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Original Article

Integration of Green Chemistry Approach for Development of Box-Behnken Design Assisted RP-HPLC Method for Estimation of Nepafenac in Ophthalmic Dosage Form: A QbD Supported Degradation Approach

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ABSTRACT

Background: NSAIDs, or Non-Steroidal Anti-Inflammatory medicines, are a large family of chemically diverse medications with a wide range of effects. Nepafenac is an anti-inflammatory medication that is used topically as an aqueous solution. **Objectives:** To develop a simple, dependable, and eco-friendly Reverse-Phase High-Performance Liquid Chromatographic (RP-HPLC) approach for the quantitative measurement of nepafenac in ophthalmic solutions using a Quality by Design (QbD) methodology. **Materials and Methods:** A Box-Behnken experimental design was used to refine the process and identify the variables influencing chromatographic performance. Chromatographic separation was achieved on a Shimadzu C18 column (250 x 4.6 mm i.d., 5 µm) using a mobile phase of methanol:0.1% orthophosphoric acid in water (75:25 v/v), adjusted to pH 3.0, at a flow rate of 1.1 mL/min and detection at 236 nm. **Results:** Nepafenac was eluted with a retention time of 4.018 min and showed great accuracy, with recovery ranging from 98.2% to 101.9%. The test for the commercial ophthalmic formulation produced a 96.16% result. Studies on forced deterioration in acidic, alkaline, oxidative, thermal, and photolytic environments validated the method's stability-indicating properties. Furthermore, the eco-friendliness of the created method was confirmed by evaluating its environmental sustainability using AGREE and the GAPI greenness assessment method. **Conclusion:** The appropriateness of the suggested method for regular quality control analysis of Nepafenac in ocular dosage forms was demonstrated through its validation in compliance with International Council for Harmonisation (ICH Q2(R2)) criteria.

Keywords: NSAID, Nepafenac, RP-HPLC, Box-Behnken design, Green Analytical chemistry, Force degradation study.

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INTRODUCTION

NSAIDs, or non-steroidal anti-inflammatory medicines, are a large family of chemically diverse medications with a wide range of effects (Ashour *et al.*, 2024). Nepafenac (Figure 1) is an anti-inflammatory medication that is used topically as an aqueous solution. During cataract surgery, it lessens discomfort, manages inflammation, and enhances patient comfort (Zokande *et al.*, 2025). Nepafenac is a prodrug of Amfenac. It is used in an ocular solution formulation to alleviate pain and inflammation (Padhye *et al.*, 2022). In chemical terms, nepafenac is a [2-(2-amino-3-benzoylphenyl)acetamide] (Gupta *et al.*, 2024). The US Food and Drug Administration (FDA) has authorised nepafenac ophthalmic suspension, 0.1%, a novel topical NSAID prodrug, to relieve pain and inflammation following cataract surgery (Lindstrom *et al.*, 2006). The production of certain proinflammatory mediators, such as prostaglandins, which have been connected to the breakdown of the blood-aqueous barrier and the rise in vascular permeability alterations linked to inflammation and edema, is prevented by the suppression of COX enzyme activity (Akbas *et al.*, 2025).

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A pharmaceutical quality management approach called Quality by Design (QbD) anticipates, manages, and methodically addresses potential hazards during the research and production phases. In order to ensure product quality, Quality by Design (QbD) urges the pharmaceutical sector to apply science-based manufacturing concepts and risk management to obtain process and product expertise (Lee *et al.*, 2022). Analytical evaluation is a crucial method for pharmaceutical product quality control. Inaccurate readings and data that might be dangerous for the medicine and product development stage could result from a faulty analytical assessment. Several pharmaceutical medication regulatory organisations, including the "US Food and Drug Administration" and the " International Council for Harmonisation" (ICH), have established Quality by Design (QbD) techniques and guidelines to address these important challenges and concerns (Suryawanshi *et al.*, 2022). The Food and Drug Administration (FDA) and the International Conference on Harmonisation (ICH) are actively promoting QbD in an effort to reduce growing development costs and regulatory obstacles to innovation and inventiveness (Mishra *et al.*, 2018). Understanding dependent variables, different factors, and their interaction effects through a desired set of experiments on the answers to be studied is a crucial part of the QbD (Patel *et al.*, 2021). The Quality by Design (QbD) idea states that during pharmaceutical or analytical development, quality should be included in the product or process. The quality of processes and products can typically be affected by a variety of input elements. In order to comprehend the impact of multidimensional input factors and their interactions on the output responses of pharmaceutical goods and analytical techniques, Design of Experiments (DoE) has become a popular tool in recent years (Fukuda *et al.*, 2018).

The goal of Green Analytical Chemistry (GAC) is to lessen the negative impacts that analytical operations have on the environment, human health, and safety (Yin *et al.*, 2024). Green chemistry gave rise to Green Analytical Chemistry (GAC) in 2000. Chemists are quite interested in this relatively new branch of green chemistry, which focuses on how analytical chemists may help make laboratory procedures more ecologically friendly (Gałuszka *et al.*, 2013). Analytical chemistry plays two separate and conflicting roles in green chemistry. It protects the environment by assessing the effects of chemical activities, but it also has the potential to exacerbate environmental issues because of the large amounts of hazardous materials used or produced during an analytical process and the high energy consumption. By addressing worker safety, energy efficiency, hazardous laboratory waste production, and solvent/reagent safety, GAC sought to redefine and review analytical processes (López-Lorente *et al.*, 2022).

Literature review revealed that there are very few chromatographic techniques for nepafenac estimation. Out-of-Trend (OOT) or Out-of-Specification (OOS) results are a common consequence of reported methods' reliance on expensive or dangerous solvents, complex processes, complex solvent systems, lack of robustness, frequent failures and long retention time. The Quality by Design (QbD) methodology has not been used in the development of nepafenac analytical procedures till date; many do not satisfy current regulatory requirements, and the main objectives of the proposed research work is to develop and optimise a simple, easy, robust, accurate RP-HPLC method by the QbD approach.

MATERIALS AND METHODS

Materials

Chemicals: Nepafenac gift sample was obtained from Deccan Nutraceuticals Pvt. Ltd., Pune, HPLC-grade methanol, 0.1N HCl, 0.1N NaOH, and 3% hydrogen peroxide were obtained from the KLE College of Pharmacy store, Belagavi; orthophosphoric acid and milli-Q water were provided by Dr. Prabhakar Kore Basic Science Research Center, Belagavi, and every chemical employed in this investigation was of analytical grade.

Instrument

In the proposed research work HPLC used was Shimadzu Sil-20ACHT, and the Shimadzu Lab Solutions software was used. And Milli-Q water collected from Thermo Scientific Smart2Pure Pro, the powdered sample of Nepafenac and the standard were weighed using a Wensar AN ISO 9001 weighing balance. In order to Brason 1800 ultrasonic cleaner sonicator was used to both degas the solvent system and dissolve the drug, and to dissolve the ophthalmic drug in the solvent.

Chromatographic conditions

The chromatographic separation of Nepafenac was achieved on a Shimadzu C18 column (250 X 4.6 mm i.d, 5 µm). A solvent system composed of methanol: 0.1% orthophosphoric acid in water proportion of 75:25v/v (pH 3.0), confirmed by QbD trial runs. The retention time of Nepafenac was obtained at 4.018 min with UV identification at 236 nm. The temperature of the column is 34.5°C with a flow rate of 1.1 mL/min. The injection volume was 10 µg/mL. For the mobile phase, 0.4 mL of orthophosphoric acid in 400 mL milli-Q water was used. It is sonicated, filtered through a 0.45 µm membrane filter (Nylon), and degassed before preparation of the solvent composition.

Preliminary analysis of drug

The colour of Nepafenac compared with the reported characteristics mentioned in the COA, and Nepafenac is fully soluble in methanol and partially soluble in water.

Preparation of solvent system

0.4 mL of orthophosphoric acid was measured and transferred to 400 mL of Milli-Q water, and it was sonicated for 10 min, and after filtering through a 0.45 µm membrane filter, degassing

was carried out, and the mobile phase used was methanol:0.1% orthophosphoric acid in water with the ratio 75:25 (v/v).

Preparation of standard stock solution

The Nepafenac stock solution was made by dissolving 10mg of Nepafenac in 10 mL of methanol, and this stock was sonicated to solubilise the Nepafenac. From this stock, 1 mL of solution was pipette out and transferred into a 10 mL volumetric flask, and it was diluted using methanol. This stock was used for method development and validation parameters. Additionally, the mentioned secondary dilution and the use of methanol as a diluent yield additional workable solutions.

Preparation of sample solution

Accurately pipette out 1 mL from 0.1% ophthalmic suspension into a 10 mL volumetric flask, and volume was made up using methanol. After 5 min to solubilise it, from this 1 mL solution was pipette out and transferred to a 10 mL volumetric flask to get a 10 µg/mL solution, and volume was made up using methanol.

Selection of wavelength

From the standard stock solution, solutions were taken, and methanol was used for dilution and scanned over a range from 800-200 nm in UV spectrophotometry, and it was observed that the drug showed absorbance at 236 nm.

Preliminary method development

In order to select the proper stationary phase, preliminary experimental runs were carried out to find possible combinations and select the column and solvent system. For the initial trials, a review of the literature was done. Methanol was chosen as a cost-effective solution. Additionally, an aqueous solvent with varying orthophosphoric acid concentrations was chosen. Additional experimental research was conducted using the C18 stationary phase.

Method optimisation based on QbD principles

QbD is described as "a systematic approach to development that starts with predetermined objectives and emphasises product and process understanding based on sound science and quality risk management." The QbD approach includes developing tests and controls based on the scientific limits of understanding during the development stage, utilising the knowledge and data generated during the product's life cycle to work on continuous improvement, and improving scientific understanding of critical parameters and qualities of the product. We have worked on several QbD components and applied them to the practical methods in this inquiry. It began with the determination of the Analytical Target Profile (ATP), then used the fishbone approach to analyse the Critical Quality Attributes (CQAs) and Risk Assessment (RA). The implementation of DOE, which aids in factor screening and RP-HPLC process optimisation, is the primary focus of the further approach (Suryawanshi *et al.*, 2022).

Analytical target profile

The first and most crucial stage in developing a QbD-based analytical technique is identifying and setting up the ATP, which is defined as gathering the data needed to create the desired quality profile. This aids in guaranteeing the analytical process's quality, safety, and practical use at the location of its commercial application in the pharmaceutical sector. The "target drug, target samples and method application, analytical method, instrument characteristics and requirements, sample preparation steps, and analytical procedure quality attributes" are some of the components that make up ATP (Park *et al.*, 2022).

Critical Quality Attributes (CQAs)

The selection of different CQAs is a crucial and crucial phase in the creation of a QbD-based method in order to satisfy the requirements of the target profile of the analytical procedure. "The measurable parameters of the chromatogram of analyte that should be within an appropriate and acceptable limit to warrant the desired quality and ability of the analytical procedure" is how the CQAs are defined.

The Tailing Factor (TF), Retention Time (RT), Peak Area (PA), Theoretical Plates (TP), assay limit, etc., are typically considered key quality attributes in chromatographic development. RT, TP, and TF are the characteristics that actually affect how well an analytical process works (Suryawanshi *et al.*, 2022).

Risk Assessment

Risk assessment is also a crucial element in the development of QbD-based procedures. Risk assessment was used in this investigation to assess the likelihood of procedure failures. An "Ishikawa Fish-Bone" (IFB) diagram was created to carry out a cause-and-effect model for the relationship of "critical material attributes" and "critical method parameters" with the Critical Analytical characteristics (CAAs) (Gaudin *et al.*, 2016).

Factor Screening by Experiment Avenue Design with Box-Behnken Design (BBD)

Design Expert software was used to optimise a new chromatographic process for Nepafenac. This software used the Box-Behnken design to optimise the "Critical Process Parameters" (CPPs) or "Critical Method Parameters" (CMPs) and evaluate how these parameters interacted with the CQAs. BBD requires fewer experimental runs because it is a three-factor, two-level design.

Box-Behnken Study Design for Optimisation

Three factors and two levels of BBD were used in the chromatographic optimisation of parameters to assess the

primary impacts and quadratic effects of crucial factors on the variables of identified responses. The following procedures are part of the screening phase of the BBD, which consists of 17 experiment runs. The flow rate, organic phase (% methanol), and detection wavelength were all chosen during the initial step of CMP selection. The selection of CQAs is part of the study's second phase. The CQAs used for this investigation are RT, PA, TP, and TF.

Throughout the practical experiments, these reactions were seen (Suryawanshi *et al.*, 2022).

Development of Methods Based on Experimental Design

As per the factor screening method, the selection of the CMPs which are affecting the performance of the procedure was optimised employing 3 factors at two equidistant levels (low (-1) and high (+1) levels). A 10 µg/mL of Nepafenac was used for all chromatographic runs, and analysed for CQAs (RT, PA, TP, and TF) (Beg *et al.*, 2012).

Data Analysis and validation of methods

Completing the proposed trial, the responses were added to the software, and other graphs, such as "graph plots" and "3D response surface plots" were attained. These charts indicate how CMPs affect the chosen CQAs. The evaluation of these plots was used to determine which parameter produced the most logical answers. These data were used to determine the procedure's final CMPs and choose the ideal conditions. For each answer, a statistical instrument such as "Analysis of Variance" (ANOVA) was employed to determine he significance of each research parameter using the *p*-value.

Method validation

Method validation was done in accordance with ICH principles. The quality assessment of Nepafenac in its marketed formulation was then successfully conducted using the suggested RP-HPLC approach.

System suitability

"A crucial component of any analytical approach is system and testing appropriateness". replicates of a 10 µg/mL standard Nepafenac solution were injected, and each chromatogram was then assessed based on its RT, TP, and TF (Shivabasappa *et al.*, 2019).

Specificity and selectivity

The HPLC peak of the Nepafenac solution (10 µg/mL) and blank chromatogram of the solvent system were obtained in order to determine the procedure's specificity and selectivity. It aids in determining the peak purity, analyte, and interference from other peaks (Bhanusali *et al.*, 2022).

Linearity and range

The procedure linearity was evaluated between 2 and 10 µg/mL of Nepafenac, by preparing various concentrations, the linearity response was determined and injecting sample was injected three times into HPLC, recording chromatograms and noting the peak area of each graph. The peak area of the standard calibration curve was plotted against the Nepafenac concentration in µg/mL (Shivabasappa *et al.*, 2019).

Detection and Quantification Limit

The lowest concentration of analyte in a sample that can be identified but may not always be quantified as an exact number is known as the detection limit of a particular analytical technique. The lowest amount of analyte in a sample that can be quantitatively identified with appropriate precision and accuracy is known as the quantitation limit of a particular analytical process". A parameter of quantitative assays for low concentrations of chemicals in sample matrices, the quantitation limit is especially useful for identifying contaminants and/or degradation products (Sanjay *et al.*, 2019).

$$LOD = 3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

where "σ" is the standard deviation of the regression line's Y-intercept and "S" is the slope of the calibration curve.

Precision

The Relative Standard Deviation (RSD) is used to estimate the precision. Three replicate injections at concentrations of 2,6,10 µg/mL were used to estimate the precision. Three replicates of Nepafenac concentrations were injected for the intraday and interday precision investigation, and the percentage RSD was calculated (It was evaluated on three separate days in addition to the same day) (Shivabasappa *et al.*, 2019).

Robustness

The method's robustness was assessed by analysing distinct wavelengths (i.e., 236±0.2) and mobile phase (75:25±0.5). The corresponding absorbances were recorded, and the percentage RSD was used to show the outcome (Shetti *et al.*, 2021).

Ruggedness

The method's ruggedness was assessed by having two distinct analysts perform the analysis and record the corresponding absorbances. % RSD was used to represent the outcome (Shetti *et al.*, 2022).

Accuracy

The method's accuracy was assessed by creating solutions with varying concentrations (80%, 100%, 120% respectively) and

the amount of pure drug varied (8 µg/mL, 10 µg/mL, 12 µg/mL for 80%, 100%, 120% respectively) and amount of marketed formulation remain constant (10 µg/mL). The accuracy was shown by the percentage of recovery when solutions were produced in triplicate (Akula *et al.*, 2021).

Assay

To perform the analysis of the pharmaceutical dosage form containing Nepafenac, a suspension comprising an equivalent amount of 10 mg of the drug was transferred to a 10 mL volumetric flask. After adding methanol to achieve the necessary volume, the mixture was sonicated for three minutes. Next, 1 mL of this solution was measured, transferred to a 10 mL volumetric flask, and the volume was adjusted using methanol. Similarly, 1 mL of the produced solution was measured, transferred to a 10 mL volumetric flask, and the volume was adjusted using methanol. For the subsequent chromatographic analysis, 10 µL of the solution was injected into the system, and the assay's percentage was calculated (Patel *et al.*, 2024).

Forced degradation study

For the forced degradation investigations of Nepafenac, a number of degradation conditions were employed, including hydrolysis, oxidation, photolysis (UV light), and thermal degradation (Patel *et al.*, 2024). 10 mL volumetric flasks were filled with precisely weighed 10 mg of Nepafenac and made up to volume using methanol. From the above solution, 1 mL was measured and transferred to a 10 mL volumetric flask (100 µg/mL), which was used as a stock solution to investigate the effects of forced deterioration.

Acid degradation

A 10 mL volumetric flask was filled with 1 mL of a 100 µg/mL Nepafenac standard stock solution, followed by 1 mL of 0.1N HCl. This flask was left at room temperature for 3 hr. The mixture was then neutralised, and the process was stopped by adding 1 mL of newly made 0.1N NaOH. A liquid chromatography apparatus was used to analyse the resultant solution after it had been diluted with mobile phase up to 10 mL.

Base degradation

Similarly, a 10 mL volumetric flask was filled with 1 mL of a 100 µg/mL Nepafenac standard stock solution to conduct the base degradation experiment. 1 mL of 0.1N NaOH was then added. To allow for base degradation, the flask was left at room temperature for 3 hr. 1 mL of 0.1N HCl was injected to finish the neutralisation process after the allotted time. A liquid chromatographic apparatus was used to analyse the base-degraded sample after it had been diluted with mobile phase to a final volume of 10 mL.

Oxidation degradation

A 10 mL volumetric flask was filled with 1 mL of a 100 µg/mL Nepafenac standard stock solution to conduct the oxidation degradation experiment. 1 mL of H_2O_2 was added, the flask was left for 2 hr at room temperature, and the solution was injected into a liquid chromatographic system after being diluted to 10 mL with the mobile phase.

Photodegradation

A sunlight source was used to expose 10 mg of Nepafenac powder in a petri dish for 4 hr in order to measure photolytic degradation. For the tests that followed, the resultant solution, which had a concentration of 10 µg/mL, was used. The solution was put into a liquid chromatography apparatus for examination following photodegradation.

Thermal degradation

For thermal degradation, 10 mg of Nepafenac powder was weighed, put in a petri dish, and heated to 70°C for 3 hr in a hot air oven. A 10 µg/mL concentration was prepared for the tests using the resultant solution, which was subjected to thermal stress for the designated amount of time. The liquid chromatography apparatus was then filled with the thermal degradation solution (Patel *et al.*, 2024).

Greenness assessment

Since several factors need to be taken into account, determining the best method for evaluating an analytical procedure's green nature is difficult. One, it is widely acknowledged that in order to declare a method or product to be sustainable, concrete evidence of actual environmental consequences must be provided. Here,

Table 1: QTPP, CQAs and CMPs of proposed RP-HPLC Method.

Sl. No.	QbD Element	Description
1.	Quality Target Product Profile (QTPP)	Flow rate, mobile phase composition, detection. Wavelength, pH, column temperature, injection. Volume, system pressure and buffer characteristics.
2.	Critical Quality Attributes (CQAs)	Retention time, peak area, and theoretical plate. Count, purity and tailing factor
3	Critical Method Parameters (CMPs)	Specificity, selectivity, linearity, precision. Accuracy, robustness, ruggedness, Limit of Detection (LOD) and limit of quantification.

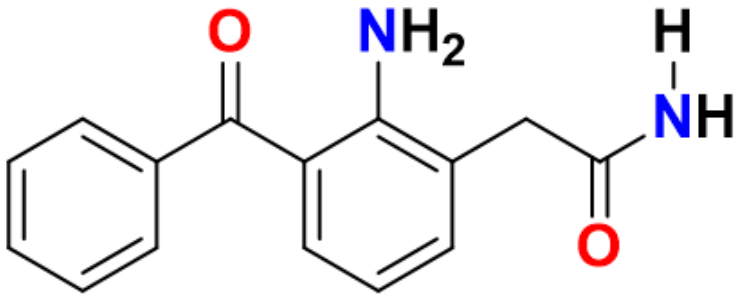

Figure 1: Chemical structure of Nepafenac.

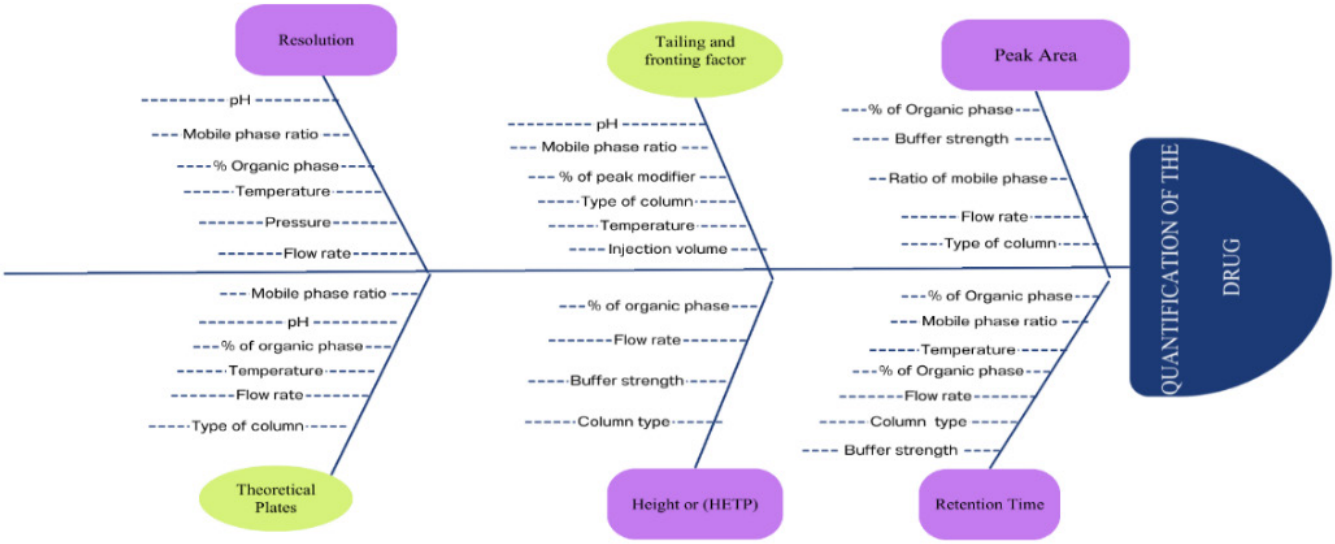

Figure 2: Ishikawa Fishbone diagram.

the created instruments for evaluating how environmentally friendly the specified analytical processes are come to the rescue (Płotka-Wasylka *et al.*, 2021).

AGREE® Tool

AGREE is a straightforward and all-inclusive instrument that evaluates the environmental friendliness of analytical tests and yields answers that are clear and easy to understand. The 12 GAC principles are converted into a 0-1 scale in order to calculate the AGREE metric. Assessing the solvents' toxicity, the energy used, and the trash generated during the testing generates a net score. Based on energy consumption, all other activities receive good marks and while waste of any form receives a bad score (Rabadiya *et al.*, 2025).

Green Analytical Procedure Index (GAPI)

The five pentagrams of the GAPI pictogram can be used to assess and disclose the greenness of various stages of the full analytical test. The GAPI pictogram uses a colour scale with three levels (green, yellow, and red hues imply low, medium, and high impact of the analytical process on the environment and human health, respectively) to show the greenness of various stages of the overall analytical method (Yin *et al.*, 2024).

RESULTS AND DISCUSSION

Preliminary analysis of the drug

Nepafenac has a yellowish colour, a solid state. A solubility test revealed that nepafenac is only marginally soluble in water and totally soluble in methanol.

Method development

Preliminary experiments based on literature search and trial and error were conducted in order to optimise a straightforward, economical, precise, and accurate approach for assessment and quality control of the Nepafenac in its bulk as well as pharmaceutical ophthalmic dosage form. Initially, other combinations of solvents, including methanol and o-phosphoric acid, were used to determine the mobile phase. To improve the Nepafenac separation, the buffer and methanol were tested in various concentrations. The choice of orthophosphoric acid at 0.1% concentration, shown in the first runs to result in quicker and more efficient drug separation with elution of Nepafenac at lower RT, along with low peak tailing and satisfactory peak symmetry.

Analytical Quality by design approach and its application

The proposed RP-HPLC method, which is included in the methods section, was subjected to a number of QbD techniques and approaches. Table 1 presents the ATP, CQAs, and process parameters of the method that were examined, determined, and identified.

Risk Assessment Screening Studies

Risk assessment research was conducted to examine the CMPs, which are high-risk variables that significantly impact the CAAs. The IFB diagram (Figure 2) was created for this investigation in order to identify significant risk variables that might affect the method's effectiveness. The elements that increase risk mentioned may include instrument-related factors like injection volume, flow rate, chromatographic mode, Temperature, and solvent system ratio. By using an appropriate experimental optimisation strategy, the high-risk procedure variables were identified and subjected to further investigation. The mobile phase, flow rate, the percentage of organic phase (methanol) in the mobile phase, and the column temperature were chosen as the high-risk technique factors.

Design of Box-Behnken Optimisation

In order to identify the primary, interactional, and quadratic impacts of these parameters on RT, PA, TP, and TF, BBD optimised them. After the 17 runs were completed, software was used to statistically evaluate the findings. RT, PA, TP, and TF were chosen as the dependent responses, whereas flow rate of mobile phase, percentage of organic phase in mobile phase, and column temperature were chosen as the independent factors. Table 2 displays the BBD optimisation, along Table 3 shows the confirmation location after 17 runs. The program performed statistical optimisation and Analysis of Variance (ANOVA). Table 4 displays the results of the ANOVA. This suggests the validity of the model.

The "3D-surface plots were also examined to establish design space and show how independent parameters affected the

Table 2: Box-Behnken Design for Optimization.

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Std	Run	A: OP	B: FR	C: TP	RT	area	TF	NTP
		%	mL/min	°C				
15	1	75	1.1	34.5	4.018	550204	1.233	2515
11	2	75	0.9	37	4.852	668538	1.235	4097
17	3	75	1.1	34.5	4.018	550204	1.239	3177
10	4	75	1.3	32	3.454	469855	1.224	3099
6	5	80	1.1	32	3.862	649644	1.241	5061
14	6	75	1.1	34.5	4.015	550723	1.233	3035
8	7	80	1.1	37	4.213	673015	1.231	7046
1	8	70	0.9	34.5	5.375	659026	1.248	8922
7	9	70	1.1	37	4.236	545782	1.249	7810
4	10	80	1.3	34.5	3.468	707887	1.225	4047
13	11	75	1.1	34.5	4.011	550850	1.233	3925
5	12	70	1.1	32	3.657	543109	1.27	5935
9	13	75	0.9	32	4.804	679894	1.355	7992
3	14	70	1.3	34.5	4.138	466541	1.213	2144
2	15	80	0.9	34.5	4.287	709165	1.247	8231
16	16	75	1.1	34.5	4.013	554844	1.215	2106
12	17	75	1.3	37	3.502	468723	1.215	4068

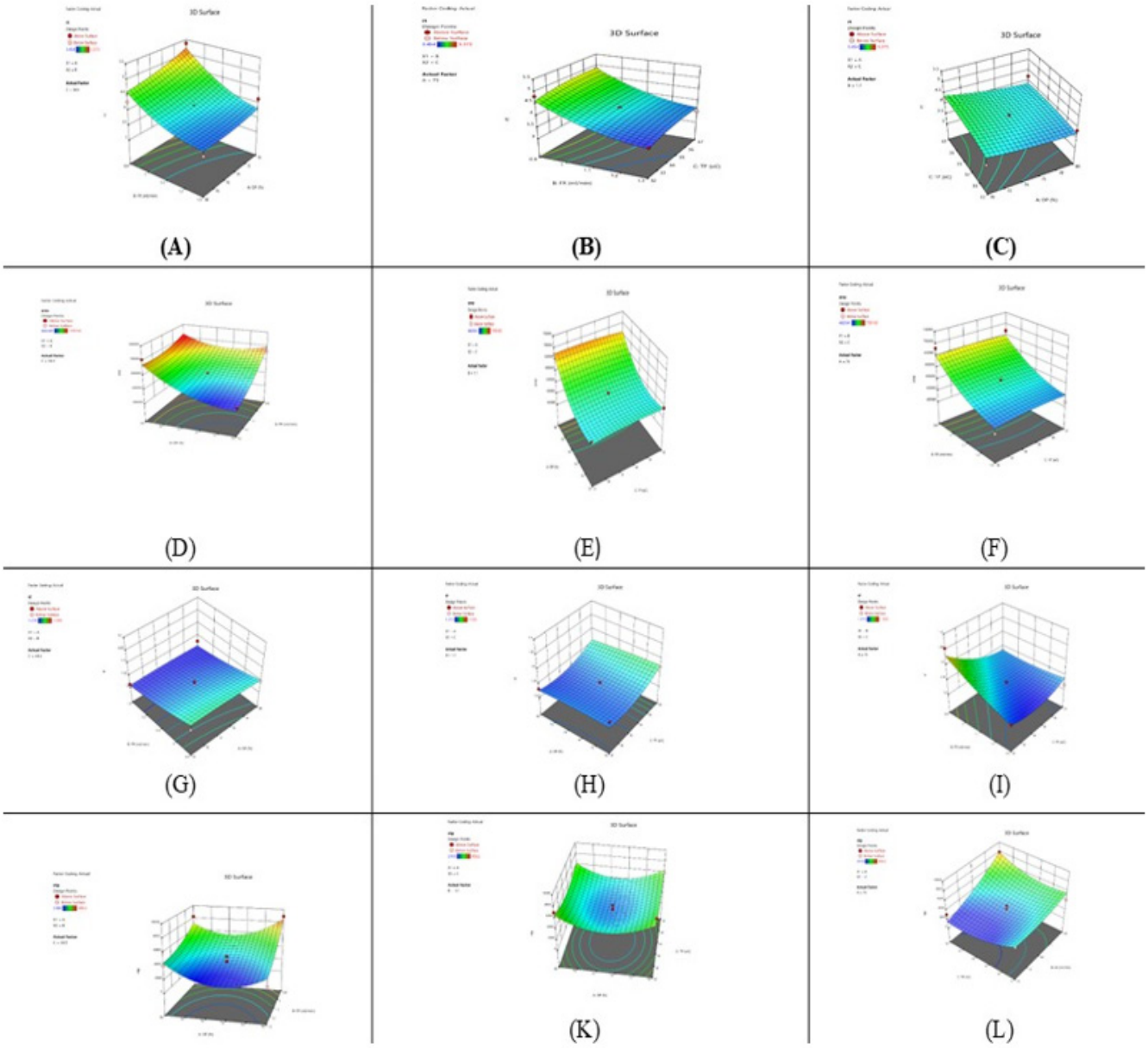

Figure 3: shows how the flow rate and % of organic phase (A), Flow rate and temperature (B), and % of organic phase and temperature (C), Impact the retention time in 3D surface plots. Peak area is also influenced by the % of organic phase and flow rate (D), the % of organic phase and temperature (E), and the flow rate and temperature (F) Additionally, the tailing factor is affected by 3D surface plots showing the % of organic phase and flow rate (G), the % of organic phase and temperature (H), and the flow rate and temperature (I), also % of organic phase and flow rate (J), % of organic phase and temperature (K), Flow rate and temperature (L), Affect the theoretical plates.

corresponding response variables. One element was maintained constant for every plot because the suggested model had more than two independent factors. The 3D surface plot map in Figure 3 illustrates how flow rate, organic phase composition percentage, and wavelength affect RT, PA, TP, and TF.

Method development

Figure 4 displayed the typical Nepafenac HPLC chromatogram with an RT of 4.018 min.

Method validation

System testing parameters, including RT, TP, and TF, were assessed, and %RSD was calculated before the analysis.

System suitability

After injecting 6 replicates of a standard Nepafenac solution containing 10 µg/mL, each chromatogram was evaluated using its Area. Table 5 shows system suitability data and % RSD.

Specificity and selectivity

The specificity and selectivity of the procedure were assessed by obtaining the HPLC peak of the Nepafenac solution (10 µg/mL) and the blank chromatogram of the solvent system. Figures 5 and 6 show the blank and standard peak.

Linearity and Range

By producing different concentrations of Nepafenac, the linearity response was ascertained, and the procedure's linearity was assessed between 2 and 10 µg/mL. Figure 7 displays a plot of the peak area of the standard calibration curve against the concentration of Nepafenac in µg/mL, and R^2 was found to be 0.998.

Table 3: Confirmation location after 17 runs.

A: OP	B:FR	C:TP
%	mL/min	°C
75	1.1	34.5

Detection and Quantification Limit

The LOD and LOQ values were obtained as 0.284 and 1.93, respectively, by using these formulas:

$$\text{LOQ is } 10 \times \sigma/S \text{ and LOD is } 3 \times \sigma/S$$

Precision

For intraday and interday precision, three replicates of nepafenac concentrations were injected, and % RSD was falls within acceptable range, and the precision data is displayed in Table 6.

Robustness

The robustness parameter was determined by doing the study at various wavelengths (i.e., 236±0.2) and Mobile phase (75:25 ±5) and the % RSD was falls within acceptable range. Robustness data is displayed in Table 7.

Table 4: Results from statistical analysis

ANOVA Parameter	RT	Peak Area	TP	TF
R-Square	0.8554	0.9431	0.8438	0.7658
Adjusted R-square	0.6696	0.87	0.643	0.4739
Predicted R-square	-1.3127	0.0922	-1.1733	-2.6687
Standard Deviation	0.295	30365.36	1375.43	0.0203
% C.V	7.17	5.16	28.1	1.63
F-value	4.6	12.9	4.2	4.14
P-value	0.0283	0.0283	0.0358	0.0289

R-squared = Determination Coefficient.F-value = Value on the F Distribution.*p*-value = Probability of falsely detecting a significant effect.C.V.% = Percent Coefficients of variance.

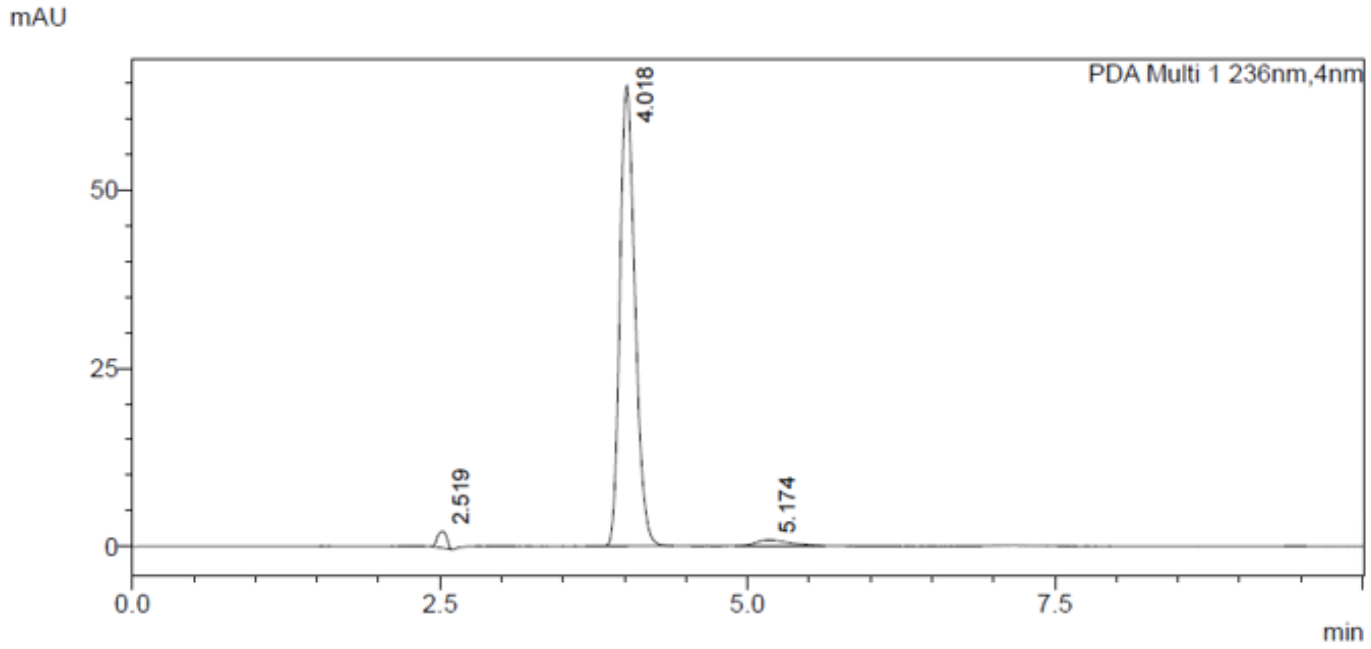

Figure 4: Chromatogram depicting the method development peak.

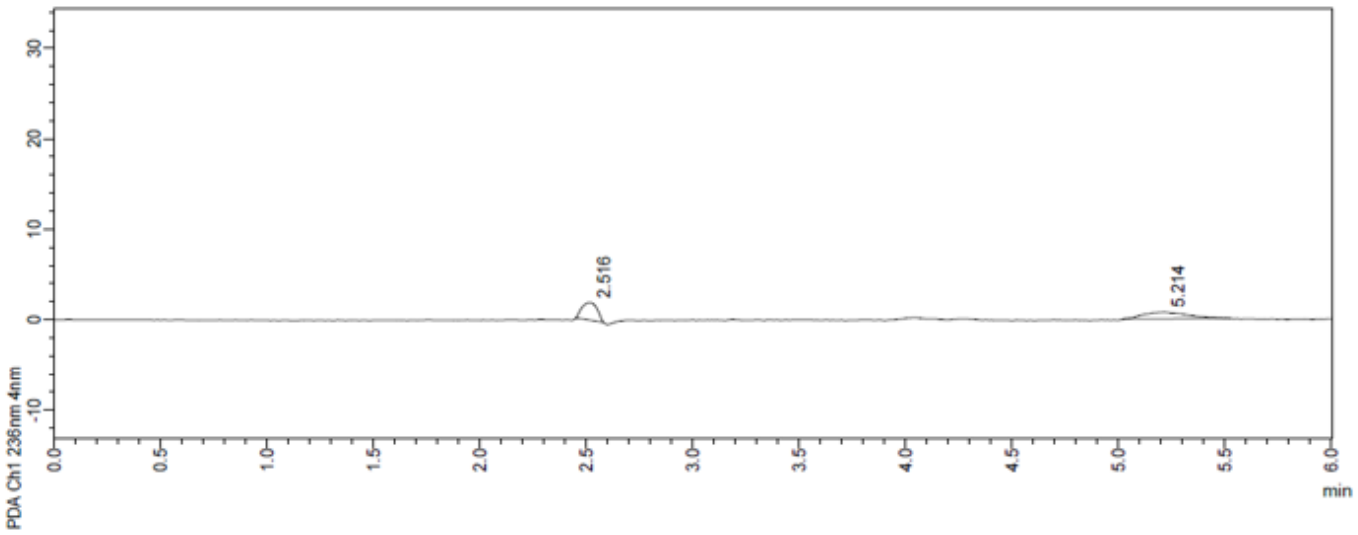

Figure 5: Chromatogram displays the blank.

Table 5: System suitability study data.

Concentration 10 µg/mL	Retention Time	Peak Area	Tailing Factor	Theoretical Plates
Mean	4.039	478715	1.234	4471
SD	0.004	7277.33	0.006	15.95
%RSD	0.112	1.520	0.545	0.356

Table 6: Intraday and interday precision data assay of Nepafenac.

Precision	Intraday		Interday1		Interday2		Interday3	
Conc.	Peak area	% RSD	Peak area	RSD	Peak area	RSD	Peak area	RSD
2 µg/mL	108790	0.109	108388	0.157	108801	0.484	109366	0.153
6 µg/mL	312425	0.862	311124	0.306	319206	0.164	315957	0.991
10 µg/mL	474133	0.184	474470	0.306	472705	0.317	475817	0.526

Ruggedness

The ruggedness parameter was determined by doing study by two analysts, and % RSD was falls within acceptable range. Ruggedness data is displayed in Table 8.

Accuracy

At 80%, 100%, and 120% of the sample concentration, accuracy was evaluated using the conventional spike technique, and % RSD falls within acceptable range. Table 9 displays the outcomes of preparing and injecting each level in triplicate.

Assay

A suspension containing an equal quantity of 10 mg of drug was determined. A standard stock solution containing 1000 µg/mL of Nepafenac was prepared this stock solution was used to make various concentrations of solution, and peak areas were recorded for the Nepafenac estimation. and % assay was found to be 96.16% i.e., within the acceptable range, as displayed in Table 10. The nepafenac chromatogram at retention time 3.99 is displayed in Figure 8.

Degradation study

Heat, UV radiation, hydrogen peroxide, bases, and acids were among the stress conditions to which nepafenac was vulnerable; oxidative and acidic environments showed the most deterioration. Nepafenac broke down and changed into degradation products due to oxidation, acid hydrolysis, and base hydrolysis. Additionally, increased oxidative and base degradation took place. Table 11 presents study results on force deterioration.

Greenness assessment

AGREE® Tool

according to the Figure 9 pictogram, the overall score in the center is 0.62, which is in the light green area and acceptable environmental performance of the evaluated method. The colour and score of each numbered segment represent the procedure's

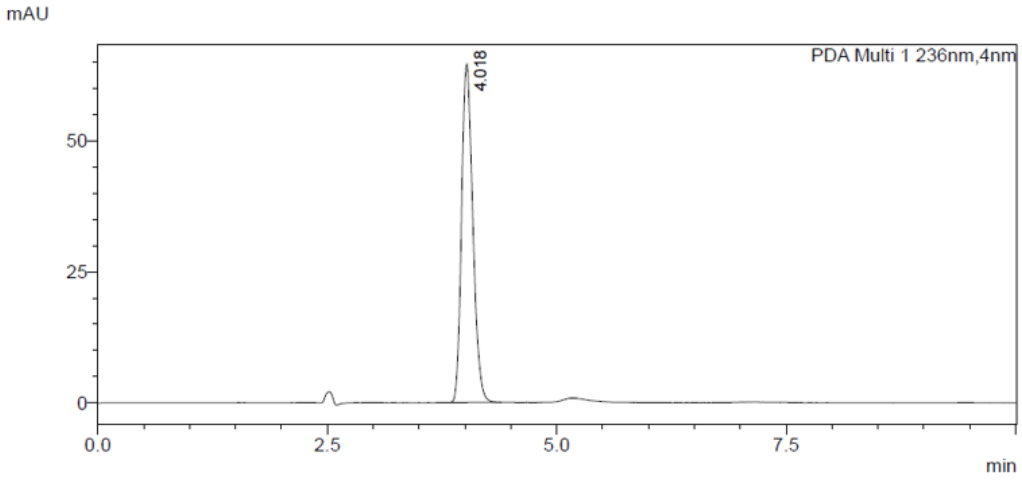

Figure 6: Chromatogram displays a standard peak.

Table 7: Robustness data of Nepafenac obtained by RP-HPLC.

Robustness												
Peak Area												
	Change in wavelength						Change in Mobile phase					
Conc.	234 nm	%RSD	236 nm	%RSD	238 nm	%RSD	70:30	%RSD	75:25	%RSD	80:20	%RSD
2 µg/mL	108955	0.692	108934	0.727	105921	0.337	1000894	0.534	108720	0.149	119246	0.315
6 µg/ mL	310501	0.115	308007	0.114	301071	0.335	306234	0.171	310814	0.125	326625	0.313
10 µg/mL	477751	0.326	474289	0.247	463629	0.295	488142	0.331	473620	0.602	487890	0.521

Table 8: Ruggedness data of Nepafenac obtained by RP-HPLC.

Ruggedness				
Change in Analyst				
Conc.	Analyst 1	%RSD	Analyst 2	%RSD
2 µg/mL	108944	0.687	109164	0.499
6 µg/mL	315994	0.946	311102	0.903
10 µg/mL	476163	0.585	472703	0.642

Table 9: Accuracy study data of Nepafenac obtained by RP-HPLC

Level	Sample conc. (µg/mL)	Std. conc. (µg/mL)	Total conc. (µg/mL)	Mean of peak area	Amount Recovery	Mean %	%RSD
80%	10 µg/mL	8 µg/mL	18 µg/mL	842924	17.68	98.20%	0.082
100%	10 µg/mL	10 µg/mL	20 µg/mL	950430	19.94	99.70%	0.158
120%	10 µg/mL	12 µg/mL	22 µg/mL	1069448	22.42	101.90%	0.049

Table 10: Assay data of Nepafenac.

Drug	Conc.	An area found	% RSD	% Assay
Nepafenac	10 µg/mL	4574 459874	0.22 0.22	96. 96.16%

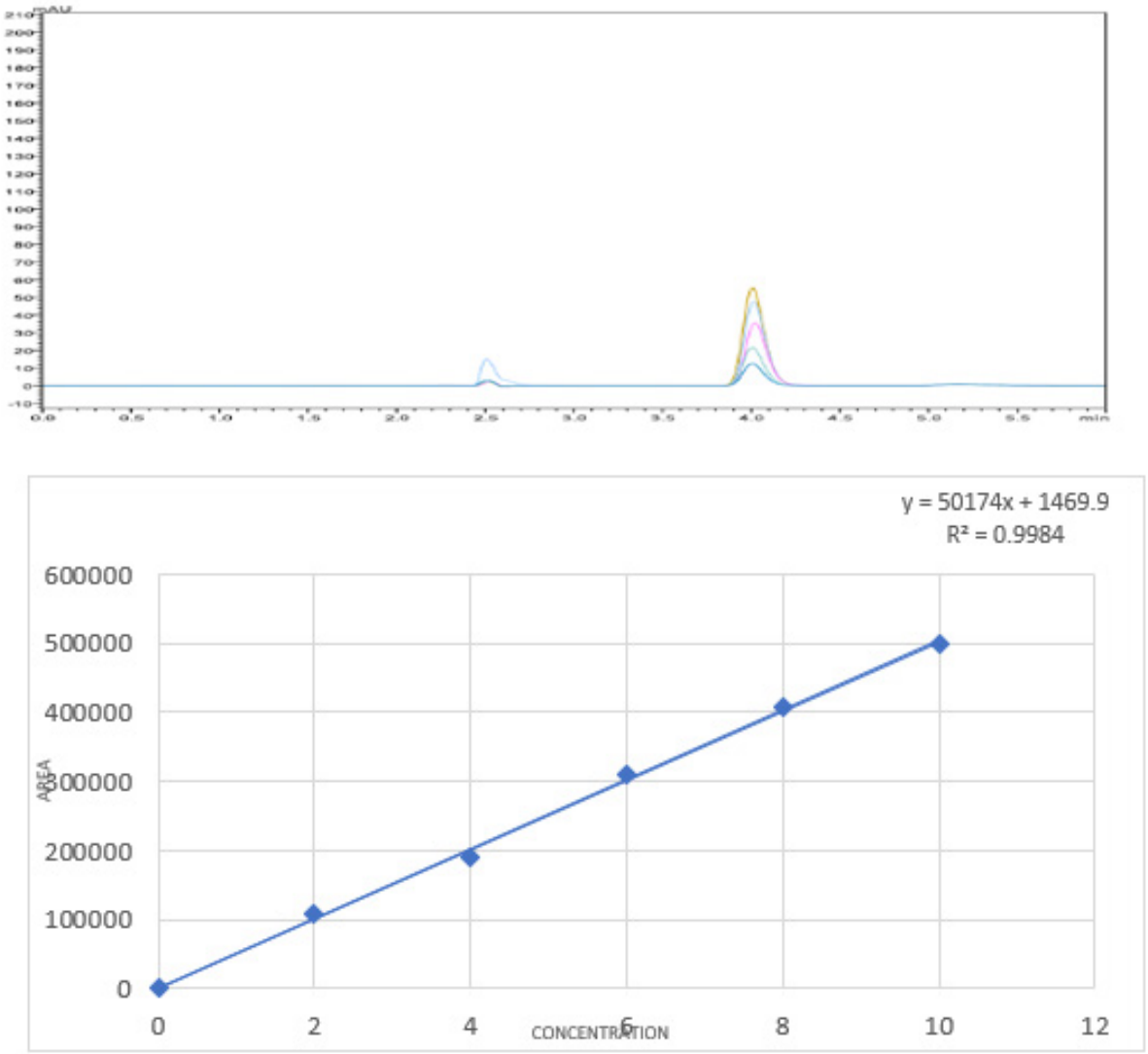

Figure 7: Displays overlay peaks and Calibration Curve of Nepafenac.

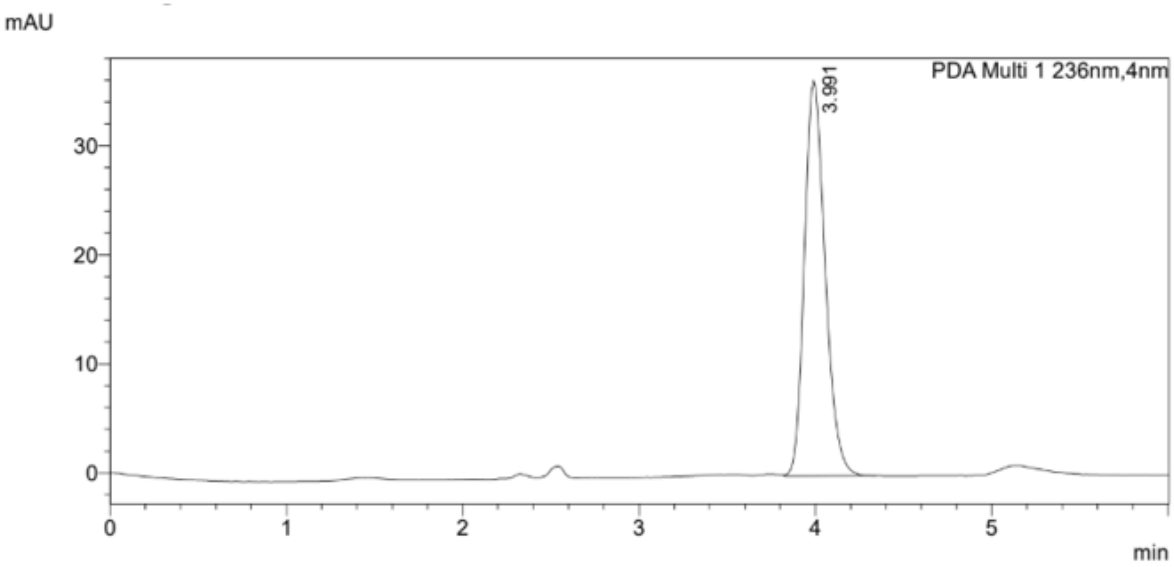

Figure 8: Chromatogram depicting Nepafenac sample peak.

Figure 9: AGREE score for the determination of Nepafenac.

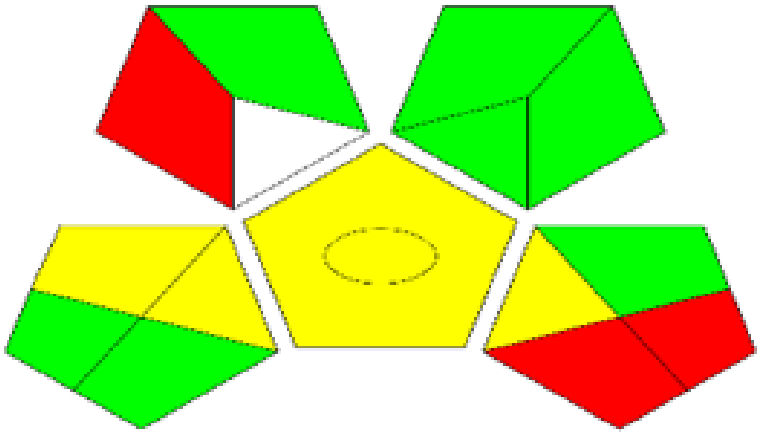

Figure 10: GAPI score for the determination of Nepafenac.

Table 11: Degradation data of Nepafenac.

Type of Degradation	Stress condition	Times (Hr)	Area	% Degradation
Acid degradation	0.1 N HCl (Room temperature)	3 hr	432207	10.33%
Base degradation	0.1N NaOH (Room temperature)	3 hr	397561	19.95%
Oxidation degradation	3%H_2O_2 (Room temperature)	2 hr	409927	16.33%
Photolytic degradation	Sun light	4 hr	453143	5.23%
Thermal degradation	Water bath 70°C	3 hr	428488	11.29%

performance of the evaluated method. The colour and score of each numbered segment represent the procedure's performance across distinct GAC principles. Principles 2, 4, 5, and 10 all exhibit excellent performance with a score of 1.0, while principles 1(0.80) and 6(0.80) also exhibit high performance. Principles 3(0.66), 8(0.51) and 12(0.40) exhibit poor performance, indicating significant flaws.

GAPI Tool

By analysing the complete process, the GAPI pictogram (Figure 10) offers a comprehensive assessment of the environment in which an analytical procedure operates, which is summarised by the yellow-coloured centre pentagon. The five surrounding pentagons represent important phases of the analytical process. A yellow colour denotes modest environmental performance, red sectors show poor performance, and green sectors show criteria with good to exceptional performance.

CONCLUSION

A simple, robust, and eco-conscious RP-HPLC method for the quantitative estimation of Nepafenac in ophthalmic preparation was successfully developed using a Quality by Design (QbD) approach. Application of the Box-Behnken experimental design enabled systematic optimisation of critical method parameters, ensuring consistent chromatographic performance and enhanced method understanding. The optimised method provided good peak symmetry, short retention time, and efficient separation using a relatively simple mobile phase composition. Validation studies performed in accordance with ICH guidelines ICHQ2(R2) confirmed that the method demonstrated good specificity, precision, linearity, and accuracy, with recovery values ranging from 98.2% to 101.9%. The commercial ophthalmic formulation's assay yielded a result of 96.7%. Additionally, analyses of forced degradation under acidic, alkaline, oxidative, thermal, and photolytic stress conditions were conducted to assess the method's capacity to indicate stability. In addition, the new method's environmental sustainability was assessed using the AGREE and GAPI greenness evaluation methods, demonstrating its eco-friendliness through a limited environmental impact and reduced solvent use. Owing to its simplicity, reliability, reduced analysis time, and environmentally considerate solvent usage, the developed RP-HPLC method is well-suited for routine quality analysis of Nepafenac in ophthalmic dosage forms.

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ABBREVIATIONS

ANOVA: Analysis of variance; **ATP:** Analytical Target Profile; **BBD:** Box-Behnken Design; **CAAs:** Critical Analytical Characteristics; **COA:** Certificate of analysis; **COX:** Cyclooxygenase inhibitor; **CMPs:** Critical Method Parameters; **CPP:** Critical Process Parameters; **CQAs:** Critical quality attributes; **DoE:** Design of Experiments; **FDA:** Food and Drug Administration; **GAC:** Green analytical chemistry; **IFB:** Ishikawa Fish-Bone; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **NSAIDs:** Non-steroidal anti-inflammatory drugs; **OOS:** Out-of-specification; **OOT:** Out-of-trend; **PA:** Peak area; **QbD:** Quality by Design; **RSD:** Relative standard deviation; **RT:** Retention time; **TF:** Tailing factor; **TP:** Theoretical plates.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

In order to establish a Box-Behnken design-assisted RP-HPLC technique inside a QbD-supported degradation framework, DRP conceived the project and oversaw the integration of a green chemistry methodology. MSP made a substantial contribution to the manuscript's arrangement and composition. SSS was in charge of the manuscript's critical evaluation and intellectual improvement. PPS offered technical know-how for maintaining, optimising, and operating the HPLC equipment. The final draft of the work has been reviewed and approved by all authors.

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