New Bioanalytical HPLC Method for the Determination of Cyproheptadine Hydrochloride in Human Plasma and its Application to Rat Pharmacokinetic Study

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ABSTRACT
Background: Cyproheptadine HCl is a serotonin antagonist and histamine H1 blocker agent. Objective: The objective of the present work was to establish a rapid, sensitive and validated bioanalytical HPLC method using Liquid extraction technique for the determination of Cyproheptadine Hydrochloride in human plasma and its application to rat pharmacokinetic study. Methodology: For development of analytical method, Cyproheptadine HCl was estimated in human plasma after liquid liquid extraction using 20 mm ammonium formate buffer and n-hexane as extracting solvents and Oxcarbazepine as internal standard. For separation of Cyproheptadine HCl from plasma components, Hypersil BDS C-18 column (250×4.6 mm i.d, 5µ particle size) at ambient temperature, 224 nm as detection wavelength and (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 at a flow rate of 1 ml/min was used. Results: Newly developed method showed good calibration curve in the concentration range of 100–800 ng/ml with excellent correlation coefficient (r²>0.998) and giving recovery more than 99%. The %RSD for both intraday and interday was less than 2%. Conclusion: The newly developed and validated HPLC-PDA method was easy, fast and can be effectively utilized for pharmacokinetic studies in rats after oral administration of Cyproheptadine HCl.

Key words: Cyproheptadine HCl, Oxcarbazepine, Bioanalytical method, LLE, HPLC.

INTRODUCTION
Bioanalytical method is employed for the quantitative estimation of drugs and their metabolites in biological media and plays an important role in estimation and interpretation of bioequivalence, pharmacokinetic and toxic kinetic studies.1 Cyproheptadine hydrochloride is a serotonin antagonist and histamine H1 blocker used as antipruritic, appetite stimulant, antiallergic, the post-gastrectomy dumping syndrome, cushing syndrome, Nelson’s syndrome, Parkinsonism etc.2,3 Its IUPAC name is 4-((Hdibenzo [a, d] - cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride. Its molecular formula is C₂₁H₂₁N. HCl and molecular weight is 323.86 g/mol. Development of quantitative analytical method for the estimation of Cyproheptadine hydrochloride in plasma is very important for studying its pharmacokinetic effects and drug-drug interactions. Different analytical methods have been described for the quantification of Cyproheptadine hydrochloride from formulations and biological samples, including Colorimetric methods,5,6 Ion pair complexometric UV spectroscopic method,4 HPLC,7 GC-MS6 and LC/MS/MS8 methods. Novak et al, have reported a HPLC method for quantification of CPH in serum or plasma.9 Another quantification procedure for CPH in plasma and urine by HPLC has been reported by Foda et al.10 RP-HPLC
method for the determination of CPH in urine was developed by Kountourellis and Ebete.\textsuperscript{12} Another GLC method for determination of cyproheptadine in urine and plasma using nitrogen sensitive detector has been reported by Hucker and Hutt.\textsuperscript{13} Despite the fact that this much literature is available for the said drug substance, till date no bioanalytical method employing simple LLE procedure as extraction method along with its pharmacokinetic studies in rat plasma have been reported for the drug substance Cyproheptadine HCl. As previously said bioanalytical method development plays a very important role for studying the pharmacokinetic as well as toxicokinetic studies it was decided to carry out the research regarding development of simple LLE procedure for extraction of drug from rat plasma, development of sensitive HPLC-PDA analytical method for its estimation and its application for studying pharmacokinetics in rat plasma.

**MATERIALS AND METHODS**

**Reagents and Chemicals**

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\textsuperscript{®} N66\textsuperscript{®} from Pall Life Sciences, USA; prior to use. Drug free EDTA human plasma was procured from Surat Blood Bank, Vadodara.

**Apparatus and Analytical Conditions**

UV Spectroscopy analysis for deriving the suitable wavelength for estimation of drugs were identified 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) and consisting of following components 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. The chromatographic analysis was performed using Empower 3 software on a Hypersil BDS C\textsubscript{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. 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\textsuperscript{®}, PALL life sciences (40 mm) and degassed by ultrasonic vibrations prior to use. 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. 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.

**Sample Pretreatment\textsuperscript{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. 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 min. After that optimized amount of precipitating solvent methanol (900μl) was added and then it was subjected to the vortexing for 5 min. Then the solutions were centrifuged at 4000 RPM for 10 mins. 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 ppm 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.

**Preparation of Standard solutions**

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 ppm). From the above stock solution I, 1 ml was diluted up to 10 ml to get 40 ppm 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 with the same for OXZ stock solution I (400 ppm). From the above stock solution I, 0.5 ml was diluted upto 100 ml to get 2 ppm stock solution II. For preparation of calibration standards, appropriate aliquots (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 ml) of CPH stock solution II were taken in 6 different volumetric flasks and diluted up to the mark 2 ml with acetonitrile to obtain final concentrations of 2-16 ppm. 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) of calibration curve 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 300 (LQC), 500 (MQC), 800(HQC) ng/ml.

**Bioanalytical Studies**

Mobile phase trials were taken on unextracted samples. Various mobile phases like water: methanol (50:50), (20:80), water: acetonitrile (30:70), water: acetonitrile pH-3 (60:40), phosphate buffer: acetonitrile ph-3(70:30), 10 mM formate buffer: acetonitrile pH-3(70:30), 10 mM formate buffer: methanol: acetonitrile (60:15:25) and 20 mM formate buffer: methanol: acetonitrile (50:10:40, pH-5.5 adjusted with 0.2% Formic acid) were tried in which 20 mM formate buffer: methanol: acetonitrile (50:10:40, pH-5.5 adjusted with 0.2% Formic acid) gave the best peak. Also, 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 dacrabazepine and oxcarbazepine were undertaken with conditions described earlier in which oxcarbazepine gave good peak. As described above under sample pretreatment, various trials were undertaken for appropriate sample pretreatment procedure from which the optimized liquid liquid extraction method parameters were used for the study as described above under sample pretreatment.
Method Validation

The optimized method was validated as per the recommendations of ICH\textsuperscript{17,18} and USP\textsuperscript{19,20} for the parameters like accuracy, linearity, precision, detection limit, quantitation limit and robustness. 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. Specificity and selectivity was carried out using six plasma samples. Blank (without IS) and zero sample (with IS) were analyzed for specificity and selectivity study respectively. The linearity of the method was determined over calibration range of 100 ng/ml to 800 ng/ml (Table 3). The calibration standards were prepared by spiking known concentration of CPH working standard solution. A linearity curve containing eight non-zero concentrations was analyzed. (Figure 1) The concentration was calculated at each level and a graph of concentration versus area was plotted. The slope, y-intercept and correlation coefficient curve were calculated by linear regression analysis as stated in (Table 1). Accuracy was measured on the sample spiked with known amounts of the analyte. Accuracy study was carried out on marketed formulation by appropriate dilution and preparing 100 ng/ml concentration of it. (Quality control sample) Accuracy was determined by replicate analysis of six determinations of low (80%), medium (100%) and high (120%) quality control sample. To 100 ng/ml quality control sample, 80% spiking was done of pure API that is CPH and thus 80 ng/ml of API was spiked, similarly it was done for 100% and 120%. Recovery of spiked API was then checked.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conc (ng/ml)</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
<th>Std 6</th>
<th>Std 7</th>
<th>Std 8</th>
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<td>400</td>
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<td>370</td>
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<td>C2</td>
<td>110</td>
<td>198</td>
<td>260</td>
<td>372</td>
<td>505</td>
<td>593</td>
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<tr>
<td>C3</td>
<td>108</td>
<td>199</td>
<td>278</td>
<td>406</td>
<td>506</td>
<td>593</td>
<td>593</td>
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<td>593</td>
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<tr>
<td>Mean</td>
<td>104.333333</td>
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<td>382.666667</td>
<td>504.666667</td>
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<td>673.333333</td>
<td>810.333333</td>
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</tr>
<tr>
<td>%RSD</td>
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<td>1.60000000</td>
<td>1.14583333</td>
<td>0.98717948</td>
<td>0.85333333</td>
<td>1.13793103</td>
<td>1.64940028</td>
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<td>1.64940028</td>
</tr>
<tr>
<td>%Mean/acc</td>
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<td>1.00000000</td>
<td>1.00000000</td>
<td>0.99999999</td>
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<td>1.00000000</td>
<td>1.00000000</td>
<td>1.00000000</td>
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</tr>
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</table>

Figure 1: Overlaid 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). In the chromatogram the peak at retention time of 4.7 min denotes internal standard and peak at retention time of 6.8 min denotes analyte.
Results of the study are represented in Table 5. 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, 7). Bench top stability was performed at LQC and HQC level. Three replicates of LQC and HQC were withdrawn from deep freezer and were kept at room temperature for 12 hr. These samples were preferred as stability samples. After 12 hr, prepared fresh samples of LQC and HQC concentrations of CPH in three replicates. These samples were referred as fresh or comparison samples. The freeze and thaw stability of analyte was determined after three FT cycles. The three sets of LQC and HQC samples were stored at -70±5°C and subjected to three freeze thaw cycles at interval of 24 hr. After the completion of three cycles of 12 to 24 hr the samples were analyzed. 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. Room temperature stock solution stability was performed by analyzing 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. Refrigerated stock solution stability was performed by analyzing three replicates of aqueous solution prepared from freshly weighed stock solution (Freshly prepared) against three replicates of aqueous solution prepared from aliquots of aqueous solution stored at 2-8°C after 5 days as stability samples (Table 2).

**Pharmacokinetic Studies- Experimental Procedure**

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 compufuge at 3000 rpm for 10 min. Plasma (1000 μl) samples were spiked with 100 μl 2000 ppm 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 kinetic PK/PD analysis software (version 5.0 Thermo Fisher Scientific). The peak plasma concentration (Cmax) and the corresponding time (Tmax) were directly obtained from the raw data. The other pharmacokinetic parameters were obtained using non-compartment model. AUC total was calculated using mixed log linear model. The pharmacokinetic data is represented in Table 3.

**RESULTS AND DISCUSSION**

For sample pretreatment 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. The extracted samples were subjected to high performance liquid chromatography. In the HPLC method optimized on extracted samples, mobile phase consisted of ammonium formate buffer: methanol: acetonitrile (50:10:40) pH 5.5 adjusted with 0.2% Formic acid, at 1ml/min flow rate which gave two sharp, well-resolved peaks with minimum tailing factor for CPH and OXZ in human plasma as shown in Figure 2. The retention times for CPH and OXZ were 6.8 min and 4.7 min, respectively. 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. The calibration curve for CPH was found to be linear over the range of 100-800 ng/ml (Figure 1). The calibration range obtained was quite wide and showed that samples can be analyzed 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 4. The proposed method was successfully applied to the determination of CPH in biological matrix. 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 3 is of blank (Unspiked) rat plasma sample extracted using LLE extraction procedure been optimized. The chromatogram depicted in Figure 4 shows two small peaks, well separated from the drug peak, which as per literature 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.

Table 4: Summary of Validation and SST Parameters.

<table>
<thead>
<tr>
<th>Parameter (Units)</th>
<th>CPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng/ml)</td>
<td>100-800</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9991±0.002754</td>
</tr>
<tr>
<td>Recovery of CPH (%)</td>
<td>99.14</td>
</tr>
<tr>
<td>Recovery of IS (%)</td>
<td>99.19</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td></td>
</tr>
<tr>
<td>Interday (n=3)</td>
<td>0.51</td>
</tr>
<tr>
<td>Intraday (n=3)</td>
<td>0.53</td>
</tr>
<tr>
<td>Robustness</td>
<td>Robust</td>
</tr>
<tr>
<td>Retention Time allowable time (min) for CPH</td>
<td>6.8±0.00744</td>
</tr>
<tr>
<td>Retention Time allowable time (min) for OXZ</td>
<td>4.7±0.00920</td>
</tr>
<tr>
<td>LOD (ng/ml)</td>
<td>26.29</td>
</tr>
<tr>
<td>LOQ (ng/ml)</td>
<td>79.66</td>
</tr>
</tbody>
</table>

SST- System suitability test parameters; RSD- Relative standard deviation; ng/ml- nanogram per milliliters.
ture 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. As per the literature study, the M1 metabolite is assumed to be quaternary ammonium glucuronide like conjugate of Cyproheptadine. Figure 5 depicts the overlay chromatograms in which the changes in drug concentrations at various designated time points (1, 2, 4, 6, 8 and 10 hr) can be seen from the changes in peak area. The assay developed is specific, accurate, precise and reproducible for the analysis of CPH in rat plasma. The accuracy data is presented in Table 5 and precision data is presented in Table 6, 7. 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.

CONCLUSION

A simple, precise and rapid bioanalytical method was developed for estimation of Cyproheptadine HCl in human plasma along with application to rat pharmacokinetic studies. The procedure selected for extraction of drug from biological matrix was liquid liquid extraction. The procedure thus optimized was easy and rapid for routine bioanalysis of Cyproheptadine HCl in drug testing laboratories and firms. On application of the developed bioanalytical method in rats, we were able to calculate various pharmacokinetic parameters and also got to know about a Metabolite (M1) being formed from Cyproheptadine HCl in rat plasma.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

CPH: Cyproheptadine HCl; OXZ: Oxcarbazepine; SPE: Solid phase extraction; CDER: Center for drug evaluation and research; H: hours; Min: minutes; M1: Metabolite 1; RT: Retention time; LLE: Liquid liquid extraction; Nm: nanometer; Cmax: Peak plasma concentration; Tmax: Time required to reach Cmax; AUC total: Total area under curve; LQC: Low quality control concentration; HQC: High quality control concentration; MQC: Medium quality control concentration; ppm: Parts per million; RIA vial: Radioimmunoassay vial; °C: Degree Celsius.

REFERENCES

Bioanalytical HPLC method was developed for analysis of Cyproheptadine HCl. For extraction of drug from biological samples, LLE method was utilized. The analysis inferred T<sub>max</sub> to be 4 hr 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.