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## Research Article

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**Posted Date:** April 17th, 2026

**DOI:** <https://doi.org/10.21203/rs.3.rs-8872875/v1>

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**Additional Declarations:** No competing interests reported.

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**Version of Record:** A version of this preprint was published at Journal of Pharmaceutical Innovation on May 20th, 2026. See the published version at <https://doi.org/10.1007/s12247-026-10744-2>.

## **A Robust RP-HPLC Method for Simultaneous Estimation of Paracetamol and Nefopam in Human Plasma: Design of Experiments Approach**

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**Abstract:**

A rapid, sensitive, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Paracetamol, and Nefopam, in human plasma. The method development was carried out using a Quality by Design (QbD) approach to systematically optimize critical chromatographic parameters for improved resolution and reproducibility. Chromatographic separation was achieved on a C18 column using a mobile phase comprising Acetonitrile and 0.1% orthophosphoric acid in a specified ratio, delivered at a flow rate of 0.7 mL/min, with detection at 255 nm. The retention times for Paracetamol and Nefopam were found to be approximately 6.220 and 7.410 minutes, respectively, indicating excellent peak symmetry and resolution. Method validation was carried out by evaluating key bioanalytical parameters, including selectivity, linearity, accuracy, precision, recovery, matrix effect, and stability under various conditions. The method exhibited a linear response over the concentration range of 10–50 µg/mL for Paracetamol and 1–5 µg/mL for Nefopam with %RSD values within acceptable limits. No significant interference from plasma components was observed at the retention times of the analytes. This validated method is suitable for pharmacokinetic, bioequivalence, and routine therapeutic drug monitoring studies involving these NSAIDs in human plasma.

Keywords: Bioanalytical, RP-HPLC, Quality by design, NSAID, validation, Plasma Analysis

## Introduction:

Inflammation is a crucial biological process of the defence mechanism against several traumas or microbial infections, which disrupted tissue homeostasis. It is triggered due to Chemicals, including prostaglandins, leukotrienes, bradykinins, platelet-activating factor, and interleukin-1, which are released from tissue and migrating cells and produce inflammation. [1-3].

Numerous illnesses, including arthritis, cardiovascular disease, inflammatory bowel disease (IBD), diabetes, asthma, and cancer, are brought on by inflammation. Chronic inflammation also plays a pivotal role in immunological disorders, persistent pain conditions, and neurodegenerative diseases such as Alzheimer's. Pain associated with these conditions is commonly managed with drugs like Paracetamol and NSAIDs. These agents exert their therapeutic effect primarily by inhibiting cyclooxygenase (COX)-1 and /or COX-2 enzymes, thereby reducing the synthesis of prostaglandins and other lipid mediators involved in inflammation. This mechanism not only alleviates pain and fever but also contributes to their immunomodulatory effects.

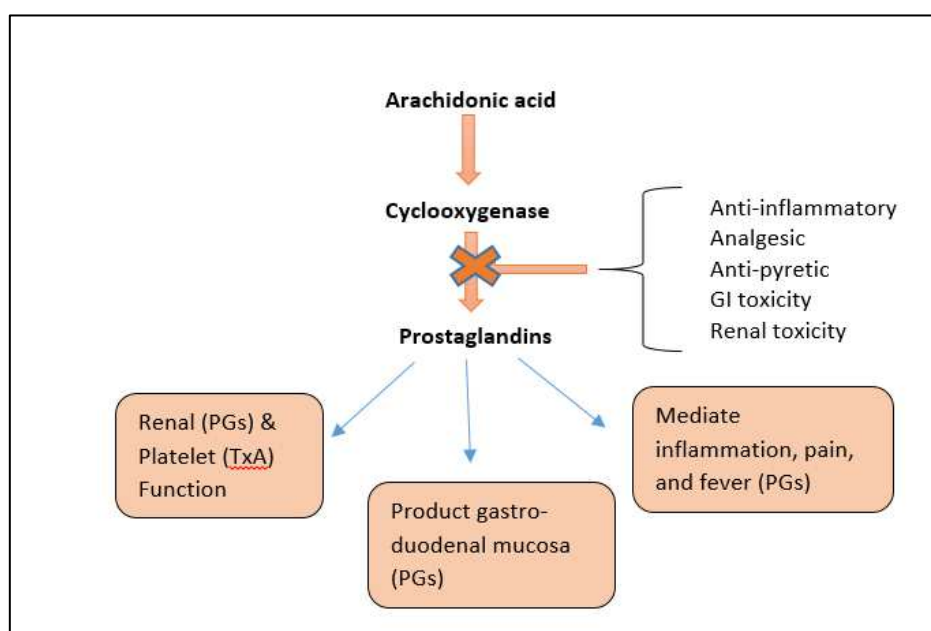


**Fig. No.1 Mechanism action of Cyclooxygenase pathway and role of Prostaglandin**

Paracetamol, chemically known as N-acetyl-p-aminophenol (APAP), is one of the most widely used medications globally. It is a p-aminophenol derivative and the active principal metabolite of phenacetin [4]. Unlike non-steroidal anti-inflammatory drugs (NSAIDs), which primarily act by reducing tissue inflammation, paracetamol exerts its effect by inhibiting cyclooxygenase (COX)-mediated prostaglandin synthesis [5]. Owing to its analgesic and antipyretic properties, paracetamol is widely prescribed for pain management across a variety of conditions, including cancer pain [6], asthma pathogenesis [7], osteoporosis [8], and Parkinson's disease [9].

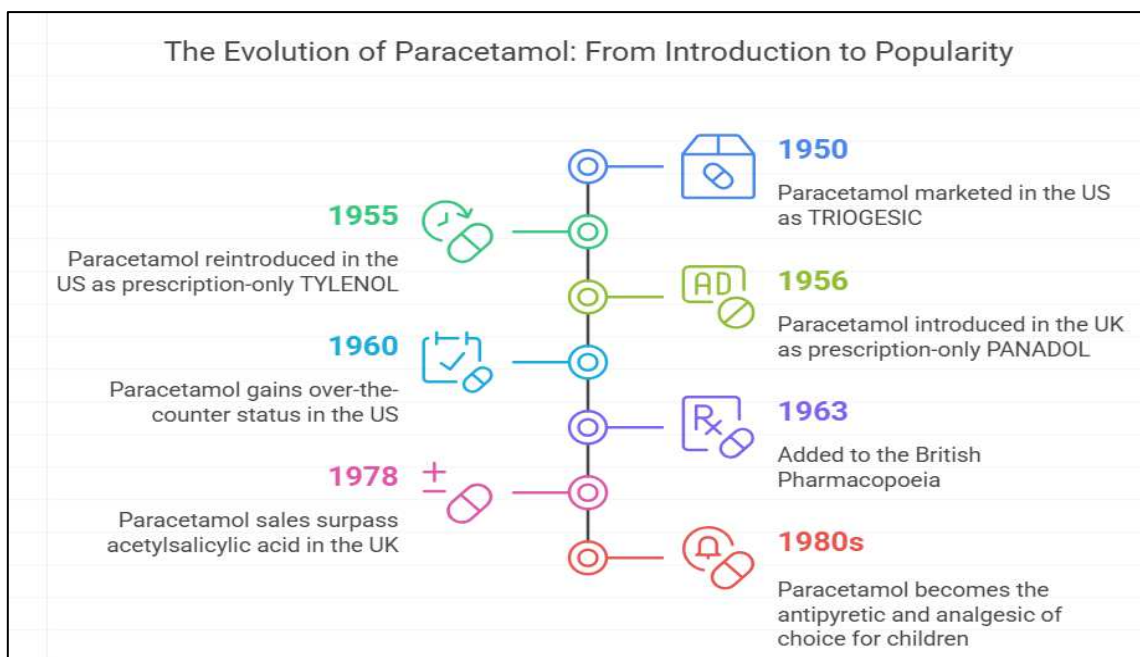


**Fig. No.2 The Evolution of Paracetamol: From Introduction to Popularity**

Orally administered Paracetamol exhibits rapid absorption through the gastrointestinal tract, exhibiting a dose-dependant systemic bioavailability ranging from 70% to 90%. The rate of absorption is largely determined by gastric emptying, and it can be delayed by factors such as food intake, propantheline, pethidine, and diamorphine. Conversely, metoclopramide accelerates this process. Paracetamol demonstrate efficient absorption via the rectal route and distributes rapidly and uniformly across most tissues and body fluids [10]. Over the years, several analytical techniques have been developed for its quantification, including spectrophotometry [11], spectrofluorimetry [12], voltammetry spectroscopy [16], high-performance liquid chromatography (HPLC), and gas chromatography-mass spectroscopy (GC-MS) [17].

Nefopam, a benzoxazocine derivative that was initially known as fenazoxine, is shares structural similarity with orphenadrine and diphenhydramine and its molecular formula is  $C_{17}H_{19}NO$ , and its systemic name is 5-methyl-1-phenyl-1,3,4,6-tetrahydro-2,5-benzoxazocine [18]. Nefopam is centrally acting, non-opioid analgesic commonly used as an alternative to opioid medications for managing pain ranging from moderate to severe, such as cancer-related pain [19], musculoskeletal and dental pain, and postoperative pain [20], as well as in end-stage renal disease [21]. Its mechanism of action involves inhibiting the reuptake of dopamine, serotonin, and norepinephrine at spinal and supraspinal levels [23]. The pharmacokinetics and bioavailability of Nefopam have been well documented: when administered orally, it exhibits a terminal half-life of approximately 4-5.1 hours, reaches peak plasma concentration within 1-3 hours and demonstrates limited bioavailability, primarily due to first-pass metabolism [24].

The use of Nefopam may be associated with several adverse effects, including cardiovascular complications such as tachycardia, rare allergic reactions characterized by swelling, itching and related symptoms and seizures in cases of overdose. Additionally, Nefopam can produce anticholinergic effects such as dry mouth, blurred vision, constipation, and urinary retention, while rare instances of hallucination and confusion have also been reported [25]. Analytical methods for Nefopam determination have been described in the literature, including thin-layer

chromatography (TLC) with densitometric detection [26], gas chromatography [27], and UV spectrophotometry [28].

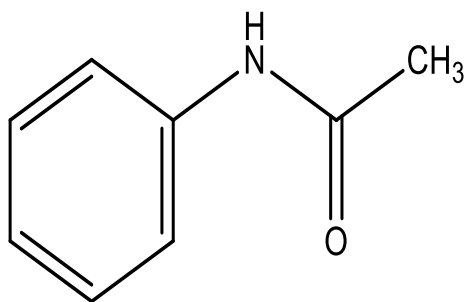


**Fig No.3 structure of Paracetamol**

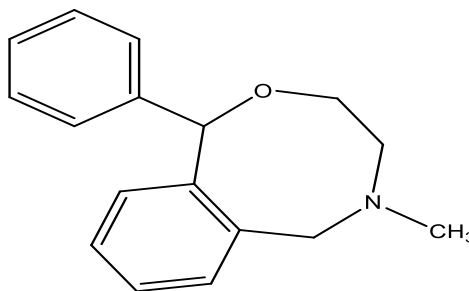


**Fig No.4 structure of Nefopam HCL**

Quality by design (QbD) is a regulatory-endorsed approach that integrates quality into pharmaceutical development by emphasizing process understanding and control rather than relying solely on end-product testing. In analytical method development, QbD enables the identification and management of critical method parameters (CMP) affecting critical Quality Attributes such as theoretical plates, peaks area, retention time, and resolution. This systematic strategy ensures robust, reliable methods capable of delivering consistent results under diverse operational conditions [29].

The Quality by Design approach is increasingly utilized in RP-HPLC method development to ensure robust performance by understanding and controlling method variability. Using Design of Experiment, a key QbD tool, allows for the systematic identification and optimization of critical method variables with minimal time, efforts, and cost. This approach enhances method efficiency, flexibility, and making it highly valuable in drug development, formulation and routine in vitro and in vivo analysis.

In the references study [30], a bioanalytical method was developed and validated using a C18 column with a mobile phase of methanol and phosphate buffer (50:50, v/v) at a flow rate of 1.0 ml/min, employing rat plasma as the biological matrix. The reported retention times for Paracetamol, Tramadol HCL, and Domperidone were 3.3, 5.3, and 8.9 minutes, respectively. The method was shown to provide satisfactory accuracy and linearity during validation and is cited here solely as a reference for the analytical determination of Paracetamol. Another investigation [31] reported an RP-HPLC method for simultaneous determination of paracetamol and cefixime in rabbit plasma, employing cefochlor as an internal standard. In this case, the chromatographic separation was achieved using a mobile phase of phosphate buffer and acetonitrile (80:20, v/v), resulting in retention times of 3.618 minutes for cefochlor, 4.068 minutes for paracetamol, and 5.914 minutes for cefixime. Both studies demonstrated acceptable accuracy and linearity, highlighting the applicability of RP-HPLC techniques in multi-drug analysis from biological samples. As reported in paper [32], an RP-HPLC method was developed for the estimation of paracetamol and etodolac in human plasma using tinidazole as an internal standard. The separation was performed on a C18 column with a mobile phase consisting of potassium phosphate buffer and acetonitrile at a flow rate of 1.0 mL/min. The method exhibited excellent linearity, with a correlation coefficient ( $r^2$ ) greater than 0.99. As presented in research paper [33], a RP-HPLC method was developed and validated for the estimation of nefopam hydrochloride in bulk and pharmaceutical dosage forms. The retention time of nefopam hydrochloride was approximately 5.313 minutes. The method exhibited excellent linearity within the concentration range of 16–120  $\mu\text{g/mL}$ , with a correlation coefficient ( $r^2$ ) of 0.999. As detailed in research paper [34], an RP-HPLC method

was established for concurrent estimation of desmethyl-nefopam (DMN) and nefopam (NEF) in urine, plasma, and globule samples, using imipramine as an internal standard. Separation was carried out using a C18 Symmetry column (150 × 4.6 mm I.D., 5 µm particle size; Waters) with a mobile phase comprising acetonitrile and buffer (15 mM KH<sub>2</sub>PO<sub>4</sub> containing 5 mM octane sulfonic acid, pH 3.7) in a ratio of 77:33 (v/v). The analysis was operating at a flow rate of 1.5 mL/min with UV detection at 210 nm.

After reviewing numerous previously published studies on the analytical determination of Paracetamol, Nefopam Hydrochloride, and other related drugs, it was observed that most methods focused on the estimation of individual drugs or their combination with different agents, primarily in pharmaceutical formulations rather than biological matrices. Very few studies address simultaneous estimation in biological matrices such as human plasma, and those that exist often face limitations such as matrix interference, lack of robustness, or the need for separate sample preparation and analysis for each compound. To date, no validated RP-HPLC method has been reported for the simultaneous quantification of Paracetamol and Nefopam hydrochloride in human plasma. This represent a significant analytical gap, particularly for clinical and pharmacokinetic studies where accurate and simultaneous measurement of both drugs is essential. The present study addresses this gap by developing and validating a QbD-based RP-HPLC method for the combined estimation of Paracetamol and Nefopam hydrochloride in plasma. The method is designed to be robust, accurate, and precise, with minimized variability through systematic optimization of critical parameters. This approach enhances its applicability for pharmacokinetic profiling, bioequivalence studies, and therapeutic drug monitoring, thereby providing a novel and efficient tool for both clinical and research settings.

## **Material and Method:**

### **Chemical and reagent:**

All substance, solvent, and chemical was of HPLC grade. Nefopam Hydrochloride (C<sub>17</sub>H<sub>19</sub>ClNO) and Paracetamol (C<sub>8</sub>H<sub>9</sub>NO<sub>2</sub>) reference samples were generously supplied by Reliable Shree Industrial Training and Research Centre (RSITC), Jalgaon. HPLC-grade Acetonitrile (ACN) and orthophosphoric acid (OPA), were also supplied by R.S.I.T.C. Jalgaon. The human plasma, which was gratefully donated by Lifeline Blood Bank in Nagpur, was marked as drug and disease-free. Initially, the plasma was stored at -20°C and subsequently maintained at -4°C for routine use.

### **Instrumentation and conditions:**

Analytical Technology Limited's UV spectrophotometer and HPLC equipment (Agilent 1100) were utilized to develop the bioanalytical method. Chromatographic separation was carried out at a 250 °C temperature with a RP C-18 (Agilent) 4.6 x 250 mm column under isocratic conditions. The mobile phase consisted of Acetonitrile and 0.1% OPA at a volumetric ratio of 80:20, delivered at a flow rate of 0.7 mL/min. A 20 µl sample volume was injected and the detection was carried out at 255nm using a multiple wavelength detector.

### **Experimental work:**

#### **Extraction of Human Plasma by Protein Precipitation Technique:**

Direct injection of human plasma into the HPLC system is not feasible, as it can lead to column blockage and damage, rendering the column unsuitable for further use. Therefore, prior to analysis, the drug must be extracted into suitable solvent, concentrated by evaporation, and then injected into the HPLC system. For sample preparation, 1mL of human plasma was mixed

with 3 mL of acetonitrile in an Eppendorf tube and vortexed for 5 min to ensure complete protein precipitation. The mixture was centrifuged at 3000 rpm, followed by a second centrifugation for 20 min to achieve clear phase separation. The supernatant was collected, refrigerated until analysis, reconstituted with the selected diluent, and then injected into HPLC column [34].

#### Preparation of individual standard solution:

After mixing 5 mg of NEFO and 50 mg of PARA with 25 mL of methanol and 1 mL of untreated human plasma, the mixture was shaken vertically for 30 minutes, centrifuged for 1 hour at 5000 rpm, and filtered through a membrane filter to get a clear organic solution. This yields 200 µg/mL of NEFO and 2000 µg/mL of PARA. This solution was designated as Stock 1 and used for HPLC analysis. For calibration, aliquots of 0.05, 0.1, 0.15, 0.2, and 0.25 mL from Stock 1 were diluted to 10 mL with mobile phase to obtain final concentrations of 10–50 µg/mL for Paracetamol (PARA) and 1–5 µg/mL for Nefopam Hydrochloride (NEFO). The calibration curve was constructed by plotting peak area against the corresponding drug concentrations.

#### Selection of analytical Wavelength:

A solution with a concentration of 20 µg/mL was scanned in the range of 200–400 nm to determine the maximum absorbance. Paracetamol and Nefopam hydrochloride showed individual  $\lambda_{\text{max}}$  values at 242 nm and 265 nm, respectively, in methanol. Based on the overlap of both drugs, 255 nm was selected as the analytical wavelength for simultaneous estimation.

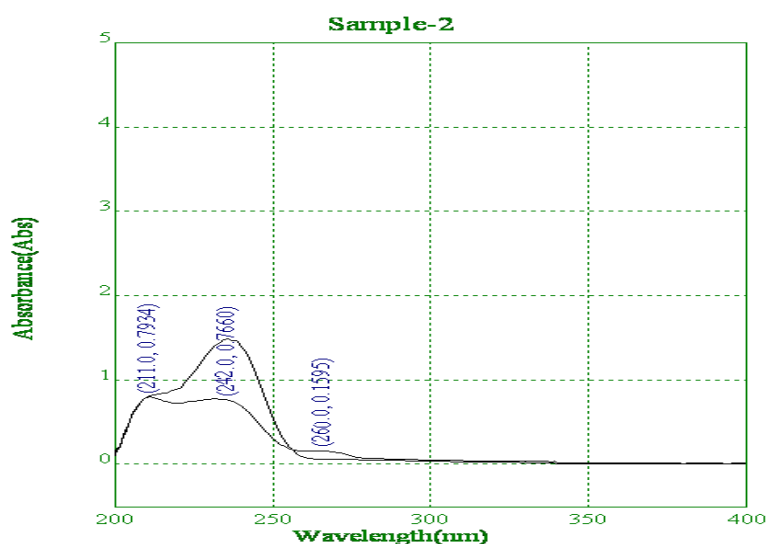


Fig. No. 5 Analytical Wavelength selection by UV

#### Initial Chromatographic Conditions:

The chromatographic method was optimized through a trial-and-error approach, and the following parameters were maintained consistently throughout the experimental work:

Table No. 1 Chromatographic condition for RP-HPLC method development

| Parameter   | Condition                                      |
|-------------|------------------------------------------------|
| HPLC System | Agilent 1100 Gradient System with MWD detector |
| Software    | Chem-Station                                   |
| Column      | C18, 4.6 × 250 mm (Agilent)                    |

|                      |                                                                           |
|----------------------|---------------------------------------------------------------------------|
| Particle Size        | 5.0 $\mu\text{m}$                                                         |
| Stationary Phase     | Reverse Phase C18                                                         |
| Mobile Phase         | Acetonitrile (ACN): 0.1% Orthophosphoric Acid (OPA) in water (80:20, v/v) |
| Detection Wavelength | 255 nm                                                                    |
| Flow Rate            | 0.7 mL/min                                                                |
| Column Temperature   | 25°C                                                                      |
| Injection Volume     | 20 $\mu\text{l}$                                                          |

### Experimental Design:

A central composite design (CCD), a widely used Response Surface Methodology (RSM) approach, was employed to systematically optimize the chromatographic conditions for the simultaneous estimation of Paracetamol and Nefopam Hydrochloride by RP-HPLC in alignment with Quality by Design (QbD) principles. CCD was selected as it effectively evaluates the linear, interaction, and quadratic effects of critical method parameters while requiring a relatively lower number of experimental runs. This design facilitates a comprehensive understanding of the method behavior and the establishment of a robust design space to ensure method reliability and consistency under varying operational conditions.

Two independent variables were studied: mobile phase composition (%ACN) (Factor A) and flow rate (mL/min) (Factor B). Each factor was investigated at three levels- low (-1), medium (0), and high (+1) to assess their influence on the chromatographic performance. The specific factor levels are summarized in table 2.

**Table No. 2 Experimental Factors and their levels**

| Factor | Parameter                        | Low (-1) | Medium (0) | High (+1) |
|--------|----------------------------------|----------|------------|-----------|
| A      | Mobile Phase Composition (% ACN) | 70       | 80         | 90        |
| B      | Flow Rate (mL/min)               | 0.6      | 0.7        | 0.8       |

The responses evaluated for both Paracetamol and Nefopam Hydrochloride included:

R1 & R4: Retention time (RT1 for Paracetamol and RT2 for Nefopam)

R2 & R5: Peak Area (Area 1 for Paracetamol and Area 2 for Nefopam)

R3 & R6: Theoretical Plates (TP1 for Paracetamol and TP2 for Nefopam)

A total of 13 experimental runs, consisting of factorial points, axial points, and centre points, were conducted to generate a reliable predictive model and ensure reproducibility. These experiments enabled systematic exploration of the design space and optimization of method parameters for robust performance.

The experimental data were analyzed using Design-Expert software (version 13). Regression equations were generated for each response to describe the relationship between the independent variables and the critical analytical attributes. The data were fitted to both quadratic and linear polynomial models, with the quadratic model expressed as:

$$Y=b_0+b_1A+b_2B+b_3AB+b_4A^2+b_5B^2$$

And the linear model as:

$$Y=b_0+b_1A+b_2B+b_3AB$$

Where Y represents the predicted response (CAA), A and B are the coded values of the CMPs,  $b_0$  is the intercept, and  $b_1$  to  $b_5$  represent the coefficient for main effects, interaction effects, and quadratic terms.

The model fitting results demonstrated excellent predictive capability, with correlation coefficient ( $R^2$  values) greater than 0.98 for all responses, indicating a strong relationship between the selected factors and the chromatographic performance. To visualize these interactions, three-dimensional (3D) response surface plots and two-dimensional (2D) contour plots were generated. These graphical tools provided valuable insights into the influence of each parameter and their interactions, thereby supporting the selection of optimal chromatographic conditions for the simultaneous estimation of both drugs.

### **Method Validation:**

The International Conference on Harmonization (ICH) guidelines and EMA guidelines were used to validate the suggested approach. Selectivity, sensitivity, linearity range, accuracy, and precision were all evaluated as validation parameters for the suggested method.

### **Linearity:**

Linearity was determined by using the calibration curve. Calibration curve was prepared by injecting appropriate quantities of working solution into untreated plasma to obtain a final concentration of 10-50 µg/ml of Paracetamol and 1-5 µg/ml of Nefopam Hydrochloride. Spiked plasma conc. were plotted on the X-axis, while the Y-axis represented the corresponding peak areas to establish the calibration curve.

### **Precision:**

The Precision was verified through intra-day and inter-day evaluation using validation quality control samples at concentrations of 20±2, 30±3, 40±4 µg/ml. Intra-day precision was determined by analyzing three replicates of the same solution of pure drugs and calculating the standard deviation (SD) and coefficient of variation to assess the consistency of the method.

### **Accuracy:**

Accuracy which reflects the closeness between expected and actual value, was evaluated by calculating the percentage recovery of two different concentration of paracetamol and nefopam hydrochloride in spiked human plasma. The results confirmed the reliability and accuracy of the accuracy of proposed method.

### **Robustness:**

A method is robust if it remains reliable under small, deliberate changes. The developed RP-HPLC method was tested by slightly varying the mobile phase ratio, flow rate, detection

wavelength. % RSD value for Paracetamol (PARA) and Nefopam Hydrochloride (NEFO) remained below 2%, confirming robustness of the method.

#### **Ruggedness:**

Ruggedness of the developed RP-HPLC method was evaluated to ensure its reliability and reproducibility under varied conditions. The study was performed by analyzing the same sample solutions under different experimental setups, including variations in analysts and instruments. This assessment helps determine whether minor changes in operational conditions affect the performance of the method. The %RSD of the assay results was calculated, and values were found to be within the acceptable limit of not more than 2%, as per ICH Q2 (R1) guidelines. These results confirm that the developed method is rugged and suitable for routine quality control analysis of Paracetamol and Nefopam hydrochloride in human plasma.

#### **System Suitability Parameter:**

System suitability testing was performed to ensure the performance and reliability of the developed RP-HPLC method. Key parameters, including column efficiency, resolution, and capacity factor, were evaluated. These parameters were determined by repeated injections of standard solutions under optimized chromatographic conditions. The study was conducted in compliance with the guidelines outlined in the United States Pharmacopoeia (USP), confirming that the system was suitable for accurate and precise analysis.

#### **Assay of marketed tablet:**

Twenty Nefopam tablets were weighed, yielding an average weight of 0.604 g. A quantity of powder equivalent to 50 mg of Paracetamol (~95mg) and 10 mg of Nefopam hydrochloride (~131.56 mg) was dissolved in 25 mL methanol, sonicated, centrifuged, and filtered. The filtrate was diluted with mobile phase and analyzed by RP-HPLC under optimized conditions. Drug content was calculated from the calibration curve and expressed as a percentage of the label claim.

#### **Results:**

##### **Chromatographic analysis of blank human plasma:**

The chromatogram illustrates a blank human plasma sample analyzed under the optimized RP-HPLC conditions. Detection was performed at 255nm with photodiode array detection. The blank plasma sample shows no significant chromatographic interference was detected at the retention times of the target analytes, Paracetamol and Nefopam hydrochloride. Minor endogenous peaks appeared at retention times 2.177, 3.390, 4.272, and 5.100 min, corresponding to plasma matrix components. However, their intensities were negligible and did not interfere with analyte quantification. These findings confirm the specificity and selectivity of the method that is established for determination of Paracetamol and Nefopam in human plasma.

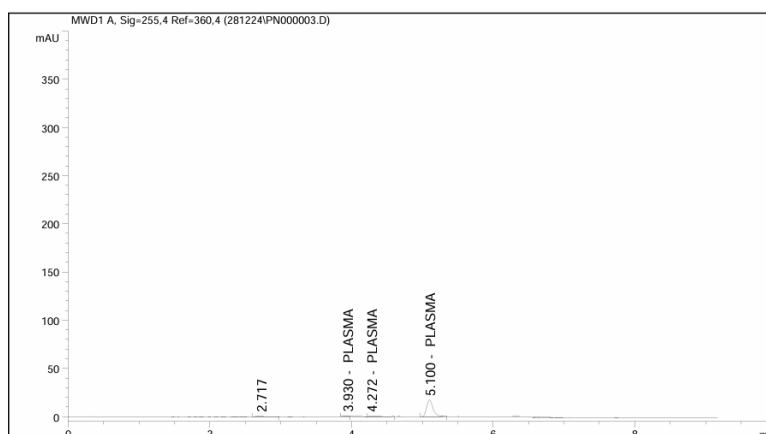


**Figure 6: Chromatogram of Blank Human Plasma**

**Table 1: Optimization of Mobile Phase Composition and Plasma Volume for RP-HPLC Separation of Paracetamol and Nefopam**

| Sr.No. | Ratio of Mobile Phase                        | Flow Rate (ml/min) | Retention time (min) |       | Inference |
|--------|----------------------------------------------|--------------------|----------------------|-------|-----------|
|        |                                              |                    | PARA                 | NEFO  |           |
| 1      | Methanol +0.1% (OPA) (80:20)                 | 0.7                | 4.032                | -     | Rejected  |
| 2      | Methanol +0.1% (OPA) (80:20)                 | 0.7                | 3.584                | -     | Rejected  |
| 3      | Methanol +0.1% (OPA) (80:20)                 | 0.7                | 3.644                | -     | Rejected  |
| 4      | ACN +0.1% (OPA) (80:20)                      | 0.7                | 4.375                | 5.514 | Rejected  |
| 5      | ACN +0.1% (OPA) (80:20)                      | 0.7                | 6.243                | 7.547 | Rejected  |
| 6      | ACN +0.1% (OPA) (80:20) + 0.2ml human plasma | 0.7                | 6.229                | 7.431 | Rejected  |
| 7      | ACN +0.1% (OPA)(80:20) + 1ml human plasma    | 0.7                | 6.220                | 7.416 | Accepted  |

During method development, different mobile phase compositions and plasma volumes were evaluated to achieve effective separation of Paracetamol and Nefopam. The flow rate kept constant at 0.7 mL/min while assessing peak response and system suitability. Among the tested conditions, the 7th trial using acetonitrile and 0.1% orthophosphoric acid (80:20 v/v) with 1 mL plasma provided optimal results, with Paracetamol and Nefopam eluting at 6.220min and

7.416 min, respectively. Minor early peaks between 3.639 and 5.042 min were observed, attributed to endogenous plasma components, but they did not interfere with the target analytes. The method demonstrated good specificity, consistent retention times, proper peak symmetry, and adequate resolution, confirming its suitability for reliable bioanalytical quantification.

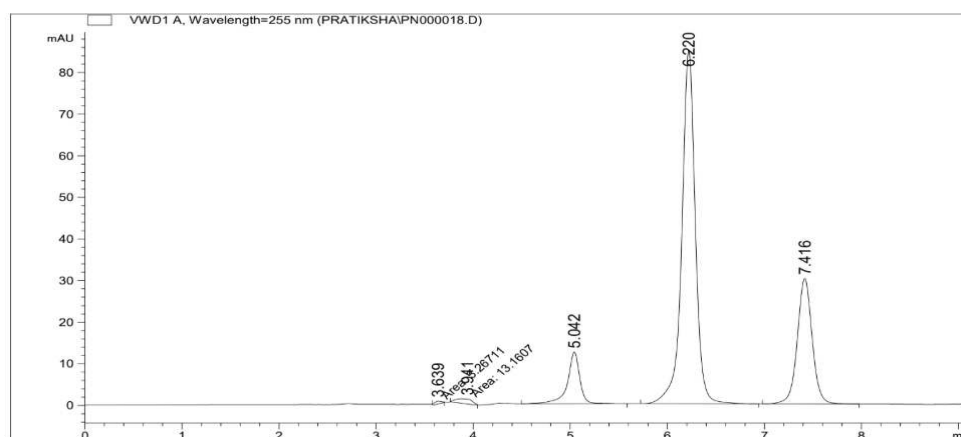


**Fig. No.7 Optimized RP-HPLC chromatogram of Paracetamol and Nefopam in human plasma under selected conditions (ACN: 0.1% OPA, 80:20 v/v, flow rate 0.7ml/min, detection at 255nm).**

#### CCD experimental runs and observed response:

Table illustrate the central composite design matrix, along with corresponding experimental responses, including retention time, peak area, and theoretical plate count for both Paracetamol (PARA) and Nefopam Hydrochloride (NEFO)

|     |     | Factor 1       | Factor 2    | Response 1 | Response 2 | Response 3 | Response 4 | Response 5 | Response 6 |
|-----|-----|----------------|-------------|------------|------------|------------|------------|------------|------------|
| Std | Run | A:mobile phase | B:flow rate | R1(RT 1)   | R2(AREA 1) | R3(TP 1)   | R4(RT 2)   | R5(AREA 2) | R6(TP 2)   |
|     |     | %              | ml/min      | min        | AUC        | TP         | min        | AUC        | TP         |
| 9   | 1   | 80             | 0.7         | 6.288      | 690.118    | 12322      | 7.556      | 232.707    | 13453      |
| 10  | 2   | 80             | 0.7         | 6.279      | 688.901    | 12287      | 7.543      | 230.74     | 13608      |
| 1   | 3   | 75             | 0.6         | 8.217      | 812.44     | 11506      | 10.406     | 272.531    | 13182      |
| 4   | 4   | 85             | 0.8         | 4.937      | 596.468    | 13065      | 5.637      | 202.161    | 14187      |
| 12  | 5   | 80             | 0.7         | 6.267      | 692.848    | 12237      | 7.514      | 231.12     | 13572      |
| 2   | 6   | 85             | 0.6         | 6.593      | 792.616    | 13547      | 7.537      | 269.616    | 14196      |
| 8   | 7   | 80             | 0.841421    | 5.247      | 579.298    | 11873      | 6.3        | 193.4      | 13454      |

|    |    |             |              |       |         |       |       |         |       |
|----|----|-------------|--------------|-------|---------|-------|-------|---------|-------|
| 11 | 8  | 80          | 0.7          | 6.254 | 692.295 | 12188 | 7.505 | 231.939 | 13664 |
| 3  | 9  | 75          | 0.8          | 6.134 | 602.01  | 10982 | 7.773 | 203.32  | 13077 |
| 13 | 10 | 80          | 0.7          | 6.267 | 691.312 | 12241 | 7.517 | 231.698 | 13708 |
| 6  | 11 | 87.0<br>711 | 0.7          | 5.462 | 676.63  | 13825 | 6.138 | 229.551 | 14464 |
| 7  | 12 | 80          | 0.5585<br>79 | 7.397 | 875.615 | 12564 | 9.507 | 295.289 | 13710 |
| 5  | 13 | 72.9<br>289 | 0.7          | 7.42  | 684.439 | 11280 | 9.633 | 230.089 | 12733 |

**Table No.2 Central Composite Design (CCD) for PARA and NEFO**

Based on statistical evaluation and response analysis, the following optimized conditions were selected.

**Table No.3 Optimized Design trial for QbD**

| Sr. no. | Composition of mobile phase                           | Flow Rate (ml/min) | Retention Time (min) |       | Inference |
|---------|-------------------------------------------------------|--------------------|----------------------|-------|-----------|
| 1.      | ACN + (0.1% OPA) Water (80:20 v/v) + 1ml human plasma | 0.7                | PARA                 | NEFO  | Accepted  |
|         |                                                       |                    | 6.220                | 7.416 |           |

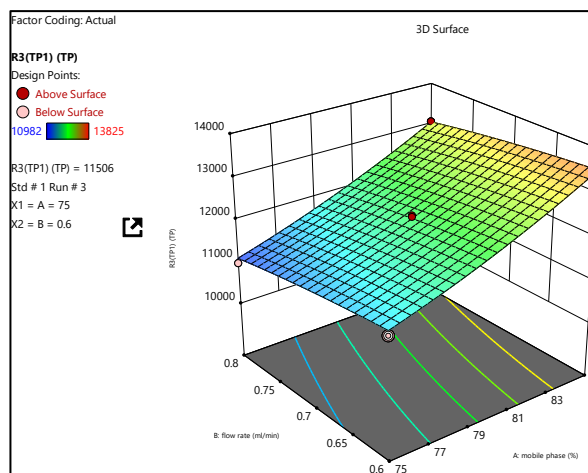
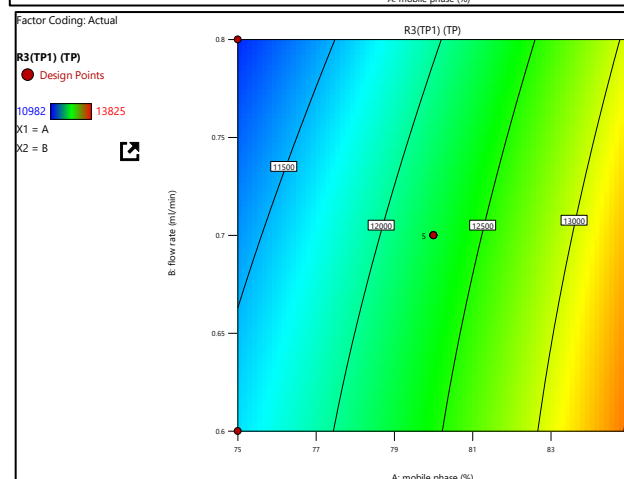
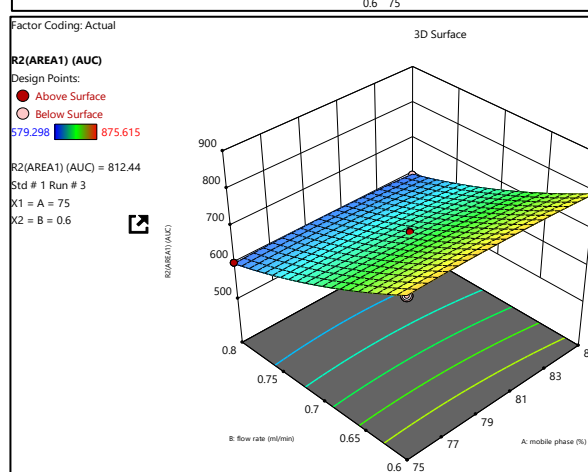
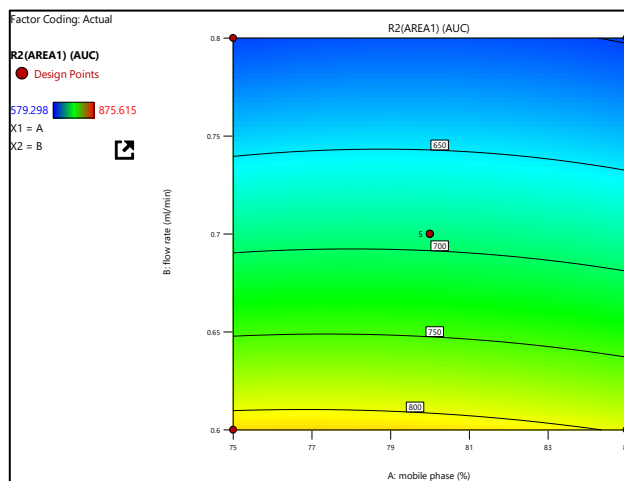
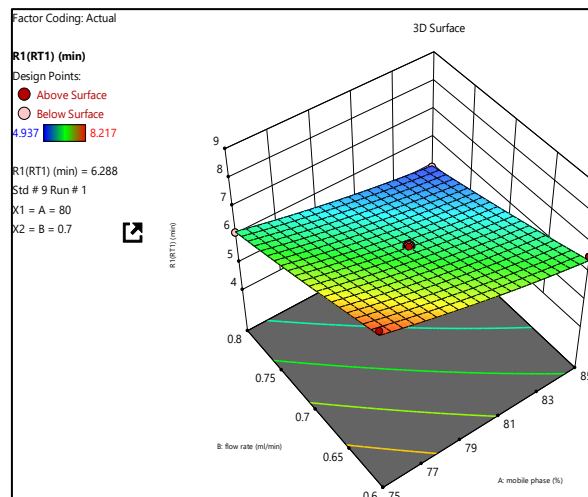
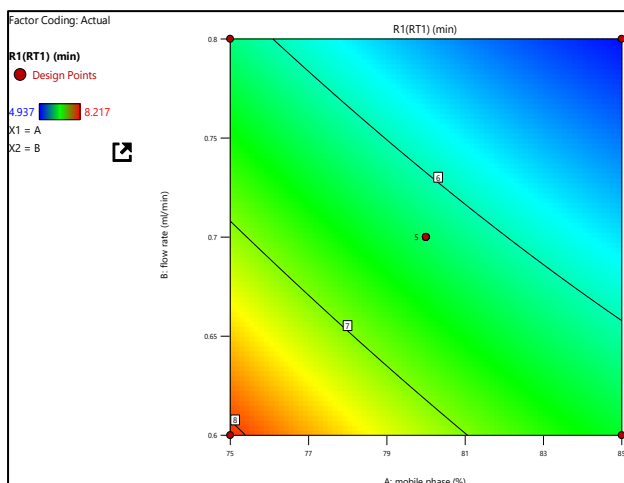
These condition show ideal retention times and satisfactory peak with minimal variation. These conditions were applied for the simultaneous estimation of Paracetamol and Nefopam Hydrochloride in spiked human plasma samples. The method yielded optimal retention times and reproducible peak responses, with minimal variability observed across replicates. These findings confirm the robustness and reliability of the method within the defined design space, in accordance with Quality by Design (QbD) principles.

**Table No.4 Optimized chromatographic parameters of Paracetamol and Nefopam Hydrochloride by RP-HPLC**

| Sr. No. | Parameter                | Description         |
|---------|--------------------------|---------------------|
| 1       | Chromatographic System   | Agilent 1100 system |
| 2       | Column                   | RP C-18( AGILENT)   |
| 3       | Column temperature       | 25°C                |
| 4       | Particle size            | 5.0 µm              |
| 5       | Detector                 | DAD                 |
| 6       | Mobile phase composition | ACN:0.1%OPA (80:20) |

|   |                     |                                 |
|---|---------------------|---------------------------------|
|   |                     | Sample in mobile phase was used |
| 7 | Flow Rate           | 0.7ml/min                       |
| 8 | Wavelength          | 255 nm                          |
| 9 | Volume of injection | 20μL                            |

This above table summarizes the final optimized chromatographic condition used for the RP-HPLC method development and validation.



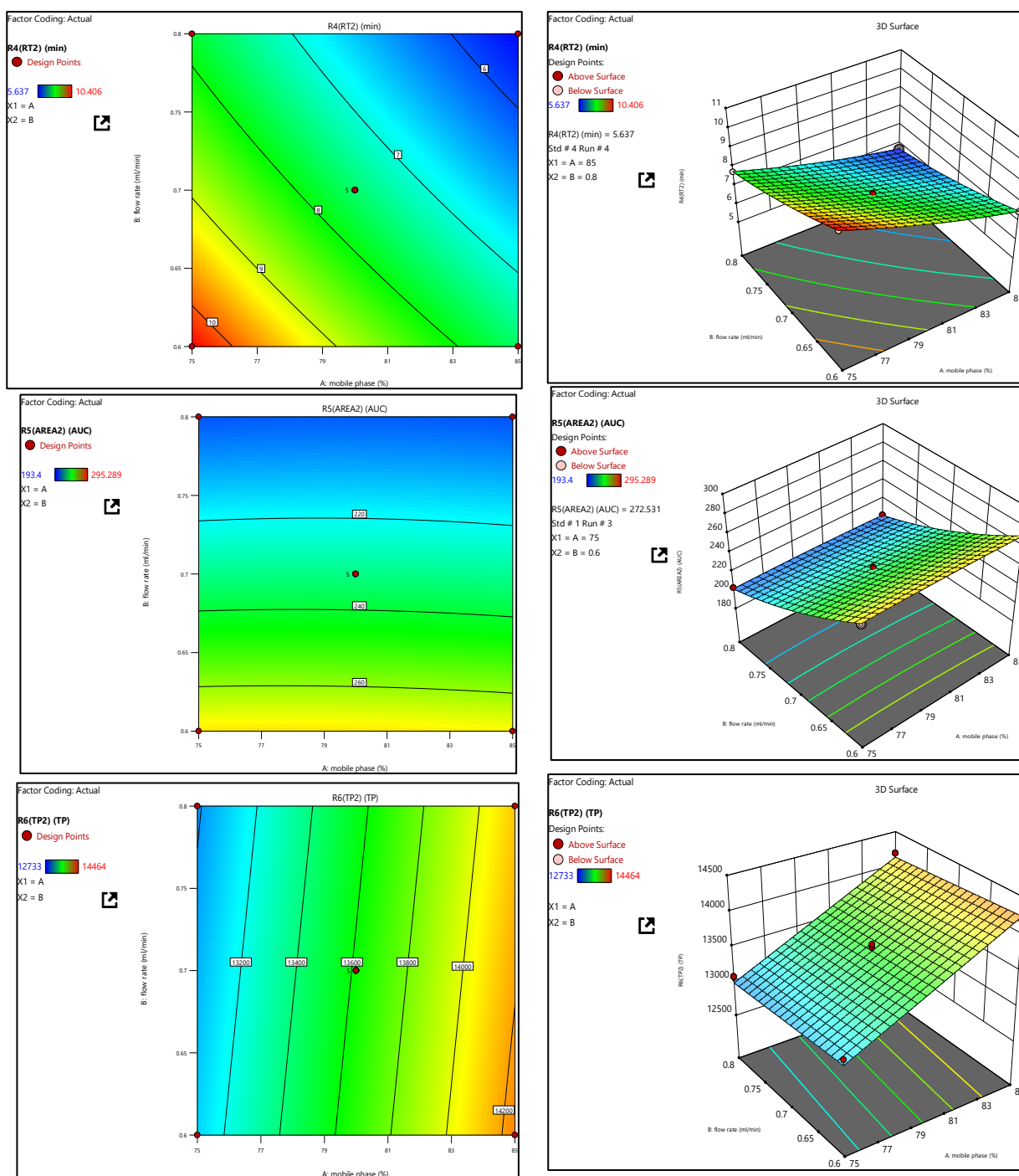


**Figure No 8: 2D contour and 3D response surface plots showing the effect of mobile phase composition (A) and flow rate (B) on (a) retention time (RT) and (b) peak area (AUC) for simultaneous estimation of Paracetamol and Nefopam Hydrochloride.**

### Model Adequacy and Statistical Validation:

The adequacy of the model for Response 1 (R1: Retention Time of Paracetamol) was evaluated based on statistical parameters derived from the regression analysis. The final regression equation in terms of coded factors is presented below:

$$R1 (RT1) = 6.27 - 0.6988A - 0.8474B + 0.1068AB + 0.1072A^2 + 0.0477B^2$$

The predictive performance and statistical reliability of the model developed for Response 2 (R2: peak area of Paracetamol) were evaluated using regression analysis. The final coded equation derived for this response is as follows:

$$R2 (AREA1) = 691.09 - 4.55A - 103.20B + 3.57AB - 6.06A^2 + 17.40B^2$$

The quadratic model developed for Response 3 (R3: Theoretical Plate Count of Paracetamol, TP1) was statistically validated to assess its predictive performance and reliability. The final regression equation in terms of coded factor is presented below:

$$R3 (TP1) = 12255.00 + 965.40A - 247.90B + 10.50AB + 121.12A^2 - 45.87B^2$$

The quadratic model for Response 4 (R4: Retention Time of Nefopam Hydrochloride, RT2) was developed and statistically validated to evaluate the effect of critical chromatographic variables. The final regression equation in terms of coded factors is as follows:

$$R4 (RT2) = 7.53 - 1.24A - 1.13B + 0.1833AB + 0.1652A^2 + 0.1742B^2$$

The quadratic model obtained through CCD for R5 (AREA2) demonstrates the influence of key chromatographic variables on peak area. The negative linear coefficients for factor A (organic modifier) and B (buffer concentration) indicate a decrease in response with increasing levels of these factors. A mild positive interaction (AB) suggests combined optimization benefits, while the quadratic terms ( $A^2$  and  $B^2$ ) highlight curvature in the response surface. This model aids in understanding the nonlinear behavior of the system and optimizing method conditions for improved analytical performance.

$$R5 (AREA2) = 231.64 - 0.6044A - 35.09B + 0.4390AB - 0.9542A^2 + 6.31B^2$$

In this coded equation of R6, factor levels are expressed using standardized values, where +1 and -1 represent the high and low levels of each variable, respectively. This approach enables a clear comparison of the relative effects of each factor on the response. The magnitude and sign of the coefficients indicate the strength and direction of each variable's influence on the theoretical plates. A positive coefficient (e.g., for A) suggests an increasing effect on the response, while a negative coefficient (e.g., for B) indicates a decreasing effect.

The regression model obtained for R6 (TP2) is:

$$R6 (TP2) = 13616.00 + 571.50A - 59.50B$$

**Table no. 5 Model fitting for all response**

| Response | Best Model | Adjusted R <sup>2</sup> | Predicted R <sup>2</sup> | Lack of Fit (p) | Status    |
|----------|------------|-------------------------|--------------------------|-----------------|-----------|
| R1       | Quadratic  | 0.9865                  | 0.9443                   | 0.0001          | Suggested |
| R2       | Quadratic  | 0.9985                  | 0.9946                   | 0.0341          | Suggested |
| R3       | Quadratic  | 0.9854                  | 0.9465                   | 0.0408          | Suggested |
| R4       | Quadratic  | 0.9994                  | 0.9978                   | 0.0791          | Suggested |
| R5       | Quadratic  | 0.9982                  | 0.9938                   | 0.0818          | Suggested |
| R6       | Linear     | 0.9676                  | 0.9554                   | 0.7188          | Suggested |

Based on the statistical comparison of different model types, the quadratic model was identified as the most suitable for the majority of responses due to its high Adjusted R<sup>2</sup>, Predicted R<sup>2</sup>, and acceptable lack of fit. It offered a strong balance between model accuracy and predictability without the over fitting risk associated with cubic models. For one response (R6), the linear model was preferred based on performance and simplicity. Overall, the quadratic model proved optimum for further optimization and design space exploration in method development.

### Overlay Plot for Determination of Optimal Chromatographic Conditions:

The overlay plot shows the design space for method optimization, where mobile phase composition (A) and flow rate (B) meet the acceptance criteria for retention time and peak area. The optimal conditions were identified as 80.365% mobile phase and 0.70 mL/min flow rate, ensuring well-resolved peaks and robust method performance.

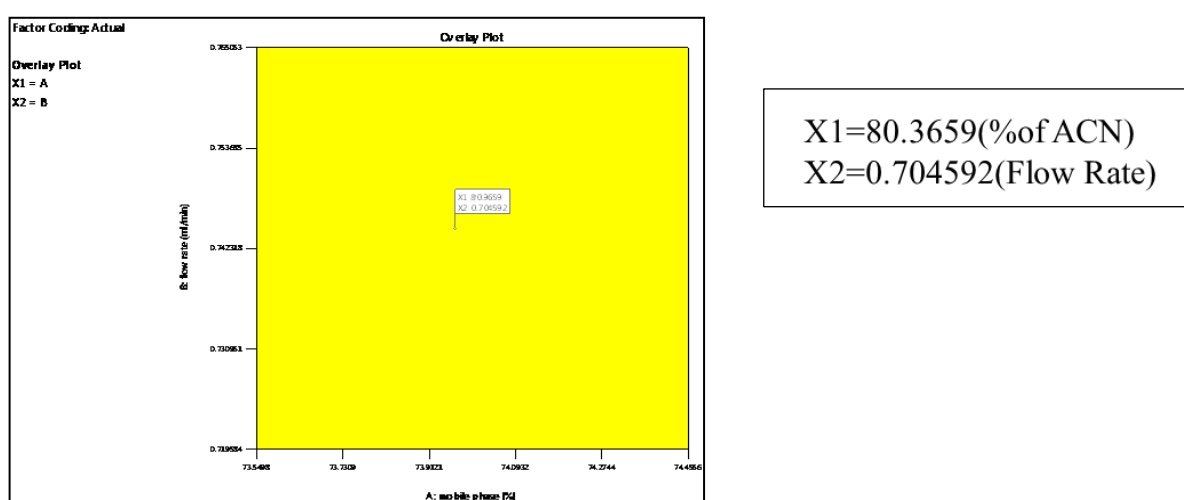


**Fig No 9 Overlay Plot Depicting the Design Space for Mobile Phase Composition and Flow Rate**

The optimization process was carried out using two independent variables: mobile phase composition (factor A) and flow rate (factor B). Each factor was evaluated at three levels: low, medium, and high. The ranges for the mobile phase and flow rate were 75-85% and 0.60-0.80 mL/min, respectively. The details of these factors and their corresponding levels are presented in table below:

**Table No. Independent variables with their levels for RP-HPLC method Optimization**

| Factor | Name         | Level  | Low Level | High Level | Std. Dev. | Coding |
|--------|--------------|--------|-----------|------------|-----------|--------|
| A      | mobile phase | 80.00  | 75.00     | 85.00      | 0.0000    | Actual |
| B      | flow rate    | 0.7000 | 0.6000    | 0.8000     | 0.0000    | Actual |

This table establishes the experimental design space for systematic optimization of chromatographic conditions using the Central Composite Design (CCD).

## Method validation:

### Linearity:

Linear regression equations of PARA and NEFO were found to be  $y = 39.191x + 54.992$  and  $y = 106.72x + 65.478$ , respectively and the regression coefficient values (r) were calculated to be 0.9999 and 1 for PARA and NEFO, respectively. This result indicates a direct proportional relationship between concentration and peak area, establishing the method as highly linear and suitable for quantitative analysis of both drugs in human plasma.

| Conc | Area I | II     | Mean       | SD   | %RSD |
|------|--------|--------|------------|------|------|
| 10   | 173.64 | 172.40 | 173.02     | 0.88 | 0.51 |
| 20   | 277.69 | 278.88 | 278.28     | 0.85 | 0.30 |
| 30   | 385.16 | 383.33 | 384.25     | 1.29 | 0.34 |
| 40   | 494.98 | 492.94 | 493.96     | 1.44 | 0.29 |
| 50   | 598.72 | 598.94 | 598.83     | 0.16 | 0.03 |
|      |        |        | Average SD | 0.92 |      |

**Table No.6 : System Suitability Evaluation of Paracetamol: Mean Peak Area, Standard Deviation, and %RSD at Low Concentration Levels (10–50 µg/mL).**

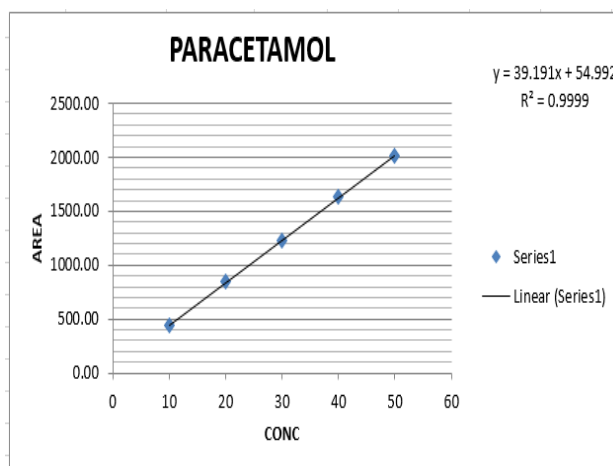


**Fig No.10 Calibration Curve of Paracetamol Showing Linearity between Concentration and Peak Area (10–50 µg/mL)**

| Conc | Area I  | II       | Mean       | SD   | %RSD |
|------|---------|----------|------------|------|------|
| 1    | 449.986 | 440.692  | 445.34     | 6.57 | 1.48 |
| 2    | 846.467 | 841.204  | 843.84     | 3.72 | 0.44 |
| 3    | 1224.38 | 1219.15  | 1221.76    | 3.69 | 0.30 |
| 4    | 1635.92 | 1627.434 | 1631.68    | 6.00 | 0.37 |
| 5    | 2013.39 | 2008.522 | 2010.96    | 3.44 | 0.17 |
|      |         |          | Average SD | 4.69 |      |

**Table No.7 : System Suitability Evaluation of Nefopam Hydrochloride: Mean Peak Area, Standard Deviation, and %RSD at Therapeutic Concentration Range (1–5 µg/mL).**

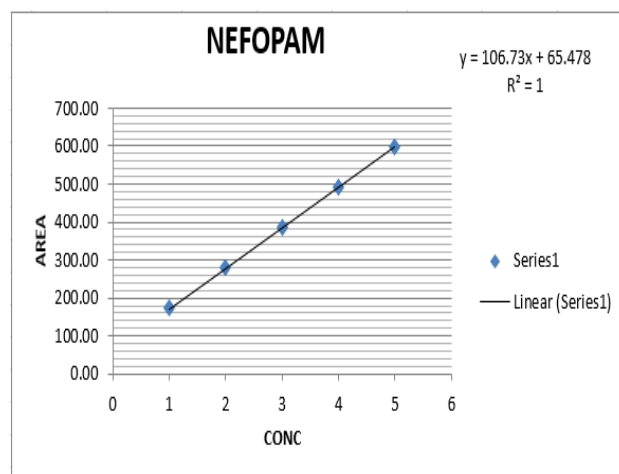


**Fig No.10 Calibration Curve of Nefopam HCL Showing Linearity between Concentration and Peak Area (1–5 µg/mL)**

**Precision:**

Precision was evaluated at three different concentration levels of Paracetamol (20, 30, and 40 µg/mL) and Nefopam Hydrochloride (2, 3, and 4 µg/mL) for both intraday and inter-day studies. The results are summarized in Table 8 & 9.

**Table No.8 Intraday and Inter-day Precision Data for Paracetamol at Selected Concentrations**

| Drug | Conc.<br>(µg/ml) | Intraday Precision |                 |       | Inter-day Precision |                 |       |
|------|------------------|--------------------|-----------------|-------|---------------------|-----------------|-------|
|      |                  | Mean ± SD          | % Amt.<br>Found | %RSD  | Mean ± SD           | % Amt.<br>Found | % RSD |
| PARA | 20               | 846.91±3.66        | 101.04          | 0.432 | 842.06±2.25         | 100.42          | 0.27  |
|      | 30               | 1221.34±1.50       | 99.21           | 0.12  | 1227.65±1.08        | 99.74           | 0.09  |
|      | 40               | 1618.20±1.54       | 99.72           | 0.095 | 1615.88±3.45        | 99.57           | 0.21  |

**Table No. 9 Intraday and Inter-day Precision Data for Nefopam Hydrochloride at Selected Concentrations**

| Drug | Conc.<br>(µg/ml) | Intraday Precision |                 |      | Inter-day Precision |                 |       |
|------|------------------|--------------------|-----------------|------|---------------------|-----------------|-------|
|      |                  | Mean ± SD          | % Amt.<br>Found | %RSD | Mean ± SD           | % Amt.<br>Found | % RSD |
| NEFO | 2                | 282.25             | 101.58          | 0.63 | 280.99              | 100.99          | 0.27  |
|      | 3                | 387.99             | 100.76          | 0.16 | 384.29              | 99.60           | 0.40  |
|      | 4                | 490.27             | 99.53           | 0.06 | 491.72              | 99.87           | 0.05  |

For Paracetamol, the intraday %RSD values were found to be between 0.095-0.432, while the inter-day %RSD ranged from 0.09-0.27.

For Nefopam Hydrochloride, the intraday %RSD was between 0.06-0.63, and the inter-day %RSD ranged from 0.05-0.40.

As per ICH Q2 (R1) guidelines, all intraday and inter-day %RSD values for both Paracetamol and Nefopam HCL were well below the acceptance limit of 2%. This indicates that the developed RP-HPLC method is highly precise, providing consistent and reproducible results, and is therefore suitable for routine quality control and bioanalytical applications.

**Accuracy:**

To confirm the accuracy of the method, 0.1ml of the tablet stock solution was mixed with the standard stock solution at concentration of 8, 10, 12 µg/mL. The mixture were then diluted to final volume of 10 ml using the mobile phase resulting in sample concentration 80%, 100%, 120 %.

**Table No.9: Preparation of Accuracy Samples for Paracetamol and Nefopam Hydrochloride at 80%, 100%, and 120% Levels**

| Accuracy Level | Tablet Solution Volume (mL) | Standard Solution Volume (mL) | Final Volume (mL) | Final Concentration (µg/mL) |
|----------------|-----------------------------|-------------------------------|-------------------|-----------------------------|
| 80%            | 0.1                         | 0.08                          | 10                | 1.6 (PARA), 16 (NEFO)       |
| 100%           | 0.1                         | 0.10                          | 10                | 2.0 (PARA), 20 (NEFO)       |
| 120%           | 0.1                         | 0.12                          | 10                | 2.4 (PARA), 24 (NEFO)       |

**Table No.10: Accuracy and Recovery Study of Paracetamol and Nefopam Hydrochloride at 80%, 100%, and 120% Spiking Levels**

| Drug | Level (%) | Amt. Taken (µg/ml) | Amt. Added (µg/ml) | Amt. Found (Mean ± SD, µg/ml) | Amt. Recovered (Mean ± SD, µg/ml) | %Recovery (Mean ± SD) |
|------|-----------|--------------------|--------------------|-------------------------------|-----------------------------------|-----------------------|
| PARA | 80        | 20                 | 16                 | 35.81 ± 0.106                 | 15.81 ± 0.106                     | 98.84 ± 0.66          |
|      | 100       | 20                 | 20                 | 39.66 ± 0.048                 | 19.66 ± 0.048                     | 98.32 ± 0.24          |
|      | 120       | 20                 | 24                 | 43.75 ± 0.045                 | 23.75 ± 0.045                     | 98.97 ± 0.19          |
| NEFO | 80        | 2                  | 1.6                | 3.58 ± 0.003                  | 1.58 ± 0.003                      | 98.63 ± 0.22          |
|      | 100       | 2                  | 2.0                | 3.97 ± 0.013                  | 1.96 ± 0.013                      | 98.60 ± 0.65          |
|      | 120       | 2                  | 2.4                | 4.37 ± 0.010                  | 2.37 ± 0.010                      | 98.62 ± 0.42          |

The method demonstrated excellent accuracy, with % Recovery values for both Paracetamol and Nefopam Hydrochloride ranging between 98-99% across all concentration levels (80%, 100%, and 120%). The low standard deviation indicates minimal variation, confirming the methods reliability for accurate quantification of both drugs.

**Robustness:**

Robustness are evaluated by altering the parameter included saturation time, analytical mobile phase composition, detection wavelength, analyte concentration, and flow rate. The optimized chromatographic conditions were confirmed by varying the flow rate by  $\pm 0.1$  mL/min, adjusting the mobile phase composition by  $\pm 1\%$ , and modifying the detection wavelength by  $\pm 1$  nm.

**Table No 11: Robustness Evaluation of the Developed RP-HPLC Method for Paracetamol and Nefopam Hydrochloride**

| Sr. No | Drug | Parameter | Area    | Mean    | SD   | RSD  |
|--------|------|-----------|---------|---------|------|------|
| 1      | PARA | 79+21     | 1178.25 | 1177.8  | 0.66 | 0.06 |
|        |      |           | 1177.32 |         |      |      |
|        |      | 81+19     | 1178.91 | 1178.97 | 0.08 | 0.01 |
|        |      |           | 1179.03 |         |      |      |
|        |      | 254       | 1089.61 | 1088.4  | 1.68 | 0.15 |
|        |      |           | 1087.24 |         |      |      |
|        |      | 256       | 1148.67 | 1148.06 | 0.87 | 0.08 |
|        |      |           | 1147.06 |         |      |      |
| 2      | NEFO | 79+21     | 368.34  | 367.7   | 0.86 | 0.23 |
|        |      |           | 367.12  |         |      |      |
|        |      | 81+19     | 369.78  | 370.47  | 0.97 | 0.26 |
|        |      |           | 371.15  |         |      |      |
|        |      | 254       | 350.7   | 350.6   | 0.16 | 0.05 |
|        |      |           | 350.47  |         |      |      |
|        |      | 256       | 383.66  | 383.12  | 0.76 | 0.20 |
|        |      |           | 382.58  |         |      |      |

The robustness study demonstrated that minor deliberate variation in chromatographic conditions, such as mobile phase composition (79:21 and 81:19) and detection wavelength (254 nm and 256 nm), did not significantly affect the peak areas of Paracetamol and Nefopam Hydrochloride. The %RSD values were found to be below 2% for all parameters, indicating high precision and reliability. These results confirm that the developed RP-HPLC method is robust and suitable for routine quality control analysis.

#### **System Suitability Parameter:**

The standard solutions (20 $\mu$ g/ml PARA and 2 $\mu$ g/ml NEFO) were injected into two replicates in order to determine the PARA & NEFO system appropriateness parameters. The RSD value for the chosen medicines' peak regions and retention times was calculated. The calculated values were contrasted with the ICH's suggested limits. Every value falls within reasonable bounds.

**Table No.12: System Suitability Parameters for Quantitative Analysis of Paracetamol and Nefopam Hydrochloride by RP-HPLC**

| Drug | Co nc. | Area    | Mea n  | Amoun t Found | AM      | %Amou nt Found | SD   | %RSD |
|------|--------|---------|--------|---------------|---------|----------------|------|------|
| PARA | 20     | 851.166 | 848.98 | 20.26         | 1.01300 | 101.30         | 3.09 | 0.36 |
|      |        | 846.802 |        |               |         |                |      |      |
| NEFO | 2      | 280.706 | 280.44 | 2.01          | 1.00735 | 100.74         | 0.38 | 0.13 |
|      |        | 280.171 |        |               |         |                |      |      |

The developed RP-HPLC method showed %amount found within 98-102% and %RSD values below 2% (0.36% for Paracetamol and 0.13% for Nefopam), indicating high accuracy, precision, and reliability. This confirms the methods suitability for routine quality control drugs.

### Discussion:

The present study describes the successful development and validation of a rapid, sensitive, and robust RP-HPLC method for the simultaneous estimation of Paracetamol and Nefopam in human plasma. By employing a QbD-driven approach, critical chromatographic parameters were systematically optimized to achieve improved resolution, reproducibility, and peak symmetry. The use of a C18 stationary phase in combination with a mobile phase of acetonitrile and 0.1% orthophosphoric acid enabled sharp, well-resolved peaks with retention times of 6.220 and 7.410 minutes for Paracetamol and Nefopam, respectively. These chromatographic characteristics confirm the method's suitability for routine analysis.

The method exhibited excellent linearity across the ranges of 10–50 µg/mL for Paracetamol and 1–5 µg/mL for Nefopam, with %RSD values within bioanalytical acceptance criteria. Importantly, no significant matrix interference was observed, demonstrating high selectivity in the presence of endogenous plasma components. Accuracy, precision, recovery, and stability evaluations also complied with international regulatory guidelines, ensuring robustness for bioanalytical applications.

Compared to previously reported methods for individual or simultaneous estimation of these drugs, the developed method offers several advantages. Earlier studies have reported longer run times, complex gradient elution programs, or higher flow rates, which not only reduced throughput but also increased solvent consumption and operational cost. Other reports indicated matrix interference issues in plasma samples or limited linearity ranges, restricting their clinical applicability. In contrast, the present method achieves faster analysis with shorter retention times, reduced solvent usage, and straightforward isocratic elution, making it both cost-effective and environmentally favorable. Furthermore, by integrating a QbD-based optimization strategy, the method demonstrates enhanced reproducibility and robustness, addressing limitations of conventional trial-and-error method development approaches.

Taken together, the developed RP-HPLC method not only fulfills bioanalytical validation criteria but also offers operational simplicity and efficiency, making it highly suitable for pharmacokinetic, bioequivalence, and therapeutic drug monitoring studies. Its advantages over previously published methods underscore its contribution as a reliable and practical analytical tool in clinical and regulatory settings.

### **Conclusion:**

This study successfully developed an RP-HPLC-based bioanalytical method for the simultaneous quantification of paracetamol and Nefopam in human plasma. The proposed method demonstrated excellent sensitivity, accuracy, and precision, confirming its robustness and reproducibility. Importantly, this is the first QbD-optimized method to simultaneously quantify Paracetamol and Nefopam hydrochloride in human plasma while incorporating validated green chemistry metrics, effectively addressing a critical gap in clinical bioanalysis.

By systematically optimizing critical method parameters through a QbD approach, this method ensures enhanced robustness and sustainability. Furthermore, its validated performance makes it a valuable analytical tool for a wide range of *in vivo* applications, including pharmacokinetic evaluations, drug distribution profiling, plasma protein binding assessments, and drug metabolism studies, ultimately supporting advanced therapeutic drug monitoring and bioequivalence research

### **References:**

1. Isailovic, N., Daigo, K., Mantovani, A. and Selmi, C. (2015) Interleukin-17 and innate immunity in infections and chronic inflammation. *J. Autoimmun.*, 60: 1-11.
2. Todd, I., Spickett, G. and Fairclough, L. (2015), *Lecture Notes: Immunology*. John Wiley & Sons, New York
3. Halliwell B, Hoult JR, Blake DR. Oxidants, inflammation, and anti-inflammatory drugs. *The FASEB journal*. 1988 Oct;2(13):2867-73.
4. Swierkosz TA, Jordan L, McBride M, McGough K, Devlin J, Botting RM. Actions of paracetamol on cyclooxygenases in tissue and cell homogenates of mouse and rabbit. *Medical science monitor: international medical journal of experimental and clinical research*. 2002 Dec 1;8(12):BR496-503.
5. Sharma CV, Mehta V. Paracetamol: mechanisms and updates. *Continuing Education in Anaesthesia, Critical Care & Pain*. 2014 Aug 1;14(4):153-8.
6. Nabal M, Librada S, Redondo MJ, Pigni A, Brunelli C, Caraceni A. The role of paracetamol and nonsteroidal anti-inflammatory drugs in addition to WHO Step III opioids in the control of pain in advanced cancer. A systematic review of the literature. *Palliative Medicine*. 2012 Jun;26(4):305-12.
7. Farquhar H, Stewart A, Mitchell E, Crane J, Eysers S, Weatherall M, Beasley R. The role of paracetamol in the pathogenesis of asthma. *Clinical & Experimental Allergy*. 2010 Jan;40(1):32-41.
8. Vestergaard P, Hermann P, Jensen JE, Eiken P, Mosekilde L. Effects of paracetamol, non-steroidal anti-inflammatory drugs, acetylsalicylic acid, and opioids on bone mineral density and risk of fracture: results of the Danish Osteoporosis Prevention Study (DOPS). *Osteoporosis International*. 2012 Apr;23:1255-65.
9. Golding GM. The Role of Paracetamol (acetaminophen) in the Reduction of Tremor in Parkinson's Disease—a Case Study. *British Journal of Pharmacy*. 2020 Apr 15;4(2).

10. Forrest JA, Clements JA, Prescott LF. Clinical pharmacokinetics of paracetamol. *Clinical pharmacokinetics*. 1982 Apr;7:93-107.
11. Romsing J, Moiniche S, Dahl JB. Rectal and parenteral paracetamol, and paracetamol in combination with NSAIDs, for postoperative analgesia. *Br J Anaesth* 2002; 88: 215-26
12. Shrestha BR, Pradhananga RR. Spectrophotometric method for the determination of paracetamol. *Journal of Nepal Chemical Society*. 2009;24:39-44.
13. Vilchez J, Blanc R, Avidad R, Navalón A. Spectrofluorimetric determination of paracetamol in pharmaceuticals and biological fluids. *Journal of pharmaceutical and biomedical analysis*. 1995 Aug 1;13(9):1119-25.
14. Goyal RN, Singh SP. Voltammetric determination of paracetamol at C60-modified glassy carbon electrode. *Electrochimica Acta*. 2006 Apr 1;51(15):3008-12.
15. Goyal RN, Gupta VK, Chatterjee S. Voltammetric biosensors for the determination of paracetamol at carbon nanotube modified pyrolytic graphite electrode. *Sensors and Actuators B: Chemical*. 2010 Aug 6;149(1):252-8.
16. Ruengsitagoon W, Liawruangrath S, Townshend A. Flow injection chemiluminescence determination of paracetamol. *Talanta*. 2006 Jun 15;69(4):976-83.
17. Moreira AB, Oliveira HP, Atvars TD, Dias IL, Neto GO, Zagatto EA, Kubota LT. Direct determination of paracetamol in powdered pharmaceutical samples by fluorescence spectroscopy. *Analytica Chimica Acta*. 2005 May 10;539(1-2):257-61.
18. Belal T, Awad T, Clark R. Determination of paracetamol and tramadol hydrochloride in pharmaceutical mixture using HPLC and GC-MS. *Journal of chromatographic science*. 2009 Nov 1;47(10):849-54.
19. Gassel MM, Diamantopoulos E, Petropoulos V, Hughes AC, Ballesteros ML, ReON. Controlled clinical trial of oral and parenteral nefopamhydrochloride. A novel and potent analgesic drug. *J Clin Pharmacol* 1976;16:34–41
20. Pasutharnchat K, Wichachai W, Buachai R. Analgesic efficacy of nefopam for cancer pain: a randomized controlled study. *F1000Research*. 2020 May 19;9:378.
21. Evans MS, Lysakowski C, Tramèr MR. Nefopam for the prevention of postoperative pain: quantitative systematic review. *British journal of anaesthesia*. 2008 Nov 1;101(5):610-7.
22. Mimos O, Chauvet S, Grégoire N, Marchand S, Le Guern ME, Saleh A, Couet W, Debaene B, Levy RH. Nefopam pharmacokinetics in patients with end-stage renal disease. *Anesthesia & Analgesia*. 2010 Nov 1;111(5):1146-53.
23. Tiglis M, Neagu TP, Elfara M, Diaconu CC, Bratu OG, Vacaroiu IA, et al. Nefopam and its role in modulating acute and chronic pain. *Rev Chim (Bucharest)*. 2018;69(10):2877–2880. Doi: doi.org/10.37358/RC.18.10.6644
24. Aymard G, Warot D, Démolis P, Giudicelli JF, Lechat P, Le Guern ME, Alquier C, Diquet B: Comparative pharmacokinetics and pharmacodynamics of intravenous and oral nefopam in fasting healthy volunteers. *Pharmacol Toxicol* 2003;92:279–286
25. Piercey MF, Schroeder LA. Spinal and supraspinal sites for morphine and nefopam analgesia in the mouse. *Eur J Pharmacol* 1981; 74: 135–40
26. Starek M, Dąbrowska M. Development and validation of a TLC-densitometry method for quantitative analysis of nefopam hydrochloride beside its degradation products. *Journal of Analytical Chemistry*. 2012 Aug;67:733-9.
27. Chang SF, Hansen CS, Fox JM, Ober RE. Quantitative determination of nefopam in human plasma, saliva and cerebrospinal fluid by gas—liquid chromatography using a nitrogen-selective detector. *Journal of Chromatography B: Biomedical Sciences and Applications*. 1981 Nov 13;226(1):79-89.

28. Singh SU, Sharma NE, Singla YP, Arora SA. Development and validation of UV-Spectrophotometric method for quantitative estimation of nefopam hydrochloride in polymethacrylate nanospheres. *Int J Pharm Pharm Sci.* 2016;8(1):414-19.
29. International Conference on Harmonisation (ICH). ICH Q8(R2): Pharmaceutical Development. Geneva: ICH; 2009.
30. [Kachave RN, Varungase AT, Mandlik PB, Wakchaure SR. Bioanalytical method development and validation for simultaneous determination of paracetamol, tramadol HCL, domperidone tablet formulation by RP-HPLC: Its pharmacokinetic applications. *science.* 2018;8:11
31. Babu R, Rao AL, Rao JV. Bioanalytical method development and validation for simultaneous estimation of paracetamol and cefixime by using RP-HPLC in rabbit plasma. *Oriental Journal of Chemistry.* 2016;32(1):701.
32. Gorain B, Choudhury H, Nandi U, Das A, Dan S, Pal TK. Development and validation of an HPLC method for simultaneous detection and quantification of paracetamol and etodolac in human plasma and its application to a pharmacokinetic study. *Journal of AOAC International.* 2013 May 1;96(3):573-9.
33. Gawde O, Nayak S, Vaidhun B. Analysis Of Nefopam Hydrochloride From Bulk And Dosage Form Using Validated Rp Hplc Method.
34. Aymard G, Warot D, Demolis P, Laville I, Diquet B. Sensitive determination of nefopam and its metabolite desmethyl-nefopam in human biological fluids by HPLC. *Journal of pharmaceutical and biomedical analysis.* 2002 Nov 7;30(4):1013-21.
35. European Medicines Agency (EMA). Guideline on bioanalytical method validation [Internet]. London: EMA; 2011 [cited 2025 Jul 24]. Available from: [https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf)