1. INTRODUCTION OF HPTLC:

1.1 Planar Chromatography

Planar chromatography is a mode of chromatography in which the stationary phase is spread on a flat, planar surface. TLC is an important planar chromatographic technique that is widely used as a cost-effective method for rapid analysis of simple mixtures [1]. It requires minimum sample cleanup as it uses a disposable stationary phase and has a high sample throughput because of its ability to analyze several samples in parallel. It allows greater flexibility and simplicity in sample evaluation because of the possibility of sequential detection by complementary techniques, post chromatographic derivatization for identification and quantification, archiving a separation for evaluation at a later time, and accessibility of the sample because of the planar format [2]. It can be applied to samples where analyte lack a convenient chromophores making detection by other methods difficult and can be applied for analyzing samples with minimal pre-purification [3]. In some respects, the techniques of TLC and HPLC are complementary. TLC is the most popular method because of its low cost, simplicity, and flexibility [4].

1.2 Applications of HPTLC:

- ***Pharmaceuticals and Drugs:***

Identification, purity testing and determination of the concentration of active ingredients, auxiliary substances and preservatives in drugs and drug preparations, process control in synthetic manufacturing processes, Quality control, Stability testing etc [5, 6].

- ***Clinical Chemistry:***

Determination of active substances and their metabolites in biological matrices, diagnosis of metabolic disorders, etc [7].

- ***Forensic Chemistry and Biochemistry:***

Detection of document forgery, Investigation of poisoning, Dyestuff analyses, etc [8].

- ***Cosmetology:***

Dye raw materials and end products, preservatives, surfactants, fatty acids, constituents of perfumes, screening of illegal substances, etc.

- ***Food Analysis:***

Determination of pesticides and fungicides in drinking water, residues in vegetables, salads and meat, vitamins in soft drinks and margarine, banned additives, compliance

with limit values (e.g. polycyclic compounds in drinking water, aflatoxins in milk and milk products), etc [9].

- ***Environmental Analysis:***

Groundwater analysis, determination of pollutants from abandoned armaments in soils and surface waters, decomposition products from azo dyes used in textiles, etc [10].

- ***Analysis of Inorganic Substances:***

Determinations of inorganic ions (metals) and metal complexes, etc.

- **Industrial Applications [10]:**

Process development and optimization, process monitoring, cleaning validation, etc.

Thin layer chromatography (TLC); also known as planar-chromatography or flat bed chromatography is like all other chromatographic techniques, a multi stage distribution process [11]. HPTLC is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques [12]. TLC / HPTLC are often found more troublesome than GLC / HPLC as quantitative TLC is an off-line technique, hence automation is difficult and because of its open character, is highly influenced by environmental factors [13]. It is, therefore, essential that each step which may require specific approach must be carefully validated to determine potential source of error.

1.3 Factors influencing the TLC / HPTLC separation and resolution of spots:

- Types of stationary phase
- Types of pre-coated plates (TLC/HPTLC)
- Layer thickness/ Binder in the layer
- Size of the initial spot
- Solvent level in the chamber
- Gradient
- Relative humidity
- Size of developing chamber
- Saturation of chamber
- Sample volume to be spotted
- Temperature

- Separation distance
- Mode of development
- Separation distance

Greater the distance between different spots and smaller the initial spot diameter of the sample, better the resolution [14]. While describing the result of any TLC / HPTLC procedure, various parameters and conditions under which results for a specific analysis have been obtained must be documented. This is absolutely essential for possible results [15].

1.4 Steps involved in HPTLC [16]:

- Selection of chromatographic layer
- Sample and standard preparation
- Layer pre-washing
- Layer pre-conditioning
- Chromatographic development
- Application of standard and sample
- Detection of spots
- Scanning

2. METHOD DEVELOPMETN & OPTIMIZATION:

Selection of Chromatographic Condition:

In the present work, an analytical method based on HPTLC was developed and validated for assay determination of Dronedarone hydrochloride in tablet formulation. The various steps involved in TLC/HPTLC are Sample preparation, TLC plate pretreatment, and Sample application, Drying, Evaluation and Documentation. Thin layer chromatography is an off-line technique as compared to HPLC/GC but the properties that govern the selection of a method and its components are essentially same. Following are the factors or steps involved in method development by TLC, which results in good separation, which is one of the most critical steps in qualitative and quantitative analysis.

- Selection of stationary phase,
- Selection of development mode
- Selection of vapour phase,
- Selection of other operating parameter
- Selection of suitable solvent

- Optimisation of solvent mix

Traditionally TLC is inexpensive, simple to use and requires minimal instrumentation, laboratory space and maintenance. However, to achieve good precision, accuracy and reproducibility, a certain degree of instrumentation is required and densitometric detection is necessary for quantification.

Development of experimental condition:

The extent of separation of various components of a mixture by a given Thin layer chromatography method depends on the separation efficiency and selectivity of the separating system. The various factors influencing the separation are:

- Type of stationary phase
- Size of the developing chamber
- Type of pre-coated plate
- Layer thickness
- Saturation of the chamber
- Relative humidity
- Binder in the layer
- Temperature
- Mobile phase
- Separation distance
- Solvent purity

Mobile phase optimization:

The solvent system in TLC is generally selected by controlled trial and error method. In normal phase, TLC separation is carried out on a polar stationary phase (silica gel) using a non-aqueous mobile phase. Developing solvent usually is a mixture of non-polar organic solvent with a polar modifier such as methanol, ethyl acetate, acetone, acetonitrile to control the solvent strength and selectivity.

Sometimes small amounts of third component such acetic acid, ammonia, triethylamine and formic acid are added to mobile phase because they partially modify the surface of silica gel. Keeping the acidic and the basic centers in a molecule non-ionised; leads to decrease in the tailing of polar sample components.

The selection of the mobile phase is of prime importance in the development of a

chromatographic technique for proper elution, resolution, spot definition, symmetrical peak shapes and R*f* reproducibility of the analytes.

In present research work, initial trials were done using solvents composition using solvent like chloroform, methanol, ethyl acetate and hexane. It was observed that spot homogeneity not sufficient. Hence, the method finalized using of Acetone: Methanol (8:2: v/v) for better reproducibility of R*f* value. Thus, optimized mobile phase used for separation was of Acetone: Methanol in the volume ratio of (8: 2 v/v).

Dronedarone hydrochloride is found to be soluble in methanol therefore chosen as diluents for standard as well as sample preparation. Recovery of analyte in accuracy study was good with the use of methanol as solvent.

2.1 Optimized chromatographic condition for HPTLC:

Optimized chromatographic conditions for HPTLC method as under,

Table-1: Optimized chromatographic condition:

Parameters	Chromatographic Conditions
Development chamber	CAMAG Twin Trough Chamber
Stationary phase	Silica gel GF254 precoated on aluminum sheet
Mobile phase	Acetone: Methanol (8:2 v/v)
Chamber saturation	30 minutes
Sample applicator	Camag linomat v
Band	8mm
Space	9 mm
Scanning speed	20mm/sec
Development distance	80 mm
Drying of plate	Room temperature
Densitometric scanner	CAMAG TLC SCANNER
Lamp	Deuterium
Wavelenth	290 nm
Volume	10µl

3. INSTRUMENTATION:

Traditional Thin Layer Chromatography and its modern instrumental quantitative analysis version HPTLC are very popular for many reasons such as visual chromatogram, simplicity, multiple sample handling, low running and maintenance costs, disposable layer etc. HPTLC is the fastest of all chromatographic methods. Very few HPTLC plates are required to handle very large number of samples. An important scientific reason for modest popularity of HPTLC is that it is an open system due to which atmospheric

conditions can affect the chromatographic process during development i.e. during separation. Humidity is found to be variable affecting reproduction of results. Most HPTLC's done today are performed on silica gel, a strongly hydrophilic substance. The nature of silica gel is such that it separates both by adsorption throughout its structural OH group as well as partition though its absorbed moisture content. The HPTLC comprises of three sections. The lower section holds the 20 x 10 cm twin through chamber. The upper half is robotic where the plate is held for pre-conditioning the layer before development specified in the method as well as for drying the plate uniformly after development. The top section contains the electronics. The chromatographic system used to perform development and validation of this assay method was Camag Linomat V Sample applicator, Camag Twin trough glass chamber and Camag TLC scanner III equipped with Cats 3 Version software.

Solvent preparation:

The solvent contain of Acetone: Methanol in the volume ratio of (8: 2 v/v) was employed for the elution.

Diluent preparation:

Methanol was used as a diluent.

***Standard* preparation:**

Weigh accurately 50 mg of dronedarone hydrochloride in to 50 mL volumetric flask, dissolve and diluted up to the mark with diluent. Pipette out 5 mL of this solution into another 50 mL volumetric flask and diluted up to the mark with diluents (50µg/ml).

Test preparation:

Five tablets were weighed and the average weight of tablet was determined. Tablets were well crushed and mixed well. Weigh accurately 35.37 mg crushed mixture and transfer into 50 mL volumetric flask, added 35 mL of methanol and sonicated for 5 minute and dilute it up to the mark with diluent. Pipette out 5 mL of solution into 50 mL volumetric flask and dilute up to the mark with diluents. The concentration obtained was 50 µg/ml of Dronedarone hydrochloride.

Chromatographic conditions:

Chromatographic analysis was performed on HPTLC plates pre coated with 0.25 mm layer of chromatographic silica gel mixture (Silica GF254) on aluminum sheets. After

development of the chromatographic plate, the detection was carried out using an UV scanning densitometer set at a wavelength of 290nm.

4. METHOD VALIDATION:

4.1 Specificity:

In an assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. There should be no interference of the diluents / placebo at R*f* value of drug substances.

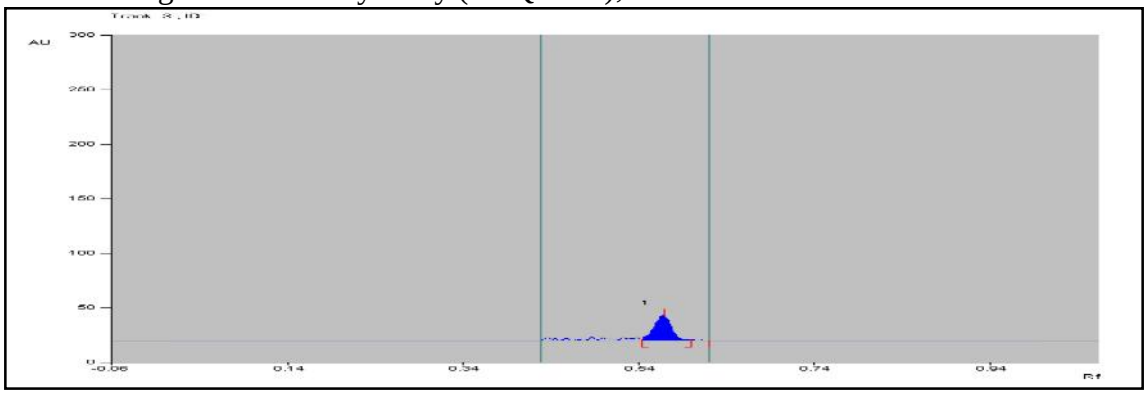
4.2 Linearity:

Standard stock solution:

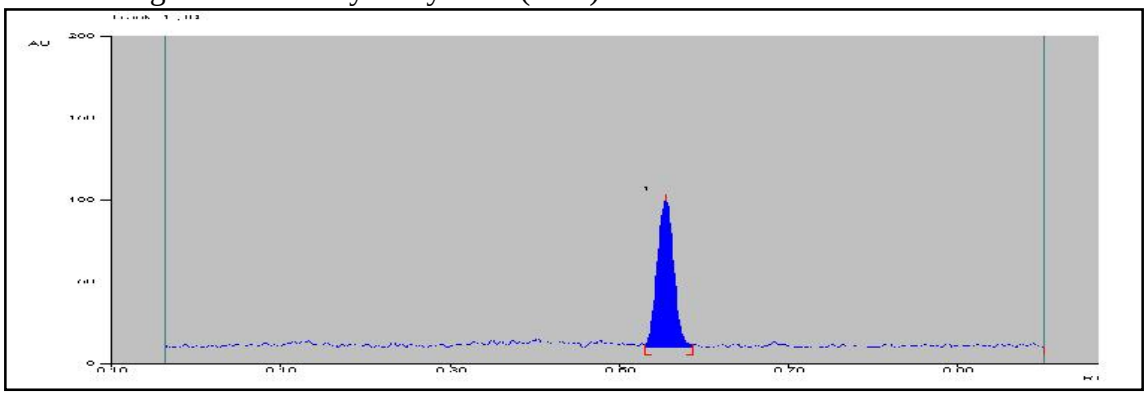
Weigh accurately 50.2 mg of dronedarone hydrochloride in to 50 mL volumetric falsk, dissolve and diluted up to the mark with diluent. Pipette out 2, 3, 4, 5, 6, 7 and 8 mL of this stock solution into another 50 mL volumetric flask and diluted up to the mark with diluents to obtained 40% to 160% concentration respectively.

For linearity seven points calibration curve were obtained in a concentration range from LOQ-160 % for Dronedarone hydrochloride. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for Dronedarone hydrochloride was y = 5.417x + 55.02 with correlation coefficient 0.999 where x is the concentration and y is the peak area in absorbance unit.

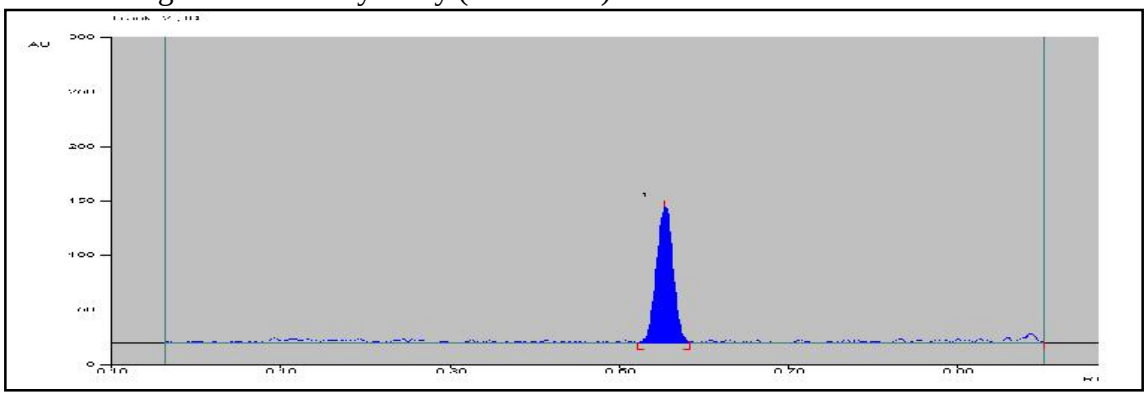
Chromatogram of linearity study (LOQ level);



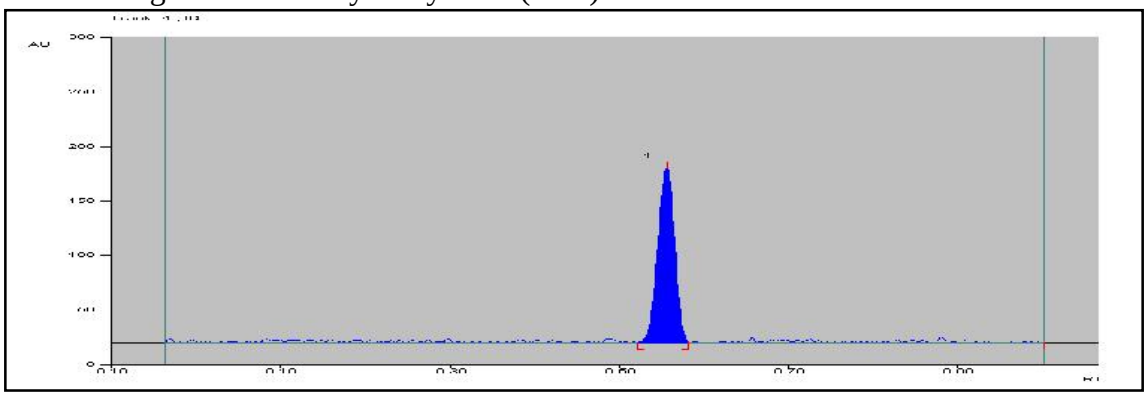
Chromatogram of linearity study level (40%):



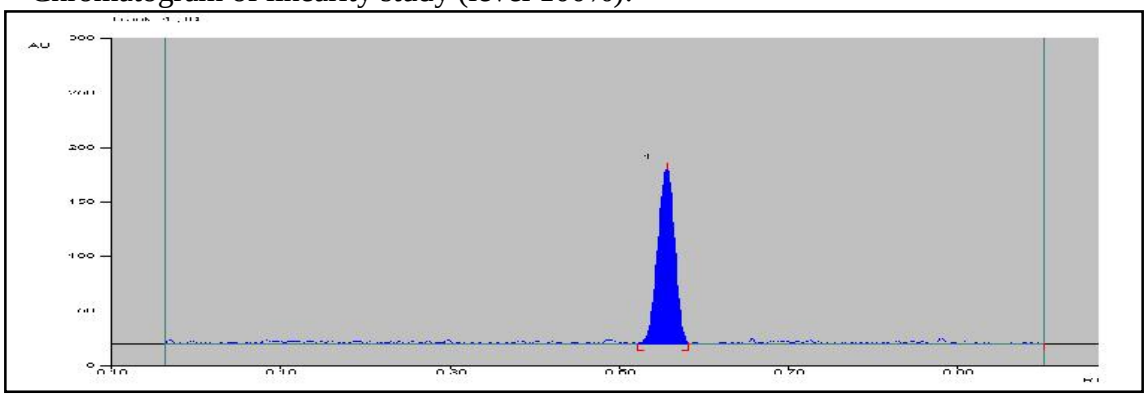
Chromatogram of linearity study (level-60%):



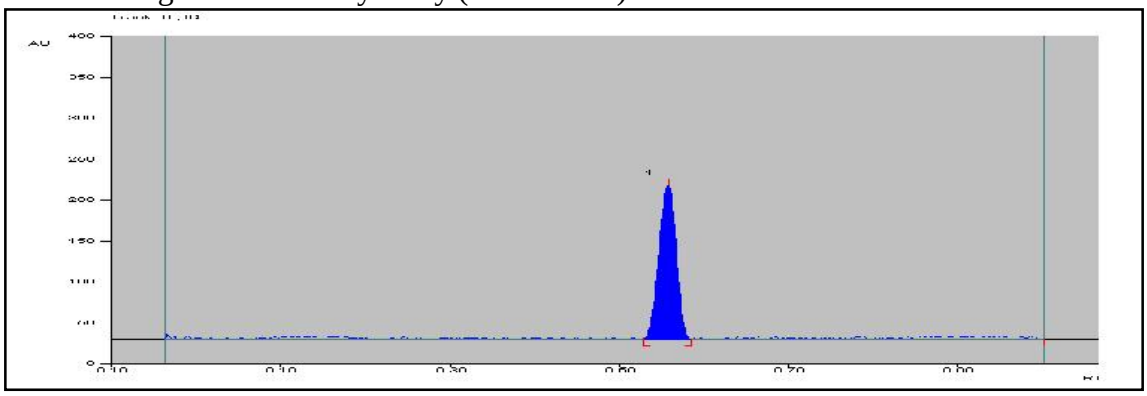
Chromatogram of linearity study level (80%):



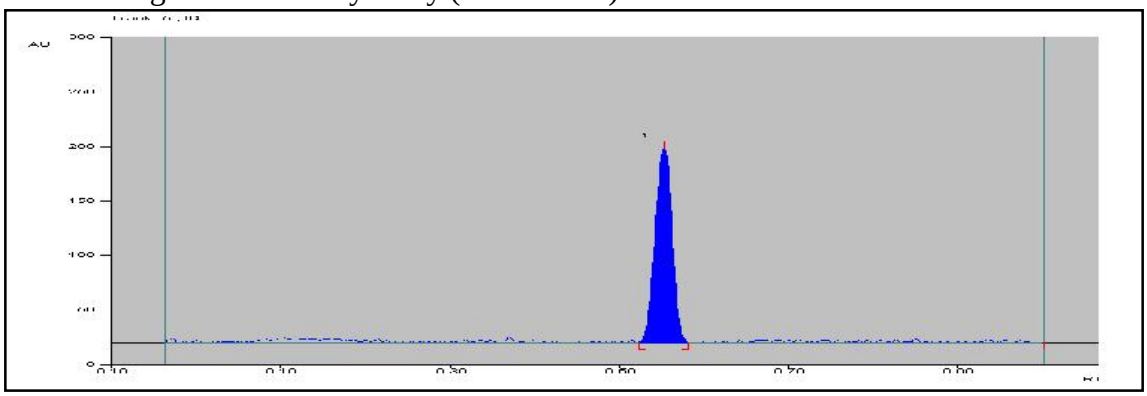
Chromatogram of linearity study (level 100%):



Chromatogram of linearity study (level-120%):



Chromatogram of linearity study (level-140%):



Chromatogram of linearity study (level 160%)

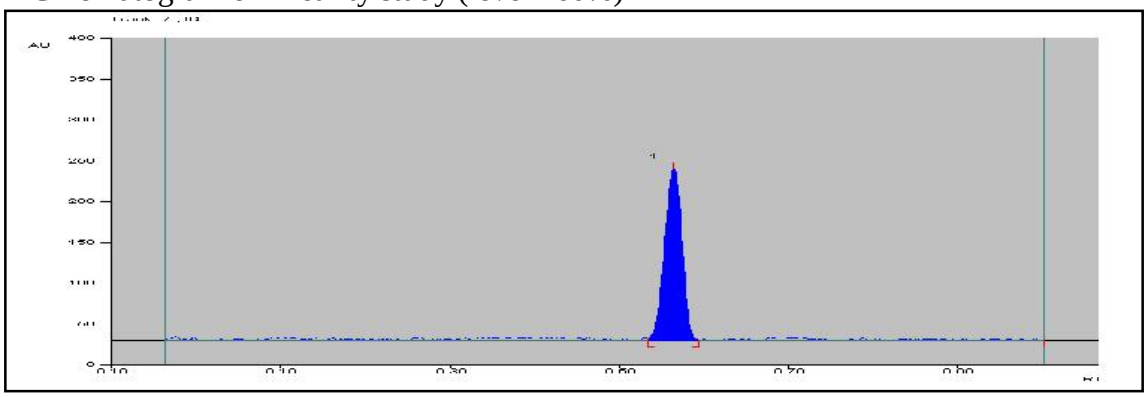


Chart-1: Linearity study of dronedarone

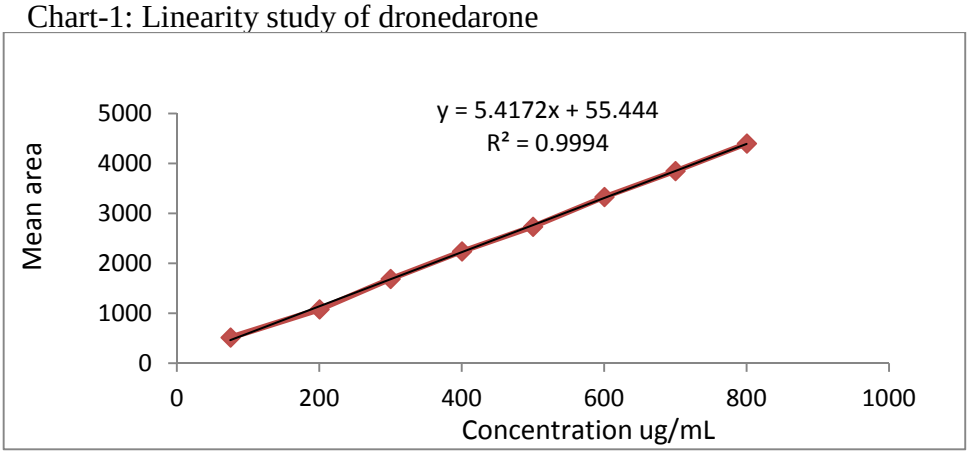


Table-2: Summary of linearity study

Linearity Level	% of Level	Concentration (ng/spot)	Mean Area
1	**LOQ**	**75.2**	**512**
2	40	200	1077
3	60	300	1686
4	80	400	2234
5	100	500	2731
6	120	600	3326
7	140	700	3847
8	160	800	4398
9			
Correlation Co-efficient			0.999
Slope			5.417
Intercept			55.44

4.3 Precision:

Standard preparation:

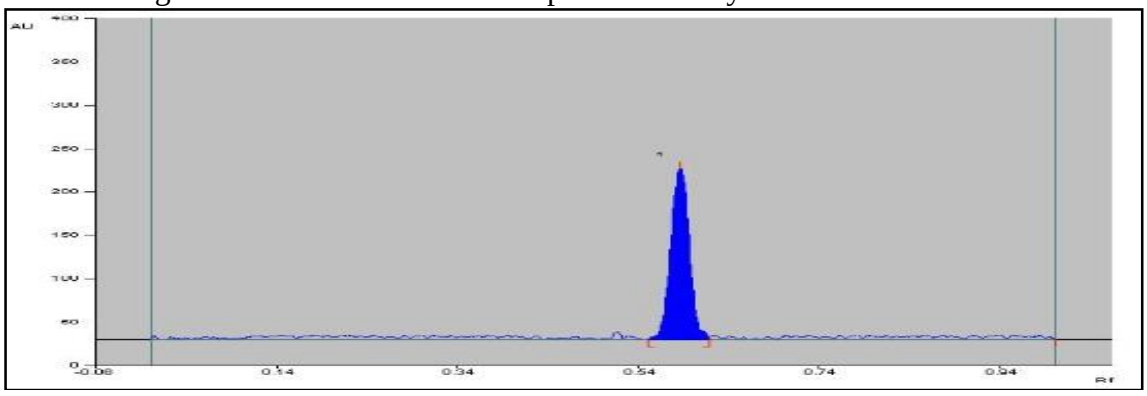
Weigh accurately 50.03 mg of dronedarone hydrochloride in to 50 mL volumetric falsk, dissolve and diluted upto the mark with diluent. Pipette out 5 mL of this solution into another 50 mL volumetric flask and diluted up to the mark with diluents (50.03µg/ml).

Sample preparation:

Five tablets were weighed and the average weight of tablet was determined. Tablets were well crushed and mixed well. Weigh accurately 35.48 mg crushed mixture and transfer into 50 mL volumetric flask, added 35 mL of methanol and sonicated for 5 minute and dilute it up to the mark with diluent. Pipette out 5 mL of solution into 50 mL volumetric flask and dilute up to the mark with diluents. The concentration obtained was 50 µg/ml of Dronedarone hydrochloride.

Data obtained from precision experiments are given in table-2 for intraday and inter day precision study for Dronedarone hydrochloride. The RSD values for intraday precision study and inter day precision study was < 2.0 % for Dronedarone hydrochloride which confirms that the method was precise.

Chromatogram of dronedarone standard precision study



Chromatogram of sample precision study

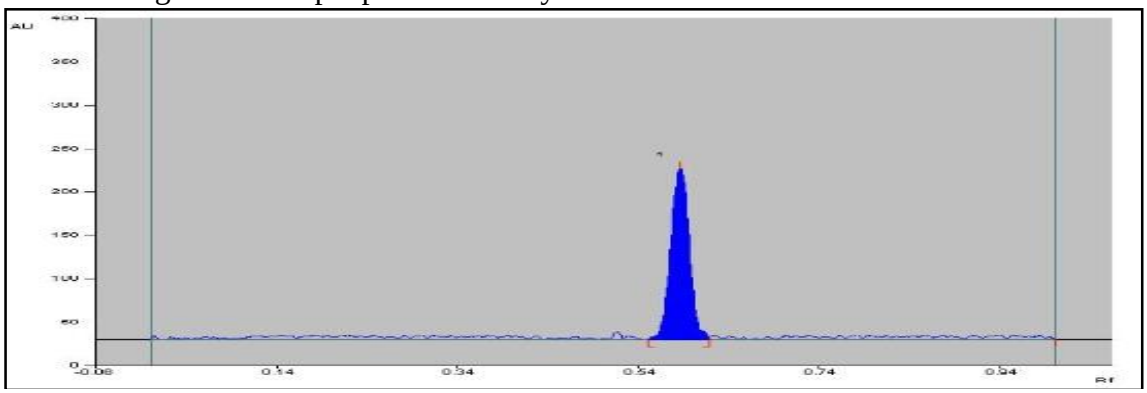


Table 3: Results of precision study

Set	%Assay [Intraday (n = 6)]	%Assay [Interday (n = 6)]
1	99.54%	99.69%
2	99.63%	99.22%
3	99.79%	100.27%
4	100.08%	100.61%
5	99.32%	99.85%
6	100.61%	99.38%
Mean	100.21%	99.92%
Standard Dev.	0.44%	0.48%
% RSD	0.44%	0.48%

4.4 Accuracy:

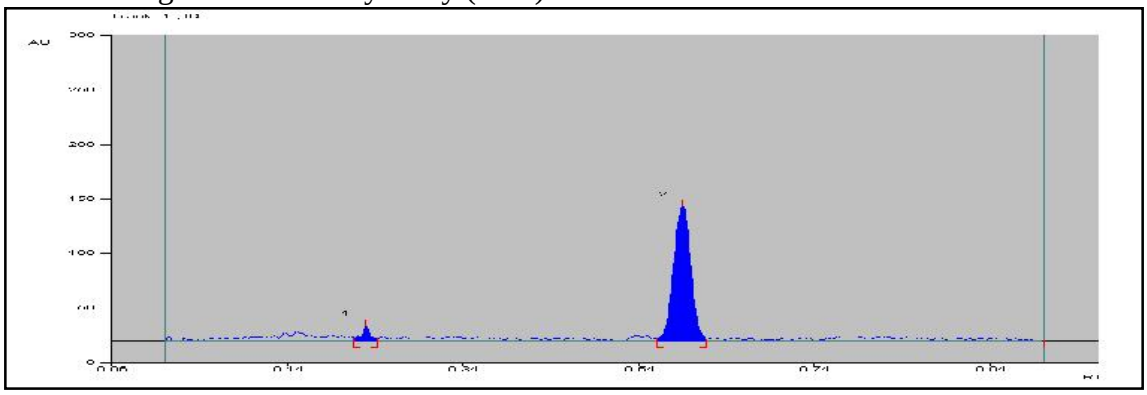
Standard preparation:

Weigh accurately 50.3 mg of dronedarone hydrochloride in to 50 mL volumetric falsk, dissolve and diluted upto the mark with diluent. Pipette out 5 mL of solution in 50 mL volumetric flask and diluted up to the mark with diluent.

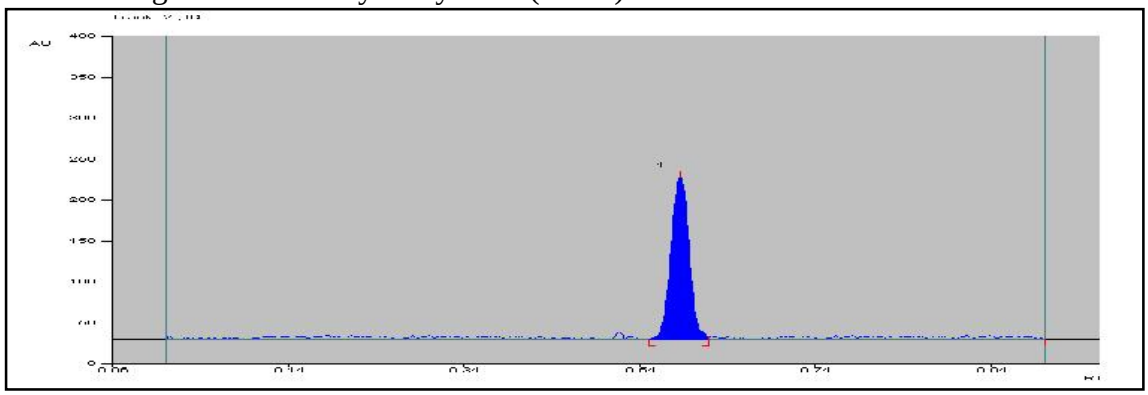
Sample preparation:

Weigh accurately 25.31, 50.26 and 75.22 mg of dronedarone hydrochloride standard and transfer into three different 50mL volumetric flask. Pipette out accurately. 5 mL of this solution into 50 mL volumetric flaks and diluted up to the mark with diluents. Concentration obtained is 50%, 100% and 150% repectively.

Chromatogram of accuracy study (50%)



Chromatogram of accuracy study level (100%)



Chromatogram of accuracy study level (150%)

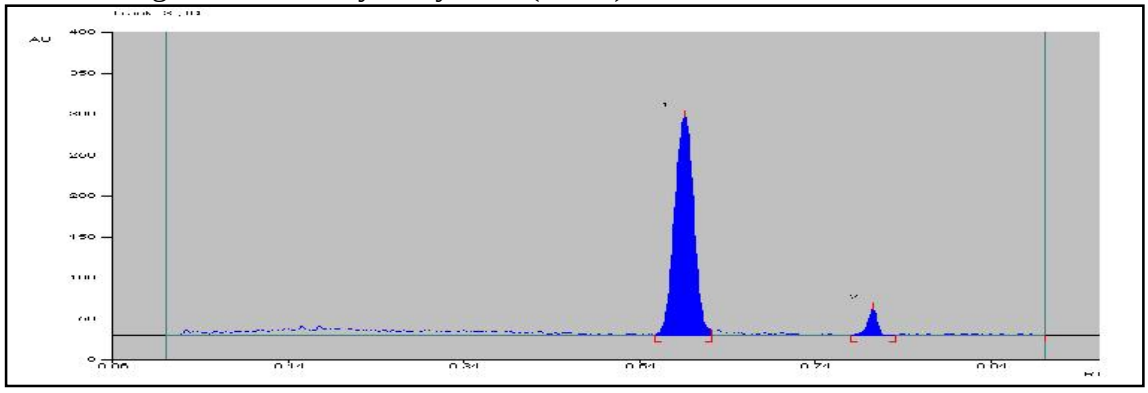


Table-4: Summary of accuracy study

Accuracy (Recovery) Study							
Accuracy Level	**Set No**	**Amount added (µg/ml)**	**Amount Found (µg/ml)**	**Recovery (%)**	**Average recovery**	**Std Dev.**	**% RSD**
I (50%)	1	25.31	25.42	100.42	100.33	0.14	0.14
	2	25.44	25.48	100.17			
	3	25.41	25.51	100.40			
II (100%)	1	50.26	49.52	99.42	99.47	0.13	0.13
	2	50.48	49.76	99.62			
	3	49.73	49.46	99.37			
II (150%)	1	75.22	74.98	99.68	99.79	0.10	0.10
	2	74.92	74.82	99.86			
	3	75.06	74.93	99.83			

4.5 Solution stability study:

Stability of sample solution was checked by using sample preparation from preparation from precision study stored at room temperature for 24 hours; withdrawn in the intervals of 2 hrs, 4hrs, 12hrs and 24hrs and applied on the chromate plate. After development, the chromatogram was evaluated for additional spots if any. There was no indication of compound instability in the sample solution.

Stability on the sorbent layer prior to development (spot stability): The time wherein the sample is left to stand on the sorbent prior to chromatographic development can influence the stability of separated spots and are required to be investigated for validation. Two-dimensional chromatography using same solvent system was used to find out any decomposition occurs during spotting and development. In case, if decomposition occurs during development, peaks of decomposition products are obtained for the analyte both in the first and second direction of run. No decomposition was observed during spotting and development.

Table-5: Summary solution stability study

SOLUTION STABILITY STUDY				
Stage	Sample solution		Standard solution	
	2-8 °C	RT	2-8 °C	RT
Initial	99.76%	99.76%	99.76%	99.76%
After 6 hours	99.62%	99.53%	99.66%	99.58%
After 12 hours	99.52%	99.41%	99.57%	99.42%
After 18 hours	99.36%	99.22%	99.30%	99.18%
After 24 hours	99.14%	98.85%	99.06%	98.87%

Table-6: Summary solution stability study

SOLUTION STABILITY STUDY				
Stage	Sample solution		Std. Solution	
	2-8 °C	RT	2-8 °C	RT
	Difference	Difference	Difference	Difference
Initial	NA	NA	NA	NA
After 6 hours	0.14%	0.23%	0.10%	0.18%
After 12 hours	0.24%	0.35%	0.19%	0.34%
After 18 hours	0.40%	0.54%	0.46%	0.58%
After 24 hours	0.62%	0.91%	0.70%	0.89%

4.6 Robustness study:

The result of robustness study of the developed assay method was established in Table 6. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

Standard preparation:

Weigh accurately 50.3 mg of dronedarone hydrochloride in to 50 mL volumetric falsk, dissolve and diluted upto the mark with diluent. Pipette out 5 mL of solution in 50 mL volumetric flask and diluted up to the mark with diluent.

Sample preparation:

Five tablets were weighed and the average weight of tablet was determined. Tablets were well crushed and mixed well. Weigh accurately 35.57 mg crushed mixture and transfer into 50 mL volumetric flask, added 35 mL of methanol and sonicated for 5 minute and dilute it up to the mark with diluent. Pipette out 5 mL of solution into 50 mL volumetric flask and dilute up to the mark with diluents. The concentration obtained was 50 µg/ml of Dronedarone hydrochloride.

Sample preparation:

Weigh accurately 25.31, 50.26 and 75.22 mg of dronedarone hydrochloride standard and transfer into three different 50mL volumetric flask. Pipette out accurately. 5 mL of this solution into 50 mL volumetric flaks and diluted up to the mark with diluents. Concentration obtained is 50%, 100% and 150% respectively.

Table-7 : Summary of robustness study

Robustness Study		
Sr. No	**Robust condition**	**% Assay**
1	Acetone:Methanol (8.5: 1.5)(v/v)	99.63%
2	Acetone :Methanol (7.5:2.5)(v/v)	99.47%
3	Analyst change	99.58%
4	Solvent volume (100mL)	100.17%
5	Solvent volume (150mL)	99.89%

4.7 System suitability:

A system suitability test of the chromatographic system was performed before each validation run to confirm the suitability and reproducibility of the system. The system suitability experiment as carried out using 100 ppm of Dronedarone hydrochloride. This solution was spotted five times on the chromatographic plate under the optimized conditions. Parameters that were studied to evaluate the suitability of the system where:-

1. % RSD of peak area
2. % RSD of retention factor of drug peak

The R*f* value of indivisual spotted should be comparable with the corresponding R*f* value of system suitability solution.The value of %Relative standard deviation of 5 replicates of peak area of bands should be well less than 5.0%. Whereas %Relative standard deviation of R*f* value should be less than 10.0 %.These conditions are fulfilled and the %Relative standard deviation for all parameter is well below the required limit for all the parameters.

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