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VALIDATION AND GREENNESS ASSESSMENT OF UPLC METHOD FOR BIOANALYSIS OF BILASTINE IN MICE PK STUDY

Drisya Ramesh PT[1], Praseetha K [1], Kathirvel S [1] & Raja Rajeswari K*[2]

[1] National College of Pharmacy, KMCT Group of Institutions, Manassery, Kozhikode-673602, Kerala, India

***[2] Department of Pharmaceutical Analysis, Sri Sivani College of Pharmacy, Srikakulam-532 402, Andhra Pradesh, India**

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Abstract

Bilastine, is a second-generation antihistamine drug which is used in the treatment of allergic rhinoconjunctivitis and urticaria. It exerts its effect as a selective histamine H_1 receptor antagonist. In this study, an ultra-high-performance liquid chromatography (UPLC) method was developed to measure the concentrations of Bilastine in mouse blood, and the method was applied in measuring the pharmacokinetics of the analyte after oral and intravenous administration. The analyte was extracted by solid phase extraction method. A UPLC BEH C18 column (2.1 mm × 100 mm, 1.8 µm particle size) was used for chromatographic separation by gradient elution using acetonitrile-water (0.1% formic acid) as the mobile phase at a flow rate of 0.4 mL/min. Bilastine was administered to the mice orally at 2 mg/kg and intravenously at 0.05 mg/kg. Blood was collected at various time intervals, and the blood samples were processed after collection and analyzed by UPLC. The intra-day and inter-day accuracy of Bilastine were 91%-103% and 85%-107%, respectively, and the precision (RSD, %) was less than 15% for both intra-day and inter-day measurements. The matrix effect ranged from 95% to 108%, and the recovery was higher than 70%. The half-life of bilastine in plasma was found to be 7.02 ± 5.21h. Bilastine has a good linear relationship in the range of 10-500 ng/mL, and the lower limit of quantification was 10 ng/mL. The precision, accuracy, extraction recovery, matrix effect, and stability meet the requirements of the guiding principles. A robust and reliable UPLC method was fully optimized and developed to detect the blood concentration of bilastine in mice and the samples were analyzed by Empower software.

Keywords: Bilastine, Solid Phase Extraction, UPLC, Validation, Antihistamine, Pharmacokinetics

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***Corresponding Author**

Dr. Raja Rajeswari Katta
Professor & HOD
Department of Pharmaceutical Analysis
Sri Sivani College of Pharmacy
Chilakapalem Junction
Srikakulam-532 402
Andhra Pradesh, India

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1. Introduction

Bilastine is a selective histamine H1 receptor antagonist [1]. During allergic response mast cells undergo degranulation which releases histamine and other subastances. By binding to and preventing activation of the H1 receptor, bilastine reduces the development of allergic symptoms due to the release of histamine from mast cells. Bilastine (Fig-1) is sold by several pharmaceutical companies under the brand names Allertine (Australia), Bilargo (Bangladesh), Blexten (Canada), Bilaxten (Switzerland), Ilaxten (UK), or Nixar (Croatia, Georgia, Macedonia and Serbia). Bilastine is approved in the European Union for the symptomatic treatment of allergic conjunctivitis and urticaria [2]. Preclinical data suggest the possibility of interactions between bilastine and drugs or food that are inhibitors or inducers of the P-glycoproteins [3]. Bilastine has an optimal benefit-to-risk ratio, meeting all conditions for contributing to safety in drivers who need antihistamines, and hence for being considered as an antihistamine of choice for drivers [4]. Several analytical methods have been reported for the analysis of bilastine individually by UV-spectroscopic [5], HPLC [6-9] and UPLC [10]. A Qbd method also published for bilasine along with its impurities [11]. Till date no bioanalytical method was reported for Bilastine by UPLC. The procurement of clinical data for bilastine [12-14] is also critical as lot of

research is still going on for this drug for its efficacy and combined dosage forms.

The objective of the current research is to develop and validate a rapid, reliable, sensitive and simple ultra-performance liquid chromatography method for the quantification of Bilastine in mouse blood. After complete validation, the method was applied to analyze study sample analysis in mice by giving a single oral dose at 2 mg/kg and intravenously at 0.05 mg/kg body weight. In this study we aimed to develop a sensitive method for estimation of Bilastine in mice blood and also to validate the method as per the ICH and USFDA Guidelines. Afterwards, the further plan is to conduct the *invivo* PK study in mice and the sample analysis by UPLC. The greenness of the method also is going to be determined.

2. Experimental

2.1 Instrumentation and Chromatographic Conditions

UPLC–UV Analysis

The LC system consisted of a Waters Acquity UPLC with Empower software equipped with a photodiode array detector. A Acquity UPLC BEH RP C18 column (2.1 mm × 100 mm, 1.8 µm particle size) from Waters was used as stationary phase and temperature maintained at 20°C. The mobile phase consisted of Acetonitrile and 0.1% formic acid in gradient mode pumped at a flow rate of 0.4 mL min-1. Analysis was performed for 5 min at the detection wavelength of 275 nm and the injection volume was 2 µL. The autosampler maintained at 4°C

2.2 Chemicals

Bilastine and internal standard (Montelukast sodium) are purchased from Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). Acetonitrile and methanol of HPLC grade and all other chemicals were obtained from Merck (Mumbai, India). Formic acid (GR grade) was purchased from Merck Chemicals Ltd., Mumbai. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Milford, MA, USA). Biological matrix (mouse blood) was obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until use.

2.3 Preparation of Calibrators and QC Samples

A standard stock solution of Bilastine was prepared by dissolving standard 50 mg of Bilastine into 50 ml volumetric flask, to this added 30 ml of methanol and sonicated for 10 minutes at a temperature not exceeding 20°C. Allowed the solution to attain room temperature and then diluted to the volume with methanol to have a solution with a concentration of 1000 µg/mL. Calibration standard and quality control (QC) samples were prepared by adding corresponding working solutions with drug-free mouse blood. A volume of 10 mL of appropriate diluted stock solution at different concentrations and 10 mL of IS at a fixed concentration were spiked into 200 µL of mouse blood to yield final concentrations of calibration samples 10, 25, 50, 100, 200, 300, 400 and 500 ng/mL. The final concentration of IS was 100 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (10 ng/mL), LQC (50 ng/mL) MQC (200 ng/mL) and HQC (400 ng/mL).

2.4 Sample preparation

Analytes were extracted from blood by employing solid phase extraction method and the extract was then cleaned for using the 3 mL Supelclean™ Ultra 2400 SPE cartridge. 2 µL of the supernatant solution was injected for UPLC analysis.

2.5 Analytical Validation

All validation experiments were performed according to the Bioanalytical Method Validation Guidance for Industry15 and the ICH guidelines16 on validation of bioanalytical methods.

Assay Specificity and Selectivity

Specificity was assessed by verifying the absence of significant interference in the biological control medium with regard to the retention time of the compound (s) to be assayed. The specificity of the method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around the retention time.

Linearity

A calibration curve was prepared within the range of 10 to 500 ng/mL Bilastine in each run. Half of the calibration samples were analyzed at the beginning of the run and half at the end. The simplest calibration model and weighting procedure were used. The calculations of the curve's parameters were based on the ratio of the peak areas of Bilastine/IS versus the concentration of Bilastine. Bilastine concentrations for samples were calculated from the curve's equation obtained by means of linear regression.

Accuracy of back-calculated calibration samples should be within ±15% of the corresponding nominal concentration, except at the lowest concentration level, where the accuracy should be within ±20%. Per calibration curve, a maximum of 33% of the calibration samples, except the LLOQ and upper limit of quantification (ULOQ, 500 ng/mL), may differ from these specifications. At least 6 concentration levels were represented in each curve.

Matrix Effect, Extraction Recovery, and Process Efficiency

The influence of the matrix on the quantification of Bilastine was monitored using a comparison of: (1) the instrument response for the low, medium, and high QCs (n = 4 per level) injected directly in mobile phase (neat solutions), (2) the same amount of analyte added to extracted blank samples (post extraction spiked samples), and (3) the same amount of analyte added to the biological matrix before extraction (pre extraction spiked samples). Total process efficiency was calculated from the ratio of mean peak areas of Bilastine in extracted validation samples versus neat unextracted samples. This term accounts for any loss in signal attributable to the extraction process or matrix effect. Extraction recovery was calculated from the ratio of mean peak areas of

Bilastine in extracted validation samples versus blank samples spiked after extraction. The absolute matrix effect was calculated from the ratio of mean peak areas of Bilastine in blank samples spiked after extraction versus neat unextracted samples. If the ratio was 85% or 115%, an exogenous matrix effect was inferred.

Matrix Variability

To confirm that the biological matrix would not interfere with the assay, the selectivity of the developed method was tested by analyzing 6 different lots of blank blood samples and also 6 different lots of blank urine samples spiked with IS at the LLOQ level (n = 3 per lot), and blank blood samples with no IS (n = 3 per lot) against a calibration curve. The results for the LLOQ samples were considered acceptable if the precision from each matrix lot was ±20% and the accuracy was within the range of 80%–120%. The acceptance criterion for the analysis of the blank samples from the 6 individual lots was based on the raw peak areas found at the retention times of Bilastine and IS. No more than 10% of the blank samples could have peak areas greater than 20% of the average peak area of Bilastine in the LLOQ QCs.

Stability studies

Stability evaluations were performed in both aqueous and matrix based samples. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). Bilastine stability in blood was evaluated by performing bench top stability, long-term stability, short term stability and freeze-thaw stability. The processed samples were studied for stability in auto sampler at 10°C. Stability in blood was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples.

3. Results and Discussion

3.1 Chromatographic and detection parameters

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed-phase C18 column. The different columns tried were Symmetry C18, Luna C18 and Zorbax C18. The best results were observed with the Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.8 µm particle size) using acetonitrile (A) and 0.1% formic acid (B) (gradient mode) as mobile phase. Gradient flow operated as 95% B upto 1 min, then 1.1–2 min, 50% A, 50% B; 2.1-3 min, 95%A, 5% B; 3.1-4 min, 95%A, 5% B; 4.1–4.5 min, 5% A, 95% B; 4.6–7 min, 5% A, 95% B.

Variation of the column temperature between 20 and 30°C did not cause significant change in the resolution, however changes in retention time were observed. The column was used at 20°C at a flow rate of 0.4 mL/min. The method allowed the separation of analyte with IS in 5 min (Figure 2) runtime.

3.2 Specificity, Linearity, Accuracy and Precision

The specificity of method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around their retention times. The eight point calibration curve for the analyte showed a linear correlation between concentration and peak area. Calibration data (Table 1) indicated the linearity (r2 > 0.99) of the detector response for all standard solutions from 10 to 500 ng/mL The limits of detection by UPLC was found to be 2 ng/mL and LOQ was found to be 10 ng/mL. All standards and samples were injected in triplicate. Multiple injections showed that the results are highly reproducible and showed low standard error. A recovery experiment was performed to confirm the accuracy of the method. Blank blood was spiked with Low QC, Mid QC and High QC levels of the standard stock solution and then extracted and analyzed under optimized conditions. The extraction recoveries of all samples from mouse blood were in the range of 97.5-108.0% with relative standard deviations less than 10.0%, which indicates the sample preparation technique is suitable for extracting. Intra- and inter-day precision of the method was determined by analyzing QC samples on two consecutive days and the obtained intra-day accuracies were in the range of 91.6–103.0% and inter-day accuracies were in the range of 96.4–106.9% (for LLOQ 85.6% was also observed which is acceptable for it). The recovery results are displayed in Table 2 and Table 3. To investigate carry-over from one sample to the other in the autosampler, each validation run containing a calibration curve included a blank sample analyzed directly after the sample at the ULOQ calibration level. The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration. To demonstrate that the method is suitable for blood sample with test compound concentration higher than the ULOQ, the dilution integrity was assessed using validation samples spiked with the test compound at 2-, 4-, and 10-fold the concentration of the high QC. The dilution test was performed by increasing the concentration of IS by the appropriate dilution factor. After extraction, the dry extract was taken up with a volume of injection solvent also multiplied by the same factor. Accuracy of the calculated concentrations within the range of 85%–115% of the nominal values would suggest that samples containing Bilastine at a higher concentration than the ULOQ can be diluted using the above tested dilution method.

Stability evaluations were performed in both aqueous and matrix-based samples. The stock solutions were stable for a period of 24 h at room temperature and for 60 days at 1–10°C. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The processed samples were stable up to 36 h in auto sampler at 10°C. The long-term matrix stability was evaluated at −20°C over a period of 60 days. No significant degradation of analytes was observed over the stability duration and conditions. The long-term stability

results presented in Table 4 were within 85–115%. Stability in blood was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples. The short-term stability of analyte at room temperature was within 85–115% upto 24 h. The stability results presented in Table 5 and Table 6. Bilastine was stable upto 10 h on bench top at room temperature and over 3 freeze–thaw cycles. In mouse blood , the freeze-thaw study was carried out and the results are presented in Table 7. The variability of the matrix effect in mouse blood has resulted a very minute changes in the recovery of middle concentration of calibration curve. The results of Matrix effect area presented in Table 8.

3.3 Application of the method to pharmacokinetic study in Mice

Mice (100±20 g) used were maintained in a clean room at a temperature between 22±2°C with 12 h light/dark cycles and a relative humidity rate of 50±5%. Rats were housed in cages with a supply of normal laboratory feed with water ad libitum. For all of the studies, the animals (n=6) were deprived of food 12 h before dosing, but had free access to water. In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the developed UPLC method was successfully applied to a pharmacokinetic study by administration of Bilastine as single solution to six mice by oral route using BD syringe attached with oral gavage needle (size 18) at the dose of 2 mg/kg body weight. Approximately, 0.2 mL of blood samples from each anesthetized (isoflurane) rat at pre-determined time intervals was collected using a capillary tube into pre-labeled eppendorf tubes containing 10% of K2EDTA anticoagulant (20 µL). The time intervals for the sample collection were 0 (predose), 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h (postdose). The total blood volume collected from each mouse was approximately 1.7 to 1.9 mL which does not exceed the maximal recommended blood volume of 20% (2.0 mL for a 200 g body weight). All the samples were extracted by solid phase extraction method and the obtained supernatant samples were transferred into pre-labeled micro vials.

The blood samples thus obtained were stored at −30 °C till analysis. Post analysis the pharmacokinetic parameters were computed using WinNonlin® software version 5.2 and SAS® software version 9.2. All the samples were analyzed by the developed method and the mean concentrations vs time profile of Bilastine is shown in Figure 4. The pharmacokinetic parameters estimated are shown in Table 9.

The pharmacokinetic parameters evaluated were Cmax (maximum observed drug concentration during the study), AUC0-48(area under the blood concentration–time curve measured 48 hours, using the trapezoidal rule), Tmax (time to observe maximum drug concentration), Kel (apparent first order terminal rate constant calculated from a semi-log plot of the blood concentration versus time curve, using the method of least square regression) and t1/2 (terminal half-life as determined by quotient 0.693/Kel).

3.4 Greenness of the method using AGREE

AGREE software produces a clockwise circular diagram in which numbers 1 to 12 are arranged in the edge, indicates 12 ideologies of green analytical chemistry. Each of the 12 principles' result is based on an aggregate scale of 0 to 1 based on inputs and weights provided.17, 18 The score is the net result of all the 12 principles Figure 5 shows the AGREE diagram of the proposed method. The representative AGREE score of the proposed UPLC was estimated at 0.77 which suggested the significant greenness for the estimation of bilastine.

Table 1. Linearity data of Bilastine

Concn in ng/mL	Area in blood
10	2005.8
50	10229
100	20058
200	43116
250	54317
300	64173
400	85332
500	105998
Y= 213.85x-230.57	
R^2 = 0.9996	

Table 2: Intra-day Precision & Accuracy Results

Bilastine								
	LLOQ QC		LOW QC		MID QC		HIGH QC	
	10 ng/mL		50 ng/mL		200 ng/mL		400 ng/mL	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
Intra-day	9.156	91.560	48.402	96.804	195.850	97.925	395.267	98.817
	9.235	92.350	48.286	96.572	194.502	97.251	381.526	95.382
	9.471	94.710	49.260	98.520	196.275	98.137	390.515	97.629
	10.303	103.030	49.977	99.954	198.665	99.333	387.419	96.855
	10.102	101.020	47.420	94.840	197.690	98.845	392.330	98.083
	9.636	96.360	48.410	96.820	201.470	100.735	394.763	98.691
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**9.650**	**96.505**	**48.626**	**97.252**	**197.409**	**98.704**	**390.303**	**97.576**
SD	**0.465**		**0.883**		**2.462**		**5.178**	
CV(%)	**4.814**		**1.815**		**1.247**		**1.327**	

Table 3: Inter-day Precision & Accuracy Results

	LLOQ QC		LOW QC		MID QC		HIGH QC	
	10 ng/mL		50 ng/mL		200 ng/mL		400 ng/mL	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
Inter-day	10.450	104.500	52.741	105.482	203.577	101.789	404.458	101.114
	9.642	96.415	51.885	103.769	200.461	100.231	408.868	102.217
	9.925	99.251	50.796	101.593	204.104	102.052	396.800	99.200
	10.694	106.941	50.991	101.982	202.120	101.060	405.031	101.258
	8.562	85.620	51.488	102.976	205.129	102.565	406.086	101.521
	9.824	98.241	48.504	97.008	204.514	102.257	393.913	98.478
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**9.849**	**98.495**	**51.067**	**102.135**	**203.318**	**101.659**	**402.526**	**100.631**
SD	**0.746**		**1.435**		**1.732**		**5.829**	
CV (%)	**7.572**		**2.810**		**0.852**		**1.448**	

Table 4: Long-term Stability studies of Bilastine in blood at two QC levels (n=6)

	LOW QC		HIGH QC	
	50 ng/mL		400 ng/mL	
	Day 0	Day-60	Day 0	Day-60
Longterm stability	48.500	50.214	408.018	427.831
	47.552	47.024	418.286	385.801
	50.960	49.251	407.326	417.382
	47.998	48.214	399.771	395.932

	51.172	50.102	394.742	403.011
	50.946	50.879	384.100	426.414
N	**6**	**6**	**6**	**6**
Mean	**49.521**	**49.281**	**402.041**	**409.395**
SD	**1.677**		**11.898**	
CV(%)	**3.387**		**2.960**	
% Change		**-0.48595**		**1.829335**

Table 5: Short-term Stability studies of Bilastine in blood at Low QC level (n=6)

Short term stability	**LOW QC-50 ng/mL**					
	0 hr		**4 hr**		**24 hr**	
	Conc found	**% Recovery**	**Conc found**	**% Recovery**	**Conc found**	**% Recovery**
	48.500	97.000	50.183	100.366	51.123	102.246
	47.552	95.104	49.610	99.220	49.579	99.158
	50.960	101.920	49.670	99.340	47.346	94.692
	47.998	95.996	50.620	101.240	46.620	93.240
	51.172	102.344	50.140	100.280	48.338	96.676
	50.946	101.892	50.620	101.240	53.551	107.102
N	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**49.521**	**99.043**	**50.141**	**100.281**	**49.426**	**98.852**
SD	**1.677**		**0.439**		**2.581**	
CV(%)	**3.387**		**0.876**		**5.222**	
% Change				**1.25034**		**-0.19222**

Table 6: Short-term Stability studies of Bilastine in blood at High QC level (n=6)

Short term stability	**HIGH QC-400 ng/mL**					
	0 hr		**4 hr**		**24 hr**	
	Conc found	**% Recovery**	**Conc found**	**% Recovery**	**Conc found**	**% Recovery**
	408.018	102.005	395.018	98.755	394.123	98.531
	418.286	104.572	394.961	98.740	390.258	97.565
	407.326	101.832	404.997	101.249	400.125	100.031
	399.771	99.943	405.062	101.266	402.563	100.641
	394.742	98.686	385.014	96.254	386.521	96.630
	384.100	96.025	399.062	99.766	390.258	97.565
N	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**402.041**	**100.510**	**397.352**	**99.338**	**393.975**	**98.494**
SD	**11.898**		**7.539**		**6.242**	
CV(%)	**2.960**		**1.897**		**1.584**	
% Change				**-1.16609**		**-2.00622**

Table 7: Freeze thaw stability (after III cycle) study Results (n-6) conducted below -20°C & -50°C for Blood samples

Freeze Thaw stability	Freeze Thaw Cycle-III below -20°C				Freeze Thaw Cycle-III below -50°C			
	LOW QC		HIGH QC		LOW QC		HIGH QC	
	50 ng/mL		400 ng/mL		50 ng/mL		400 ng/mL	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
	50.869	101.738	404.415	101.104	45.078	90.156	427.831	106.958
	51.572	103.144	398.758	99.690	55.010	110.020	385.801	96.450
	51.164	102.328	403.459	100.865	50.800	101.600	417.382	104.346
	50.497	100.994	428.419	107.105	49.526	99.052	395.932	98.983
	49.256	98.512	420.706	105.177	47.445	94.890	403.011	100.753
	50.840	101.680	424.041	106.010	48.610	97.220	426.414	106.604
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**50.700**	**101.399**	**413.300**	**103.325**	**49.412**	**98.823**	**409.395**	**102.349**
SD	**0.794**		**12.538**		**3.364**		**17.158**	
CV (%)	**1.566**		**3.034**		**6.808**		**4.191**	

Table 8: Matrix effect Results

Unit No.	Bilastine 200 ng/mL	
	Neat standard sample peak area	Extracted blank plus spiked sample peak area
Unit No.: 1	42214	45510
Unit No.: 2	42506	43529
Unit No.:3	44078	46523
Unit No.: 4	41096	43525
Unit No.: 5	43087	42532
Unit No.: 6	44196	47540
N	**6**	**6**
Mean	**42862.665**	**44859.861**
SD	**1180.921**	**1966.748**
CV(%)	**2.755**	**4.384**
Matrix effect (%)	**1.047**	

Table 9: Pharmacokinetic parameters of Bilastine in Mice (n=6, Mean ± SD)

Parameter	Bilastine
C_{max} (ng/mL)	194.111 ± 12.426
T_{max} (h)	1.50 ± 0.7071
$t_{1/2}$ (h)	7.02 ± 5.21h
K_{el} (h^{-1})	0.079 ± 0.0078

Cmax: maximum analyte concentration.
Tmax: time point of maximum concentration.
$t_{1/2}$: half life of drug elimination during the terminal phase.
K_{el}: elimination rate constant

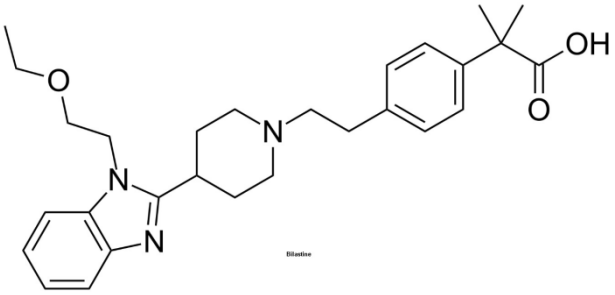

Figure 1: Chemical structure of Bilastine

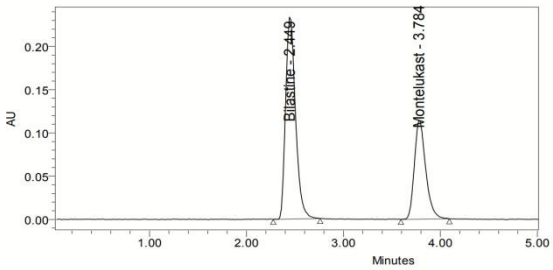

Figure 2: LLOQ chromatogram showing the separation of the analyte from IS

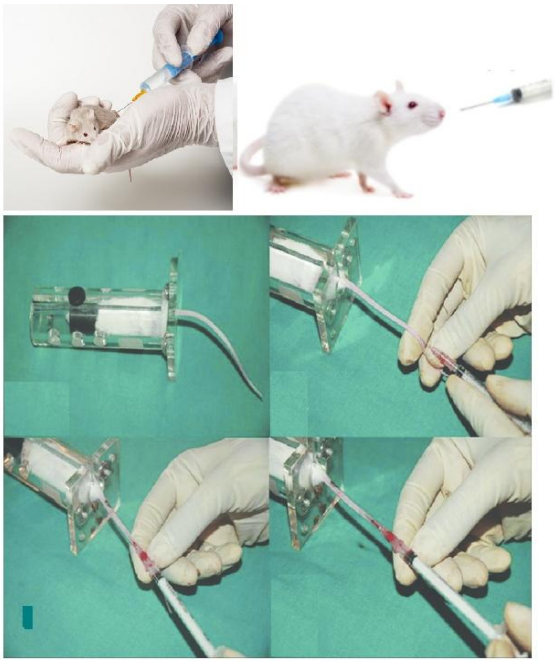

Figure 3: PK study and sample collection of Bilastine in mouse by tail vein

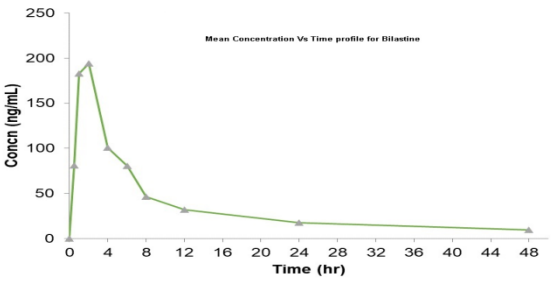

Figure 4: Mean concentration–time profile curve in mouse Blood

Figure 5: Greenness of the proposed UPLC method

4. Conclusion

The UPLC method offers significant advantages over those previously reported, interms of lower sample requirements, simplicity of extraction procedure without any matrix effect. The linear dynamic range established was adequate to measure the concentration of Bilastine in any preclinical and clinical study involving different biological species. The overall pharmacokinetic parameters were within the range of 80-125% therefore it can be concluded that, the present study provides firm evidence to support in clinical pharmacokinetic studies for further Research of selected drug. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

5. Funding

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6. Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

7. Author Contributions

All authors contributed to the study conception and design. All authors read and approved the final manuscript.

8. Inform Consent and Ethical Statement

Not Applicable

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10. References

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