Essential Chem

ISSN: 2837-8083 (Online) Journal homepage: www.tandfonline.com/journals/tess20

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To cite this article: P. Suresh , Gunjan Jadon & Panneerselvam Theivendren (2025) Development and validation of a RP-HPLC method for Milrinone quantification using analytical quality by design principles: stability, precision, and method optimization, Essential Chem, 2:1, 2502833, DOI: 10.1080/28378083.2025.2502833

To link to this article: https://doi.org/10.1080/28378083.2025.2502833

Published online: 04 Jun 2025.

ESSENTIAL CHEM
2025, VOL. 2, NO. 1, 2502833
https://doi.org/10.1080/28378083.2025.2502833

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Development and validation of a RP-HPLC method for Milrinone quantification using analytical quality by design principles: stability, precision, and method optimization

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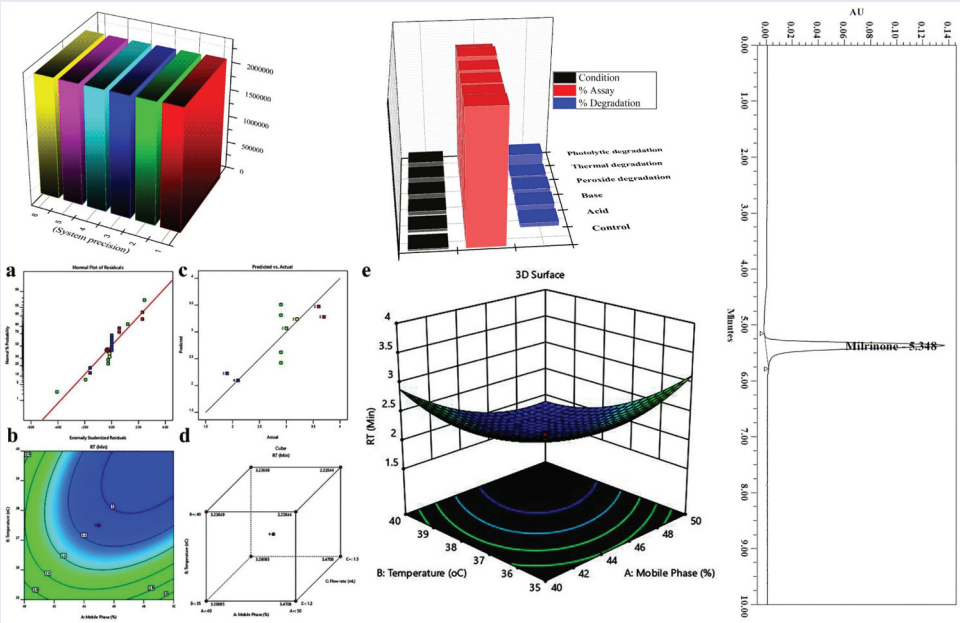
ABSTRACT

A research study demonstrates successful implementation of Analytical Quality by Design for creating a solid and dependable Reverse Phase High-Performance Liquid Chromatography technique to determine Milrinone concentrations. The study presents evidence for using AQbD principles during method development practices. Response surface methodology underwent optimization with chromatographic conditions to establish proper separation resulting in a 1.901 minutes retention time. The implementation of Reverse Phase High-Performance Liquid Chromatography demonstrates how AQbD provides method precision as well as accuracy and reliability during Milrinone quantification. The system suitability test produced smooth peaks with high efficiency that generated USP tailing factor 1.0 and plate count 10,795. The precision tests showed that the method possessed 0.3% for %RSD thus indicating strong method reliability and precision. The developed method achieved outstanding results through all test conditions which demonstrated linearity with R^2 = 0.999 alongside accuracy through recovery rates of 99.9–101.1% while performing robustly in minor variation tests. The validated tests showed excellent reliability because they proved high accuracy alongside precision and linearity. The stability experiments demonstrated different stress conditions but the main challenge was photolytic degradation.



ARTICLE HISTORY

Received March 05, 2025
Accepted May 02, 2025

KEYWORDS

Analytical quality by design; method validation; Milrinone quantification; RP-HPLC method; stability studies

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1. Introduction

The pharmaceutical industry is tasked with the critical responsibility of ensuring that all therapeutic drugs are both safe and effective for patient use. This demands not only strict regulatory standards but also the application of advanced analytical techniques to verify the quality and stability of pharmaceutical formulations. In the field of analytical chemistry, Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) has emerged as one of the most widely utilized techniques for the quantitative analysis of pharmaceutical compounds. The method's precision, sensitivity, and ability to effectively separate complex mixtures make it indispensable for the quality control of drugs. However, with the increasing complexity of pharmaceutical formulations, there is a growing need for more systematic and optimized approaches to analytical method development. AQbD is one such approach that has transformed traditional practices by incorporating risk-based methodologies, robust statistical tools, and comprehensive control strategies to improve method reliability.[1–3]

The concept of Quality by Design (QbD) has its roots in manufacturing processes but has since been extended to various aspects of pharmaceutical development, including analytical method development under the framework of AQbD. Unlike conventional analytical method development, which typically follows an empirical or trial-and-error approach, AQbD is based on a structured and systematic methodology aimed at achieving predefined objectives. The ultimate goal of AQbD is to develop robust, reliable, and reproducible analytical methods that meet stringent regulatory standards and ensure consistent drug quality.[4,5]

AQbD emphasizes the use of a science-driven and risk-based approach to method development. It begins with defining the Analytical Target Profile (ATP), which outlines the critical quality attributes (CQAs) that the method must meet, such as precision, accuracy, linearity, sensitivity, and robustness. After defining the ATP, the key parameters influencing the method's performance, known as Critical Method Attributes (CMAs) and Critical Process Parameters (CPPs), are identified. These parameters are systematically investigated using statistical tools, such as Design of Experiments (DoE), to optimize the method and understand the interactions between different variables. The ultimate aim is to ensure that the method is capable of delivering consistent results, even when there are variations in experimental conditions.[6,7]

By applying AQbD principles, analysts can develop a more profound understanding of their methods and mitigate potential risks that could impact the quality of the analysis. This approach is particularly valuable in the pharmaceutical industry, where the accuracy and precision of analytical methods are paramount for ensuring the safety and efficacy of drugs. AQbD also aligns with regulatory expectations for method validation, as it provides a clear scientific rationale for method development and offers comprehensive documentation of the method's robustness and reliability.[8–10]

The pharmacological agent Milrinone works as a powerful phosphodiesterase-3 inhibitor for treating heart failure which occurs when the heart becomes defective at blood pumping. Milrinone enhances cardiac output by increasing cAMP compounds within cells to boost myocardial contractions together with vasodilatation. The combination of inotropic and vasodilatory properties makes Milrinone an important pharmaceutical agent for treating unresponsive patients with acute heart failure. Adequate dosage control of Milrinone in cardiovascular therapy remains essential because it supports patient treatment success and protects against side effects. The exact determination of Milrinone along with its precise quantification in pharmaceutical products remains essential both for maintaining product quality and stability evaluation purposes. The analysis of Milrinone concentration requires an analytical method which presents high precision accuracy and reproducibility due to its profound effects on patient health outcomes. RP-HPLC needs a reliable method for Milrinone quantification because the drug shows sensitivity to light and oxidative environments and high temperatures. There are several different stress conditions used in forced degradation studies to test compound stability which serve as method development tools to verify the analytical method's ability to accurately measure the compound including its degradation products. Evaluation of drug shelf life needs this method to validate safety and effectiveness throughout time.[11,12] Multiple researchers developed validated RP-HPLC techniques to measure milrinone concentrations through precision-based studies that utilized analytical quality by design (AQbD) principles for stability optimization. Research teams studied three aspects of mobile phase composition and flow rates and detection wavelengths to improve both separation efficiency and measurement precision. The implementation of AQbD approaches which include method robustness evaluation and risk assessments leads to improved method reliability and better stability. The stability-indicating methods showed effective validation results which ensure degradation products will not affect the quantification process. Research studies

confirm that accurate clinical milrinone observation requires assessment of LOD, LOQ, and method reproducibility.[13–16] The previous strategies used to develop RP-HPLC methods for Milrinone detection have multiple shortcomings which include unstable methods coupled with imprecise results along with insufficient optimization steps. The implementation of analytical quality by design principles together with sensitivity-versus-reproducibility balance issues creates difficulties for the adoption of these methods on a broader scale. Enhanced optimization and precision as well as stability result from using RP-HPLC combined with Analytical Quality by Design principles for Milrinone quantification. Systematic optimization of method development through this approach leads to high reliability and reproducibility which provides a new dependable approach to quantify Milrinone in pharmaceutical formulations. In this study, AQbD principles were applied to the development of an RP-HPLC method for the quantification of Milrinone. The goal was to create a method that was not only accurate and precise but also robust enough to perform consistently under varying experimental conditions. The application of AQbD allowed for the systematic optimization of chromatographic parameters and the evaluation of the method's performance in terms of system suitability, precision, filter evaluation, forced degradation, and robustness. To conclude, the AQbD principles were effectively applied for developing a RP-HPLC method to assay Milrinone that ensures building up an accurate and precise analytical technique. It systematically optimizes the chromatographic conditions and solves critical factors, which helps to improve method consistency and applicability; in turn ensures accuracy of quantitative determination results is necessary for maintaining drug quality security.

2. Materials and materials

This research obtained reagents and materials from well-established suppliers. The complimentary milrinone sample (98% purity) was obtained from Dr. Reddy's Laboratories within Hyderabad, India. The research utilized sodium hydroxide (NaOH) and analytical grade sodium phosphate obtained from Venus, Tamil Nadu, India together with ethanol purchased from the company as well. The supplementary reagents 30% hydrogen peroxide H_2O_2 and hydrochloric acid HCl together with acetonitrile and glacial acetic acid were purchased from the vendor Venus, Tamil Nadu, India. The production of HPLC-grade water occurred within the facility using the Inhouse Lab Water system. The development of chromatographic analysis took place with an Autosampler Shimadzu HPLC SIL-20A which was equipped with a column heater system and a UV-PDA detector. The database collection and processing occurred through Empower Software delivered by Shimadzu.

2.1. Analytical method development

2.1.1. Method development

The method development for the assay of Milrinone using HPLC begins with buffer preparation by dissolving 2.5 g of sodium phosphate in 1000 mL of deionized water and adjusting the pH to 4.0. Mobile phase, consisting of pH 4.0 buffer and acetonitrile in the different proportion for method optimization. An Inertsil ODS column (250 x 4.6 mm, 5 µm) was used with a flow rate of 1.5 mL/min and a detection wavelength of 220 nm.

2.1.2. Sample preparation

To prepare a sample concentration of 100 ppm, 50 mg of the drug was dissolved in 100 mL of solvent, ensuring thorough dissolution. Subsequent dilutions were performed by transferring 5 mL and 25 mL aliquots into distinct volumetric flasks and diluting each to a final volume of 100 mL with the identical solvent. The concentrations were determined using dilution factors to guarantee precision, and the final concentrations were documented in µg/mL for subsequent analysis and evaluation.

2.1.3. Response surface methodology

The experimental procedure involves adjusting key variables mobile phase, temperature, and flow rate to evaluate their effects on runtime. Each factor is set at coded levels, and multiple experiments are conducted to measure the response, which is runtime in this case. Data is collected on both actual and predicted values, and residuals are calculated to assess accuracy. An analysis of variance (ANOVA) is then performed to determine the significance of each factor. Significant model terms are identified, and a final equation is derived to predict the runtime based on actual factor levels, which can guide further optimization.[17–21]

2.2. Analytical method validation

2.2.1. System suitability

The chromatographic parameters were assessed for system adequacy according to USP tailing, plate counts, and the similarity factor of standard solutions. A sample of the prepared standard solutions was injected, and the chromatograms were analyzed.[19]

2.2.2. System precision

In order to evaluate the precision of the system, six consecutive injections of the standard solution were carried out, and the peak areas from each injection were recorded.[20]

2.2.3. Evaluation of filters

Various filtration processes were utilized to evaluate the assay percentage and recovery of distinct sub-fractions. Unfiltered samples were then tested to determine a baseline percentage assay. Subsequently, 2 mL, 4 mL, and 6 mL sub-fractions were filtered utilizing 0.45 µm nylon, PVDF, and PTFE filters. The results were subsequently recorded, comparing the assay % and recovery across various sub-fractions, emphasizing the efficacy of each filtration procedure in preserving the sample's integrity.[21]

2.2.4. Evaluation of forced degradation studies

The HCl, NaOH, peroxide, thermal degradation, and photolytic degradation, were also utilized in the forced degradation studies that were carried out in order to assess the drug's stability under a variety of stress conditions.[21]

2.2.5. Method precision

Within the scope of this procedure, the analysis of test percentages for Milrinone. Milrinone, is carried out over a total of six samples. Tests are performed on each sample in order to determine the percentage of Milrinone is present in the sample. It is possible to compute the mean, the standard deviation (SD), and the relative standard deviation (%RSD) for the data under consideration.[22]

2.2.6. Evaluation of linearity

It is necessary to prepare five different solutions of Milrinone, each of which has a different concentration level (50%, 80%, 100%, 120%, and 150%). The use of chromatographic analysis allows for the documentation of the mean peak area response of each solution as well as linear regression analysis.[22]

2.2.7. Evaluation of solution stability

To ensure stability of the standard and sample solutions it was evaluated at different time intervals to ascertain assay percentage and response variations. The response of the standard solution was measured, and the percentage difference from the original value was computed. The assay % for the sample solution was calculated, and the percentage deviation from the first assay was documented. Measurements were conducted at consistent intervals of 2, 4, 6, 8, 10, and extending to 48 hours. The data indicate continuous assessment of assay stability throughout time, with percentage variances for both solutions documented at each evaluation time point.[23]

2.2.8. Evaluation of accuracy

The precision of Milrinone was assessed at three concentrations: 50%, 100%, and 150%. At each level, designated quantities of Milrinone were administered, and the resultant amounts were documented. The percent recovery was computed for each solution, and the average percent recovery together with the relative standard deviation (%RSD) was established for each concentration level. Triplicate were examined for each level of accuracy.[24]

2.2.9. Evaluation of robustness

To assess the impact of different conditions on chromatographic performance, particular parameters were methodically altered. The flow rate was modified to 1.35 mL/min and 1.65 mL/min, assessing tailing, plate counts, %RSD of areas in standard injections, and % similarity factor for each condition. The organic content in the mobile phase was modified by ±2%, with results documented accordingly. The column oven temperature was established between 35°C and 45°C, while the detection wavelength was adjusted between 218 nm, and 222 nm. The pH of the mobile phase was changed to 3.8, and 4.2, with all findings recorded for analysis. Utilizing optimum HPLC settings, and an evaluation of the system's applicability, precision, filtering efficacy, forced degradation, sample preparation, method development, precision, linearity, solution stability, accuracy, and robustness for Milrinone was carried out.

3. Results

The potential of AQbD to improve analytical method development is shown by its successful application to the development of the RP-HPLC method for Milrinone quantification. AQbD guarantees that analytical techniques are dependable and strong, thereby satisfying the high requirements for pharmaceutical quality control.

3.1. Milrinone

Milrinone is a small molecule compound that has the chemical formula $C_{12}H_9N_3O$ and molecular weight of 211.22 g/mol. It has pH-dependent solubility in water, and is soluble in solvents such as DMSO, ethanol, and methanol. It possesses a pKa of around 6.4 and LogP =.1 demonstrating its hydrophilic property. Milrinone is given intravenously in the clinical setting therefore, the intravenous bioavailability of

Milrinone is 100%. It has a 70% to 80% protein binding rate, and is mainly excreted unchanged in the urine, requiring dose changes for patients with renal impairment. In patients with congestive heart failure, the average elimination half-life is 2.3 hours Table 1.

Milrinone is effective for the treatment of cardiac function but its use may accompany adverse effect such as arrhythmias hypotension and headache.

3.2. Analytical method development

3.2.1. Method development

The development test for Milrinone by RP-HPLC entails the optimization of chromatographic parameters to get effective separation with minimized retention durations. A buffer solution was made by dissolving 2.5 g of sodium dihydrogenphosphate in 1000 MilliQ of deionized water and adjusting the pH to 4.0 with ortho phosphoric acid. The solution was subjected to filtration using a 0.45 µm membrane filter. The mobile phase was prepared by mixing pH 4.0 buffer and acetonitrile in the different proportion for method optimization. The mobile phase was subsequently degassed to ensure optimal performance. A diluent comprising water and acetonitrile in a 60:40 ratio was utilized for sample preparation of sample, needle wash solution, and standard solution. The chromatographic conditions utilized reverse-phase high-performance liquid chromatography in isocratic mode. An Inertsil ODS column (250 x 4.6 mm, 5 µm) was employed, operating at a flow rate of 1.5 mL/min, an injection volume of 10 µL, and a detection wavelength of 220 nm. To prepare the standard solution, 50 mg of Milrinone reference standards were measured, dissolved in 50 mL of diluent, sonicated, and subsequently diluted to a final volume of 100 mL. A 5 mL aliquot of this stock solution was diluted to 25 mL to achieve a 100-ppm standard solution.

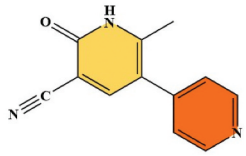
The retention times (RT) for Milrinone, in the initial method was performed with the identical with mobile phase composition buffer and acetonitrile in the ratio of 70:30, temperature 30°C and flow rate of 1.0 mL/min, resulting in a retention time of **5.384** minutes for Milrinone, as depicted in Figure 1.

The second trial was performed with the identical composition with mobile phase composition buffer and acetonitrile in the ratio of 60:40, column oven temperature 35°C and flow rate of 1.2 mL/min, resulting in a retention time of **3.006** minutes for Milrinone, as depicted in Figure 2.

3.2.2. Response surface methodology

The Analytical Target Profile (ATP) establishes critical quality attributes (CQAs) which the method needs to fulfill through the variables of accuracy, precision, sensitivity and robustness. An assessment determines and identifies all risks that could affect method performance specifically related to variations in solvent, organic modifier, flow rate, injection volume and temperature parameters. The selection of Critical Method Attributes and Critical Process Parameters takes place through systematic investigation using Design of Experiments

Table 1. Chemical structure and key physicochemical properties of 6-methyl-2-oxo-5-pyridin-4-yl-1 H-pyridine-3-carbonitrile, including molecular weight, solubility, pKa, LogP, half-life, TPSA, hydrogen bonding, and rotatable bonds.



Properties	Property
IUPAC Name	6-methyl-2-oxo-5-pyridin-4-yl-1 H-pyridine-3-carbonitrile
Chemical Formula	$C_{12}H_9N_3O$
Molecular Weight	211.22 g/mol
Solubility	Soluble in water (pH-dependent), freely soluble in DMSO, ethanol, and methanol
pKa	6.4
LogP	0.1
Half-life	2.3 hours
Topological Polar Surface Area	65.8Å^2
Heavy Atom Count	16
Hydrogen Bond Donor Count	1
Hydrogen Bond Acceptor Count	3
Rotatable Bond Count	1
Covalently-Bonded Unit Count	1
SMILES	CC1 = CC2 = C(C = NC = N2)C(=O)C3 = CC = CC = C13

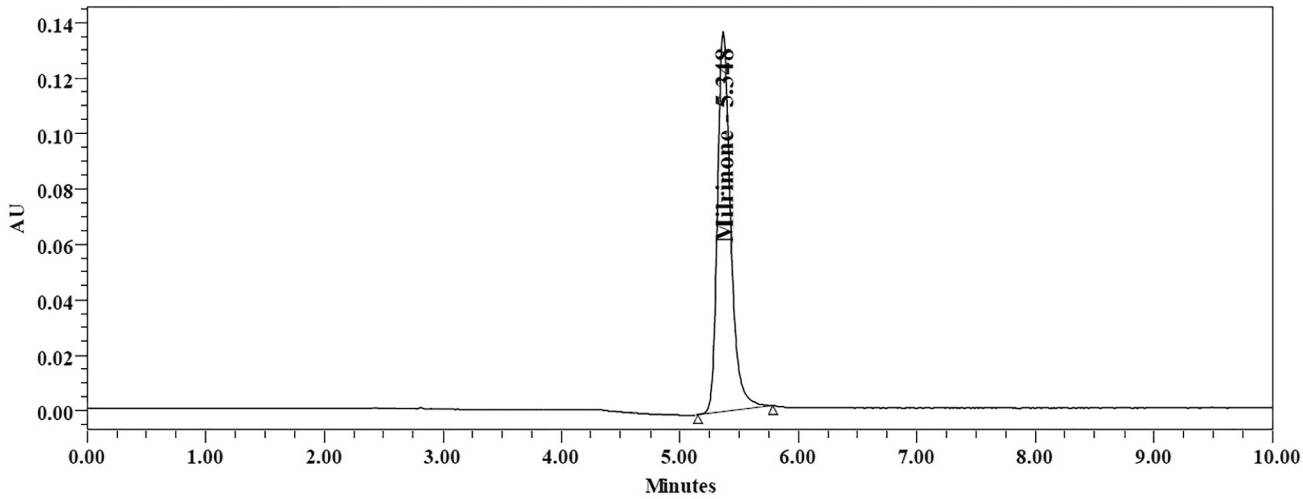


Figure 1. Chromatographic profile of Milrinone (trial-1) analyzed using Empower 3 software, showing RT at 5.384 minutes.

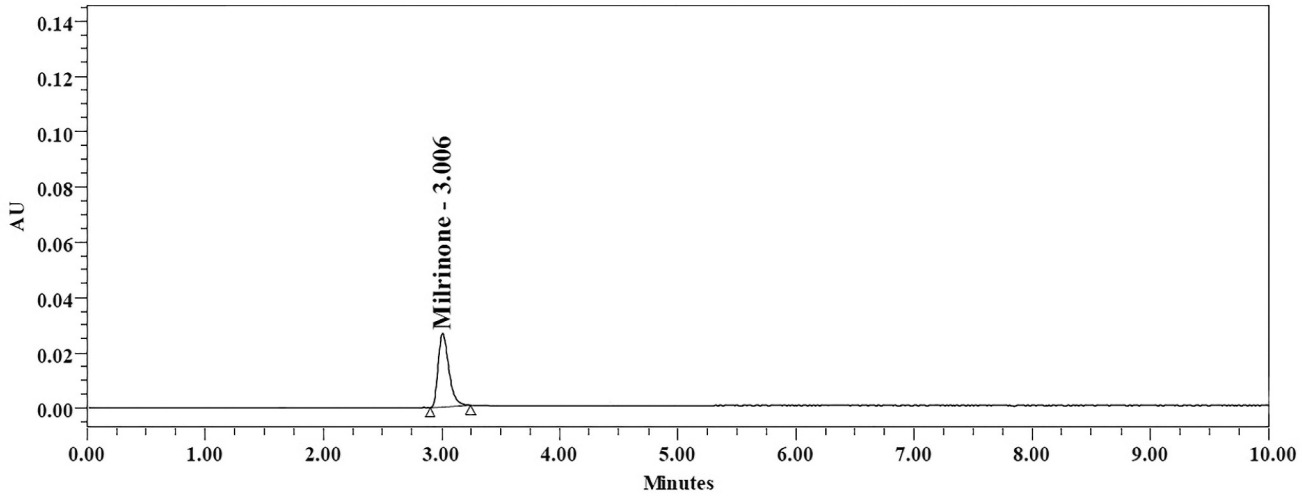


Figure 2. Chromatographic profile of Milrinone (trial-2) analyzed using Empower 3 software, showing RT at 3.006 minutes.

which ensures optimal parameter selection with 50 experimental runs. The risk-based selection procedure leads to reliable results which meet the requirements of AQbD principles. The RP-HPLC retention time of Milrinone depends on the solvent composition (A) and organic modifier (B) concentrations according to the 3D surface plot. The retention time shows minimal shift from its 1.8-minute duration as the solution temperature ranges between various concentrations of solvent together with organic modifier content. The method produced reproducible and stable measurement results for Milrinone quantification because researchers optimized testing conditions (C: Temperature = 35°C, D: Flow rate = 1.25 mL/min, E: Injection volume = 15 µL). Selective conditions have been achieved to maintain a steady zone for reliable method outcomes Figure 3(a).

The surface map demonstrates the interaction between solvent (A) concentration and organic modifier (B) concentration when determining injection volume (µL) in RP-HPLC analysis. The evaluation shows that the constant injection volume stays at 15 µL throughout all experimental conditions of solvent concentrations and organic modifier amounts. Experimental conditions associated with design points show that the ideal volume to inject is approximately 15 µL. Method reliability and consistent performance for the Milrinone measurements exist due to operating at 35°C with a flow rate of 1.25 mL/min Figure 3(b).

The flow rate (mL/min) during RP-HPLC analysis can be seen in the 3D surface plot because of solvent concentration (A) and organic modifier concentration (B). The flow rate maintains a stable value at 1.25 mL/min throughout different concentrations of solvent along with organic modifier. The designed experimental points verify the optimal flow rate for stable chromatographic operation. The flow rate

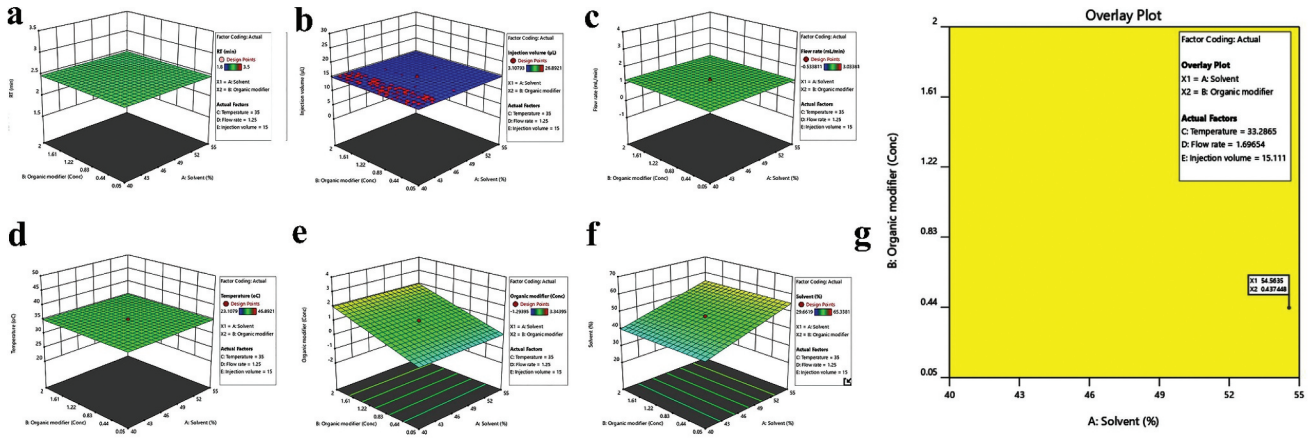


Figure 3. 3D response surface plots showing the effect of solvent percentage (a) and organic modifier (b) on various chromatographic parameters. Overlay plot (g) illustrates optimal conditions for method development.

stabilization at 1.25 mL/min combined with a temperature of 35°C in this method generates dependable Milrinone measurement outcomes while proving that solvent and organic modifier concentration changes do not alter the flow rate Figure 3(c).

The 3D surface plot illustrates the column temperature (°C) changes according to solvent concentration (A) and organic modifier concentration (B) during RP-HPLC analysis. The plot demonstrates that the temperature stays at 35°C throughout all variations of solvent concentrations plus organic modifier concentrations without showing any notable changes. Reproducible chromatographic performance together with stability results from the design points that present the best temperature settings. This method provides stable results for Milrinone measurement at 35°C temperature along with a flow rate of 1.25 mL/min and an injection volume of 15 µL Figure 3(d).

The 3D surface plot visualizes how solvent concentration (A) and organic modifier concentration (B) affect the organic modifier level in RP-HPLC analysis. The organic modifier concentration shows minimal changes during different solvent percentages in the response measurements according to the plot data. The design points display the most suitable concentration values for organic modifier control allowing reliable and consistent chromatographic results. The method delivers reliable and repeatable Milrinone results because of controlled 35°C temperature and 1.25 mL/min flow rate combined with a set injection volume of 15 µL Figure 3(e).

The 3D surface chart depicts how adjusting A (solvent concentration) and B (organic modifier concentration) impacts the RP-HPLC analysis solvent percentage. The solvent concentration shows minimal fluctuations between 40% and 50% which demonstrates that changing organic modifier amounts does not impact solvent percentage measurements. Design points in the optimized region of the solvent percentage determine the most stable chromatographic performance conditions. At 35°C temperature and 1.25 mL/min flow rate and 15 µL injection volume the method produces dependable quantification results of Milrinone Figure 3(f).

General Equation

$$RT=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_5+\beta_1X_1{}^2\beta_2X_2{}^2+\beta_3X_3{}^2+\beta_4X_4{}^2+\beta_5X_5{}^2+\beta12X1X2+\beta_3X_1X_3+\beta_4X_1X_4+\beta_5X_1X_5+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{25}X_2X_5\beta_{34}X_3X_4+\beta_{35}X_3X_5+\beta_{45}X_4X_5+\epsilon$$

β0 is the intercept (constant),

β1, β2, β3, β4, β5 are the coefficients for the factors X1 (solvent), X2 (organic modifier), X3 (temperature), X4 (flow rate), and X5 (injection volume), ϵ represents the error term.

The total explained variability by the model corresponds to the individual factors including solvent and organic modifier and temperature and flow rate produced significant impact on the response because their sum of squares measured with mean squares. The significant factor in this experiment is mobile phase, temperature, flow rate, and pH.

The 3D surface chart depicts how adjusting A (solvent concentration) and B (organic modifier concentration) impacts the RP-HPLC analysis solvent percentage. The solvent concentration shows minimal fluctuations between 40 and 50% which demonstrates that changing organic modifier amounts does not impact solvent percentage measurements. Design points in the optimized region of the solvent percentage determine the most stable

chromatographic performance conditions. At 35°C temperature and 1.25 mL/min flow rate and 15 µL injection volume the method produces dependable quantification results of Milrinone Figure 3(g). As per RSM report the final trial was performed with the identical composition with pH 4, Mobile Phase 50:50, column oven temperature 35°C and flow rate of 1.25 mL/min, resulting in a retention time of **1.901** minutes for Milrinone, as depicted in Figures 4 and 5.

3.3. Analytical method validation

3.3.1. System suitability

The system suitability test given indicates that the USP tailing factor is 1.0, which means that chromatographic analysis has a good peak symmetry. It shows an acceptable USP plate count (10795) attributed to a high column efficiency; vital for component separation. Further, the similarity percentage between standard 2 and standard 1 was found to be highest as at value of (98.9%) which clearly shows the gradation in terms of quality control during analysis by any means thus giving a accuracy feel for repetitiveness restraint condition percent on method. These criteria validate the system's appropriateness for precise and dependable quantification of Milrinone Table 2 and Figure 5.

3.3.2. System precision

The system precision demonstrates the reproducibility of the analytical method based on six injections, with

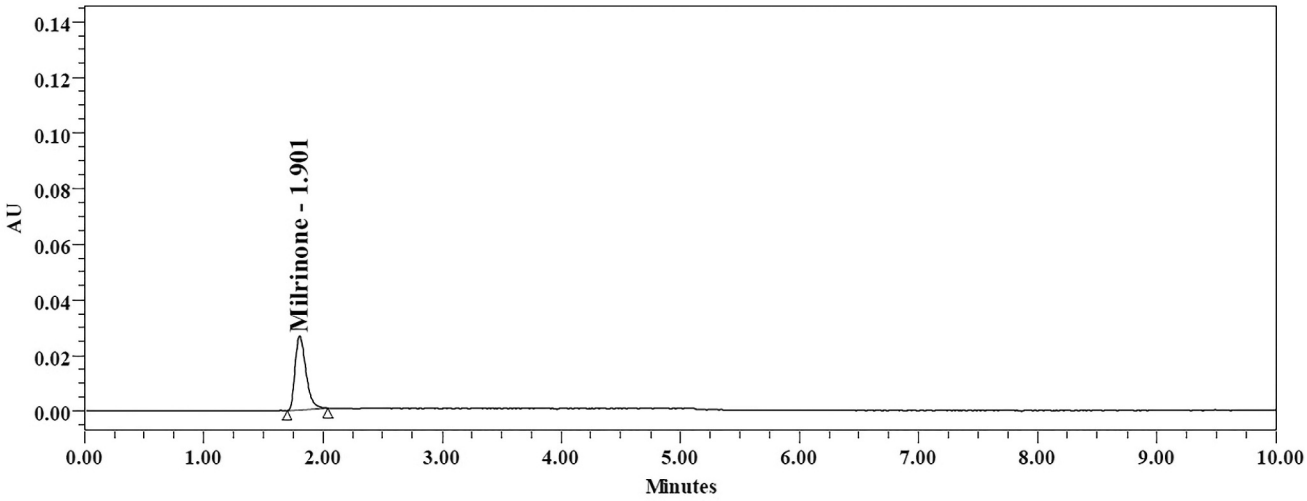


Figure 4. Chromatographic profile of Milrinone (trial-3) analyzed using Empower 3 software, showing RT at 1.901 minutes.

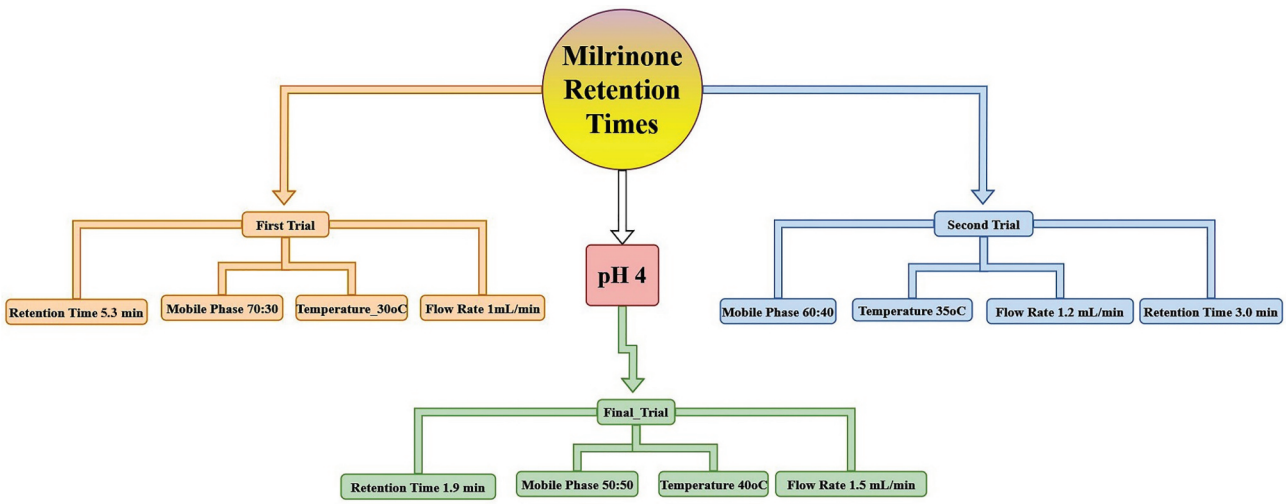
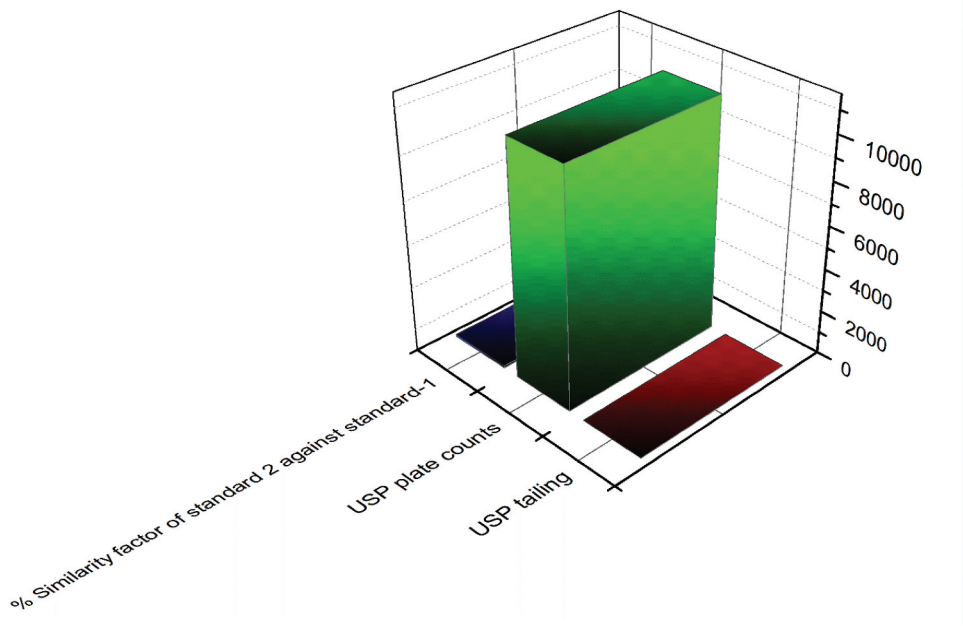


Figure 5. Flowchart illustrating Milrinone retention times under different conditions: trials with varying mobile phase ratios, temperatures, and flow rates at pH 4, detailing retention times for each setup.

Table 2 and Figure 5. System suitability parameters for Milrinone, including USP tailing (1.0), USP plate counts (10,795), and % similarity factor of standard 2 against standard 1 (98.9%).

System suitability	
USP tailing	1.0
USP plate counts	10795
% Similarity factor of standard 2 against standard-1	98.9



their respective area counts recorded. The relative standard deviation (%RSD) is 0.3%, which is very low, confirming high precision of the method. In chromatographic analyses, a %RSD below 2% is typically considered acceptable, and in this case, 0.3% signifies an exceptionally reproducible method. The data confirms that the method produces consistent results over multiple injections, making it reliable for quantitative assessments Table 3 and Figure 6.

3.3.3. Evaluation of filters

The table summarizes the % Assay and % Recovery of various filtered sub-fractions compared to an unfiltered Milrinone in an analytical test. The unfiltered sample shows a % Assay of 97.8%, which acts as the baseline for comparison. Different filters-nylon, PVDF, and PTFE-were used to analyze sub-fractions at volumes of 2, 4, and 6 mL.

3.3.3.1. For the 0.45 µm nylon filter. The % Assay ranges from 97.0% to 97.4%, with % Recovery values from 0.4% to 0.8%.

3.3.3.2. For the 0.45 µm PVDF filter. The % Assay is slightly lower, ranging from 96.3% to 97.4%, with % Recovery between 0.4% and 1.5%. This suggests slightly higher losses in recovery compared to the nylon filter.

3.3.3.3. For the 0.45 µm PTFE filter. The % Assay ranges from 96.7% to 97%, with % Recovery values from 0.8% to 1.1%.

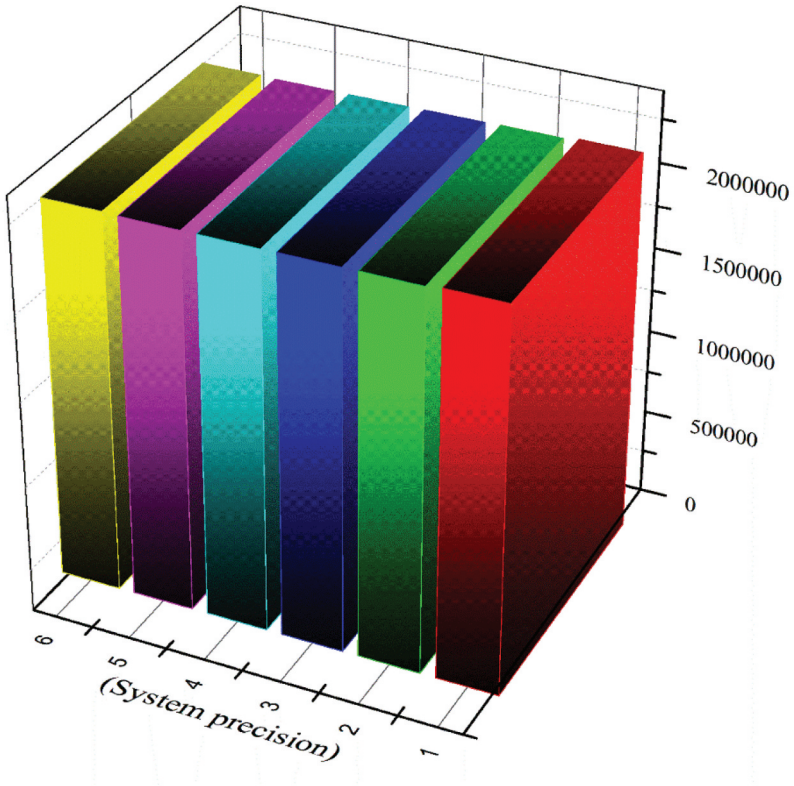
Overall, the nylon filters showed minimal impact on assay values and relatively low recovery loss, making them a more suitable choice for preserving sample integrity. PVDF filters showed the greatest recovery loss, particularly with larger sub-fractions. PTFE filters exhibited similar results to nylon but with slightly higher % Recovery values, indicating moderate loss Table 4 and Figure 7.

3.3.4. Evaluation of forced degradation studies

The Milrinone degradation study evaluates the stability of a compound under various stress conditions by comparing % Assay and % Degradation. The control sample, which serves as a baseline, shows a % Assay of 98.1%, indicating minimal degradation.

Table 3 and Figure 6. System precision results for Milrinone, showing injection areas, mean (2,218,258.67), standard deviation (6,730.36), and % relative standard deviation (%RSD = 0.3), indicating high precision.

System precision	
Injection	Area
1	2,231,219
2	2,218,219
3	2,216,219
4	2,213,369
5	2,212,763
6	2217763
Mean	**2218258.667**
SD	**6730.363012**
%RSD	**0.3**



Under acidic conditions (5 mL of 5 N HCl at 60°C for 2 hours), the % Assay is reduced to 95.8%, reflecting a 3% degradation. Similarly, exposure to a basic condition (5 mL of 5 N NaOH at 60°C for 2 hours) results in a % Assay of 94.5%, with a 4% degradation.

Peroxide-induced degradation (5 mL of 5% v/v H_2O_2 kept at room temperature for 24 hours) also leads to a % Assay of 94.6%, showing a 4% degradation. Thermal degradation (exposure to 105°C for 48 hours) results in a % Assay of 95.3%, indicating a 3% degradation similar to acid exposure.

Photolytic degradation, under intense light (1.2 million lux hours/200-watt hours per square meter), leads to the highest degradation, with a % Assay of 89.2% and a 9% degradation.

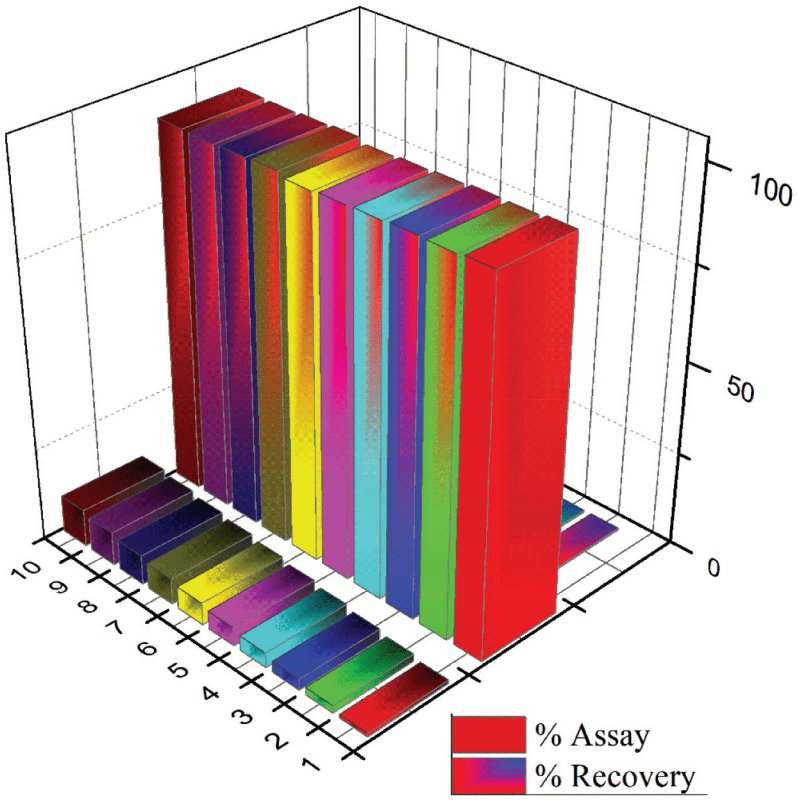
These results indicate that the compound is relatively stable under acidic, basic, thermal, and peroxide conditions but is more susceptible to photolytic degradation Table 5 and Figure 8.

3.3.5. Method precision

The table presents the % Assay values of Milrinone across six samples to evaluate the consistency and precision of the method. The % Assay values range from 98.2% to 99.3%, with an average (mean) of 98.7%, indicating the drug maintains a stable assay level across the

Table 4 and Figure 7. Filtration study results, showing % assay and % recovery across different 0.45 µm filters (nylon, PVDF, and PTFE) at varying sub-fraction volumes (2 mL, 4 mL, 6 mL).

Name	% Assay	% Recovery
Unfiltered (mean area of injections)	97.8	NA
0.45 µm nylon filter 2 mL sub-fraction	97.3	0.5
0.45 µm nylon filter 4 mL sub-fraction	97.4	0.4
0.45 µm nylon filter 6 mL sub-fraction	97	0.8
0.45 µm PVDF filter 2 ml sub-fraction	96.7	1.1
0.45 µm PVDF filter 4 mL sub-fraction	96.3	1.5
0.45 µm PVDF filter 6 mL sub-fraction	97.4	0.4
0.45 µm PTFE filter 2 ml sub-fraction	97	0.8
0.45 µm PTFE filter 4 mL sub-fraction	96.7	1.1
0.45 µm PTFE filter 6 mL sub-fraction	96.9	0.9



samples. Additionally, the relative standard deviation (% RSD) is 0.5%, which signifies excellent precision, as values below 2% are typically considered acceptable in analytical methods. This low %RSD highlights the reliability and consistency of the Milrinone assay method, ensuring that the results are reproducible and accurate. Such precision is critical in pharmaceutical analysis to maintain the quality and efficacy of the drug Table 6 and Figure 9.

3.3.6. Evaluation of linearity

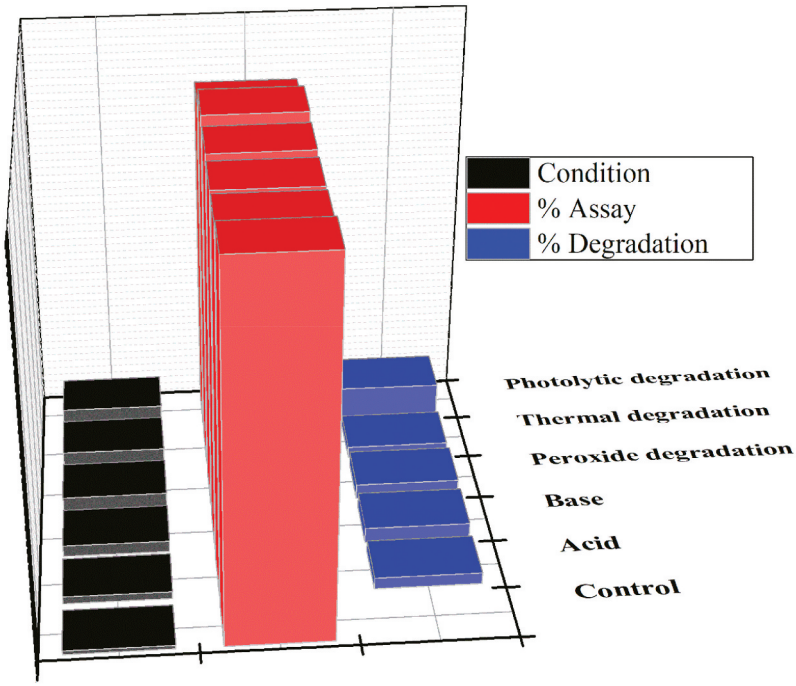
The concentration of Milrinone (µg/mL) at various levels (50% to 150%) and their corresponding mean peak area responses. As the Milrinone concentration increases from 49.65 µg/mL to 148.95 µg/mL, the mean peak area response increases proportionally and showing a strong linear relationship. The regression coefficient (R^2) was 0.999, indicating a perfect correlation between concentration and peak area response. This high degree of linearity (R^2 close to 1) suggests that the method is reliable and linear for quantifying Milrinone across different concentration levels, essential for precise analytical quantification in pharmaceutical formulations. Table 7 and Figure 9.

3.3.7. Evaluation of solution stability

The stability of Milrinone both the standard and sample solutions over a 48-hour period, with measurements

Table 5 and Figure 8. Forced degradation study of Milrinone, showing % assay and % degradation under acid, base, peroxide, thermal, and photolytic conditions.

Mode of degradation	Condition	% Assay	% Degradation
Control	NA	98.5	NA
Acid	5 mL of 5 N HCl 60°C 2 hrs.	95.5	3
Base	5 mL of 5 N NaOH 60°C 2 hrs.	94.5	4
Peroxide degradation	5 mL of 5 % v/v H_2O_2 room temperature 24 hours.	94.5	4
Thermal degradation	105°C/48 hrs.	95.5	3
Photolytic degradation	1.2million lux hours/200 watt hours square meter	89.5	9



taken at various time intervals. Over the 48-hour period, the response remains fairly consistent, with only slight fluctuations, as indicated by the small % difference (ranging from 0.5% to 0.9%). The response drops slightly to 1,905,434 at the 48th hour, showing a minor 0.8% difference from the initial measurement, indicating minimal degradation. For the sample solution, the initial % Assay is 100.2%, with the % difference being tracked across time. The assay slightly decreases to 99.1% at the 2nd hour and further to 98.7% at the 4th hour, with the highest % difference of 1.5% observed at the 4th hour. However, the assay remains stable after that, with values between 99.0% and 99.8% for the remainder of the time points. The % difference remains below 1.5% throughout the 48 hours, indicating good stability of the sample solution. Overall, both the standard and sample solutions exhibit excellent stability over the 48-hour period, with minimal variations in % Assay and response Table 8 and Figure 10.

3.3.8. Evaluation of accuracy

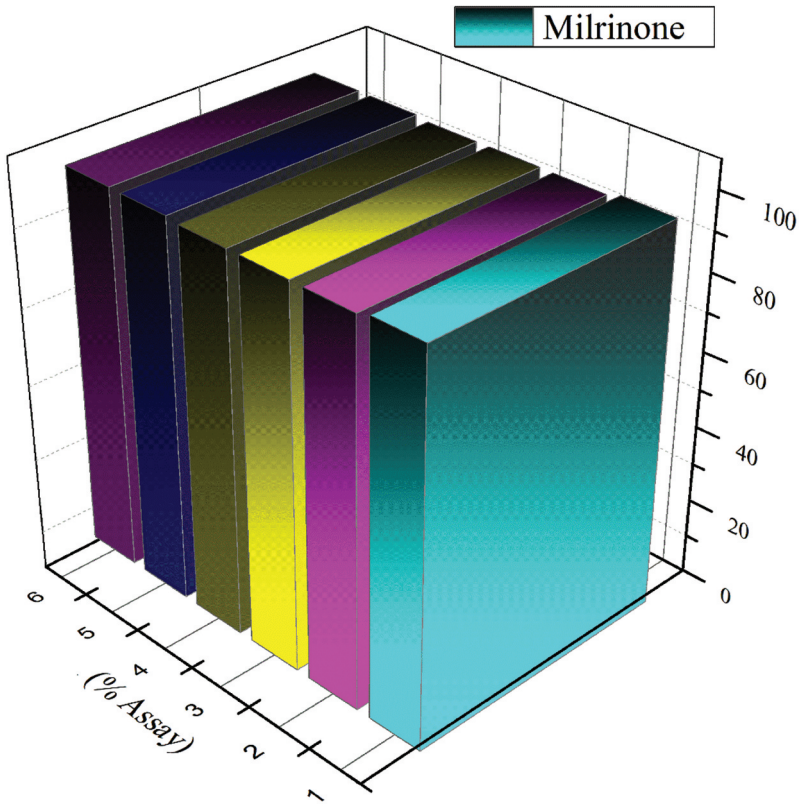
The data provided evaluates the accuracy of Milrinone recovery at three different levels: 50%, 100%, and 150%. For each level, three accuracy solutions were prepared, and the amount of Milrinone added and found was measured to calculate % Recovery, with corresponding %RSD values.

The accuracy of the analytical method was assessed by evaluating the recovery of known amounts of Milrinone at different levels (50%, 100%, and 150%). The percent recovery and %RSD were calculated to determine the accuracy and precision of the method.

3.3.8.1. Accuracy at 50% level. The recovery values for the 50% accuracy solutions ranged from 99.9% to 101.0%, with an average recovery of 100.4%. The %RSD was calculated as 0.2%, indicating high precision in the recovery at this level. The recovery within the acceptable range

Table 6 and Figure 9. Assay results for Milrinone, showing % assay values across six samples, with a mean of 98.7%, standard deviation (SD) of 0.485, and % relative standard deviation (%RSD) of 0.5, indicating consistency.

Sample No.	% Assay Milrinone
1	99.3
2	98.2
3	98.3
4	98.3
5	99.0
6	99.2
Mean	**98.7**
SD	**0.485**
%RSD	**0.5**



(100 ± 2%) indicates that the method can accurately measure Milrinone at lower concentration levels.

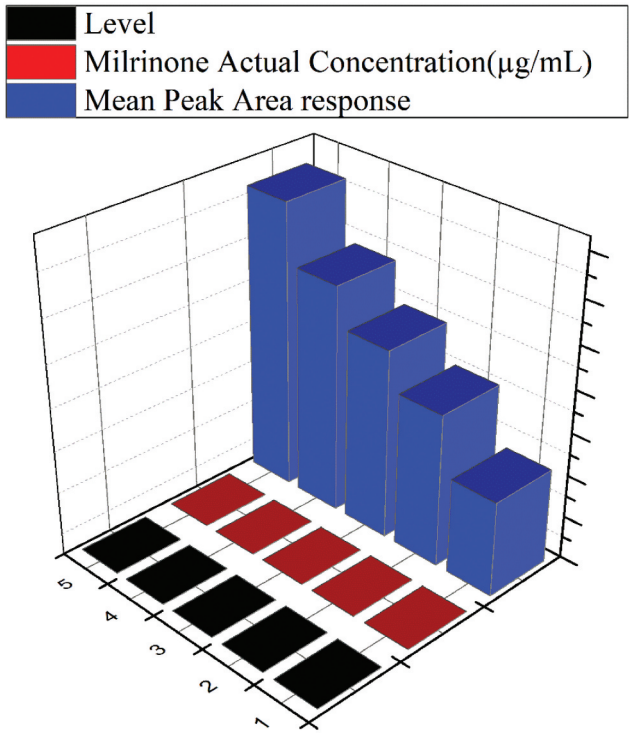
3.3.8.2. Accuracy at 100% level. For the 100% accuracy solutions, recovery values ranged from 99.9% to 101.0%, with an average recovery of 101.1%. The %RSD remained at 0.4%, demonstrating consistent and precise measurements at this concentration. The close adherence to the target recovery range confirms the method's reliability for quantifying Milrinone at its intended concentration.

3.3.8.3. Accuracy at 150% level. The 150% accuracy solutions showed recovery values between 99.9% and 101.0%, with an average recovery of 101.0%. The %RSD was again 0.3%, illustrating that the method maintains precision even at higher concentration levels. The recoveries indicate that the method is accurate for Milrinone quantification, even at elevated concentrations.

The method demonstrated excellent accuracy and precision across all three accuracy levels, with average % recoveries close to 100% and %RSD values consistently low at 0.2-0.4%. These results suggest that the method is highly reliable for the quantification of Milrinone, as it meets the acceptable recovery criteria (100 ± 2%) at multiple concentration levels. The consistent %RSD values across levels indicate robust precision,

Table 7 and Figure 9. Linearity study of Milrinone, showing actual concentrations (µg/mL) and mean peak area responses across different levels (50%–150%). The regression coefficient (R^2 = 0.99996), slope (22,682.55), and intercept (−11,162.25) indicate excellent linearity.

Solution No.	Level	Milrinone Actual Concentration(µg/mL)	Mean Peak Area response
1	50%	49.65	1,117,841
2	80%	79.44	1,796,131
3	100%	99.3	2,231,219
4	120%	119.16	2,686,388
5	150%	148.95	3,374,496
	Regression Coefficient (R^2)		0.999963412
	Slope		22682.54895
	Intercept		−11162.25009



confirming that the method is suitable for routine use in Milrinone analysis. Table 9 and Figure 11.

3.3.9. Evaluation of robustness

Table 10 and Figure 12 presents data on Milrinone how different conditions affect the performance of a chromatographic system, specifically through USP tailing, plate counts, %RSD of areas in standard injections, and % similarity factor.

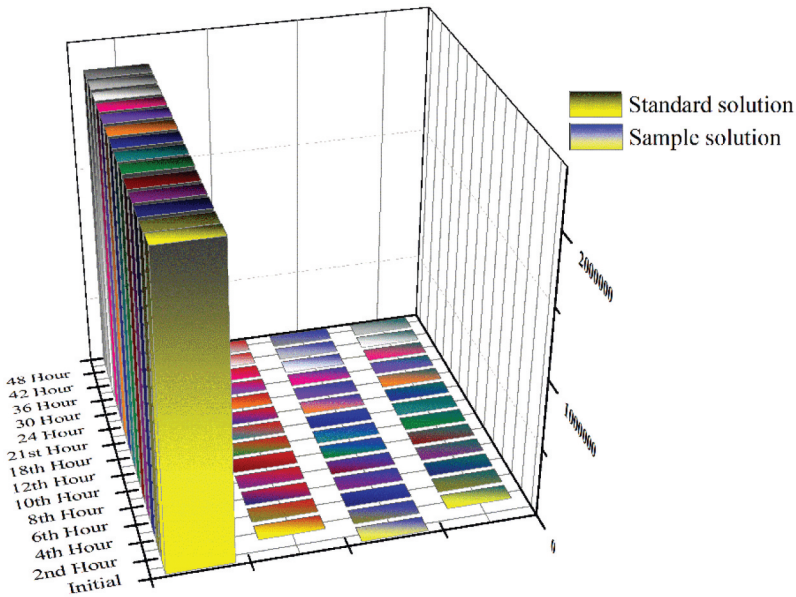
The robustness of the method was evaluated by assessing its performance under slight variations in chromatographic parameters, including flow rate, organic content, column oven temperature, wavelength, and pH. The following observations were noted based on parameters like USP tailing factor, plate count, %RSD of areas in standard injections, and % similarity factor.

3.3.9.1. USP tailing factor. The tailing factor for the control condition was 1.3, indicating acceptable symmetry. Variations in flow rate, organic content, temperature, wavelength, and pH resulted in minimal changes in tailing factors, ranging from 1.1 to 1.3. This consistency suggests that the method can maintain symmetrical peaks under slight parameter adjustments, showing good robustness regarding tailing.

3.3.9.2. Plate counts. Plate counts for all conditions were consistently high, with values close to the control (11097), indicating the method's ability to maintain efficient separation under different conditions. Minor variations were observed, with plate counts slightly lower (e.g., 11016 at a temperature of 45°C) but remaining above 11000, suggesting stable column efficiency across variations.

Table 8 and Figure 10. Stability study of Milrinone standard solution over 48 hours, showing response, % assay, and % difference at various time intervals. Results indicate minimal variation, confirming stability over the tested period.

	Standard solution		Sample solution	
Interval	Response	% Difference	% Assay	% Difference
Initial	2221123	NA	100.2	NA
2nd Hour	2206123	0.7	99.1	1.1
4th Hour	2206623	0.7	98.7	1.5
6th Hour	2205123	0.7	100.2	0.0
8th Hour	2203123	0.8	99.7	0.5
10th Hour	2211123	0.5	99.4	0.8
12th Hour	2205123	0.7	99.3	0.9
18th Hour	2206623	0.7	99.8	0.4
21st Hour	2205523	0.7	99.6	0.6
24 hour	2207623	0.6	99.5	0.7
30 hour	2207023	0.6	99.7	0.5
36 hour	2205023	0.7	99.2	1.0
42 hour	2205273	0.7	99.0	1.2
48 hour	2205434	0.7	99.3	0.9



3.3.9.3. %RSD of areas in standard injections. The % RSD for standard injections remained below 1.2% across all variations, with the control at 0.7%. This low %RSD indicates good precision, demonstrating the method's reliability for consistent area measurement under varied conditions. The highest %RSD observed was 1.1% when organic content in the mobile phase was increased, but it remained within acceptable limits.

3.3.9.4. % Similarity factor. The similarity factor for the control condition was 99.7%, which closely matched the target value, showing accurate quantitation. Variations in flow rate and temperature led to a slight decrease in the similarity factor (e.g., 99.1% with a 2% increase in organic content), but all values remained within a narrow range (99.1–100.1%). This indicates that the method can accurately quantify under different parameter settings without significant deviations.

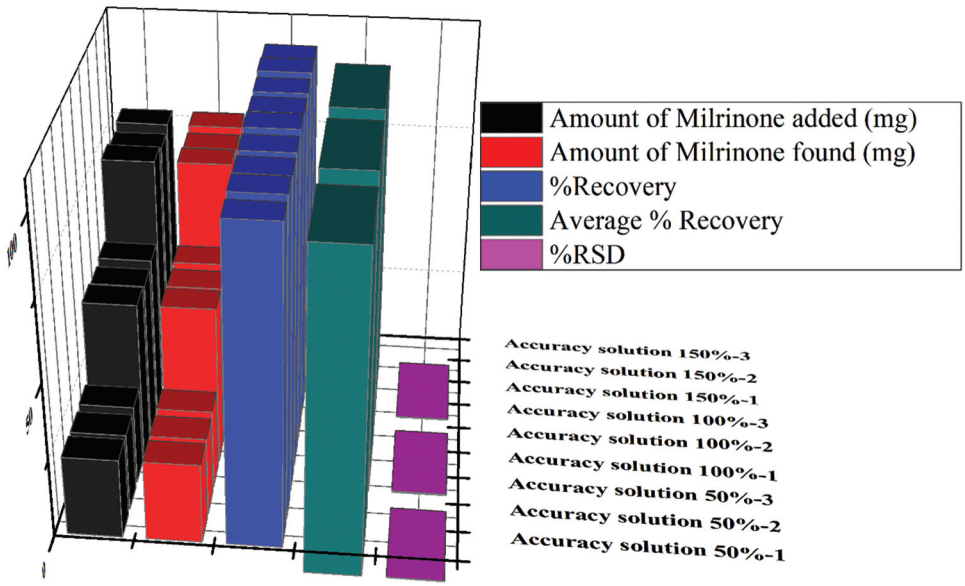
The USP tailing factor and plate counts remained stable, indicating maintained peak shape and separation efficiency. Low %RSD values for standard injections highlight method precision, while the % similarity factor reflects consistent quantitation. Overall, this method is reliable for routine analysis, showing robustness across minor variations in flow rate, organic content, temperature, wavelength, and pH.

4. Discussion

The integration of AQbD for RP-HPLC method development has shown strong potential to maintain pharmaceutical quality during quantification of

Table 9 and Figure 11. Accuracy study of Milrinone, showing amount added, amount found, % recovery, and %RSD across 50%, 100%, and 150% levels. The average recovery (100.4%-101.1%) and low %RSD (0.2%–0.4%) confirm method accuracy.

Accuracy Level	Amount of Milrinone added (mg)	Amount of Milrinone found (mg)	%Recovery	Average % Recovery	%RSD
Accuracy solution 50%-1	25.6615	25.706	100.2	100.4	0.2
Accuracy solution 50%-2	25.0735	25.3155	101.0		
Accuracy solution 50%-3	25.708	25.689	99.9		
Accuracy solution 100%-1	51.323	51.412	100.2	101.1	0.4
Accuracy solution 100%-2	50.147	50.631	101.0		
Accuracy solution 100%-3	51.416	51.378	99.9		
Accuracy solution 150%-1	76.9845	77.118	100.2	101.00	0.3
Accuracy solution 150%-2	75.2205	75.9465	101.0		
Accuracy solution 150%-3	77.124	77.067	99.9		



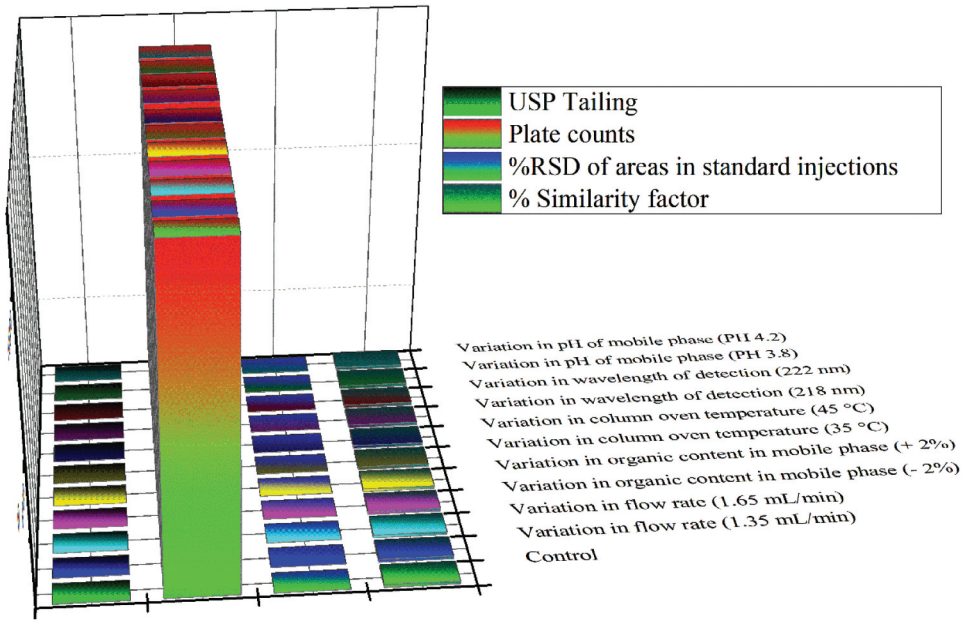
Milrinone. Method robustness combined with accuracy and precision become achievable through systematic optimization of chromatographic parameters made possible by AQbD. The method development tests reveal that the method's buffer composition together with column temperature and flow rate are essential elements in attaining optimal separation results and precise retention time performance. The final optimized method used a 50:50 mobile phase ratio at pH 4.0 while maintaining the column temperature at 40°C and setting the flow rate to 1.5 mL/min which led to an optimized retention time of 1.901 minutes. The validation tests strengthened the method's reliability status. The system suitability test results generated excellent peak symmetry together with high column efficiency which resulted in a USP tailing factor of 1.0 and a plate count of 10,795. The findings from precision tests revealed a %RSD of 0.3% which indicates high precision and reliability of the method. The results from forced degradation experiments revealed that Milrinone could be degraded through exposure to light thus requiring adequate storage measures. Under all testing conditions the developed method showed outstanding results for linearity (R^2 = 0.999) and accuracy through recovery rates from 99.9% to 101.1% and demonstrated robustness across minor variation tests. The RP-HPLC analysis method proves appropriate for regular Milrinone assessments which guarantee top-quality pharmaceutical products.

5. Conclusion

Using AQbD principles for developing and validating an RP-HPLC method to quantify Milrinone produces a reliable quantitative analysis system. The testing procedure delivered exact and reproducible readings which produced dependable Milrinone measurements in pharmaceutical preparations. By optimizing the mobile phase composition and column temperature and flow rate parameters systematically scientists obtained notable retention time reduction with enhanced separation efficiency. The method proved its excellence during validation tests by fulfilling

Table 10 and Figure 12. Robustness study of Milrinone under varied chromatographic conditions, assessing USP tailing, plate counts, %RSD of areas, and % similarity factor. Minimal variation confirms method robustness and reliability.

Conditions	USP Tailing	Plate counts	%RSD of areas in standard injections	% Similarity factor
Control	1.3	11,097	0.7	99.7
Variation in flow rate (1.35 mL/min)	1.2	11,072	0.9	99.5
Variation in flow rate (1.65 mL/min)	1.1	11,095	0.6	99.3
Variation in organic content in mobile phase (- 2%)	1.2	11,078	0.9	100.1
Variation in organic content in mobile phase (+ 2%)	1.2	11,093	1.1	99.1
Variation in column oven temperature (35 °C)	1.1	11,057	0.5	99.2
Variation in column oven temperature (45 °C)	1.3	11,016	0.3	99.5
Variation in wavelength of detection (218 nm)	1.2	11,099	0.4	99.2
Variation in wavelength of detection (222 nm)	1.3	11,093	0.5	99.4
Variation in pH of mobile phase (PH 3.8)	1.2	11,029	0.4	99.6
Variation in pH of mobile phase (PH 4.2)	1.3	11,019	0.8	99.3



essential standards of linearity and accuracy as well as precision and robustness. Analysis of Milrinone through forced degradation procedures showed that light plays a significant role in degrading the compound which demonstrates the need for proper storage requirements. When used with AQbD principles the developed RP-HPLC method delivers a dependable analysis system for Milrinone quantification that fulfills strict regulatory standards and protects pharmaceutical product quality. Research evidence demonstrates that AQbD provides great potential to build reliable and robust analytical methods which improves drug quality control systems while ensuring patient safety.

Author contributions

CRediT: **P. Suresh:** Methodology, Writing – original draft, Writing – review & editing; **Gunjan Jadon:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing; **Panneerselvam Theivendren:** Conceptualization, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Declarations

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

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