Innovations

RP-HPLC and UV Methods for Simultaneous Estimation of Fixed Dose Combinations of Cinnarizine and Piracetam: Simultaneous Equation, Experimental Design and Statistical Correlation

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Abstract : *The current study represents the development and validation of UV spectroscopic and HPLC methods for simultaneous estimation of Cinnarizine and Piracetam in their combined formulation by using design of experiments (DoE). In UV spectroscopy method, Cinnarizine and Piracetam were determined by using simultaneous equation method. Methanol was used as solvent. 250 nm and 229 nm wavelengths were selected for the estimation of Cinnarizine and Piracetam. In RP-HPLC method, three independent factors like; Organic solvent composition, Buffer pH and flow rate was employed. Central composite design (CCD) was applied and also to study the response surface methodology. Capacity factor, Retention time and Resolution were simultaneously optimized by using Desirability function for HPLC method. The proposed RP-HPLC method, the optimized and predicted data consisted of mobile phase Acetonitrile: Orthophosphoric acid (pH 2.8 with Orthophosphoric acid) in the ratio of 40:60% v/v respectively, at a flow rate of 0.7 ml/min and wavelength of 229nm.For UV, the linearity ranges from 4-24 μg/ml for cinnarizine and 80-480 μg/ml for Piracetam. For HPLC, the linearity ranges from 2-12 μg/ml for cinnarizine and 40-240 μg/ml for Piracetam .The optimized both method conditions were validated according to International Council for Harmonisation (ICH) guidelines. The Student's t-test and Two way analysis of variance (ANOVA) were used to correlate the results of Cinnarizine and Piracetam determination in dosage form by means of UV and HPLC method. The proposed methods can be routinely employed in quality control for the analysis of Cinnarizine and Piracetam in the pharmaceutical dosage forms.*

Key words: *UV, HPLC, Optimization, Design of experiment, Central composite design, Cinnarizine, Piracetam*

Introduction

Cinnarizine (CIN) (Figure 1), [1-(diphenyl methyl)-3-(4-phenylprop-2-enyl)-piperazine] is a derivative of piperazine that has antihistaminic (H1 blocker), sedative and calcium channel blocking activity. Due to calcium channel blocking activity cinnarizine acts as a nootropic drug. Piracetam (Figure 2) chemically called [2-(2-Oxopyrrolidin-1-yl)acetamide]. Piracetam may also have an impact on NMDA glutamate receptors, which are connected to memory and learning. It's believed that piracetam increases the permeability of cell membranes. Piracetam may modulate ion channels (Na+, K+) to have a global effect on brain neurotransmission. The most popular fractional factorial design for the response surface model is the central composite design. A collection of axial points known as star points is added to the centre points in this design. First-order and second-order terms can be estimated quickly with this design. **The statistical analysis tool known as analysis of variance (ANOVA) divides the observed aggregate variability present in a data set into two categories: systematic factors and random factors. When comparing more than two groups, an ANOVA is a useful tool for determining the relationships between them. The number of independent variables in the analysis of variance test determines whether it is one-way or two-way. An expansion of the one-way ANOVA is the two-way ANOVA. T tests are limited to comparing two groups at a time; in contrast, ANOVA allows for the comparison of three or more groups.**

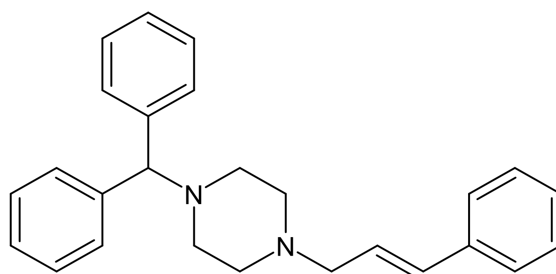


Figure 1 Structure of Cinnarizine

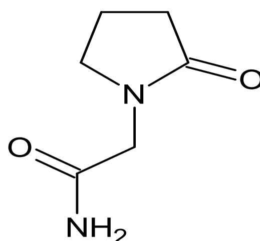


Figure 2 Structure of Piracetam

Literature review revealed that the determination of Cinnarizine alone or combined with other drugs were reported. Method development and validation of Cinnarizine by UV[1], Impurities study in HPLC [2], HPLC [3], LC and TLC [4], Stability indicting HPTLC [5], UV,Atomic absorption spectrometry and Potentiometry [6], LC-MS[7] and combined with other drugs the methods were UV [8], Spectrofluorimetry [9], HPLC [10], Stability indicating HPLC [11], UV and Spectrofluorimetry [12], HPTLC [13], RP-LC [14], UPLC [15], TLC and HPLC [16] has been reported. The determination of Piracetam alone or combined with other drugs were reported. Method development and validation of Piracetam by impurities in HPLC[17], Stability indicating UPLC[18], FTIR[19], LC-MS[20], HPLC[21],TLC[22] and combined with other drugs the methods were HPLC[23], TLC[24], Stability indicating HPLC[25], Quality by design technique[26] and UV[27] has been reported. Simultaneous determination of Cinnarizine and Piracetam by UV [28], Stability indicating HPLC [29], FTIR [30], UV, HPLC and Spectrodensitometry [31] were reported.

From the literature there was no method reported for the simultaneous determination of Cinnarizine and Piracetam by RP-HPLC method using central composite method and UV (Simultaneous equation). So an attempt was made to develop RP-HPLC (QbD) and UV (Simultaneous equation) methods.

Materials and methods

Instrumentation and software

The Agilent 1200 series was utilized for the development and validation of RP-HPLC. The Design Expert 12 ® (version 7.1.6., trial version) software was used for the Central Composite Design (CCD). The Shimadzu UV-1900 was used for the Ultra Visible Spectrophotometry.

Chemicals and reagents

Acetonitrile (HPLC grade), Methanol (HPLC grade) and ortho-phosphoric acid (Analytical grade) were obtained from the Merck. The Avicetam capsule (Cinnarizine 20mg and Piracetam 400mg) and the active pharmaceutical ingredient of Cinnarizine and Piracetam were collected from the Kausikh Therapeutics Pvt Ltd, Gerugambakkam, Chennai.

Instruments and Chromatographic conditions

High Performance Liquid Chromatography with UV detector (Agilent 1200 series), Ultra Visible Spectrophotometry (1100 series), Analytical balance (RADWAG) and Ultra sonicator (Labman) instruments were used. The High Performance Liquid Chromatography with UV detector (Agilent 1200 series) was used to achieve the chromatographic separation. The mobile phase consists of Acetonitrile and Orthophosphoric acid pH (2.8) in the ratio of 40:60 %v\v for RP-HPLC. The mobile phase was filtered through 0.45 µm membrane filter, degassed and injected onto the Hypersil column (C_{18}) (250mmX4.6mm, 5µ particle size) at a flow rate of 1.0 ml\min. The injection volume was 20µl.

Selection of wavelength

10µg/ml solution of Cinnarizine and Piracetam were prepared by using methanol as solvent. The solutions were scanned separately between the wavelength ranges of 200-400 nm by using methanol as blank. The spectrum was recorded. From this spectrum, 250 nm for Cinnarizine and 229 nm for Piracetam were selected for the further analysis.

Preparation of standard stock solution

About 10 milligram of Cinnarizine and 200 milligram of Piracetam was accurately weighed and transferred into 100 ml volumetric flask .1.2 ml solution was pipette out from the above solution and transferred into 10ml volumetric flask and volume was made by using methanol (100µg/ml for Cinnarizine and 2000 µg/ml for Piracetam). The concentration of the solution was 12µg/ml and 240µg/ml for Cinnarizine and Piracetam respectively.

For HPLC method, from the primary stock solution, 0.3 ml volume was pipetted out and transferred in to 100ml volumetric flask (200µg/ml for Cinnarizine and 4000µg/ml for Piracetam).Then volume was made with mobile phase. The final concentration of the solutions was made with 6.0µg/ml for Cinnarizine and 120µg/ml for Piracetam.

Preparation of Sample solution

10 capsules of Avicetam (20mg of Cinnarizine and 400 mg of Piracetam) were taken and their average weight was determined. Capsule powder equivalent to 20 mg of Cinnarizine and 400 mg of Piracetam was accurately weighed. Then transferred to 100 ml volumetric flask and half of the volume was made with mobile phase. Then the solution was sonicated for 10 minutes. Finally the volume was made with mobile phase (200µg/ml and 4000µg/ml). Then the solution was filtered using whatmann

filter paper No 41 with 0.45 micron filter size. For UV method, from this 0.6 ml solution was pipetted out from the above solution and transferred into 10ml volumetric flask. Then the volume was made by using methanol. The concentration of the solution was 12μg/ml and 240μg/ml for Cinnarizine and Piracetam respectively. For HPLC method, from the primary stock solution, 0.3 ml volume was pipetted out and transferred into 100ml volumetric flask. Then the volume was made with mobile phase. The final concentration of the solutions was made with 6.0μg/ml for Cinnarizine and 120μg/ml for Piracetam.

Preparation of Linearity (Calibration Graph)

For UV method, from the primary stock solutions (200μg/ml and 4000 μg/ml) of Cinnarizine and Piracetam respectively linearity was observed for both the drugs by preparing various working solutions. 0.2ml, 0.4 ml, 0.6 ml, 0.8 ml, 1.0 ml, 1.2 ml of secondary stock solutions were transferred to 10 ml volumetric flask and volume was made with methanol. The linearity concentration ranging from 80-480μg/ml and 4-24μg/ml respectively was prepared and absorbance measured at specific wavelength. The calibration curve for both of these drugs was prepared by plotting the graph between absorbance vs. Concentration.

For HPLC method, the aliquots of stock solution of Piracetam (4000μg/ml) and Cinnarizine (200μg/ml) 0.1 to 0.6 ml were transferred into six 10ml volumetric flasks and made up to mark with mobile phase. The solutions containing the concentration of 2-12μg/ml for Cinnarizine and 40-240μg/ml for Piracetam. From this solution 20μl were injected and the chromatogram were recorded at 229 nm. The above concentration range was found to be linear and obeys beer's law. The procedure was repeated for three times. The peak areas were plotted against concentration and the calibration curve was constructed.

LOD and LOQ

The linearity study was carried out for six times for both the UV and RP-HPLC methods. The LOD and LOQ values were calculated based up on the calibration curve method. The LOD and LOQ values were calculated by using average of slope and intercept.

Precision

The interday and intraday was checked by repeated analysis of the formulation for six times with the same concentrations. The amount of drug present in the capsule formulations was calculated. The percentage RSD value was calculated for both UV and RP-HPLC methods.

Recovery studies (Accuracy)

The recovery studies were done by adding known concentration of Cinnarizine and Piracetam raw material to the pre-analysed formulation for both the method. For UV method, the capsule powder equivalent to 10mg of Cinnarizine and 200 mg of Piracetam was weighed accurately and transferred into a series of three 100ml standard flask. To that raw material Cinnarizine and Piracetam (50 %, 100 % and 150 %) was added and the volume was made upto the mark with methanol. The content was kept in a sonicator for 15 minutes, after sonication the solutions were filtered through Whatmann filter paper no. 41. From the clear solution further dilutions were made by diluting 0.6 ml to 10 ml volumetric flask with methanol for UV method. Absorbance was measured at specific wavelength. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration.

For HPLC method sample (Capsule formulation) with standard different concentration (50 %, 100 %, and 150 %) (raw material) were diluted by using mobile phase for each concentration. The above said procedure was followed. From the filtrate 0.3ml was pipette out and transferred into 10ml standard flask and made upto the volume with mobile phase. For each concentration 20µl solutions were injected in to the chromatographic system then the chromatograms were recorded.

Robustness

For RP-HPLC method, the robustness was studied by evaluating the effect of small variation in the chromatographic conditions. The conditions studied were flow rate (± 0.2 ml/min), composition of mobile phase (± 2 ml) and wavelength (± 2 nm). For each condition, 20 µl solutions were injected into the chromatographic system and chromatograms were recorded.

Ruggedness

Ruggedness of the method was confirmed by the analysis of formulation performed by the different analyst. The amount of the drug and % RSD were calculated.

Results and Discussion

UV spectrophotometric method (Simultaneous equation method)

A simple, accurate and precise simultaneous equation method was developed and validated for UV. Methanol is the common solvents for both the drug Cinnarizine and Piracetam. 10µg/ml concentrations of both these drugs were scanned in the UV region and the spectra were recorded. From the spectra the λ max of the drugs were found to be 250 and 229 nm for Cinnarizine and Piracetam. The spectrum for

Cinnarizine at 250nm and 229nm were shown in figure 3,4 . Piracetam UV spectrum at 229nm and 250nm were shown in figure 5,6.

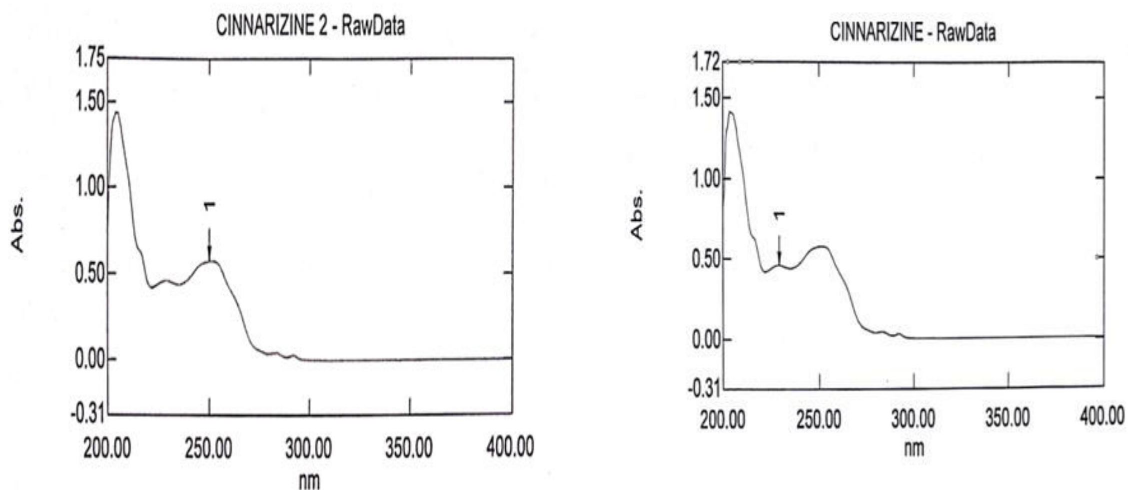
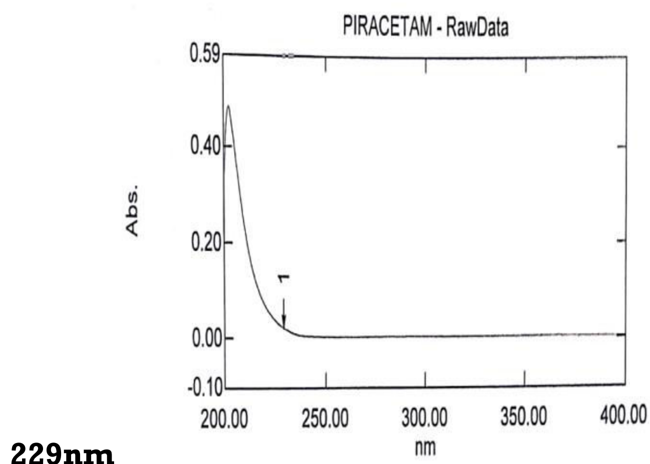


Figure 3, 4 UV spectrum for Cinnarizine at 250nm and 229nm



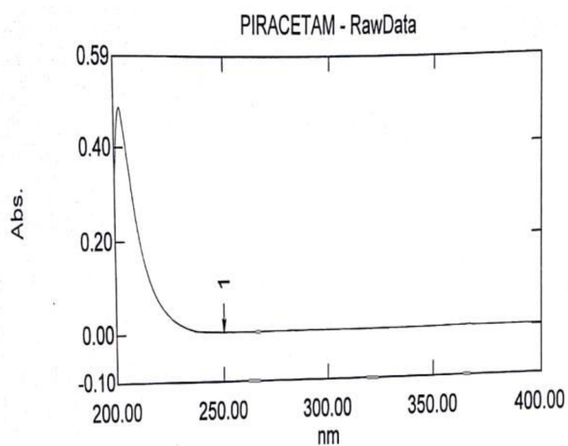


Figure 5, 6 UV spectrum for Piracetam at 229nm and 250nm

Limit of detection was found to be 0.3236µg/ml for Cinnarizine and 1.3527µg/ml for Piracetam. Limit of quantification was found to be 0.9807µg/ml for Cinnarizine and 4.099µg/ml for Piracetam. The % RSD values for intraday and inteday precision were found to be 0.3222, 1.4461 and 0.3722, 1.6474 for Cinnarizine and Piracetam respectively. Less than 2% RSD values found indicated the developed UV method was precise. The accuracy of the method was confirmed by recovery studies. The %

Parameters		**Cinnarizine**	**Piracetam**
Range (µg/ml)		4-24	80-480
Correlation Coefficient (r)		0.999	0.999
Regression equation (Y=mx+c)		y = 0.0256x + 0.2442	y = 0.0011x - 0.0307
Slope(m)		0.0256	0.0011
Intercept(c)		0.2442	0.0307
LOD (µg/ml)		0.3236	1.3527
LOQ (µg/ml)		0.9807	4.099
Precision(%RSD)	Interday	0.3722	1.6474
	Intraday	0.3222	1.4461
Assay (%)		100.4	99.96
Accuracy (%RSD)		0.2640	0.1525

Table 1 Validation parameters Report for UV method

recovery was found to be in the range of 99.9-100.8% for Cinnarizine and 99.9-100% for Piracetam. The recovery % RSD value was found to be less than 2% (0.2640% for Cinnarizine and 0.1525% for Piracetam) it indicated no interference due to excipients. Hence the method was found to be accurate. Ruggedness study was confirmed by two analyst. The % RSD value by analyst 1 and analyst 2 were found to be 0.1138, 0.2652 and 1.1445, 0.4931 for Cinnarizine and Piracetam respectively. The low %RSD value was indicated that the developed method was more rugged. Results of the validation parameters for UV were shown in Table 1.

RP-HPLC method

Chromatographic method optimization

The selectivity of the chromatographic factors such as the separation of analytes, simultaneous optimization of resolution, retention time chemometric protocol of response surface design and Derringer's desirability function were successfully employed. The central composite design could be applied to optimize the separation and to assist the development of better understanding of the interaction of several chromatographic factors on separation quality. In this work, the important chromatographic factors were selected and optimized by a central composite design experiment. Factors selected and optimized were based on risk assessment.

The process of risk assessment entails developing a methodical procedure, obtaining data from multiple sources, using instruments and strategies for risk identification, recording the risks, and evaluating the process' efficacy. It's critical to establish a control strategy subsequent to method development. In order to direct the strategy's development, this entails developing an analytical target profile. The predetermined controls in the analytical control strategy are based on risk management, analytical procedures, and suitability for purpose. Together, these elements ensure that the procedure operates efficiently and generates high-quality outcomes that are consistent with the defined analytical target profile. The reference states that the strategy includes controls for sample preparation, measurement, and replication.

According to the Quality by Design ICH Q8 (R2) guidance document, a critical quality attributes (CQAs) is any physical, chemical, biological, or microbiological property or characteristic that must fall within a suitable range, limit, or distribution in order to guarantee the desired level of product quality.[32,33] A quality target product profile (QTPP) defines the expected product performance, and defining the necessary product attributes for that performance and taking other information

sources into account are prerequisites for evaluating CQAs.[34,35] In order to define acceptance criteria and an effective control strategy, all identified CQAs must have their variability within the manufacturing process evaluated. This further connects the issue to process characterization. Finding the quality attributes that need to be under control is the aim of the CQA assessment.

The factors selected for optimization process were Acetonitrile concentration (A), Ortho phosphate buffer pH (B) and Flow rate (C). The ranges of factors used were Acetonitrile concentration (40-60% v/v), Buffer pH (2.5-2.9) and flow rate (0.3–0.7 ml / min) .The levels of each factor studied for finding out the optimum values and responses (Table 4).The capacity factor for the first eluted peak of Piracetam (K_1), retention time of second eluted peak of Cinnarizine (Rt_2) and the resolution of both the peaks between Piracetam and Cinnarizine ($Rs_{1, 2}$) were selected as responses. **The length of the retention period is determined by the structure of the particular molecule as well as other elements including the chromatographic column's dimensions, the mobile phase's flow rate, and the characteristics of the stationary and mobile phases. Retention times are typically typical for a particular compound in a particular separation. The composition of the mobile phase affects an analyte's retention. Retention will be impacted by either a high water content or a high organic solvent content in the mobile phase. The retention time of peak A will drop by a factor of two if the flow rate is doubled; in other words, if the mobile phase is moving twice as quickly as the analyte, it will take half as long for the analyte to pass through the column.**

All experiments were conducted in randomized order to minimize the effects of uncontrolled variables that might introduce a bias on the measurements. Replicates (n = 6) of the central points were performed to estimate the experimental error. For an experimental design with the three factors, including linear, quadratic and cross terms, the model can be expressed as

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X2 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32}$$

where Y is the response to be modeled, β is the regression coefficient and X_1, X_2 and X_3 represent factors A, B and C respectively. Statistical parameters obtained from ANOVA for the reduced models (Table 3). The insignificant terms ($p > 0.05$) were eliminated from the model through backward elimination process to obtain a simple and realistic model [36]. Since R^2 always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regressor variables into account, is usually selected. The adjusted R^2values were well within the acceptable limits of $R^2 \geq 0.80$, which revealed that the experimental data showed a good fit with second order

polynomial equations. For all the reduced models, p value of < 0.05 was obtained, implying these models were significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio. A ratio greater than 4 is desirable .The ratio was found to be in the range of 5.9861-9.7469 which indicated an adequate signal and therefore the model was significant for the separation process. The coefficient of variation (C.V) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10%.

Table 2 Central Composite Arrangement and Responses

Std	**Run**	**Space Type**	**Factor1 A:Acetonitrile com %v/v**	**Factor 2 B:Buffer pH**	**Factor 3 C:Flow rate ml/min**	**Response 1 Capacity factor K1**	**Response 2 Rs 1,2 min**	**Response 3 Rt2 min**
15	4	Center	50	2.7	0.5	1.44	17.37	10.428
18	9	Center	50	2.7	0.5	1.44	17.37	10.428
20	11	Center	50	2.7	0.5	1.44	17.37	10.428
16	13	Center	50	2.7	0.5	1.44	17.37	10.428
19	15	Center	50	2.7	0.5	1.44	17.37	10.428
17	19	Center	50	2.7	0.5	1.44	17.37	10.428
12	6	Axial	50	3.03636	0.5	1.43	18.27	8.63
13	7	Axial	50	2.7	0.163641	3.87	18.41	16.618
11	8	Axial	50	2.36364	0.5	1.43	18.12	7.58
10	12	Axial	58.409	2.7	0.5	1.03	17.29	6.166
14	17	Axial	50	2.7	0.836359	0.87	17	5.632
9	18	Axial	41.591	2.7	0.5	2.49	18.45	10.34
7	1	Factorial	45	2.9	0.7	1.03	17.24	5.996
4	2	Factorial	55	2.9	0.3	1.22	17.56	6.487
2	3	Factorial	55	2.5	0.3	1.23	17.63	6.438

5	5	Factorial	45	2.5	0.7	1.04	17.51	5.879
1	10	Factorial	45	2.5	0.3	1.71	18.31	7.775
6	14	Factorial	55	2.5	0.7	0.88	17.21	5.389
3	16	Factorial	45	2.9	0.3	1.72	18.2	7.711
8	20	Factorial	55	2.9	0.7	0.88	17.03	5.34

The interaction with the largest absolute coefficients among the fitted model was AC (+0.1769) of Rt_2 model (Retention time). The positive interaction between A and C was statistically significant (< 0.0001) for Rt_2. The study revealed that changing the Acetonitrile concentration from low to high resulted in a rapid decline in the retention time of Cinnarizine in the high and low levels of flow rate (ml/min).

Table 3 Reduced Response Surface Models and Statistical Parameters Obtained from ANOVA

Responses	Regression model	Adjusted R^2	Model p value	C.V (%)	Adequate Precision
K1	$+0.9635-0.0369A+0.0144B-0.0359C-0.0459AB+0.0536AC-0.0346BC+0.0260A^2+0.0006B^2+0.0494C^2$	0.9576	<0.0001	3.78	7.8510
Rs1,2	$+17.38-0.2768A-0.0277B-0.3721C+0.0163AB+0.1013AC-0.0337BC+0.09866A^2+0.2135B^2+0.0402C^2$	0.9869	<0.0001	1.34	9.7469
Rt2	$+10.52-0.7855A+0.1322B-1.78C-0.0066AB+0.1769AC+0.0104BC-1.34A^2-1.39B^2-0.3249C^2$	0.8963	<0.0001	5.76	5.9861

In order to gain a better understanding of the results, the predicted models were presented in the form of perturbation plots and 3D response surface plots .Variables giving quadratic and interaction terms with the largest absolute

coefficients in the fitted models were chosen for the axes of the response surface plots. Perturbation plot provided silhouette views of the response surface plots where it showed how the response changes as each factor moved from a chosen reference point, with all factors held constant at the reference value. The steepest slope or curvature indicated the sensitiveness of the response to a specific factor. Flow rate (factor C) had most important effect on Retention time (Rt_2) following the factor A (ACN). The rest of the factors had significant effect on capacity factor (K_1) and resolution ($Rs_{1,2}$). Retention time (Rt_2) values decreased as the level of Acetonitrile concentration increased and that resolution ($Rs_{1,2}$) values increased as the level of Buffer pH increased . The value of retention time (Rt_2) decreased with increasing levels of factor A .The value of Resolution increased with increase in the Factor B levels. Analysis of the perturbation plots and response plots of optimization models revealed that factor B and C had significant effect on the separation of the analytes. Derringer's desirability function was employed for global optimization of three responses and to select different optimal conditions for the analysis of formulation in the present study. The identified criteria for the optimization were resolution between the peaks, peak height and elution time. The Derringer's desirability function, D, is defined as the geometric mean, weighted or otherwise of the individual desirability functions. The expression that defines the Derringer's desirability function is:

$$\mathbf{D = [d_1^{p2} \times d_2^{p2} \times d_3^{p2} \times \ldots\ldots\ldots \times d_n^{pn}]^{1/n}}$$

Where pi is the weight of the response, n the number of responses and di is the individual desirability function of each response. Desirability function (D) can take values from 0 to 1. Weights can range from 0.1 to 10. Weights lower than 1 give less importance to the goal, whereas weights greater than 1 give more importance to the goal. The criteria for the optimization of each individual response (Table 4).

Table 4 Criteria for the optimization of the individual responses

Response	Lower limit	Higher limit	Criteria / Goal
K1	0.87	3.87	Minimize
Rs 1,2	17	18.45	Minimize
Rt2	5.34	16.618	Minimize

In criteria, the responses Rt_2 was in the minimize in order to shorten the analysis time, Capacity factor (K_1) was in the range for sensitivity and in order to separate the first eluting peak (Piracetam) and second eluting peak (Cinnarizine) and the Resolution factor($Rs_{1,2}$) was minimized. Following the conditions and restrictions

above, the optimization procedure was carried out. The 3D surface obtained for the Derringer's desirability function was presented in figure 13.

It could be concluded that there was a set of coordinates producing high desirability value (D = 0.974) were Acetonitrile concentration: Orthophosphoric acid (40:60), flow rate 0.7 ml/min and wavelength 229 nm. The optimized assay conditions were Acetonitrile: Orthophosphoric acid (40:60), pH 2.8 as mobile phase at a flow rate of 0.7 ml/min. The predicted response values corresponding to the later value of D were K_1=0.67, Rt_2 =6.08 min and $Rs_{1,2}$ =17.10. The observed differences between the predicted and experimental responses were found to be in good agreement, within a difference of 5.0%. The percentage of prediction error was calculated by using the following equation (Table 5).

Average error = Experimental- predicted/ predicted X 100

Table 5 Comparison of experimental and predictive values of different functions under optimal conditions

Optimum conditions	**ACN Comp (%v/v)**	**Buffer pH**	**Flow rate**	**Capacity Factor**	**Rs1,2**	**Rt2**
Predictive	55.000	2.815	0.700	1.50	3.3	3.15
Experimental	55.00	2.815	0.700	1.59	3.241	3.23
Average error				4	1.787	2.539
Desirability value (D) =0.974						

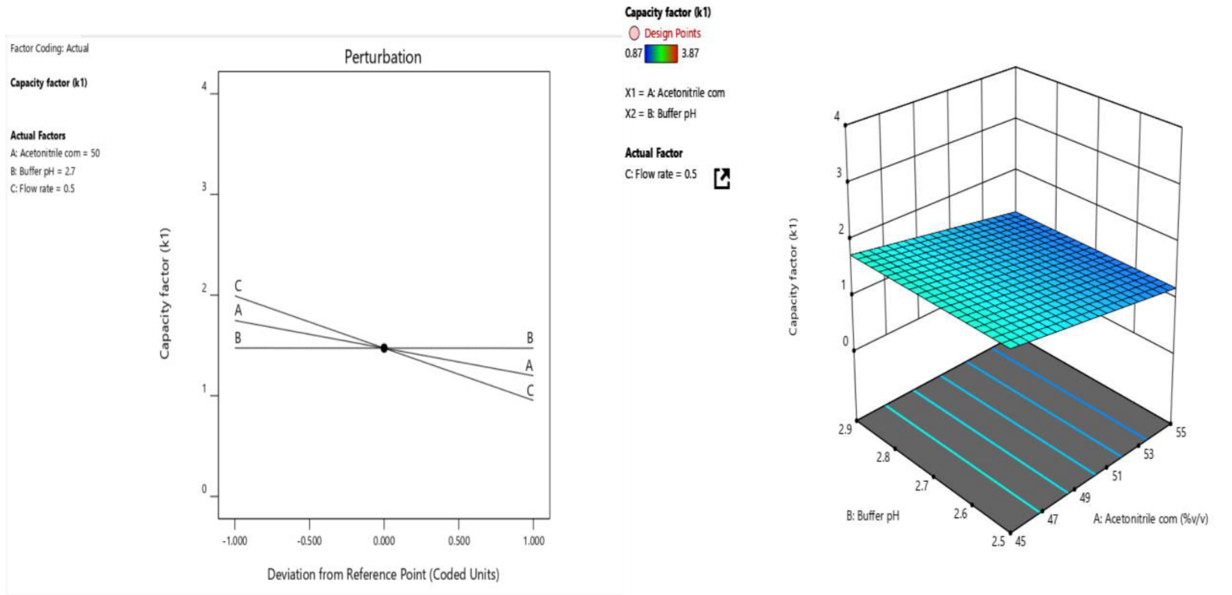


Fig.7 Perturbation plot for Capacity factor (k1) **Fig.8 3D surface for Capacity factor (k1)**

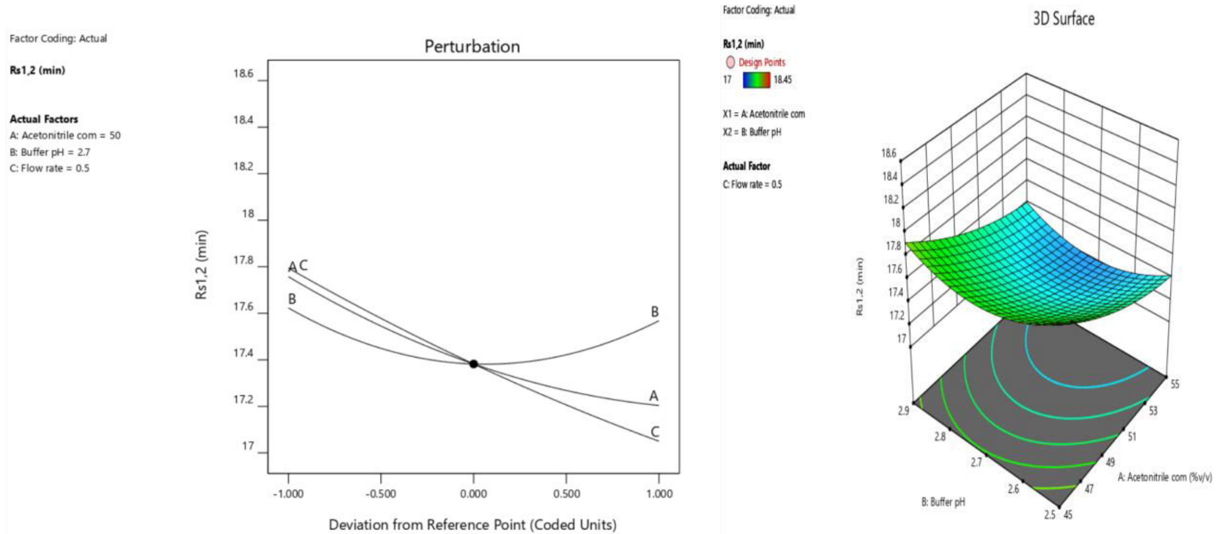


Fig.9 Perturbation plot for Resolution (Rs 1,2) **Fig.10 3D surface for Resolution (Rs 1,2)**

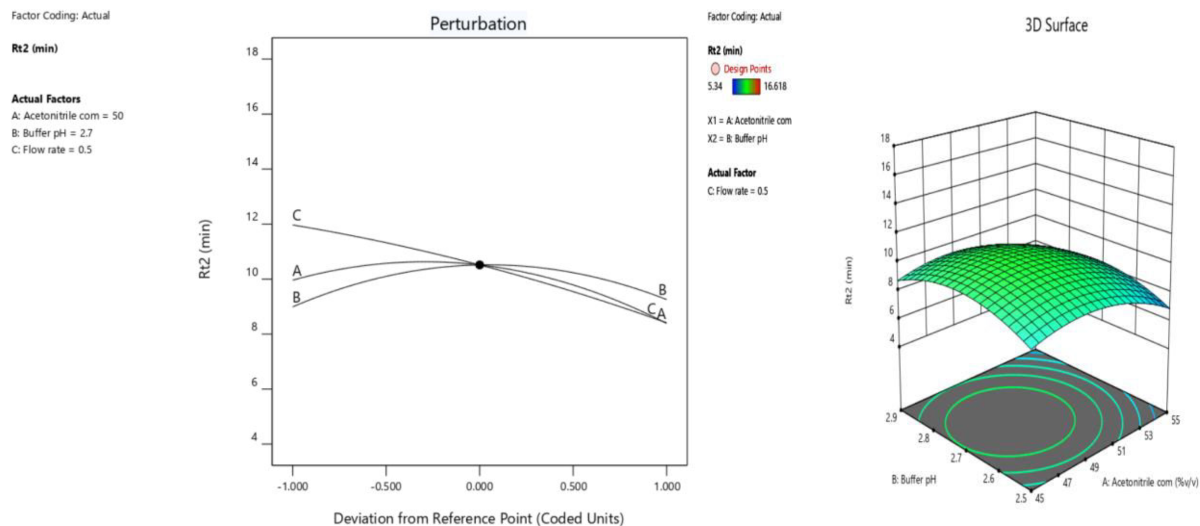


Fig.11 Perturbation plot for Retention time (Rt2) Fig.12 3D surface for Retention time(Rt2)

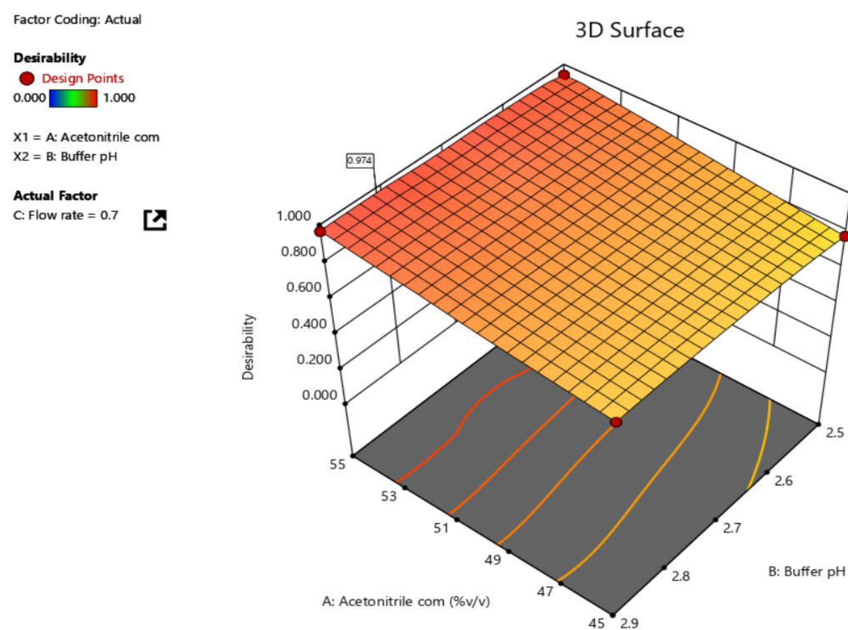


Fig.13 3D plots for Derringer's Desirability function

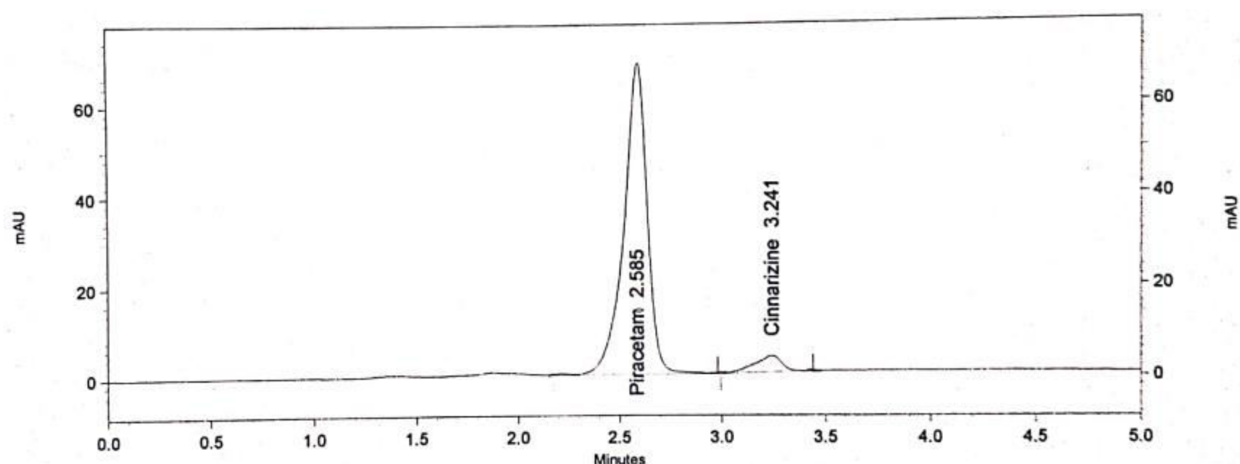


Figure 14 Optimised chromatogram for RP-HPLC

The developed HPLC method was simple, accurate and precise. The linearity range was found to be 2-12μg/ml for Cinnarizine and 40-240μg/ml for Piracetam. Limit of detection was found to be 0.0113μg/ml for Cinnarizine and 0.0181μg/ml for Piracetam. Limit of quantification was found to be 0.0345μg/ml for Cinnarizine and 0.0550μg/ml for Piracetam. The % RSD for intraday precision was found to be 1.3987 for Cinnarizine and 0.3275 for Piracetam. The % RSD values for interday precision were found to be 0.2968 for Cinnarizine and 0.0978 for Piracetam. Low % RSD values (less than 2%) indicated the developed method was precise. The percentage recovery was found to be 99.3% for Cinnarizine and 99.32% for Piracetam. The % RSD value for Cinnarizine and Piracetam were found to be 0.8547 and 0.1599 respectively. There was no interference due to excipients (%RSD value less than 2%). So, the developed method was found to be accurate. Ruggedness is also validated for the developed method. The % RSD value for analyst I found to be 1.6987 and 0.1816 for Cinnarizine and Piracetam respectively. The % RSD values for analyst II found to be 0.5589 and 0.2845 for Cinnarizine and Piracetam respectively. The developed method was more rugged. The robustness study indicated that the factors selected remained unaffected by small variation of flow rate, wavelength and the organic composition of mobile phase. Results of the validation parameters for HPLC were shown in Table 6.

Table 6 Validation parameters report for RP-HPLC method

<table>
<tr><th colspan="2">Parameters</th><th>Cinnarizine</th><th>Piracetam</th></tr>
<tr><td colspan="2">Range (μg/ml)</td><td>2-12</td><td>40-240</td></tr>
<tr><td colspan="2">Correlation Coefficient (r)</td><td>0.999</td><td>0.999</td></tr>
<tr><td colspan="2">Regression equation (Y=mx+c)</td><td>y = 136155x + 19820</td><td>y = 115684x - 32729</td></tr>
<tr><td colspan="2">Slope(m)</td><td>136155</td><td>115684</td></tr>
<tr><td colspan="2">Intercept(c)</td><td>19820</td><td>32729</td></tr>
<tr><td colspan="2">LOD (μg/ml)</td><td>0.011391</td><td>0.01817</td></tr>
<tr><td colspan="2">LOQ (μg/ml)</td><td>0.034517</td><td>0.05507</td></tr>
<tr><td rowspan="2">Precision(%RSD)</td><td>Interday</td><td>0.2968</td><td>0.0978</td></tr>
<tr><td>Intraday</td><td>1.3987</td><td>0.3275</td></tr>
<tr><td colspan="2">Assay (%)</td><td>100.51</td><td>99.91</td></tr>
<tr><td colspan="2">Accuracy (%RSD)</td><td>0.8547</td><td>0.1599</td></tr>
</table>

From the comparison table 7 shows the LOD and LOQ values for the reported method RP-HPLC was more when compared to the developed method. DL and QL values were very less when compared to the reported method so it indicated the sensitiveness of the method. Hence the method was more sensitive when compared with the reported method. Additionally, the linearity ranges of the reported RP-HPLC method were more when compared to the developed method. Both drugs retention time was found to less when compared to the reported method. So the developed method can be applied for regular quality control analysis, the least amount drug can be required.

Table 7 Comparison between the developed method and reported method for RP-HPLC

Parameters	Drugs	Developed method	Reported method [37]
Retention Time	Cinnarizine	2.548	8.103
	Piracetam	3.241	3.888
LOD(µg/ml)	Cinnarizine	0.011	1.04
	Piracetam	0.018	16.0
LOQ(µg/ml)	Cinnarizine	0.034	3.4
	Piracetam	0.055	48
Linearity(µg/ml)	Cinnarizine	2-12	10-80
	Piracetam	40-240	160-960

From the comparison table 8 between the developed method for UV and RP-HPLC method the LOD and LOQ value was less when compared with the UV method. DL and QL value was very less when compared to the UV method so it indicated the sensitiveness of the method. Hence the method was more sensitive when compared with the UV method. Additionally, the linearity ranges of the UV method were more when compared to the RP-HPLC method. So the RP-HPLC method can be applied for regular quality control analysis, the least amount drug can be required.

Table 8 Comparison between the developed method for UV and RP-HPLC method

Parameters	**Drugs**	**UV**	**RP-HPLC**
Retention Time and Absorbance	Cinnarizine	Absorbance 0.579 (250 nm) 0.462 (229 nm)	Retention time 2.548
	Piracetam	Absorbance 0.005 (250 nm) 0.023(229 nm)	Retention time 3.241
LOD(μg/ml)	Cinnarizine	0.3236	0.011
	Piracetam	1.3527	0.018
LOQ(μg/ml)	Cinnarizine	0.9807	0.034
	Piracetam	4.099	0.055
Linearity(μg/ml)	Cinnarizine	4-24	2-12
	Piracetam	8-480	40-240

Correlation between the RP-HPLC and UV method

Six different samples from the two different batches were taken, and quantification was done simultaneously to correlate the difference between the two developed methods of RP-HPLC and UV. Statistical tests were conducted to test the difference between the proposed HPLC and UV method at a 95% confidence level (P=0.05). Two way ANOVA test was applied for both method-sample interaction. In both cases, F stat was less than F crit, indicating that the method-sample interaction and the differences between the methods were not significant (Table 9).

A paired student's t-test was applied to test means between the HPLC and UV methods. From the student's t-test, it was found that the t stat<t critical, signifying there was no significant difference between the means (Table 10).

Table 9 Two-way Anova Test of Cinnarizine and Piracetam in six-independent samples by UV and HPLC

Two way ANOVA Test of Cinnarizine Determination				
HPLC[a]			**UV[a]**	
Sample	**First sampling**	**Second sampling**	**First sampling**	**Second sampling**
1	20.05	20.1	20.10	20.12
2	20.11	20.22	20.16	19.97
3	20.35	20.25	19.98	20.04
4	20.09	20.14	19.99	20.06
5	20.29	20.19	20.09	20.11
6	20.31	20.30	20.14	20.15

ANOVA: Two-Factor with Replication

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Sample	0.092504	1	0.092504	11.4285	0.002971	4.351244
Columns	0.0000417	1	0.0000417	0.000515	0.982123	4.351244
Interaction	0.0000417	1	0.0000417	0.000515	0.982123	4.351244
Within	0.161883	20	0.008094			
Total	0.254396	23				

Two way ANOVA Test of Piracetam Determination				
HPLC[b]			**UV[b]**	
Sample	**First sampling**	**Second sampling**	**First sampling**	**Second sampling**
1	400.1	399.57	400.1	400.04

2	399.43	400.02	399.95	399.98
3	399.52	399.56	400.05	400.02
4	399.48	399.47	400.03	399.96
5	399.59	399.51	400.06	399.91
6	399.58	399.54	399.97	399.93

ANOVA: Two-Factor with Replication

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0.893204	1	0.893204	33.44812	0.000017	4.351244
Columns	0.005104	1	0.005104	0.191137	0.666656	4.351244
Interaction	0.003504	1	0.003504	0.131222	0.720968	4.351244
Within	0.534083	20	0.026704			
Total	1.435896	23				

Table 10 Determination of HPLC and UV and their correlation by paired t-Test

Student t-Test for Cinnarizine

Sample	HPLC[a]	UV[a]
1	100.51	99.96
2	100.25	100.01
3	100.55	100.02
4	101.75	99.99
5	100.27	99.97
6	100.56	99.93
Average	100.64	99.98

Student t-Test: Paired two sample for Means (Cinnarizine)

	Variable 1	**Variable 2**
Mean	100.6483333	99.98
Variance	0.310416667	0.00112
Observations	6	6
Pearson Correlation	0.065430208	
Hypothesized Mean Difference	0	
Df	5	
t Stat	-2.94456799	
P(T<=t) one-tail	0.016043234	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.032086467	
t Critical two-tail	2.570581836	

Student t-Test for Piracetam

Sample	**HPLC**[b]	**UV**[b]
1	99.91	100.01
2	100.01	99.91
3	99.85	100.02
4	99.88	99.96
5	99.89	100.06
6	99.83	99.95
Average	99.89	99.98

Student t-Test: Paired two sample for Means (Piracetam)

	Variable 1	Variable 2
Mean	99.895	99.985
Variance	0.00399	0.00299
Observations	6	6
Pearson Correlation	-0.437174584	
Hypothesized Mean Difference	0	
Df	5	
t Stat	-2.204540769	
P(T<=t) one-tail	0.039321491	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.078642982	
t Critical two-tail	2.570581836	

[a] The results are presented as mg of label claim amount of Cinnarizine in capsule

[b] The results are presented as mg of label claim amount of Piracetam in capsule

Conclusion

The current research work was investigated to a develop and validated UV (Simultaneous equation) and HPLC (using QbD) methods. The developed methods was validated and found to be in conformance with ICH guidelines. Based on the reports for UV method, the method was simple, precise, economic and accurate. In HPLC method, the CCD technique was use to fit significant factors. The Derringer's desirability function was used simultaneously optimized the factors. This techniques decreases the overall assay development time and reports the interaction effects on their chromatographic factors on the attributes of separation. From the statistical correlation it can be concluded that both the methods are useful for simultaneous quantification of Cinnarizine and Piracetam with accuracy, precision, less retention time. So it can be concluded that the developed method can be successfully employed for the routine analysis of Cinnarizine and Piracetam in bulk and pharmaceutical dosage forms.

Conflict of interest

The authors declare no Conflict of interest.

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