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The use of QbD for the development and validation of a stability-indicating RP-HPLC method for the quantitation of nevirapine in bulk, tablet and niosome formulations

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Introduction: A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the quantitation of nevirapine (NVP) in bulk drug, commercial tablets, and niosome formulations using an Analytical Quality by Design (AQbD) approach.

Methods: Critical analytical attributes and method parameters were identified and optimized using a Central Composite Design (CCD), with the retention time and resolution between NVP and internal standard carbamazepine as key responses. Chromatographic separation was achieved on a C₁₈ column using an isocratic mobile phase of water and acetonitrile (57.5:42.5 v/v), a 1.0 mL/min flow rate, and detection at 280 nm. The method was validated per ICH Q2 (R1) guidelines for all parameters including repeatability, intermediate precision, accuracy (recovery/bias), and robustness, in addition to specificity, linearity, LOD and LOQ.

Results and Discussion: The method demonstrated specificity, linearity (0.5–200 µg/mL), a detection limit of 0.033 µg/mL, and quantification limit of 0.5 µg/mL. The method was precise, accurate, and robust. Stress studies suggested stability-indicating performance under the tested conditions with no observed interference at the analyte retention time at 280 nm. Application to commercial and in-house formulations confirmed its suitability for routine analysis. This work highlights the value of AQbD in developing cost-effective, high-performing analytical methods for pharmaceutical analysis.

KEYWORDS

central composite design, design of experiments, nevirapine, quality by design, response surface methodology, RP-HPLC

1 Introduction

Nevirapine (NVP) is an adipyrido-diazepinone compound, with an IUPAC name 11-cyclopropyl-4-methyl-5, 11-dihydro-6H-dipyrido (3, 2-b: 2', 3'-e) (1, 4) diazepin-6-one (Sriram et al., 2006) and is classified as a non-nucleoside reverse transcriptase inhibitor (NNRTI) which is often used in tandem with other compounds for the suppression of HIV to halt development of AIDS (Lange, 2002). NVP was the first NNRTI approved by the Food

and Drug Administration (FDA) for the treatment of HIV-1 infections and requires frequent dosing. NVP has a toxicity profile which includes serious side effects such as dermatological reactions, Stevens-Johnson Syndrome (SJS) and serious hepatotoxicity (Sulkowski et al., 2002). Presently, most NVP-based regimens require twice daily dosing and are associated with an inability to maintain drug levels at a sufficiently high concentration which, in part, has contributed to the occurrence of multi-drug resistant strains of HIV. To overcome some of these therapeutic challenges novel drug delivery systems have been proposed for the delivery of NVP. Our research group developed and optimised NVP-containing niosomes stabilised using dihexadecyl phosphate (DCP) and cholesterol in combination with Span® 20 or Span® 80 (Witika and Walker, 2019; 2021).

The analysis of nevirapine has been previously achieved using gradient elution (Mustafa et al., 2014), ion-pair RP-HPLC (Van Heeswijk et al., 1998), isocratic RP-HPLC (Pav et al., 1999; Hollanders et al., 2000; Kappelhoff et al., 2003; Ramachandran et al., 2006; Hamrapurkar et al., 2010; Kumar et al., 2010), gas chromatography-mass spectrophotometry (Vogel et al., 2010) and liquid chromatography-mass spectrophotometry (Rentsch, 2003; Sichilongo et al., 2014). Most of these methods have been used for the determination and quantitation of NVP in plasma (Pav et al., 1999; Hollanders et al., 2000; Kappelhoff et al., 2003), as bulk drug (Kappelhoff et al., 2003; Hamrapurkar et al., 2010; Ravisankar and Rao, 2013) and in dosage forms (Kappelhoff et al., 2003; Ravisankar and Rao, 2013) and require long analysis times which may not be suitable for routine laboratory use where a large number of samples may be required to be analysed within a short period.

Traditionally, the steps involved in method development entail a trial-and-error approach executed by evaluating the impact of one-factor-at-a-time (OFAT) on a separation. This approach generally requires a large set of experiments to be conducted and does not permit the evaluation of interactions between the factors evaluated, which may compromise the development and optimization process of a method (Bezerra et al., 2008; Fukuda et al., 2018). To circumvent the limitations of using an OFAT approach, a design of experiments (DoE) model, which is capable of generating results, including interaction effects, with fewer experimental runs (Bezerra et al., 2008) was used in this study.

The concept of analytical quality by design (AQbD) in analytical method development is a key aspect in the area of pharmaceutical analysis. The AQbD approach places emphasis on science- and risk-based understanding of critical parameters which may affect method performance and results in the development of a robust method (Beg et al., 2012; 2015). The use of DoE is the cornerstone of pharmaceutical and analytical QbD (Bezerra et al., 2008).

A Central Composite Design (CCD) used for the optimization of response surface methodology (RSM) tools is among the most popular second-order designs as five (5) levels of each input factor are possible to evaluate using a reduced number of experiments when compared to three-level full factorial designs (Bezerra et al., 2008).

The present study aimed to develop and optimise a rapid, simple, selective, accurate and precise stability indicating RP-HPLC method for the analysis of NVP in bulk and tablet that is better than existing methods and capable of determining the NVP content in novel drug delivery platforms such as niosomes using CCD and RSM.

2 Materials and methods

2.1 Materials

NVP was donated by Aspen Pharmacare® (Port Elizabeth, South Africa). The methanol (MeOH) UV cut off 215 nm used to prepare stock solutions of NVP and internal standard and the HPLC grade acetonitrile 200 far UV Romil-SpS™ Super Purity Solvent (ACN) used to prepare mobile phase were purchased from Microsep® Ltd. (Port Elizabeth, South Africa). Carbamazepine (CBZ), which was used as the internal standard, was purchased from Sigma Aldrich® (St. Louis, United States of America). A Milli-Q Academic A10 water purification system, consisting of an Ion-X® ion-exchange cartridge and a quantum EX-ultrapore Organex® cartridge equipped with a 0.22 µm Millipak® 40 sterile filter (Burlington, MA, United States of America) was used to purify the HPLC grade water.

2.2 Methods

2.2.1 Instrumentation

The HPLC system utilised was a Waters® Alliance Model 2695 separation module, which included a solvent delivery module, auto-sampler, online degasser, and a model 2489 UV-vis detector (Milford, United States of America). The acquisition, processing, and reporting of data were conducted utilising Waters® Empower 3 software (Milford, United States of America). The stationary phase utilised was a Phenomenex Luna® C₁₈ (5 µm, 250 mm × 4.6 mm i. d.) column (Separations, Randburg, South Africa). Detection was performed at 280 nm to provide a strong and stable UV response for nevirapine and the internal standard with an acceptable baseline under the selected chromatographic conditions. A Phenomenex Luna® C₁₈ (5 µm, 250 mm × 4.6 mm i. d.) column was selected as a robust reversed-phase stationary phase offering sufficient efficiency and resolution for separating nevirapine, carbamazepine, formulation/excipient components, and potential degradants, which is required for a stability-indicating assay.

2.2.2 Preparation of stock solutions

A standard stock solution was prepared by precisely weighing 200 mg of NVP with a Mettler® Model AE163 top-loading balance (Zurich, Switzerland) and quantitatively transferred to a 200 mL A-grade volumetric flask. The volumetric flask was subsequently filled to volume with MeOH. The solution underwent sonication for a duration of 10 min with a Branson® B12 sonicator (Shelton, United States of America), resulting in a concentration of 1 mg/mL. The standard stock solution facilitated the preparation of solutions within the concentration range of 0.5–200 µg/mL through a methodical serial dilution process utilising A-grade glassware.

2.2.3 Selection of internal standard

To reduce system and procedural variability that could occur during sample preparation, as a result of analytical procedure, or because of equipment variability, internal standards are utilised during quantitative analysis (Akpino, 1982). An internal standard

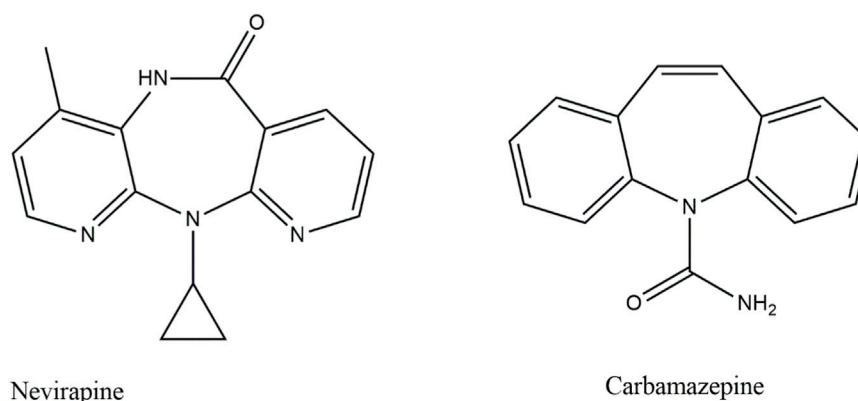


FIGURE 1
Chemical structures of NVP and CBZ.

enhances the precision of an analytical process by offsetting daily instrument fluctuations and inconsistent injection volumes (Lindholm et al., 2003).

Carbamazepine (CBZ) has commonly been used as an internal standard (Hamrapurkar et al., 2010; Mustafa et al., 2014) and oxcarbamezepine has also been used (Sarkar et al., 2006). Possible internal standards that were considered included efavirenz, tenofovir disoproxil fumarate and CBZ. CBZ resolved well and had a retention time of 7.12 min prior to method optimisation. Consequently, CBZ was chosen as the IS for these studies. The structures of NVP and CBZ are provided in Figure 1.

2.2.4 Preparation of internal standard solution

A stock solution of internal standard was prepared by accurately weighing 200 mg carbamazepine (CBZ) using a Mettler® Model AE163 top-loading analytical balance (Zurich, Switzerland) and quantitatively transferred to a 200 mL A-grade volumetric flask. The volumetric flask was then made up to volume with MeOH and sonicated for 10 min using a Branson® B12 sonicator (Shelton, USA) to produce a solution of 1 mg/mL. Subsequently, 10 mL of the stock solution was transferred to a 100 mL A-grade volumetric flask and diluted with the mobile phase to achieve a concentration of approximately 100 µg/mL. A 0.75 mL aliquot of the 100 µg/mL CBZ was combined with 0.75 mL of the nevirapine (NVP) solution and stirred before analysis.

2.2.5 Preparation of mobile phase

The mobile phase used was comprised of acetonitrile and HPLC-grade water. The individual solvents were then transferred to a 1000 mL Scott® Duran bottle (Wertheim, Germany) and placed on separate lines of the HPLC system and mixed online for the analysis. The mobile phase was not recycled during analysis.

2.2.6 Experimental design

A CCD design was selected for the optimisation of the approach developed for NVP analysis. Initial studies suggested that three distinct input parameters, namely flow rate (X_1), organic solvent composition (X_2), and column temperature (X_3), were significant. The observed responses were the retention times of NVP (Y_1) and the resolution factor (R_s) (Y_2). The experiments conducted for the

CCD are summarized in Table 1. To minimise any potential bias, all trials were performed in triplicate and randomised order.

Factor coding was defined as A = flow rate, B = organic solvent (acetonitrile) content in the mobile phase, and C = column temperature. Model terms AB, AC and BC represent two-factor interactions, while A^2 , B^2 and C^2 represent quadratic effects.

2.2.6.1 Method optimisation

Numerical optimisation was performed to identify method conditions that best satisfy the predefined analytical goals and constraints for the chromatographic performance responses. A desirability-based approach was implemented in Design-Expert® to explore the factor space and select an overall solution. The following factor constraints were applied with equal weights and importance set to 3: flow rate (A) targeted at 1.00 mL/min organic solvent fraction (B) constrained within 40%–60% (v/v), and column temperature (C) constrained within 25 °C–35 °C. Response goals were defined as minimising the retention time of nevirapine (RT NVP; 4.00–6.36 min) and maximising chromatographic resolution (R_s ; 1.96–24.07) to support separation and method robustness. Although carbamazepine was used as an internal standard in the chromatographic method, its retention time (RT CBZ) was treated as a system suitability/control criterion with the upper limit set to 10 min rather than a primary optimisation response and is therefore not emphasised as a formal response variable. The recommended operating conditions were verified experimentally by running the method in replicate ($n = 5$) under the selected set-point.

2.2.7 Method validation

Method validation was performed in accordance with ICH guidance to confirm that the developed RP-HPLC procedure is suitable for its intended purpose in the quantitation of nevirapine in bulk material, tablets and niosome formulations. Validation parameters included precision, linearity and range, accuracy, limits of detection and quantitation, specificity and assay applicability (Causey et al., 1990; Green, 1996; Wood, 1999; Food and Drug Administration, 2015).

All validation experiments were conducted using the CCD-optimised chromatographic conditions. Unless stated otherwise, measurements were performed in triplicate ($n = 3$) and results are reported as mean values.

TABLE 1 Summary of CCD experiments performed, actual code and responses observed.

Std no.	Run	Flow rate mL/min	Organic solvent %	Temp °C
18	1	0.88	50.00	30.00
1	2	0.75	40.00	25.00
11	3	0.88	33.18	30.00
3	4	0.75	60.00	25.00
4	5	1.00	60.00	25.00
7	6	0.75	60.00	35.00
12	7	0.88	66.82	30.00
2	8	1.00	40.00	25.00
8	9	1.00	60.00	35.00
13	10	0.88	50.00	21.59
20	11	0.88	50.00	30.00
14	12	0.88	50.00	38.41
16	13	0.88	50.00	30.00
19	14	0.88	50.00	30.00
9	15	0.66	50.00	30.00
17	16	0.88	50.00	30.00
15	17	0.88	50.00	30.00
6	18	1.00	40.00	35.00
10	19	1.09	50.00	30.00
5	20	0.75	40.00	35.00

2.2.7.1 Precision

Precision describes the degree of agreement among individual results obtained when the method is applied repeatedly to multiple samples of a homogeneous matrix under prescribed conditions (Causey et al., 1990; Clarke, 1994). In this study, precision was assessed as repeatability (intra-day) and inter-day precision. Results were expressed as %RSD of NVP-to-IS peak area ratios. The acceptance criterion was set at ≤ 5 %RSD, which is appropriate for pharmaceutical dosage forms where biological-matrix variability is not applicable (Food and Drug Administration, 2015).

Repeatability (intra-day): Standard solutions across the calibration range were analysed on the same day in triplicate ($n = 3$), and %RSD of peak area ratios was calculated.

Inter-day precision: The same calibration standards were analysed in triplicate ($n = 3$) across three consecutive days, and %RSD values were determined to assess inter-day variability.

Reproducibility (between laboratories/operators) was not evaluated because the method was applied within a single laboratory by one analyst using the same instrument, and inter-day precision sufficiently addressed precision requirements for this context.

2.2.7.2 Linearity and range

Linearity was evaluated using least-squares regression of NVP concentration versus response (peak area ratio) over the specified

working range. The coefficient of determination (R^2) and regression equation were used to confirm proportionality across the calibration range (Causey et al., 1990; Food and Drug Administration, 2001). The range was defined as the interval between the lowest and highest concentrations that could be quantified with acceptable precision and accuracy.

2.2.7.3 Accuracy

Accuracy reflects the closeness of the measured value to the true value and was assessed using recovery experiments at three concentration levels (low, medium and high) spanning the calibration range (Causey et al., 1990; Green, 1996; Wood, 1999; Food and Drug Administration, 2001; Ermer and John, 2005). Samples were prepared at each level and analysed in triplicate ($n = 3$), with results reported as % recovery, % bias, and % RSD (Hokanson, 1994; Green, 1996).

2.2.7.4 Limits of detection and quantitation

The LOQ was defined as the lowest concentration that could be quantified with acceptable precision ($\leq 5\%$ RSD) and accuracy (Hokanson, 1994). The LOD was defined as the lowest concentration producing a measurable response but not meeting the requirements for accurate quantitation (Hokanson, 1994; Paino and Moore, 1999). LOQ was determined experimentally using the precision-based approach ($\leq 5\%$ RSD), and the LOD was calculated as 30% of the LOQ by convention (Paino and Moore, 1999).

2.2.7.5 Specificity

Specificity was assessed by demonstrating the absence of interference at the retention time of nevirapine and the internal standard in chromatograms of blank/mobile phase, reference standard solutions, and tablet sample solutions. Chromatograms of nevirapine standards were compared with chromatograms obtained from commercially available Aspen[®] nevirapine tablets to confirm that excipients did not interfere with the analyte response (Vessman, 1996; Rosing et al., 2000).

2.2.7.6 Assay and entrapment efficiency

2.2.7.6.1 Assay of commercial and in-house developed sustained release tablets. The suitability of the method for analysing NVP in dosage forms was confirmed by analyzing commercially available Aspen[®] NVP 200 mg tablets as well as in-house developed 400 mg sustained release tablets (Mwila, 2013) using the proposed technique. Twenty tablets were weighed and pulverised using a mortar and pestle. A quantity of powder equal to 100 mg was transferred into a 100 mL A-grade volumetric flask. Approximately 50 mL of methanol was added, and the mixture was subjected to sonication for 10 min before adjusting the volume with the mobile phase. The solution was then filtered with 0.45 µm HVLP Millipore[®] filter membrane and diluted with mobile phase to 100 µg/mL prior to analysis. The experiments were performed in triplicate.

2.2.7.6.2 Preparation of niosomes niosome samples for analysis. NVP niosomes were manufactured using a previously described thin layer hydration method (Azmin et al., 1985; Baillie et al., 2011) with slight modification and optimized using a non-rotatable Box-Behnken Design (BBD) to investigate the impact of four formulation and process variables on the Critical Quality Attributes (CQA) of NVP niosomes (Witika, 2017; Witika and Walker, 2019; 2021).

Briefly, a weight equivalent to 40 mg of NVP was accurately weighed and transferred to a round-bottomed flask and different molar ratios of surfactant, cholesterol and DCP were added. The powder was dissolved using 10 mL chloroform and methanol in a 90/10% v/v solution. The solvent was evaporated under vacuum using a Buchi[™] R-215 rotary evaporator (Buchi[™] Laboratories, Switzerland) for 45 min. Thereafter, the dried lipid layer was hydrated with 10 mL phosphate buffered saline (PBS) at pH 7.4. Hydration was conducted by rotating the sample at a temperature of 70 °C at a set speed for a specific period, as defined by the BBD. Following hydration, the suspension was annealed overnight at 4 °C (Witika, 2017; Witika and Walker, 2019; 2021).

Entrapment efficiency (EE) was determined by centrifuging 1 mL of each suspension using an Eppendorf 3154-C centrifuge (Hamburg, Germany) at 14,000 x g for 1 h. The supernatant was then decanted into a 10 mL A-grade volumetric flask and the pellet was washed using Milli-Q[®] water after which the sample was centrifuged at 14,000 g for a further 30 min to ensure all free NVP was removed. The supernatant was then decanted into the same A-grade volumetric flask, diluted with methanol and analysed. The entrapment efficiency was confirmed by destroying the niosome pellet at the base of the centrifuge tube with n-propanol and sonicating for 1 h using a Branson 8510 sonicator (Connecticut, USA). The solution was then decanted into a 100 mL A-grade

volumetric flask and made up to volume with MeOH prior to analysis. Stability studies.

2.2.8 Forced degradation studies

2.2.8.1 Oxidative, acidic and alkali

Three separate 100 mL A-grade volumetric flasks were each filled with 100 mg samples of NVP. Forty millilitres of MeOH were added to each flask, and the solutions were subjected to sonication for 10 minutes. The solutions were prepared to a final volume using 30% v/v H₂O₂ (Allied Drug Company Ltd., Durban, South Africa), 0.1 M HCl, and 0.1 M NaOH for the oxidative, acidic, and alkaline degradation studies, respectively. The samples underwent reflux for 8 h at a temperature of 90 °C. Aliquots of 1.2 mL were collected at 2, 4, 6, and 8 h and diluted to a theoretical concentration of 50 µg/mL using mobile phase in 10 mL A-grade volumetric flasks. Aliquots of 0.75 mL were combined with 0.75 mL of the internal standard solution and vortexed before analysis via the RP-HPLC method outlined in this document. The experiments were conducted in triplicate.

2.2.8.2 Neutral hydrolytic studies

Neutral hydrolytic studies involved weighing 100 mg of NVP and transferring it into a 100 mL A-grade volumetric flask. Subsequently, 40 mL of MeOH was added, the solution was sonicated for 10 min, and the volume was adjusted with HPLC grade water. The sample was refluxed at 90 °C for 8 h, with 1.2 mL aliquots collected at 2, 4, 6, and 8 h, subsequently diluted to a concentration of 50 µg/mL using the mobile phase in a 10 mL A-grade volumetric flask. Aliquots of 0.75 mL were collected and combined with 0.75 mL of the IS solution, followed by vortexing before analysis using RP-HPLC.

2.2.8.3 Photodegradation studies

Approximately 100 mg NVP was accurately weighed and transferred into a 100 mL A-grade volumetric flask. Methanol (40 mL) was added and the solution was sonicated for 10 min and then made up to volume with mobile phase. The sample was exposed to light of 500 W/m² at a temperature of 27 °C using a model CPS + SUNTEST[®] Weathering unit (Linsengericht, Germany) for 8 h. Sample aliquots of 1.2 mL were collected at 2, 4, 6 and 8 h and diluted to a concentration of 50 µg/mL with mobile phase in a 10 mL volumetric flask. Sample aliquots of 0.75 mL were then collected and 0.75 mL of internal standard solution added and the solutions vortexed prior to analysis by HPLC.

3 Results

3.1 Central Composite Design

Twenty experiments were performed as required for the CCD and a summary of the experiments and response factors are listed in Table 2. All experiments were conducted in a randomised fashion which resulted in simplified data sets being obtained.

The responses presented in Table 2 were assessed utilising version 8.0.2 of Design Expert[®] software (Minneapolis, MN, USA), and the data were analysed using various models. The effectiveness of each model in representing the relationship

TABLE 2 CCD experiments and results for analysis of NVP.

Std no.	Run	Flow rate mL/min	Org sol %	Temp °C	RT NVP min	R _s
18	1	0.88	50.00	30.00	4.082	8.89
1	2	0.75	40.00	25.00	5.861	17.06
11	3	0.88	33.18	30.00	6.360	24.07
3	4	0.75	60.00	25.00	4.294	3.29
4	5	1.00	60.00	25.00	3.177	2.52
7	6	0.75	60.00	35.00	4.294	3.29
12	7	0.88	66.82	30.00	3.424	1.96
2	8	1.00	40.00	25.00	4.429	15.27
8	9	1.00	60.00	35.00	3.177	2.52
13	10	0.88	50.00	21.59	4.102	8.83
20	11	0.88	50.00	30.00	4.082	8.89
14	12	0.88	50.00	38.41	3.987	8.91
16	13	0.88	50.00	30.00	4.082	8.89
19	14	0.88	50.00	30.00	4.082	8.89
9	15	0.66	50.00	30.00	5.416	9.94
17	16	0.88	50.00	30.00	4.082	8.89
15	17	0.88	50.00	30.00	4.082	8.89
6	18	1.00	40.00	35.00	4.429	15.26
10	19	1.09	50.00	30.00	3.329	8.05
5	20	0.75	40.00	35.00	5.861	17.06

Values are reported as mean of replicate injections (n = 3) for each run; centre-point runs were replicated to estimate pure error.

TABLE 3 Summary of model parameters and values used to evaluate adequacy of the model for resolution factor.

Parameter	Value	Parameter	Value
Std. Dev.	0.100	R ²	0.9934
Mean	4.33	Adj R ²	0.9874
C.V. %	2.30	Pred R ²	0.9497
PRESS	0.76	Adeq Precision	43.637
F-Value	166.80		

between the independent input variables and the monitored responses was assessed, with ANOVA employed to identify significant parameters.

3.1.1 Evaluation of model adequacy for retention time of NVP

Table 3 summarises the values of the factors considered to determine the model's adequacy. The most critical metrics for determining model adequacy are the model F-value, coefficient of variance, acceptable precision, PRESS, and R² values.

Table 4 summarises the results of the ANOVA analysis performed following the CCD trials, as well as the major factors

influencing NVP retention. Values of "P > F" < 0.0500 suggest that the model terms are significant.

3.1.2 Evaluation of model accuracy for R_s

Table 5 provides a summary of the findings used to determine R_s. Every parameter taken into account when evaluating the model's suitability showed that the chosen quadratic model was suitable for navigating the design space.

Table 6 presents the results of the ANOVA data analysis for the CCD experiments of the parameters that substantially affect the resolution between NVP and CBZ. The R_s for CBZ and NVP was shown to be influenced by the organic solvent concentration and flow rate, and model terms with a p-value < 0.05 were deemed significant. The resolution factor was also significantly impacted by concurrent variations in the concentration of organic solvent and flow rate and the quadratic term B² was also significant, indicating curvature in the relationship between organic solvent composition and R_s across the explored range.

3.1.3 Method optimisation

The optimised chromatographic conditions yielded a separation with retention times of NVP and CBZ at 4.10 ± 0.014 min and 6.7 ± 0.014 min, respectively, and a resolution

TABLE 4 ANOVA for Response Surface Quadratic Model Analysis of variance table (Partial sum of squares - Type III) for the retention time of NVP.

Source	Sum of squares	Df	Mean square	F-value	P-value Prob > F
Model	14.93	9	1.66	166.80	<0.0001
A-flow rate	5.43	1	5.43	545.49	<0.0001
B-organic solvent	8.19	1	8.19	823.40	<0.0001
C-column temp	2.739E-003	1	2.739E-003	0.28	0.6112
AB	0.050	1	0.050	4.99	0.0496
AC	0.000	1	0.000	0.000	1.0000
BC	0.000	1	0.000	0.000	1.0000
A ²	0.14	1	0.14	14.20	0.0037
B ²	1.15	1	1.15	115.77	<0.0001
C ²	4.149E-003	1	4.149E-003	0.42	0.5329
Residual	0.099	10	9.946E-003		
Lack of Fit	0.099	5	0.020		
Pure Error	0.000	5	0.000		
Cor Total	15.03	19			

TABLE 5 Model parameters affecting resolution factor navigate the design space for this separation.

Parameter	Value	Parameter	Value
Std. Dev.	0.48	R ²	0.9963
Mean	9.57	Adj R ²	0.9930
C.V. %	5.03	Pred R ²	0.9723
PRESS	17.45	Adeq Precision	65.351
F-Value	301.52		

factor of 13 ± 0.012 ($n = 5$). The optimal chromatographic conditions established for the quantification of NVP as well as the predicted and actual responses are presented in Table 7. These conditions were selected based on UV response at 280 nm and C₁₈ column performance to achieve adequate retention, peak shape and resolution. Figure 2 illustrates a conventional chromatogram depicting the separation of NVP and CBZ.

3.1.4 Method validation

3.1.4.1 Linearity

A graph illustrating the peak area ratio of NVP/CBZ against the concentration of NVP produced a calibration curve with a slope of 0.0059, a y-intercept of 0.0066, and a correlation coefficient of 0.9994, as shown in Supplementary Figure S2. The results demonstrate that the approach exhibited linearity across the examined concentration range. The exceptional precision of the data rendered the standard deviation bars invisible on the plot.

3.1.4.2 Precision

3.1.4.2.1 Repeatability. The results for repeatability are summarized in Table 8. The % RSD for results is <5% which was the acceptable value for these experiments.

3.1.4.2.2 Intermediate precision. The data obtained for intermediate precision are provided in Table 9. An RSD % <5% suggests that this approach is suitable for analysing NVP on different days.

3.1.4.2.3 Accuracy. A summary of the results of accuracy studies is listed in Table 10 and indicates the method is accurate, with all % RSD and % Bias values <5%.

3.1.4.3 LOQ and LOD

The LOQ was determined to be 0.5 µg/mL, with a corresponding % RSD of 0.65%. By convention, the LOD was set at 0.033 µg/mL.

3.1.4.4 Specificity

The specificity studies indicated that the NVP peak was not affected by the excipients present in both commercially available Aspen® nevirapine tablets and the in-house developed nevirapine tablets (Figure 3). Consequently, the method is considered precise for the examination of NVP in tablets and niosomal formulations.

3.1.4.5 Assay and entrapment efficiency

3.1.4.5.1 Commercial and in-house sustained release tablet assay. The mean amount of NVP was found to be 98.35% ± 0.7435% of the label claim for the Aspen® NVP 200 mg tablets assessed while in the in-house developed sustained release tablet the label claims was found to be 99.13% ± 0.8325%.

TABLE 6 ANOVA for Response Surface Quadratic Model Analysis of variance table [Partial sum of squares - Type III] for the resolution factor.

Source	Sum of squares	Df	Mean square	F value	p-value Prob > F
Model	627.40	9	69.71	301.52	<0.0001
A-flow rate	5.05	1	5.05	21.86	0.0009
B-organic solvent	595.94	1	595.94	2577.63	<0.0001
C-column temp	1.136E-003	1	1.136E-003	4.913E-003	0.9455
AB	0.53	1	0.53	2.27	0.1626
AC	1.250E-005	1	1.250E-005	5.407E-005	0.9943
BC	1.250E-005	1	1.250E-005	5.407E-005	0.9943
A ²	0.29	1	0.29	1.24	0.2912
B ²	23.62	1	23.62	102.15	<0.0001
C ²	0.49	1	0.49	2.14	0.1741
Residual	2.31	10	0.23		
Lack of Fit	2.31	5	0.46		
Pure Error	0.000	5	0.000		
Cor Total	629.72	19			

TABLE 7 Optimised chromatographic conditions for the analysis of NVP and Predicted vs. Experimental values.

Parameter	Setting		
Flow rate	1 mL/min		
Injection Volume	10 µL		
Wavelength	280 nm		
Temperature	25 °C		
Mobile phase composition	57.5:42.5% v/v ACN: H ₂ O		
Total run time	10 min		
Response	Predicted	Actual	Prediction error (%)
RT NVP (min)	4.176	4.10	-1.82
Rs	13.485	13.00	-3.60

The typical chromatogram of the tablet assay is provided in [Figure 3](#).

3.1.4.5.2 Niosome entrapment efficiency. To demonstrate applicability of the proposed RP-HPLC method to vesicular matrices, the method was applied to quantify nevirapine (NVP) during determination of niosome entrapment efficiency (EE). Free (unentrapped) NVP was quantified in the supernatant, while entrapped NVP was quantified after disruption of the recovered pellet. Entrapment efficiency was calculated as:

$$\%EE = \frac{\text{Entrapped NVP}}{\text{Total NVP}} \times 100$$

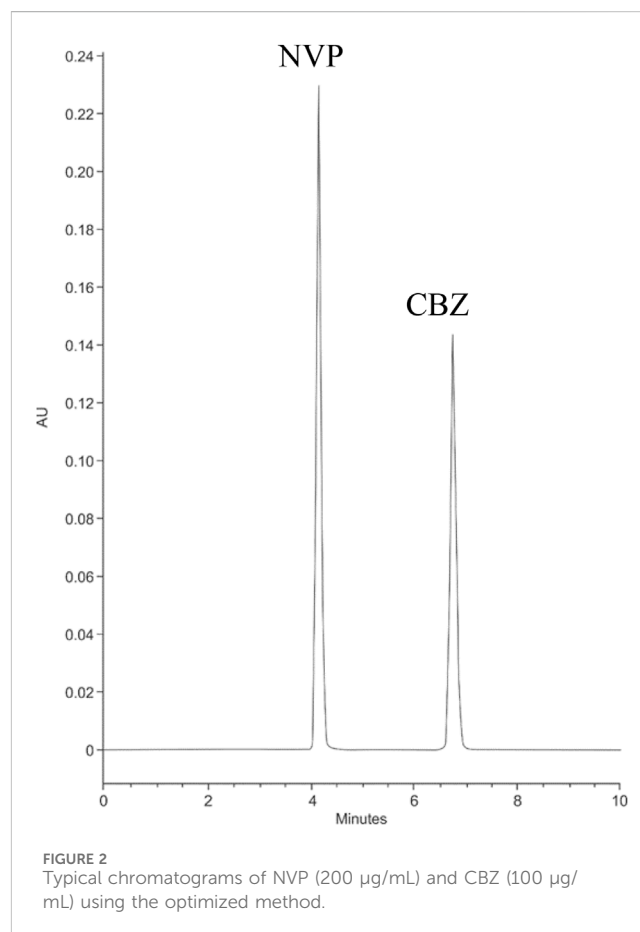


FIGURE 2
Typical chromatograms of NVP (200 µg/mL) and CBZ (100 µg/mL) using the optimized method.

TABLE 8 Results of repeatability studies.

Concentration	Average PAR	Std. Dev	% RSD
200	1.1859	0.0021	0.1750
150	0.8897	0.0014	0.1521
100	0.6274	0.0001	0.0138
20	0.1250	0.0001	0.0488
10	0.0645	0.0003	0.4612
5	0.0288	0.0000	0.0781
0.5	0.0081	0.0001	0.6459

PAR, peak area ratio; %RSD, relative standard deviation; SD, standard deviation.

Using this approach, the optimised NVP niosomes previously developed using Span-based systems achieved high %EE values (e.g., 96.8% and 98.0% for optimised Span[®] 20 and Span[®] 80 systems, respectively), confirming that the method is suitable for quantitation of NVP in niosome formulations (Witika and Walker, 2019).

In complementary excipient-screening work, %EE remained high even in the presence of cholesterol (e.g., 92.4%–94.1% at

50 μmol cholesterol, depending on surfactant), further supporting method applicability to niosomal matrices (Witika and Walker, 2021). A typical chromatogram of the quantitation of NVP from a niosome is depicted in Supplementary Figure S1.

3.1.5 Forced degradation studies

3.1.5.1 Oxidative degradation

NVP degraded by $15.0\% \pm 1.12\%$ following the reflux for 8 h in 30% v/v H_2O_2 . However, the chromatograms revealed no evidence of degradation peaks in all samples which agrees with previously reported results (Mwila, 2013).

3.1.5.2 Acidic degradation

NVP exhibited a degradation of $8.7\% \pm 0.87\%$ in 0.1 M HCl following refluxing at 90°C for 8 h, with no degradation peaks seen during the study period.

3.1.5.3 Alkali degradation

Following exposure to 0.1 M NaOH and refluxing at 90°C for 8 h, degradation by approximately $11.9\% \pm 1.07\%$ was observed with no degradation peaks observed for the duration of the study.

TABLE 9 Results of intermediate precision studies.

Day	Concentration ($\mu\text{g}/\text{mL}$)	Average PAR	SD	% RSD (across 3 days)
1	200	1.1852	0.0005	0.1755
	150	0.8912	0.0005	0.1497
	100	0.6274	0.0002	0.0092
	50	0.3020	0.0000	0.0382
	20	0.1250	0.0000	0.0462
	5	0.0288	0.0000	0.0000
	0.5	0.0081	0.0000	0.7157
2	200	1.1842	0.0008	
	150	0.8894	0.0028	
	100	0.6274	0.0002	
	50	0.3020	0.0001	
	20	0.1250	0.0001	
	5	0.0288	0.0001	
	0.5	0.0081	0.0000	
3	200	1.1882	0.0038	
	150	0.8886	0.0045	
	100	0.6275	0.0001	
	50	0.3022	0.0002	
	20	0.1251	0.0001	
	5	0.0288	0.0000	
	0.5	0.0080	0.0000	

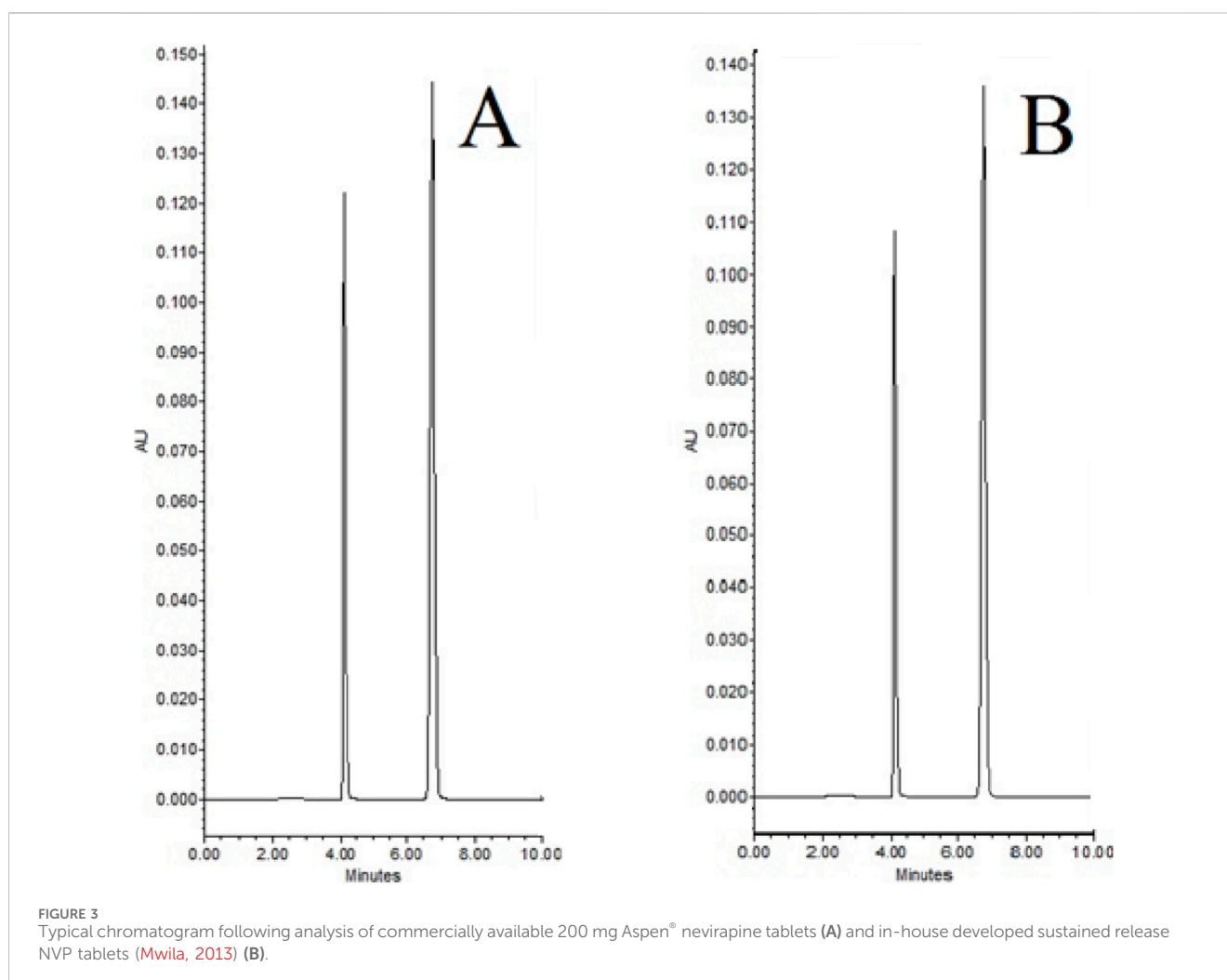
PAR, peak area ratio; %RSD, relative standard deviation; SD, standard deviation.

Overall %RSD (3 days) was calculated from the three daily mean PAR, values at each concentration level.

TABLE 10 Results of accuracy studies.

Theoretical concentration $\mu\text{g/mL}$	Actual concentration $\mu\text{g/mL}$	SD	% RSD	% Recovery	% Bias
1	1.0152	0.00035	0.80079	101.52	-1.52
75	74.323	0.00059	0.08798	99.097	-0.903
175	176.846	0.00145	0.11213	101.054	1.054

%RSD, relative standard deviation; SD, standard deviation.



3.1.5.4 Neutral hydrolytic degradation

NVP demonstrated stability when subjected to reflux under neutral conditions at an elevated temperature of 90 °C.

3.1.5.5 Photodegradation

NVP demonstrated stability after being subjected to light conditions of 500 w/m^2 at 27 °C for a duration of 8 h.

The findings indicate a reduction in the % recovery of NVP following exposure to 30% v/v H_2O_2 , 0.1 M HCl, and 0.1 M NaOH, along with refluxing at 90 °C for 8 h, signifying that deterioration had occurred. The % recovery of NVP remained mostly constant following exposure to neutral hydrolytic conditions at 90 °C and 500 w/m^2 at 27 °C for 8 h, indicating that NVP was stable under these conditions.

The outcomes of stability experiments regarding % recovery under various stress conditions at distinct time intervals are shown in Table 11.

4 Discussion

4.1 Evaluation of model accuracy for retention time of NVP

Regarding the ANOVA components (Table 4), the Residual sum of squares represents unexplained variability after fitting the model and was very small relative to the Cor Total variability, indicating that the fitted quadratic model explains the majority of the retention-time

TABLE 11 Stability of NVP following stress testing.

Time (h)	Recovery %			
	Stress conditions			
	30% v/v H ₂ O ₂	0.1 M HCL	0.1 M NaOH	Neutral hydrolytic
0	99.7	99.4	99.2	99.4
2	95.2	97.2	96.4	99.3
4	91.4	94.8	92.9	99.3
6	87.4	92.2	89.6	99.2
8	84.7	90.7	87.3	99.2

variation. The Lack of Fit term reflects any systematic deviation between the model and experimental data beyond replicate variability, whereas Pure Error represents replicate-to-replicate variability at identical factor settings. Due to the pure error being effectively zero in this dataset, due to very high repeatability at replicated points, the formal lack-of-fit partitioning becomes less informative. Overall model adequacy is therefore supported by the strong statistical diagnostics viz $R^2 = 0.9934$, $\text{Adj-}R^2 = 0.9874$, $\text{Pred-}R^2 = 0.9497$, low CV = 2.30%, and high Adeq Precision = 43.637 (Table 3).

The correlation between the independent input variables and the retention time of NVP is a function of the interaction of the stated parameters and is explained by Equation 1.

$$RT_{NVP} = 4.08 - 0.63A - 0.77B - 0.014C + 0.079AB + 0.000AC + 0.000BC + 0.099A^2 + 0.28B^2 - 0.017C^2 \quad (1)$$

The results generated review linear contributions of mobile phase organic composition and flow rate, as well as quadratic interactions thereof, had significant and antagonistic effects on the retention time of NVP as indicated by the negative sign of the model terms in the equation.

The flow rate, A, and organic solvent content, B, significantly influenced the retention time of NVP. Raising the organic solvent content leads to a decrease in retention time, as anticipated. This phenomenon can be explained by a diminished hydrophobic interaction between NVP and the stationary phase of the column (Wheeler et al., 1993; Snyder et al., 1997; Peiró-Vila et al., 2024). The increase in flow rate correspondingly decreased the retention time of NVP, a phenomenon that can be explained by the enhanced elution rate associated with a higher mobile phase flow rate.

The influence of column temperature on retention time was minimal. The equation indicates a negative sign on the parameter $-0.014C$, implying that an increase in temperature leads to decreased retention times. This phenomenon can be linked to a reduction in the viscosity of the mobile phase (Snyder et al., 1997).

The quadratic terms A^2 and B^2 indicate measurable curvature (non-linearity) in the response surface, meaning that the magnitude of the A and B effects is not strictly linear across the full investigated ranges which is expected in chromatographic DoE/retention modelling when factor ranges are wide enough for curvature to become detectable (den Uijl et al., 2021). The AB interaction term is small but non-zero,

suggesting a modest dependence of the effect of organic solvent on RT NVP on the flow rate setting (and vice versa). In contrast, AC and BC are effectively zero in this model, indicating negligible interaction contributions involving temperature within the explored range.

4.1.1 Response surface model plots for retention time of NVP

The correlation among the key factors and the retention time of NVP can be illustrated through 3-D plots, as depicted in Figure 4. An increase in the concentration of organic solvent, while maintaining a constant flow rate, led to a decrease in retention time. The retention time of NVP decreases in a nearly linear fashion as the concentration of the organic solvent increases, assuming the column temperature remains constant. The 3-D plots demonstrate that the column temperature has a negligible impact on the retention time of NVP. An increase in both organic solvent concentration and column temperature results in a reduction of retention time.

4.2 Evaluation of model accuracy for R_s

As with retention time, the near-zero pure error reflects high repeatability for replicated runs, limiting the interpretability of the lack-of-fit partitioning. The selected quadratic model is nevertheless strongly supported by its overall performance indicators: $R^2 = 0.9963$, $\text{Adj-}R^2 = 0.9930$, $\text{Pred-}R^2 = 0.9723$, CV = 5.03%, and Adeq Precision = 65.351 (Table 5), together with a highly significant model ($p < 0.0001$) (Table 6).

The fitted quadratic model describing the effect of the coded factors on the resolution response is given in Equation 2:

$$R_s = 8.9349 - 0.5969A - 6.6097B + 0.0091C + 0.2570AB - 0.0008AC + 0.0013BC - 0.1391A^2 + 1.2762B^2 - 0.1889C^2 \quad (2)$$

Within the investigated design space, resolution was primarily governed by organic solvent content (B) and flow rate (A), while column temperature (C) exerted a negligible effect. Response surface analysis (Figure 5) confirmed that increasing the organic solvent fraction reduced R_s , and that this relationship was non-linear across the studied range, consistent with the significant B^2 term. Increasing flow rate likewise decreased R_s , reflecting reduced time for

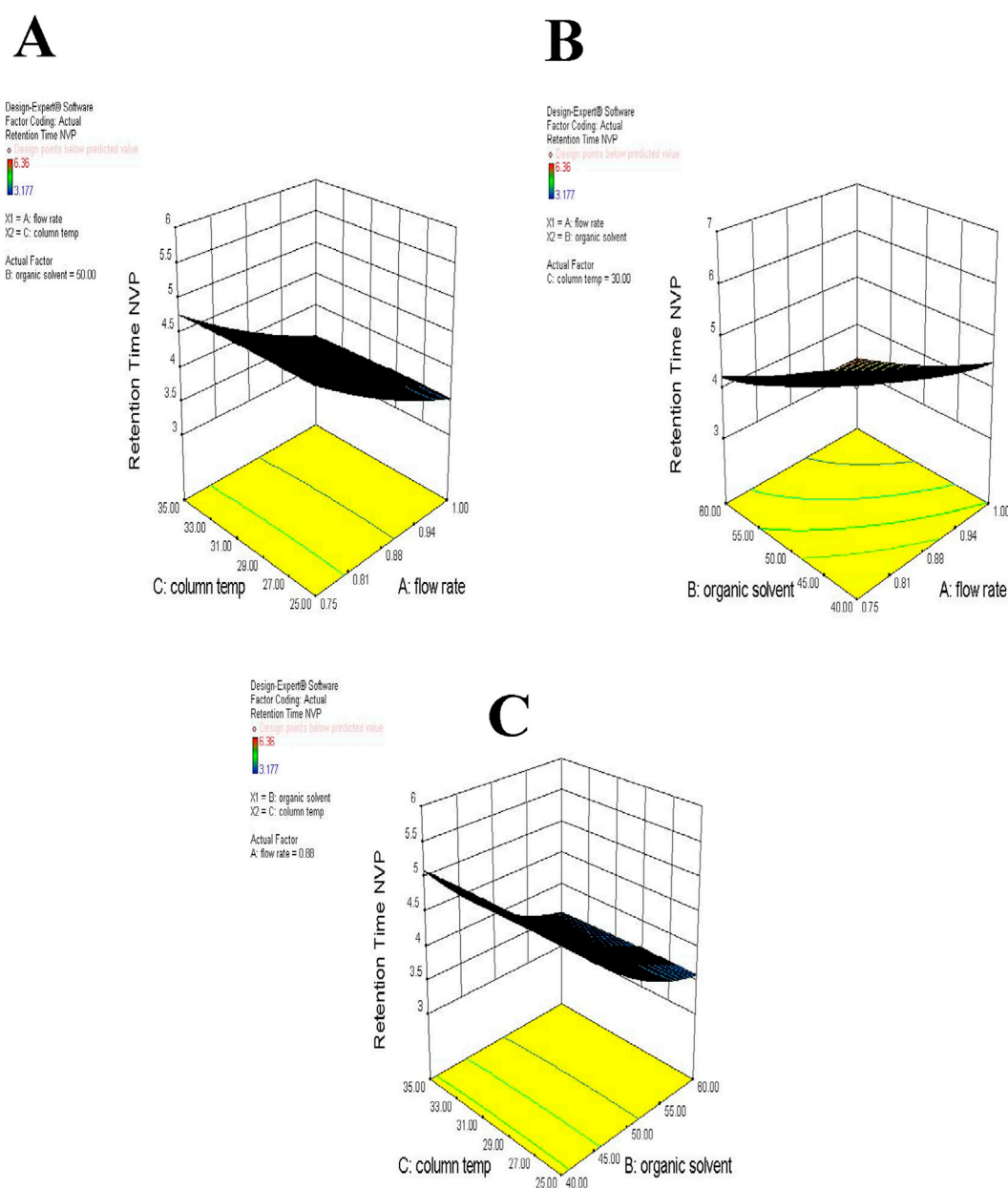


FIGURE 4 3D response surface plot depicting the impact of column temperature and flow rate (A) organic solvent content and flow rate (B) and column temperature and organic solvent content (C) on the retention time of NVP.

separation under faster elution conditions. Interactions involving temperature (AC and BC) were minimal, in agreement with their non-significant contributions in the ANOVA. Overall, the model and response surfaces highlight organic solvent composition as the main lever for maintaining adequate resolution, with flow rate acting as a secondary but significant contributor.

4.3 Comparative evaluation with published HPLC methods for nevirapine

Several RP-HPLC methods for the determination of nevirapine in bulk drug and dosage forms have previously been reported

(Table 12). The majority of reported RP-HPLC techniques for nevirapine predominantly utilise buffer-based mobile phases (phosphate or acetate) and, in certain instances, gradient elution systems. Although successful, these methods are operationally intricate: buffer preparation is laborious, may reduce column longevity due to salt precipitation, and produces excess laboratory waste. Conversely, the current technique employs a straightforward isocratic system comprising solely acetonitrile and water, so mitigating these disadvantages and improving both sustainability and cost-efficiency.

Equally significant, the majority of previously documented approaches were formulated through trial-and-error optimisation and failed to integrate systematic Design of Experiments (DoE) or

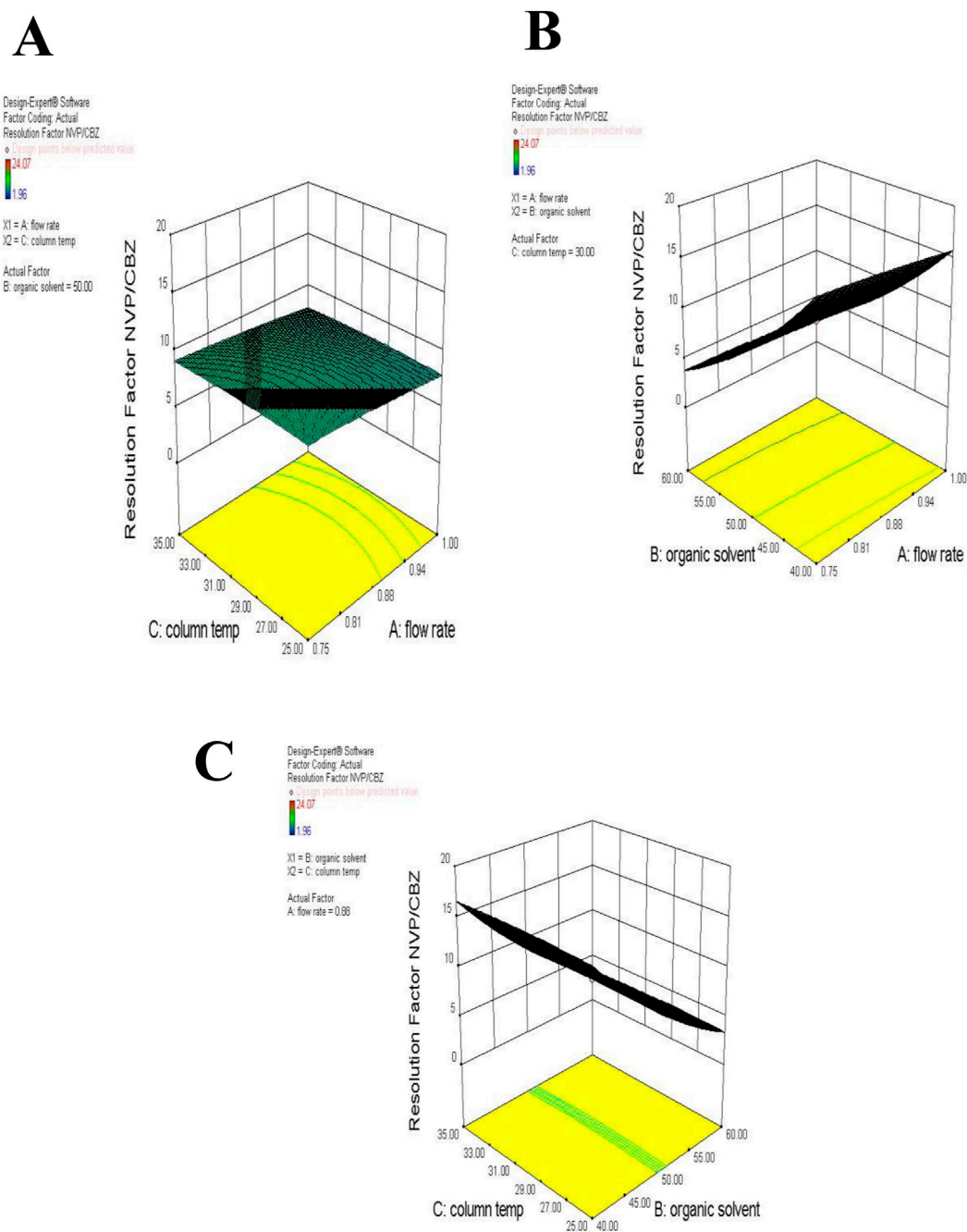


FIGURE 5 3D response surface plot depicting the impact of column temperature and flow rate (A) organic solvent content and flow rate (B) and column temperature and organic solvent content (C) on the resolution factor.

Analytical Quality by Design (AQbD) concepts. The lack of a specified design space constrains their reliability when utilised beyond restricted laboratory environments. In contrast, the current investigation utilised Central Composite Design (CCD) and Response Surface Methodology (RSM) to delineate factor interactions and guarantee consistent performance amidst minor fluctuations in flow rate, temperature, and solvent ratio.

With regard to elution efficiency, gradient methods often suffer from long run times and equilibration periods (≥ 14 min in (Kapoor et al., 2006; Samee et al., 2007)). Even isocratic buffer-based systems

typically report retention times > 7 min (Chan Li et al., 2000; Navaneethan et al., 2012). The present method achieves a shorter run time of < 8 min, allowing high sample throughput in quality control environments. Furthermore, unlike most earlier methods, an internal standard (carbamazepine) was utilized here, significantly improving reproducibility and accuracy.

Analytical performance comparisons also favour the current method. While some reports demonstrated sensitivity at low $\mu\text{g/mL}$ levels (e.g. LOQ = $0.24 \mu\text{g/mL}$ (Ravisankar and Rao, 2013)), most published methods had narrow linearity ranges (e.g. $2\text{--}10 \mu\text{g/mL}$,

TABLE 12 Summary of existing HPLC methods NVP that analyse Tablet or Bulk.

Sample	Elution mode	Mobile phase	Buffer	Internal standard	RT NVP Min	Linearity range $\mu\text{g/mL}$	Notes	Ref
Tablet/bulk	Isocratic	Methanol, acetonitrile and water in ratio of (50:30:20 v/v) with pH adjusted to 4.6 with o-phosphoric acid	Yes	-	3.660	2–10	Stability-indicating; impurities A & B resolved	Ravisankar and Rao (2013)
Combined tablet	Isocratic	20 mM sodium phosphate buffer (containing 8 mM 1-octanesulphonic acid sodium salt); acetonitrile (4:1, v/v) with pH adjusted to 3.5 using phosphoric acid	Yes	-	8.39	66.6–333.2	Complex mobile phase; long run	Anbazhagan et al. (2005)
Combined tablet	Gradient	(A) comprising of 80% of 10 mM acetate buffer adjusted to pH 3.5 with glacial acetic acid and 20% methanol and mobile phase (B) comprising of 50% acetonitrile with 50% isopropyl alcohol	Yes	-	14.254	5–100	Impurity profiling; stability-indicating	Kapoor et al. (2006)
Tablet/bulk	Isocratic	(20:80 v/v) Acetonitrile–25 mM $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 5.0)	Yes	BIRH-44 BS BIRG 616 BS	7.44	0.3–1.0	Sensitive but narrow range	Chan Li et al. (2000)
Bulk	Isocratic	0.05 M Monobasic Ammonium phosphate buffer (pH 4.5) and Acetonitrile (7:3, v/v)	Yes	-	8.77	120–360	Ion-pairing system; long run	Navaneethan et al. (2012)
Bulk form and tablets	Gradient	Acetonitrile (A) and water (B) 5%–10% A from 0 to 3 min, 10%–16% A from 3 to 6 min, 16%–25% A from 6 to 8 min, 25% A from 8 to 11 min and 25%–80% A from 11 to 13 min. For column re-equilibration, 5% A was maintained from 13 to 16 min. Different flowrates (0.1, 0.2 and 0.25 mL/min)	Yes	-	9.7	20–100	Stability-indicating (acid degradation)	Reis et al. (2016)
Combined Tablet	Gradient	20 mM sodium phosphate buffer with pH adjusted to 3.5 with phosphoric acid and methanol	Yes	-	14.8	5–30	XR tablets; stability-indicating	Samee et al. (2007)
Tablets	Isocratic	Ammonium formate buffer 5 mM, pH 5.4/acetoneitrile/methanol (60:20:20)	Yes	-	6.568	200–300	Monte Carlo simulation together with DoE to construct method operable design region (MODR).	Jesus do Nascimento Lopes et al. (2024)
Bulk, tablets, niosomes	Isocratic	ACN:H ₂ O (57.5:42.5)	No	CBZ	4.10	0.5–200	Buffer-free, AQbD-optimised, fast, robust, stability-indicating, applicable to novel formulations	

120–360 $\mu\text{g/mL}$). The present method covers a broad range of 0.5–200 $\mu\text{g/mL}$ with excellent linearity ($R^2 = 0.9994$) and relatively low LOQ (0.5 $\mu\text{g/mL}$), making it versatile for both low-dose and high-concentration analyses.

Finally, while stability-indicating capability has been reported in selected studies these were primarily impurity-focused and not combined with AQbD principles. This method provides a distinctive blend of simplicity, robustness, sensitivity, and stability-indicating performance, and is further distinguished by its applicability to both commercial dosage forms and advanced drug delivery systems (niosomes), which are infrequently discussed in prior literature. A

summary of how prior studies compare to this method is provided in Table 12.

5 Conclusion

The application of RSM for method optimization to determine the ideal chromatographic conditions for separating NVP with the IS, CBZ, facilitated the development of a method utilising a straightforward mobile phase without buffer, resulting in a more cost-effective approach compared to previously documented

methods (Pav et al., 1999; Chan Li et al., 2000; Lopez et al., 2001; Anbazhagan et al., 2005; Kapoor et al., 2006; Shah et al., 2012; Ravisankar and Rao, 2013).

The absence of interfering peaks during the analysis of commercially available Aspen[®] NVP tablets as well as the in-house developed sustained release tablets is an indication that the method is specific for the analysis of NVP analysis in these tablets and can be used for niosomal formulations. The LOQ and LOD values obtained were very low indicating the sensitivity of the method developed over the existing HPLC methods (Pav et al., 1999; Chan Li et al., 2000; Lopez et al., 2001; Anbazhagan et al., 2005; Kapoor et al., 2006; Shah et al., 2012) for the analysis of NVP. The low values for % RSD of <5.0% for the accuracy and intra and inter-day precision studies reflect the adequacy of the analytical method.

The application of the concepts of AQbD to method development and validation is efficient and has universal application for the development of simple, reproducible, selective and rapid analytical methods.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

Author contributions

BW: Visualization, Formal Analysis, Data curation, Writing – original draft, Software, Investigation. RW: Resources, Writing – original draft, Supervision.

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Glossary

A	Flow rate (coded DoE factor)	R_s	Resolution
A²	Quadratic term for factor A	RT	Retention time
AB	Interaction term between factors A and B	RSM	Response surface methodology
AC	Interaction term between factors A and C	SD	Standard deviation
ACN	Acetonitrile	SJS	Stevens-Johnson syndrome
Adj R²	Adjusted coefficient of determination	UV	Ultraviolet
AIDS	Acquired immunodeficiency syndrome	v/v	Volume/volume
ANOVA	Analysis of variance		
AQbD	Analytical Quality by Design		
B	Organic solvent content (coded DoE factor; acetonitrile fraction)		
B²	Quadratic term for factor B		
BBD	Box–Behnken design		
BC	Interaction term between factors B and C		
C	Column temperature (coded DoE factor)		
C²	Quadratic term for factor C		
C₁₈	Octadecylsilane (C ₁₈) stationary phase		
CBZ	Carbamazepine		
CCD	Central composite design		
CQA	Critical quality attribute(s)		
DCP	Dicetyl phosphate		
df	Degrees of freedom		
DoE	Design of experiments		
EE	Entrapment efficiency		
FDA	U.S. Food and Drug Administration		
HIV	Human immunodeficiency virus		
HPLC	High-performance liquid chromatography		
ICH	International Council for Harmonisation		
IS	Internal standard		
LOD	Limit of detection		
LOQ	Limit of quantitation		
MeOH	Methanol		
MODR	Method operable design region		
NNRTI	Non-nucleoside reverse transcriptase inhibitor		
NVP	Nevirapine		
OFAT	One-factor-at-a-time		
PBS	Phosphate-buffered saline		
Pred R²	Predicted coefficient of determination		
QbD	Quality by Design		
R²	Coefficient of determination		
RSD	Relative standard deviation		
%RSD	Percent relative standard deviation		
RP-HPLC	Reversed-phase high-performance liquid chromatography		