6.1. SELECTION OF DRUG

First approved in early 1960's, Cyproheptadine has been one of the widely used antihistaminic drugs without any serious adverse effects reported till date. It is also widely used as appetite stimulant as it competes with serotonin at its receptor sites in intestine. Its NDA was filed by Merck in 1961. Generic version of the drug is also widely available. Due to its induction in early era, various studies were reported for the said drug approximately before 30 years. Despite the fact that sufficient literature is available for the said drug substance, till date no bioanalytical method employings simple LLE procedure as extraction method along with its pharmacokinetic studies in rat plasma has been reported for the drug substance Cyproheptadine HCl. As bioanalytical method development plays a very important role for studying the pharmacokinetic as well as toxic kinetic studies, it was decided to carry out the research regarding development of simple LLE procedure for extraction of drug from human plasma, development of sensitive HPLC-PDA analytical method for its estimation and its application for studying pharmacokinetics in rat plasma.

6.2 DRUG PROFILE

The drug profile of Cyproheptadine HCl is already discussed in Section 3.2.

Chemical name: Oxcarbazepine (IS) [2] It is used as internal standard

IUPAC Name: 5-oxo-6H-benzo[b][1]benzazepine-11-carboxamide

Molecular Formula: $C_{15}H_{12}N_2O_2$

Molecular Weight: 252.273 g/mol

Chemical Structure:

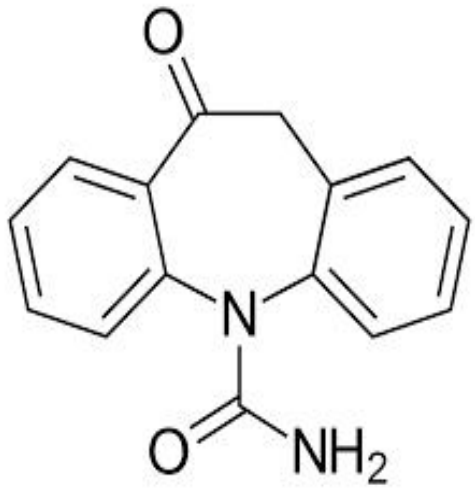


Figure 6.1: Structure of Oxcarbazepine

Appearance: White to slightly yellowish crystalline powder

Melting point: 201-215 °C

pKa: 0.65

Log p: 1.5

Solubility: Slightly soluble in chloroform, dichloromethane, acetone and methanol. Practically insoluble in ethanol, ether.

Drug Category: Antiepileptic

Mechanism of action: It blocks voltage-gated sodium channels, thereby stabilizing hyper-excited neural membranes, inhibiting repetitive neuronal firing, and decreasing the propagation of synaptic impulses.

Uses: Antiepileptic, anticonvulsant

6.3 LITERATURE REVIEW

Different analytical methods have been described for the quantification of Cyproheptadine hydrochloride from formulations and biological samples, including Ion pair complexometric UV spectroscopic method [4], Colorimetric methods [5, 6], HPLC [8], GC-MS [9] and LC/MS/MS [7] methods. Novak et al, have reported a HPLC method for quantification of CPH in serum or plasma. [10] Another quantification procedure for CPH in plasma and urine by HPLC has been reported by Foda et al. RP-HPLC method for the determination of CPH in urine was developed by Kountourellis and Ebete. [12] A GLC method for determination of cyproheptadine in urine and plasma using nitrogen sensitive detector has been reported by Hucker and Hutt. [13] Despite of sufficient literature available for bioanalysis of CPH, no bioanalytical method using simple procedure of LLE extraction giving % recovery of >99% from plasma was available in literature best to our knowledge. Thereby, it was decided to make efforts to develop a bioanalytical method for CPH and to study its application by rat pharmacokinetic studies.

6.4 SECTION –A

6.4.1 Experimental

6.4.1.1 Chemicals and materials

Cyproheptadine HCl (CPH) was obtained as a gift sample from HealthCare Pharmaceuticals Pvt. Ltd. (Vadodara, India) whereas Oxcarbazepine (OXZ) of pharmaceutical grade was kindly supplied as a gift sample by Sun Pharmaceuticals Pvt Ltd. HPLC grade acetonitrile and methanol were procured from Fischer Scientific Pvt Ltd. (India). Ammonium formate and formic acid were purchased from Lobachem chemicals Pvt Ltd (Mumbai, India) and Merck (India) respectively. Unless otherwise specified, all solutions were filtered through a 0.2 µm Nylon 6, 6 membrane filter, Ultipor® N66® from Pall Life Sciences, USA; prior to use. Drug free EDTA human plasma was procured from Suraktam Blood Bank, Vadodara.

6.4.1.2 Equipments and analysis conditions

The suitable wavelength for estimation of drug was determined by scanning over the range of 200–400 nm with a Shimadzu UV-1700 double beam spectrophotometer (Shimadzu, Japan). Chromatographic analysis was carried out on a Waters, Ahmedabad (from Waters Acquity Corporation, Milford, MA, USA) which consisted of a gradient pump, PDA detector, a manual injection facility with 20 µl fixed loop, low pressure gradient flow control valve, column oven, solvent delivery module. In addition, a Solid Phase Extractor (OROCHEM, Ezypress HT48), SPE cartridges (Phenomenex, Oasis HLB Cartridges), an electronic balance (Shimadzu AX120ELB300), a pH meter (Lab India Pico+), a sonicator (Spectra Lab, Selec XT 543), a hot air oven (SK Industries), vortex shaker (SPINIX), membrane filter 0.22 micron (Pall life sciences, Ultipor Nylon), deep freezer(EIE Instruments), micropipette (Tarsons, accupipete), refrigerated centrifuge (Remi), refrigerator (Godrej, Pantacool) were used in this study.

6.4.1.3 Sample preparation

10 mg CPH was weighed accurately transferred into 25 ml volumetric flask and dissolved in acetonitrile and double distilled filtered water (1:9). The volume was diluted up to the mark with the same for CPH stock solution I (400 µg/ml). From the above CPH stock solution I, 1 ml was diluted up to 10 ml to get 40 µg/ml CPH stock solution II. 10 mg OXZ (Internal standard) was weighed accurately, transferred into 25 ml volumetric flask and dissolved in double distilled filtered water. The volume was made up to the mark for OXZ stock solution I (400 µg/ml). From the above OXZ stock solution I, 0.5 ml was diluted up to 100 ml to get 2 µg/ml OXZ stock solution II. For preparation of calibration standards, appropriate aliquots (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 ml) of CPH stock solution II were taken in 8 different volumetric flasks and diluted up to the mark 5 ml with acetonitrile to obtain final concentrations of 2-16 µg/ml. For preparation of calibration standard samples in biological matrix appropriate aliquots (0.1 ml each) of CPH calibration standards were taken and appropriate aliquots (0.1 ml) of internal standard were spiked and the final volume of 2 ml was made up with plasma to obtain final concentration of 100-800 ng/ml of CPH. The quality Control samples of CPH in biological matrix consisted of 100(LLQC), 300(LQC), 500 (MQC), 800(HQC) ng/ml.

6.4.1.4 Sample pre-treatment for extraction of drug from biological matrix [14, 15]

Sample preparation technique used for the study plays a significant role with respect to bioanalytical samples. Sample preparation is applied to remove matrix of interfering biological compounds. It is essential to reduce the effect of the matrix formed due to biological and buffer components. As a bonus, analytes can be concentrated during the extraction processes. Sample preparation procedure is tedious and time consuming. However, the cleanliness of the samples affects the overall performance of the analysis. Different extraction techniques tried were protein precipitation, liquid liquid extraction and solid phase extraction.

6.4.1.5 HPLC method

The chromatographic analysis was performed using Empower 3 software on a Hypersil BDS C_{18} column (250×4.6 mm, 5 µm particle size). The analysis was carried out using acetonitrile: methanol: 20 mM ammonium formate (pH 5.5 adjusted with 0.2% formic acid) (40:10:50, v/v/v) as the mobile phase and 224 nm as detection wavelength. The mobile phase flow rate and typical pressure of the system were maintained at 1.00 ml/min and 2000 psi respectively. The analysis was performed at ambient temperature with injection volume of 20 µL. The mobile phase was filtered through 0.2 µm disposable filters from Ultipore®, PALL life sciences (40 mm) and degassed by ultrasonic vibrations prior to use.

6.4.1.6 Preparation of buffer solution

Ammonium formate (20 mM) was prepared by dissolving 0.63 gm of anhydrous ammonium acetate in 500 ml of double distilled water and adjusted to pH 5.5 using formic acid which was finally filtered with 0.2 µm Nylon membrane filter and degassed by ultra-sonication for 5 minutes.

6.4.1.7 Pharmacokinetic study

The pharmacokinetic study was carried out in Male Wistar Albino rats. The experimental procedure for it was approved by Institutional animal ethics committee (IAEC), Pharmacy department under protocol number (MSU/IAEC/2017-18/1723) on 10 November 2017.

6.4.1.8 Method Validation [19]

The method was validated using International Conference on Harmonization (ICH), Q2 (R1) guideline (ICH, 2005) for linearity, range, precision, limit of detection, limit of quantification, robustness, selectivity and specificity.

6.4.2 Results and discussion

6.4.2.1 Determination of suitable wavelength

UV overlain spectra of both CPH and OXZ showed that both drugs absorbed appreciably at 224 nm, so this wavelength was selected as the detection wavelength as presented in figure 6.2.

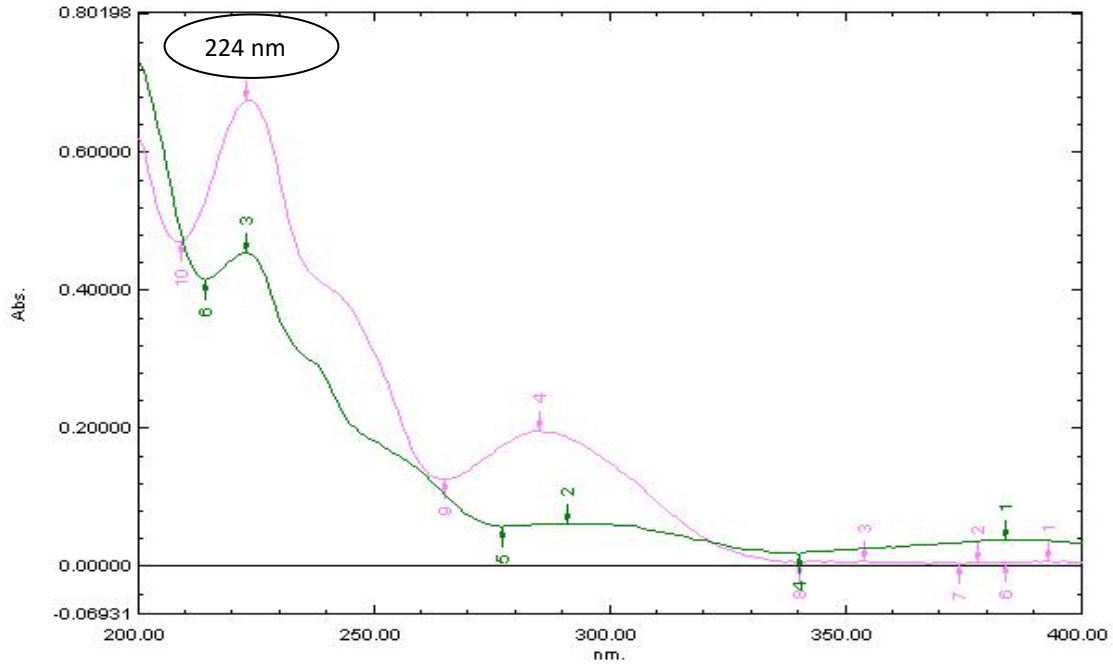


Figure 6.2 UV overlain spectra of CPH and OXZ for selection of detection wavelength

6.4.2.2 Selection of internal standard

Internal standard is a different compound from the analyte but one that is well resolved in the separation. One of the main reasons for using an internal standard is for samples requiring significant pretreatment or preparation. Real samples may undergo unknown transformations, reactions, metabolism in physiological conditions. Moreover, sample preparation steps that include precipitation, filtration, result in the sample losses. When added prior to sample pretreatment, a properly chosen internal standard can be used to correct for these sample losses. For internal standardization the compound selected as IS is added to the sample prior to sample pretreatment and ratio of response of analyte to that of 'IS' is plotted against concentration of the analyte. Trials for selection of appropriate internal standard were taken in which screening was done on the basis of structural resemblance, log P value, pKa value and availability. Chromatographic trials for dacarbazepine and oxcarbazepine were undertaken. Dacarbazepine gave chromatographic peak at retention time of 6.5 min which was merging with drug peak and also tailing was observed with it whereas Oxcarbazepine gave sharp and symmetric peak at retention time of 4.7 min with good resolution with the drug peak.

6.4.2.3 Sample extraction methods [14, 15]

For selection and optimization of particular extraction techniques various trials were taken as described below.

Initially protein precipitation method was tried using acetonitrile, methanol, trichloroacetic acid, perchloric acid and acetone as precipitating agents. For

protein precipitation aliquots ranging from 10 – 80 µl (from stock solution) of drug solution were taken and spiked with 100µl of plasma followed by vortexing for 5 minutes. After that optimized amount of precipitating solvent methanol (900µl) was added and then it was subjected to the vortexing for 5 minutes. Then the solutions were centrifuged at 4000 RPM for 10 minutes. Clear supernatant was used for the HPLC analysis. 100 ng/ml was the final concentration of sample being used for optimization of all three extraction methods. The above mentioned steps for protein precipitation methods were repeated for other precipitating solvents utilized in the method viz., ACN and acetone. Also effect on acidifying agents were tried along with precipitating agent which includes 0.1% trichloroacetic acid in water (450 µl) along with (450 µl) methanol and 0.1% perchloric acid in water (450 µl) along with (450 µl) methanol, but none of the agents gave satisfactory sample clean up. It showed greater plasma interference, greater sample transfer and greater sample evaporation steps. Samples obtained were unclean which can be harmful to life of analytical instrument in long run. Though the protein precipitation method was not used, however best recovery of about 60-65% was obtained by using methanol as protein precipitating agent.

Solid phase extraction (SPE) technique was tried in which interference due to plasma matrix was reduced. The procedure followed for SPE was to 0.5 ml plasma sample, 0.5 ml drug diluted in water in ratio of 1:2 was taken for sample pretreatment. For conditioning of cartridges to be used for extraction 0.5 ml methanol was used along with 0.5 ml water for equilibration of system. 0.5 ml pretreated sample was loaded onto the equilibrated cartridges. Rinsing of cartridges was done by 0.5 ml water. For drying of the sample thus eluted into the RIA vial, nitrogen purging for 1-2 min was carried out. The dried and eluted sample (100 ng/ml) was then reconstituted using 0.5 ml mobile phase. This procedure for SPE was tried using 2 brands of cartridges namely Oasis HLB SPE cartridges (Waters), Orochem SPE cartridges. However, best recovery of about 80% was obtained by using Orochem SPE cartridges, but the procedure was much tedious.

Finally liquid liquid extraction (LLE) technique was tried. Various solvents and their combinations tried for this technique included chloroform, n-pentane, n-hexane, MTBE (methyl tert-butyl ether), ethyl acetate, phosphate buffer and ammonium formate buffer. In this technique interference due to plasma matrix was less. The procedure followed for LLE method was 100 µl of 2000 µg/ml drug and internal standard was taken in a 12 ml RIA vial. To it 1 ml plasma, 1 ml ammonium formate buffer (pH=4), 500 µl n-hexane was added. Then the vial was kept for vortexing for 15 min and then kept in a centrifuge for 15 min at 5000 rpm. The upper layer obtained after centrifugation was let to evaporate and residue was dissolved in methanol and then sample (100 ng/ml) was ready for injection into the developed HPLC system. Best recovery of above 99% was obtained with this method.

The method was easy, fast and didn't require any special equipment for extraction; also it gave better recovery than SPE technique.

6.4.2.4 Method optimization and development

For sample pre-treatment, LLE method was finalized. LLE was preferred over protein precipitation and SPE, as it gave clean samples with high recovery and was rapid. The extracted samples of plasma were retrieved from prelabeled sample tubes stored in deep freezer at -20 °C and then subjected to LLE.
Mobile phase trial was taken on unextracted samples using method discussed in section 3.4.2.2. The method optimized for CPH in section 3.4.2.2 was also compatible for OXZ analysis and thus was continued for bioanalytical studies of CPH. The details about the scheme for mobile phase optimization is given in Table 6.1

Table 6.1: Mobile phase optimization trials

Mobile phase	**Ratio (%v/v/v)**	**Column**	**Flow rate (ml/ min)**	**Cyproheptadine HCl**		**Oxcarbazepine**	
				Rt (min)	Peak shape	Rt (min)	Peak shape
20 mM formate buffer: methanol: acetonitrile, pH-5.5, adjusted with 0.2%formic acid), 0.1%TEA	50:10:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.00	6.80	Sharp and symmetric peak	4.70	Sharp and symmetric peak

This optimized analytical method was applied for analysis of samples extracted from biological matrix using optimised LLE method.
In the HPLC method optimized on unextracted samples, satisfactory detection was obtained in extracted samples and gave two sharp, well-resolved peaks with minimum tailing factor for CPH and OXZ in human plasma as shown in Figure 6.3. The retention times for CPH and OXZ were 6.80 min and 4.70 min, respectively. The calibration curve for CPH was found to be linear over the range of 100-800 ng/ml. Figure 6.4 represents the overlay chromatogram and figure 6.5 represents the calibration curve for CPH. The calibration range obtained was quite wide and showed that samples can be analysed at low concentration. Also the range of calibration curve was selected such that the peak plasma concentration can be measured and thus can be applied for estimation of the pharmacokinetic parameters from the study. The data of regression analysis of the calibration curves is shown in Table 6.2.

Figure 6.3: Chromatogram of analyte Cyproheptadine HCl and internal standard Oxcarbazepine spiked in human plasma

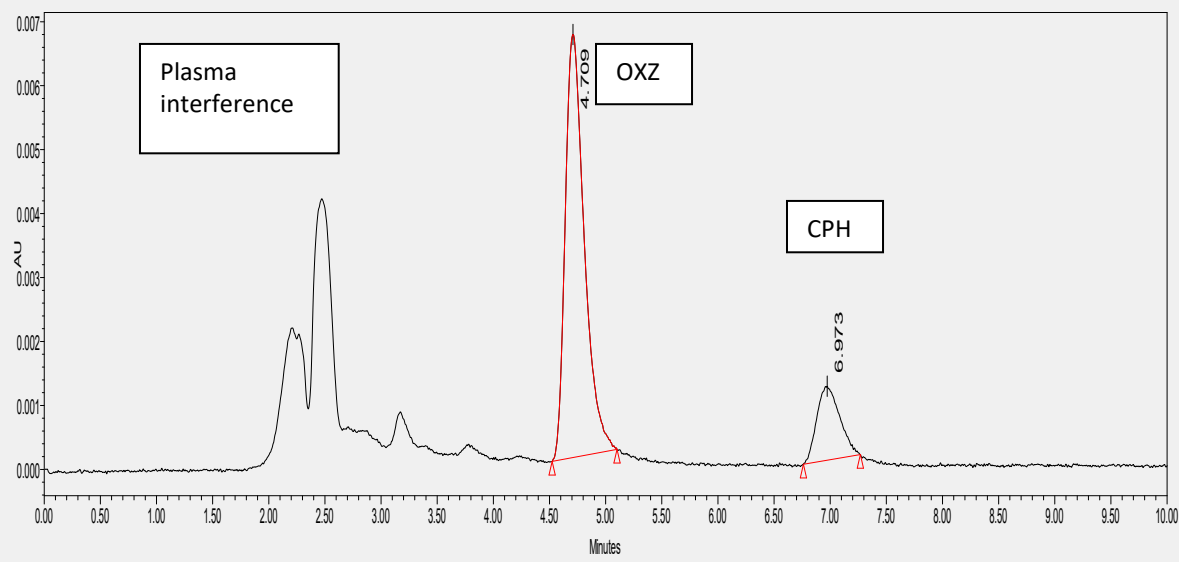


Figure 6.4: Overlain chromatogram of analyte Cyproheptadine HCl (CPH) and Oxcarbazepine (OXZ) used as Internal standard in developed HPLC method (800ng/ml - 100ng/ml is the concentration range of analyte whereas the concentration of Internal standard used is 100 ng/ml).

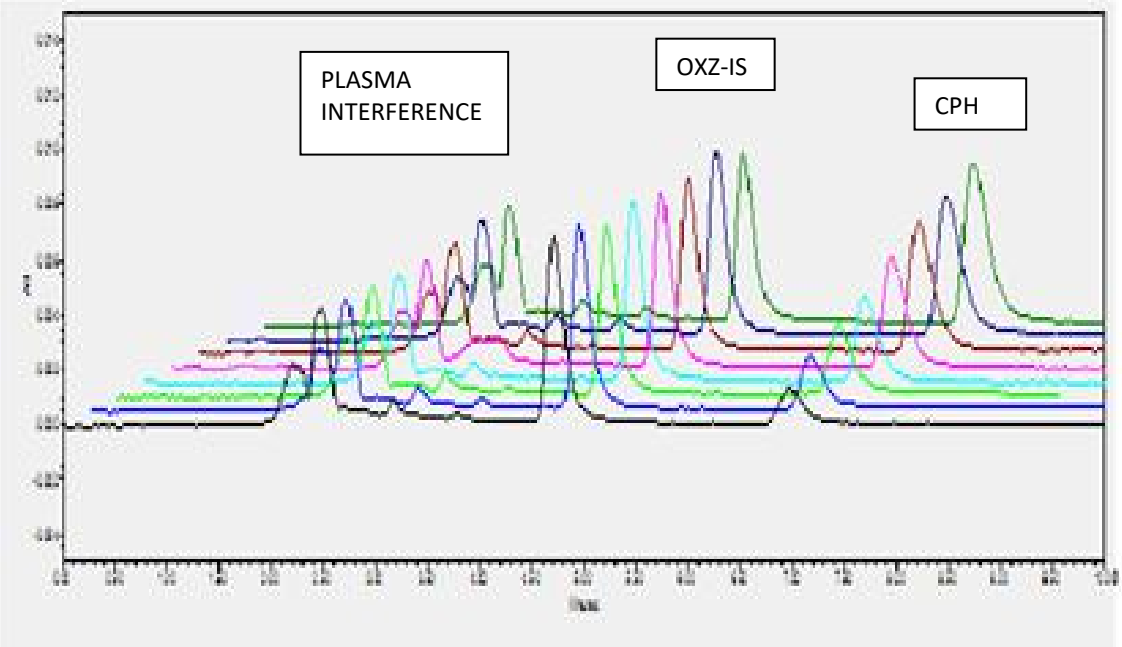
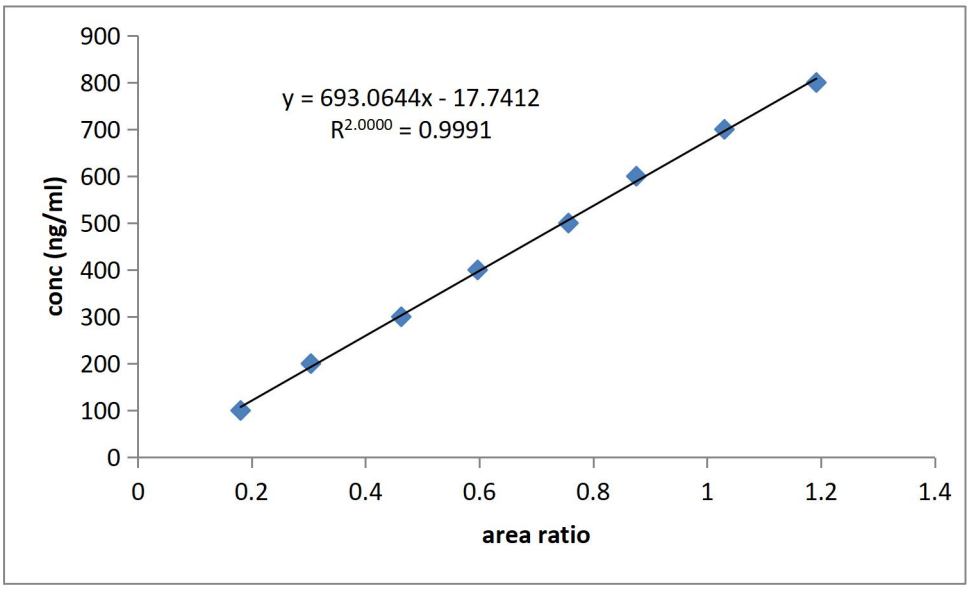


Figure 6.5: Calibration curve for CPH in range 100-800 ng/ml

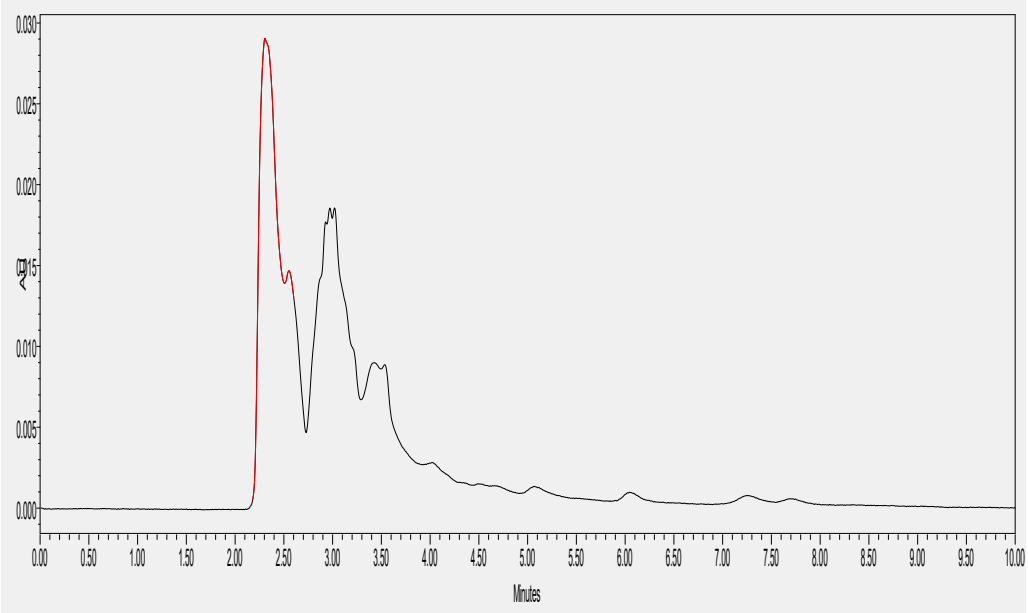


6.4.2.5 Method validation using ICH Q2 (R1) guideline [16]

The optimized method was validated as per the recommendations of ICH [18, 20] and USP [17, 19] for the parameters like accuracy, linearity, precision, detection limit, quantitation limit, robustness and specificity.

Specificity is defined as the lack of interfering peaks at the retention time of the assayed drugs and the internal standard in the chromatograms.
Specificity was done using six batches of plasma and the result was found within the acceptance limits. The peaks were of good shape, completely resolved from plasma components. No significant interferences from the plasma matrix were observed at the retention time of the analyte and IS. The developed method was also found to be specific, since it was able to separate drug in the biological matrix. The chromatogram presented in Figure 6.6 is of blank (unspiked) rat plasma sample extracted using optimized LLE extraction procedure.

Figure 6.6: Chromatogram of blank unspiked rat plasma sample manifests the selectivity of the method which displays the lack of interfering peaks at the retention time of the assayed drugs and the internal standard in the chromatogram



Recovery in plasma was evaluated by comparing the mean peak responses of at least six injection of external spiked low (300 ng/ml), medium (500 ng/ml) and high (800 ng/ml) matrix extracted sample, prepared in plasma to mean peak responses of non-spiked samples prepared in elution solvent. Recovery of analyte were evaluated by injecting three replicates of the aqueous QC samples at each LQC, MQC and HQC concentrations and three replicates of the extracted QC samples at each LQC, MQC and HQC concentrations. Recovery of IS were evaluated by injecting three replicates of the aqueous QC samples at each LQC, MQC and HQC concentrations and six replicates of the extracted QC samples at each LQC, MQC and HQC concentrations. Results are presented in table 6.2 and table 6.3.

Table 6.2 Absolute Recovery of CPH

Replicate Number	HQC		MQC		LQC	
	Extracted peak area	Unextracted peak area	Extracted peak area	Unextracted peak area	Extracted peak area	Unextracted peak area
1	92456	93326	59678	60027	35987	35897
2	92135	93452	60056	60189	36109	36968
3	91909	93313	59678	60067	35089	35906
Mean	92166	93363	59804	60094	35728	36017
SD	274.87	76.77	218.23	114.55	557.02	200.102
%CV	0.29	0.082	0.36	0.19	0.97	0.107
%Mean Recovery	98.71		99.51		99.19	

%Overall Recovery	99.14
%Overall CV	0.44

Table 6.3 Absolute Recovery of IS

Replicate Number	HQC		MQC		LQC	
	Extracted peak area	Unextracted peak area	Extracted peak area	Unextracted peak area	Extracted peak area	Unextracted peak area
1	77568	78456	77582	78145	77856	78256
2	77125	78413	77025	77256	77865	78123
3	76542	77125	76451	76845	76542	77581
Mean	77078.33	77998	77019.33	77415.33	77421	77986.67
SD	514.58	756.34	565.52	664.48	761.25	357.56
%CV	0.67	0.97	0.74	0.86	0.99	0.46
%Mean Recovery	98.82		99.49		99.27	
%Overall Recovery	99.19					
%Overall CV	0.34					

SD: Standard deviation, CV: Coefficient of variation

Precision is expressed as the % coefficient of variation (% CV). Precision study was done for intraday as well as interday variations for verifying the reproducibility of method. (Table 6.4 and Table 6.5 show the intraday as well as interday precision calculations for the optimised method. The values of %CV below 2 conforms method to be valid and reproducible. Figure 6.8 denotes the chromatogram of analyte and IS spiked in human plasma at LQC, MQC and HQC levels.

Table 6.4: Intra Day Precision of CPH

Sr. No.	Run ID	Back Calculated Conc.(ng/ml)			
	Nominal Conc.(ng/ml)	LLOQ/QC 100 ng./ml	LQC 300 ng./ml	MQC 500 ng./ml	HQC 800 ng./ml
1	PA1	100.31	300.20	500.29	800.68
		101.84	298.73	498.37	799.10
		99.32	301.66	501.83	801.20
2	PA2	98.42	300.82	502.11	802.10
		101.45	301.33	500.92	798.48
		100.89	302.91	501.33	800.83
3	PA3	99.72	298.39	500.88	798.30
		100.23	299.68	499.10	799.76
		102.33	300.29	498.92	802.34
	Mean	100.50	300.44	500.41	800.31
	S.D	1.25	1.42	1.33	1.40
	%CV	1.25	0.47	0.26	0.17

Table 6.5 Inter Day Precision of CPH

Sr. No.	Run ID	Back Calculated Conc.(ng/ml)			
	Nominal Conc.(ng/ml)	LLOQ/QC 100 ng./ml	LQC 300 ng./ml	MQC 500 ng./ml	HQC 800 ng./ml
1	**PA1**	101.29	300.18	500.29	798.19
		100.79	299.19	499.71	799.10
		102.37	302.23	498.17	802.33
2	**PA2**	100.87	302.56	502.32	800.81
		100.38	300.28	501.18	798.72
		100.67	301.32	500.91	799.26
3	**PA3**	99.26	300.98	499.56	800.80
		99.22	303.11	498.39	800.37
		99.01	298.30	499.25	801.38
	Mean	100.42	300.90	499.97	800.10
	S.D	1.10	1.58	1.34	1.36
	%CV	1.09	0.52	0.26	0.17

LQC: Low quality control sample
MQC: Medium quality control sample
HQC: High quality control sample
ng/ml- nanogram per millilitres

For LOD and LOQ, calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated.
Robustness was performed by making small deliberate changes in method like change in analytical wavelength, flow rate and change in % of organic phase by 2 unit plus and minus in selected variables of the method. No significant change in system suitability parameters of method was observed signifying method to be robust. Refer Table 6.8 for robustness studies.
Ruggedness was checked by change in operator in which also no significant change was observed in system suitability parameters of method signifying method to be rugged. System suitability was performed before start of every new batch. It was performed by injecting six replicates of aqueous MQC of CPH. The mean standard deviation and % CV for the peak area ratio and for the retention time of analyte and IS were calculated. Table 6.7 denotes the system suitability parameters for analyte.
Acceptance criteria:
1. % CV for peak area ratio should be ≤ 5.0%
2. Asymmetric factor should be < 2 for both analyte and IS.
3. The number of Theoretical Plate Should is > 3000 for both analyte and IS.

The summary of validation parameters are represented in Table 6.6.

Table 6.6: Summary of validation parameters

Parameter (Units)	**CPH**
Linearity range (ng/ml)	100-800
Correlation coefficient	0.9991
Recovery of CPH (%)	99.14
Recovery of IS (%)	99.19
Precision (%RSD)	
Interday (n=3)	0.51
Intraday (n=3)	0.53
Robustness	Robust
Retention Time allowable time (min) for CPH	6.8±0.01
Retention Time allowable time (min) for OXZ	4.7±0.04
LOD (ng/ml)	26.29
LOQ (ng/ml)	79.66

RSD-Relative standard deviation
ng/ml- nanogram per millilitres

Table 6.7 Summary of system suitability parameters

Parameter	**Data obtained***
Retention time of CPH (min) ± SD	6.8 ± 0.01
Retention time of OXZ (min) ± SD	4.7±0.04
Theoretical plate of CPH ± SD	4598± 57.98

Asymmetry factor of CPH ± SD	1.27 ± 0.02
Theoretical plate of OXZ ± SD	6027± 76.39
Asymmetry factor of OXZ ± SD	1.19 ± 0.01
Resolution ± SD	5.46 ± 0.07

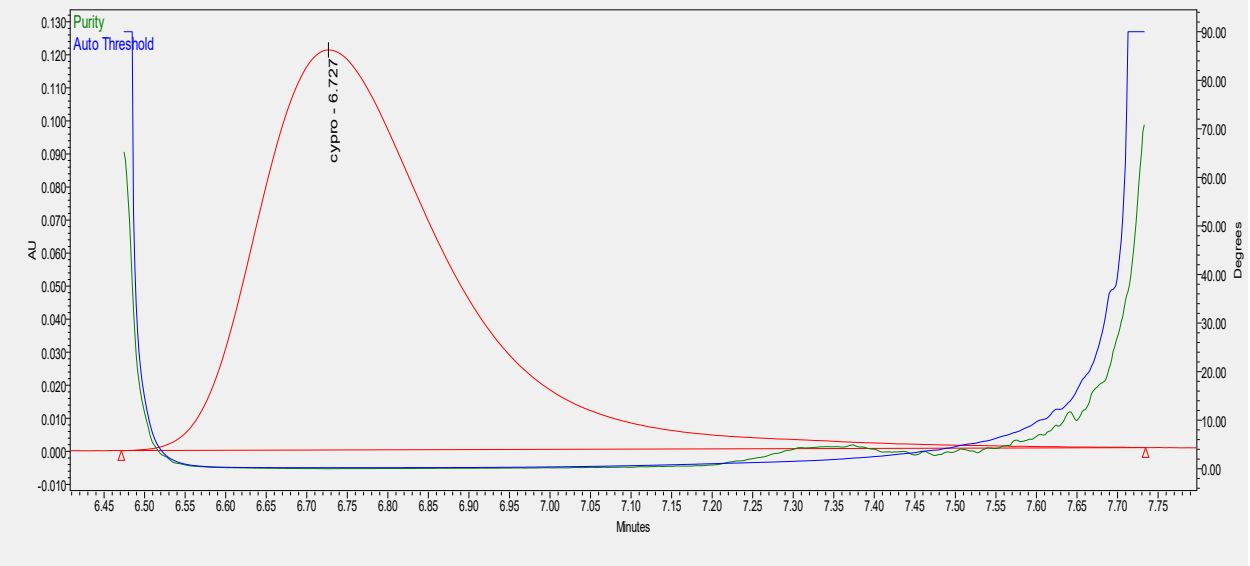
*Data obtained of six replicates

6.4.2.6 Peak purity studies

Peak purity studies were undertaken for confirming the presence of only one analyte at the retention time of peaks obtained by HPLC-PDA method. The extracted peak purity plots of standard drug along with internal standard used for study are presented in Figure 6.7 and further the values of purity threshold higher than the values of purity angles for each peak signify the peaks to be pure. (Table 6.8)

Figure 6.7 Peak purity studies

a) Peak purity of CPH



b) Peak purity plot of OXZ

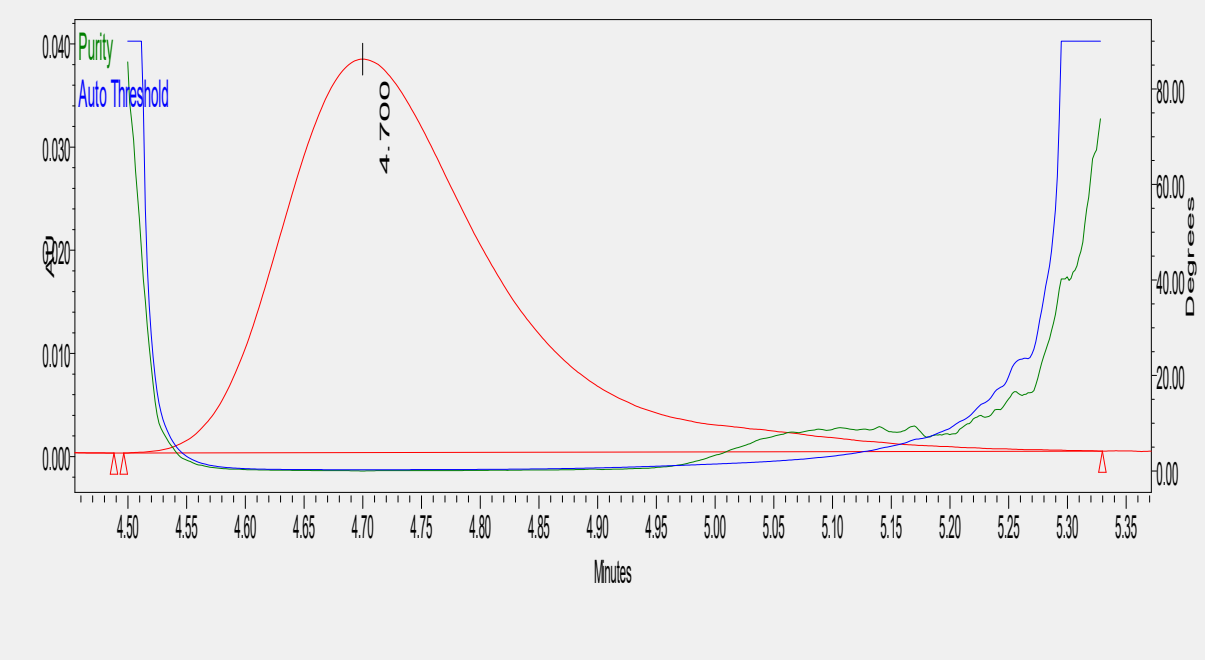


Table 6.8 Peak purity studies

Sr. No	Peak	Peak RT	Peak purity angle	Purity threshold
1	CPH	6.7	0.122	0.261
2	OXZ	4.7	0.403	0.821

6.4.2.7 Stability studies

The stability studies were undertaken as follows:
Refrigerated stock solution stability was performed by analysing three replicates of aqueous solution prepared from freshly weighed stock solution (freshly prepared) against three replicates of aqueous solution prepared from aliquots of analyte and IS stored at 2-8°C after 5 days as stability samples. The % change (Bias) in area response of analyte and I.S. for 5 days of freshly prepared and stability samples at 2 - 8 ºC was measured. The results are within ± 10%, presented in table 6.90.

Table 6.9 Refrigerated stock solution stability of CPH and OXZ at 2-8ºC after 5 days

Long term stock solution stability (5 days)				
Replicate no	**Drug stock(1000 µg/ml)**		**IS stock(1000 µg/ml)**	
	Drug nominal conc.(100 ng/ml)		**IS nominal conc.(100 ng/ml)**	
	Area		**Area**	
	Comparison samples	**Stability samples**	**Comparison samples**	**Stability samples**
1	15534	15478	78166	78156
2	15490	15439	78564	78321
3	15467	15399	78256	77999

Mean	15497	15438	78328.66	78158.66
SD	31.11	39.50	208.71	161.02
%CV	0.20	0.25	0.27	0.21
% Mean stability	99.62		99.78	

Conclusion: % change is within ± 10 % for fresh samples, while compared with stability samples, hence it is concluded that CPH and IS stock solutions were stable for 5 days at 2-8℃.

Room temperature stock solution stability was performed by analysing three replicates of aqueous solutions prepared from freshly weighed stock solution against three replicates of aqueous solution prepared from aliquots of analyte and IS stored at room temperature for eight hrs (stability samples). The stability of CPH and IS in the stock solution were determined at 2-8°C after 5 days. The results are within ± 10%, presented in table 6.10.

Table 6.10 Room Temperature Stock Solution Stability of CPH and OXZ for 8 hours

Room temperature stock solution stability (8 hours)				
Replicate no	**Drug stock(1000 µg/ml)**		**IS stock(1000 µg/ml)**	
	Drug nominal conc.(100 ng/ml)		**IS nominal conc. (100 ng/ml)**	
	Area		**Area**	
	Comparison samples	**Stability samples**	**Comparison samples**	**Stability samples**
1	15459	15367	78456	77586
2	15589	15410	77568	77125
3	15528	15394	78456	78125
Mean	15525.33	15390.33	78160	77612
SD	65.04	21.73	512.68	500.51
%CV	0.41	0.14	0.65	0.64
% Mean stability	99.13		99.29	

Conclusion: % change is within ± 10 % for fresh samples, while compared with stability
samples, hence it is concluded that CPH and IS stock solutions were stable for 8 hrs at room temperature.

The freeze thaw stability of analyte was determined after three FT cycles. The three sets of LQC and HQC samples were stored at -70±5 and subjected to three freeze thaw cycles at interval of 24 hrs. After the completion of three cycles of 12 to 24 hrs the samples were analysed. Stability of samples was compared against freshly prepared samples. The stability of CPH and IS in the stock solution were determined at room temperature for 8 hrs. The results are within 15 % as presented in table 6.11.

Table 6.11 Freeze-Thaw Stability of CPH after Three Freeze Thaw Cycles

Freeze-Thaw Stability					
Nominal conc. (LQC) 300ng/ml			Nominal conc. (HQC) 800 ng/ml		
Sample ID	Calculated conc. (ng/ml)	% Accuracy	Sample ID	Calculated conc. (ng/ml)	% Accuracy
FT1HQC	297.43	99.14	FT1LQC	798.30	99.78
FT2HQC	301.34	100.45	FT2LQC	800.83	100.10
FT3HQC	298.22	99.41	FT3LQC	801.39	100.17

Conclusion: % difference is within ± 15% for both QC levels, hence it is concluded that CPH plasma samples are stable after three freeze thaw cycles.

The bench top stability of CPH at room temperature was examined by keeping three sets of LQC and HQC at room temperature for 12 hours. After 12 hrs, prepared fresh samples of LQC and HQC concentrations of CPH in three replicates. These samples were referred as fresh or comparison samples. % Mean change was within the acceptance criteria of ± 15%. (Table 6.12)

Table 6.12 Bench Top Stability

Bench Top Stability					
Nominal conc. (LQC) 300ng/ml			Nominal conc. (HQC) 800 ng/ml		
Sample ID	Calculated conc. (ng/ml)	% Accuracy	Sample ID	Calculated conc. (ng/ml)	% Accuracy
FT1HQC	301.78	99.14	FT1LQC	802.29	99.79
FT2HQC	302.82	100.45	FT2LQC	801.42	100.10
FT3HQC	299.20	99.41	FT3LQC	801.19	100.17

Conclusion: % difference is within ± 15% for both QC levels, hence it is concluded that CPH plasma samples are stable for 12 hrs on bench top at room temperature.

Carry over effect:
Carry-over should be addressed and minimized during method development. During validation, carry-over was assessed by injecting blank samples after a high concentration sample. Carry over in the blank sample following the high concentration standard was not greater than 20% of the lower limit of quantification (LLOQ) and 5% for the internal standard**.**

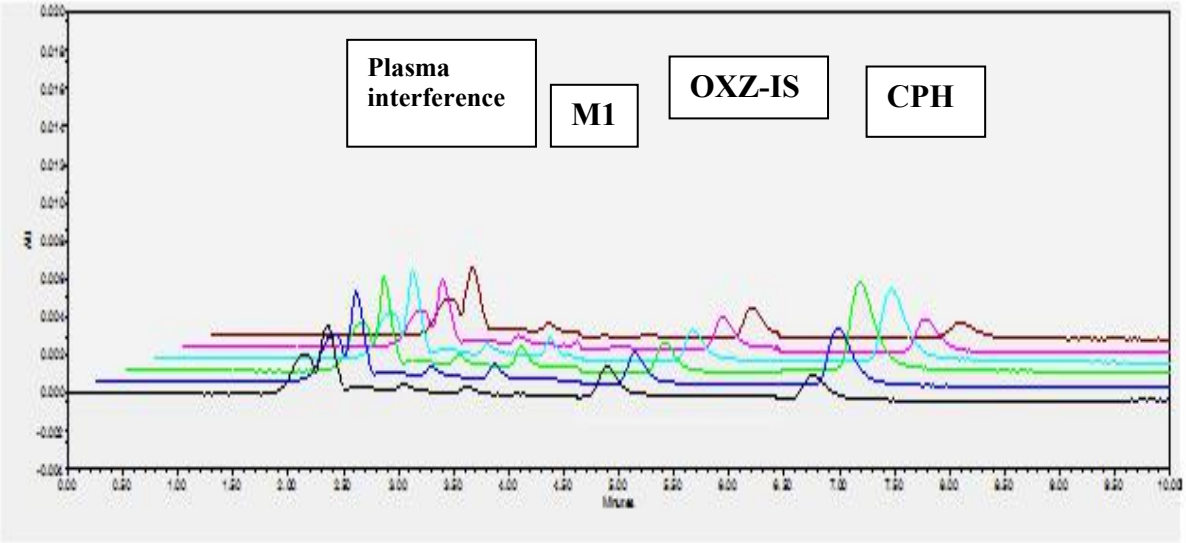
6.4.2.8 Pharmacokinetics study

The pharmacokinetic study was carried out in Male Wistar Albino rats. The six healthy animals were selected for the study. The animals were fasted overnight (~14 h) and had free access to water throughout the experimental period. CPH was administered by oral gavage at a dose of 45 mg/kg, as 2% CMC (Carboxy methyl cellulose) suspension of drug in double distilled water. Blood samples (0.5 ml) were collected from the retro orbital plexus sinus at designated time points (1, 2, 4, 6, 8 and 10 h) into micro centrifuge tubes containing 100 µl of heparin. Plasma was harvested by centrifuging the blood using cold centrifuge at 3000 rpm for 10 min. Plasma (1000 µl) samples were spiked with 100 µl 2000 µg/ml IS and processed same as standards

as described above. The experimental procedure was approved by Institutional animal ethics committee (IAEC), Pharmacy department under protocol number (MSU/IAEC/2017-18/1723) on 10 Nov 2017. The pharmacokinetic parameters were calculated with a non-compartmental model using Thermo kinetica PK/PD analysis software (version 5.0 Thermo Fisher Scientific). Figure 6.10 represents Mean plasma concentration Vs time profile of CPH after oral administration of drug to rat. The peak plasma concentration (*C*max) and the corresponding time (*T*max) were directly obtained from the raw data. The other pharmacokinetic parameters were obtained using non compartment model. AUCtotal was calculated using mixed log linear model. The pharmacokinetic data is represented in Table 6.14. The chromatogram depicted in Figure 6.8 shows two small peaks, well separated from the drug peak, which as per literature [21] at RT of 3.6 min shows a pattern similar to drug peaks, and thus can be considered as metabolite peak 1, (M1) and other small peak at RT of 3.0 min doesn't show any pattern thus cannot be inferred to be a metabolite peak but also doesn't interfere with the drug peak, thus doesn't interfere in bioanalytical chromatographic analysis. It also depicts the overlay chromatograms in which the changes in drug concentrations at various designated time points (1, 2, 4, 6, 8 and 10 h) can be seen from the changes in peak area. As per the literature study, the M1 metabolite is assumed to be quaternary ammonium glucuronide like conjugate of Cyproheptadine. [21]

The assay developed is specific, accurate, precise and reproducible for the analysis of CPH in rat plasma. The use of the method can easily enable the characterization of CPH pharmacokinetics after single oral dose. According to pharmacology and toxicology review by CDER, based on plasma profiles, overall pattern of metabolism in humans most closely approximated the metabolite pattern seen in rats. So, the assay can be easily extended to quantitate CPH in plasma for routine monitoring of levels of CPH in laboratories.

Figure 6.8: Overlain chromatogram for pharmacokinetic study at different time intervals

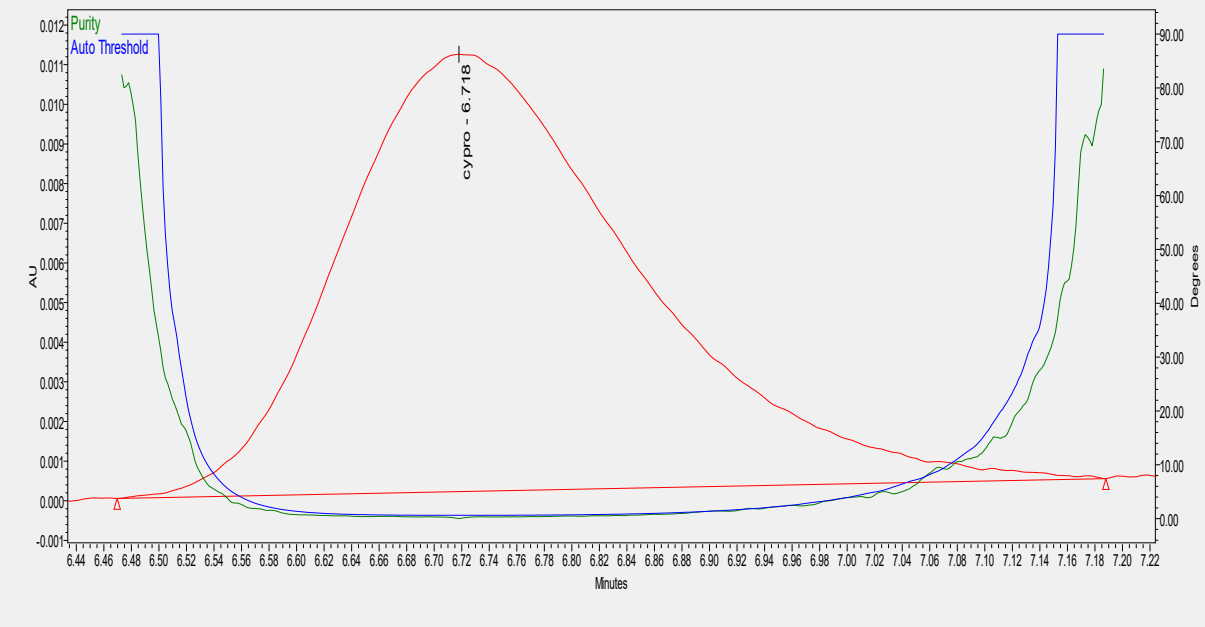


6.4.2.9 Peak purity studies after pharmacokinetic studies

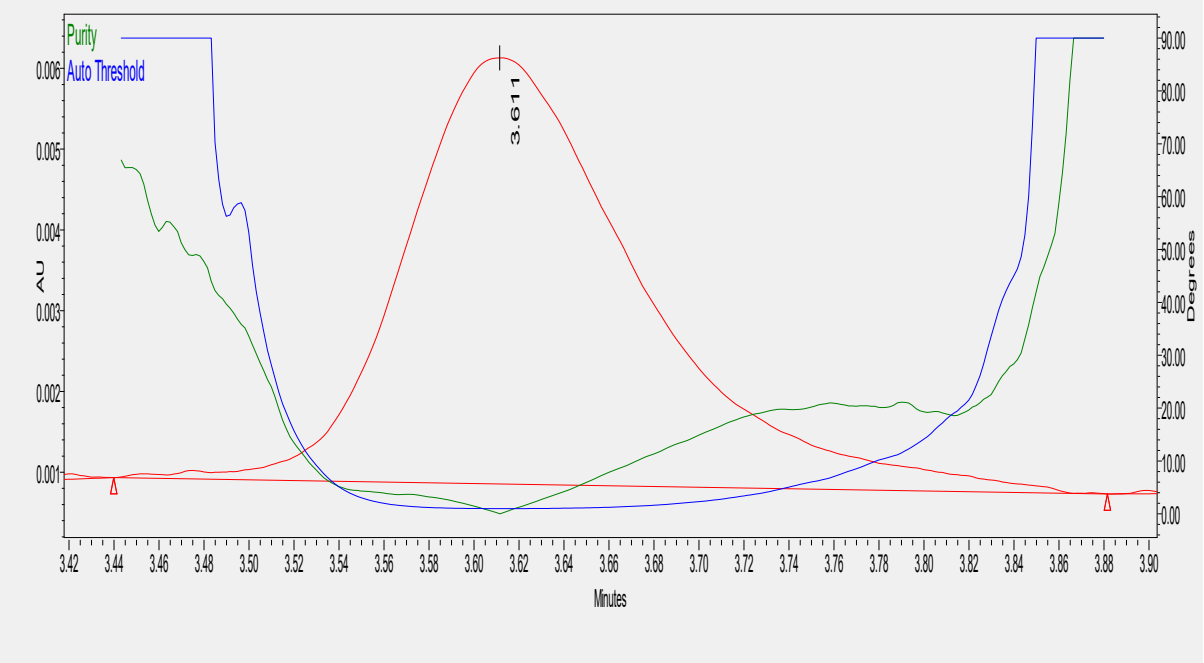
Peak purity studies were undertaken for confirming the presence of only one analyte at the retention time of peaks obtained by HPLC-PDA method. The extracted peak purity plots of both standard drugs are presented in Figure 6.9 and further the values of purity threshold higher than the values of purity angles for each peak signify the peaks to be pure. (Table 6.13)

Figure 6.9 Peak purity studies

a) Peak purity plot of CPH



b) Peak purity plot of M1



c) Peak purity plot of IS (OXZ)

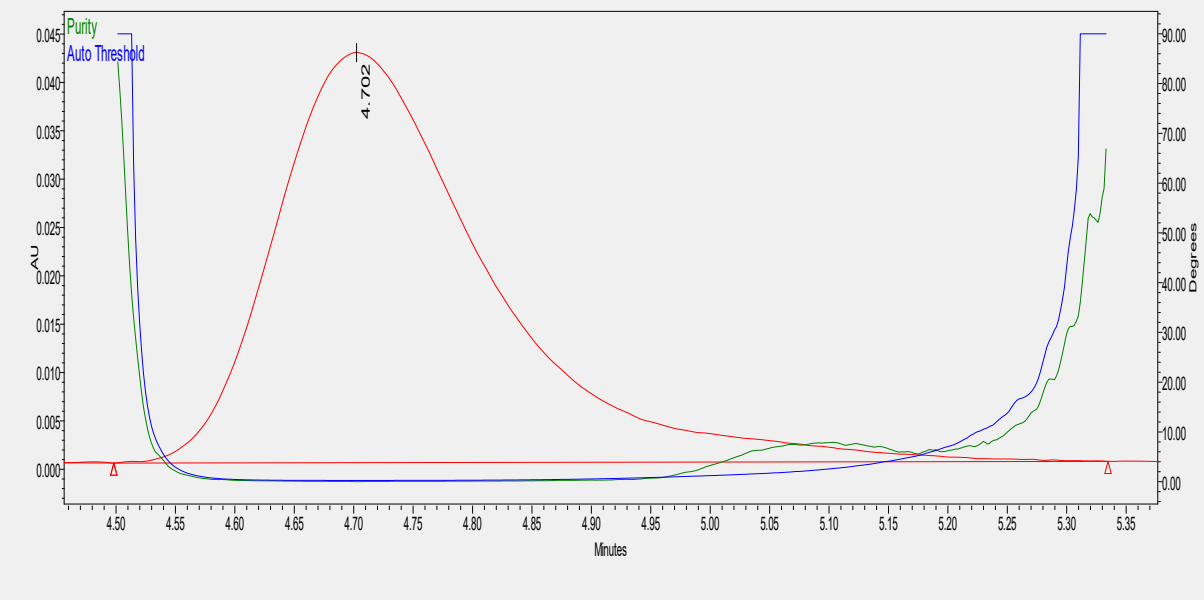


Table 6.13 Peak purity studies

Sr. No	Peak	Peak RT	Peak purity angle	Purity threshold
1	CPH	6.7	0.709	0.880
2	M1	3.7	0.306	0.309
3	IS (OXZ)	4.7	0.237	0.259

Figure 6.10: Mean plasma concentration Vs time profile of CPH after oral administration of drug to rat showing the application of analytical developed method for analysing the pharmacokinetics parameters in rat. The graph shows Tmax of 4 hours and Cmax of 583.83 ng/ml.

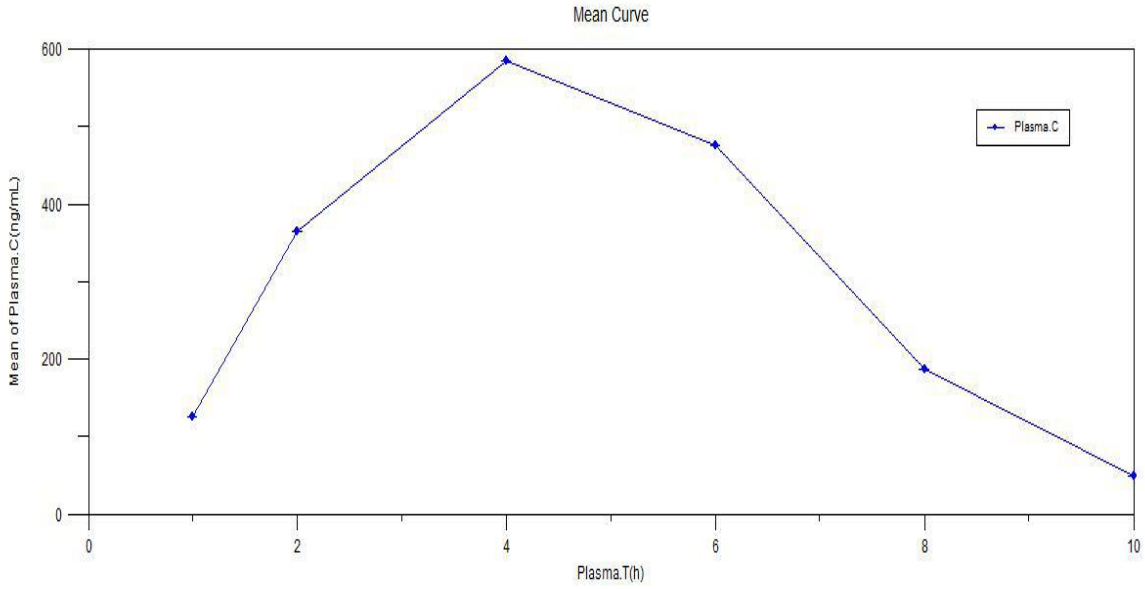


Table 6.14: Pharmacokinetic parameter for bioanalytical method

Parameters	Observed Value	Reported Value	Unit
Cmax	583.82	Dose dependent	ng/ml
Tmax	4	4	Hr
AUC tot	3.22	Dose dependent	ng/l. h
T half	1.2	Dose dependent	Hr
MRT	4.91	Dose dependent	Hr
AUMC	1.47	Dose dependent	$ng/l.h^2$

Cmax-Peak Plasma concentration
Tmax-Time required to reach Cmax
AUC tot-Total Area under curve
T half-Half life
ng/ml- nanogram per milliliters
Hr-Hour
µg/ml. h-microgram per milliliters hour

6.5 CONCLUSION

Bioanalytical HPLC method was developed for analysis of Cyproheptadine HCl. For extraction of drug from biological samples, LLE method was utilized. The analysis inferred Tmax to be 4 h which helps in selection of dosage regimen for the said drug. It also illustrates that a metabolite (M1) is formed on introduction of Cyproheptadine HCl to rats. The developed method is easy, fast and sensitive and thus can be used for routine bioanalytical analysis of drug.

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