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Development and Validation of Analytical Methods for the Estimation of Milrinone Lactate

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ABSTRACT

The invention of an analytical method and the validation of the substance milrinone using UV visible spectrometry, HPLC, and HPTLC.

This drug is commonly employed in situations where traditional heart failure treatments may not be effective. Administered intravenously in a hospital setting due to its short half-life, Milrinone is particularly beneficial in acute heart failure instances, such as post-cardiac surgery or when standard therapies prove insufficient.

To create a successful analytical technique for milrinone, one must have a solid understanding of its chemical structure and characteristics. Milrinone, also known as 1,6-dihydro-2-methyl-6-oxo-(3,4'-bipyridine)-5-carbonitrile, is a positive inotropic and vasodilator drug. It is a white to off-white crystalline powder with a molecular weight of 211.22 g/mol.

The unique properties of milrinone and the analytical needs determine which analytical method—such as high-performance thin layer chromatography (HPTLC) or high-performance liquid chromatography (HPLC)—is best. It is necessary to thoroughly examine elements including the choice of stationary phases, the makeup of the mobile phase, flow rates, column temperatures, and detection wavelengths.

Any proposed method should also follow legal requirements and undergo validation for attributes including robustness, specificity, linearity, accuracy, and precision. For biological sample analysis, such that of plasma or urine, validation is essential to guarantee the practicality and dependability of the technique.

Milrinone's value in pharmaceutical and clinical contexts can be increased by accurately detecting and quantifying it by careful consideration of these aspects and optimization of the analytical procedure.

KEYWORDS

Milrinone
HPLC method development (high-performance liquid chromatography)
HPTLC method development (high-performance thin layer chromatography)
UV (ultraviolet-visible spectrophotometry)
Chromatographic conditions
Mobile phase composition
Stationary phase selection
Sample preparation techniques
Detection methods
Method validation
Retention time
Peak resolution
Method sensitivity
Method selectivity
Linearity and range
Robustness and precision

INTRODUCTION

Milrinone is a crystalline compound with an off-white to tan appearance, having a molecular weight of 211.2 and an empirical formula of C12H9N3O. It displays limited solubility in methanol and chloroform, and very slight solubility in water.

Milrinone is a medication classified as a phosphodiesterase inhibitor, primarily utilized in the management of heart failure. It functions by elevating the levels of cyclic adenosine monophosphate (cAMP) within cardiac muscle cells, leading to increased cardiac contractility and vasodilation. These effects contribute to enhanced cardiac output and improved blood flow.

Milrinone is primarily used for its positive inotropic and vasodilator effects, making it valuable in the management of certain cardiovascular conditions. The main use of Milrinone is in the treatment of heart failure.

Precise analytical methods play a vital role in drug development, guaranteeing pharmaceutical product quality, safety, and efficacy through accurate measurement, impurity identification, and compliance with regulatory standards.

The key objectives of analytical method development include ensuring accuracy, precision, sensitivity, selectivity, and robustness. Additionally, the method should demonstrate linearity, reproducibility, and compliance with regulations, while being cost-effective, user-friendly, and well-documented.

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Developing an analytical method for the drug milrinone is essential for several reasons:

1. Quality Assurance: Creating an analytical method ensures that the drug can undergo thorough testing to confirm its identity, purity, and potency, crucial for maintaining quality standards.

2. Pharmacokinetic Studies: An analytical method is necessary for studying the drug's absorption, distribution, metabolism, and excretion in both preclinical and clinical pharmacokinetic studies.

3. Formulation Development: The creation of an analytical method assists in determining the compatibility of milrinone with different excipients and in formulating robust dosage forms.

4. Stability Studies: Analytical methods help assess the drug's stability under various conditions (e.g., temperature, humidity, light) to determine storage requirements and shelf life.

5. Regulatory Compliance: Adequate analytical methods are vital to ensuring that milrinone meets the quality, safety, and efficacy standards set by regulatory agencies.

In summary, analytical method development for milrinone is crucial to guarantee its quality, effectiveness, and safety, and to comply with regulatory requirements.

Importance of Accurate Quantification in Pharmaceutical Research:

Key Significance
Precise Dosing and Administration
Assessment of Drug Effectiveness
Preventing Adverse Effects
Ensuring Clinical Trial Integrity and Reliability
Support for Regulatory Compliance and Approvals
Bioavailability and Pharmacokinetics Assessment
Rigorous Quality Control Measures
Facilitating Precision Medicine and Tailored Dosing
Contributing to Research Reproducibility
Optimizing Drug Development Processes and Resource Utilization

Accurate quantification is pivotal in pharmaceutical research, influencing dosing precision, patient safety, regulatory compliance, and the overall advancement of drug development.

High Performance Liquid Chromatography

Importance of High-Performance Liquid Chromatography (HPLC) in pharmaceutical method development

1. **Precision and Accuracy**: HPLC allows for precise and accurate quantification of drug substances and impurities in pharmaceutical formulations, aiding in ensuring the safety and efficacy of the final products.

2. **Separation of Complex Mixtures:** HPLC is capable of separating and analysing complex mixtures of compounds, making it invaluable in pharmaceutical research and quality control where multiple components need to be separated, identified, and quantified.

3. **Regulatory Compliance:** HPLC methods are widely accepted and recommended by regulatory authorities for pharmaceutical analysis, ensuring that the methods developed are in compliance with industry standards and regulations.

4. **Method Validation:** HPLC methods can be robustly validated, providing evidence of the reliability, accuracy, and consistency of the analytical data generated, which is crucial for pharmaceutical method development.

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5. **Impurity Profiling:** HPLC allows for the detection and quantification of impurities in pharmaceutical products, ensuring that the medications meet strict quality standards.

6. **Pharmacokinetic Studies:** HPLC is used to determine drug concentrations in biological samples, supporting pharmacokinetic studies essential for the development of pharmaceutical formulations.

7. **Research and Development:** HPLC is a fundamental tool in pharmaceutical research and development, aiding in the identification of new drug candidates, their optimization, and the analysis of stability and degradation products.

8. **Quality Control:** HPLC plays a critical role in the quality control of pharmaceutical products, ensuring batch-to-batch consistency and the absence of contaminants and impurities.

High Performance Thin Layer Chromatography
Importance of High-Performance Thin-Layer Chromatography (HPTLC) in pharmaceutical method development

1. **Rapid Analysis:** HPTLC offers fast and efficient separation and analysis of pharmaceutical samples, making it a valuable tool for high-throughput screening and routine analysis in pharmaceutical laboratories.

2. **Cost-Effectiveness:** HPTLC is cost-effective compared to other chromatographic techniques, making it an attractive option for pharmaceutical method development, especially when large numbers of samples need to be analyzed.

3. **Sensitivity:** HPTLC methods can be highly sensitive, allowing for the detection and quantification of trace amounts of compounds, including impurities, in pharmaceutical formulations.

4. **Quantitative Analysis:** HPTLC can be used for quantitative analysis of drugs and their impurities, providing valuable information for pharmaceutical formulation and quality control.

5. **Simple Sample Preparation**: HPTLC generally requires minimal sample preparation, reducing the time and resources needed for analysis, making it a convenient method for pharmaceutical laboratories.

6. **Suitability for Multiple Compounds:** HPTLC is suitable for the simultaneous analysis of multiple compounds in a single sample, which is beneficial when assessing complex pharmaceutical formulations.

7. **Method Transferability:** HPTLC methods are relatively easy to transfer between laboratories, making them convenient for pharmaceutical companies with multiple sites or external collaborations.

8. **Complementary Technique:** HPTLC can complement other analytical techniques in pharmaceutical method development, providing additional information and results that can enhance the overall analysis process.

OBJECTIVE OF THE STUDY
To Qualify and Quantify High-Performance Liquid Chromatography and High-Performance Thin Layer Chromatography.

Analyses of milrinone drugs by HPLC and HPTLC.
Methods of Development of Milrinone Drugs by Using Different Solvents.
Survey of Milrinone Drugs for Patients and the Dose Frequency.
Finding Retention Time, Area Findings, and All the Validation Parameters.
Establish accurate quantitative and qualitative analysis techniques for Milrinone.
Demonstrate the high specificity, sensitivity, precision, accuracy, and linearity of the developed methods.
Provide a well-defined and validated analytical methodology for efficient analysis of milrinone in various pharmaceutical formulations and research studies.

EXPERIMENTAL WORK:
MATERIAL AND METHODS:

- **Reagents and chemicals:**
- Methanol (HPLC Grade),
- Formic Acid (AR Grade)
- HPLC grade water.

All chemicals and reagents that is Methanol, Phosphoric acid, were purchased from S. D. Fine Chemicals Ltd., Mumbai.

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Instruments:

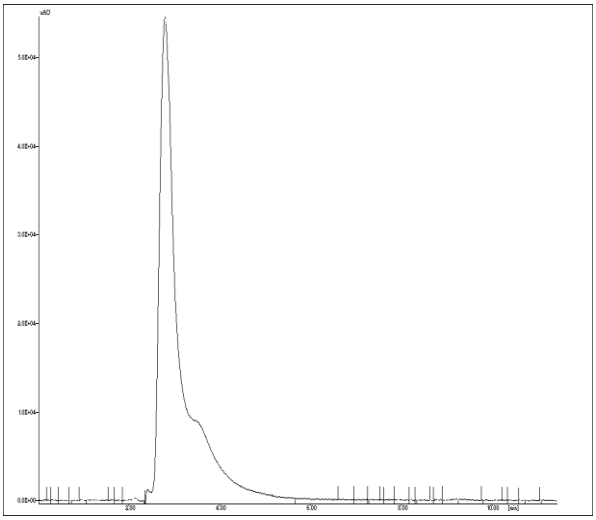
1. HPLC:
- Borwin chromatography software (version 1.50)
- Model PU 2080 Plus Intelligent HPLC pump
- Rheodyne sample injection port with 20µl loop
- Grace C_{18} column (150 x 4.6 mm, 3.5 µm)
- JASCO UV-2075 UV-VIS detector

2. Double Beam UV-Vis Spectrophotometer (Jasco V 730)
3. Shimadzu (model AY-120) Electronic weighing balance
4. Sonicator: PRAMA solutions for laboratory
5. Extrapure lab link water purification system
6. Electronic pH meter
7. Calibrated Glassware's.

Development and validation of HPLC Method for estimation of Milrinone Lactate

1. Experimental, Results and Discussion:

Table 1:Trials of mobile phase for HPLC method development of Milrinone Lactate:

Sr. No	Mobile phase	Observations	Chromatogram
1.	MeOH: water (75:25 v/v) Column: SunQSil C18 Column (250 mm × 4.6 mm, 5 µm)	Peak shape was not proper. Peak Tailing RT = 4.900 min.	

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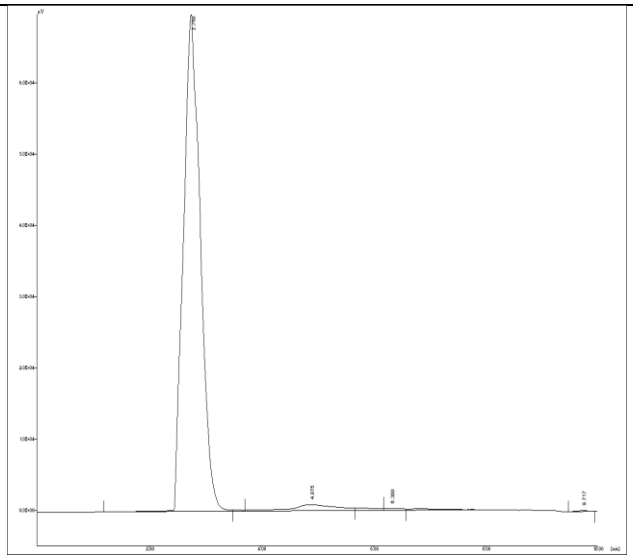
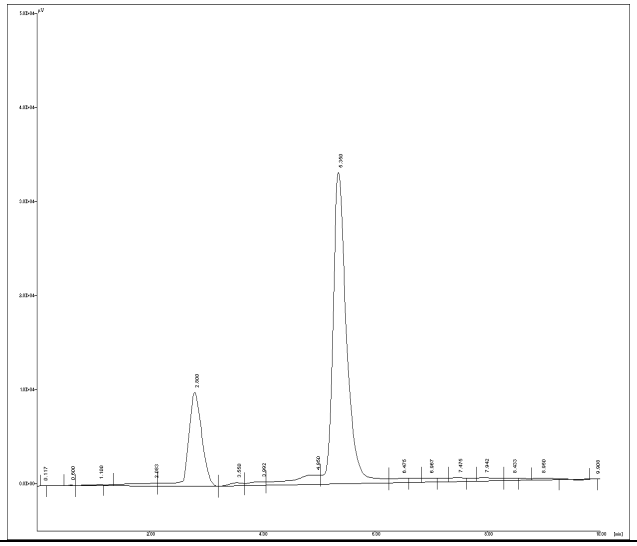
2.	MeOH: 0.1 % Formic Acid (30:70 v/v) Grace C_{18} column (150 x 4.6 mm, 3.5 µm)	Peak eluted earlier RT = 2.750 min.	
3.	MeOH: 0.1 % Formic Acid (60:40 v/v) Grace C_{18} column (150 x 4.6 mm, 3.5 µm)	Good Peak Shape, RT at 5.350 min.	

A. Selection of mobile phase and chromatographic conditions:

Chromatographic separation studies were carried out on the working standard solution of Milrinone Lactate10 µg/ml. Initially, trials were carried out using methanol and buffer in various proportions of varying pH, to obtain the desired system suitability parameters. After few trials, MeOH: 0.1 % Formic Acid in the ratio of 60:40 v/v was chosen as the mobile phase, which gave good resolution and acceptable peak parameters.

B. Preparation of Standard stock solution:

Standard stock solution of drug was prepared by dissolving 10 mg of drug in 10 ml of methanol to get concentration of 1000 µg/ml (A). From the corresponding standard stock solution, working standard solution was prepared containing 100 µg/ml of Milrinone Lactate in methanol(B). This solution was further diluted with methanol to get final solution of Milrinone Lactate (10 µg/ml).

C. Selection of Detection Wavelength:

From the standard stock solution further dilutions were done using methanol and scanned over the range of 200 - 400 nm and the spectra was obtained (Fig. 1). It was observed that drug showed considerable absorbance at 235 nm.

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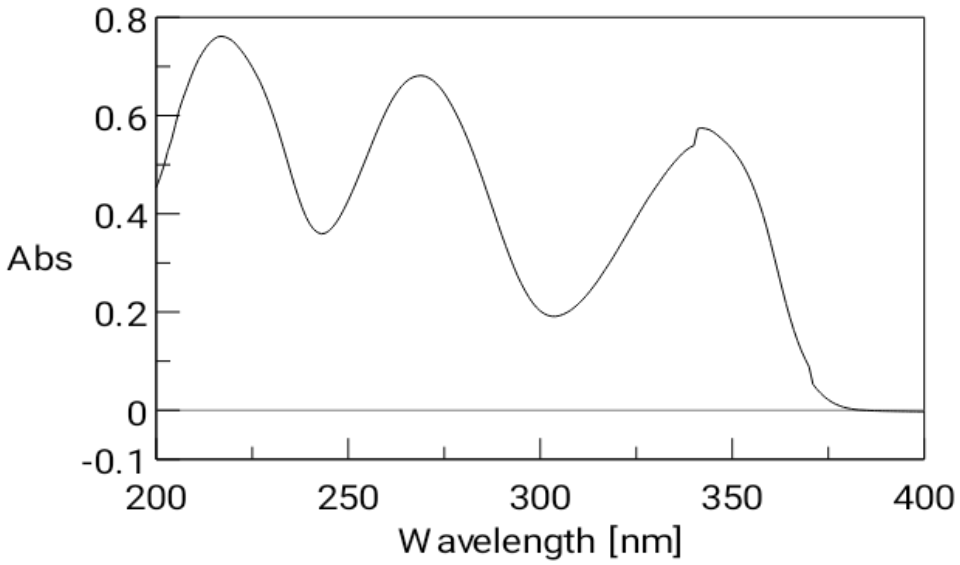


Fig1: UV-VIS Spectra of Milrinone Lactate (10 µg/ml)

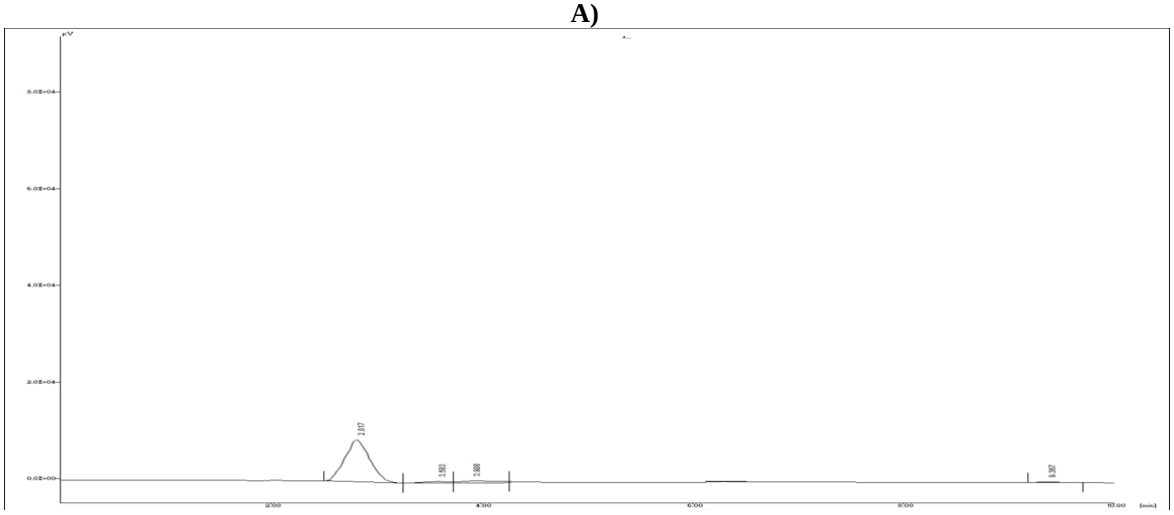
D. Preparation of sample solution:(Injection formulation)

Injection containing 10 mg of Milrinone Lactate (Milrican, American Remedies, Label Claim: Each ml contains Milrinone Lactate … 1 mg) was pipetted and was transferred to 10 ml volumetric flask and volume was made up with methanol to get concentration (100µg/ml). Solution was filtered, from this solution 0.4 ml was taken in 10 ml volumetric flask and volume was made up with methanol to get final concentration 4µg/ml.

E. Chromatogram and system suitability parameter of drug:

The column was equilibrated with the mobile phase (indicated by constant back pressure at desired flow rate). Working standard solution of drug (10 µg/ml) was injected on system. The retention time for the drug was found 4.906 min. Chromatogram of Milrinone Lactate shown in Fig.2

A)



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B)

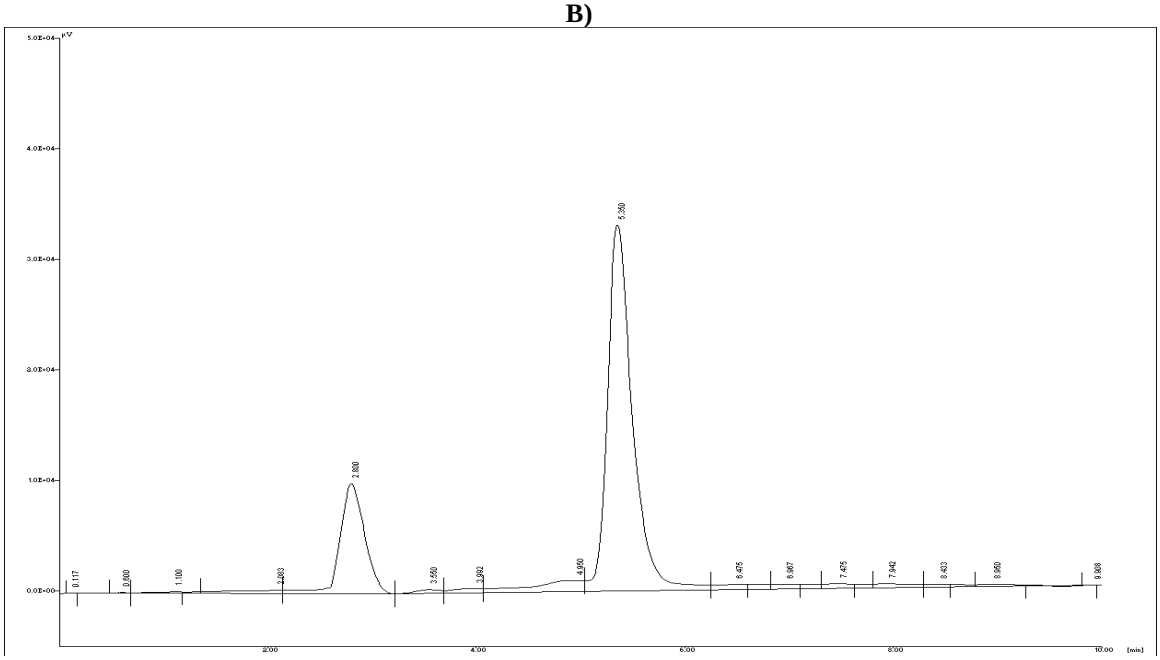

Fig 2: Chromatogram of A) Blank B) Milrinone Lactate (10 µg/ml)

Table2: System suitability parameters for Milrinone Lactate

Drug	Concentration (µg/ml)	RT ±% RSD (Min)	Area	Plates	Asymmetry
MILRINONE LACTATE	10	5.364 ± 0.214	1257521.608	3279.67	0.99

F. Summary of Chromatographic parameters selected:

Table3: Summary of Chromatographic parameters

Sr. No.	Parameter	Conditions used for Analysis
1	Column	Grace C_{18} column (150 x 4.6 mm, 3.5 µm)
2.	Mobile phase	MeOH: 0.1 % Formic Acid (60:40 v/v)
3.	Flow rate	1 ml/min
4.	Detection Wavelength	270 nm
5.	Sample injector	20 µl loop
6.	Column temperature	Ambient

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2. Validation of Analytical Method

A. Linearity

From the standard stock solution (1000 µg/ml) of Milrinone Lactate, solution was prepared containing 100 µg/ml of Milrinone Lactate with methanol. This solution was further used to prepare range of solutions containing six different concentrations. The linearity (relationship between peak area and concentration) was determined by analyzing six solutions over the concentration range of2-12µg/ml.The results obtained are shown in Table4.Linearity curve of Milrinone Lactate is shown in Fig. 3 and calibration curve shown in Fig. 4.

Table 4: Linearity study of Milrinone Lactate

Replicates	Concentrations of Milrinone Lactate					
	2 µg/ml	4 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml	12 µg/ml
	Peak Area					
1	291973.256	564961.121	808759.368	1016635.990	1260142.582	1443349.031
2	293737.736	555720.423	805648.436	1033834.332	1257521.608	1475018.060
3	291749.680	556289.202	796499.796	1010397.496	1278581.974	1458296.084
4	294050.760	555417.183	798521.974	1008133.216	1255320.735	1447118.664
5	292743.708	558489.170	780586.888	1022602.612	1280412.149	1466657.072
6	294206.414	582818.106	811241.044	1028906.252	1255320.735	1464968.968
Mean	293076.926	562282.534	798503.341	1020084.983	1264549.964	1459234.647
Std. Dev.	1072.518	10671.350	11189.582	10215.533	11727.629	12141.842
%RSD	0.366	1.898	1.401	1.001	0.927	0.832

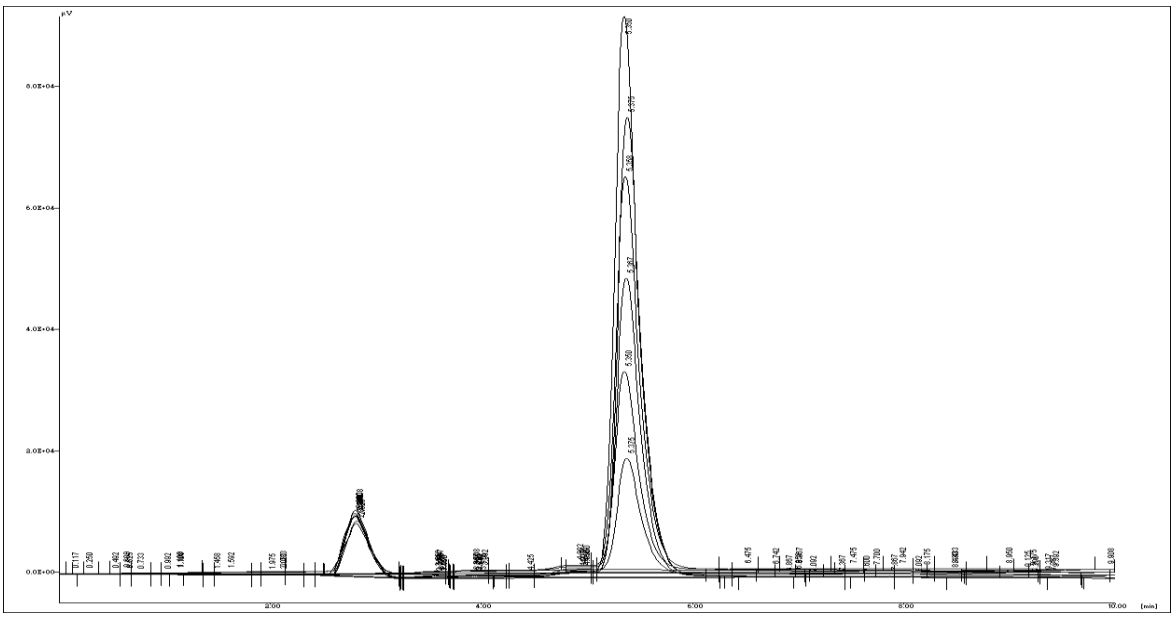

Fig 3: Linearity curve of Milrinone Lactate (2-12 µg/ml)

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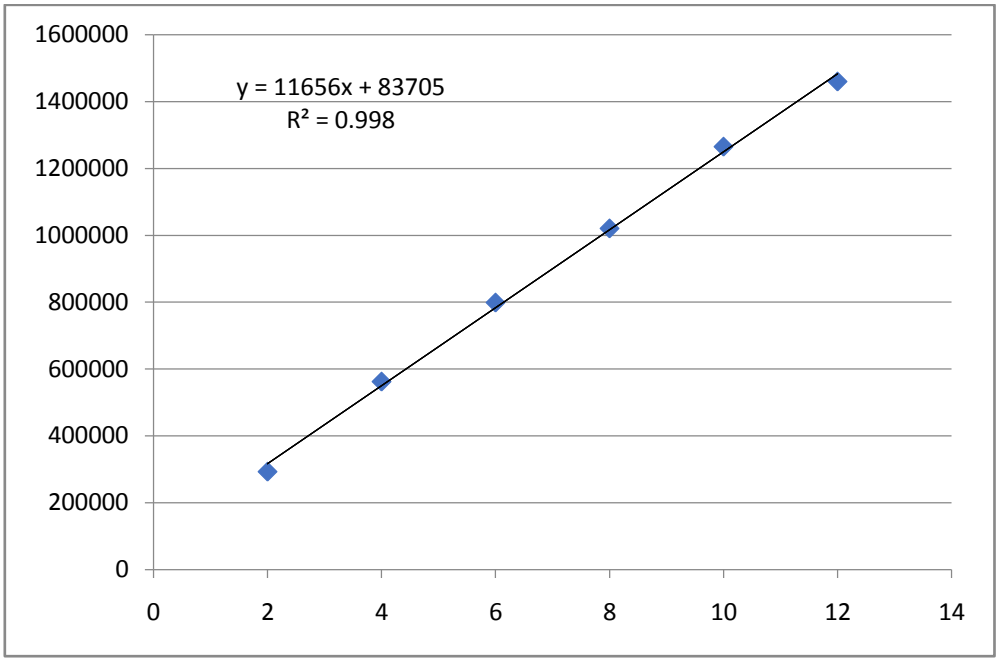

Fig 4: Calibration curve of Milrinone Lactate

B. Range:
2-12 µg/ml.

C. Limit of Detection (LOD) and Limit of Quantification (LOQ)
LOD and LOQ are calculated from the formula: -

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\ \sigma}{S}$$

Where,
σ = standard deviation of Y intercept = 9499.733
S = slope of the calibration curve = 116535.233

LOD = 0.269 µg/ml
LOQ = 0.815 µg/ml

D. Precision:
The precision of the method was demonstrated by intra-day and inter-day variation studies. In the Intra-day studies, 3 replicates of 3different concentrations were analyzed in a day and percentage RSD was calculated. For the inter day variation studies, 3 different concentrations were analyzed on 3 consecutive days and percentage RSD were calculated. The results obtained for Intraday and Inter day variations are shown in Table 5 and Table 6, respectively.

Table 5: Intra-day Precision Results

Conc (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	Average % Recovery	SD	%RSD
4	551237.774	4.011	100.277			
4	556229.910	4.054	101.348	101.348	0.835	0.824
4	548561.603	3.988	99.703			
8	1025504.756	8.080	100.999			
8	1009340.736	7.941	99.266	99.266	1.226	1.235
8	1031422.746	8.131	101.634			
10	1249487.641	10.002	100.016			
10	1245977.701	9.971	99.715	99.715	0.226	0.227
10	1244328.564	9.957	99.573			

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Table 6: Inter-day Precision Results

Conc (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	Average % Recovery	SD	%RSD
4	550853.857	4.008	100.195			
4	550717.553	4.007	100.166	100.166	0.489	0.488
4	546839.843	3.973	99.334			
8	1013723.048	7.979	99.736			
8	1026280.732	8.087	101.083	101.083	0.928	0.918
8	1009693.890	7.944	99.304			
10	1245697.192	9.969	99.690			
10	1241157.440	9.930	99.301	99.667	0.355	0.357
10	1249431.316	10.001	100.011			

E. Specificity

The specificity of the method was ascertained by injecting blank (mobile phase) as well as standard stock solution of Milrinone Lactate. There are no peaks in blank at retention time of drug. Also,peak purity values were found to be more than 996, indicating the no interference of any other peak of degradation product, impurity or matrix. (Table 7).

Table7: Peak purity of Milrinone Lactate

Drug	**Purity tail**	**Purity front**
Milrinone Lactate	996.348	998.569

F. Assay

MILRICAN injection formulation analysis was carried out as mentioned under section1D. Procedure was repeated for six times. Sample solution was injected and area was recorded. Concentration and % recovery was determined from linear equation. (Table8)

Table 8: Assay of marketed formulation

Sr. No.	**Peak area**	**Amount Recovered (µg/ml)**	**% Recovery**
1	552219.677	4.020	100.488
2	553127.188	4.027	100.683
3	548272.979	3.986	99.641
4	551245.508	4.011	100.279
5	550659.365	4.006	100.153
6	553439.433	4.030	100.749
Mean	551494.025	4.013	100.332
SD	1903.258	0.016	0.408
%RSD	0.345	0.407	0.407

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A. Accuracy

To check accuracy of the method, recovery studies were carried by spiking the standard drug to the sample solution, at three different levels around 50, 100 and 150 %.Basicconcentration of sample solution chosen was 10 µg/ml. % recovery was determined from linearity equation. The results obtained are shown in (Table 9)

Table 9: Recovery studies of Milrinone Lactate

Level	Conc. of Sample solution (µg/ml)	Conc. of Standard solution spiked (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	Mean % recovery ± RSD
50%	4	2	786697.876	6.031	100.519	100.426 ± 0.461
			782536.570	5.995	99.924	
			788906.252	6.050	100.835	
100%	4	4	1014724.747	7.987	99.843	100.093 ±0.692
			1012084.904	7.965	99.560	
			1024357.232	8.070	100.876	
150%	4	6	1264897.002	10.134	101.338	100.135 ± 1.112
			1248461.868	9.993	99.928	
			1239269.020	9.914	99.139	

B. Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase composition, detection wavelength, flow rate was altered and the effects on the area were noted. The results obtained are shown in Table 10.

Table 10: Robustness study

% RSD Found For Robustness Study(PeakArea)		
DETECTION WAVELENGTH(± 1 nm)		
269	270	271
566921.873	562300.952	552599.489
566886.002	556751.801	544461.845
574499.981	556614.040	540453.215
569435.952	558555.598	545838.183
4385.614	3244.304	6189.000
0.770	0.581	1.134

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FLOW RATE(± 0.05 ml/min)

0.95	1	1.05
570446.240	558405.908	564744.355
564813.395	551237.303	558601.231
561310.078	549307.415	556823.466
565523.2379	552983.5417	560056.3506
4609.260	4794.023	4156.098
0.815	0.867	0.742

MP COMPOSITION(± 2 ml in Composition)

62:38	60:40	58:42
543230.896	566503.345	564237.313
541837.661	572341.097	560643.210
546925.583	568176.214	561552.246
543998.0466	569006.8852	562144.2565
2629.282	3006.218	1868.757
0.483	0.528	0.332

Summary of validation study:

Table 11: Summary of validation study by HPLC method

Sr. No.	Validation Parameter	Milrinone Lactate
1.	Linearity	y = 116560x + 83705 R^2 = 0.994
2.	Range	2-12µg/ml
3.	Precision	(%RSD)
	A) Intraday precision	0.227–1.235
	B) Interday precision	0.357 – 0.918
4.	Assay ± %RSD	100.332± 0.407
5.	Accuracy	Mean % recovery ± %RSD
	50 %	100.426 ± 0.461
	100 %	100.093 ±0.692
	150 %	100.135 ± 1.112
6.	Limit of Detection	0.269
7.	Limit of Quantitation	0.815
8.	Robustness	Robust
9.	Specificity	Specific

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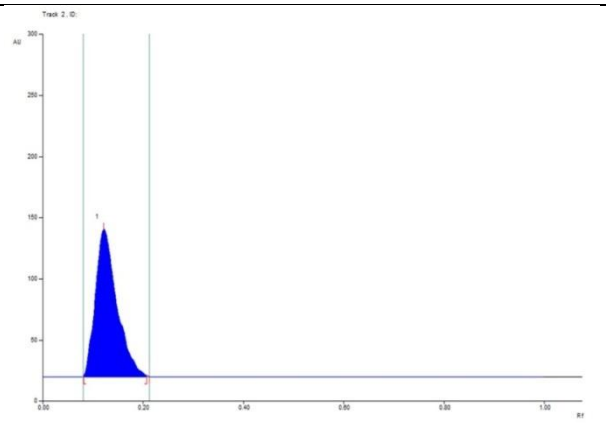
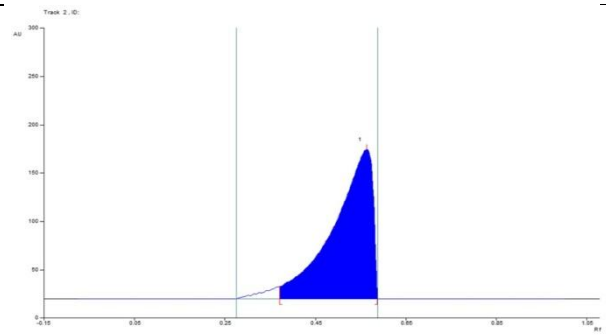
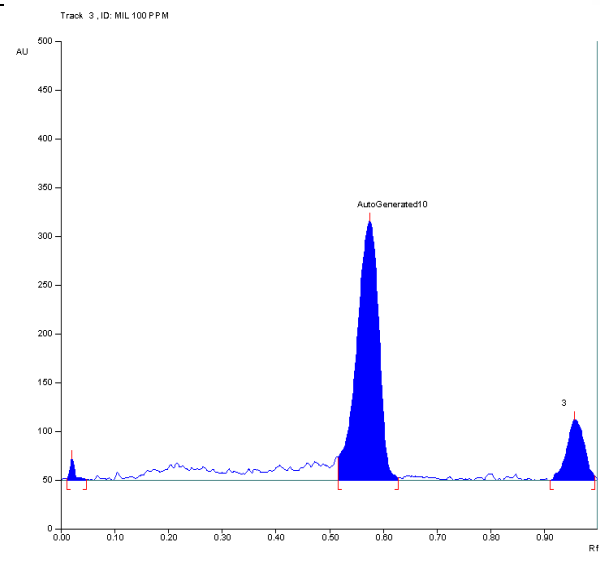
Development and validation of HPTLC method for estimation of Milrinone Lactate

Experimental, Results and Discussion:

Table 1: Trials of mobile phase for Milrinone Lactate

Sr.No.	Mobile phase	Observation	Densitogram
1.	n-Hexane: Ethyl acetate (8:2 v/v).	Peak retained at start position. peak Tailing	
2.	Chloroform: MeOH (7:3 v/v).	Peak Fronting	
3.	Diethyl Ether: Methanol (6.5: 3.5 v/v)	R_f – 0.57 with good peak shape, and acceptable peak parameters.	

A. Instrument Details

1. Camag HPTLC System

- Linomat - 5 sample applicator
- Camag TLC Scanner 3
- Win CATS software V- 1.4.2
- Hamilton syringe (100µl)

2. UV-Visible Double beam spectrophotometer (Jasco V-730) with single Monochromator.

3. Calibrated Glasswares

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- **Selection of mobile phase and chromatographic conditions**

Chromatographic separation studies were carried out on the working standard solution of Milrinone Lactate 100µg/ml. Initially, trials were carried out using solvents in various proportions on normal TLC plates to obtain the desired $R_f$and shape for drug peak. After few trials, Diethyl Ether: Methanol (6.5: 3.5 v/v)was chosen as the mobile phase, which gave acceptable peak parameters.Other chromatographic conditions like chamber saturation time, run length, sample application volume were optimized.

- **Preparation of Standard stock solution**

Standard stock solution of drug was prepared by dissolving 10 mg of the drug in 10 ml of methanol to get concentration of 1000 µg/ml. From the standard stock solution, working standard solution was prepared containing 100 µg/ml of Milrinone Lactate.

- **Preparation of sample solution:**

Injectioncontaining 10 mg of Milrinone Lactate(Milrican, American Remedies, Label Claim: Each ml contains Milrinone Lactate … 1 mg) was pipetted and was transferred to 10 ml volumetric flask and volume was made up with methanol to get concentration (100µg/ml).2 µl of this solution was applied on TLC plate

- **Selection of detection wavelength**

From the standard stock solution (1000 µg/ml) further dilutions were made using methanol and scanned over the range of 200-400 nm and the spectra was obtained. It was observed that the drug showed considerable absorbance at 270 nm. Representative UV spectrum of Milrinone Lactate is shown in Fig 1.

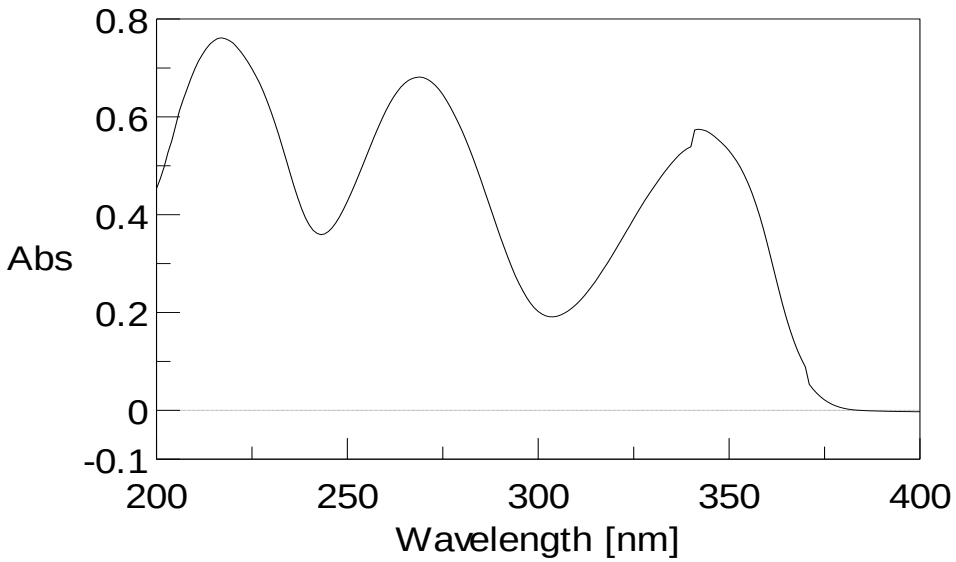

Fig. 1: The UV spectrum of Milrinone Lactate (10 µg/ml)

- **Densitogram**

Chromatographic separation of drug was performed on aluminum plates pre-coated with silica gel 60 GF_{254}, (10 cm × 10 cm with 250 µm layer thickness). From the standard stock solution (1000 µg/ml), working standard solution was prepared containing 100 µg/ml of Milrinone Lactate.2 µl of the resultant solution was applied on TLC plate as a band of 6 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland) with a linomat 5 applicator (Camag, Switzerland) to get concentration of 200 ng/band. The mobile phase was composed of Diethyl Ether: Methanol (6.5: 3.5 v/v). 10 cm × 10 cm Camag twin trough glass chamber was used for linear ascending development of TLC plate under 10 min saturation condition and 10 ml of mobile phase was used per run. Migration distance was 80 mm. Densitometric scanning was performed at 270 nm using Camag TLC scanner 3, operated by winCATS software, slit dimensions were 5.00×0.45 mm and Deuterium lamp was used as a radiation source. The retention factor was found to be 0.572 ± 0.652. Representative densitogram of Milrinone Lactate (200 ng/band) is shown in Fig. 2.

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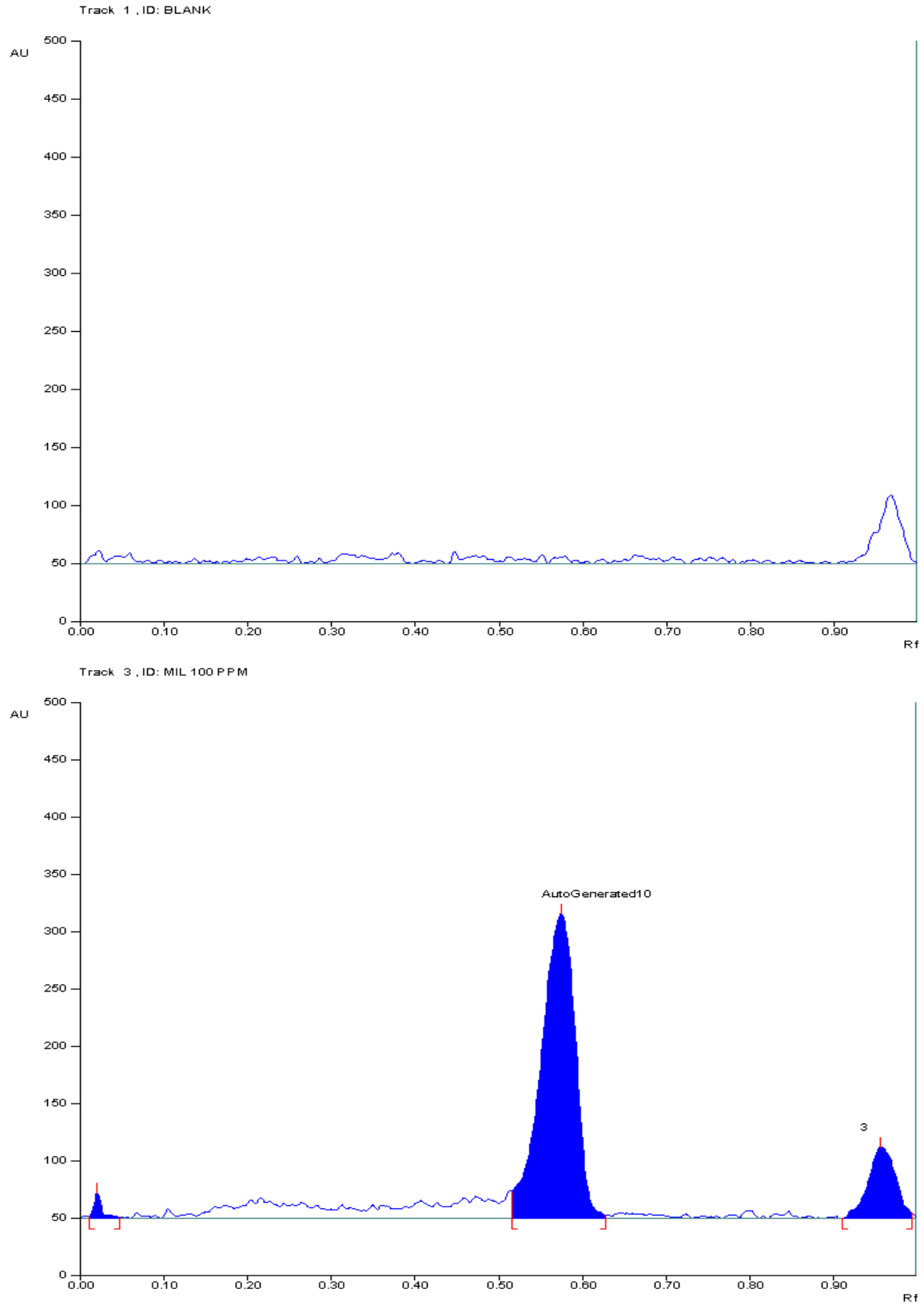

Fig 2: Densitogram of blank and standard solution of Milrinone Lactate (200 ng/band)

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- **Summary of chromatographic parameters selected:**

Chromatographic parameters are summarized in Table 2

Table 2 Chromatographic parameters

Sr. No.	Parameter	Conditions used for Analysis
1	Stationary phase	TLC aluminum plate precoated with silica gel 60 F_{254}
2.	Mobile phase	Diethyl Ether: Methanol (6.5: 3.5 v/v)
3.	Detection Wavelength	270 nm
4.	Saturation time	10 min
5.	Band width	6 mm

2.VALIDATION OF ANALYTICAL METHOD

- **Specificity:**

The specificity of the method was ascertained by peak purity profile studies. The peak purity values were found to be more than 0.997, indicating the no interference of any other peak of degradation product, impurity or matrix.

- **Linearity**

From the standard stock solution (1000 µg/ml) of Milrinone Lactate, solution was prepared containing 100 µg/ml of Milrinone Lactate. This solution was further used for spotting. Six replicates per concentration were spotted. The linearity (relationship between peak area and concentration) was determined by analyzing six concentrations over the concentration range 100-600 ng/band for Milrinone Lactate to obtain calibration curve. The results found to be linear with regression equation of y = 16.46x + 1483.52 and R^2= 0.995. Thedensitogram of linearity of Milrinone Lactate (100-600 ng/band) is shown in Fig. 3. Linearity study of Milrinone Lactate is shown in Table 3. The calibration curve is shown in Fig. 4.

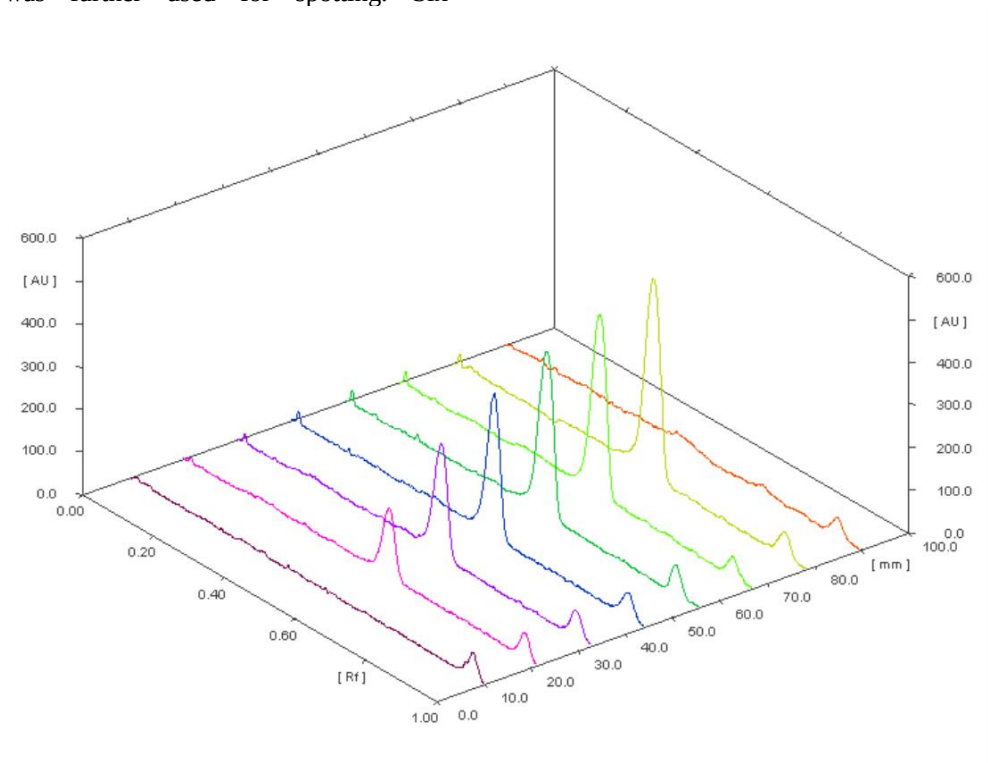

Fig 3: 3 D of the HPTLC densitogram of calibration bands

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Table 3: Linearity study of Milrinone Lactate

Replicate	Concentrations of Milrinone Lactate (ng/band)					
	100	200	300	400	500	600
	Peak area					
1	2906.68	4987.68	6375.30	8225.28	9939.06	10835.70
2	2859.19	4899.67	6301.98	8388.06	9930.78	11231.94
3	2900.17	4971.01	6465.96	8108.34	9781.08	11130.35
4	2902.53	4986.03	6513.96	8386.38	9738.84	11148.66
5	2858.51	4955.23	6450.75	8216.52	9835.60	11138.70
6	2910.53	4972.22	6347.04	8117.28	9931.56	11156.70
Average	2889.603	4961.972	6409.165	8240.309	9859.486	11107.008
SD	24.083	33.767	80.538	123.702	87.044	137.818
% RSD	0.833	0.681	1.257	1.501	0.883	1.241

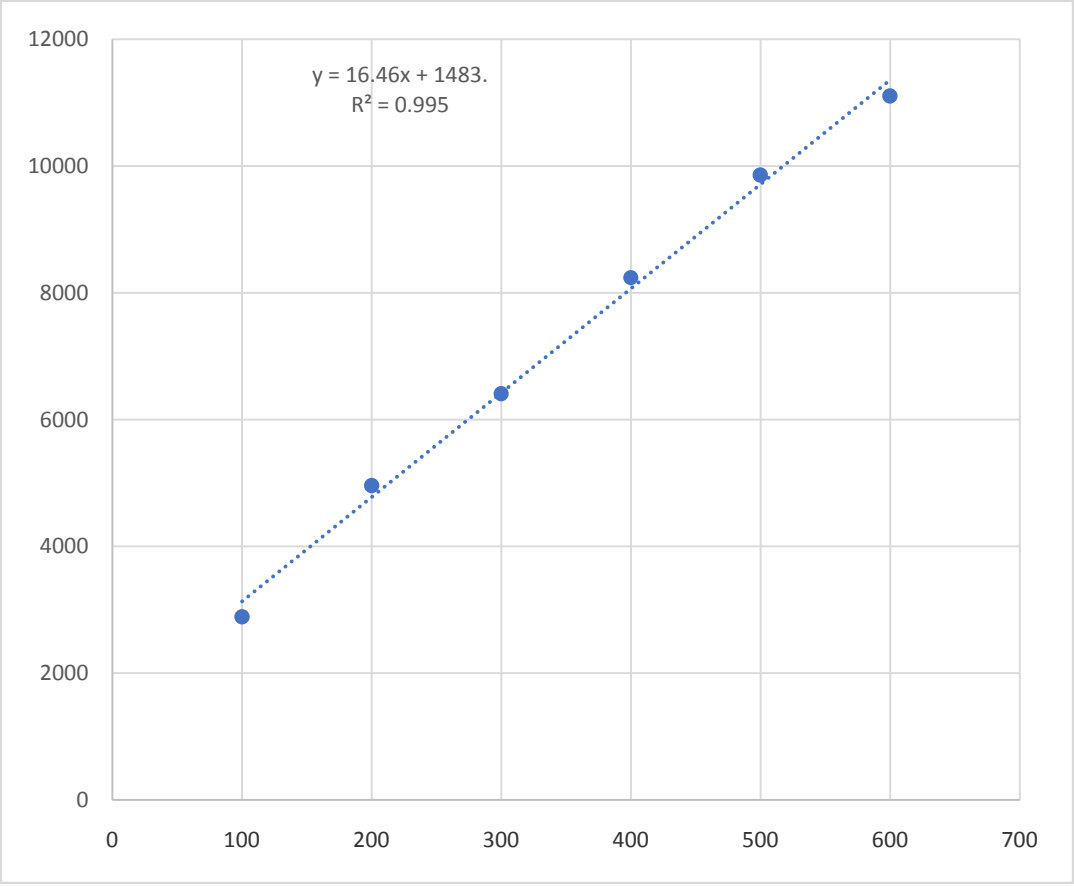

Fig 4: Calibration curve of Milrinone Lactate (100-600ng/band) reference standard

- **Range:**

Milrinone Lactate =100-600 ng/band

- **Precision:**

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the inta-day studies 3 replicates of 3 concentrations were analyzed on the same day, and % RSD was calculated. For the interday variation studies, 3 concentrations were analyzed on 3 consecutive days and % RSD was calculated. For intraday precision and interday precision results obtained are shown in Table 4 and 5.

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Table 4: Intraday variation studies data for Milrinone Lactate

Conc. (ng/band)	Area	% recovery	Average	SD	%RSD
200	4789.68	100.430			
	4748.66	99.184			
	4803.54	100.851	100.155	0.867	0.866
300	6399.04	99.545			
	6424.38	100.058			
	6393.54	99.433	99.679	0.333	0.334
400	8067.28	99.996			
	8066.08	99.978			
	8098.52	100.471	100.148	0.279	0.279

Table 5: Interday variation studies data for Milrinone Lactate

Conc. (ng/band)	Area	% recovery	Average	SD	%RSD
200	4780.62	100.155			
	4757.88	99.464			
	4766.84	99.736	99.785	0.348	0.349
300	6415.36	99.875			
	6425.96	100.090			
	6397.04	99.504	99.823	0.296	0.297
400	8057.31	99.845			
	8108.34	100.620			
	8107.28	100.604	100.356	0.443	0.441

- **Limit of Detection (LOD) and Limit of quantitation (LOQ):**

LOD and LOQ are calculated from the formula: -

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\ \sigma}{S}$$

Where,
σ = S.D of the response at lowest concentration or standard deviation of Y intercept;
S = Average of slope of the calibration curve

Table6: LOD and LOQ of Milrinone Lactate

Method	Average slope	S. D	LOD (ng/band)	LOQ (ng/band)
1.Using S.D of the response at lowest concentration	16.46	24.083	4.828	14.631
2. Using S.D of y-intercept	16.46	75.35	15.107	45.778

- **Assay:**

Injection formulation analysis was carried out as mentioned under section preparation of sample solution. Procedure was repeated for six times. 2µl volume of sample solution was applied and area was recorded. Basic concentration of

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sample chosen was 200 ng/band from. Concentration and % recovery was determined from linear equation. Assay results obtained are shown in Table 7. The representative densitogram is given in Fig. 5

Table 7: Assay of marketed formulation

Sr. No.	Peak area	Amount recovered (ng/band)	% recovery
1	4793.88	201.115	100.558
2	4768.56	199.577	99.789
3	4761.92	199.174	99.587
4	4779.28	200.228	100.114
5	4790.04	200.882	100.441
6	4782.42	200.419	100.210
Mean	4779.350	200.233	100.116
SD	12.289	0.747	0.373
%RSD	0.257	0.373	0.373

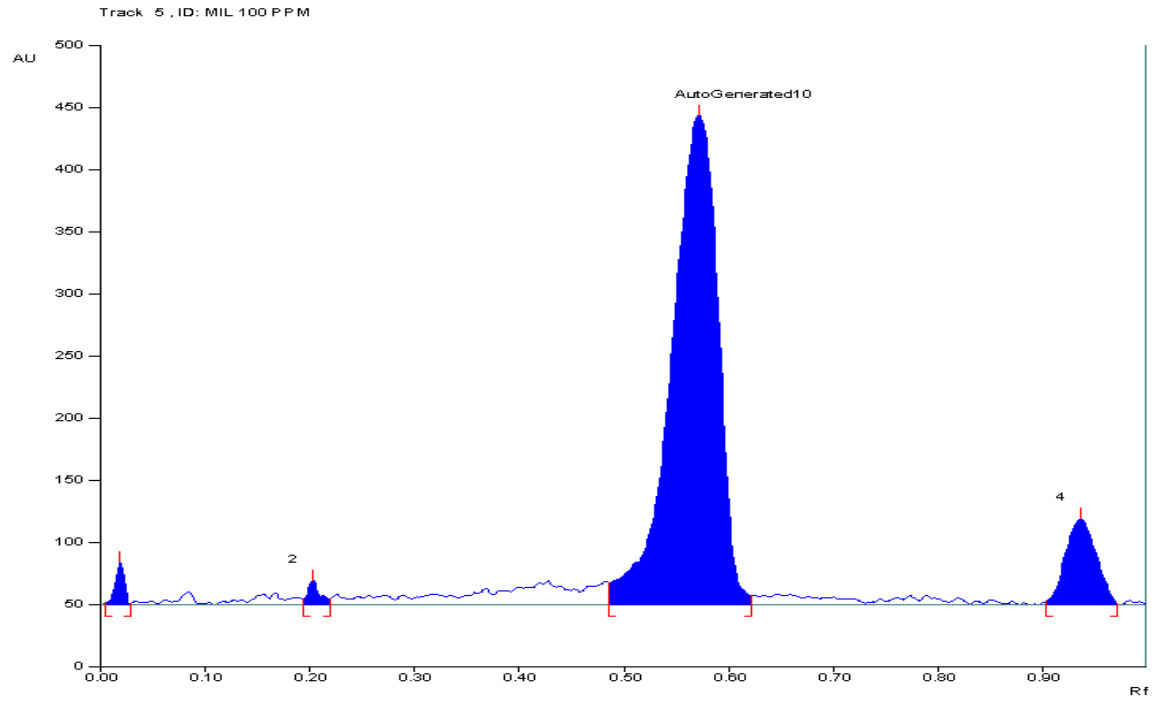

Fig. 5: Densitogram of sample solution of Milrinone Lactate (200 ng/band).

- **Accuracy:**

To check accuracy of the method, recovery studies were carried out by spiking the standard drug to the tablet solution, at three different levels 50, 100 and 150%. Basic concentration of sample chosen was 200 ng/band. % recovery was determined from linear equation. Accuracy results obtained are shown in Table 8.

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Table 8: Accuracy studies of Milrinone Lactate

Level	Amount of sample taken (ng/band)	Amount of standard spiked (ng/band)	Area	% recovery	Mean % Recovery ± RSD
50%	200	100	6420.76	99.985	100.166 ± 0.210
			6441.12	100.397	
			6427.32	100.117	
100%	200	200	8088.92	100.325	100.262 ± 0.495
			8115.16	100.724	
			8050.26	99.738	
150%	200	300	9719.73	100.075	100.215 ± 0.237
			9720.16	100.081	
			9753.84	100.490	

- **Robustness**

Robustness of the method was determined by carrying out the analysis under conditions during which chamber saturation time, mobile phase composition, time from spotting to development, time from development to scanning and wavelength change (+/- 1 nm) was changed and the effect on the area was noted. It was found that method is robust. The results obtained are shown in Table9.

Table9: Robustness study

Sr. No.	Parameters	Robust condition	%RSD
1	Saturation time (10 min)± 1 min	9 min	0.549
		10 min	0.627
		11 min	0.503
2	Time from spotting to development	Immediate	0.458
		After 30 min	0.869
		After 1 hr	0.486
3	Time from development to scanning	Immediate	1.039
		After 30 min	1.165
		After 1 hr	0.634
4.	Wavelength Change (+/- 1 nm)	269 nm	1.300
		270 nm	0.222
		271 nm	0.976

- **Summary of validation study**

Table 10: Summary of Validation Parameters

Sr. No.	Validation parameters	Milrinone Lactate
1.	Linearity equation R^2 Range	y = 16.46X + 1483.52 R^2= 0.995 100-600ng/band
2.	Precision	(%RSD)
	Intraday	0.279 – 0.866
	Interday	0.297 – 0.441
3.	Assay	100.116 ± 0.373
4.	Accuracy	(Mean ± % RSD)
	50 %	100.166 ± 0.210
	100 %	100.262 ± 0.495
	150 %	100.215 ± 0.237
5.	Limit of detection	15.107 ng/band
6.	Limit of quantitation	45.778 ng/band
7.	Specificity	Specific
8.	Robustness	Robust

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