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Sensitive LC-MS/MS assay method for the estimation of desloratadine in human plasma: Application to a bioequivalence study in Indian populations

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ABSTRACT

Purpose: Sensitive and selective analytical method is required for the estimation of desloratadine in human plasma. After absorption in in vivo system, desloratadine reach maximum plasma concentration (C_{max}) with low value and have high intra-subject variability. Hence, to estimate the accurate plasma concentration in clinical samples and characterization of pharmacokinetic profile, it was essential to develop a sensitive method with low limit of quantification for 5 mg formulation.
Methods: A specific, sensitive and reproducible high performance liquid chromatography-tandem mass spectrometric method was developed and validated for determination of desloratadine in human plasma, using desloratadine-d_5 as an internal standard. Desloratadine and desloratadine-d_5 were extracted from human plasma using solid phase extraction and compounds are separated on BDS Hypersil C8 (100 mm × 4.6 mm, 5 µm) column with mobile phase consisting of acetonirile and ammonium formate (pH 4.3; 5mM) (60:40,*v/v*). Quantification was achieved by monitoring transitions of *m/z* 311.2→259.2 for desloratadine and 316.2→264.3 for desloratadine-d_5 in multiple reaction monitoring, using turbo ion source in positive polarity.
Results: No matrix effect was observed within the linearity range of 40.1-8005.0 pg/mL ($r > 0.999$). The degree of matrix effect (ion-suppression) for desloratadine was determined as 0.5% and it had no impact on incurred samples analysis with run time of 6.50 min. The intra-and inter-day precision values were within 6.5% and 4.3% respectively, for desloratadine at the lower limit of quantification level.
Conclusions: Stability and incurred sample reanalyzis data indicated that desloratadine are stable under various handling conditions. The method was successfully applied for the bioequivalence study of desloratadine after oral administration of 5 mg tablet in healthy Indian volunteer and analytical phase was conduted without any failures of analytical run/batch and its support the ruggedness as well as robustness of the proposed method.

KEYWORDS: Bioequivalence study; Liquid chromatography-mass spectrometry; Solid phase extraction; Desloratadine.

ABBREVIATIONS: HPLC: High Pressure Liquid Chromatography; UPLC: Ultra Performance Liquid Chromatography; ISR: Incurred Sample Reanalysis; LOQ: Limit of Quantitation; LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry; MRM: Multiple Reaction Monitoring; DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Collision cell Exit Potential; SPE: Solid Phase Extraction; CAD: Collision Activated Dissociation; CUR: Curtain Gas; GS1: nebulizer gas; CC: Calibration Standards; QC: Quality Control; LOQQC: Lower Limit of Quantification; LQC: Low Quality Control; MQC: Medium Quality Control.

INTRODUCTION

Desloratadine [IUPAC name: 8-chloro-6,11-dihydro-11-(4-piperdinylidene)-5H-benzo[5,6]cyclohepta[1,2-b]pyridine] the principal metabolite of loratadine, is an orally active, nonsedating, peripheral histamine H1-receptor antagonist indicated for the relief of the symptoms of seasonal allergic rhinitis, skin hives and itching in people with chronic skin reactions, perennial allergic rhinitis and chronic idiopathic urticaria.[1] Studies in mice have shown that desloratadine is approximately four times as potent as loratadine, whereas human *in vitro* studies have indicated that it is up to 10 times more active.[2] After absorption from the gut, desloratadine reach blood stream with low C_{max} value The major metabolite of desloratadine in human plasma and urine is the glucuronide conjugate of 3-hydroxydesloratadine.[3]

Literature survey revealed that there are many methods for the qualitative and quantitaion estimation of desloratadine in biological (human and animal) fluids and pharmaceutical formulations, such as spetroflurometry,[4] capillary electrophoresis,[5] UV spectroscopy,[6,7] ion-pair chromatography,[8] gas-liquid chromatography,[9] high pressure liquid chromatography (HPLC) couple with UV detection,[7,10] ultra performance liquid chromatography (UPLC)[11] and liquid chromatography-tandem mass spectrometry (LC-MS/MS).[12-19] However, all these methods have their own disadvantages and which includes complicated sample pretreatment, time consuming chromatographic separation for HPLC-UV methods, low sensitivity (>0.1 ng/mL) for HPLC methods, poor chromatography (tailing observed), narrow calibration curve range for UPLC method, poor performance of selectivity not established with different donors for reported LC-MS/MS methods and matrix effect (absolute as well as relative matrix effect) not evaluated in different types of plasma for reported LC-MS/MS methods and large volume of plasma (>500 µL) for all reported analytical methods.

To conduct bioequivalence study of desloratadine of 5 mg strength, it was essential an assay method with low limit of quantitation (LOQ) (40 pg/mL) to evaluate all the pharmacokinetic parameters at elimation phase. Thus, liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed and has numerous advantages over other existing methods. The pros of the developed method includes: less aliquot volume (200 µL), adequate sensitivity (40 pg/mL), easy protocol for sample preparation, highly selective towords different plasma lots (different donor, n=50), effect of co-elute matrix on target analytes is null, which was evaluated with different types of plasma (like normal plasma, hemolyzed plasma and lipemic plasma) using optimized chromatographic conditions. The detailed study of matrix effect in different types of plasma was not investigated previously.

To demonstrate the selectivity experiment during development phase, processed human plasma was analyzed with all the chromatpographic conditions (with short analysis time) mentioned in the published articles. But, selectivity data was not meet the acceptance criteria at desired LOQ level as it affected by plasma lots of different donors. This problem was not reported by any of the published assay methods where short analysis time was reported[17,19] and hence reported short analysis time. During invstigation, it was identified that an endogenous peak was eluted at the retention time of desloratadine and causes the significant interference and this endogenous peak is not separated with short analysis time. After re-optimization of the chromatographic conditions, the endogenous peak is eluted at different retention time and separeated from the desloratadine peak. Hence, no interference was observed at retention time of desloratadine in the processed plasma sample. As a result accurate estimation of desloratadine in incurred samples was possible.

EXPERIMENTAL

Standards and Chemicals

Desloratadine [Batch No: SP(H-447)031, purity: 99.2%] and desloratadine-d_5 (internal standard, IS, Lot No: 1138-054A1, purity: 97.2%) were procured from sun pharmaceutical industries limited and TLC Pharma Chem., Inc. respectively (Figure 1). Ammonium formate, acetonitrile and methanol were procured from Fluka (Sigma-Aldrich, steinheim, USA). Formic acid and liquor ammonia were obtained from Fischer Scientific, India. All reagents used were of ACS grade or higher, with solvents of LC-MS grade. Milli-Q water (Millipore, Moscheim Cedex, France) was used in the preparation of solutions. Oasis® HLB, 30 mg/1cc, solid phase extraction (SPE) cartridges were procured from Waters Corporation, USA and human K_3 EDTA plasma lots were obtained from Biological Specialty Corporation, PA, USA.

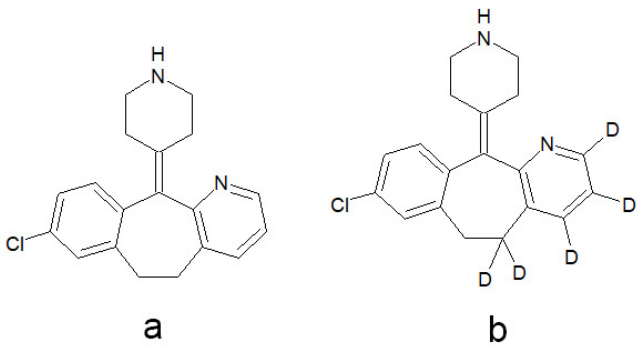

Figure 1: Chemical structures of desloratadine (1a) and desloratadine-d5 (1b).

LC-MS/MS Instrument and Conditions

An HPLC system (Shimadzu, Kyoto, Japan) equipped with a BDS Hypersil C8 analytical column (Dimension: 100 mm × 4.6 mm, 5 µm), a binary LC-20AD prominence pump, an auto sampler (SIL-HTc) and a solvent degasser (DGU-20A3) was used for the study. Aliquot of 25 µL of the processed samples were injected into the column, which was maintained at 45 ºC ± 1 ºC temperature. A isocratic mobile phase composed with of acetonirile and ammonium formate (pH 4.3; 5 mM) (60:40,*v/v*) was delivered at a flow rate of 0.8 mL/min. The autosampler (in injector) was maintained at 10 ºC ± 1 ºC and the injection needle was rinsed with rinsing solution (methanol-water, 50:50, *v/v*) between two successive injections. The ionization and detection were carried out on a triple quadruple mass spectrometer, MDS Sciex API-4000 (Sciex Division of MDS, Toronto, Ontario, Canada), equipped with electrospray ionization operated in positive polarity using multiple reaction monitoring (+MRM). The compound and source parameters were optimized by infusing individual neat solution (5.0 ng/mL) of desloratadine and IS into the mass spectrometer. The mass transitions (m/z) were selected as 311.2→259.2 and 316.2→264.3 for desloratadine and IS, respectively. The optimized compound parameters for monitoring desloratadine and IS were set as follows: declustering potential (DP), 60.0 V; entrance potential (EP), 10.0 V; collision energy (CE), 30.0 V; and collision cell exit potential (CXP), 15.0 V. The source parameters of the mass spectrometer were optimized and maintained as follows: collision activated dissociation gas (CAD), 10 psi; curtain gas (CUR), 20 psi; nebulizer gas (GS1), 50 psi; heater gas (GS2), 50 psi; turbo ion spray voltage, 4000 V and source temperature, 650 ºC. Quadrupole 1 and quadrupole 3 were both maintained at unit resolution, and dwell time was set at 400 millisec for all analytes. The data acquisition and processing were performed by Analyst software (Version 1.4.2; MDS Sciex, Toronto, Canada). For quantification, the peak area ratios (analyte peak area/IS peak area) were compared with weighted $1/X^2$ (where, X = drug concentration) least squares calibration curves in which the peak area ratios of the calibration standards were plotted *versus* their concentrations.

Preparation of stock solution and spiked plasma samples

Two separate stock solutions of desloratadine were prepared for bulk spiking of calibration standards (CC) and quality control (QC) samples for method validation exercises as well as incurred sample analysis. Stock solution of desloratadine and desloratadine-d_5 was prepared in methanol at a concentration of 1000 µg/mL and 500 µg/mL, respectively. Working solutions for CC and QC samples were prepared by appropriate dilution in methanol-water (50:50, *v/v*). Human K_3EDTA plasma was screened prior to spiking to ensure that there is no significant endogenous interference at the retention time (RT) of desloratadine and IS. An eight-point CC and QC samples at four concentration levels were prepared by spiking (2%, *v/v*) of the secondary solutions of desloratadine in human K_3EDTA plasma. Calibration standards were prepared at concentrations of 40.1, 110.7, 204.9, 512.3, 1280.8, 3202.0, 6404.0 and 8005.0 pg/mL and QC samples at lower limit of quantification (LOQQC), low quality control (LQC), medium quality control (MQC) and high quality control (HQC) at concentrations of 40.1, 110.9, 3204.1 and 6408.3 pg/mL respectively. The bulk spiked CC and QC samples were stored below -15 ºC and protected from light till use. The working solution of IS (4000.0 pg/mL) for routine use, was prepared by diluting the stock solution of IS in methanol-water (50:50, *v/v*).

Extraction Technique

Solid phase extraction (SPE) technique was used to extract desloratadine and it IS from the human plasma. For this purpose, 200 µL of plasma sample and 50µL of IS working solution (4000.0 pg/mL of IS) was added in labeled polypropylene tubes. After that, 400µL of solution-1 (5% formic acid in water, *v/v*) was added and then samples were vortexed for 10 sec. The pretreated samples were loaded onto the SPE cartridge (Oasis® HLB, 30 mg/1cc) that was pre-conditioned with 0.5 mL of methanol followed by 0.5 mL of water. The cartridges were washed using 1mL of solution-1 followed by 1mL of water. The analyte and IS were eluted with 1mL of elution solution (2% liquor ammonia in methanol, *v/v*). During extraction, refrigerated centrifuge spun at 2000 rcf for 1 min for each step. The extracted samples were evaporated to dryness in Zymark TurboVap LV nitrogen evaporator (Caliper, Hopkinton, MA, USA) at 50 ºC and at 20 psi and the dried residue was reconstituted with 400 µL of mobile phase. The reconstituted samples of 25 µL volume were used for injection in LC-MS/MS system.

Method Validation

A thorough and complete method validation of desloratadine in human K_3EDTA plasma was carried out, as per the USFDA bioanalytical guideline 2001[20] and EMEA bioanalytical guideline 2011.[21] The method was validated for selectivity, sensitivity, linearity, precision, accuracy, process efficiency, matrix effect and stability of desloratadine during both short term sample processing and long-term storage.

Selectivity and S/N ratio: The selectivity of the method towards endogenous plasma matrix components was assessed after screening ten lots (6 normal plasma, 2 haemolyzed plasma and 2 lipemic plasma) of human K_3EDTA plasma, free from all analyte of interest. These samples were processed using the proposed extraction protocol and analyzed with optimized chromatographic conditions set for desloraratine and IS. The peak area response at the RT of desloraratine and IS in the processed plasma lots due to the co-elute matrix components or any other interferences should be less than 20% and 5% of mean peak area of desloraratine and IS observed in spiked LOQ sample respectively. The sensitivity of the developed method was established by signal to noise (S/N) ratio. The S/N ratio of spiked LOQ samples was calculated using following formula:

$$\frac{S}{N} = \frac{\text{Signal to noise ratio of LOQ}}{\text{Mean of signal to noise ratio of blanks}} > 5$$

Linearity, precision and accuracy: Three calibration curves were used to demonstrate the linearity of the method. The ratio of area responses for desloraratine was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/X^2$) linear regression (obtained by best fit method). Back-calculations were made from these curves to determine the concentration of desloraratine in each calibrator. A correlation coefficient r>0.99 was desirable for all the calibration curves. The analyte peak of LOQ sample should be identifiable, discrete and reproducible with a precision (%CV) should be <20.0 and accuracy within ±20.0%. The deviation of standards other than LOQ from the nominal concentration should not be more than ±15.0%.

The intra- and inter-day precision and accuracy were performed for desloraratine in K_3EDTA plasma. The intra-run (within a day) and inter-run (between days) accuracy was determined by replicate analysis of QC samples at LOQQC, LQC, MQC and HQC. The precision of the method was determined by calculating the coefficient of variation (% CV) for each QC level. The deviation at each concentration level from the nominal concentration was expected to be <15.0 except for the LOQQC, for which it should be <20.0. Similarly the mean accuracy should be within ±15.0% except for at the LOQQC, for which it should be ±20.0% of the nominal concentration.

Recovery and matrix effect: The absolute recovery (ARE) was estimated by measuring the mean peak area response of desloratadine and its IS in six replicates of extracted QC samples (at LQC, MQC and HQC level) against the mean peak area response of desloratadine in neat (aqueous) samples. The six replicates injection of neat samples was analyzed from the same auto injector vial. At LQC, MQC and HQC levels, the relative recovery (RRE) was also estimated by measuring the mean peak area response of desloratadine in six replicates of extracted QC samples against the mean peak area response of post extracted samples. The concentration of desloratadine and IS in post-extracted samples and neat samples was equivalent to those obtained in the final extracted QC samples.

Absolute recovery (ARE) was calculated by the following formula:

$$\% \text{ ARE} = \frac{\text{Mean peak area response of analyte in extracted samples}}{\text{Mean peak area response of analyte in neat samples}} \times 100$$

Relative recovery (RRE) was calculated by the following formula:

$$\% \text{ RRE} = \frac{\text{Mean peak area response of analyte in extracted samples}}{\text{Mean peak area response of analyte in post extracted samples}} \times 100$$

In bioanalysis, the effect of co-elute matrix on ionization of molecule is qualitatively determined by 'T'-joint experiment.'T'-joint experiment is a post column infusion technique. To perform this experiment, a working solution containing desloratadine and IS (~500 ng/mL of each) was infused at a flow rate of 10µL/min through infusion pump into the mobile phase (post column *via* a 'T' connector) and 25 µL of extracted blank plasma sample was injected from the autosampler through LC column. In Analyst software (version 1.4.2), chromatograms were acquired and monitored for both the analyte and IS. From the 'T-Joint' experiment, it was concluded that co-elute matrix has no role on ionization of desloratadine and IS in ion source.

The absolute matrix effect (AME) was estimated by the following equation:

$$\% \text{ RRE} = [1- \frac{\text{Mean peak area response of analyte in post extracted samples}}{\text{Mean peak area response of analyte in neat solution}}] \times 100$$

Case I: when AME =1 it indicates there is no matrix effect on ionization of target analyte(s)
Case II: when AME value is <1, ionization of the analyte in mass spetrometer ion source is suppressed by the co-elute matrix components (i.e. ion-suppression)
Case III: when AME value is >1, ionization of the analyte in mass spetrometer is enhanced by the co-elute matrix components (i.e. ion-enhancement)

Required number of aliquots of human plasma with different lots were processed as per our developed sample processing protocol till drying step and after drying reference dilutions (containing desloratadine and IS) were added into the dried samples to prepared the post-extracted samples and analyzed in LC-MS/MS system along with the neat samples. The reference dilutions were prepared by assuming the 100% extracted concentrations for analyte as well as internal standard in LQC, MQC and HQC levels.

Relative Matrix effect (RME) was evaluated in ten lots of human K_3EDTA plasma (including two hemolyzed and two lipemic plasma lots). Matrix effect QC (MEQC) samples were prepared in ten acceptable plasma lots (at LOQC and HQC level) and processed in duplicate. The back-calculated concentration of MEQC samples was used to check the acceptability of the result. At each QC level, the %accuracy of MEQC sample in each matrix was calculated along with %CV. The % accuracy of the MEQC sample should not be more than ±15% of their respective nominal concentration and at least 90% of the matrix lots at each QC level should be within the aforementioned criteria.

Stability exercises: Stability experiments were carried out to study the stability of desloratadine in stock solution and in plasma samples under different conditions. Stock solution stability was performed by comparing peak area response of desloratadine and IS in old stock solution, with the peak area response in fresh stock solution. Stability studies were performed in human plasma at two QC levels (i.e. LQC and HQC) using four replicates at each level. The analyte was considered stable if the % change is less than 15, as per US FDA/EMEA guidelines and was calculated by using the following formula:

$$\% \text{ Change } = \left[\frac{S}{F} - 1\right] \times 100$$

Where, S=Mean calculated concentration of stability samples and F=Mean calculated concentration of freshly spiked samples.

The bench top stability was determined by stored spiked QC samples for ~7.5 hr at room temperature before processing. The auto sampler stability was determined by stored reconstituted QC samples for ~120 hr under auto sampler condition (at 10 ºC) before being analyzed. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -15 ºC and thawed at room temperature three times, with freshly spiked QC samples. Four aliquots each of LQC and HQC concentration level were used for the freeze-thaw stability evaluation. For long term stability evaluation, the concentrations obtained after 76 days were compared with initial concentrations. All stability exercises were performed against freshly spiked CC.

Prior to perform the blood stability, human K_3EDTA whole blood was incubated at 37 **ºC** for about 15 min. After incubation, the secondary solutions of desloratadine were spiked in human whole blood at LQC and HQC level and after spiking samples were stored for a period of approximately 2 hrs at room temperature and these samples were served as stability samples. After completion of the storage period, secondary solutions of desloratadine were again spiked in blood to prepared comparison samples. Comparison samples were allowed to reach equilibrium between RBC and plasma and after that all the samples were centrifuged together at 4±2 **ºC** and 4000 rpm for 15 mins to separate plasma from the blood. Four aliquots of all samples were processed and analyzed in LC-MS/MS system. The stability duration was calculated as the difference between the times of spiking of comparison samples less the time of spiking of stability samples. % Stability was calculated by using the following equation:

$$\% \text{ Stability } = \frac{\text{Mean area ratio of stability samples}}{\text{Mean area ratio of comparison samples}} \times 100$$

Method Application

An open label, balanced, randomized, two-treatment, two-period, two-sequence, single-dose, crossover design was used for the assessment of pharmacokinetics and bioequivalence. Thirty two healthy volunteers who gave written informed consent took part in this study. The study was approved by Ethics Committee of Institutional Review Board (IRB) at Jamia Hamdard Institutional Review Board, New Delhi, India. A single oral dose of desloratadine 5 mg tablet of sun pharmaceutical industries limited and Aerius® tablet of Schering-Plough Labo NV, Belgium was given to the volunteers during each period of the study. Blood samples were collected before (pre-dose) and at 0.333, 0.667, 1.000, 1.333, 1.667, 2.000, 2.333, 2.667, 3.000, 3.333, 3.667, 4.000, 4.333, 4.667 5.000, 6.000, 8.000, 12.000, 16.000, 24.000, 36.000, 48.000, 72.000, 96.000 and 120.000 hr post dose in each period. After separation of plasma from blood by centrifugation, plasma samples were stored frozen below -15 ºC until analysis.

Incurred sample reanalysis (ISR) reinforces the confidence in a bioanalytical method by demonstrating reproducibility in the measurement of study data. The possible causes of irreproducibility in ISR can be due to many reasons, which may include: Stability issues-conversion of metabolite to its parent compound, isomeric changes or effect of pH; drug-protein binding differences in subject samples; issues related to matrix interference-general and those associated with phospholipids; concomitant medications; process efficiency issue. Therefore, reproducibility of the method was confirmed by performing ISR. ISR was assessed using a total of 154 incurred samples. After completion of original anaysis, ISR samples selection carried out for both the phases i.e. at absorption phase as well as elimination phase. At absorption phase time point with maximum plasma concentration (C_{max}) and at elimination phase, time points with concentration greater than 3 times of LOQ were selected for ISR analysis. The acceptance criterion for the ISR analytical run was 67% (two-thirds of the total sample size) and should lie within 20% difference.[22] The % difference from the original analysis was calculated as:

$$\% \text{ Difference } = \frac{\text{Reanalyzed concentration} - \text{Original concentration}}{\text{Mean concentration}} \times 100$$

RESULTS AND DISCUSSION

Optimization of Mass Parameters

Desloratadine, is reported to be highly variable drug in terms of pharmacokinetic (PK) behavior with low C_{max} value. For accurate and reliable characterization of PK profile, it is essential to develop a sensitive method with low LOQ (40.0 pg/mL) for 5 mg formulation. In order to develop a method with the desired LOQ, it was necessary to use MS-MS detection. Pyridine and piperidine nucleus are present in the chemical moiety of desloratadine, hence electrospray in positive ion mode was evaluated. Likewise, atmospheric pressure chemical ionization (APCI) was also assessed but provided no advantages over electrospray. During product ion scan, the major product ions at *m/z* 259.1, 282.2, 294.0 were observed. The predominant product ion of *m/z* 259.1 is specific for desloratadine corresponding deuterated analog. During ramping of the compound parameters, optimization it was noted that CE and CAD are most critical parameter to achieve highest sensitivity and stable response for desloratadoine. Product ion spectra of desloratadine and IS are shown in Figure 2.

Optimization of Chromatographic Conditions

Chromatographic optimization of desloratadine and IS was carried out under isocratic conditions to obtain adequate response, sharp and symmetrical peak shape with short analysis time. The

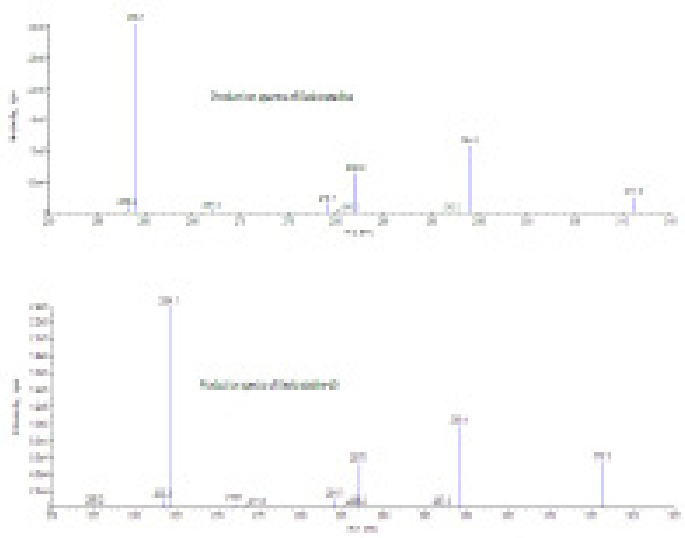

Figure 2: Product ion spectra of desloratadine and desloratadine-d5.

use of volatile buffers like ammonium formate and ammonium acetate (in combination of methanol-acetonitrile) for the separation of desloratadine had been evaluated also. It was observed that the pH of mobile phase and selection of column were critical parameters. Chromatographic separation was tried using various combinations of methanol-acetonitrile, acidic buffers and additives (like formic acid, glacial acetic acid and liquor ammonia) on different reversed phase columns with 5 µm particle size [viz., XBridge C8 (75 mm × 4.6 mm, 5 µm), Xterra C18 (150 mm × 4.6 m,5 µm), Chromolith RP-18 (100 mm × 4.6 mm), Atlantis HILLIC (100 mm × 4.6 mm, 5 µm), Ascentis C8 (100 mm × 4.6 mm), Zorbax SB C8 (100 mm × 4.6 mm,5 µm), BDS Hypersil C8 (100mm×4.6mm,5µm) and BDS Hypersil C18 (50 mm × 4.6 mm, 5 µm)] to optimize liquid chromatographic parameters. In presence of acetonirile as organic phase, peak area counts is increased. Hence, acetonitrile alone was selected as an organic phase. The analytes showed nonlinear behavior on Chromolith RP-18 column while HILLIC column was marked unsuitable due to co-eluting matrix compounds especially with haemolysed plasma samples. In most of the C18 columns, tailing was observed in chromatography for both desloratadine and IS and in BDS Hypersil C8 column satisfactory peak shape (S/N >90) was observed. During optimization method selectivity data was not established at desired LOQ level, as significant peak area was observed at RT of desloratadine in the processed plasma lots of different donors. After performing an invstigation, an endogenous peak was identified which was eluted at the RT of desloratadine and causes the significant interference. After re-optimization of the mobile phase composition (i.e. organic/aqueous phase ratio and ammonium formate buffer strength), the endogenous peak is eluted at different RT and separeated from desloratadine peak. From now, no interference was observed at RT of desloratadine in all the processed plasma lots (different donors, n=50). The mobile phase consist of acetonirile-5mM ammonium formate (pH 4.3±0.1) buffer (60:40,*v/v*) was found most suitable for consisting of acetonitrile eluting the desloratadine and IS from BDS Hypersil C8 (100 mm × 4.6 mm, 5 µm) column within run time of 6.5 min.

Sample Preparation

Due to hydrophobic nature of desloratadine, different polymeric cartridges like Oasis HLB, Bond Elut Plexa, Cleanert PEP-H and Cleanert PEP-3 were tried for extraction during development phase. % Recovery is high when plasma sample extracted with HLB cartridge as compared to other SPE cartridges. At basic condition (pH>7.0), the % of unionized form of desloratadine and IS is more in cartridge bed and elution facilitate with methanol as an organic solvent. Hence, using basified methanol as an elution solvent, recovery of desloratadine and IS are increased. Sample pretreatment step was optimized to increase the recovery of analyte and reduce the matrix effect. Upon re-optimization, it was observed that samples pretreated with formic acid solution (5%,*v/v*) showed higher and consistent recovery across all QC levels. In addition, washed the HLB cartridge with acidic solution (5% formic acid solution, *v/v*) followed by water helped to eliminate the effect of co-elute matrix components.

Selection of Internal Standard

Selection of internal standard with similar chromatographic and mass spectrometric behavior to that of analyte is of extreme priority for LC-MS/MS analysis. For LC-MS/MS assay method, usage of structure related isotopic labeled compound (SIL, desloratadine-d_5) as an internal standard have some key advantages over the other compounds. Due to similar physicochemical properties with desloratadine, desloratadine-d_5 shows similar retention time in reverse phase liquid chromatography, similar fragmentation pattern in mass spectroscopy and equivalent extraction efficiency with that of desloratadine. Therefore, desloratadinel-d_5 was selected as an IS.

Method Validation

Selectivity and S/N ratio: There was no significant interference observed at the RT of desloratadine and IS in screened plasma lots. The maximum interference observed at the RT of deslo-

ratadine and its IS was 3.07% and 0.03% respectively (Table 1). The typical chromatograms of blank sample, blank processed with IS, LOQ, ULOQ and incurred sample in human plasma are shown in Figure 3. We observed that S/N ratio was >90 during method validation and bioanalysis.

Linearity, precision and accuracy: The limit of quantitation was 40.1 pg/mL of desloratadine in plasma. The precision and relative error at LOQQC level were 6.5% and 1.5% respectively. The calibration curve was linear from 40.1 to to 8005.0 pg/mL for desloratadine in human plasma. Calibration curve was constructed using peak area ratio of analyte to IS and by applying linear, weighted least squares regression analysis with weighting factor of 1/(concentration)^2. The 'r' was greater than 0.99 during the course of validation and bioanalysis. The results of three precision and accuracy batches are summarized in Table 2. The intra-day precision and inter-day precision (%CV) ranged from 2.4 to 8.6 and the intra-day and inter-day relative error (% bias) ranged from -2.9 to 3.8%.

Matrix effect and recovery: In LC-MS/MS method, ionization of analyte is affected by the co-elute matrix components spe-

Plasma lot	Double blank sample		% Interference		Peak area response in LOQ sample (n= 6)	
	at the RT of desloratadine	at the RT of IS	at RT of desloratadine	at the RT of IS	at the RT of desloratadine	at the RT of IS
LOT-1	92	18	0.58	0.00	15550	383936
LOT-2	23	35	0.14	0.01	16245	385818
LOT-3	490	102	3.07	0.03	15443	400155
LOT-4	57	38	0.36	0.01	16396	400227
LOT-5	72	16	0.45	0.00	15796	420268
LOT-6	96	50	0.60	0.01	16335	400743
LOT-7[a]	18	22	0.11	0.01		
LOT-8[a]	55	62	0.34	0.02		
LOT-9[b]	49	30	0.31	0.01		
LOT-10[b]	113	0	0.71	0.00		
Mean					15961	398525

[a] hemolyzed plasma; [b] lipemic plasma % Interference in the processed plasma lot = (peak arae response at RT of analyte or IS in plasma lot/mean peak arae response of analyte or IS in LOQ samples)×100

Table 1: Selectivity.

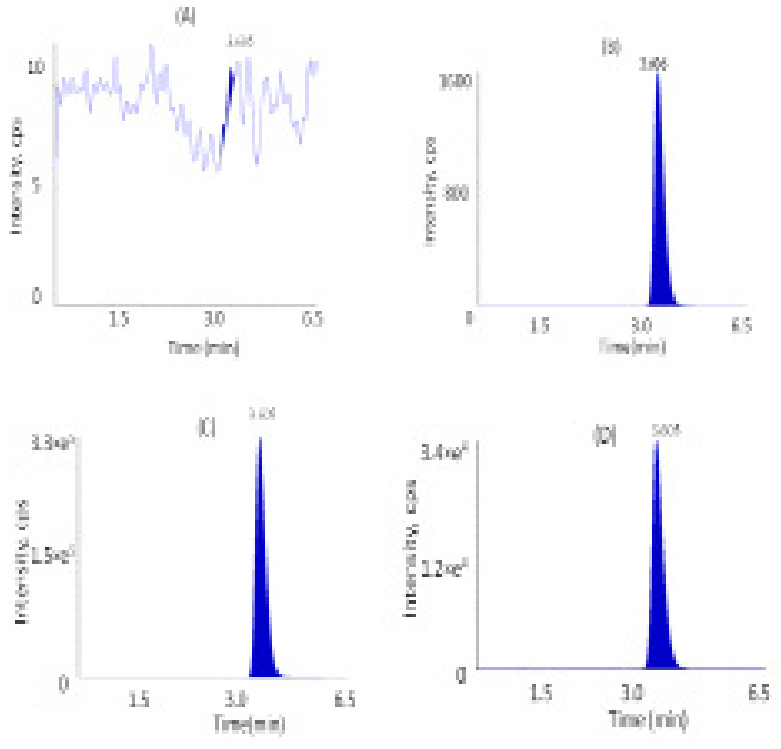

Figure 3: Representative chromatograms (A) Double blank sample- at RT of desloratadine, (B) LOQ- at RT of desloratadine, (C) ULOQ-at RT of desloratadine and (D) Single blank sample at RT of IS.

QC sample	Spiked concentration (pg/mL)	Intra-run (n =12)			Inter-run (n = 18)		
		Mean observed concentration (pg/mL)	% Relative error or bias	%CV	Mean observed concentration (pg/mL)	% Relative error or bias	%CV
LOQQC	40.1	39.5	1.5	6.5	40.7	-1.4	4.3
LQC	110.9	106.7	3.8	8.2	109.5	1.2	8.4
MQC	3204.1	3173.7	0.9	2.4	3159.6	1.4	2.6
HQC	6408.3	6544.3	-2.1	7.0	6594.3	-2.9	8.6

Table 2: Intra-and inter-run results.

cially when, ESI is applied as an ionization mode. Hence, to estimate the accurate concentration of desloratadine in the clinical samples, it is important to eliminate the role of co-elute matrix components on ionization of target analyte in ion source. So, the two most important parameters like, matrix effect and recovery (ARE and RRE) were evaluated during validataion. Simmilar peak area response was observed for desloratadine and IS in neat aqueous solution as well as post extracted samples (for all types of matrix) used for matrix effect analysis (Table 3). The % ion suppression was varies from -0.45 to 1.03 between three QC levels. The results indicated that there was no significant effect of matrix upon ionization of desloratadine and IS in mass spectrometer. The mean absolute recovery (ARE) of desloratadine was 79.88% (%CV<2) across the QC levels. The mean relative recovery (RRE) of desloratadine was 80.26% (%CV< 3) across the QC levels. The mean absolute and relative recovery of IS was 81.47% and 84.06% respectively. Both the recoveries are consistant across the QC levels for desloratadine and IS. The results of relative matrix effect (RME) and ISTD-normalized matrix factor are presented in Table 4.

Stability exercises: Stability of desloratadine and IS in stock solution were established for 14 days at refrigerated storage conditions (1-10 ˚C) and % stability of desloratadine and IS were 98.76 and 99.68 respectively. Desloratadine was proved to be stable in plasma for three freeze-thaw cycles. Bench top stability of desloratadine was established for ~7.5 hr in human plasma at room temperature and under low light conditions. Auto sampler stability was assessed for ~121 hr and long term stability was established at -15 ˚C for 76 days. The observed mean calculated concentration of desloratadine was found to be within ±15% of their respective nominal concentration and % CV was less than 15 at LQC and HQC levels (Table 5). Desloratadine was stable in human K_3EDTA whole blood for ~2.0 hr.

QC level	LQC			MQC			HQC		
Analyte Concentration (pg/mL)	**55.5**	**55.2**	**55.2**	**1602.1**	**1607.9**	**1607.9**	**3204.2**	**3215.7**	**3215.7**
Serial No.	Extracted sample	Post extracted sample	Neat sample	Extracted sample	Post extracted sample	Neat sample	Extracted sample	Post extracted sample	Neat sample
1	48022	60059	64517	1428030	2085182	2064952	3234730	4149709	4114607
2	51986	65774	64901	1706034	2030580	2068838	3190828	3981939	4055130
3	53397	64071	63196	1632089	2042684	2030595	3216621	4207229	4063122
4	55453	66319	66281	1697771	2127310	2084132	3432663	3780955	4016763
Mean	52215	64056	64724	1615981	2071439	2062129	3268711	4029958	4062406
%CV	6.01	4.42	1.96	8.02	2.12	1.10	3.39	4.75	0.99
Mean peak area with C.F		64404	65076		2063967	2054691		4015546	4047878
% Absolute Recovery	80.24			78.65			80.75		
% Relative Recovery	81.07			78.29			81.40		
% Ion suppression	1.03			-0.45			0.80		
Correction factor (C.F) = concentration of analyte in extracted sample/concentration of analyte in neat or post extracted sample									

Table 3: Recovery and ion suppression.

Plasma lot	Relative matrix effect						IS-MF [d]
	LOQQC			HQC			
	Mean calculated concentration (pg/mL)	%CV	% bias[c]	Mean calculated concentration (pg/mL)	%CV	% bias[c]	
LOT-1	40.3	3.7	-0.4	6454.0	1.1	-0.7	1.01
LOT-2	38.4	9.8	4.4	6465.7	3.3	-0.9	1.03
LOT-3	41.7	1.7	-4.0	6596.2	0.3	-2.9	1.02
LOT-4	38.5	5.5	4.0	6435.9	2.4	-0.4	1.00
LOT-5	43.7	0.8	-8.9	6456.2	1.7	-0.7	1.00
LOT-6	40.6	2.3	-1.1	6427.3	0.8	-0.3	1.00
LOT-7[a]	40.9	2.3	-1.9	6363.3	0.6	0.7	1.01
LOT-8[a]	40.8	2.8	-1.7	6411.3	1.0	0.0	1.00
LOT-9[b]	41.7	1.0	-4.0	6466.7	3.3	-0.9	1.00
LOT-10[b]	38.9	1.8	3.0	6336.9	4.3	1.1	1.01
[a] hemolyzed plasma; [b] lipemic plasma [c] Bias = (spiked concentration-observed concentration) / spiked concentration ×100							
[d] Overall Mean IS-normalized matrix factor at LQC, MQC and HQC levels							

Table 4: Relative matrix effect and ISTD normalized matrix factor.

Stability parameter	Level	Comparison sample		Stability sample		
		Calculated concentration (pg/mL)	%CV	Calculated concentration (pg/mL)	%CV	% Change
Auto sampler stability	LQC	108.1	2.5	108.0	4.5	-0.1
(~121.0 hr, 10ºC)	HQC	6442.4	1.1	6592.3	3.8	2.3
Bench top stability	LQC	108.1	2.5	108.1	3.7	0.0
(~7.58 hr, at room temperature)	HQC	6442.4	1.1	6330.8	2.4	-1.7
Freeze-thaw stability	LQC	108.1	2.5	106.4	2.5	-1.6
(Thee freeze-thaw cycle)	HQC	6442.4	1.1	6454.3	0.5	0.2
Long term stability	LQC	110.7	5.7	104.7	6.3	-5.4
(76 days, below -15ºC)	HQC	6855.4	7.4	6622.2	2.7	-3.4

Table 5: Stability experiment (n = 4).

Method Application

Following analysis, pharmacokinetic parameters like peak plasma concentration (C_{max}), time (T_{max}) to reach C_{max}, $t_{1/2,}$ $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\infty}$ were calculated by non-compartmental analysis using WinNonlin Professional software (Version 5.0, Pharsight Corp., Mountain View, CA, USA). The pharmacokinetic parameters summarized in Table 6 are the mean estimates obtained from 27 subjects, who completed all periods of the study. The linear plot of mean plasma concentration (pg/mL) *versus* time (hr) and semi-log plot of mean plasma concentration (pg/mL) versus time (hr) are shown in Figure 4. ISR results demonstrated that the samples with percentage difference within ±20% was 75% (Table 7).

CONCLUSION

In summary, a rapid, selective, specific, reproducible and high-throughput LC-MS/MS method was developed and validated to estimate desloratadine in human plasma using desloratadine-d_5 as an internal standard. The proposed assay method showed good performance with respect to all the validation parameters tested, demonstrated optimized working conditions for desloratadine in human plasma. Stability experiment data and ISR data confirmed that desloratadine is stable in all the working conditions. The assay method was successfully employed for a bioequivalence study of desloratadine after oral administration of 5 mg desloratadine tablet.

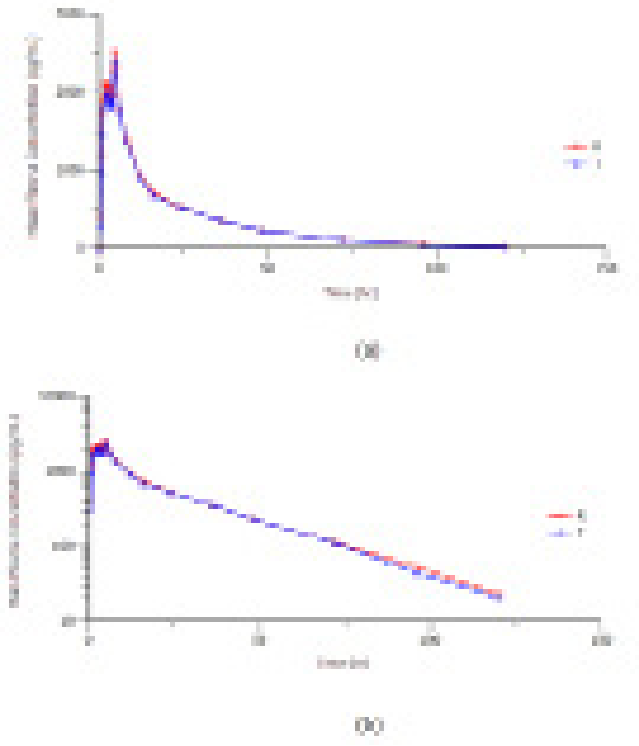

Figure 4: Plasma profile of desloratadine (N=27) R: Reference drug; T:Test drug; (a) Linear plot of mean plasma concentration (ng/mL) versus time (hr) and (b) Semi log plot of mean plasma concentration (ng/mL) versus time (hr).

Parameters	(Mean±SD)	
	Reference product	Test product
T_{max} (hr)	3.864±1.402	3.889±1.184
C_{max} (pg/mL)	2807.00±788.889	2566.50±602.140
$AUC_{0\rightarrow t}$ (hr.pg/mL)	42589.93± 15648.613	41399.39±16216.084
$AUC_{0\rightarrow\infty}$ (hr.pg/mL)	44952.56±16649.515	43654.73±17187.085

Table 6: Pharmacokinetic parameters of desloratadine.

Subject No.	Period	Time point (hr)	Original concentration (pg/mL)	Repeat concentration (pg/mL)	% Difference
2	I	12.000	976.6	994.6	1.83
3	II	120.000	150.9	152.8	1.25
6	I	48.000	156.5	164.3	4.86
9	II	4.667	1972.2	1849.6	-6.42
16	I	4.667	3003.2	3118.7	3.77
18	I	4.667	2161.5	2065.3	-4.55
8	I	16.000	2592.1	2534.4	-2.25
11	I	72.000	136.3	150.2	9.70
13	II	1.000	2559.5	2725.6	6.29
21	I	4.667	1764.3	1895.2	7.15
22	I	48.000	121.6	119.2	-1.99
25	II	1.333	2210.3	2117.6	-4.28
26	II	4.667	2596.4	2727.6	4.93
29	II	16.000	2409.1	2564.7	6.26
32	I	12.000	831.1	851.6	2.44

Table 7: Representative ISR data with I is the first period and II is the second period.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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