml/min), the ratio of mobile phase (A: B) that is, ACN: buffer at (34:64, 32:68, and 30:70 v/v) and wavelength (203, 205, and 207 nm) in Six replicates of the method's mid concentration, i. e., 6 $\mu\text{g/ml}$, were injected and their peak area percentage recovery, and retention time were noted [22].

Specificity

The specificity of the developed method was estimated by injecting acetonitrile (ACN) and 0.72% anhydrous sodium sulphate with pH 2.3 (31.5:68.5 v/v). If peaks appear at the retention times of Sel and BE, then the method is not specific, and vice versa [23].

Statistical analysis

The results of all the experimental data were noted as the average and standard deviation (SD). All data values for average, standard deviation (SD), and relative SD were calculated using a Microsoft Excel worksheet [24].

Assessing the environmental sustainability of the HPLC method

This work was developed using the AGREEprep and AGREE tools (available online). The "AGREEprep" and "AGREE" are calculators for evaluating analytical greenness based on online resources offered by the Universidade de Vigo (University of Vigo) in Pontevedra, Galicia, Spain (<https://agreeprep.anvil.app/>, accessed on June 15, 2025). These calculators assess environmental sustainability using 12 greenness assessment criteria (GAC) and their corresponding significance scores. The color coding, ranging from deep green to red, represents the transition from environmentally friendly to non-environmentally friendly methodologies, as illustrated in the calculator's circular representation of the 12 GACs. AGREEprep provides a matrix for analytical greener sample preparation, while AGREE estimates the greenness of analytical methods based on the scores and CAGs. This approach utilized ethyl acetate (a green solvent), methanol (another green solvent), n-hexane, and toluene. The methodology incorporated QbD principles to decrease energy consumption and minimize the unnecessary use of harmful organic solvents during mobile phase development, contrasting with traditional methods that adopt a hit-and-trial approach. The process avoids hazardous operations and the generation of toxic waste. It features a low sample size, minimal injection volume, in-line sample measurement, no derivatization, and employs non-toxic reagents, thus addressing safety issues. This methodology conserves energy, allows the potential integration of multiple analytes for evaluation, and can be automated [9]. The greenness clock was established considering the aforementioned CAGs and the experimental conditions of the developed validation process for analyzing the targeted drugs.

RESULTS AND DISCUSSION

A preliminary study for analysis

Determination of the isosbestic point

The isosbestic point of Sel and BE was found to be 205 nm. The intersection of the UV spectrum of both the drugs was observed at this wavelength, indicating it to be the most suitable wavelength for the simultaneous analysis of both the drugs. The advantage of this is that at this point, we can use a unified calibration curve for the quantitative analysis to estimate the combined purity of the mixture. The advantage of an isosbestic point is that it simplifies data interpretation, ensures accuracy, and meets system suitability requirements. About Brahmi and selegiline, this is important as the combination is showing enhanced efficacy in the management of Parkinson's disease. Therefore, simultaneous analysis will be faster and direct quantification of the molecules in various samples. Furthermore, it provides greater accuracy in identifying the two compounds loaded in a single formulation. Hence, it is used to estimate both drugs simultaneously, as depicted in fig. 2.

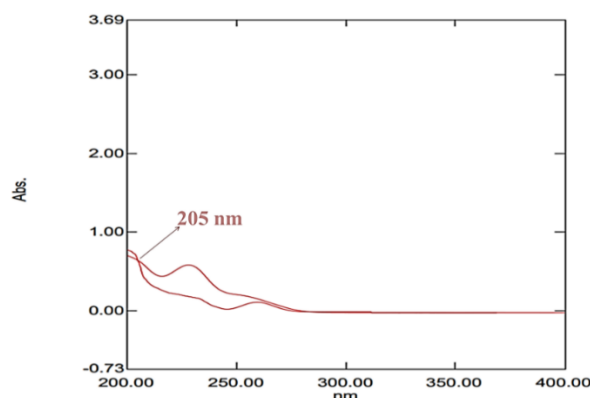


Fig. 2: Determination of the isosbestic point

Selection of the mobile phase for the simultaneous estimation of Sel and BE

The Different trials were performed using different solvents and flow rates to estimate Sel and BE simultaneously. Trials 1 to 7

showed that neither peak appeared in combination, or if they did, they were not sharp or well-resolved. Therefore, those trials were rejected; however, Trial 8 showed a well-resolved, sharp peak (fig. 3). Hence, this ACN: Buffer (31.5:68.5%, v/v) trial at a flow rate of 1 ml/min was used throughout the study.

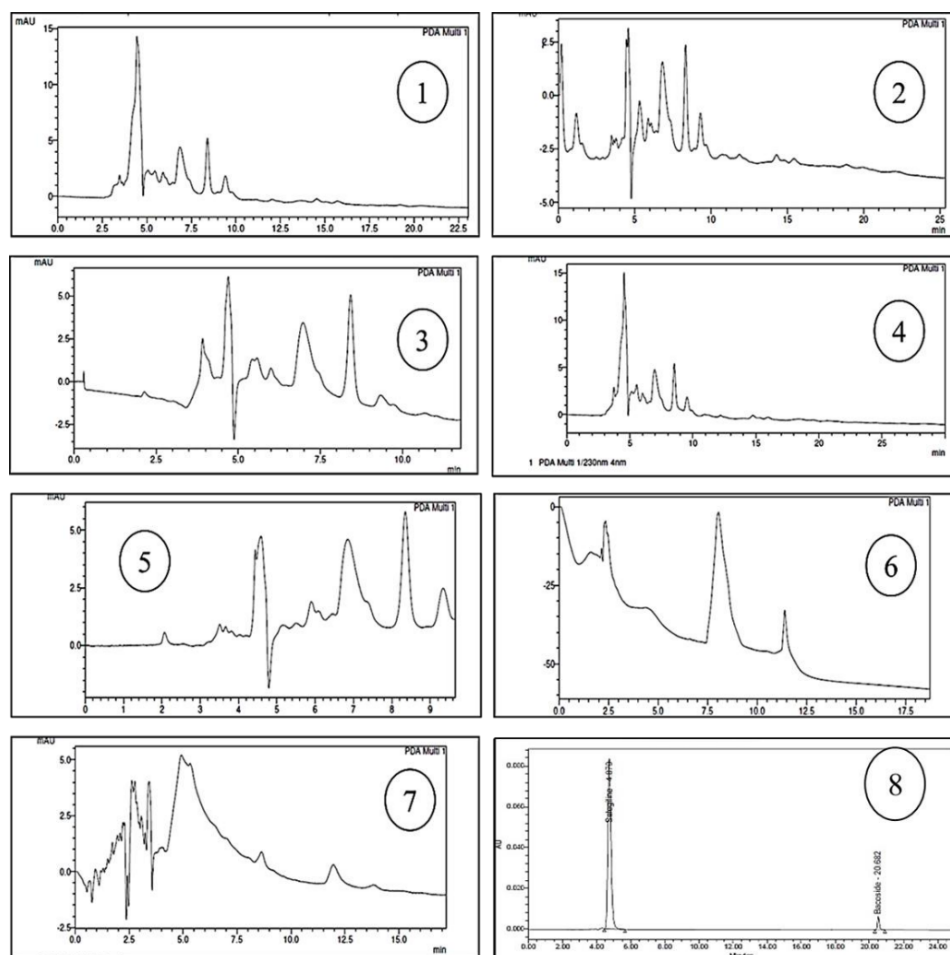


Fig. 3: Trials for the selection of the mobile phase

Along with BE, Selegiline is also present in the formulation for which the chromatogram has been shown in fig. 4. Brahmi extract (containing 20% Bacoside A) was used as a source of Bacoside A. To

confirm the presence of bacoside A in the BE, it was compared with the standard bacoside A marker. The results of the study showed that BE had retention time (fig. 6) similar to that of bacoside A (fig. 5).

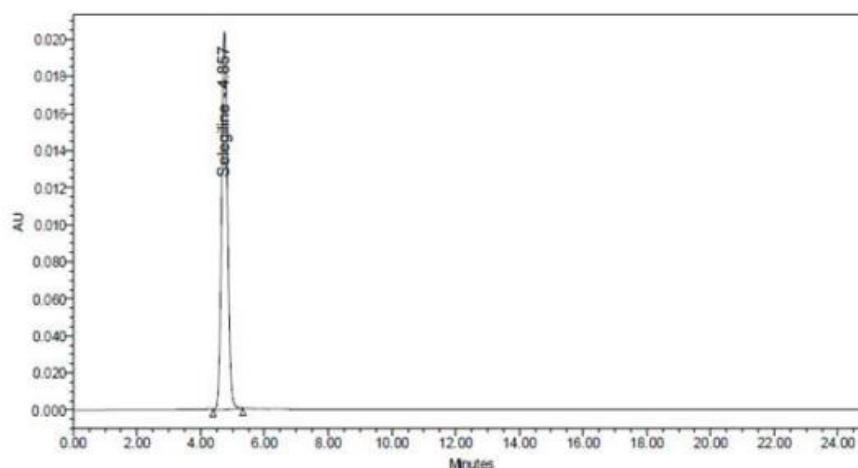


Fig. 4: Chromatogram of selegiline

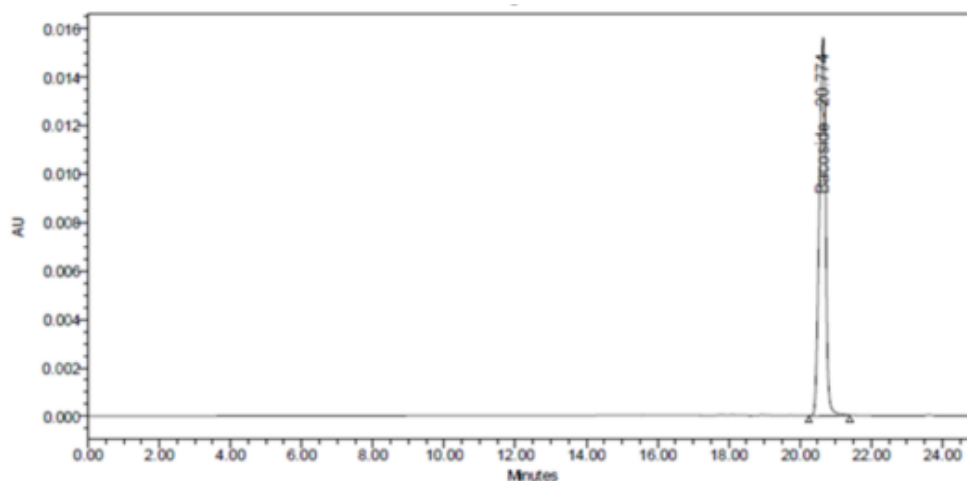


Fig. 5: Chromatogram of standard bacoside A

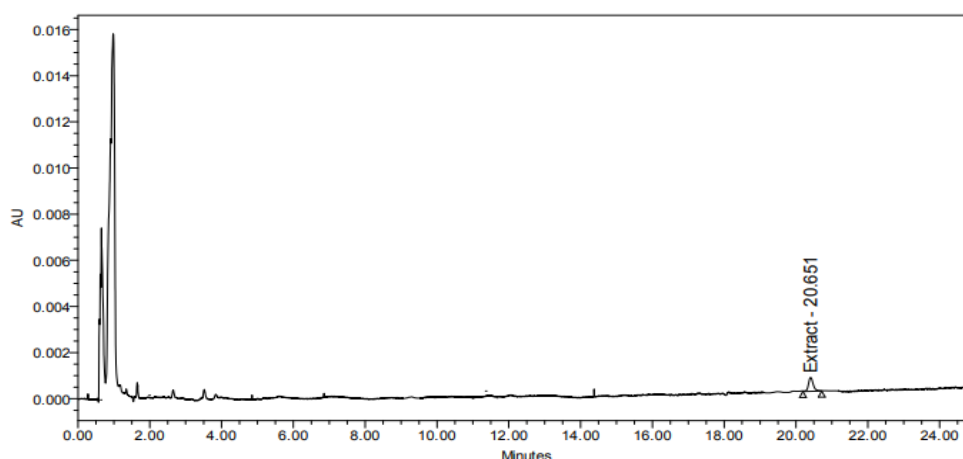


Fig. 6: Chromatogram of BE

Risk assessment studies

The risk assessment studies based on the FMEA analysis were summarised and are mentioned in table 5. For optimisation purposes, the factors with associated risk factors in the medium

and high range were selected for optimisation. Out of these 2 factors, were having the value in the mid and high range, therefore were optimised using the CCD design. The table explicitly shows high and medium-risk associated factors in red and yellow fill, respectively.

Table 5: FMEA analysis

Factors	Severity (S)	Occurrence (O)	Detectability (D)	RPN score (SXOXD)
MPR	8	8	7	448
Flow rate	8	8	6	384
Column temp	5	4	5	100
Type of flow	4	4	3	48
	Low: 1-300	Medium: 301-700	High: 701-1000	

Response surface optimization

Response surface methodology and subsequent data analysis, using a second-order quadratic polynomial model, facilitated the identification of main and interaction effects, as well as the performance of ANOVA and other model-fitting parameters (table 6). High correlation coefficient values coupled with low type I error probabilities (p-values) suggest that the model is well-chosen and exhibits a strong level of fit, as noted by Khurana *et al.* [18]. Table 6 provides a detailed summary of the coefficients for the polynomial model across all Critical Quality Attributes (CQAs).

The data fitting was conducted using a polynomial as described in equation 4.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2 \quad \text{Eq. 4}$$

The linear model variables, β_3 , indicate the interaction term in the model, and β_4 and β_5 represent the quadratic components.

To enhance the understanding of the relationships between factors and responses, three-dimensional response surfaces and their associated two-dimensional contour graphs (fig. 7) were created for all the examined CAAs.

Table 6: Response surface optimization MLRA model polynomials in analytical method optimized for various CAA

	RT BE (min)	RT Sel (min)	PA BE (AU)	PA Sel (AU)	TF BE	TF Sel	PC Sel	PC BE
β_0	41.59	27.09	-2092969.15	4089847.98	4089847.98	9.19	-6879.04	-9180.54
β_1	-0.58	-0.58	65946.77	-41179.88	-41179.88	-0.06	81.74	170.30
β_2	-5.62	-5.62	4287171.02	-4893094.90	-4893094.90	-14.66	26769.08	20303.39
β_3	0.03	0.03	957.89	6987.13	6987.13	0.00	3.41	4.19
β_4	0.00	0.00	-736.55	470.12	470.12	0.00	-1.23	-1.87
B_5	0.64	0.64	-2080896.26	1776122.54	1776122.54	7.77	-13123.59	-10385.93
R^2	1.00	1.00	0.78	0.99	0.95	0.89	0.84	0.81
PRESS	0.06	0.06	2.504	1.001	0.049	0.79	1.622	3.284
p-value	<0.0001	<0.0001	<0.05	<0.001	<0.001	<0.01	<0.05	<0.05

In the above table, MLRA = Multiple Linear Regression Analysis, PRESS = Prediction Error Sum of Squares, PA stands for Peak Area, RT = Retention time, TF = Tailing Factor, PC = Plate count, Sel = Selegiline, BE = Brahmi Extract, p-value = Probability Value

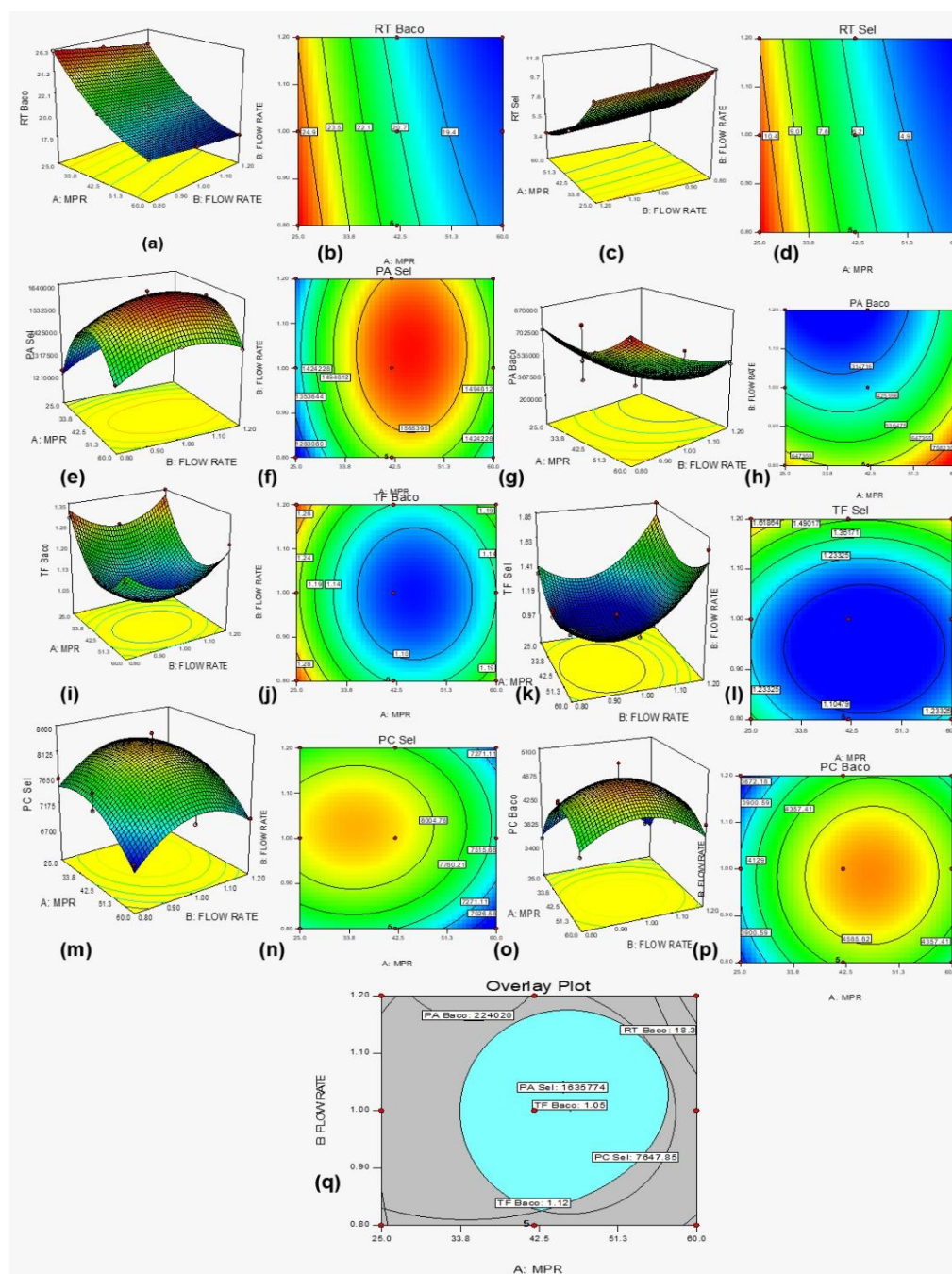


Fig. 7: Response surface optimization study outcomes illustrated as 3-D as well as corresponding 2-D contour plots

Design space verification using monte carlo simulation

Monte Carlo simulations were employed to verify the robustness of the design space, using parameters modelled as normal distributions. The simulation yielded a mean value of the capability metric along with its associated standard deviation. The analysis indicated that all values remained well within the predefined design space, confirming its robustness and reliability. Specifically, the results showed minimal variability, with the majority of simulated values concentrated near the mean. This distribution demonstrated high precision and consistency in the design parameters.

Additionally, no data points extended beyond the design space limits, reinforcing the design framework's ability to handle practical variability without compromising performance. The findings demonstrate that the design space can accommodate potential fluctuations and uncertainties

inherent to the process, thereby validating its suitability for implementation in real-world conditions. This result highlights the importance of employing computational simulations as a critical step in verifying the design space. Furthermore, the Monte Carlo simulation results indicate that variability or random fluctuations in key parameters do not result in deviations outside the design space. This confirms that the design space is robust-it can accommodate these fluctuations while still ensuring the system remains functional and achieves its intended objectives.

Furthermore, in the matrix plot, the average correlation coefficient was 0.012, indicating no multicollinearity. Secondly, the VIF value ranges between 1.000 and 1.703. As the value is <3 , this indicates a lack of multicollinearity, suggesting the appropriateness of the chosen response. These simulations have been depicted in fig. 8, and the quantitative output data have been compiled in table 7 below.

Table 7: Quantitative outputs for Monte Carlo simulation

Chart label	Lower bound (LB/ISL)	Upper bound (UB/USL)	Sample mean	Sample N	Std. Dev. (Overall)	Std. Dev. (Within)
RT BE	3	*	7.04262	1000	2.56432	2.56496
RT Sel	*	10	7.12086	1000	2.81236	2.81306
PA BE	10000	*	547725	1000	244564	244626
PA Sel	10000	*	1450920	1000	185564	185610
TF BE	*	2	1.18207	1000	0.12756	0.127592
TF Sel	*	2	1.27946	1000	0.313512	0.268284
PC BE	2000	*	4219.64	1000	585.058	517.469
PC Sel	2000	*	7561.96	1000	688.619	597.639

(*) indicate that the specific bound (Upper or Lower) was not defined or visible for that process in the data boxes

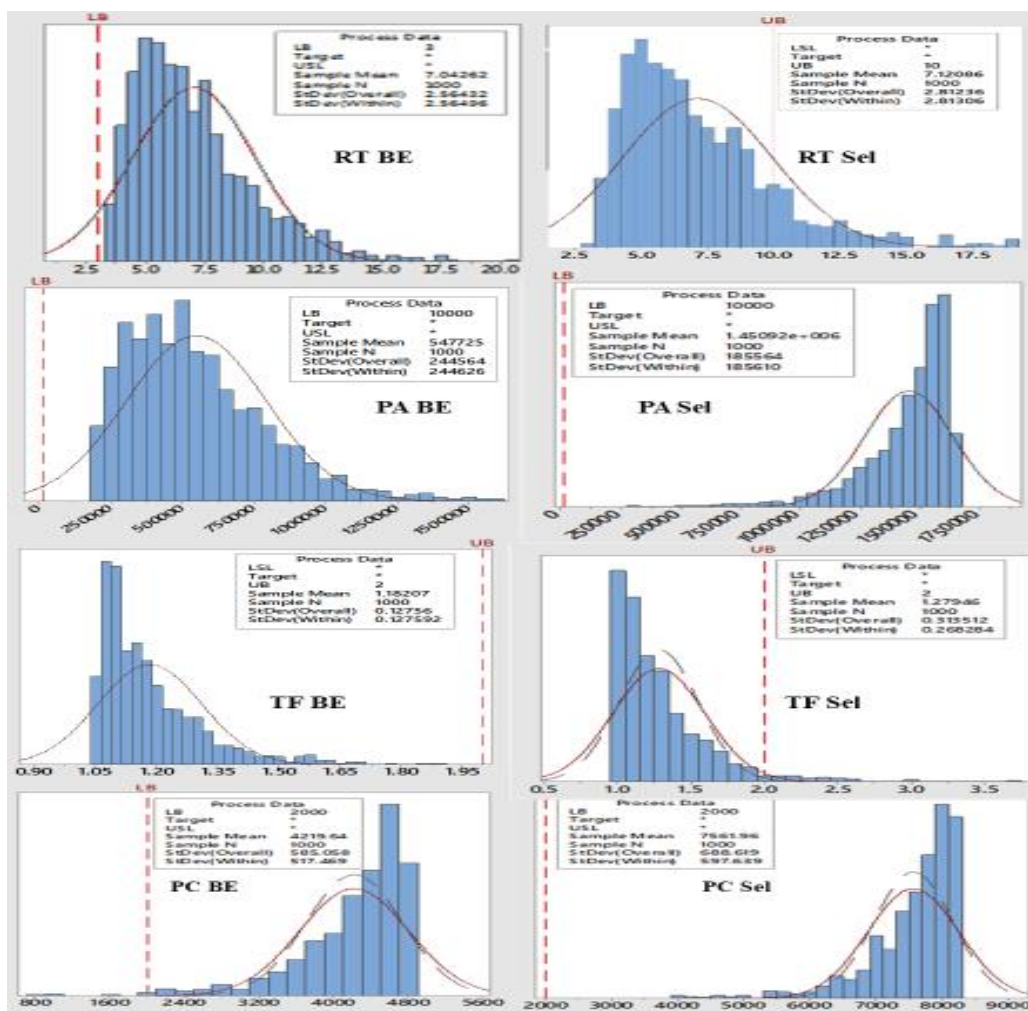


Fig. 8: Monte carlo simulations for design space validation for various CQAs studied, PA, RT, TF, and PC

Partial least squares (PLS) regression results

The Partial Least Squares (PLS) regression analysis, conducted after the factor optimization studies, provided comprehensive insights into the multivariate relationships between the experimental factors and the chromatographic responses. This advanced statistical approach was employed to elucidate how factors jointly influenced all responses, offering a holistic understanding of the LC method's

behavior across the design space, particularly beneficial in the presence of correlated responses and multicollinear predictors.

In case of PABE, PLS loading plot shows a wide angle between the line of high degree of orthogonality in the factors, Actual vs predicted shows good correlation, referring to the veracity of devised pls model, and Residuals are random and do not exhibit any pattern, indicating validity of the model (fig. 9A, 9B, 9C, 9D, 9E, 9F, 9G, 9H, 9I).

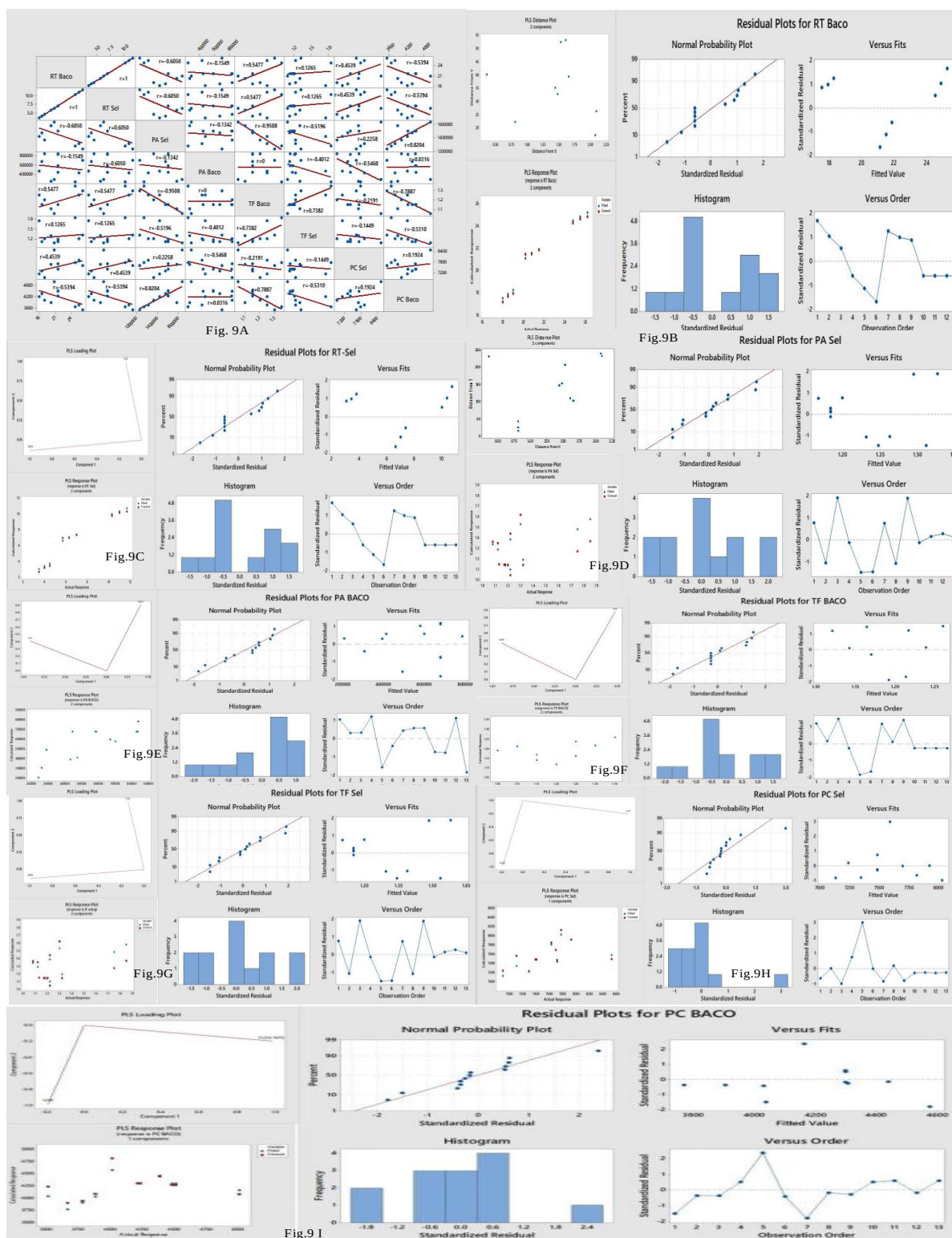


Fig. 9A: Matrix plot for various CAAs, fig. 9B: PLS results for RT BE, fig. 9C: PLS results for RT Sel, fig. 9D: PLS results for PA Sel, fig. 9E: PLS results for PA BE, fig. 9F: PLS results for TF BE, fig. 9G: PLS results for TF Sel, fig. 9H: PLS results for PC Sel, fig. 9I: PLS results for PC BE

When the angle between the lines of two variables in a pls loading plot is nearly 90 degrees, it indicates that these two variables are uncorrelated or almost uncorrelated with respect to the latent components they project, i. e., they are orthogonal. Random scatter is observed in the residual plot. Observed vs. predicted responses are quite overlapping, indicating high prognostic ability of the model. The histogram shows only a slight bias in the data, further corroborating the results.

The comprehensive analysis of the Partial Least Squares (PLS) data strongly supports the good prognostic ability of the applied Central Composite Design (CCD) model. The loading plot's near 90-degree angles between components indicate that the model effectively extracts distinct and independent latent variables, enhancing interpretability. The random distribution of residuals further confirms that the model accurately captures the underlying

systematic relationships within the data, leaving no unmodeled patterns.

Moreover, the close overlap between actual and predicted response values across the observed range demonstrates the model's high predictive accuracy. While the histogram of residuals shows a consistent peak around +0.6, indicating a slight systematic under-prediction, this bias is generally uniform and does not negate the model's overall strong predictive power. The residuals remain within acceptable standard deviation limits, with only one isolated run (Run 6) showing an abnormally high residual, suggesting it as a potential outlier for further investigation. Overall, these findings underscore the CCD model's robust ability to provide a reliable prognosis within the studied system. The VIP scores for chromatographic responses have been compiled in the table 8 below.

Table 8: Compiled VIP scores for chromatographic responses

Response variable	VIP: MPR (Factor 1)	VIP: FLR (Factor 2)	Dominant factor
Peak RT BE	1.24	0.71	MPR
Peak RT Sel	1.18	0.78	MPR
Tailing Factor (TF)	0.45	1.22	FLR
Plate Count (PC)	0.32	1.45	FLR

Comprehensive interpretation of the design space

1. Retention Time Control (MPR-Driven)

For both RT BE and RT Sel, the VIP scores for MPR exceed 1.0. This confirms that the organic modifier concentration is the "Master Switch" for the elution speed. To meet the revised goal of RT < 5 min, adjusting the MPR will yield much faster results than increasing the Flow Rate alone.

2. System Suitability (FLR-Driven)

The Tailing Factor and Plate Count show high VIP scores for FLR. This is a classic chromatographic behavior where the linear velocity (Flow Rate) directly impacts the HETP (Height Equivalent to a Theoretical Plate) and the peak symmetry.

1. Factor Interaction Summary

- MPR is the primary tool for Selectivity and Retention.
- FLR is the primary tool for Efficiency and Peak Shape

Greenness

The developed HPLC method demonstrated excellent greenness characteristics. Based on the AGREE (Analytical Greenness) score calculations, the method achieved a highly favorable rating, as shown in fig. 10. The AGREE assessment, which considers various environmental impact factors, confirmed its eco-friendly nature. Specifically, the method's use of greener solvents and reduced waste

generation contributed significantly. Calculations showed a low environmental footprint, aligning with sustainable analytical practices. The AGREE score of 1.0 indicated minimal consumption of hazardous reagents. This validated the method as a greener alternative compared to conventional HPLC approaches. The favorable AGREE score suggests a reduced impact on operator safety and the environment. Overall, the quantitative AGREE evaluation score of 0.62 unequivocally classified this HPLC method as green, making it a highly desirable, environmentally responsible analytical tool for routine use. Moreover, the solvents used are environmentally safe. The AGREE assessment data have been given in table 9.



Fig. 10: AGREE prep score for devised analytical method

Table 9: AGREE score for devised analytical method

S. No.	Criterion	Details/Value	Score	Weight
1.	Sample preparation placement:	Not set	1.00	3
2.	Hazardous materials	Not set [g or ml]	1.00	5
3.	Sustainability, renewability, and reusability of materials:	>75% of reagents and materials are sustainable or renewable	0.75	2
4.	Waste	15 [g or ml]	0.19	4
5.	Size economy of the sample	Mass or volume of the sample: 0.1 [g or ml]	1.00	2
6.	Sample throughput:	10 [samples/h]	0.54	3
7.	Integration and automation	Sample prep. steps: 3 steps, Semi-automated systems	0.38	2
8.	Energy consumption	317 [W]	0.11	4
9.	Post-sample preparation configuration for analysis:	Liquid chromatography, gas chromatography with quadrupole detection, etc.	0.25	2
10.	Operator's safety:	Not set	1.00	3

Analytical method validation

Linearity and range

The corresponding residual plot between % residual and observed concentration indicates no outliers and a selection rate for linearity for the drugs shown in fig. 11.

Limit of detection, limit of quantification, and system suitability

The LOD and LOQ for Sel were found to be 0.86 µg/ml and 2.35 µg/ml, respectively. Similarly, the LOD and LOQ for BE were 0.71

µg/ml and 2.35 µg/ml, respectively, indicating a sensitive method.

Accuracy

The accuracy of the method was assessed by determining the average percentage recovery of the drug from the low-quality control (LQC), mid-quality control (MQC), and high-quality control (HQC) samples. The results indicated that the average percentage recovery across all three levels fell within the accepted range of 95%–105% (table 10). This shows that the developed method is accurate.

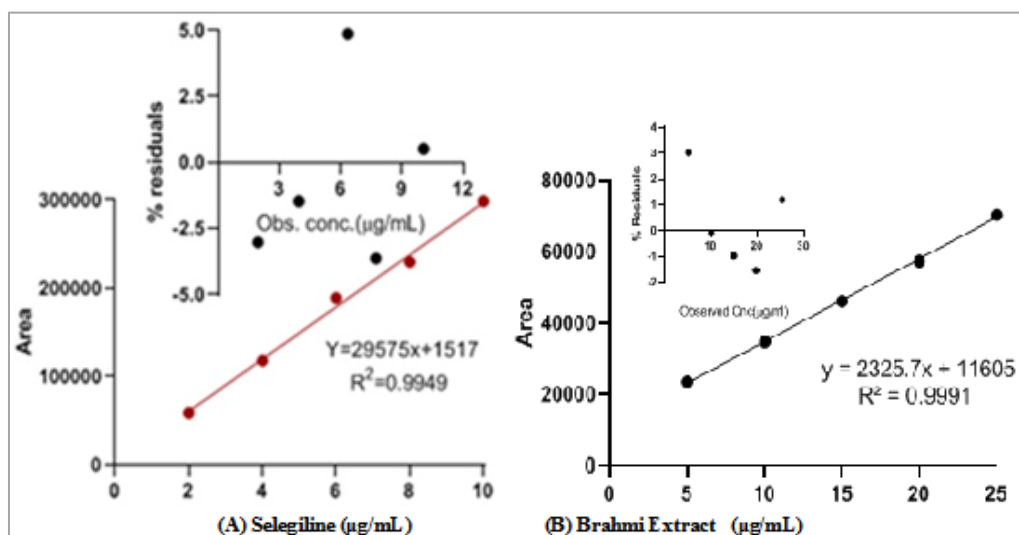


Fig. 11: Calibration curves of (A) Selegiline and (B) Brahmi extract

Table 10: Accuracy study of selegiline and BE

Drug	Level	Concentration (µg/ml)	Avg. Area.	Concentration (µg/ml)	% Recovery	%RSD
Selegiline	LQC	4.8	145442.33	4.866	101.385	0.43
	MQC	6.0	188989.00	6.339	105.649	0.52
	HQC	7.2	225474.00	7.573	105.175	0.63
BE	LQC	4.8	26486.67	4.720	98.340	0.49
	MQC	6.0	35393.33	6.340	105.666	0.66
	HQC	7.2	39399.67	7.069	98.174	0.84

In the above table, LQC=Low Quality Control, MQC=Mid Quality Control, HQC=High Quality Control

Table 11: Precision study of selegiline

Parameters	Level	Concentration (µg/ml)	Avg. Area	Std. Dev.	%RSD
Interday	LQC	4.8	145442.33	734.05	0.504
	MQC	6.0	188989	735.05	0.388
	HQC	7.2	225474	736.05	0.326
Intraday 1	LQC	4.8	144942.73	737.05	0.508
	MQC	6.0	186689	738.05	0.395
	HQC	7.2	224386	739.05	0.329
Intraday 2	LQC	4.8	145184	740.05	0.509
	MQC	6.0	188459	741.05	0.393
	HQC	7.2	225184	742.05	0.329
Intraday 3	LQC	4.8	147354	753.12	0.511
	MQC	6	1878012	751.33	0.040
	HQC	7.2	226093	746.02	0.330
Analyst 1	LQC	4.8	145662.33	746.05	0.331
	MQC	6.0	188189	747.05	0.396
	HQC	7.2	225169	748.05	0.332
Analyst 2	LQC	4.8	145274	749.05	0.514
	MQC	6.0	188744	750.05	0.397
	HQC	7.2	223284	751.05	0.336
Analyst 3	LQC	4.8	146254	752.05	0.514
	MQC	6.0	179959	753.05	0.418
	HQC	7.2	226054	754.05	0.333

In the above table, LQC=Low Quality Control, MQC=Mid Quality Control, HQC=High Quality Control; n = 6

Precision

The precision of the established method was assessed by calculating the percentage relative standard deviation (RSD) for six replicate measurements of the LQC, MQC, and HQC solutions at intraday, interday, and interanalyst levels, under the same experimental conditions. The %RSD was <2%, indicating that the developed method is precise for intraday, interday, interanalyst, and intraanalyst applications (table 11 and table 12).

Robustness

The results for % RSD and % recovery after minute changes in the flow rate, the mobile phase ratio, and the chromatogram wavelength were <2% and 95-105%, respectively. This indicates that the developed method was robust and was not markedly altered by the changes in operating conditions listed in table 13.

System suitability

The system suitability tests showed that the theoretical plate values for both drugs exceeded 2000, the tailing factor was less than 2, and the resolution between the two peaks was 30.76.

A USP Resolution value greater than 1.5 is considered suitable for elucidating, detecting, and analyzing a multi-component system using HPLC (table 14).

Specificity

The results of the study showed that the blank injection of the mobile phase, i. e., ACN and 0.72% anhydrous sodium sulphate with a pH of 2.3 (31.5:68.5 v/v), did not show any peak at the retention time of Sel and BE. This indicated that the developed method is specific (fig. 12).

Table 12: Precision study of BE

Parameters	Level	Conc. (µg/ml)	Avg. Area	Std. Dev.	%RSD
Intraday 1	LQC	4.8	26486.67	121.33	0.458
	MQC	6	35393.33	122.33	0.345
	HQC	7.2	39399.67	123.33	0.313
Intraday 2	LQC	4.8	25486.67	124.33	0.487
	MQC	6	34383.33	125.33	0.364
	HQC	7.2	38399.67	126.33	0.328
Intraday 3	LQC	4.8	26514	127.33	0.480
	MQC	6	35548	128.33	0.361
	HQC	7.2	39421	129.33	0.328
Analyst 1	LQC	4.8	26592	130.33	0.490
	MQC	6	35384	131.33	0.371
	HQC	7.2	39254	132.33	0.337
Analyst 2	LQC	4.8	26886.17	133.33	0.495
	MQC	6	34393.33	134.33	0.390
	HQC	7.2	38979.47	135.33	0.347
Analyst 3	LQC	4.8	26584	136.33	0.512
	MQC	6	35733	137.33	0.384
	HQC	7.2	39841	138.33	0.347
Analyst 3	LQC	4.8	25998	139.33	0.535
	MQC	6	36199	140.33	0.387
	HQC	7.2	38754	141.33	0.364

In the above table, LQC=Low Quality Control, MQC=Mid Quality Control, HQC=High Quality Control; n = 6

Table 13: Robustness studies

Parameters	Values	Conc. (µg/ml)	Mean area (AU)	SD	%RSD	% Recovery	RT (Sel+ BE)	Resolution
Flowrate (ml/min)	0.8	6	188558.7	734.0534	0.504	103.05	Sel 4.909, BE 20.756	28.81
	1.0	6	189254.67	862.02	0.457	99.30	Sel 4.936, BE 20.936	26.89
	1.2	6	188459.56	769.9	1.339	96.59	Sel 4.923, BE 20.730	31.3
Ratio of mobile phase (ACN: Buffer)	33.5:66.5	6	193486.33	2821.51	0.943	95.06	Sel 4.931 BE 20.749	29.02
	31.5:68.5	6	198489.16	1159.23	0.370	99.54	Sel 4.927 BE 20.781	29.09
	29.5:70.5	6	199837816	2335.97	0.719	103.46	Sel 4.938, BE 20.741	31.29
Wavelength (nm)	203	6	188459.56	1718.99	0.554	98.72	Sel 4.937 BE 20.761	27.28
	205	6	188459.56	1017.43	0.325	99.48	Sel 4.951 BE 20.746	29.8
	207	6	188558.79	769.9	0.459	99.95	Sel 4.933, BE 20.926	29.62

*n = 6

Table 14: System suitability studies

System suitability parameters	Experimental value (Sel)	Experimental value (BE)	Recommended value as per CDER
Capacity factor (k')	1.47	1.65	1-20
Precision Injection repeatability (RSD)	0.39	0.44	≤1.0% for 5 replicates
Tailing factor (TF)	1.389	1.286	≤2.0
Theoretical plate number (N)±SD	7001±77	3254 ±55	>2000 (plate count)
Asymmetry factor (As)	1.33	1.24	<1.5

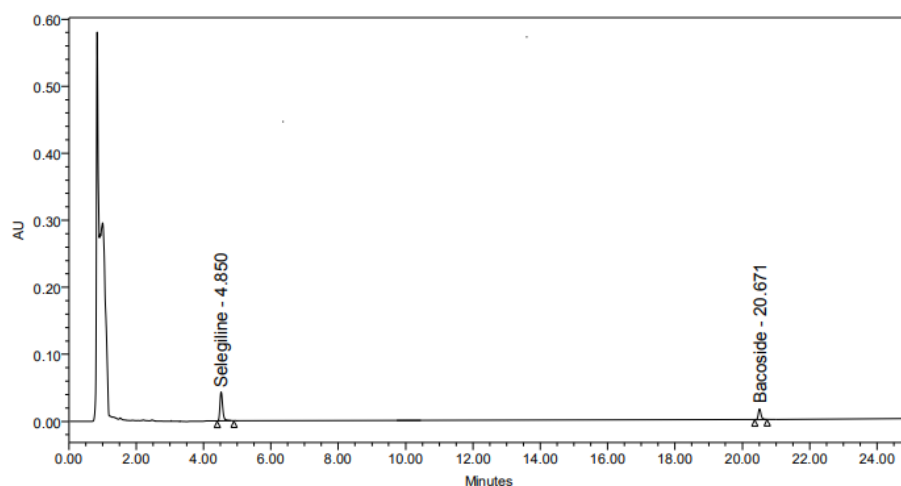


Fig. 12: Chromatogram of the mobile phase for specificity

CONCLUSION

The current LC method was developed to detect and analyze Selegiline and Brahmi extract simultaneously. The method is precise, sensitive, and robust, and is suitable for use. There are numerous HPLC methods that researchers have used to estimate the individual Sel and BE. These methods were developed using methanol or ACN as solvents, along with phosphate buffer. In the current study, a simultaneous estimation of Sel and BE was performed using a mobile phase of ACN and 0.72% anhydrous sodium sulphate with a pH of 2.3 (31.5:68.5 v/v). The validation study results, in accordance with ICH Q2(R2) guidelines, revealed that the developed method is simple, precise, accurate, robust, and specific. Most importantly, the developed method can be used in the formulation development and/or quality control of combined drug products.

ACKNOWLEDGEMENT

The authors are thankful to Lovely Professional University for providing the facilities to conduct the experimental work.

FUNDING

No financial support was obtained for this project.

DECLARATION ON USE OF AI TOOLS

No generative AI was-based tools were used for the generation of data. During the preparation of this work, the author(s) used a free version of Grammarly software for language polishing and gmar correction. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

AUTHORS CONTRIBUTIONS

Rakesh Kumar: Methodology, Investigation, Formal Analysis, Writing-Original Draft; Sumant Saini: Methodology, Formal analysis, Data Curation, Writing-Reviewing and Editing; Rajesh Kumar: Supervision, Conceptualization, Formal analysis, Data Curation; Vijay Mishra: Methodology, Formal Analysis, Writing-Reviewing and Editing.

CONFLICT OF INTERESTS

The authors have no conflict of interest

REFERENCES

- Mishra N, Sharma S, Deshmukh R, Kumar A, Sharma R. Development and characterization of nasal delivery of selegiline hydrochloride loaded nanolipid carriers for the management of Parkinson's disease. *Central Nervous System Agents in Medicinal Chemistry*. 2019;19(1):46-56. doi: 10.2174/1871524919666181126124846.
- Tabi T, Vecsei L, Youdim MB, Riederer P, Szoko E. Selegiline: a molecule with innovative potential. *J Neural Transm (Vienna)*. 2020;127(5):831-42. doi: 10.1007/s00702-019-02082-0, PMID 31562557.
- Banerjee S, Anand U, Ghosh S, Ray D, Ray P, Nandy S. Bacosides from *Bacopa monnieri* extract: an overview of the effects on neurological disorders. *Phytother Res*. 2021;35(10):5668-79. doi: 10.1002/ptr.7203, PMID 34254371.
- Walker EA, Pellegrini MV. *Bacopa monnieri*. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2023 Mar 17. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK589635/>.
- Stojanovic Z, Cajic E, Turkic E, Ibrsimovic I. Parkinson's detector application. *Int J Curr Pharm Rev Res*. 2022;14(2):13-23.
- Kumar R, Singh T, Kumar D, Singh M, Kaur S, Garg R. Estimation of bacoside-a in *Bacopa monnieri* aerial parts using TLC densitometry. *Int J Pharm Pharm Sci*. 2019;11(2):115. doi: 10.22159/ijpps.2019v11i2.32236.
- Raval K, Srinivasa U. Development and validation of HPLC method for the simultaneous estimation of pioglitazone and alogliptin in bulk and dosage form. *Int J Res*. 2014;6(11):10201-7.
- PubChem. Selegiline. Bethesda (MD): National Library of Medicine (US); 2026. Available from: <https://pubchem.ncbi.nlm.nih.gov/compound/selegiline>. [Last accessed on 11 Mar 2026].
- PubChem. Bacoside A. Bethesda (MD): National Library of Medicine (US); 2026. Available from: <https://pubchem.ncbi.nlm.nih.gov/compound/bacoside-a>. [Last accessed on 11 Mar 2026].
- Kurangi B, Jalalpure S, Jagwani S. A validated stability-indicating HPLC method for simultaneous estimation of resveratrol and piperine in cubosome and human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2019;1122-1123:39-48. doi: 10.1016/j.jchromb.2019.05.017, PMID 31150952.
- Gangishetty S, Verma S. RP-HPLC method development and validation for simultaneous estimation of clarithromycin and paracetamol. *ISRN Anal Chem*. 2013;2013:1-5. doi: 10.1155/2013/948547.
- Vancha H, Tewari D, Kumar R, Govindaiah P, Mohd S, Singh SK. Analytical quality by design driven development and validation of UV-visible spectrophotometric method for quantification of Xanthohumol in bulk and solid lipid nanoparticles. *Turk J Pharm Sci*. 2023;20(3):165-75. doi: 10.4274/tjps.galenos.2022.05335, PMID 37417199.
- Saini S, Kaur R, Gulati M, Singh B, Hussain A. QbD and green metric based HPLC method development and validation of bromocriptine mesylate in bulk product and biological samples. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2025;1258:124566. doi: 10.1016/j.jchromb.2025.124566, PMID 40262358.
- Boyes B, Dong M. Modern trends and best practices in mobile-phase selection in reversed-phase chromatography. *LCGC North Am*. 2018;36(10):752-68.
- Dupuis DJ, Victoria Feser MP. Robust VIF regression with application to variable selection in large data sets. *Ann Appl Stat*. 2013;7(1):319-41. doi: 10.1214/12-AOAS584.

16. Laky D, Xu S, Rodriguez JS, Vaidyaraman S, Garcia Munoz S, Laird C. An optimization-based framework to define the probabilistic design space of pharmaceutical processes with model uncertainty. *Processes*. 2019;7(2):96. doi: [10.3390/pr7020096](https://doi.org/10.3390/pr7020096).
17. Rajesh R, Jithu J. A validated RP-HPLC method for simultaneous estimation of pyrantel pamoate and praziquantel in bulk and pharmaceutical dosage form. *Int J Pharm Pharm Sci*. 2019;11(5):62-7. doi: [10.22159/ijpps.2019v11i5.30488](https://doi.org/10.22159/ijpps.2019v11i5.30488).
18. Khurana K, Beg S, Lal D, Katare OP, Singh B. Analytical quality by design approach for development of a validated bioanalytical UPLC method of docetaxel trihydrate. *Curr Pharm Anal*. 2015;11(3):180-92. doi: [10.2174/1573412911666150121234150](https://doi.org/10.2174/1573412911666150121234150).
19. Pukngam P, Burana-osot J. Development and validation of a stability-indicating HPLC method for determination of bromocriptine mesylate in bulk drug and tablets. *Curr Pharm Anal*. 2013;9(1):92-101. doi: [10.2174/157341213804806106](https://doi.org/10.2174/157341213804806106).
20. Rayudu S, Manoranjani M, Anitha D. Analytical method development and validation of sodium phenylbutyrate and taurursodiol by using RP-HPLC in bulk and pharmaceutical dosage form. *Int J Appl Pharm*. 2026;18(1):380-8. doi: [10.22159/ijap.2026v18i1.55081](https://doi.org/10.22159/ijap.2026v18i1.55081).
21. International Council for Harmonisation (ICH). Validation of analytical procedures Q2 (R2). Geneva, Switzerland: ICH; 2022. p. 1.
22. Panda SS, Bera RK, Behera AK, Beg S. Chemometrics-assisted development of a liquid chromatography method for estimation of lapatinib in tablets: a case study on a novel quality concept. *Separation Science Plus*. 2020;3(1-2):12-21. doi: [10.1002/sscp.201900096](https://doi.org/10.1002/sscp.201900096).
23. Saini S, Sharma T, Patel A, Kaur R, Tripathi SK, Katare OP. QbD-steered development and validation of an RP-HPLC method for quantification of ferulic acid: rational application of chemometric tools. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2020;1155:122300. doi: [10.1016/j.jchromb.2020.122300](https://doi.org/10.1016/j.jchromb.2020.122300), PMID 32771967.
24. Sharma P, Kapoor B, Hussain MS, Singh G, Rani P, Saini B. Development and validation of reverse-phase high-performance liquid chromatography method for simultaneous estimation of doxorubicin and clotrimazole. *Assay Drug Dev Technol*. 2024;22(2):86-96. doi: [10.1089/adt.2023.057](https://doi.org/10.1089/adt.2023.057), PMID 38150558.
25. Hardeep PNK, Pandey NK, Singh SK, Kumar B, Corrie L, Goutam U. Development and validation of reverse-phase high-performance liquid chromatography-based bioanalytical method for estimation of simvastatin in Rat's plasma. *Assay Drug Dev Technol*. 2022;20(8):349-58. doi: [10.1089/adt.2022.080](https://doi.org/10.1089/adt.2022.080), PMID 36473162.