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Research Article

Development and Validation of a Novel and Simple Isocratic HPLC Method for Simultaneous Estimation of Nelfinavir and Quercetin in Patented Pharmaceutical Formulation

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ARTICLEINFO

ABSTRACT

Objective: A novel, selective, precise, and accurate reverse phase High Performance Liquid Chromatographic method for estimation of Nelfinavir (NEL) and (Quercetin) was developed and validated according to ICH guidelines

Method: HPLC method was developed using C18, (150mm × 4.6mm, 3.5µm) column with 1% Acetic acid in water: Methanol (45:55 v/v) as a mobile phase at a flow rate of 0.8mL/min and eluents were detected at 284 nm.

Results: The calibration curves were linear over the concentration range of 10 to 70 ng/mL (R^2 =0.9999) for Nelfinavir and 1 to 13 ng/mL (R^2 = 0.9996) for Quercetin. The average retention time of Nelfinavir and Quercetin was found to be 12.96 min and 13.29 min respectively. Average percentage recoveries of Nelfinavir and Quercetin were 99.99 ± 0.34 and 100.52± 0.28 %, respectively. LOD and LOQ of proposed method were found to be 0.1195 ng/ml and 0.3623 ng/ml for Nelfinavir respectively whereas for Quercetin, said values were 0.0372 ng/ml and 0.1129 ng/ml respectively. Intra- and inter-day precision values (% RSD) of proposed method were less than 2%.

Conclusion: A simple, precise, accurate, linear, and rapid RP-HPLC method was developed for simultaneous estimation of Nelfinavir and Quercetin and validated as per ICH guidelines. The results suggest that the developed method can be applicable in routine estimation of Nelfinavir and Quercetin in bulk as well as pharmaceutical formulation.

Keywords: Nelfinavir; Quercetin; Validation; RP- HPLC; Calibration curves; ICH guidelines

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1. Introduction

Nelfinavir is an antiretroviral medication with the chemical composition of (3S,4aS, 8aS)-N-tert-butyl-2-[(2R,3R)-2-hydroxy-3-[(3-hydroxy-2-methylphenyl) formamide]-4-(phenylsulfonyl) butyl] decahydroisoquinoline-3-carboxamide. [1]. Nelfinavir is classified as a protease inhibitor drug, & it effectively inhibits the protease enzymes of both HIV-1 & HIV-2 [2]. Nelfinavir has demonstrated the ability to substantially decrease viral load & elevate CD4+ T cell counts in both adults & children with HIV infection, particularly when used in combination with other anti-HIV medications, including nucleoside analogues, non-nucleoside analogues of reverse transcriptase inhibitors, or protease inhibitors [3]. Quercetin is the chemical, 2-(3,4-dihydroxy phenyl0-3,5,7-trihydroxy-4H-chromen-4-on [5]. A phytochemical Quercetin plant-derived flavonoid is obtained from onions, grapes, cherries, broccoli, & citrus fruits [6]. Flavonoids constitute a broad category of naturally occurring polyphenolic compounds with a relatively small molecular size. They are ubiquitously found throughout the plant kingdom & serve various vital roles, including acting as antioxidants & exhibiting chelating properties [4]. Quercetin is commonly employed for therapeutic purposes in the management of allergic conditions, including asthma, hay fever, as well as eczema & hives [7]. Currently, a new approach utilizing pure phytochemicals as bio-enhancers is gaining the attention of pharmaceutical companies. Quercetin was recently discovered to boost the absorption of Nelfinavir [8]. Considering the therapeutic importance & the future need of pharmaceutical industries for Nelfinavir & Quercetin combination, it is envisaged that the development & validation of RP-HPLC method for simultaneous estimation of Nelfinavir & Quercetin will be worthwhile [9].

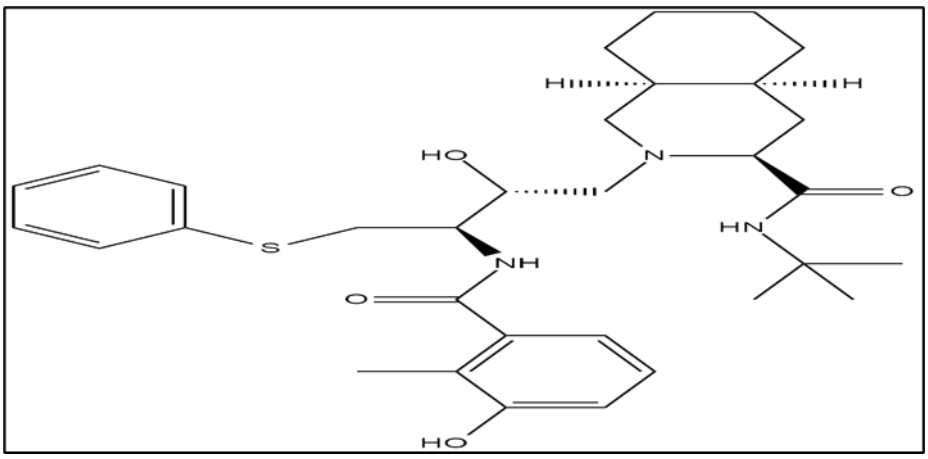

Fig.1: Chemical structure of NEL

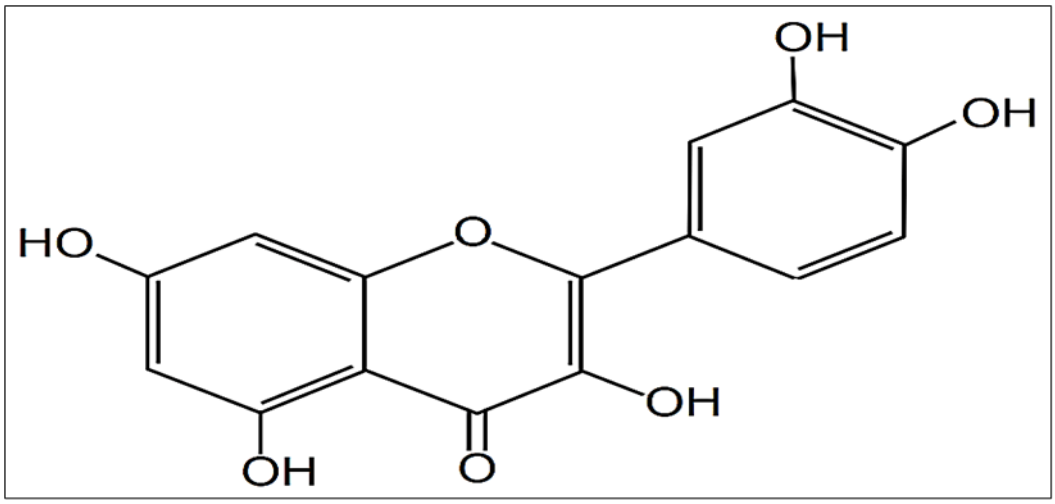

Fig.2: Chemical structure of QUE

2. Materials and Methods

Chemicals and Reagent

NEL and QUE was obtained from TCI chemicals (India) Pvt. Ltd. All the chemicals and reagent used were of at least analytical grade. HPLC grade methanol and water were used for the proposed study.

Instruments

Chromatographic analysis was performed using an Agilent HPLC system that consisted of a G1311C quaternary HPLC pump (Agilent Technologies, Palo Alto, CA), G1329B autosampler system (Agilent Technologies) and G1315F variable wavelength detector (Agilent Technologies). HPLC grade water was obtained from "Extrapure" water purification system (Lablink). Mobile phase was degassed by using Ultrasonicator (PCi Analyticals). For weighing purpose, Vibra HT(Essae) analytical balance was used.

Optimization of RP-HPLC Method

Chromatographic conditions were optimized by injecting standard solution (10 ng/mL NEL and 2 ng/mL QUE) into HPLC system and allowed to run in different mobile phases so as obtain optimum conditions for separation of both drugs.

Preparation of Mobile Phase

1 % Acetic acid in water was prepared by dissolving 10 mL of Acetic acid in 1000ml of HPLC grade water. It was filtered through 0.22µm filter and degassed by ultrasonication for 10 min. HPLC grade methanol was used in combination with 1% acetic acid as a mobile phase.

Preparation of standard stock solution

Stock solutions (1 mg/mL) of NEL (Stock I) and QUE (Stock II) were separately prepared in HPLC grade methanol andfiltered through 0.45-m nylon membrane syringe filter. The solution of QUE was protected from light using aluminum foil.

Preparation of standard calibration curve

Stock I & II were diluted suitably with methanol and mixed together to achieve 7 calibration standards (CAL STD) containing NEL and QUE in combination: CAL STD-1: NEL 10 ng/mL + QUE 1 ng/mL; CAL STD-2: NEL 20 ng/mL + QUE 3 ng/mL; CAL STD-3: NEL 30ng/mL + QUE 5 ng/mL; CAL STD-4: NEL 40 ng/mL + QUE 7 ng/mL; CAL STD-5: NEL 50ng/mL + QUE 9 ng/mL; CAL STD-6: NEL 60 ng/mL + QUE 11

ng/mL; CAL STD-7: NEL 70 ng/mL + QUE 13 ng/mL. All the solutions were injected into HPLC column and the peak area of each solution was measured. The standard calibration curves of peak area Vs concentration (ng) were plotted.

Method Validation

Developed method was validated as per ICH guidelines. Various analytical method validation parameters viz. system suitability, linearity, range, LOD, LOQ, accuracy, precision and stability were assessed [8,10].

System Suitability

Before performing the main analysis, the system suitability test was carried out using freshly prepared standard working solution consisting of 10 ng/mL of NEL and 2 ng/mL of QUE. During the test, five replicates of above-mentioned solution were analyzed for retention time, peak area, and the theoretical plates. Qualification parameters for the system suitability tests were less than 2% relative standard deviation (RSD) for retention time and peak area and more than 1500 theoretical plates for both NEL as well as QUE. The resolution (acceptance criteria > 3) was calculated using the following formula.

$R = 1.18 [(t2-t1)/(W2+W1)]$

Where $t1$ and $W1$ are retention time and peak width at half height of NEL

$t2$and $W2$ are retention time and peak width at half height of QUE.

Validation Parameter

Linearity

Linearity of the proposed method was calculated by using seven different CAL STDs. After analyzing CAL STDs, calibration curves representing concentration vs. peak area were plotted and linear regression analysis was performed.

Accuracy (% Recovery)

To ensure the accuracy of method, recovery studies were performed by standard addition method using 80%, 100% and 120% levels of drug concentrations. Percent recovery was calculated from the amount found and the actual amount added.

Precision

Precision of proposed method was evaluated at three different levels i.e., LQC (NEL 10 ng/mL + QUE 2 ng/mL), MQC (NEL 35 ng/mL + QUE 5 ng/mL) and HQC (NEL 60 ng/mL + QUE 10 ng/mL). Intra-day and Inter-day precision was determined by analyzing the solutions at different time intervals on the same day and on three consecutive days.

LOD and LOQ

ASTM LOD and LOQ were calculated by analyzing the CAL STD-1. Chromatogram of CAL STD-1 was processed using HPLC software settings "Annotations" and obtained values were reported as LOD and LOQ for NEL and QUE.

Stability

The stability of NEL and QUE solutions (Standard as well as formulation) was determined by keeping MQC and the formulation in tightly closed volumetric flasks at room temperature for 48 hrs. The solutions were analyzed at 12 hr intervals. The % assay and the RSD values were reported.

Estimation of NEL and QUE in Patented Pharmaceutical formulation

To achieve the improvised oral bioavailability of Nelfinavir, a pharmaceutical formulation comprising Nelfinavir and Quercetin was developed by the Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. The details of composition of the formulation are as follows

Nelfinavir was dissolved in dimethyl sulfoxide (Solution-A). Accurately weighed polyethylene glycol 4000 was dissolved in water and propylene glycol was added into it (Solution-B). Solution-A was added into solution-B and mixed well using vortex mixer to obtain an oral solution as comparative example in table 1. Similarly, Nelfinavir was dissolved in dimethyl sulfoxide (Solution-C). Accurately weighed polyethylene glycol 4000 was dissolved in water. Quercetin and propylene glycol was added into water containing polyethylene glycol (Solution-D). Solution-C was added into solution-D and mixed well using vortex mixer to obtain oral compositions.

One ml of pharmaceutical formulation was diluted suitably with methanol (final concentration: NEL-50 µg/mL and QUE-10 µg/mL) and filtered through 0.45 µm syringe filter. Predefined volume of solution was analyzed using pre-optimized HPLC conditions (n = 3). Contents of pharmaceutical formulation were calculated by comparing mean peak area of sample with that of the standard.

3. Results and Discussion

Optimization of RP-HPLC Method

Resolution was considered to be the most important criteria for the method and was imperative to achieve good resolution among the both compounds. Based on pKa and solubility of both the compounds, various compositions of mobile phase were tried and best resolution was obtained with mobile phase consisting of 1% acetic acid in water and methanol in the ratio of 45:55v/v. better resolution of the peaks with clear base line was found. Detection was carried out at 284 nm. Optimized chromatographic conditions are given in Table 1. Under these conditions retention time for NEL and QUE were 12.96 min and 13.29 min, respectively (Fig. 3).

Table 1. The optimized chromatographic conditions

Separation variable	**Optimized conditions**
Chromatography	Agilent HPLC system
Column	C18, (150mm × 4.6mm, 3.5µm)
Mobile phase	1% Acetic acid in water: methanol (45:55 v/v)
Flow rate	0.8 mL/min
Temperature	Ambient
Detection wavelength	284nm
Retention time (NEL)	12.96 min
Retention time (QUE)	13.29 min

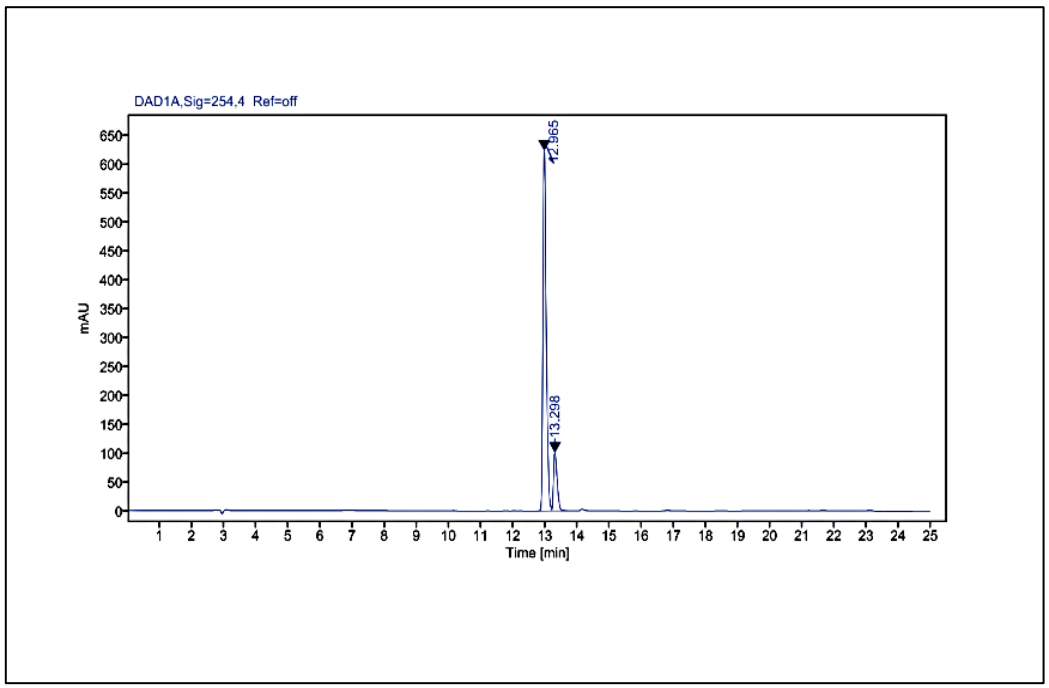

Fig. 3: A typical RPLC chromatogram of NEL and QUE

System suitability

During system suitability test, RSD of all parameter were calculated to evaluate the suitability of the developed method. From the results, it was found that %RSD for retention time and peak area was less than 2 and the number of theoretical plates were more than 2000 (Table 2).

Table No. 2: System suitability parameters for NEL and QUE

Sr.No.	Parameter	Acceptance criteria	NEL		QUE	
			Observed Value	%RSD	Observed Value	%RSD
1	Retention Time	%RSD ≤ 2%	12.96	0.31	13.29	0.91
2	Area	%RSD ≤ 2%	4369.53	0.51	794.24	0.33
3	Theoretical plates	≥ 2000	5585	0.46	6139	0.29

Adequate resolution of 13.12 was obtained between NEL and QUE using developed HPLC method.

Method validation

Linearity and Range

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven-point calibration curve of NEL (10-70 ng/mL) and QUE (2-13 ng/ mL) were constructed. Different concentrations and peak area values are depicted in Table 3. Calibration curve when subjected to least square regression analysis yielded an equation; y = 438.05x + 73.143 for NEL and y = 393.54x+ 38.96

for QUE with correlation coefficient 0.9999 and 0.9996 respectively (Fig. 4 and 5). From the linearity study, it was revealed that, there is a linear relationship between response and amount of drug within the range 10-70 ng/mL for NEL and 2-13 ng/mL for QUE.

Table No. 3: Linearity of NEL and QUE

Sr.No.	NEL		QUE	
	Conc. (ng/mL)	Peak Area	Conc. (ng/mL)	Peak Area
1	10	4269	1	397
2	20	8729	3	1291
3	30	13018	5	1985
4	40	17578	7	2779
5	50	21746	9	3574
6	60	26217	11	4368
7	70	30586	13	5162
8	**Slope**	**73.143**	**Slope**	**38.96**
9	**y-intercept**	**438.05**	**y-intercept**	**393.54**
10	**R^2**	**0.9999**	**R^2**	**0.9996**

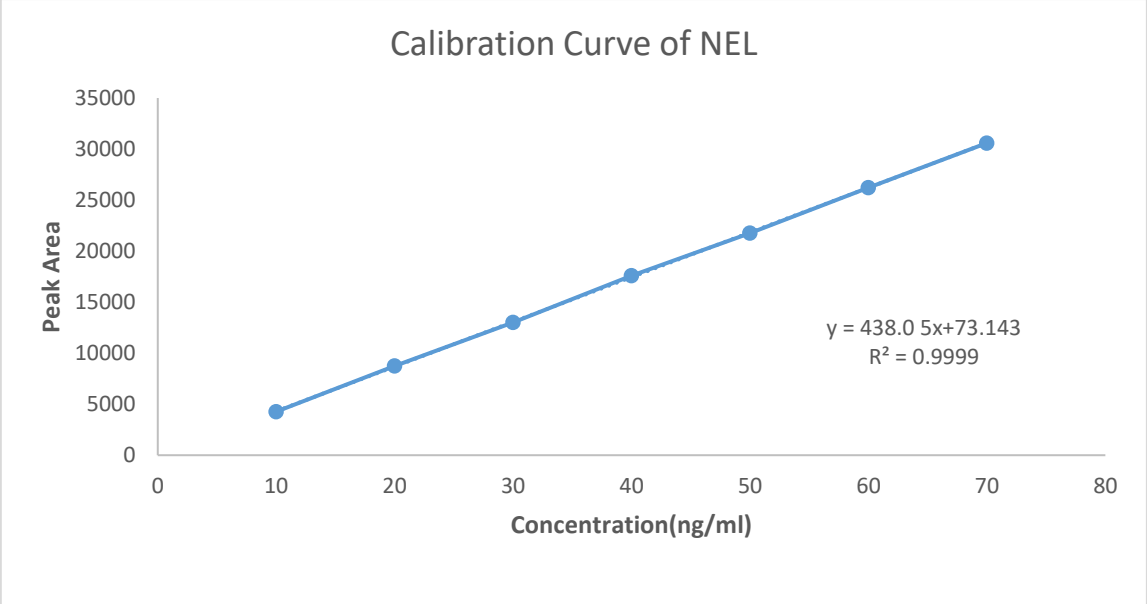

Fig. 4: Calibration curve of NEL

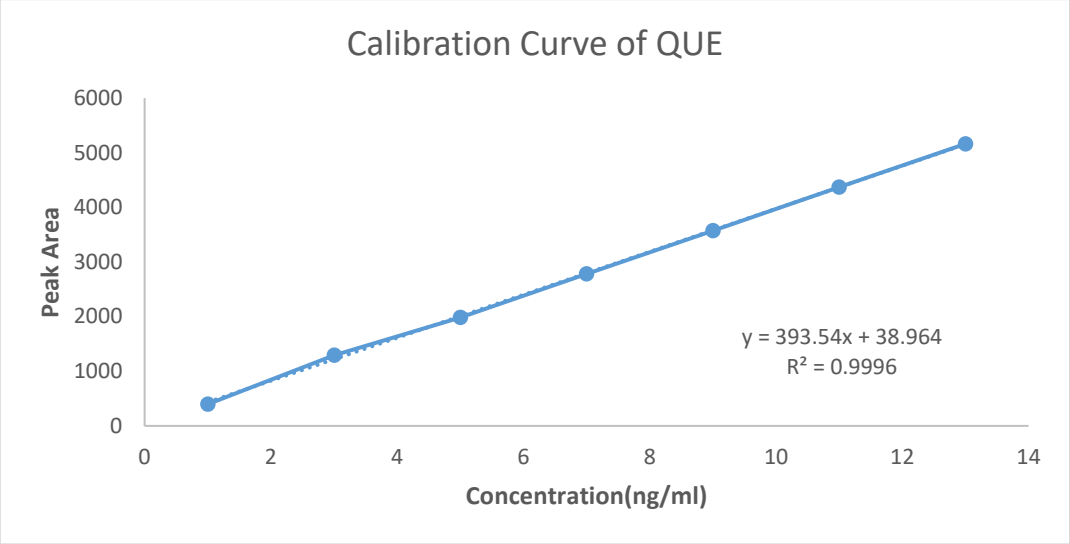

Fig. 5: Calibration curve of QUE

Accuracy (% Recovery)

Accuracy is the closeness of test results to the true value obtained by proposed method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For NEL and QUE, accuracy was determined using recovery studies. At 80, 100 and 120 % standard addition, mean recovery of NEL and QUE was found to be 99.99 and 100.52 % respectively. The relative standard deviation (% RSD) was found to be less than 2 (Table 4). From the results of accuracy studies, it was concluded that the analytical technique showed good accuracy

Table No. 4: Recovery studies of NEL and QUE

Sr. No.	Sample	Spiked level	Amount present (ng/mL)	Amount recovered (ng/mL)	% Recovery	Mean % Recovery	% RSD
1	NEL	80%	10	9.98	99.86	99.99	0.70
		100%	35	35.07	100.02		
		120%	60	59.95	99.93		
2	QUE	80%	2	2.03	101.57	100.52	0.49
		100%	5	5.01	100.29		
		120%	10	9.97	99.71		

Precision

Precision was studied by analysis LQC, MQC and HQC STDs containing both the drugs at concentrations covering the entire calibration range**.** The results expressed in terms of % RSD for the intra- and inter-day precision study (Table 4 and 5). Percent RSD values of intra-day precision study were found to be 0.87 and 0.69 for NEL and QUE respectively, whereas inter-day precision was 0.64

and 0.55 respectively. It was concluded that the analytical technique showed good repeatabilit

Table No 5: Intra-day precision data for NEL and QUE

Sr. No.	NEL				QUE			
	Amount present (ng/mL)	Amount recovered (ng/mL)	% Assay	% RSD	Amount present (ng/mL)	Amount recovered (ng/mL)	% Assay	% RSD
1	10	9.88	98.80	1.44	2	1.96	98.00	0.89
2	35	35.54	101.54	0.59	5	5.07	101.14	0.75
3	60	59.21	98.68	0.58	10	10.04	100.04	0.44

Table No 6: Inter-day precision data for NEL and QUE

Sr. No.	NEL				QUE			
	Amount present (ng/mL)	Amount recovered (ng/mL)	% Assay	% RSD	Amount present (ng/mL)	Amount recovered (ng/mL)	% Assay	% RSD
1	10	9.94	99.40	1.02	2	1.97	98.50	0.85
2	35	35.29	100.82	0.43	5	5.03	100.6	0.61
3	60	59.61	99.35	0.48	10	10.02	100.2	0.21

LOD and LOQ

Limit of detection LOD (signal-to- noise ratio of 3) and limit of quantification LOQ (signal-to- noise ratio of 10) were measured based on the signal-to noise ratio. The LOD and LOQ values for NEL were 0.1195 and 0.3623 ng/mL, respectively and these values for QUE were 0.0372 and 0.1126 ng/mL.

Stability

The stability of NEL and QUE in the prepared sample was determined by analyzing LQC (NEL 10 ng/mL + QUE 2 ng/mL) at 1, 12, 24 and 48 h (Table No. 7). During stability testing, no significant change was observed in the content of NEL and QUE. Percent RSD was less than 2% indicating a good stability of the sample. Hence it was concluded that NEL and QUE solutions are stable for 48 h

Table No. 7: Stability study of NEL and QUE

Sr.No.	Time (h)	NEL			QUE		
		Amount recovered	% Assay	% RSD	Amount recovered	% Assay	% RSD

		(ng/mL)			(ng/mL)		
1	1	9.98	99.86	0.2596	2.02	101.00	0.5678
2	12	9.81	98.13	1.4589	1.98	99.20	0.2679
3	24	10.00	100.06	0.3597	9.94	99.46	0.5891
4	48	10.08	100.80	1.0597	2.01	100.93	1.5698

Estimation of NEL and QUE in Pharmaceutical formulation

Proposed validated analytical method was successfully applied to the determination of NEL and QUE in pharmaceutical formulation. The results of the assay (n = 5) yielded 100.29 % for NEL and 99.89% QUE. The observed concentration of NEL was found to be 50.14 ± 0.076 ng/mL (mean ± SD) these values for the QUE was 9.98 ± 0.016 ng/mL (Table no.9). The results of the assay indicate that the method is selective for the analysis of NEL and QUE without interference of the excipients.

Table no. 9: Analysis of pharmaceutical formulation

Sr.No.	Amount present (ng/mL)		Amount recovered (ng/mL)	
	NEL	QUE	NEL	QUE
1	50	10	50.07	9.97
2	50	10	50.22	10.0
3	50	10	50.14	9.98
4	**Average**		**50.14**	**9.98**
5	**± S.D.**		**0.076**	**0.016**
6	**% Assay**		**100.29**	**99.89**

4. Conclusion

An accurate, precise, simple yet sensitive RP-HPLC method was developed and validated for the simultaneous determination of NEL and QUE. Further, it was found that developed method could be used for the routine analysis of pharmaceutical composition containing NEL and QUE.

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Declaration of Competing Interest

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

Authorship contribution statement

Sachin Bhusari: Supervision, Validation, Methodology, Writing – original draft, **Akshay Thorat:** Conceptualization, Investigation **Pravin Wakte**: Administration, Funding, Data Curation.

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