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DISOPYRAMIDE BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION USING RP-UPLC CHROMATOGRAPHIC METHOD FOLLOWING ICH M10 GUIDELINES

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ABSTRACT

A simple, Accurate, precise method was developed for the estimation of Disopyramide in Rabbit plasma was developed and validated. By using precipitation method, the sample preparation was prepared. Chromatogram was run through Std Hibar C18 (100 x 2.1 mm, 2µ) Mobile phase containing Buffer Ammonium Acetate: Methanol taken in the ratio 60:40 was pumped through column at a flow rate of 0.2ml/min. For the separation of Disopyramide, Internal Standard [IS] used was Darunavir. The Temperature was maintained at 30°C. Optimized wavelength selected was 215nm. Retention time of Disopyramide and Internal Standard were found to be 1.281 min and 1.535 min. The standard curve was linear (R2 >0.995) over the concentration range of 0.15-6 ng/ml. All the analytical validation parameters were determined as per ICH guidelines The bioanalytical method developed approach was selective, robust, and reliable, as accuracy, precision, recovery, and other validation parameters were all within the recommendation's limitations. The peaks produced for the drug of interest and the internal standard were well separated from one another without any plasma interferences, and the peaks were symmetrical with an adequate tailing factor. The method has the potential to be very beneficial in therapeutic drug monitoring (TDM), bioequivalence research, pharmacokinetics studies, toxicology, and biomedical investigations.

KEYWORDS: Disopyramide, Internal Standard, RP UPLC, Bioanalysis, Rabbit Plasma.

INTRODUCTION

Bioanalytical techniques, employed for the quantitative determination of drugs and their metabolites in biological fluids and creates a specific procedure to enable a coalesce of interest to be identified and at the same time to be quantified in a matrix. A coalesce is measured by several procedures. The choice of analytical procedures involve many considerations, such as: concentration levels, chemical properties of the analyte, specimen matrix, cost of the analysis, experimental speed, quantitative or qualitative measurement, required precision and necessary equipment.[2] Bioanalytical method validation comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery, sensitivity, and stability.[1]

"UPLC is an emerging area of analytical separation science which retains the practicality and principles of UPLC while increasing the overall interlaced attributes of speed, sensitivity and resolution. Speed and peak capacity can be extended to new limits, termed Ultra Performance Liquid Chromatography, or UPLC by using fine particles. UPLC takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rates for increased speed, sensitivity and superior resolution.[3-5]

Disopyramide is a monocarboxylic acid amide that is butanamide substituted by a diisopropylamino group at position 4, a phenyl group at position 2 and a pyridin-2-yl group at position 2. It is used as a anti-arrhythmia drug. It has a role as an anti-arrhythmia drug. It is a monocarboxylic acid amide, a member of pyridines and a tertiary amino compound. It is chemically called as 4-[bis(propan-2-yl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide.[6]

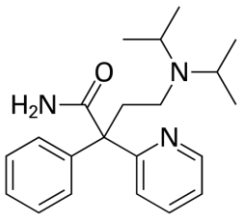

Figure 1: Chemical Structure of Disopyramide.

EXPERIMENTAL WORK

MATERIALS AND METHODS

Materials

Table 1: Chemicals and Solvents.

S. no	Chemical name	Grade	Manufacturing company
1	Distilled water	HPLC Grade	Rankem, Avantor performance material India limited
2	Water	Analytical Reagent	Rankem, Avantor performance material India limited
3	Acetonitrile	Analytical Reagent	Rankem, Avantor performance material India limited
4	Phosphate buffer	Analytical Reagent	Rankem, Avantor performance material India limited
5	Methanol	Analytical Reagent	Rankem, Avantor performance material India limited
6	Sodium dihydrogen phosphate	Analytical Reagent	Rankem, Avantor performance material India limited
7	Ortho-phosphoric acid	Analytical Reagent	Rankem, Avantor performance material India limited

4. Instruments

Table 2: Instruments and Equipment's.

S. no	Instrument	Company name	Brand name
1	Electronic balance	Sartorious	Denver
2	pH meter	Metsar	BVK enterprises
3	Sonicator	Lab man	BVK enterprises
4	Centrifuge	Thermo Fisher	-
5	Vertex	Remi CM101	-
6	Water	Acquity	UPLC Acquity

Methodology[7-10]

Diluent: Based up on the solubility of the drugs, diluent was selected, 0.01N Potassium dihydrogen phosphate and acetonitrile taken in the ratio of 60:40.

Extraction procedure

Take 750µl of plasma and 500µl of internal standard, 200µl of Disopyramide from the spiking solutions of both into a centrifuging tube and add 1 ml of Acetonitrile go for cyclomixer for 15 sec. Then vertex for 2 min and finally centrifuge for 5 min at 3200 rpm speed. After the centrifugation collect the sample and filter it directly inject 10 µL into into UPLC System.

Preparation of Disopyramide Stock solution (200 µg/ml)

Take 10 mg of Disopyramide in 50 ml volumetric flask and make the volume with diluent to produce 200 µg/ml.

Preparation of Disopyramide Spiking Solutions

From the above Disopyramide stock solution 0.05ml, 0.1ml, 0.15ml, 0.6ml, 1.0ml, 1.2ml, 1.6ml and 2.0 ml was pipette and transferred to 8 individual 10 ml volumetric flask and make up the volume up to the mark with diluent to produce 0.15 µg/ml, 0.3µg/ml, 0.45µg/ml, 1.2 µg/ml, 3 µg/ml, 3.6 µg/ml, 4.8 µg/ml and 6µg/ml.

Calibration standards and test drug sample were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.15 µg/ml, 0.3µg/ml, 0.45µg/ml, 1.2 µg/ml, 3 µg/ml, 3.6 µg/ml, 4.8 µg/ml and 6µg/ml.

Preparation of internal standard Solution (Darunavir)

Stock solution -1: Take 50 mg of Darunavir in 100 ml volumetric flask and make up the volume with diluent to produce 500µg/ml.

Stock Solution -2: From the above solution, take 1ml of solution into 10 ml volumetric flask and make up the volume with diluent to produce 50µg/ml solutions.

Final concentration: From the above solution, take 0.5ml of solution and spiking blank plasma with working stock dilutions of analyte to produce 10µg/ml ISD concentration.

Validation of optimized bioanalytical Method[11-20]

System Suitability Parameter

System Suitability test is performed that the test mixture is essential to check the specifications of a liquid chromatographic system. the System suitability testing limits are acceptance criteria that must be prior to sample analysis**.** The test is carried out by injecting six samples of quality control samples of MQC and check the criteria

acceptance accordingly as the % CV of the retention time (RT) should be ≤ 2.00 %.

Auto Sampler Carryover

Carry-over is an alteration of a measured concentration due to residual analyte from a preceding sample that remains in the analytical instrument, during validation carry-over should be assessed by analysing blank samples after the calibration standard at the ULOQ. Carry-over in the blank samples following the highest calibration standard should not be greater than 20% of the analyte response at the LLOQ and 5% of the response for the IS.

Specificity and Screening of Biological matrix

Specificity is the ability of a bioanalytical method to detect and differentiate the analyte from other substances, including its related substances (e.g., substances that are structurally similar to the analyte, metabolites, isomer, impurities, degradation products formed during sample preparation or concomitant medications that are expected to be used in the treatment of patients with the intended indication). Specificity is determined by the injecting six samples of standard solution and the LLOQC sample solution and check the % Interference Response of interfering peaks in STD Bulk at the retention time of analyte should be ≤20.00 % of that in LLOQ and At least 80 % of the matrix lots (B/iological Sample) with intended anticoagulant should be within the acceptance criteria.

Sensitivity

Sensitivity is often interpreted as related to the detection/determination ability, LLOQ based on precision and accuracy (bias) data, this is probably the most practical approach and defines the LLOQ as the lowest concentration of a sample that can still be quantified with acceptable Limit. the sensitivity is performed by injecting six injections of lower concentration of sample (LLOQ) the acceptance criteria of sensitivity of LLOQ are At least 67 % (4 out of 6) of samples should be within 80.00-120.00 %.

Matrix Factor evaluation

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation it is necessary to evaluate the matrix effect between different independent sources/lots. The matrix effect should be evaluated by analyzing at least 3 replicates of **low and high QCs (LQC and HQC),** each prepared using matrix from at least 6 different sources/lots. The accuracy should be within ±15% of the nominal concentration and the precision (per cent coefficient of variation (%CV)) should not be greater than 15% in all individual matrix sources/lots.

Linearity (Calibration Curve and Range)

the relationship between the nominal analyte concentration and the response of the analytical platform to the analyte, Calibration standards, prepared by spiking matrix with a known quantity of analyte, span the calibration range and comprise the calibration curve. Calibration standards should be prepared in the same biological matrix as the study samples. The calibration range is obtained by injecting 6 concentrations of calibration standards not including blank and zero samples and establishing the concentration-response relationship by the sample regression model method and the % accuracy for all CC standards except of LLOQ /8*96(STD 1) standard should be within 85.00-115.00 %. The % accuracy for LLOQ standard should be within 80.00-120.00 %.

Rugged Linearity

Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis, The calibration range is obtained by injecting 6 concentrations of calibration standards not including blank and zero samples and establishing the concentration-response relationship by the sample regression model method and The % accuracy for all CC standards except of LLOQ (STD 1) standard should be within 85.00-115.00 %.The % accuracy for LLOQ standard should be within 80.00-120.00 %.

Precision and Accuracy (Intra-day)

Accuracy and precision should be determined by analysing the QCs within each run (within-run) and in different runs (between-run). Accuracy and precision should be evaluated using the same runs and data. The test is performed injecting the QC samples were injected 6 replicates at each qc concentration level in each analytical run the overall accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Rugged Precision and Accuracy (Inter-Day)

Accuracy and precision should be evaluated using the same runs and data. The test is performed injecting the QC samples were injected 6 replicates at each qc concentration level in each analytical run the overall accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Recovery

Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Drug The recoveries for Disopyramide at LQC, MQC and HQC levels the results demonstrated that the bioanalytical method had good extraction efficiency by injecting the

six samples of LQC, MQC and HQC with the main drug and check the interference with unextracted and extracted, The % CV of recovery at each QC level should be ≤ 15.00 %. The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

Recovery of Internal Standard

The measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with Internal Standards containing the same area with known amount of Drug, the recoveries for IS at 6 replicates the results demonstrated that the bioanalytical method had good extraction efficiency by injecting the six samples and check the interference with unextracted and extracted, The % CV of recovery at each QC level should be ≤ 15.00 %. The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

Reinjection Reproducibility

Reproducibility of the method is assessed by replicate measurements of the QCs and is usually included in the assessment of precision and accuracy. However, if samples could be reinjected (e.g., in the case of instrument interruptions or other reasons such as equipment failure), reinjection reproducibility should be evaluated and included in the Validation Report or provided in the Bioanalytical Report of the study where it was conducted. The reproducibility was performed by injecting the qc samples in 6 replicates and check the acceptance limits the % mean accuracy for LQC, MQC and HQC samples should be within 85.00-115.00 % and for the LLOQ QC sample it should be within 80.00-120.00 %.

Stabilities[20-21]

Stability evaluations should be carried out to ensure that every step taken during sample preparation, processing and analysis as well as the storage conditions used do not affect the concentration of the analyte. The stability is assessed by long term stock solution stability and Matrix samples stability at -28±5 °C for 37 days & -80±5 ^{0}C, stability testing is performed by injecting the QC samples of high and low concentrations (HQC and LQC) with taken biological matrix The mean concentration at each QC level should be within ±15% of the nominal"

RESULTS AND DISCUSSIONS

Method development

Based on drug solubility and P^{ka} Value following conditions has been used to develop the method estimation of Disopyramide as per current ICH guidelines.

Optimization of the chromatographic conditions

"For developing the method for the assay of Disopyramide, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. A hypurity advance C_{18}column was chosen as the stationary phase for this study. The mobile phase and the flow rate in order to get sharp peaks and base line separation of the components, a number of experiments was carried out by varying the commonly used solvents, their compositions and flow rate. To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a C_{18} stationary phase. A binary mixture of acetonitrile and 0.01N Potassium dihyrogen ortho phosphate buffer in a ratio of 60:40 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 0.2 mL/min was found to be suitable. The drug molecule was tuned on the UPLC for the detection of Disopyramide and by injecting 0.15ng/mL and 6ng/ml concentration respectively. All the optimized system suitability parameters within the limits results".

Optimized method for the Disopyramide

Chromatographic conditions

Mobile phase: Methanol: Ammonium acetate (60:40)
Flow rate: 1.0ml/min
Column: Hibar (150mm x 4.6 mm, 3.5µ)
Detector wavelength: 215nm
Column temperature: 30^{0}C
Injection volume: 0.5µL
Run time: 3 min.

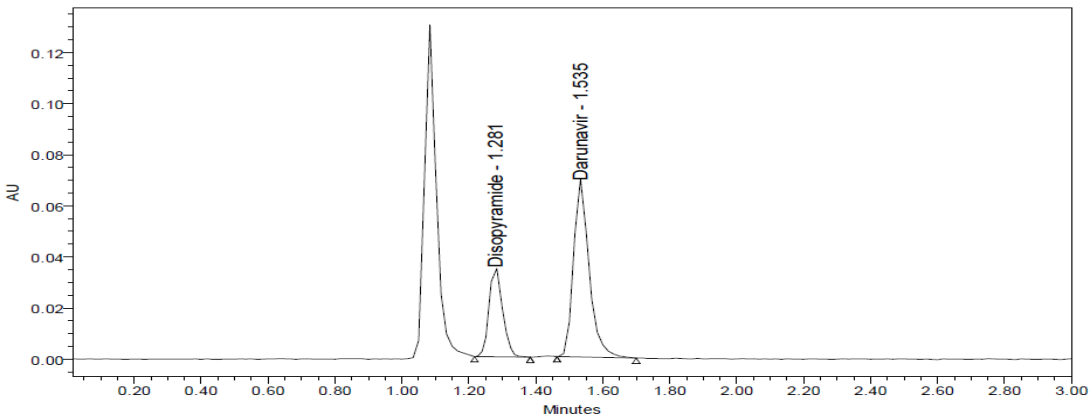

Fig no. 2: Optimized Chromatogram of Disopyramide.

Table no 9: Observation of Optimized Chromatogram.

S. No	Drug Name	RT	Area	USP plate count	USP tailing	USP resolution
1	Darunavir	1.281	102596	4552.6	1.2	3.0
2	Disopyramide	1.535	228320	4882.5	1.3	

Disopyramide and Internal Standard Darunavir were eluted at 1.535 min, 1.281min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. The Disopyramide and Darunavir (ISD) were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits.

METHOD VALIDATION

System suitability of Disopyramide

This system suitability method is intended to guarantee that the UPLC system is working in such a way that correct and reproducible data may be submitted to regulatory agencies with confidence. This procedure includes signal stability, carryover, and instrument response tests.

Table no 10: System Suitability of Disopyramide.

System Suitability					
S.No	Disopyramide		Darunavir		Area Ratio
	Analyte Area	Analyte RT (min)	ISTD Area	ISTD RT (min)	
1	91563	1.23	484562	1.525	0.1890
2	91745	1.22	487896	1.536	0.1880
3	91639	1.21	487453	1.535	0.1880
4	91856	1.21	487863	1.532	0.1883
5	91746	1.22	487562	1.536	0.1882
6	91786	1.21	487456	1.553	0.1883
MEAN		1.217		1.536	0.1882
SD		0.0082		0.0092	0.00035
%CV		0.67		0.60	0.19

Acceptance Criteria

The % CV of the retention time (RT) should be ≤ 2.00 %.

The % CV of the area ratio should be ≤ 5.00 %

Plate count, tailing factor, resolution of Disopyramide was According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. The % CV of the retention time (RT) should be ≤ 2.00 %. based on the results system suitability was passed.

Auto sampler carryover of Disopyramide

The carryover was tracked back to the injection valve and eradicated by converting from a partial loop injection to a full loop injection, which allowed more effective cleansing of the sample flow channel. The UPLC system's susceptibility to carryover was shown to be dependent on the detection method's absolute sensitivity and the mass of analyte injected at the assay's lower limit of quantitation (LLOQ). The results shows that the area obtained is less than 20 % of extracted LLOQ standard area to unextracted area by injected of replicate manner.

Table 11: Auto sampler carryover of Disopyramide.

Sample ID	Peak Area		% Carryover	
	Drug	ISTD	Drug	ISTD
Unextracted samples				
RS	0	0	N/A	N/A
AQ ULOQ	178134	487965	**0.00**	**0.00**
RS	0	0		
AQ LLOQ	4765	487652	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	176354	486523	**0.00**	**0.00**
STD Blk	0	0		
LLOQ	4736	486521	N/A	N/A

Acceptance Criteria

The carryover area response in subsequent injections of RS or STD Bulk after aqueous or extracted ULOQ should be ≤ 20.00 % of the equivalent aqueous or extracted LLOQ standard area.

Specificity and Screening of Biological Matrix

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The results were shows that the no interfering peaks were not Observed in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific.

Table 12: Specificity and Screening of Biological Matrix of Disopyramide.

S. No.	Sample ID	Response		% Interference		Pass/Fail
		Drug	ISTD	Drug	ISTD	
1	STD Blk1	0	0	0.00	0.00	Pass
2	LLOQ1	4756	483685			
3	STD Blk2	0	0	0.00	0.00	Pass
4	LLOQ2	4763	487632			
5	STD Blk3	0	0	0.00	0.00	Pass
6	LLOQ3	4796	487632			
7	STD Blk4	0	0	0.00	0.00	Pass
8	LLOQ4	4746	487632			
9	STD Blk5	0	0	0.00	0.00	Pass
10	LLOQ5	4738	487632			
11	STD Blk6	0	0	0.00	0.00	Pass
12	LLOQ6	4796	487632			

Acceptance Criteria

Response of interfering peaks in STD Blk at the retention time of analyte should be ≤20.00 % of that in LLOQ.
Response of interfering peaks in STD Blk at the retention time of ISTD should be ≤ 5.00 % of that in LLOQ.

At least 80 % of the matrix lots (excluding haemolysed, heparinised and lipemic matrix lots) with intended anticoagulant should be within the acceptance criteria. retention times of these drugs in this method. So, this method was said to be specific.

Sensitivity

A sensitivity is defined as "the lowest analyte concentration that can be measured with acceptable accuracy and precision i.e., LLOQ Nominal Concentration 0.150 ng/mL and Nominal Concentration Range 0.120ng/ml -0.180 ng/ml.

Table 13: Sensitivity of Disopyramide.

S.No	Calculated Concentration (ng/mL)
1	0.154
2	0.139
3	0.163
4	0.174
5	0.126
6	0.144
n	6
Mean	0.1500
SD	0.01729
% CV	**11.52**
% Mean Accuracy	**100.00**

Acceptance Criteria

At least 67 % (4 out of 6) of samples should be within 80.00-120.00 %.
% Mean accuracy should be within 80.00-120.00 %.
% CV accuracy should be ≤ 20.00 %.

The LLOQ concentration was found between 80 -120 % and % Coefficient of variation found to be 11.52% and mean of 6 injections was found to be 100.00 % within the acceptance limits. As the limit of Sensitivity % CV was less than "20%" the system Sensitivity was passed in this method.

Matrix factor evaluation

The Evaluation of Matrix by injecting the samples of high and low concentrations in 6 lots the %Mean obtained was 99.18% and 99.91 of HQC and LOQ and % CV obtained are 11.35% and 8.92% of HQC and LOQ. As the limit of CV was less than "20%" the system Matrix was passed in this method.

Table no 14: Matrix factor evaluation (absence of matrix factor).

S. No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration (ng/mL)	
		4.800	0.450
		(4.080-5.520)	(0.383-0.518)
		Calculated Concentration (ng/mL)	
1	LOT1	4.092	0.472
		4.084	0.396
		5.490	0.506
2	LOT2	5.287	0.454
		4.099	0.394
		4.258	0.418
3	LOT3	4.953	0.431
		5.435	0.510
		5.254	0.500
4	LOT4	4.454	0.415
		4.539	0.485
		4.124	0.429
5	LOT5	4.191	0.404
		5.425	0.426
		5.139	0.462
6	LOT6	4.738	0.421
		5.369	0.509
		4.761	0.461
N		18	18
Mean		4.7607	0.4496
SD		0.54057	0.04010
% CV		**11.35**	**8.92**
% Mean Accuracy		**99.18**	**99.91**

Acceptance Criteria

At least 67 % (2 out of 3) of samples at each level should be within 85.00-115.00 %.At least 80 % (5 out of 6) of the matrix lot should be within the acceptance criteria.

The % mean accuracy of back calculated concentration of LQC and HQC samples prepared from different biological matrix lots should be within 85.00-115.00 %.

Linearity

Table 17: Linearity of Disopyramide.

Acquisition Batch ID	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							
	0.150	0.300	0.450	1.200	3.000	3.600	4.800	6.000
	Nominal Concentration Range (ng/mL)							
	(0.120-0.180)	(0.255-0.345)	(0.383-0.518)	(1.020-1.380)	(2.550-3.450)	(3.060-4.140)	(4.080-5.520)	(5.100-6.900)
	Back Calculated Concentration (ng/mL)							
P&A1	0.128	0.275	0.396	1.143	2.784	3.139	4.387	5.358
P&A2	0.153	0.298	0.452	1.162	2.863	3.654	4.823	5.594
P&A3	0.165	0.324	0.483	1.286	3.264	3.965	5.118	6.447
n	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
Mean	0.1487	0.2990	0.4437	1.1970	2.9703	3.5860	4.7760	5.7997
SD	0.01888	0.02452	0.04409	0.07766	0.25737	0.41718	0.36776	0.57289
%CV	**12.70**	**8.20**	**9.94**	**6.49**	**8.66**	**11.63**	**7.70**	**9.88**
% Mean Accuracy	**99.11**	**99.67**	**98.59**	**99.75**	**99.01**	**99.61**	**99.50**	**96.66**

Acceptance Criteria

The % accuracy for all CC standards except of LLOQ (STD 1) standard should be within 85.00-115.00 %.The % accuracy for LLOQ standard should be within 80.00-120.00 %.

At least 75 % of CC standards should meet the acceptance criteria, including the LLOQ and highest CC standard (ULOQ). Any two consecutive points shall not be excluded.

Response of interfering peaks in STD Blk and STD ZERO at the retention time of analyte should be ≤ 20.00 % of that in LLOQ.

Response of interfering peaks in STD Blk at the retention time of ISTD should be ≤ 5.00 % of that in LLOQ**.**

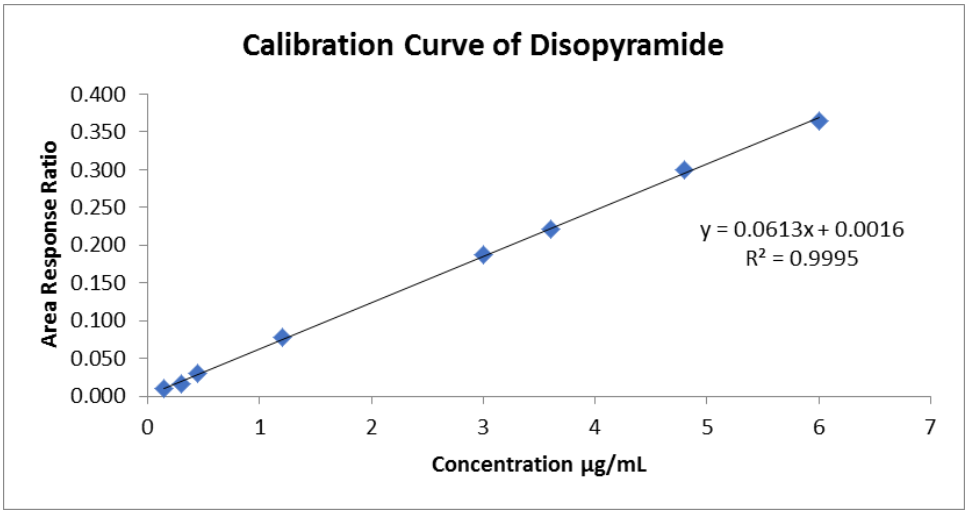

Fig. 5: Representative Calibration Curve for Regression Analysis.

The Calibration was found to be linear over the concentration range of 0.15 to 6 µg /ml. The coefficient correlation (r^2) value was found consistently greater than 0.999 in all the cases. This indicating linearity of results and an excellent correlation between peak area ratios for each concentration of analytes.

Precision and accuracy (intra-day runs of Disopyramide)

Table no. 18: Precision data for intra-day runs of Disopyramide.

S. No.	HQC	MQC	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	4.800	**3.000**	**0.450**	**0.150**
	Nominal Concentration Range (ng/mL)			
	(4.080-5.520)	(2.550-3.450)	(0.383- .518)	(0.120-0.180)
	Back Calculated Concentration (ng/mL)			
1	4.126	2.296	0.396	0.125
2	4.251	2.864	0.428	0.139
3	4.531	2.987	0.434	0.142
4	4.854	3.125	0.451	0.149
5	5.135	3.254	0.482	0.150
6	5.298	3.367	0.506	0.168
n	**6**	**6**	**6**	**6**
Mean	**4.6992**	**2.9822**	**0.4495**	**0.1455**
SD	**0.47543**	**0.38137**	**0.03954**	**0.01424**
%CV	**10.12**	**12.79**	**8.80**	**9.79**
% Mean Accuracy	**97.90**	**99.41**	**99.89**	**97.00**
1	4.096	2.654	0.395	0.123
2	4.238	2.753	0.413	0.135
3	4.655	2.864	0.425	0.143
4	4.543	2.564	0.453	0.147
5	5.253	3.135	0.493	0.156
6	5.423	3.351	0.509	0.176
n	**6**	**6**	**6**	**6**
Mean	**4.7013**	**2.8868**	**0.4480**	**0.1467**
SD	**0.53544**	**0.30130**	**0.04546**	**0.01821**
%CV	**11.39**	**10.44**	**10.15**	**12.41**
% Mean Accuracy	**97.94**	**96.23**	**99.56**	**97.78**
1 2	4.125	2.642	0.389	0.129

3	4.298	2.725	0.408	0.132
4	4.566	2.810	0.415	0.140
5	4.632	2.631	0.447	0.145
6	5.185	3.123	0.486	0.151
	5.510	3.392	0.515	0.168
n	6	6	6	6
Mean	4.7193	2.8872	0.4433	0.1442
SD	0.52989	0.30609	0.04894	0.01422
%CV	11.23	10.60	11.04	9.86
% Mean Accuracy	98.32	96.24	98.52	96.11
Between Batch Precision and Accuracy				
n	18	18	18	18
Mean	4.7066	2.9187	0.4469	0.1454
SD	0.48319	0.31490	0.04218	0.01475
%CV	10.27	10.79	9.44	10.14
% Mean Accuracy	98.05	97.29	99.32	96.96

Acceptance Criteria

The within and between batch precision for LQC, MQC and HQC samples should be ≤ 15.00 % and for the LLOQ QC, it should be ≤ 20.00 %.

Intra batch

At least 67 % (16 out of 24) of total QC samples and 50 % (3 out of 6) at each level should be within 85.00-115.00 % except LLOQ QC. LLOQ QC should be within 80.00-120.00 %.

% Mean accuracy for LQC, MQC and HQC samples should be within 85.00-115.00 % and for the LLOQ QC sample it should be within 80.00-120.00 %.

Inter batch

% Mean accuracy between batch for LQC, MQC and HQC samples should be within 85.00-115.00 % and for the LLOQ QC sample it should be within 80.00-120.00 %.

Rugged Precision and Accuracy (inter-day runs of Disopyramide)

The intraday and inter day accuracy and precision was assessed by analysing six replicates at five different QC levels like LLOQ, LQC, MQC and HQC. Accuracy and precision method performance was evaluated by determined by six replicate analyses for Disopyramide at four concentration levels, i.e.,0.15µg/ml(LLOQ), 0.45 µg/ml (LQC), 3 µg/ml (MQC) and 4.8 µg/ml HQC The intra-day and inter day accuracy of plasma samples were assessed and excellent mean % accuracy was obtained with range varied from 99.96-100.35%, and 98.99%-99.93 % for intraday and 99.06%-100.02 and 98.91%-100.24 for inter day respectively. The precision (%CV) of the analytes and plasma samples were calculated and found to be 0.38-11.54% and 0.76%-13.49% for intraday and 0.66%-14.23% and 0.77 %-13.16% for inter day respectively.

Table no 19: precision data for inter-day runs of Disopyramide.

P&A ID	**HQC**	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	4.800	**3.000**	**0.450**	**0.150**
	Nominal Concentration Range (ng/mL)			
	(4.080-5.520)	(2.550-3.450)	(0.383-0.518)	(0.120-0.180)
	Calculated Concentration (ng/mL)			
Different Column	4.183	2.623	0.395	0.129
	4.452	2.952	0.415	0.132
	4.632	2.573	0.426	0.145
	4.852	3.152	0.470	0.152
	5.126	3.251	0.475	0.162
	5.365	3.321	0.512	0.178
N	**6**	**6**	**6**	**6**
Mean	**4.7683**	**2.9787**	**0.4488**	**0.1497**
SD	**0.43640**	**0.32037**	**0.04402**	**0.01855**
% CV	**9.15**	**10.76**	**9.81**	**12.40**
% Mean Accuracy	**99.34**	**99.29**	**99.74**	**99.78**
Different Analyst	4.187	2.552	0.392	0.132
	4.456	2.877	0.399	0.136
	4.623	2.937	0.413	0.139

	4.825	3.120	0.445	0.148
	5.162	3.228	0.489	0.156
	5.325	3.181	0.515	0.174
N	**6**	**6**	**6**	**6**
Mean	**4.7630**	**2.9825**	**0.4422**	**0.1475**
SD	**0.43000**	**0.25184**	**0.05047**	**0.01562**
% CV	**9.03**	**8.44**	**11.41**	**10.59**
% Mean Accuracy	**99.23**	**99.42**	**98.26**	**98.33**

Acceptance Criteria

The within and between batch precision for LQC, MQC and HQC samples should be ≤ 15.00 % and for the LLOQ QC, it should be ≤ 20.00 %.

At least 67 % (16 out of 24) of total QC samples and 50 % (3 out of 6) at each level should be within 85.00-115.00 % except LLOQ QC. LLOQ QC should be within 80.00-120.00 %.

% Mean accuracy for LQC, MQC and HQC samples should be within 85.00-115.00 % and for the LLOQ QC sample it should be within 80.00-120.00 %.

Acceptance criteria

Precision: Low, medium & high QC concentrations should be within 15% & 20% for LLOQ conc.

Accuracy: Low, medium & high QC concentrations should be within ±15% & ±20% for LLOQ conc of nominal value.

Recovery of Disopyramide

Table no 20: Recovery of Disopyramide.

S. No.	HQC		MQC1		LQC	
	Un extracted Response	**Extracted Response**	**Un extracted Response**	**Extracted Response**	**Un extracted Response**	**Extracted Response**
1	147865	145763	91586	90999	14762	14625
2	145486	143215	91632	91245	14732	14563
3	148753	147632	91745	91563	14738	14532
4	149856	148874	91463	91325	14536	14363
5	147542	147375	91463	91562	14732	14663
6	145632	144365	91785	91563	14746	14532
n	**6**	**6**	**6**	**6**	**6**	**6**
Mean	147522	146204	91612	91376	14708	14546
SD	1720.37	2147.08	136.45	230.84	84.85	104.02
% CV	**1.17**	**1.47**	**0.15**	**0.25**	**0.58**	**0.72**
% Mean Recovery	**99.11**		**99.74**		**98.90**	
Overall % Mean Recovery	**99.251**					
Overall SD	**0.4378**					
Overall % CV	**0.44**					

Acceptance Criteria

The % CV of recovery at each QC level and for ISTD should be ≤ 15.00 %.

The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

Recovery - Internal standard

Table no 21: Recovery of Darunavir (IS).

S.No.	Un extracted Area Ratio	Extracted Area Ratio
1	485236	487632
2	486584	486215
3	485523	487634
4	487632	486252
5	487635	486589
6	489486	486932
n	6	6
Mean	487016.0	486875.7

SD	1577.82	641.55
% CV	0.32	0.13
% Mean Recovery	**99.97**	

Acceptance Criteria

The % CV of recovery at each QC level and for ISTD should be ≤ 15.00 %.

The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

DISCUSSION

Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Disopyramide and . The overall % mean recovery for was found to be 99.251% at LQC, MQC and HQC levels and % CV ranged from 0.32- 0.13 for IS, 1.17 1.47, 0.15, 0.25, 0.58,0.72 LQC, MQC and HQC(Extracted & UnExtracted). The results demonstrated that the bioanalytical method had good extraction efficiency. The results demonstrated that the bioanalytical method had good extraction efficiency

Acceptance criteria

The C.V% of mean analyte & ISTD recoveries must be ≤15% for each QC conc level.

The difference of % recovery between the lowest % recovery & highest % recovery should not be more than 25%.

Ruggedness Linearity

Table no 22: Rugged Linearity of Disopyramide.

Ruggedness Linearity								
Analyte	**Disopyramide**					**ISTD**	**Darunavir**	
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							
	0.150	0.300	0.450	1.200	3.000	3.600	4.800	6.000
	Nominal Concentration Range (ng/mL)							
	(0.120-0.180)	(0.255-0.345)	(0.383-0.518)	(1.020-1.380)	(2.550-3.450)	(3.060-4.140)	(4.080-5.520)	(5.100-6.900)
Different Column	Calculated Concentration (ng/mL)							
	0.158	0.310	0.465	1.225	3.032	3.621	4.823	6.123
Different Analyst								
	0.179	0.322	0.479	1.834	3.214	3.963	4.935	6.724

Acceptance Criteria

The % accuracy for all CC standards except of LLOQ (STD 1) standard should be within 85.00-115.00 %.The % accuracy for LLOQ standard should be within 80.00-120.00 %.

At least 75 % of CC standards should meet the acceptance criteria, including the LLOQ and highest CC standard (ULOQ). Any two consecutive points shall not be excluded.

Response of interfering peaks in STD Bulk and STD ZERO at the retention time of analyte should be ≤ 20.00 % of that in LLOQ.

Response of interfering peaks in STD Blk at the retention time of ISTD should be ≤ 5.00 % of that in LLOQ.

Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis, The calibration range is obtained by injecting 6 concentrations(0.15 ng/ml-6ng/ml) of calibration standards not including blank and zero samples and establishing, The calibration curves were appeared linear and the coefficient of correlation was found to be 0.999 for Disopyramide.

Reinjection Reproducibility

Table no 23: Reinjection Reproducibility of Disopyramide.

S. No	**HQC**	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	4.800	3.000	0.450	0.150
	Nominal Concentration Range (ng/mL)			
	(4.080-5.520)	(4.080-5.520)	(4.080-5.520)	(0.120-0.180)
	Calculated Concentration (ng/mL)			
1	4.096	2.621	0.396	0.125
2	4.252	2.762	0.425	0.139
3	4.565	2.951	0.435	0.142
4	4.932	3.025	0.462	0.148

5	5.285	3.135	0.470	0.159
6	5.365	3.320	0.510	0.175
n	6	6	6	6
Mean	4.7492	2.9690	0.4497	0.1480
SD	0.53057	0.25226	0.03977	0.01730
% CV	**11.17**	**8.50**	**8.84**	**11.69**
% Mean Accuracy	**98.94**	**98.97**	**99.93**	**98.67**
Note: Individual sample calculated concentration which appears in bold are out of acceptance criteria but included in statistical calculations.				
Reinjection Reproducibility has been proven at 2-8˚C for 70 Hr(s) 6 min(s).				
Acceptance Criteria				
At least 67 % (16 out of 24) of total QC samples and 50 % (3 out of 6) at each level should be within 85.00-115.00 % except LLOQ QC. LLOQ QC should be within 80.00-120.00 %.				
The % mean accuracy for LQC, MQC and HQC samples should be within 85.00-115.00 % and for the LLOQ QC sample it should be within 80.00-120.00 %.				
The % CV for LQC, MQC and HQC samples should be ≤ 15.00 % and for the LLOQ QC it should be ≤ 20.00 %.				

The % mean accuracy for LQC, MQC and HQC samples was found to be 98.94, 98.97, 99.93 and % CV was found to be 11.17, 8.50, 8.84 and LLOQ was found 98.67 and % CV was found to be 11.69. The results demonstrated that the bioanalytical method had good extraction efficiency.

Stabilities

In bench-top stability, six replicates of LQC & HQC samples (0.09 and 0.96 µg/ml) were analyzed for 9 hours at room temperature on the laboratory bench. The % mean stability was calculated and found to 99.52% for LQC and 99.48% for HQC respectively.

Long term stock solution stability

Table no 24: stability of Disopyramide (zero days).

S.No.	**HQC**	**LQC**
	Nominal Concentration (ng/mL)	
	4.800	**0.450**
	Nominal Concentration Range (ng/mL)	
	(4.080-5.520)	**(0.383-0.518)**
	Calculated Concentration (ng/mL)	
1	4.185	0.399
2	4.365	0.425
3	4.621	0.431
4	4.852	0.463
5	5.214	0.475
6	5.412	0.494
n	6	6
Mean	4.7748	0.4478
SD	0.47840	0.03547
% CV	**10.02**	**7.92**
% Mean Accuracy	**99.48**	**99.52**
Note: Individual sample calculated concentration which appears in bold are out of acceptance criteria but included in statistical calculations.		
Acceptance Criteria		
At least 67 % (8 out of 12) of total QC samples and 50 % (3 out of 6) at each level should be within 85.00-115.00 %.		
The % mean accuracy of LQC and HQC should be within 85.00-115.00 %.		
The % CV of LQC and HQC samples should be ≤ 15.00 %.		

Matrix sample stability at -28°C& -80°C for 37 days

Long term stock solution stability for the Disopyramide was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28°C& -80°C in refrigerator. The % mean stability of the Disopyramide was found to be 101.68%, 99.93% at -28 ± 5°C and 101.31%, 99.89% at -80 ± 5°C respectively. Long term stock solution stability for the was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28°C& -80°C in refrigerator. The % mean stability of the was found to be 99.98%, 99.52% at 28 ± 5°C. Long Term Stability of Analyte in Matrix of Disopyramide shows stability at Temperature -28 ± 5°C and 80 ± 5°C for 37 Days

Matrix samples stability at -28±5 °C for 37 days.

Table no 25: Matrix samples stability at -28±5 °C for 37 days.

S.No	HQC		LQC	
	Nominal Concentration (µg/mL)			
	0.000	4.800	0.450	0.450
	Nominal Concentration Range (µg/mL)			
	(4.080-5.520)	(4.080-5.520)	(0.383-0.518)	(0.383-0.518)
	Calculated Concentration (µg/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	4.125	4.257	0.383	0.392
2	4.362	4.365	0.422	0.421
3	4.481	4.456	0.438	0.434
4	4.936	4.623	0.464	0.461
5	5.126	5.241	0.481	0.472
6	5.329	5.412	0.505	0.516
n	6	6	6	6
Mean	4.7265	4.7257	0.4488	0.4493
SD	0.47359	0.48366	0.04380	0.04340
% CV	10.02	10.23	9.76	9.66
%Mean Accuracy	98.47	98.45	99.74	99.85
% Mean Stability	99.98		100.11	
Note: Individual sample calculated concentration which appears in bold are out of acceptance criteria but included in statistical calculations.				

Acceptance Criteria

At least 67 % (8 out of 12) of total QC samples and 50 % (3 out of 6) at each level in stability and comparison samples should be within 85.00 -115.00 %.

The % mean accuracy of back calculated concentration of LQC and HQC samples should be within 85.00-115.00 %.

The % CV of LQC and HQC samples should be ≤ 15.00 %.The % Mean Stability of LQC and HQC samples should be within 85.00-115.00 %.

Matrix samples stability at -80±5 °C for 37days

Table no 22: Matrix samples stability at -80±5 °C for 37 days.

S. No.	HQC		LQC	
	Nominal Concentration (µg/mL)			
	4.800	4.800	0.450	0.450
	Nominal Concentration Range (µg/mL)			
	(4.080-5.520)	(4.080-5.520)	(0.383-0.518)	(0.383-0.518)
	Calculated Concentration (µg/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	4.18	4.19	0.398	0.389
2	4.34	4.37	0.411	0.416
3	4.67	4.67	0.431	0.448
4	4.88	4.88	0.458	0.452
5	5.17	5.15	0.480	0.479
6	5.32	5.45	0.514	0.516
n	6	6	6	6
Mean	4.7597	4.7845	0.4487	0.4500
SD	0.45266	0.47605	0.04390	0.04490
% CV	9.51	9.95	9.78	9.98
%Mean Accuracy	99.16	99.68	99.70	100.00
% Mean Stability	100.52		100.30	

Acceptance Criteria

At least 67 % (8 out of 12) of total QC samples and 50 % (3 out of 6) at each level in stability and comparison samples should be within 85.00 -115.00 %.

The % mean accuracy of back calculated concentration of LQC and HQC samples should be within 85.00-115.00 %.

The % CV of LQC and HQC samples should be ≤ 15.00 %.The % Mean Stability of LQC and HQC samples should be within 85.00-115.00 %.

SUMMARY

Identification of Disopyramide Using UV absorption Spectrum was run with 200 nm – 400 nm the λmax was found at 215 nm with methanol as Solvent. The solubility of the Disopyramide was done by using the different solvents. Disopyramide was highly soluble in methanol and buffer ammonium acetate. Based on drug solubility and P^{Ka} Value following conditions has been used to develop the method estimation of Disopyramide as per current ICH guidelines. For developing the method for the assay of Disopyramide, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. A Hiber advance C_{18} Column was chosen as the stationary phase for this study. The mobile phase and the flow rate in order to get sharp peaks and base line separation of the components, carried out a number of experiments by varying the commonly used solvents, their compositions and flow rate. To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a C_{18} stationary phase. A binary mixture of Methanol and 0.01N Ammonium Acetate buffer in a ratio of 60:40 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 0.3 mL/min was found to be suitable. The drug molecule was tuned on the UPLC for the detection of Disopyramide and by injecting 0.15µg/mL All the optimized system suitability parameters within the limits results. Disopyramide and Internal Standard were eluted at 1.281 min, 1.535min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. Drugs were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits.

The carryover was tracked back to the injection valve and eradicated by converting from a partial loop injection to a full loop injection, which allowed more effective cleansing of the sample flow channel. The UPLC system's susceptibility to carryover was shown to be dependent on the detection method's absolute sensitivity and the mass of analyte injected at the assay's lower limit of quantitation (LLOQ). The area obtained is less than 20 % of extracted LLOQ standard area to unextracted area by injected of replicate manner. The response areas obtained of analyte and internal standard are less than 20% and 5 % of LLOQ area. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific. The LLOQ concentration was found between 80 -120 % and % Coefficient of variation found to be 11.52% and mean of 6 injections was found to be 100.00 % within the acceptance limits. As the limit of Sensitivity % CV was less than “20%” the system Sensitivity was passed in this method. The Evaluation of Matrix by injecting the QC samples of high and low concentrations in 6 lots the %Mean accuracy obtained are 99.18% and 99.91% of HQC and LQC and % CV obtained are 11.35% and 8.92% of HQC and LQC. As the limit of % CV was less than “15%” the system Matrix was passed in this method. Calibration was found to be linear over the concentration range of 0.15 µg /mL to 6 µg /mL. The coefficient correlation (R^2) value was found consistently greater than 0.999 in all the cases. This indicating linearity of results and an excellent correlation between peak area ratios for each concentration of analytes.

The intraday and inter day accuracy and precision was assessed by analyzing six replicates at four different QC levels like LLOQ, LQC, MQC and HQC. Accuracy and precision method performance was evaluated by determined by six replicate analyses for Disopyramide at four concentration levels, i.e.0.15 µg/mL (LLOQ), 0.45 µg/mL (LQC), 3 µg/mL (MQC) and 4.8 µg/mL HQC The intra-day and inter day accuracy of plasma samples were assessed and excellent % mean accuracy was obtained with range varied from 96.96%,99.32%, 97.29%, 98.05% for intraday and 98.33%, 98.26%, 99.42%, 99.23% for inter day respectively. The precision (%CV) of the analytes and plasma samples were calculated and found to be 10.14%, 9.44%, 10.79%, 10.27% for intraday and 10.59%, 11.41%, 8.44%, 9.03% for inter day respectively.

The overall % mean recovery for was found to be 99.251% for Disopyramide and 99.97% IS at LQC, MQC and HQC levels and % CV ranged from 1.17%, 1.47%, 0.15%, 0.25%, 0.58%, 0.72% for Disopyramide and 0.32%, 0.13% for IS at HQC, MQC and LQC (Extracted & Unextracted). The results demonstrated that the bioanalytical method had good extraction efficiency. Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis, The calibration range is obtained by injecting 6 concentrations (0.15µg/mL-6µg/mL) of calibration standards not including blank and zero samples and establishing, the calibration curves were appeared linear and the coefficient of correlation was found to be 0.999 for Disopyramide.

The % mean accuracy for HQC, MQC and LQC samples was found to be 98.94%, 98.97%, 99.93% and % CV was found to be 11.17%, 8.50%, 8.84% and for LLOQ the % mean accuracy was found 98.67% and % CV was found to be 11.69%. The results demonstrated that the bioanalytical method had good extraction efficiency. In bench-top stability, six replicates of LQC & HQC samples (0.45µg/mL & 4.80 µg/mL) were analyzed for 9 hours at room temperature on the laboratory bench. The % mean stability was calculated and found to 99.52% for LQC and 99.48% for HQC respectively. Long term stock solution stability for the Disopyramide was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28 ± 5°C & -80°C ± 5°C in freezer. The % mean stability of the Disopyramide was found to be 100.11%, 99.98 % at -28 ± 5°C and 100.3 %, 100.52 % at -80 ± 5°C respectively.

CONCLUSION

A simple, Accurate, precise method was developed for the estimation of Disopyramide in Rabbit plasma was developed and validated. By using Precipitation method, the sample preparation was extracted. Chromatogram was run through Std Hibar C_{18} (100 x 2.1 mm, 2µ) Mobile phase containing Buffer Ammonium Acetate: Methanol taken in the ratio 60:40 was pumped through column at a flow rate of 0.3ml/min. Buffer used Ammonium Acetate in this method was buffer. For the separation of Disopyramide Internal Standard [IS] used is Darunavir. The Temperature was maintained at 30°C. Optimized wavelength selected was 215nm. Retention time of Disopyramide and Internal Standard were found to be 1.281 min and 1.535 min. The standard curve was linear (R^2 >0.995) over the concentration range of 0.15-6 µg/ml. All the analytical validation parameters were determined as per ICH guidelines The bioanalytical method developed approach was selective, robust, and reliable, as accuracy, precision, recovery, and other validation parameters were all within the recommendations' limitations. The peaks produced for the drug of interest and the internal standard were well separated from one another without any plasma interferences, and the peaks were symmetrical with an adequate tailing factor.

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