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Bio- Method Development And Validation Of Ketotifen Fumarate In Rabbit Plasma Using RP-HPLC

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Abstract

A simple, Accurate, precise method was developed for the simultaneous estimation of Ketotifen Fumarate in human plasma was developed and validated. By using Protein Precipitation, the sample preparation was prepared. Chromatogram was run through Discovery C_{18} (150x 4.6 mm, 5µ) Mobile phase containing Buffer Na_2HPO_4: Methanol taken in the ratio 55:45 was pumped through column at a flow rate of 1.0ml/min. Buffer used Sodium Phosphate Buffer in this method was buffer. For the separation of Ketotifen Fumarate Internal Standard [IS] used is Remogliflozin. The Temperature was maintained at 30°C. Optimized wavelength selected was 222.0nm. Retention time of Ketotifen Fumarate and Internal Standard were found to be 2.980 min and 2.344 min. The standard curve was linear (R2 >0.995) over the concentration range of 9-360 ng/ml. According to ICH guidelines, each analytical validation parameter was determined. As accuracy, precision, recovery, and other validation parameters were all within the guidelines' constraints, the bioanalytical technique created approach was selective, robust, and reliable. Without any interference from plasma, the peaks generated for the target substance and the internal standard were adequately separated from one another and had a sufficient tailing factor. Therapeutic drug monitoring (TDM), bioequivalence research, pharmacokinetics studies, toxicology, and biological investigations might all greatly benefit from the technique.

Key Words: Ketotifen Fumarate, RP-HPLC, Rabbit Plasma, Internal Standard, Bio-Analytical method, Validation.

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 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]

Drug Analysis in Various Biological Media

Blood, urine, and faeces are the most commonly acquired samples for biopharmaceutical analysis, especially if the drug or metabolite is poorly absorbed or substantially eliminated in the bile.Saliva, breath, and tissue are examples of other media that can be used. The nature of the investigation heavily influences the selection of sampling media. In a clinical pharmacokinetic investigation, for example, medication levels necessitate the use of blood, urine, and saliva. A bioavailability study may necessitate drug level data in blood and/or urine, but a drug identification or drug addiction concern may only necessitate one type of biological sample.

The nature of the drug investigation heavily influences the selection of sample media. In a clinical pharmacokinetic study, for example, medication levels necessitate the use of blood, urine, and perhaps saliva. Bioavailability research may necessitate medication level measurements in blood or urine. The steps involved in estimating medicines in biological fluid are sample collection, sample treatment, separation of the compound of interest from the matrix, and analysis. [2-3]

Ketotifen Fumarate is the fumarate salt of ketotifen, a cycloheptathiophene derivative with anti-allergic activity. Ketotifen selectively blocks histamine (H1) receptors and prevents the typical symptoms caused by histamine release. This agent also interferes with the release of inflammatory mediators from mast cells involved in hypersensitivity reactions, thereby decreasing chemotaxis and activation of eosinophils. It chemically called as (E)-but-2-enedioic acid;2-(1-methylpiperidin-4-ylidene)-6-thiatricyclo [8.4.0.03,7]tetradeca-1(14),3(7),4,10,12-pentaen-8-one. Ketotifen selectively blocks histamine (H1) receptors and prevents the typical symptoms caused by histamine release. This agent also interferes with the release of inflammatory mediators from mast cells involved in hypersensitivity reactions, thereby decreasing chemotaxis and activation of eosinophils. The Pka 7.15 [4].

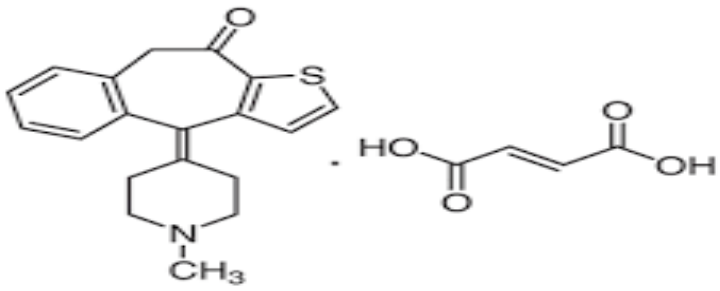

Figure 1: chemical structure of Ketotifen Fumarate

Experimental work: [5-6]

2. Materials and Methods:

Ketotifen Fumarate API was obtained as a gift sample from Jai Ram Biosciences, Kukatpally, Hyderabad, Internal Standard from Akrivis Pharma pvt Ltd. K2 EDTA control plasma Deccan Pathological labs, Hyderabad Acetonitrile, Phosphate buffer, Methanol, Sodium dihydrogen phosphate, Ortho-phosphoric acid were AR Grade and manufacturing company were Rankem and Avantor performance material India limited purchased from Srinivasa life sciences Hyderabad.

METHOD DEVELOPMENT [7-11]

Diluent: Based up on the solubility of the drugs, diluent was selected, 0.01N Potassium dihydrogen phosphate and acetonitrile taken in the ratio of 55:45.

Extraction procedure

Take 750µl of plasma and 0.5µl of internal standard, 0.25µl of Ketotifen Fumarate 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 HPLC.

Preparation of Ketotifen Fumarate Spiking Solutions:
From the above Ketotifen Fumarate stock solution 0.010ml, 0.020ml, 0.030ml, 0.160ml, 0.200ml, 0.240ml, 0.320ml and 0.400 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 0.009 µg/ml, 0.018 µg/ml, 0.027µg/ml, 0.144 µg/ml, 0.180 µg/ml, 0.216 µg/ml, 0.288 µg/ml and 0.360µg/ml.
quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.009 µg/ml, 0.018 µg/ml, 0.027µg/ml, 0.144 µg/ml, 0.180 µg/ml, 0.216 µg/ml, 0.288 µg/ml and 0.360µg/ml

Final concentration: From the above solution, take 0.5ml of solution and spiking blank plasma with working stock dilutions of analyte to produce 50µg/ml ISD concentration

Validation Methodology in bioanalytical method
System Suitability Parameter
System Suitability test are 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 analyzing 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, 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). 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 (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. 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 analysing 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 (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%.

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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 Ketotifen Fumarate 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 [12-15]

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

3. Results And Discussions

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

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

Optimized Method:
Chromatographic conditions
Mobile phase : Sodium hypo phosphate: Methanol (55:45)
Flow rate : 1.0 ml/min
Column : DiscoveryC_{18} (150mm x 4.6 mm, 5µ)
Detector wavelength : 222 nm
Column temperature : 30^{0}C
Injection volume : 10µL
Run time : 5 min

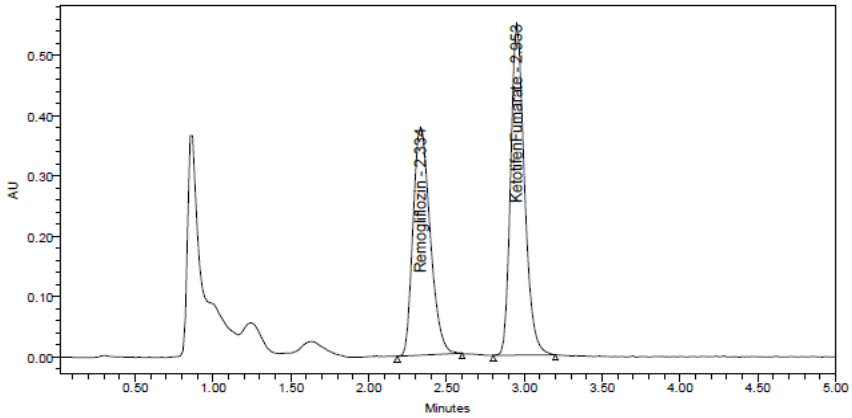

Figure 2 : optimised Chromatogram for Ketotifen Fumarate

S.No	Peak Name	RT	Area	USP Plate count	USP resolution	USP tailing
1	Remogliflozin	2.334	1486636	2698.7		1.3
2	Ketotifen fumarate	2.953	1482367	5328.5	3.3	1.3

Table 1 System suitability for the Ketotifen Fumarate

Observation: Both peaks eluted with good peak shape and retention time and tailing was passed.

Discussion: Ketotifen Fumarate and Internal Standard were eluted at 2.953 min, 2.334min 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

System suitability values of Ketotifen Fumarate
This system suitability method is intended to guarantee that the HPLC 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.

	Analyte	Ketotifen Fumarate	ISTD	Remogliflozin	
Sample Name	**Analyte Area**	**Analyte RT (min)**	**ISTD Area**	**ISTD RT (min)**	**Area Ratio**
AQ MQC	886366	2.96	1427070	2.33	0.6211
AQ MQC	889349	2.96	1469094	2.33	0.6054
AQ MQC	889606	2.96	1475360	2.33	0.6030
AQ MQC	884477	2.97	1461622	2.35	0.6051
AQ MQC	886890	2.98	1479873	2.35	0.5993
AQ MQC	885340	2.98	1476166	2.35	0.5998
MEAN		2.968		2.337	0.60561
SD		0.0087		0.0094	0.008019
%CV		0.29		0.40	1.32
System Suitability Status		Suitable			
Acceptance Criteria:					
The % CV of the retention time (RT) should be ≤ 2.00 %.					
The % CV of the area ratio should be ≤ 5.00 %					

Table 2: System Suitability of Ketotifen Fumarate

Auto sampler carryover of Ketotifen Fumarate

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 HPLC 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).

Autosampler Carryover				
Analyte	Ketotifen Fumarate		**ISTD**	Remogliflozin
Sample ID	Peak Area		% Carryover	
	Drug	ISTD	Drug	ISTD
Unextracted samples				
RS	0	0	N/A	N/A
AQ ULOQ	1769854	1478652	**0.00**	**0.00**
RS	0	0		
AQ LLOQ	88269	1448652	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	1753256	1420635	**0.00**	**0.00**
STD Blk	0	0		
LLOQ	88186	1407865	N/A	N/A
Acceptance Criteria:				
The carryover area response in subsequent injections of RS or STD Blk after aqueous or extracted ULOQ should be ≤ 20.00 % of the equivalent aqueous or extracted LLOQ standard area.				

Table 3: Auto sampler carryover of Ketotifen Fumarate

METHOD VALIDATION

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

Specificity and Screening of Biological Matrix						
Analyte	**Ketotifen Fumarate**			**ISTD**	**Remogliflozin**	
S.No.	Sample ID	Response		% Interference		Pass/Fail
		Drug	ISTD	Drug	ISTD	
1	STD Blk1	0	0	0.00	0.00	Pass
2	LLOQ1	88654	1465821			
3	STD Blk2	0	0	0.00	0.00	Pass
4	LLOQ2	88326	1496524			
5	STD Blk3	0	0	0.00	0.00	Pass
6	LLOQ3	88249	1435876			
7	STD Blk4	0	0	0.00	0.00	Pass
8	LLOQ4	88697	1493258			
9	STD Blk5	0	0	0.00	0.00	Pass
10	LLOQ5	88549	1432658			
11	STD Blk6	0	0	0.00	0.00	Pass
12	LLOQ6	88065	1496587			
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.						

Table 4: Specificity and Screening of Biological Matrix of Ketotifen Fumarate

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.

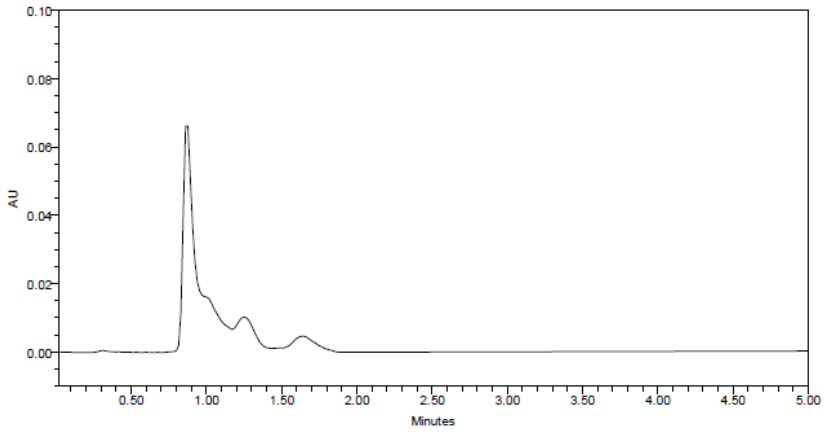

Figure 3 Representative Chromatogram of a Blank Plasma Sample

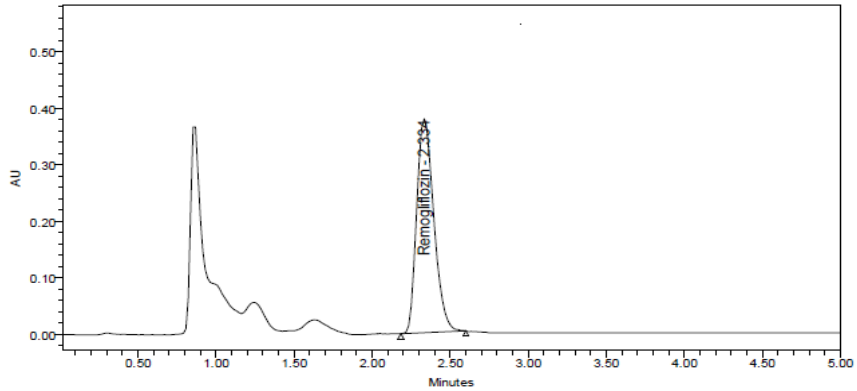

Figure 4 Representative Chromatogram of Blank Plasma with Internal Standard

Sample
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

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

Sensitivity	
Analyte : Ketotifen Fumarate , Remogliflozin (Standard)	
S.No	**LLOQ**
	Nominal Concentration (ng/mL)
	9.000
	Nominal Concentration Range (ng/mL)
	(7.200-10.800)
	Calculated Concentration (ng/mL)
1	**8.890**
2	**8.840**
3	**8.870**
4	**8.950**
5	**8.950**
6	**9.020**
n	**6**
Mean	**8.9200**
SD	**0.06573**
% CV	**0.74**
% Mean Accuracy	**99.11**
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 %.	

Table 5: Sensitivity of Ketotifen Fumarate

Discussion: - The LLOQ concentration was found between 80 -120 % and % Coefficient of variation found to be 0.74% and mean of 6 injections was found to be 99.11 % within the acceptance limits. As the limit of Sensitivity % CV was less than "20%" the system Sensitivity was passed in this method.

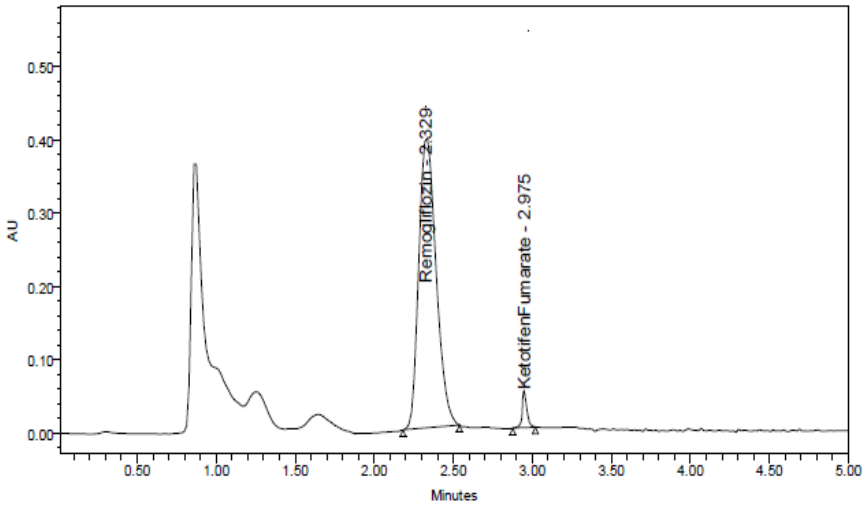

Figure 6 LLOQ Chromatogram of Ketotifen fumarate

5)Matrix factor evolution

Matrix Effect			
Analyte	**Ketotifen Fumarate**	**ISTD**	**Remogliflozin**
S. No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration (ng/mL)	
		288.000	27.000
		Nominal Concentration Range (ng/mL)	
		(244.800-331.200)	(22.950-31.050)
		Calculated Concentration (ng/mL)	
1	LOT1	286.63	26.48
		282.75	27.02
		287.26	26.78
2	LOT2	288.25	26.74
		287.84	27.65
		288.63	26.48
3	LOT3	286.25	27.08
		285.15	26.45
		286.38	26.65
4	LOT4	285.42	26.85
		287.20	26.65
		288.21	26.05
5	LOT5	286.24	26.35
		287.29	26.74
		288.52	27.06
6	LOT6	286.74	27.54
		287.56	26.51
		288.95	26.25
n		18	18
Mean		286.9596	26.7406
SD		1.50860	0.41550
% CV		0.53	1.55
% Mean Accuracy		99.64	99.04
No. of QC Failed		0	0
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 %.			

Table 6: Matrix factor evaluation (absence of matrix factor)

Discussion- The Evaluation of Matrix by injecting the QC samples of high and low concentrations in 6 lots the %Mean obtained was 99.64% and 99.04% of HQC and LQC and % CV obtained are 0.53% and 1.53% of HQC and LOQ. As the limit of CV was less than "20%" the system Matrix was passed in this method.

6) Linearity:

Linearity								
Analyte	**Ketotifen Fumarate**						**ISTD**	**Remogliflozin**
Acquisition Batch ID	**STD1**	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
	Nominal Concentration (ng/mL)							
	9.000	**18.000**	**27.000**	**144.000**	**180.000**	**216.000**	**288.000**	**360.000**
	Nominal Concentration Range (ng/mL)							
	(7.200-10.800)	**(15.300-20.700)**	**(22.950-31.050)**	**(122.400-165.600)**	**(153.000-207.000)**	**(183.600-248.400)**	**(244.800-331.200)**	**(306.000-414.000)**

	Back Calculated Concentration (ng/mL)							
P&A1	**8.954**	**17.846**	**26.854**	**143.860**	**179.840**	**215.470**	**287.965**	**359.580**
P&A2	**9.025**	**17.980**	**25.987**	**143.750**	**178.850**	**214.954**	**288.025**	**363.587**
P&A3	**8.956**	**18.024**	**27.620**	**143.850**	**180.240**	**214.680**	**286.954**	**359.846**
n	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
Mean	**8.9783**	**17.9500**	**26.8203**	**143.8200**	**179.6433**	**215.0347**	**287.6480**	**361.0043**
SD	**0.04043**	**0.09271**	**0.81702**	**0.06083**	**0.71557**	**0.40113**	**0.60177**	**2.24061**
%CV	**0.45**	**0.52**	**3.05**	**0.04**	**0.40**	**0.19**	**0.21**	**0.62**
% Mean Accuracy	**99.76**	**99.72**	**99.33**	**99.88**	**99.80**	**99.55**	**99.88**	**100.28**
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.								

Table 7: Linearity curve values of Ketotifen Fumarate

S.No	conc in ng/ml	ISD(area)	Drug(area)	Area response ratio
1	0	0	0	0
2	9	1497070	88207	0.0589
3	18	1409094	165267	0.1173
4	27	1475360	218373	0.1480
5	144	1401622	708688	0.5056
6	180	1479873	887468	0.5997
7	216	1476166	1086666	0.7361
8	288	1496523	1416839	0.9468
9	360	1496582	1754096	1.1721

Table 8: Linearity of Ketotifen Fumarate

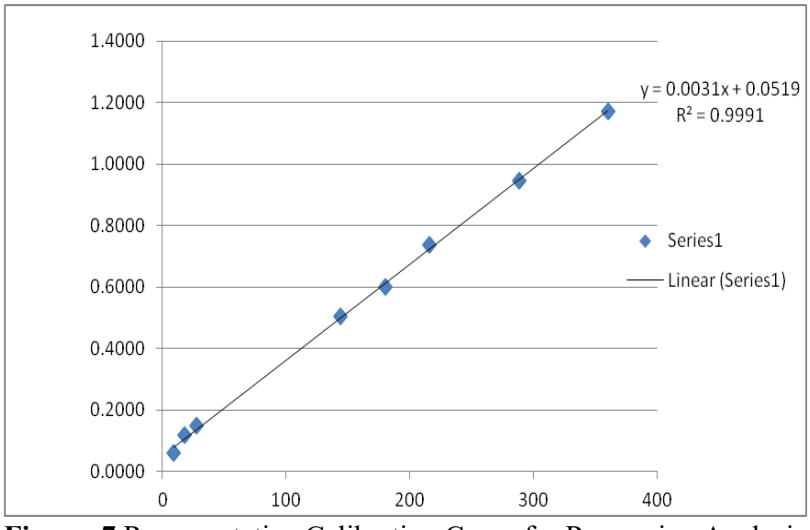

Figure 7 Representative Calibration Curve for Regression Analysis

Discussion: - Calibration was found to be linear over the concentration range of 9 to 360ng /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 Ketotifen Fumarate)

	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	288.000	180.000	27.000	9.000
	Nominal Concentration Range (ng/mL)			
	(244.800-331.200)	(153.000-207.000)	(22.950-31.050)	(7.200-10.800)
	Back Calculated Concentration (ng/mL)			
	286.510	178.954	26.385	8.955
	287.658	179.845	26.745	8.756
	288.245	180.745	26.984	9.025
	299.548	180.325	27.325	8.956
	287.658	179.568	26.954	9.085
	288.056	178.546	27.685	8.962
n	6	6	6	6
Mean	289.6125	179.6638	27.0131	8.9564
SD	4.90454	0.82480	0.45163	0.11066
%CV	1.69	0.46	1.67	1.24
% Mean Accuracy	100.56	99.81	100.05	99.52
	287.654	179.845	27.840	9.026
	286.954	180.652	26.990	8.965
	288.035	180.569	26.856	8.622
	287.645	180.995	26.954	9.211
	289.674	179.584	27.845	9.625
	286.954	179.632	26.385	8.764
n	6	6	6	6
Mean	287.8193	180.2128	27.1450	9.0354
SD	1.00415	0.59993	0.58235	0.35467
%CV	0.35	0.33	2.15	3.93
% Mean Accuracy	99.94	100.12	100.54	100.39
	287.654	174.258	26.845	8.652
	286.358	180.250	26.846	9.056
	288.475	179.654	26.521	8.964
	286.541	180.657	26.984	9.056
	288.541	180.652	27.035	8.763
	286.451	179.658	26.159	8.977
n	6	6	6	6
Mean	287.3367	179.1883	26.7317	8.9115
SD	1.02198	2.45656	0.33284	0.16612
%CV	0.36	1.37	1.25	1.86
% Mean Accuracy	99.77	99.55	99.01	99.02
Between Batch Precision and Accuracy				
n	18	18	18	18
Mean	288.2562	179.6883	26.9632	8.9678
SD	2.94850	1.50547	0.47305	0.22692
%CV	1.02	0.84	1.75	2.53
% Mean Accuracy	100.09	99.83	99.86	99.64

Table 9: precision data for intra-day runs of Ketotifen Fumarate

Rugged Precision and Accuracy (inter-day runs of Ketotifen Fumarate)

	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	288.000	**180.000**	**27.000**	**9.000**
	Nominal Concentration Range (ng/mL)			
	(244.800-331.200)	**(153.000-207.000)**	**(22.950-31.050)**	**(7.200-10.800)**
	Calculated Concentration (ng/mL)			
	288.562	**179.685**	**26.541**	**8.745**
	287.658	**180.520**	**26.845**	**8.993**
	288.023	**179.485**	**26.956**	**8.952**
	287.659	**179.562**	**26.745**	**9.048**
	286.452	**173.548**	**26.953**	**8.963**
	288.065	**180.658**	**27.056**	**9.046**
n	**6**	**6**	**6**	**6**
Mean	**287.7366**	**178.9097**	**26.8493**	**8.9576**
SD	**0.71183**	**2.67414**	**0.18483**	**0.11170**
% CV	**0.25**	**1.49**	**0.69**	**1.25**
% Mean Accuracy	**99.91**	**99.39**	**99.44**	**99.53**
Different Analyst	**285.056**	**174.658**	**27.956**	**8.956**
	287.654	**177.965**	**26.845**	**8.962**
	286.984	**179.786**	**26.845**	**9.066**
	288.054	**178.956**	**27.956**	**8.968**
	286.398	**180.856**	**26.456**	**8.542**
	284.785	**178.625**	**26.942**	**9.079**
n	**6**	**6**	**6**	**6**
Mean	**286.4885**	**178.4743**	**27.1667**	**8.9287**
SD	**1.34290**	**2.11997**	**0.63381**	**0.19705**
% CV	**0.47**	**1.19**	**2.33**	**2.21**
% Mean Accuracy	**99.48**	**99.15**	**100.62**	**99.21**

Table no 10: precision data for inter-day runs of Ketotifen Fumarate

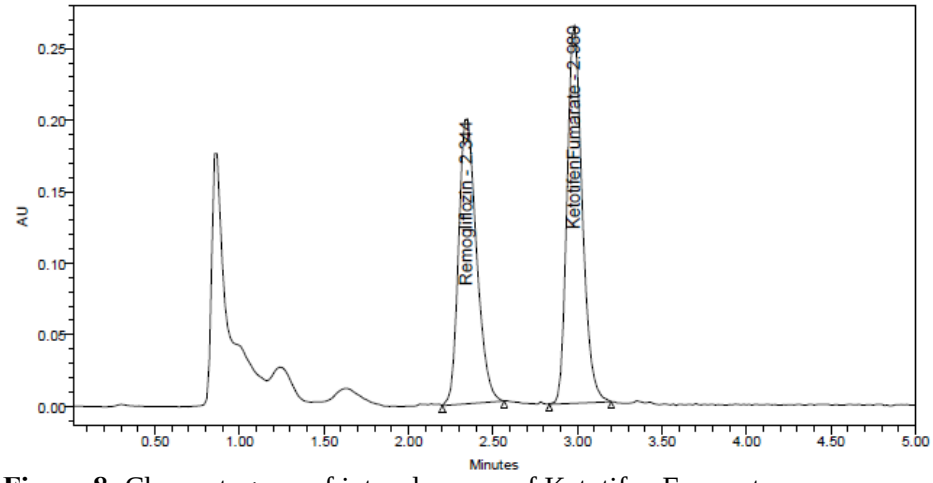

Figure 8: Chromatogram of inter-day runs of Ketotifen Fumarate

Discussion: - The intraday and inter day accuracy and precision was assessed by analyzing 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 Ketotifen Fumarate at four concentration levels, i.e.,0.009µg/ml(LLOQ), 0.027 µg/ml (LQC), 0.180 µg/ml (MQC) and 0.288 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.

S. No.	HQC		MQC1		LQC	
	Un extracted Response	Extracted Response	Un extracted Response	Extracted Response	Un extracted Response	Extracted Response
1	1431938	1417034	8896386	885688	219904	217153
2	1490578	1428013	8894152	885432	216362	212675
3	1424564	1387578	8887372	884586	219457	218601
4	1497459	1453966	8891005	888411	217477	217038
5	1454973	1408183	8913195	889598	212372	214573
6	1468890	1414126	8893489	887370	220506	218723
n	6	6	6	6	6	6
Mean	1461400	1418150	8895933	886848	217680	216461
SD	29912.92	22052.17	8998.92	1936.25	3034.58	2383.54
% CV	2.05	1.55	0.10	0.22	1.39	1.10
% Mean Recovery	97.04		9.97		99.44	
Overall % Mean Recovery	68.817					
Overall SD	50.9774					
Overall % CV	**74.08**					

Table 11: Recovery of Ketotifen Fumarate

Recovery - Internal standard

S.No.	Un extracted Area Ratio	Extracted Area Ratio
1	1495061	1477070
2	1468849	1409094
3	1481550	1475360
4	1422395	1401622
5	1496072	1479873
6	1504540	1476166
n	6	6
Mean	1478077.8	1453197.5
SD	30034.21	37162.77
% CV	2.03	2.56
% Mean Recovery	**98.32**	

Table 12: Recovery of Remogliflozin (IS)

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 Ketotifen Fumarate and. The overall % mean recovery for was found to be 98.32% % CV ranged from 1-3 for IS(Extracted & Unextracted). The results demonstrated that the bioanalytical method had good extraction efficiency. The results demonstrated that the bioanalytical method had good extraction efficiency

Rugged Linearity:

Analyte	Ketotifen Fumarate					ISTD	Remogliflozin	
P&A ID	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							
	9.000	18.000	27.000	144.000	180.000	216.000	288.000	360.000
	Nominal Concentration Range (ng/mL)							
	(7.200-10.800)	(15.300-20.700)	(22.950-31.050)	(122.400-165.600)	(153.000-207.000)	(183.600-248.400)	(244.800-331.200)	(306.000-414.000)
	Calculated Concentration (ng/mL)							

Different Column	**8.955**	**17.568**	**26.845**	**143.578**	**179.658**	**216.628**	**287.451.**	**358.469**
Different Analyt	**8.746**	**18.054**	**27.658**	**144.025**	**179.860**	**217.045**	**287.656**	**358.746**

Table 13: Rugged Linearity of Ketotifen Fumarate

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 (9 ng/ml-360ng/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 Ketotifen Fumarate.

Ruggedness Precision and Accuracy

P&A ID	**HQC**	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (µg/mL)			
	2.080	**1.300**	**0.195**	**0.065**
	Nominal Concentration Range (µg/mL)			
	(1.768-2.392)	**(1.105-1.495)**	**(0.166-0.224)**	**(0.052-0.078)**
	Calculated Concentration (µg/mL)			
P&A01	**1.799**	**1.115**	**0.169**	**0.056**
	1.821	**1.169**	**0.174**	**0.059**
	1.862	**1.268**	**0.189**	**0.063**
	2.193	**1.285**	**0.191**	**0.066**
	2.298	**1.398**	**0.214**	**0.071**
	2.390	**1.482**	**0.220**	**0.073**
n	**6**	**6**	**6**	**6**
Mean	**2.0605**	**1.2862**	**0.1928**	**0.0647**
SD	**0.26370**	**0.13727**	**0.02062**	**0.00665**
% CV	**12.80**	**10.67**	**10.70**	**10.29**
% Mean Accuracy	**99.06**	**98.94**	**98.89**	**99.49**

Table 14: Ruggedness Precision and Accuracy of Ketotifen Fumarate

Reinjection Reproducibility

Reinjection Reproducibility				
Analyte	**Ketotifen Fumarate**		**Temperature**	
ISTD	**Remogliflozin**		**2-8˚C**	
P&A ID	**HQC**	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	288.000	**180.000**	**27.000**	**9.000**
	Nominal Concentration Range (ng/mL)			
	(244.800-331.200)	**(153.000-207.000)**	**(22.950-31.050)**	**(7.200-10.800)**
	Calculated Concentration (ng/mL)			
P&A01	**286.58**	**179.86**	**26.84**	**8.956**
	289.65	**180.25**	**26.85**	**8.841**
	288.51	**179.63**	**27.06**	**9.056**
	287.62	**178.21**	**26.95**	**9.074**
	286.64	**179.25**	**26.88**	**9.159**
	284.76	**173.26**	**26.75**	**9.055**
n	**6**	**6**	**6**	**6**
Mean	**287.2944**	**178.4110**	**26.8882**	**9.0236**
SD	**1.70450**	**2.61822**	**0.10737**	**0.11022**
% CV	**0.59**	**1.47**	**0.40**	**1.22**
% Mean Accuracy	**99.75**	**99.12**	**99.59**	**100.26**

Table 15: Reinjection Reproducibility of Ketotifen Fumarate

Discussion: - The % mean accuracy for LQC, MQC and HQC samples was found to be 99.75, 99.12, 99.59 and % Cv was found to be 0.59, 1.47, 0.40 and LLOQ was found 100.26and % Cv was found to be 1.22. The results demonstrated that the bioanalytical method had good extraction efficiency.

Stabilities

a) **long term stock solution stability**

DAY ZERO ASSESSMENT BATCH		
Analyte	**ISTD**	**Remogliflozin**
Replicate No.	**HQC**	**LQC**
	Nominal Concentration (ng/mL)	
	288.000	**27.000**
	Nominal Concentration Range (ng/mL)	
	(244.800-331.200)	**(22.950-31.050)**
	Calculated Concentration (ng/mL)	
1	**278.524**	**26.854**
2	**288.695**	**26.954**
3	**286.642**	**26.325**
4	**285.650**	**27.096**
5	**285.632**	**26.854**
6	**287.560**	**27.963**
n	**6**	**6**
Mean	**285.4503**	**27.0077**
SD	**3.59002**	**0.53600**
% CV	**1.26**	**1.98**
% Mean Accuracy	**99.11**	**100.03**

Table 16: stability of Ketotifen Fumarate (zero days)

Discussion- In bench-top stability, six replicates of LQC & HQC samples (0.027 and 0.288 µg/ml) were analyzed for 9 hours at room temperature on the laboratory bench. The % mean stability was calculated and found to 99.11% for LQC and 100.03% for HQC respectively.

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

Long Term Analyte Stability in Matrix				
Analyte Name	**Ketotifen Fumarate**	**Temperature**	**-28**	**±5 ˚C**
Replicate No.	**HQC**		**LQC**	
	Nominal Concentration (ng/mL)			
	288.000	**288.000**	**27.000**	**27.000**
	Nominal Concentration Range (ng/mL)			
	(244.800-331.200)	**(244.800-331.200)**	**(22.950-31.050)**	**(22.950-31.050)**
	Calculated Concentration (ng/mL)			
	Comparison Samples	**Stability Samples**	**Comparison Samples**	**Stability Samples**
1	**288.65**	**288.56**	**26.854**	**26.958**
2	**287.65**	**286.69**	**26.745**	**26.845**
3	**288.65**	**285.66**	**27.124**	**26.956**
4	**285.26**	**288.97**	**27.066**	**27.038**
5	**287.97**	**285.62**	**27.163**	**26.845**
6	**287.63**	**284.37**	**26.845**	**27.054**
n	**6**	**6**	**6**	**6**
Mean	**287.6358**	**286.6443**	**26.9662**	**26.9493**
SD	**1.24863**	**1.80347**	**0.17309**	**0.09023**
% CV	**0.43**	**0.63**	**0.64**	**0.33**

%Mean Accuracy	**99.87**	**99.53**	**99.87**	**99.81**
% Mean Stability	**99.66**		**99.94**	

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

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

Long Term Analyte Stability in Matrix				
Analyte Name	**Ketotifen Fumarate**	**Temperature**	**-80**	**±5 ˚C**
Replicate No.	**HQC**		**LQC**	
	Nominal Concentration (ng/mL)			
	288.000	**288.000**	**27.000**	**27.000**
	Nominal Concentration Range (ng/mL)			
	(244.800-331.200)	**(244.800-331.200)**	**(22.950-31.050)**	**(22.950-31.050)**
	Calculated Concentration (ng/mL)			
	Comparison Samples	**Stability Samples**	**Comparison Samples**	**Stability Samples**
1	**288.26**	**287.06**	**26.756**	**26.755**
2	**286.26**	**286.58**	**26.065**	**26.856**
3	**285.28**	**286.55**	**25.965**	**27.956**
4	**284.37**	**286.33**	**26.857**	**26.845**
5	**285.80**	**284.04**	**26.765**	**27.856**
6	**288.00**	**282.26**	**27.966**	**26.963**
n	**6**	**6**	**6**	**6**
Mean	**286.3272**	**285.4670**	**26.7290**	**27.2051**
SD	**1.53259**	**1.89899**	**0.71731**	**0.54784**
% CV	**0.54**	**0.67**	**2.68**	**2.01**
%Mean Accuracy	**99.42**	**99.12**	**99.00**	**100.76**
% Mean Stability	**99.70**		**101.78**	

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

Discussion: -Long term stock solution stability for the Ketotifen Fumarate was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28°C& -80°C in refrigerator.

Summary

In the present work we have developed a simple, accurate, precise method was for the estimation of Ketotifen Fumarate in Rabbit plasma and the method was validated. The sample preparation in this study was conducted using the protein-precipitation method. The chromatogram was passed through a Discovery C18 column (150x 4.6 mm, 5m) using a mobile phase consisting of a mixture of Buffer Na2HPO4 and Methanol in a ratio of 55:45. The column was pumped at a flow rate of 1.0ml/min. The buffer employed Sodium Phosphate. The buffer used in this procedure was a buffer. The Remogliflozin is utilized as the Internal Standard [IS] for the separation of Ketotifen Fumarate. The temperature was consistently maintained at 30°C. The wavelength selected for optimization was 222.0nm. The retention time for Ketotifen Fumarate and the internal standard were determined to be 2.953 minutes and 2.344 minutes, respectively. The standard curve exhibited a linear relationship (R2 >0.995) across the concentration range of 9-360 ng/ml. This system suitability method is intended to guarantee that the HPLC 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. 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 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 Evaluation of Matrix by injecting the QC samples of high and low concentrations in 6 lots the %Mean obtained was 99.64% and 99.04% of HQC and LQC and % CV obtained are 0.53% and 1.53% of HQC and LOQ. As the limit of CV was less than "20%" the system Matrix was passed in this method. The intraday and inter day accuracy and precision was assessed by analyzing 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 Ketotifen Fumarate at four concentration levels, i.e.,0.009µg/ml(LLOQ), 0.027 µg/ml (LQC), 0.180 µg/ml (MQC) and 0.288 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 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 Ketotifen Fumarate and. The overall % mean recovery for was found to be 98.32% % CV ranged from 1-3 for IS(Extracted & Unextracted). The results demonstrated that the bioanalytical method had good extraction efficiency. The results demonstrated that the bioanalytical method had good extraction efficiency. The % mean accuracy for LQC, MQC and HQC samples was found to be 99.75, 99.12, 99.59 and % Cv was found to be 0.59, 1.47, 0.40 and LLOQ was found 100.26and % Cv was found to be 1.22. The results demonstrated that the bioanalytical method had good extraction efficiency. In bench-top stability, six replicates of LQC & HQC samples (0.027 and 0.288 µg/ml) were analyzed for 9 hours at room temperature on the laboratory bench. The % mean stability was calculated and found to 99.11% for LQC and 100.03% for HQC respectively. Long term stock solution stability for the Ketotifen Fumarate was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28°C& -80°C in refrigerator.

4. Conclusion

As per the ICH recommendations, every analytical validation parameter was established. The bioanalytical procedure developed was deemed selective, robust, and reliable as it met the recommendations' requirements for accuracy, precision, recovery, and other validation metrics. In the absence of plasma interference, the peaks produced for the target material and the internal standard were effectively distinguished from one other and exhibited a satisfactory tailing factor. The technique could provide significant advantages for therapeutic drug monitoring (TDM), bioequivalence research, pharmacokinetics studies, toxicology, and biological investigations.

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