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Bioanalytical Method Development & Validation Of Propafenone In Rabbit Plasma By RP-UPLC Using Vildagliptin As Internal Standard

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Abstract

A simple, Accurate, precise method was developed for the estimation of Propafenone in Rabbit plasma was developed and validated. By using Centrifugation technique, the sample preparation was prepared. Chromatogram was run through Std ACQUITY UPLC HSS C18 Column, 1.8 µm, 2.1 mm X 100 mm, Mobile phase containing Buffer DiSodium Hydrogen Phosphate: Acetonitrile taken in the ratio 70:30 was pumped through column at a flow rate of 0.3ml/min. Buffer used Na2HPO4.in this method was buffer. For the separation of Propafenone Internal Standard [IS] used is Vildagliptin. The Temperature was maintained at 30°C. Optimized wavelength selected was 248nm. Retention time of Propafenone and Internal Standard were found to be 1.273 min and 0.972 min. The standard curve was linear (R2 >0.995) over the concentration range of 15-600 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 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.

Keywords: Propafenone, Internal Standard, RP UPLC, Bioanalysis, Rabbit Plasma

1. 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 involves 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 equipment2. Bioanalytical method validation comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery, sensitivity, and stability.

Drug Analysis in Various Biological Media

Biopharmaceutical analysis usually uses blood, urine, and feces, especially if the medication or metabolite is poorly absorbed or removed in the bile. Other mediums include saliva, breath, and tissue. Investigation type strongly determines sampling media choices. Clinical pharmacokinetic studies require blood, urine, and saliva to measure medication levels. Bioavailability studies may require blood and/or urine drug levels, however drug identification or addiction concerns may just require one biological sample.

Drug investigation types substantially influence sample media selection. Clinical pharmacokinetic studies require blood, urine, and maybe saliva to measure medication levels. Bioavailability studies may require blood or urine drug levels. Sample collection, treatment, separation of the compound of interest from the matrix, and analysis are the procedures in quantifying medications in biological fluid.

UPLC is a leading approach for liquid chromatography. It can decrease the length of the column, which saves time and reduces solvent consumption. It also decreases noise and improves the signal to noise ratio. UPLC is one of the most important tools in analytical chemistry. It increases the speed, resolution, and sensitivity of the chromatographic analysis, and decreases the time, solvent consumption, and cost involved. To fully capitalize on the speed afforded by UPLC, a fast injection cycle time is needed, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to increase sensitivity.

Propafenone Hydrochloride is the hydrochloride salt form of propafenone with class 1C antiarrhythmic effects. Propafenone hydrochloride stabilizes the neuronal membrane by binding to and inhibiting voltage-gated sodium channels, thereby reducing sodium influx required for the initiation and conduction of impulses in Purkinje and myocardial cells. This agent produces a marked depression of phase 0 and prolonged effective refractory period in the atrio-ventricular nodal and His-Purkinje tissue, resulting in a profound decrease in excitability, conduction velocity and automaticity, thereby counteracting atrial and ventricular arrythmias.it is chemically called as 1-{2-[2-hydroxy-3-(propylamino) propoxy] phenyl}-3-phenylpropan-1-one.

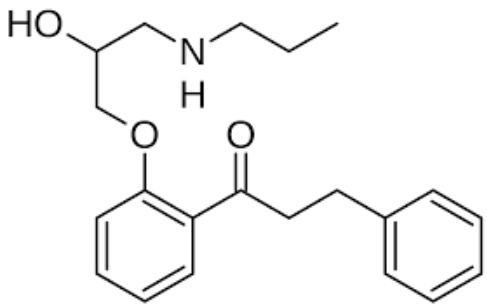

Figure1: Chemical Structure of Propafenone

Experimental Work:

2. MATERIALS AND METHODS:

Materials:

Propafenone API was obtained as a gift sample from Jai Ram Biosciences, Kukatpally, Hyderabad, Internal Standard from Akrivis Pharma pvt Ltd. K_2 EDTA control plasma Deccan Pathological labs, Hyderabad. Distilled water, Acetonitrile, Phosphate buffer, Methanol, Sodium dihydrogen phosphate, Ortho-phosphoric acid of the analytical grade was purchased from Rankem, Avantor performance material India limited. Electronic balance, pH meter, Sonicator, Centrifuge, Vertex, UPLC used Waters Acquity were well calibrated.

Methodology:

Preparation of solutions

All solutions performed sonication, were stored at room temperature, and were utilized within 24 hours after their production.

The next section outlines the methodology for preparing buffers and possible solutions.

Preparation of diluent (v/v):

Based up on the solubility of the drugs, diluent was selected, Water and Acetonitrile taken in the ratio of 50:50.

Preparation of Buffer (v/v):

0.01N Na_2HPO4 Buffer: Accurately weighed 1.41gm of Disodium phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 4.8 with dil. Orthophosphoric acid solution.

Preparation of stock solutions: -
Standard Preparation: Accurately Weighed and transferred 6mg of Propafenone working Standards into a 200ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents, and filter the solution with HPLC nylon 0.5µm size filters (30 ppm/µg/ml of Propafenone)

Standard Working Solution: From the above Propafenone stock solution 0.05ml, 0.1ml, 0.15ml, 0.4, 1.0ml, 1.2ml, 1.6ml and 2.0 ml was pipette and transferred to 8 individual of 10 ml volumetric flask and make up the volume up to the mark with diluent to produce 0.015 µg/ml, 0.030 µg/ml, 0.045µg/ml, 0.120 µg/ml, 0.030 µg/ml, 0.360 µg/ml, 0.480 µg/ml and 0.600µg/ml.

Stock solution of internal standard (Vildagliptin**): -**
Standard Preparation: Accurately Weighed and transferred 10mg of Vildagliptin working Standards into a 100ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents, and filter the solution with HPLC nylon 0.5µm size filters (100 ppm/µg/ml of Propafenone).

Final concentration: From the above solution, take 1ml of solution and spiking blank plasma with working stock dilutions of analyte to produce 10µg/ml ISD concentration.

Preparation of calibration curve (CC) standards and quality control (QC) samples
Quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.015 µg/ml(Standard-1/LLOQ), 0.030 µg/ml(Standard-2), 0.045µg/ml(Standard-3/LQC), 0.120 µg/ml(Standard-4), 0.300 µg/ml(Standard-5/MQC), 0.360 µg/ml(Standard-6), 0.480 µg/ml(Standard-7/HQC) and 0.600µg/ml(Standard-8/ULOQ).

Table no-1 CC spiking solutions of Propafenone:

Spiking solution	pippetout in ML	make up in ML	spiking in ML	make upon ML	final conc in ng/ml
Standard-1	0.05	10	0.25	2.5	15
Standard-2	0.1	10	0.25	2.5	30
Standard-3	0.15	10	0.25	2.5	45
Standard-4	0.4	10	0.25	2.5	120
Standard-5	1.0	10	0.25	2.5	300
Standard-6	1.2	10	0.25	2.5	360
Standard-7	1.6	10	0.25	2.5	480
Standard-8	2.0	10	0.25	2.5	600

Table no-2 Preparation of QC spiking solutions:

Spiking solution	pippetout in ml	make up in ml	spiking in ml	make upon ml	final conc in ng/ml
LLOQ	0.05	10	0.25	2.5	15
LQC	0.15	10	0.25	2.5	45
MQC	1.0	10	0.25	2.5	300
HQC	1.6	10	0.25	2.5	480
ULOQ	2.0	10	0.25	2.5	600

The solutions containing CCs and QCs were stored in a deepfreeze at a temperature of -20°C. A volume of 0.25 mL of spiked samples was tightly closed and stored in multiple pre-labeled vials at a temperature of -20°C.

- CC standards.
- QC samples.
- Standard blank (with spiking IS and analyte).
- Standard zero sample (spiking of IS working solution to blank plasma during sample processing).
- These Samples were subsequently used for conducting various validation experiments and analyzing animal study samples.

Extraction procedure for Bio-Sample analysis.
The protein precipitation method was employed to extract Propafenone from rat plasma, utilizing Vildagliptin as an internal standard (IS), in the subsequent procedure.
In this experiment, a total of 750µl of plasma was combined with 500µl of internal standard and an additional 250µl of Propafenone. The mixture was subjected to a 15-second cyclo mixing process. Following this, 1 ml of acetonitrile was added to the mixture, and the resulting solution was subjected to vertexing for a duration of 2 minutes. Subsequently, the solution was centrifuged at a speed of 3200 rpm for a period of 5 minutes, allowing for the collection of the supernatant sample. To ensure the removal of any impurities, the sample was then filtered using a polyvinylidene fluoride or polyvinylidene difluoride 0.45µ filter. Finally, 10 µL of the filtered sample was injected into the ultra-performance liquid chromatography (UPLC) system for further analysis.

Data analysis
The Analyst software version empower 2 was used to data acquisition and analysis, and additionally, a validated excel sheet was used to compute the statistics like mean, SD and %CV for analytical values generated during method validation.

Validation Methodology in bioanalytical method: -
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**.**

Methodology: The experiment involves the administration of six quality control samples of MQC (specifically, 3.000 ng/ml) from a single vial at the beginning of the study.

Acceptance criteria: The criteria acceptance accordingly as the % CV of the retention time (RT) should be ≤ 2.00 %., The % CV of the area ratio should be ≤ 5.00 %.

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 analyzing blank samples after the calibration standard at the ULOQ.

Methodology: The high-performance liquid chromatography (UPLC) technology was evaluated in order to investigate the potential occurrence of carry-over. The carryover was evaluated by injecting the following samples in a sequential manner.
- Blank refers to a solution that is used as a mobile phase and contains water as the solvent.
- Standard_QC (ULOQ).
- Blank
- Standard_QC (ULOQ)
- lower standard (AQ LLOQ)

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 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, and degradation products formed during sample preparation or concomitant medications that are expected to be used in the treatment of patients with the intended indication).

Methodology: Specificity is determined by the injecting six samples of standard solution and the LLOQC sample solution and

Acceptance criteria: - check the % Interference Response of interfering peaks in STD Blk at the retention time of analyte should be ≤20.00 % of that in LLOQ and At least 80 % of the matrix lots (Biological 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.

Methodology: - the sensitivity is performed by injecting six injections of lower concentration of sample (LLOQ).

Acceptance criteria: -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.

Methodology: - 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.

Acceptance criteria: - 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.

Methodology: - 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

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 %.

Rugged Linearity
Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis,

Methodology: -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
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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.

Methodology: -
The test is performed injecting the QC samples were injected 6 replicates at each qc concentration level in each analytical run.

Acceptance criteria: - 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)
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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.

Methodology: -The recoveries for Propafenone 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

Acceptance criteria:
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.

Methodology: -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.

Acceptance criteria: 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.

Methodology: -The reproducibility was performed by injecting the qc samples in 6 replicates and check the acceptance limits.

Acceptance criteria: 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
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.

Methodology: -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

Acceptance criteria: The mean concentration at each QC level should be within ±15% of the nominal.

3. RESULTS AND DISCUSSION

Method Development

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

Optimization of the chromatographic conditions

For developing the method for the assay of Propafenone, 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 high purity 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, the author has 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 acetonitrile and 0.01N Sodium dihyrogen ortho phosphate buffer in a ratio of 70:30 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 Propafenone and by injecting 15ng/mL and 600ng/ml concentration respectively. All the optimized system suitability parameters within the limits result

Optimized method:

Chromatographic conditions

Mobile phase	: Disodium hydrogen Phosphate : Acetonitrile (70:30)
Columm	: ACQUITY UPLC HSS C18 Column, 1.8 µm, 2.1 mm X 100 mm
Flow rate	: 0.3ml/min
Detector wavelength	: 248nm
Column temperature	: 30^0C
Injection volume	: 0.50µL
Run time	: 2.0min

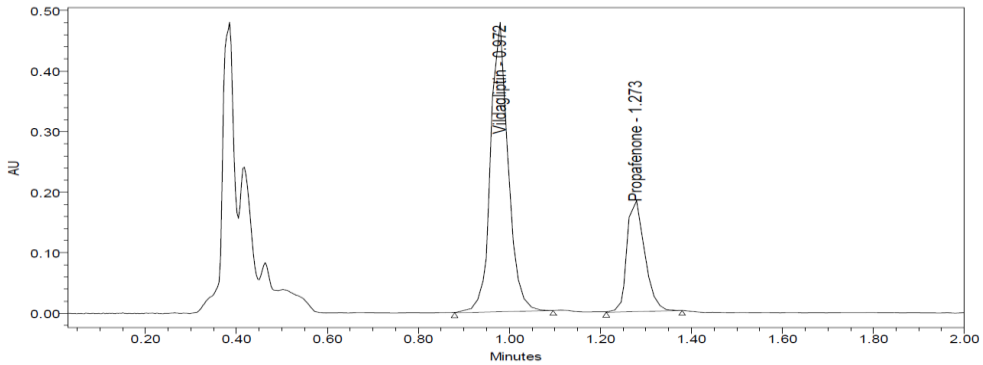


Figure 2: Chromatogram of Optimized chromatogram of Propafenone

Table no 3: Observation of Optimized Chromatogram

S.NO	Name of the Peak	Retention Time	Area	USP Plate count	USP Resolution	USP Tailing
1	Vildagliptin	0.972	723338	2875.8	-	1.1
2	Propafenone	1.273	745587	3109.8	3.5	1.2

Observation: Propafenone and Internal Standard were eluted at 1.273 min, 0.972min 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

METHOD VALIDATION

System suitability of Propafenone

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 4: System Suitability of Propafenone

Sample Name	Analyte Area	Analyte RT (min)	ISTD Area	ISTD RT (min)	Area Ratio
AQ MQC	380262	1.28	722152	0.977	0.5266
	387813	1.28	721906	0.979	0.5372
	383647	1.28	726578	0.981	0.5280
	385309	1.28	721121	0.981	0.5343
	386650	1.28	729529	0.981	0.5300
	381412	1.28	728777	0.982	0.5234
MEAN		1.281		0.980	0.52991
SD		0.0014		0.0018	0.005106
%CV		**0.11**		**0.19**	**0.96**

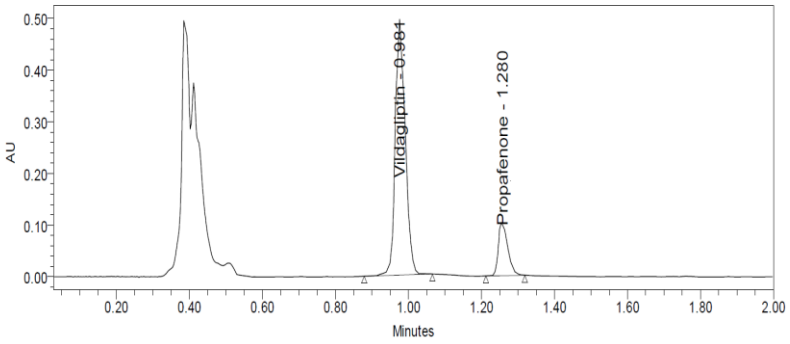


Figure 2: system suitability chromatogram of Propafenone

Discussion: plate count, tailing factor, resolution of Propafenone 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 %.

Quality Control Samples

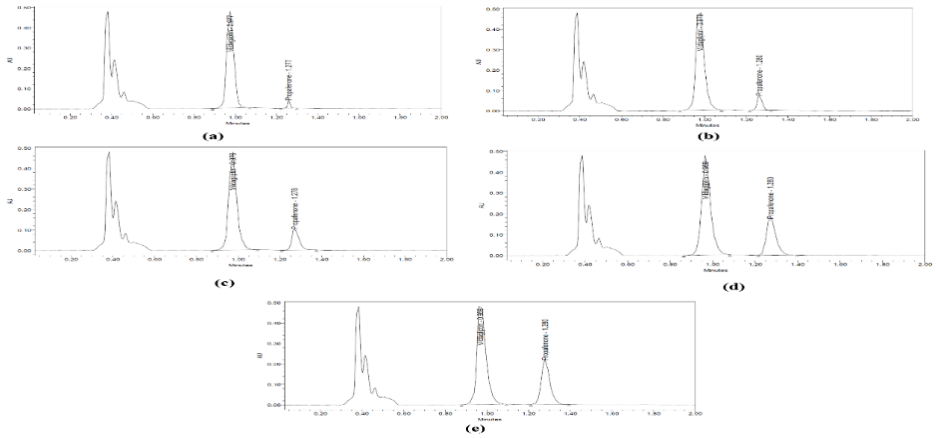


Figure 3 (a): Chromatogram of LLOQ (b): Chromatogram of LQC (c): Chromatogram of MQC (d): Chromatogram of HQC (e): Chromatogram of ULQC

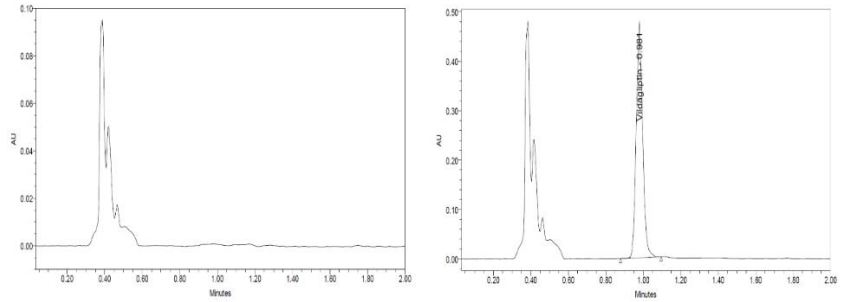
Auto sampler carryover of Propafenone

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).

Table 5: Auto sampler carryover of Propafenone

Parameters	**Peak Area**		**% Carryover**	
	Drug	**ISTD**	**Drug**	**ISTD**
	Unextracted samples			
RS	0	0	N/A	N/A
AQ ULOQ	769874	748765	**0.00**	**0.00**
RS	0	0		
AQ LLOQ	39875	728739	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	744706	726450	**0.00**	**0.00**
STD Blk	0	0		
LLOQ	36295	720483	N/A	N/A

Discussion: - The area obtained is less than 20 % of extracted LLOQ standard area to unextracted area by injected of replicate manner



(a) (b)

Figure 4 a) Chromatogram of a Blank Plasma Sample b) Chromatogram of Blank Plasma with Internal Standard Sample

Table 6: Specificity and Screening of Biological Matrix of Propafenone

S.No.	**Parameters**	**Response**		**% Interference**		**Pass/Fail**
		Drug	**ISTD**	**Drug**	**ISTD**	
1	STD Blk1	0	0	0.00	0.00	Pass
2	LLOQ1	36546	729837			
3	STD Blk2	0	0	0.00	0.00	Pass
4	LLOQ2	36982	720983			
5	STD Blk3	0	0	0.00	0.00	Pass
6	LLOQ3	36297	729863			
7	STD Blk4	0	0	0.00	0.00	Pass
8	LLOQ4	36739	724563			
9	STD Blk5	0	0	0.00	0.00	Pass
10	LLOQ5	36328	727647			
11	STD Blk6	0	0	0.00	0.00	Pass
12	LLOQ6	36982	727651			

Observation: 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.
Discussion – 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

Sensitivity

A sensitivity is defined as "the lowest analyte concentration that can be measured with acceptable accuracy and precision i.e., LLOQ

Table 7: Sensitivity of Propafenone

S.No	**LLOQ**
	Nominal Concentration (ng/mL)
	15.000
	Nominal Concentration Range (ng/mL)
	(12.000-18.000)
	Calculated Concentration (ng/mL)
1	14.854
2	14.839
3	14.989
4	14.874
5	14.926
6	14.944
n	6
Mean	14.9043
SD	0.05820
% CV	0.39
% Mean Accuracy	99.36

Discussion: - The LLOQ concentration was found between 80 -120 % and % Coefficient of variation found to be 0.39% and mean of 6 injections was found to be 99.36% % 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

Table no 8: Matrix factor evaluation (absence of matrix factor)

S. No.	**Plasma Lot No.**	**HQC**	**LQC**
		Nominal Concentration (ng/mL)	
		480.000	**45.000**
		Nominal Concentration Range (ng/mL)	
		(408.000-552.000)	**(38.250-51.750)**
		Calculated Concentration (ng/mL)	
1	LOT1	475.092	44.472
		476.084	43.396
		479.490	43.506
2	LOT2	476.287	44.454
		480.020	43.394
		476.258	44.418
3	LOT3	479.953	45.431
		478.435	44.510
		472.254	45.300
4	LOT4	479.454	44.915
		476.539	45.485
		476.124	46.429
5	LOT5	472.191	44.404
		486.425	45.426
		480.014	45.462
6	LOT6	476.738	44.421
		476.369	44.509
		482.761	44.861

n	**18**	**18**
Mean	**477.8049**	**44.7107**
SD	**3.46580**	**0.80219**
% CV	**0.73**	**1.79**
% Mean Accuracy	**99.54**	**99.36**
No. of QC Failed	**0**	**0**

Discussion- The Evaluation of Matrix by injecting the QC samples of high and low concentrations in 6 lots the %Mean obtained was 99.54% and 99.36 of HQC and LOQ and % CV obtained are 0.73% and 1.79% of HQC and LOQ. As the limit of CV was less than "20%" the system Matrix was passed in this method.

Linearity:

Table 9: Linearity of Propafenone

	STD1	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
	Nominal Concentration (ng/mL)							
	15.000	**30.000**	**45.000**	**120.000**	**300.000**	**360.000**	**480.000**	**600.000**
	Nominal Concentration Range (ng/mL)							
	(12.000-18.000)	**(25.500-34.500)**	**(38.250-51.750)**	**(102.000-138.000)**	**(255.000-345.000)**	**(306.000-414.000)**	**(408.000-552.000)**	**(510.000-690.000)**
	Back Calculated Concentration (ng/mL)							
P&A1	14.923	29.756	44.520	119.562	298.650	359.985	479.246	599.860
P&A2	14.826	29.865	44.632	118.658	297.620	357.956	478.654	599.860
P&A3	14.856	30.210	44.856	119.650	296.300	360.520	480.000	599.741
n	3	3	3	3	3	3	3	3
Mean	14.8683	29.9437	44.6693	119.2900	297.5233	359.4870	479.3000	599.8203
SD	0.04966	0.23700	0.17108	0.54909	1.17798	1.35260	0.67462	0.06870
%CV	0.33	0.79	0.38	0.46	0.40	0.38	0.14	0.01
% Mean Accuracy	99.12	99.81	99.27	99.41	99.17	99.86	99.85	99.97

final conc in ng/ml	**ISD (area)**	**Drug(area)**	**Area response ratio**
0	0	0	0
15	722152	36295	0.050
30	721906	73323	0.102
45	726578	101642	0.140
120	721121	173611	0.241
300	729529	389526	0.534
360	728777	456302	0.626
480	724587	597706	0.825
600	726581	746201	1.027

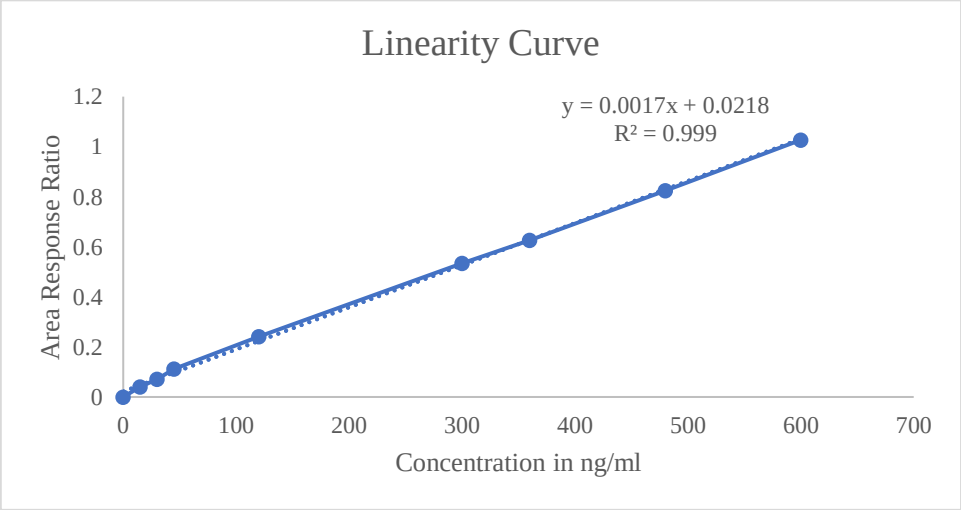


Figure:5 Linearity curve for the Propafenone

Discussion: - Calibration or linearity curve was found to be linear over the concentration range of 15 to 600 ng /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 Propafenone)

Table no10: precision data for intra-day runs of Propafenone

	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	480.000	300.000	45.000	15.000
	Nominal Concentration Range (ng/mL)			
	(408.000-552.000)	(255.000-345.000)	(38.250-51.750)	(12.000-18.000)
	Back Calculated Concentration (ng/mL)			
	479.256	296.965	44.256	14.956
	480.020	299.954	43.259	14.765
	479.652	300.230	44.985	14.956
	478.025	297.658	44.653	14.856
	479.652	296.856	44.987	14.985
	478.562	298.652	45.320	14.952
n	6	6	6	6
Mean	479.1945	298.3858	44.5766	14.9117
SD	0.75784	1.47104	0.73937	0.08432
%CV	0.16	0.49	1.66	0.57
% Mean Accuracy	99.83	99.46	99.06	99.41
	480.250	300.020	44.856	14.658
	476.580	298.650	44.075	15.065
	482.650	297.652	44.856	14.865
	479.658	298.645	44.058	14.756
	478.650	296.352	44.956	14.852
	480.360	297.562	44.963	14.965
n	6	6	6	6
Mean	479.6913	298.1468	44.6273	14.8602
SD	2.01389	1.25047	0.43692	0.14475
%CV	0.42	0.42	0.98	0.97
% Mean Accuracy	99.94	99.38	99.17	99.07
	476.225	296.560	44.756	14.852
	480.632	298.456	44.675	14.965
	478.630	297.650	44.653	14.852
	479.620	296.350	45.036	14.784
	476.185	298.456	44.987	14.985
	480.510	299.456	44.698	14.856
n	6	6	6	6
Mean	478.6337	297.8213	44.8008	14.8823
SD	2.01417	1.20523	0.16747	0.07691
%CV	0.42	0.40	0.37	0.52
% Mean Accuracy	99.72	99.27	99.56	99.22
Between Batch Precision and Accuracy				
n	18	18	18	18
Mean	479.1732	298.1180	44.6683	14.8847
SD	1.65911	1.25709	0.48471	0.10229
%CV	0.35	0.42	1.09	0.69
% Mean Accuracy	99.83	99.37	99.26	99.23

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

Table no 11: precision data for inter-day runs of Propafenone

	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentrations (ng/mL0			
	480.000	300.000	45.000	15.000
	Nominal concentration Range (ng/mL)			
	(408.000-552.000)	(255.000-345.000)	(38.250-51.750)	(12.000-18.000)
	Calculated Concentration (ng/mL)			
Different Column	478.650	298.650	44.875	14.856
	478.658	297.568	44.786	14.765
	479.658	298.658	44.965	14.965
	480.658	299.856	44.856	14.856
	479.658	297..685	44.865	14.965
	480.230		44.875	14.956
n	6	4	6	6
Mean	479.5853	298.6830	44.8703	14.8938
SD	0.81383	0.93468	0.05719	0.08180
% CV	0.17	0.31	0.13	0.55
% Mean Accuracy	99.91	99.56	99.71	99.29
Different Analyst	480.690	298.657	44.850	14.856
	478.650	296.350	44.698	14.985
	480.650	297.652	44.980	14.763
	476.520	299.658	45.026	15.065
	479.320	300.250	45.856	14.895
	480.520	297.652	45.785	14.965
n	6	6	6	6
Mean	479.3917	298.3698	45.1992	14.9215
SD	1.63364	1.44133	0.49512	0.10643
% CV	0.34	0.48	1.10	0.71
% Mean Accuracy	99.87	99.46	100.44	99.48

Discussion: - 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 Propafenone at four concentration levels, i.e.,15µg/ml(LLOQ), 45 µg/ml (LQC), 300 µg/ml (MQC) and 480 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.

Recovery of Propafenone-

Table no 12: Recovery of Propafenone

S. No.	**HQC**		**MQC1**		**LQC**	
	Un extracted Response	**Extracted Response**	**Un extracted Response**	**Extracted Response**	**Un extracted Response**	**Extracted Response**
1	609387	596384	398124	389167	104568	101546
2	599734	592563	397653	386724	102454	101658
3	602367	592570	397643	385409	102673	101487
4	609836	593274	398726	384986	101879	101376
5	607823	597445	395672	381934	103984	101839
6	609378	598263	392384	387634	104837	101497
n	6	6	6	6	6	6
Mean	606421	595083	396700	385976	103399	101567
SD	4296.97	2581.55	2350.09	2495.21	1225.33	161.55

% CV	0.71	0.43	0.59	0.65	1.19	0.16
% Mean Recovery	**98.13**		**97.30**		**98.23**	
Overall % Mean Recovery	**97.885**					
Overall SD	**0.5120**					
Overall % CV	**0.52**					

Recovery - Internal standard

Table no 13: Recovery of Vildagliptin (IS)

S.No.	**Un extracted Area Ratio**	**Extracted Area Ratio**
1	739827	722152
2	729837	721906
3	729386	726578
4	725463	721121
5	730928	729529
6	730234	728777
n	6	6
Mean	730945.8	725010.5
SD	4756.18	3741.59
% CV	0.65	0.52
% Mean Recovery	**99.19**	

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 Propafenone. The overall % mean recovery for was found to be 99.01% at LQC, MQC and HQC levels and % CV ranged from 0.65% for IS, The results demonstrated that the bioanalytical method had good extraction efficiency. The results demonstrated that the bioanalytical method had good extraction efficiency

Rugged Linearity:

Table no 14: Rugged Linearity of Propafenone

STD1	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
Nominal Concentration (ng/mL)							
15.000	30.000	45.000	120.000	300.000	360.000	480.000	600.000
Nominal Concentration Range (ng/mL)							
(12.000-18.000)	(25.500-34.500)	(38.250-51.750)	(102.000-138.000)	(255.000-345.000)	(306.000-414.000)	(408.000-552.000)	(510.000-690.000)
Calculated Concentration (ng/mL)							
14.856	29.680	44.850	118.600	298.650	358.658	478.256	598.650
14.965	29.650	45.980	118.630	297.300	360.250	479.650	599.680

Discussion: - 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 (15 ng/ml-600ng/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 Propafenone.

Reinjection Reproducibility

Table no 15: Reinjection Reproducibility of Propafenone

P&A01	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	480.000	300.000	45.000	15.000
	Nominal Concentration Range (ng/mL)			
	(408.000-552.000)	(255.000-345.000)	(38.250-51.750)	(12.000-18.000)
	Calculated Concentration (ng/mL)			
	478.950	299.850	44.856	14.856
	480.054	300.250	44.756	14.856
	480.120	298.650	44.856	15.000
	478.658	288.650	44.856	15.032

	479.856	296.654	44.862	14.856
	480.520	298.745	45.030	14.976
n	6	6	6	6
Mean	479.6930	297.1332	44.8693	14.9293
SD	0.72752	4.34024	0.08859	0.08227
% CV	0.15	1.46	0.20	0.55
% Mean Accuracy	99.94	99.04	99.71	99.53

Discussion: - The % mean accuracy for LQC, MQC and HQC samples was found to be 99.94, 99.04, 99.71 and % Cv was found to be 0.15, 1.46, 0.20 and LLOQ was found 99.53 and % CV was found to be 0.55. The results demonstrated that the bioanalytical method had good extraction efficiency.

Stabilities

Long term stock solution stability

Table no 16: stability of Propafenone (zero days)

S. No.	**HQC**	**LQC**
	Nominal Concentration (ng/mL)	
	480.000	**45.000**
	Nominal Concentration Range (ng/mL)	
	(408.000-552.000)	**(38.250-51.750)**
	Calculated Concentration (ng/mL)	
1	**479.652**	**44.860**
2	**476.580**	**44.850**
3	**476.580**	**45.860**
4	**479.865**	**44.865**
5	**465.745**	**44.986**
6	**476.580**	**44.874**
n	**6**	**6**
Mean	**475.8337**	**45.0492**
SD	**5.18235**	**0.40037**
% CV	**1.09**	**0.89**
% Mean Accuracy	**99.13**	**100.11**

Discussion- In bench-top stability, six replicates of LQC & HQC samples (45 and 480 ng/ml) were analyzed for 9 hours at room temperature on the laboratory bench. The % mean stability was calculated and found to 99.13% for LQC and 100.11% for HQC respectively.

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

Table no 17: Matrix samples stability at -28±5 °C for 37 days

S. No.	**HQC**		**LQC**	
	Nominal Concentration (ng/mL)			
	480.000	**480.000**	**45.000**	**45.000**
	Nominal Concentration Range (ng/mL)			
	(408.000-552.000)	**(408.000-552.000)**	**(38.250-51.750)**	**(38.250-51.750)**
	Calculated Concentration (ng/mL)			
	Comparison Samples	**Stability Samples**	**Comparison Samples**	**Stability Samples**
1	**476.950**	**478.650**	**44.560**	**44.650**
2	**486.750**	**478.658**	**44.026**	**44.985**
3	**489.650**	**478.680**	**44.850**	**44.870**
4	**478.658**	**476.320**	**44.658**	**44.965**
5	**480.654**	**480.620**	**45.260**	**44.856**
6	**478.658**	**480.035**	**44.056**	**44.856**
n	**6**	**6**	**6**	**6**
Mean	**481.8867**	**478.8272**	**44.5683**	**44.8637**

SD	**5.11195**	**1.48598**	**0.47381**	**0.11900**
% CV	**1.06**	**0.31**	**1.06**	**0.27**
%Mean Accuracy	**100.39**	**99.76**	**99.04**	**99.70**
% Mean Stability	**99.37**		**100.66**	

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

Table no 18: Matrix samples stability at -80±5 °C for 37 days

S.No.	**HQC**		**LQC**	
	Nominal Concentration (ng/mL)			
	480.000	**480.000**	**45.000**	**45.000**
	Nominal Concentration Range (ng/mL)			
	(408.000-552.000)	**(408.000-552.000)**	**(38.250-51.750)**	**(38.250-51.750)**
	Calculated Concentration (ng/mL)			
	Comparison Samples	**Stability Samples**	**Comparison Samples**	**Stability Samples**
1	**478.65**	**474.33**	**44.856**	**44.574**
2	**479.52**	**472.31**	**44.856**	**44.856**
3	**478.65**	**480.06**	**44.856**	**45.033**
4	**476.25**	**478.36**	**44.856**	**44.856**
5	**480.04**	**478.05**	**44.795**	**44.153**
6	**476.52**	**480.65**	**44.652**	**45.325**
n	**6**	**6**	**6**	**6**
Mean	**478.2718**	**477.2922**	**44.8118**	**44.7994**
SD	**1.55637**	**3.29308**	**0.08202**	**0.40141**
% CV	**0.33**	**0.69**	**0.18**	**0.90**
%Mean Accuracy	**99.64**	**99.44**	**99.58**	**99.55**
% Mean Stability	**99.80**		**99.97**	

Discussion: -Long term stock solution stability for the Propafenone 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 Propafenone was found to be 99.37 %, 100.06% at 28 ± 5°C and 99.80%, 99.97% 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.

Summary

The bioanalytical method development and validation of the propafenone using the rabbit plasma using RP UPLC was performed by using the ACQUITY UPLC HSS C18 Column, 1.8 µm, 2.1 mm X 100 mm, Mobile phase containing Buffer DiSodium Hydrogen Phosphate: Acetonitrile taken in the ratio 70:30 was pumped through column at a flow rate of 0.3ml/min. Buffer used Na2HPO4.in this method was buffer. For the separation of Propafenone Internal Standard [IS] used is Vildagliptin. The Temperature was maintained at 30°C. Optimized wavelength selected was 248nm. Retention time of Propafenone and Internal Standard were found to be 1.273 min and 0.972 min. The standard curve was linear (R2 >0.995) over the concentration range of 15-600 ng/ml. the results of the validated parameters were tabulated in table 19

Table 19: validation results of propafenone

Analyte Parameters	**Propafenone**		**Internal standard**	**Acceptance Criteria**	
	%Nominal	precision		%	Precision
Biological Matrix	Rabbit Plasma		Rabbit Plasma	N/AP	N/AP
Analytical Range	15ng/ml-600ng/ml		N/AP	N/AP	N/AP
Minimum Quantifiable	600ng/ml		N/AP	N/AP	≤ 20%
Matrix Effect LQC HQC	99.54%& 99.36%		N/AP	85% - 115%	≤ 15%
Coefficient of correlation	0.999		N/AP	r2 ≥ 0.98	
Accuracy and Precision for Sensitivity	100.0		N/AP	80% - 120%	≤ 20%

Within Batch Accuracy and Precision	96.23% LLOQ QC), 99.32% to 98.05 %.(L, M, H).	N/AP	85%- 115% (L, M1, M2, H) 80%- 120%	≤15%% (L, M1, M2,H) ≤20%(LL

4. CONCLUSION

A simple, accurate, precise method was developed for the estimation of the Propafenone in Rabbit plasma using the Vildagliptin as internal standard. Retention time of Propafenone was found to be 1.273min., the internal standard found in Rabbit plasma. Further, the reported method was validated as per the ICH guidelines and found to be well within the acceptable range. The proposed method is simple, rapid, accurate, precise, and appropriate for pharmacokinetic and therapeutic drug monitoring in the clinical laboratories.

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