LC–MS-ESI for the Determination of Loratadine and Descarboethoxyloratadine in Human Plasma

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Abstract

A rapid, sensitive, and accurate liquid chromatography–tandem mass spectrometry assay for simultaneous determination of loratadine (L) and its active metabolite, descarboethoxyloratadine (DCL), in human plasma is developed using desipramine as internal standard (IS). The analytes and IS are separated on a Betabasic cyano (100 mm × 2.1 mm, 5 μm) column and detected by tandem mass spectrometry with a turbo ion spray interface operating in positive ion and multiple reaction monitoring acquisition mode. The total chromatographic runtime is 3.0 min with retention time for L, DCL, and IS at 0.82, 1.58, and 1.97 min, respectively. The method is validated over a dynamic linear range of 0.05–15.00 ng/mL for both L and DCL with a correlation coefficient of r^2 0.9984 and 0.9979, respectively. The intra-batch and inter-batch precision (%CV) across five levels (LLOQ, LQC, MQC, HQC, and ULOQ) is less than 9%. The method is successfully applied to a bioequivalence study of 10 mg loratadine tablet formulation in 28 healthy Indian male subjects under fasted condition.

Introduction

Loratadine (L), a piperidine derivative, is a potent long-acting, non-sedating tricyclic antihistamine with selective peripheral H_1-receptor antagonist activity. It is used for relief of nasal and non-nasal symptoms of seasonal allergies and skin rashes (1). Due to partial distribution in central nervous system, it has less sedating power compared to traditional H_1 blockers. L is rapidly absorbed from the gastrointestinal tract after oral administration and reaches peak plasma concentration (T_{max}) in ~ 1–2 h (2,3). It undergoes extensive first-pass metabolism in the liver, forming an active metabolite, descarboethoxyloratadine (DCL). Compared to loratadine, DCL shows higher affinity for histamine H_1 human receptors. DCL is less extensively bound to plasma proteins (~ 75%) in comparison to L (~ 98%). The elimination half-life of L in normal volunteers is 8–14 h and that of DCL is 7–24 h (4,5).

Literature reveals the use of different analytical techniques to determine L and/or DCL in plasma, viz. voltammetry (6), radioimmunoassay (7), gas chromatography with nitrogen-phosphorous (8), high-performance liquid chromatography (HPLC)-UV (9,10) and fluorescence detection (11–15), and liquid chromatography–tandem mass spectrometry (LC–MS–MS) (16–25). Ghoneim et al. (6) have studied the polarographic behaviour of L in Britton-Robinson (BR) buffer solution. They have optimized a differential pulse cathodic adsorptive stripping voltammetry procedure for direct determination of the drug in pharmaceutical and human plasma samples. The calibration graph for L was rectilinear within the concentration range $1 \times 10^{-6} – 2 \times 10^{-5}$M in plasma. The radioimmunoassay method (7) was used specifically to study multiple dose pharmacokinetics of L. The HPLC–UV and fluorescence methods for L (9,11,12) and DCL (10) have low sensitivity (≥ 0.5 ng/mL) to conduct a pharmacokinetic study of 10 mg dose. Four LC–MS–MS methods (18–21) have been reported for the determination of only L in human plasma. These methods employ either large plasma volume for processing, have low sensitivity, or require long run time for analysis. Yang et al. (22) have determined DCL and 3-hydroxydesloratadine in human plasma using 96-well solid phase extraction, followed by LC–MS–MS detection with an lower limit of quantitation (LLOQ) of 25 pg/mL.

All the reported procedures determine either L or DCL in plasma samples. However, simultaneous determination of L and DCL is more beneficial for clinical pharmacokinetic studies, as they have similar pharmacological activity. Based on this criterion, one gas chromatography (GC) (8), three HPLC with fluorescence detection (13–15), and five LC–MS–MS (16,17,23–25) methods for the simultaneous determination of L and DCL in human plasma can be compared with the present study. The GC method (8) was proposed to determine L and DCL in plasma with nitrogen-phosphorous detector. It required a lengthy and cumbersome process for extraction and the sensitivity achieved was 0.1 ng/mL. The HPLC-fluorescence methods (13–15) had low sensitivity (0.5 ng/mL) and long chromatographic run times. This could be a limitation for routine analysis where large a number of samples need to be quantified. Yang et al. (16,17) have presented two method on LC–MS–MS to quantify L and DCL in rat, rabbit, mouse, and dog plasma. Their assay had a dynamic range of 1–1000 ng/mL with on-column loading of 5.56 pg for each analyte at the LLOQ level. A sensitive (0.1 ng/mL for both the analytes) method was given by Sutherland et al. (23) with a long run time of 6 min. The drawback of this method was a lengthy four step liquid–liquid extraction procedure, which

involved a tedious workup to get a recovery of 61% and 100% for L and DCL, respectively. An interesting work has been proposed by Naidong et al. (24) on a silica column using acidic aqueous–organic mobile phase for L and DCL in human plasma. Their lower limit for quantification was 10 pg/mL (L) and 25 pg/mL (DCL) and an equally encouraging chromatographic run time of 3.0 min. But the recoveries for L and DCL at the three quality control (QC) levels had a very low precision (%CV), ranging from 15–25%. Recently, Vlase et al. (25) have reported an LC–MS–MS method using protein precipitation for the extraction of plasma samples with a dynamic concentration range of 0.52–52.3 ng/mL for L and DCL. The chromatographic run time per sample was 8 min, which may not be favourable for high throughput analysis. A summary of the salient features of methods reported for simultaneous determination of L and DCL in plasma is presented in Table I.

As an effort to develop a simple, cost effective, and an accurate method for the simultaneous determination of L and DCL, we have presented a fast LC–MS–MS method with adequate sensitivity for routine subject sample analysis. The chromatographic separation of the analytes and IS was achieved in a run time of 3.0 min, giving a high turnaround for the analysis. Also, the flash freezing of aqueous phase in the liquid–liquid extraction considerably reduced labour, cost, and time for analysis. The method was successfully applied in studying the pharmacokinetics/bioequivalency of 10 mg L tablet formulation in 28 healthy Indian male subjects under fasted condition.

Experimental

Chemicals and materials

Reference standard material of L (99.4%) and DCL (99.6%) were procured from Cadila Healthcare (Ahmedabad, India), and desipramine (IS, 99.0%) was purchased from Sigma (St. Louis, MO). HPLC-grade ethyl acetate, n-hexane, acetonitrile, and methanol were procured from S.D. Fine Chemicals (Mumbai, India). Ammonium trifluoroacetate used in mobile phase was of Acros Organics (Morris Plains, New Jersey), and AR-grade disodium hydrogen orthophosphate was obtained from S.D. Fine Chemicals (Mumbai, India). Water used for the LC–MS–MS was prepared using Milli-Q water purification system from Millipore (Bangalore, India). Control buffered (K3 EDTA) human plasma was procured from Clinical Department, BA Research India Limited (Ahmedabad, India) and was stored at –20°C. Platform shaker and Centrifuge were of Innova 2100 from New Brunswick Scientific (Edison, New Jersey) and Eppendorf 5810 (Hamburg, Germany) respectively. Buffer solution: 50 mM disodium hydrogen orthophosphate solution.

LC–MS–MS Instrumentation and conditions

The LC system (Shimadzu, Kyoto, Japan) consisted of a LC-10ADvp pump, an autosampler (SIL-HTc) and an on-line degasser (DGU-14A). Chromatographic column used was Betabasic cyano, 100 mm length × 2.1 mm i.d., with 5.0-µm particle size. The mobile phase consisted of 700 mL methanol, 300 mL deionized water, and 1.0 mL, 1.0M ammonium trifluoroacetate. Separation of analytes and IS was performed under isocratic condition at a flow rate of 500 µL/min. The auto-sampler temperature was maintained at 4°C, and the injection volume was 5 µL. The total LC run time was 3 min. Detection of analytes and IS was performed on a triple quadrupole mass spectrometer, API-4000 equipped with Turbo Ion spray, manufactured by MDS SCIEX (Toronto, Canada) operating in the positive ion mode. Quantitation was done using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transition of m/z 383.3 → 337.1 for L; 311.2 → 259.2 for DCL and 267.4 → 72.1 for IS (Figures 1, 2 and 3 respectively). All the parameters of LC and MS were controlled by Analyst software version 1.4.1.

For L, DCL, and desipramine (IS) the source parameters maintained were: Gas 1 (GS1), 55.0 psi; Gas 2 (GS2), 65.0 psi; ion spray voltage (ISV), 4500.0 V; turbo heater temperature (TEM), 550.0°C; interface heater (Ihe), ON; entrance potential (EP), 10.0 V; collision activation dissociation (CAD), 7.0 psi; curtain gas (CUR), 25.0 psi. The compound dependent parameters like declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimized at 86.0, 35.0, and 22.0 V for L, 65.0, 30.0, and 20.0 V for DCL and 56.0, 14.0, and 11.0 V for desipramine respectively. Quadrupole 1 and quadrupole 3 were maintained at unit resolution. Dwell time set was 500 ms for both the analytes.

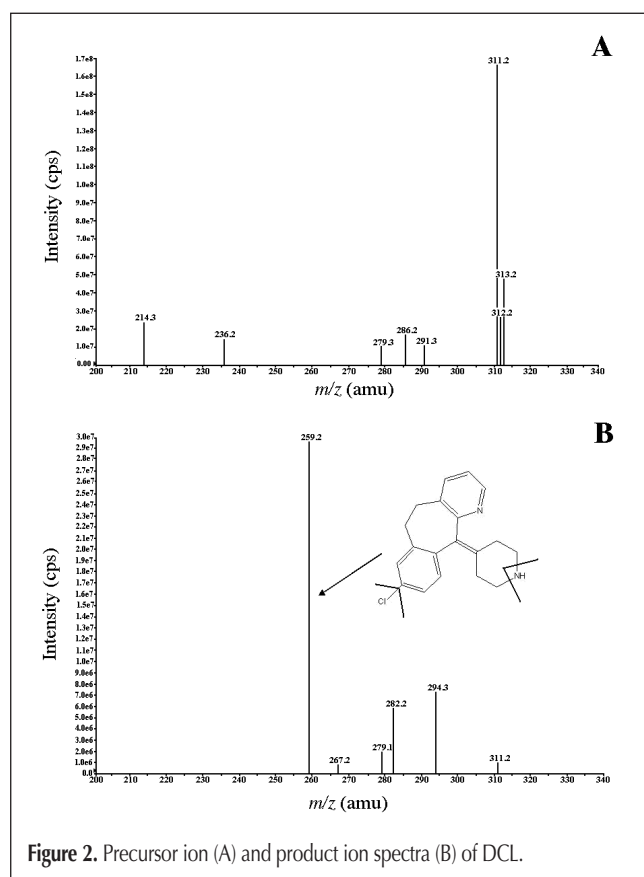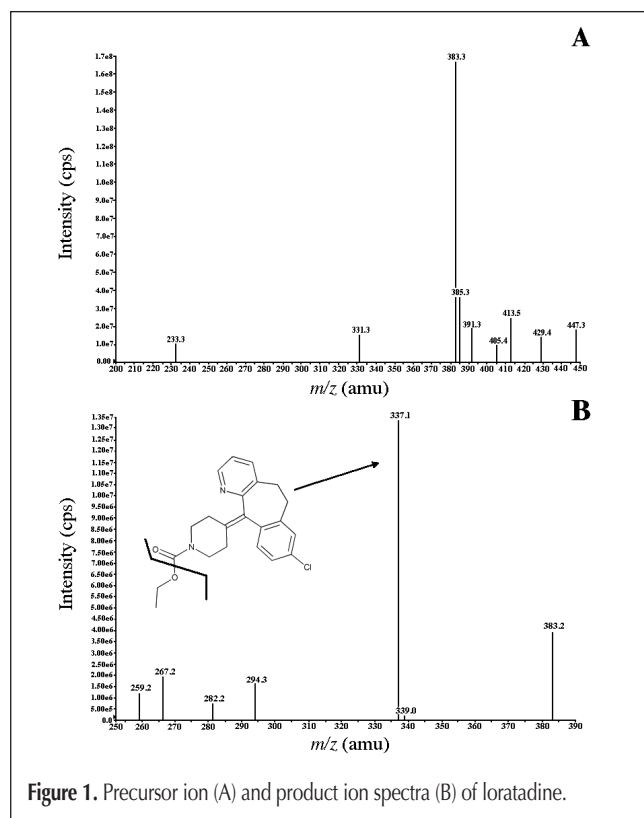### Preparation of standard stocks and plasma samples

The standard stock solutions of 100.0 µg/mL were prepared by dissolving requisite

Table I. Comparison of Analytical Methods Developed for Simultaneous Determination of L and DCL in Plasma*

Sr. No.	EP (human plasma volume)	Elution; MP; injection volume	Max. on-column loading‡	Analytical run time	Detection Technique	LLOQ	Ref. No.
1	Two step LLE with toluene (1 mL)	Gradient; Methanol/ACN/ 0.05 M KH$_2$PO$_4$ buffer; 100 µL	8 ng for L and DCL	12 min	HPLC–UV	0.5 ng/mL for L and DCL	14
2	96-well SPE (0.5 mL)‡	Isocratic; Methanol: 25mM AF (85:15 v/v); 5µL	5.55 ng for L and DCL	3.5 to 0.35 min	LC–MS–MS	1.0 ng/mL for L and DCL	16, 17
3	LLE with toluene, back extraction in FA (1 mL)	Gradient; ACN-0.1% FA (10 to 90% ACN); 30 µL	3.03 ng for L 3.04 ng for DCL	6.0 min	LC–MS–MS	0.10 ng/mL for L and DCL	23
4	LLE with hexane (1 mL)	Isocratic; ACN–H$_2$O–0.1% TFA; 35 µL	175 pg for L and 437.5 pg for DCL	3.0 min	LC–MS–MS	0.01 ng/ml L 0.025 ng/mL DCL	24
5	PP with ACN (0.3 mL)	Isocratic; 0.4% FA in ACN (92:8, v/v); 10 µL	523 pg for L and DCL	8.0 min	LC-MS/MS	0.52 ng/ml for L and DCL	25
6	LLE with 30% EA in hexane (0.5 mL)	Isocratic; Methanol: H$_2$O (70:30 v/v) + 1 mL, 1.0 M ATF; 5 µL	125 pg for L and DCL	3.0 min	LC-MS/MS	0.05 ng/mL for L and DCL	PM

* EP = extraction procedure; MP = mobile phase; PP: protein precipitation; LLE: liquid–liquid extraction; SPE: solid-phase extraction; ACN: acetonitrile; AF: ammonium formate; PM = present method; FA: formic acid; TFA: trifluoroacetic acid; ATF: ammonium trifluoroacetate; ‡Rat, rabbit, mouse and dog plasma; ‡At ULOQ level.

amount of L, DCL, and desipramine in methanol. These stock solutions were further diluted appropriately to get an intermediate concentration of 1000 ng/mL for both the analytes.

Figure 1. Precursor ion (A) and product ion spectra (B) of loratadine.

Figure 2. Precursor ion (A) and product ion spectra (B) of DCL.

Combined working solutions of L and DCL, required for spiking plasma calibration and quality control samples were subsequently prepared using the standard and intermediate stock solutions in methanol–water (80:20 v/v). IS working solution of 75.00 ng/mL was prepared diluting the stock of 100.0 µg/mL in deionized water. All the standard stock, intermediate stock and working stock solutions were prepared and stored at $4 \pm 6°C$ until use. Drug free plasma [i.e., control (blank) plasma] was withdrawn from the deep freezer and allowed to get completely thawed before use. The calibration standards (CS) and QC samples [lower limit of quantitation (LLOQ); low quality control (LQC); middle quality control (MQC); high quality control (HQC); upper limit of quantitation (ULOQ)] were prepared by spiking blank plasma with respective working solutions (5% of total volume of plasma). Calibration standards were made at 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 4.00, 9.00, 12.00, and 15.00 ng/mL for both the analytes. Quality controls were prepared at 0.05 ng/mL (LLOQ), 0.15 ng/mL (LQC), 1.30 ng/mL (MQC), 11.50 ng/mL (HQC), and 15.00 ng/mL (ULOQ) for L and DCL. The spiked plasma samples at all the levels were stored at –20°C for validation and subject sample analysis.

Procedure for sample extraction

Prior to analysis, spiked plasma samples were withdrawn from –20°C freezer and thawed for 30–45 min at room temperature. The samples were vortexed adequately using a vortex mixer before pipetting. Aliquots of 500 µL plasma were transferred into 15 × 125 mm screw cap extraction tubes; 25 µL deionized water along with 100 µL working solution of IS (75.00 ng/mL) was added and vortexed to mix. To the same tubes, 500 µL of 50mM disodium

Figure 3. Precursor ion (A) and product ion spectra (B) of desipramine (IS).
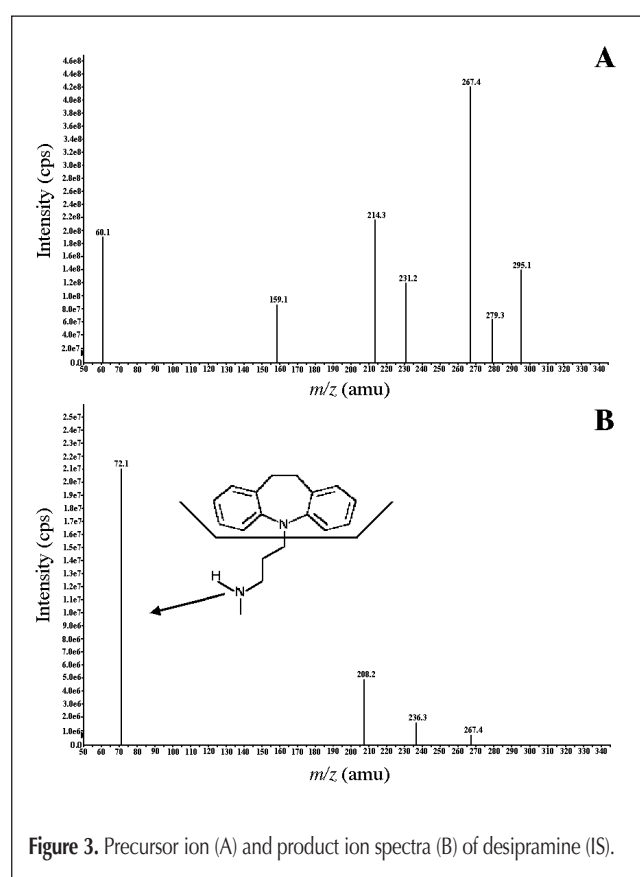
hydrogen orthophosphate buffer solution was added and vortexed again. Further, 5.0 mL of 30% ethyl acetate in hexane solution was added to all the tubes, capped, and shaken for 10 min in a platform shaker at 150 rpm for 10 min. The tubes were then centrifuged for 5 min at 3000 rpm. The organic layer was transferred to glass tubes by freezing the aqueous part in dry ice bath and evaporated at 40°C under gentle stream of nitrogen (15 psi) for 20 min. The residue was taken up in 300 µL of mobile phase, and 5 µL was used for injection in LC–MS–MS in partial loop mode.

Methodology for validation

A thorough and complete method validation of L and DCL in human plasma was done following the U.S. FDA guidelines (28). The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, stability, and dilution integrity.

The selectivity test was carried out in 10 different lots of blank plasma (with K3 EDTA as anticoagulant), processed by the same liquid–liquid extraction protocol, and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at the retention time of analytes and the internal standard. In this experiment, from each of these 10 different lots, two replicates each of 475 µL were spiked with 25 µL methanol–water solution (80:20, v/v). In the first set, the blank plasma was directly injected after extraction (without analyte and IS), while the other set was spiked with only IS before extraction (total 20 samples). Further, one system suitability sample (SSS) at CS-2 concentration and two replicates of LLOQ concentration (CS-1) were prepared by spiking blank plasma (5% of total volume of plasma) with combined working aqueous standards of L and DCL. The blank plasma sample used for spiking of SSS and LLOQ were chosen from one of these 10 lots of plasma.

The linearity of the method was determined by analysis of standard plots associated with a 10-point standard calibration curve. Five linearity curves containing 10 non-zero concentrations were analyzed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x^2$) for L and DCL. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level. The peak area ratio values of calibration standards were proportional to the concentration of the drugs in plasma over the range tested.

Intra-batch and inter-batch accuracy and precision was evaluated at five different concentrations levels (LLOQ, LQC, MQC, HQC, and ULOQ) in six replicates for both the analytes. Mean values were obtained for calculated drug concentration over these batches. The accuracy and precision was calculated and expressed in terms of % bias and coefficient of variation (% CV), respectively.

Recovery of the analytes from the extraction procedure was performed at LQC, MQC, and HQC levels. It was evaluated by comparing peak area of extracted samples (spiked before extraction) to the peak area of unextracted samples (quality control working solutions spiked in extracted plasma).

To study the effect of matrix on analyte quantitation with respect to consistency in signal (suppression/enhancement), the matrix effect was checked in six different lots of K3 EDTA plasma. Four replicates, each at LQC and HQC levels were prepared from these lots of plasma (total 48 QC samples) and checked for the accuracy in terms of % bias in all the QC samples. The specificity experiment was conducted for L, DCL, and IS at ULOQ level by comparing the peak area at their respective retention times.

Stability experiments were performed to evaluate the analyte stability in stock solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed by comparing area response of stability sample of analytes and internal standard with the area response of sample prepared from fresh stock solutions. Bench top stability (BTS), room temperature stability, refrigerated stability of extracted sample (RSS), and freeze-thaw stability were performed at LQC, MQC, and HQC levels using six replicates at each level.
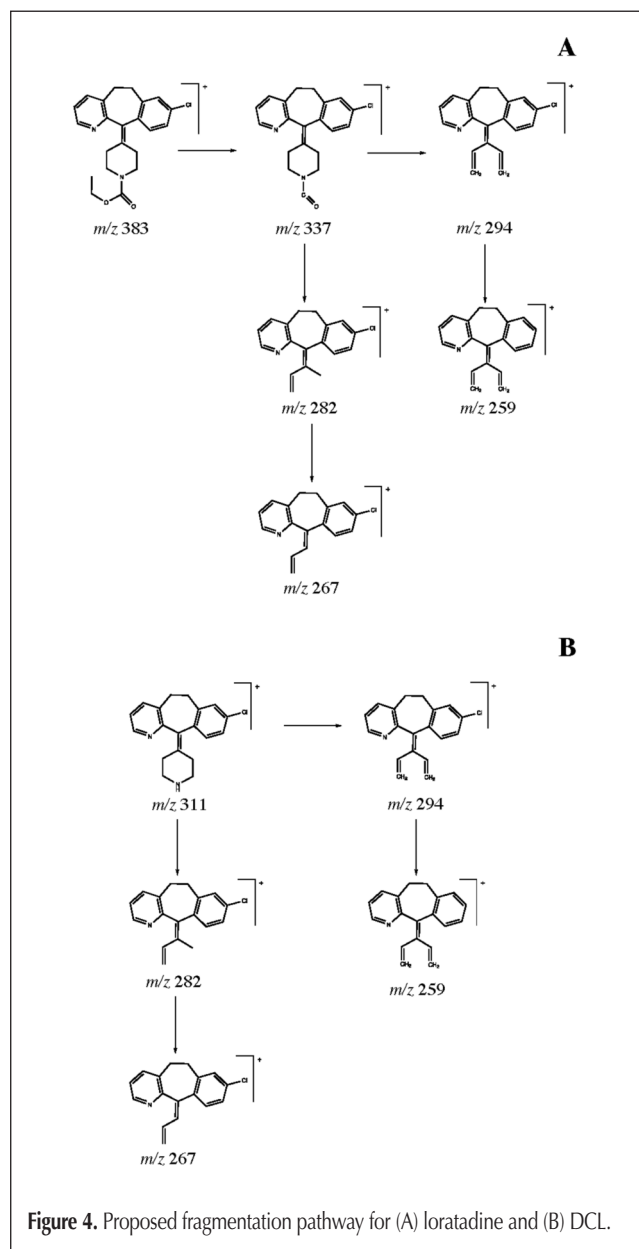

Figure 4. Proposed fragmentation pathway for (A) loratadine and (B) DCL.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (more than the ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at five times the ULOQ concentration (i.e., 75 ng/mL for L and DCL and also at HQC level for both the analytes). Six replicate samples each of 1/10 of 5 × ULOQ and 1/10 of HQC concentration were prepared and their concentrations were calculated, by applying the dilution factor of 10 against the freshly prepared calibration curve for L and DCL.

Bioequivalence study design

The design of study comprised of an open label randomized, two period, two treatment, two sequence, single dose, crossover study, comparative evaluation of relative bioavailability of test (10 mg L tablets) and reference formulation (CLARITYNE, 10 mg L tablets) in 28 healthy Indian subjects under fasting conditions. All the subjects were informed of the aim and risk involved in the study and written consent forms were obtained. Ethics committee approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by the International Conference on Harmonization and U.S. FDA (29). Health check up for all subjects was done by general physical examination, ECG, and laboratory tests like hematology, biochemistry, and urine examination. All subjects were found negative for serological tests. They were orally administered a single dose of test and reference formulation after recommended wash out period with 240 mL of water. Drinking water was not allowed and supine position was restricted 2 h post dose. Standardized meals were provided as per schedule. Blood samples were collected in vacutainers containing K3 EDTA before (0.00 h) and at 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 12.0, 24.0, 48.0, 72.0, 96.0, 120.0, and 144 h of administration of drug. Blood samples were centrifuged at 3200 rpm for 10 min and plasma was separated, stored at –20°C until use.

Results and Discussion

Method development

This bioanalytical method was developed and validated for assaying L and DCL in therapeutic concentration range for the analysis of routine samples. It was important to develop a simple and accurate method for simultaneous extraction of L and DCL from human plasma as they have different physicochemical properties (4).

MS

During method development, tuning of MS parameters in positive and negative ionization modes was carried out for L, DCL, and desipramine (IS) using 50.0 ng/mL tuning solution. However, the response observed was much higher in positive ionization mode for all three compounds compared to the negative mode due to their basic nature. Moreover, use of ammonium trifluoroacetate (1.0M) in the mobile phase further enhanced the response for both the analytes and IS with low background noise, resulting in higher sensitivity. The analytes and IS gave predominant singly charged precursor $[M]^+$ ions at m/z of 383.3, 311.2, and 267.4 for L, DCL, and IS, respectively in Q1 MS full-scan spectra. Further, fragmentation was initiated using sufficient nitrogen for collision-activated dissociation and by applying 15 V collision energy to break the precursor ions. However, the most abundant ions found in the product ion mass spectra were m/z 337.1, 259.2, and 72.1 at 35, 30, and 14V collision energy for L, DCL, and IS respectively. The fragmentation pattern for L and DCL followed a similar trend following elimination of CH_3CH_2OH from L. The pro-
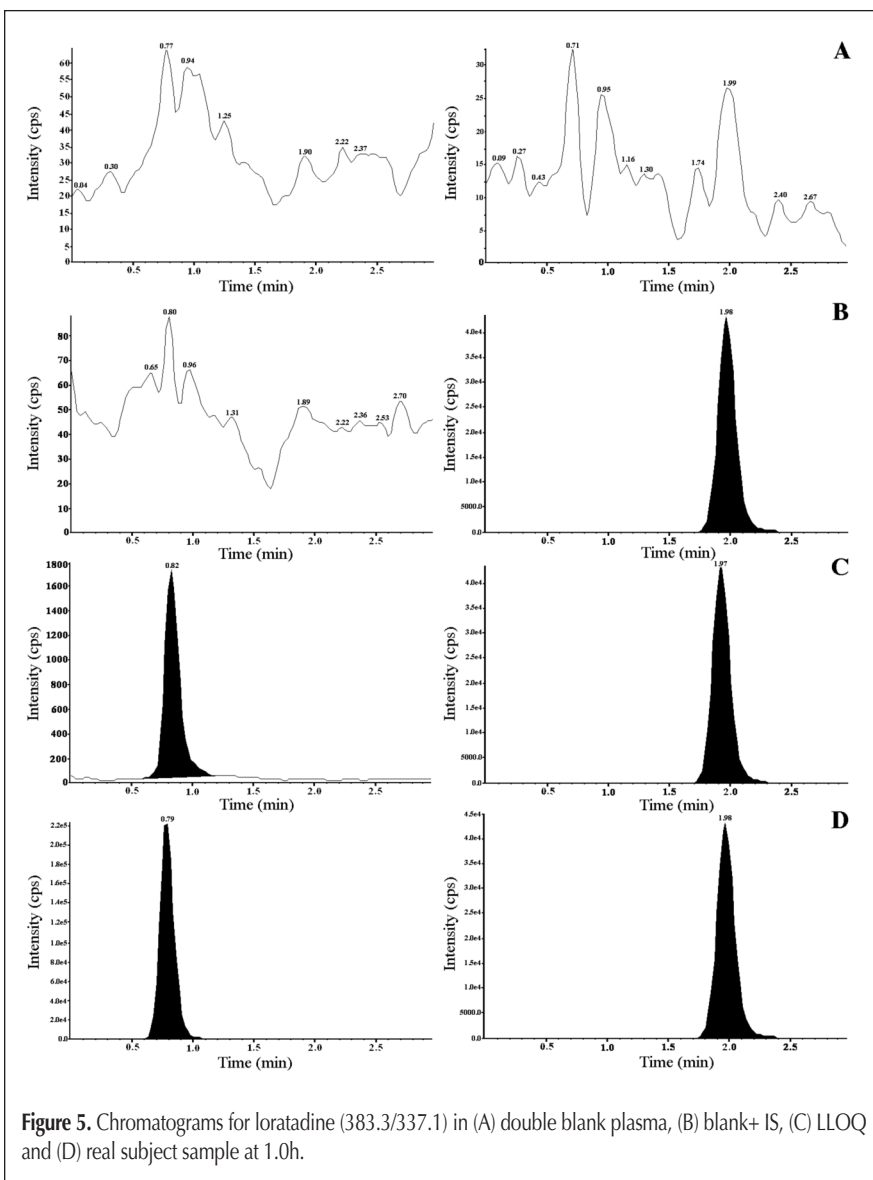

Figure 5. Chromatograms for loratadine (383.3/337.1) in (A) double blank plasma, (B) blank+ IS, (C) LLOQ and (D) real subject sample at 1.0h.

posed fragmentation pattern for L and DCL is given in Figures 4A and 4B, respectively. To attain an ideal Taylor cone and a better impact on spectral response, nebuliser gas pressure (GS1) was optimized at 55 psi due to the high flow rate (500 µL/min). Fine tuning of GS1 (nebuliser gas), GS2 (heater gas), and CAD gas was done to get a consistent and stable response. Ion spray voltage and temperature did not have significant impact on analyte response and, hence, were maintained at 4500 V and 550°C, respectively. A dwell time of 500 ms was adequate and no cross talk was observed between the MRMs of analytes and IS. Dimerization study was conducted specifically for DCL as it had one pka in the acidic range and the other in alkaline range, but no dimer formation was observed.

Sample extraction

Quantitative extraction of both the analytes and IS was difficult as they have different pka values and polarities. DCL has two pka values, pka_1 4.2 and pka_2 9.7 due to pyridine and piperidine units respectively. Naidong et al. (19) have tested different extraction procedures [liquid–liquid extraction (LLE), protein precipitation (PP), and solid phase extraction (SPE)] for L estimation in plasma. They concluded that LLE was the best extraction procedure compared to PP and SPE where the noise level was significantly high. Also, matrix suppression was observed up to 64% and 15% with PP and SPE on Oasis HLB cartridge, respectively. We verified their findings by conducting SPE on Oasis HLB cartridge for L, DCL, and IS. Indeed, the recoveries were poor and inconsistent for the analytes (50%) and IS (60%), with higher background and hence very low sensitivity. Thus, LLE was tried with different solvent systems viz. hexane, 30% ethyl acetate in hexane, dichloromethane, diethyl ether, and methyl tert-butyl ether (MTBE); still, the recoveries were not encouraging in any of these solvents. Addition of disodium hydrogen orthophosphate buffer helped in giving consistent and reproducible response for analytes and IS in 30% ethyl acetate in hexane compared to other solvents. The buffer solution assisted in breaking the drug protein binding, at the same time, maintaining the basic analytes in a nonionic lipophilic form. Significant efforts were then aimed at improving the method ruggedness during LLE and transferability by introducing flash freezing step, which helped in retaining the polar matrix in frozen aqueous phase. The mean recoveries obtained were quantitative for L and IS but were low for DCL (40.3%). The reason for this low recovery of DCL could be due to alkaline buffer which renders it partially ionized due to low pka_1 (4.2) value. Multiple extractions for quantitative recoveries were deliberately avoided as it was time consuming and was less suitable for high throughput analysis. Also, according to the U.S. FDA guidelines (27), the recovery need not be 100%, but the extent of recovery of an analyte should be consistent and reproducible at each QC level. Moreover, the validation results and subject sample analysis support this extraction methodology and hence was accepted in the present study.

LC

Because L and DCL have different pka values and polarities, it was difficult to set chromatographic conditions that produced sharp peak shape and adequate response. This included mobile phase selection, pH of buffer solution, flow rate, column type, and injection volume. Different volume ratios of methanol–water and acetonitrile–water combination were tried as mobile phase, along with ammonium trifluoroacetate, ammonium acetate, and ammonium formate buffers in varying strength on Aquasil C_{18} (100 mm × 2.1 mm i.d., 5 µm), Hypurity cyano (50 mm × 4.6 mm i.d., 5 µm), and Betabasic cyano (100 mm × 2.1mm i.d.,
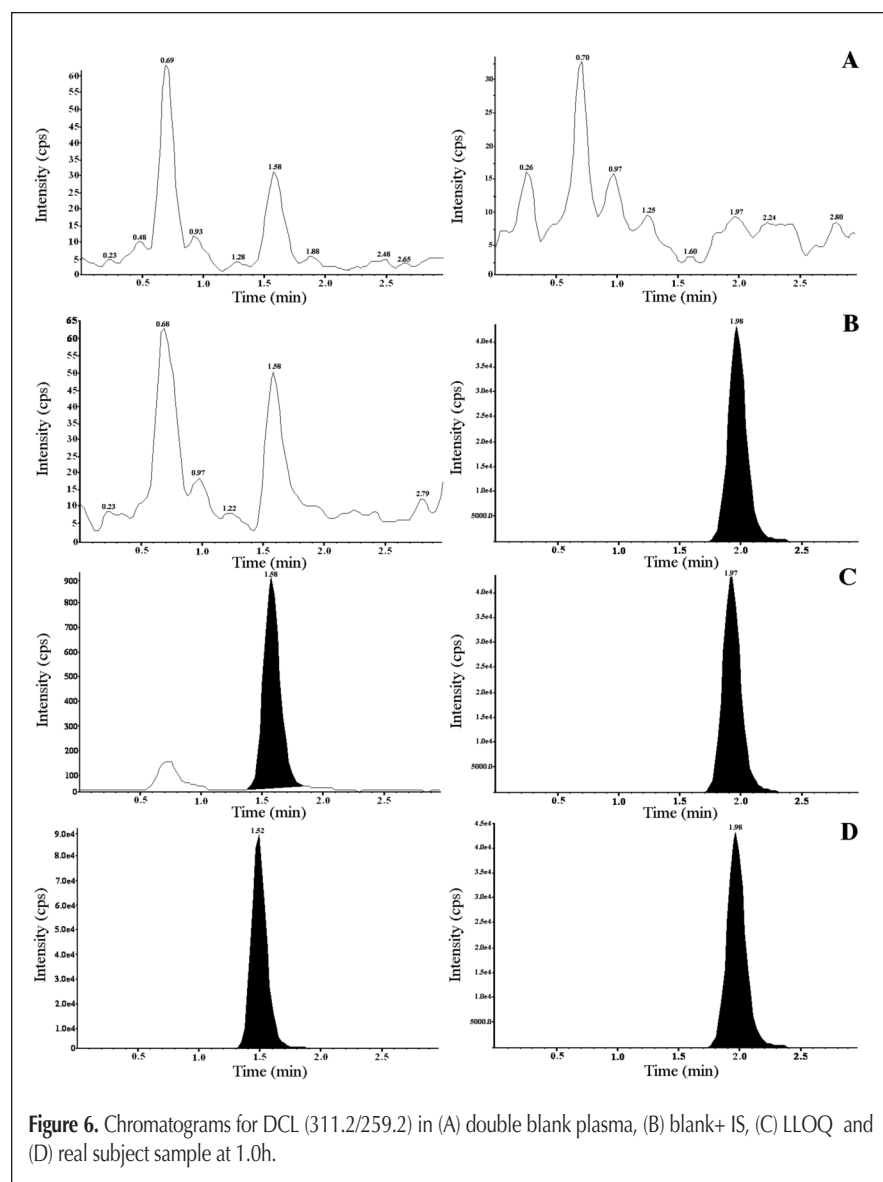

Figure 6. Chromatograms for DCL (311.2/259.2) in (A) double blank plasma, (B) blank+ IS, (C) LLOQ and (D) real subject sample at 1.0h.

5 μm). It was observed that 1.0 mL, 1M ammonium trifluoroacetate in 700 mL methanol and 300 mL water mixture as the mobile phase was most appropriate for faster elution and better efficiency and peak shape. 30% aqueous part was adequate to retain the relatively polar compound L. The use of Betabasic cyano chromatography column helped in the separation and elution of all three compounds in a very short time. The maximum on-column loading of L and DCL per sample injection was 125 pg. The total chromatographic run time was 3.0 min for each run using 500 μL/min flow rate.

As per regulatory guideline, an ideal IS should be a structurally similar analog, stable, and a labeled compound. Initially, azatadine, belonging to the same class as L was used IS in the present study, but the analyte response was significantly affected (suppressed) due to its presence. Thus, desipramine, though belonging to a different class of compounds but with some structural similarity with the analytes was tested as an IS. All three compounds had similar chromatographic behavior and were extracted easily with 30% ethyl acetate in hexane in the liquid–liquid extraction. Moreover, there was no matrix effect of IS on both the analytes. Also, the validation results obtained from this LC–MS–MS methodology encouraged its selection as an IS for the present study.

Selectivity and sensitivity

The aim of performing selectivity check with 10 different types of plasma batches (healthy Indian male subjects with K3 EDTA as an anticoagulant) was to ensure the authenticity of the results for study sample analysis. Figures 5 and 6 demonstrate the selectivity results with the chromatograms of double blank plasma (without IS), blank plasma (with IS), and the peak response of L and DCL at LLOQ (0.05 ng/mL for both) concentration. Also, the real subject sample chromatograms are presented for L and DCL at 1.5 h after oral administration of 10 mg L in these figures. The liquid–liquid extraction method employed gave very good selectivity for the analytes and IS in the blank plasma. The chromatograms show excellent peak shape for both the analytes and IS. No endogenous interferences were found at the retention times of L (0.82 min), DCL (1.58 min), and IS (1.97 min) in the blank plasma. The retention time was short for both the analytes, which makes it suitable for routine analysis. The area observed at the retention time

of L, and DCL was less than 20% of their LLOQ area, whereas it was less than 5% IS area observed in the LLOQ sample.

Linearity

The calibration curves for L and DCL were linear from 0.05–15.00 ng/mL with correlation coefficient of r^2 0.9972 within five calibration curves. The standard deviation values obtained for slope and correlation coefficient 'r' from five linearties were 0.0379 and 0.0006 for L; 0.0230 and 0.0008 for DCL, respectively. Their observed mean back calculated concentrations with accuracy (%) and precision (% CV) of five linearties are given in Table II.

Table II. Summary of Calibration Curves for L and DCL with Back Calculated Conc. (n = 5)*

ID No.	CS-1 0.0500	CS-2 0.1000	CS-3 0.2000	CS-4 0.5000	CS-5 1.000	CS-6 2.000	CS-7 4.000	CS-8 9.000	CS-9 12.00	CS-10 15.00	Slope	Intercept	r
Loratadine (conc. in ng/mL)													
1	0.0492	0.1008	0.2132	0.4990	0.9631	1.854	4.119	8.933	12.36	15.04	0.5142	0.00391	0.9991
2	0.0507	0.0965	0.2057	0.5860†	0.9388	1.889	4.328	8.463	11.92	16.41	0.5042	0.01400	0.9978
3	0.0483	0.1096	0.1861	0.5124	1.052	1.874	3.778	8.711	12.58	15.50	0.5506	0.00964	0.9979
4	0.0493	0.1057	0.1867	0.5116	1.045	1.870	3.787	8.679	12.40	16.13	0.6008	0.01214	0.9982
5	0.0493	0.1039	0.1923	0.5239	1.046	1.860	3.775	8.947	12.33	15.38	0.5435	0.01060	0.9988
M	0.0494	0.1033	0.1968	0.5117	1.009	1.869	3.957	8.747	12.32	15.69	0.5427	0.01006	0.9984
SD	0.0009	0.0050	0.0121	0.0102	0.0537	0.014	0.254	0.201	0.24	0.56	0.0379	0.00381	0.0006
%CV	1.7	4.8	6.1	2.0	5.3	0.7	6.4	2.3	2.0	3.6			
%B	–1.3	3.3	–1.6	2.3	0.9	–6.6	–1.1	–2.8	2.7	4.6			
Descarboethoxyloratadine (conc. in ng/mL)													
1	0.0514	0.0969	0.1938	0.4418	1.109	2.096	4.239	9.147	11.04	14.96	0.3322	0.00648	0.9972
2	0.0499	0.1029	0.1887	0.4937	1.032	2.070	4.410	8.468	11.28	14.89	0.3348	0.00371	0.9983
3	0.0514	0.0957	0.1964	0.4810	0.9986	2.020	4.240	9.267	11.39	15.34	0.3350	0.00066	0.9991
4	0.0531	0.0889	0.1976	0.4804	0.9798	2.060	4.314	9.251	11.39	15.53	0.3866	0.00041	0.9979
5	0.0537	0.0870	0.1956	0.4820	0.9703	2.069	4.324	9.413	11.35	15.56	0.3545	0.00113	0.9972
M	0.0519	0.0943	0.1944	0.4758	1.018	2.063	4.305	9.109	11.29	15.26	0.3486	0.00248	0.9979
SD	0.0015	0.0064	0.0035	0.0198	0.056	0.028	0.071	0.371	0.15	0.31	0.0230	0.00260	0.0008
%CV	2.9	6.8	1.8	4.2	5.5	1.3	1.6	4.1	1.3	2.1			
% B	3.8	–5.7	–2.8	-4.8	1.8	3.2	7.6	1.2	–5.9	1.7			

* CV = coefficient of variance; SD = standard deviation; M = Mean; B = Bias; r = correlation coefficient; n = total number of observation
† Value not included in calculation as it did not meet the acceptance criteria (± 15% bias).

Table III. Intra-batch and Inter-batch Accuracy and Precision*

Level	Conc. added (ng/mL)†	n	Intra-batch Mean Conc. found (ng/mL)	Bias (%)	% CV	n	Inter-batch Mean Conc. found (ng/mL)‡	Bias (%)	% CV
Loratadine									
LLOQ	0.050	6	0.047	–6.0	5.3	18	0.048	–4.0	7.7
LQC	0.150	6	0.135	–10.0	1.6	18	0.142	–5.3	6.0
MQC	1.300	6	1.194	–8.2	3.0	18	1.288	–0.9	8.1
HQC	11.50	6	11.06	–3.8	4.5	18	11.73	2.0	7.5
ULOQ	15.00	6	14.67	–2.2	6.9	18	15.52	3.5	8.6
Descarboethoxyloratadine									
LLOQ	0.050	6	0.047	–6.0	2.1	18	0.051	2.0	7.7
LQC	0.150	6	0.147	–2.0	5.6	18	0.142	–5.3	7.6
MQC	1.300	6	1.194	–8.2	3.7	18	1.211	–6.8	4.3
HQC	11.50	6	11.11	–3.4	4.4	18	10.89	–5.3	5.9
ULOQ	15.00	6	15.03	0.2	1.7	18	14.77	–1.5	5.1

* CV = coefficient of variation; n = total number of observation. † Mean of six replicate observations at each concentration.
‡ Mean of eighteen replicate observations over three different analytical runs.

Accuracy and precision

The intra-assay precision and accuracy were evaluated in five replicate analyses for L and DCL at five concentration levels viz. LLOQ, LQC, MQC, HQC, and ULOQ each on the same analytical run. Inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. Concentrations were calculated from calibration curve and the intra-batch and inter-batch precision was less than 9% for both the analytes. Accuracy expressed in terms of %bias was within –10.0 to + 3.5%. The comprehensive results for intra-assay and inter-assay accuracy and precision are given in Table III.

Table IV. Matrix Effect in Human Plasma at LQC and HQC Levels (n = 4)

	LQC (0.1500 ng/mL)		HQC (11.50 ng/mL)	
	Mean Calc. conc.	% Bias	Mean Calc. conc.	% Bias
Loratadine				
Lot-1	0.1499	–0.1	11.87	3.2
Lot-2	0.1481	–1.3	12.08	5.0
Lot-3	0.1515	1.0	12.05	4.8
Lot-4	0.1532	2.1	12.20	6.1
Lot-5	0.1521	1.4	12.22	6.3
Lot-6	0.1518	1.2	12.54	9.0
Descarboethoxyloratadine				
Lot-1	0.1452	–3.2	11.60	0.9
Lot-2	0.1415	–5.7	12.29	6.9
Lot-3	0.1436	–4.3	12.25	6.5
Lot-4	0.1490	–0.7	12.38	7.7
Lot-5	0.1481	–1.3	12.42	8.0
Lot-6	0.1415	–5.7	12.04	4.7

* n = total number of observation.

Table V. Stability Results for L and DCL (n = 6)*

Level	Loratadine			Descarboethoxyloratadine		
	A (ng/mL)	% CV	% Bias	A (ng/mL)	% CV	% Bias
Stability in biological matrix (SBM): Room temp. (48 h)						
LQC	0.1433	3.1	–4.5	0.1420	3.6	–5.3
MQC	1.288	7.5	–0.9	1.223	3.7	–5.9
HQC	11.49	6.6	–0.1	10.72	3.2	–6.8
Refrigerator stability of extracted samples (RSS): Autosampler (4°C, 93 h)						
LQC	0.1340	1.5	–10.7	0.1373	3.6	–8.5
MQC	1.169	3.0	–10.1	1.226	2.2	–5.7
HQC	10.86	4.3	–5.6	10.97	5.5	–4.6
Bench top stability of extracted samples (BTS) Room temperature (93 h)						
LQC	0.1314	2.5	–12.4	0.1424	7.0	–5.1
MQC	1.180	3.6	–9.2	1.239	2.2	–4.7
HQC	10.84	5.1	–5.7	10.99	6.0	–4.4
Freeze and thaw stability (FTS): After 6 th cycle at –20°C						
LQC	0.1399	1.3	–6.7	0.1421	3.4	–5.3
MQC	1.127	4.5	–5.6	1.268	3.1	–2.5
HQC	11.40	4.1	–0.9	11.53	6.3	0.3
Long term stability (LTS): 76 days at –20°C						
LQC	0.1312	8.9	–12.5	0.1459	4.4	–2.7
MQC	1.172	4.4	–9.8	1.256	2.9	–3.4
HQC	10.21	3.7	–11.2	11.15	2.1	–3.0

* A = mean comparison concentration;
CV = coefficient of variance; *n* = total number of observation.

Recovery

The overall mean recovery for L at LQC, MQC, and HQC levels were 89.6, 95.3, and 90.7% and that for DCL was 40.6, 42.5, and 37.8% respectively with variability (%CV) between the three quality control levels of 3.3% for L and 5.9% for DCL. The recovery of IS was found to be 61.5%. Thus, the consistency in recoveries of L, DCL, and IS upholds the extraction procedure for its application to routine sample analysis.

Matrix effect

Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy, and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS–MS method for supporting pharmacokinetics studies. Assessment of matrix effect was done with the aim to see the effect of different lots of plasma on the back calculated value of QCs nominal concentrations. The results found were well within the acceptable range (Table IV). Moreover, the minor suppression of analyte signal due to endogenous matrix does not affect the quantitation of analytes and IS. The specificity experiment indicated no inter-conversion between L and DCL, as the area of DCL observed at the retention time of L, present in the ULOQ sample was negligible (< 0.1%) and vice-versa. Also, the extraction method was rugged enough and gave accurate and consistent results when applied to healthy male subjects.

Stability

The stability experiments were performed thoroughly to evaluate their stability in stock solutions and in plasma samples under different conditions. The stability of spiked QC samples was compared with freshly prepared quality control samples. The results obtained were well within the acceptable limits. Stock solution of L was found stable at room temperature up to 6 h, while DCL and IS were stable at room temperature for 7 h. The refrigerated (4°C ± 6°C) stability for L and DCL was 38 days, while it was 16 days for IS with mean % change well within ± 7%. The intermediate solution of L and DCL in methanol–water

Table VI. Pharmacokinetic Parameters of L and DCL in 28 Indian Subjects After Oral Dose of 10 mg L Tablet Formulation

Parameter	Test Mean ± % RSD	Reference Mean ± % RSD
Loratadine		
C_{max} (ng/mL)	5.450 ± 113.969	5.467 ± 121.731
T_{max} (h)	1.202 ± 31.141	1.144 ± 29.099
$t_{1/2}$ (h)	11.044 ± 167.463	12.354 ± 170.145
$AUC_{0-144\,h}$ (h.ng/ml)	17.733 ± 129.126	19.636 ± 158.014
AUC_{0-inf} (h.ng/ml)	18.945 ± 122.905	20.977 ± 150.335
Kel	0.199 ± 74.230	0.171 ± 76.106
Descarboethoxyloratadine		
C_{max} (ng/mL)	3.159 ± 26.924	3.114 ± 24.673
T_{max} (h)	1.519 ± 65.314	1.462 ± 25.460
$t_{1/2}$ (h)	21.149 ± 24.438	22.231 ± 30.032
$AUC_{0-144\,h}$ (h.ng/ml)	41.098 ± 35.380	42.710 ± 39.964
AUC_{0-inf} (h.ng/ml)	43.261 ± 33.941	45.068 ± 38.342
Kel	0.035 ± 22.928	0.034 ± 26.139

(80:20 v/v) was stable for 13 days. Both the analytes were found stable in controlled plasma at room temperature up to 48 h and for at least six freeze and thaw cycles. The analytes in extracted plasma samples were stable for 93 h under refrigerated conditions of $4°C \pm 6°C$. Bench-top stability of extracted samples was also up to 93 h. The L and DCL spiked plasma samples stored at $-20°C \pm 10°C$ for long term stability were found stable for minimum period of 76 days. The values for the percent change for the above stability experiments are compiled in Table V.

Dilution integrity

The mean back-calculated concentrations for 1/10 dilution samples were within 85–115% of their nominal values. The precision (%CV) for 1/10 dilution samples was < 5.0 for both the analytes.

Application of the method on human subjects

Pharmacokinetic studies of L and DCL in Caucasian (3,7,15) and Chinese (26,27) subjects have been reported, but no literature on Indian subjects is available. Thus, the proposed validated method was applied for the assay of L and DCL in 28 healthy Indian adult male subjects who received 10 mg test and reference formulations of L under fasted condition. The samples were processed based on the proposed extraction protocol for quantification of L and DCL. The method was sensitive enough to monitor their plasma concentration up to 144 h. All 1440 samples, including the calibration, QC, and volunteer samples were run and analyzed in only eight days and the precision accuracy for calibration and QC samples were well within the acceptable limits. The mean pharmacokinetic profile for the treatment, under fasting condition, is presented in Figure 7. The pharmacokinetic parameters viz. maximum plasma concentration C_{max}, area under the plasma co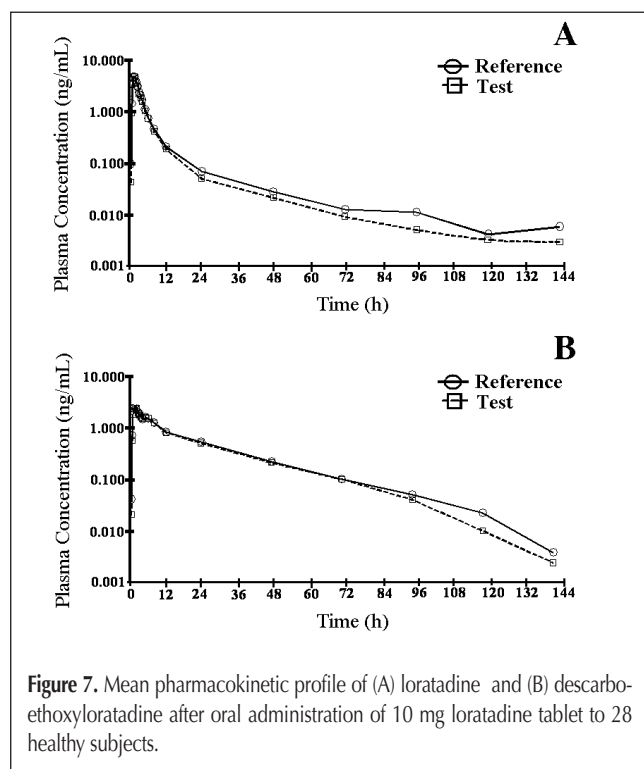ncentration-time curve from zero hour to the last measurable concentration AUC_{0-t}, area under the plasma concentration-time curve from zero hour to infinity $AUC_{0-\infty}$, time point of maximum plasma concentration curve T_{max}, elimination rate constant K_{el}, and half life of drug elimination during the terminal phase $t_{1/2}$ were calculated for L and DCL. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table VI. These observations confirm the bioequivalence of 10 mg test sample with the reference product in terms of rate and extent of absorption. It is known from earlier studies (27) that the parameters such as AUC, C_{max}, and $t_{1/2}$ for L are extremely variable and exhibit significant inter-individual variability due to its first-pass metabolism as compared to DCL. In this study, the results for T_{max} values of L and DCL were 1.202 and 1.519 h, respectively, which are in agreement with the data reported for Chinese (27) and Caucasian (3) subjects. The $t_{1/2}$ values for L and DCL were more close to Caucasian than Chinese subjects. However, the AUC for both the analytes were much lower in Indian subjects compared to others for 10 mg dose. Similarly, C_{max} was much lower in Indian subjects compared to Caucasian and Chinese subjects. Further, there was no adverse event during the course of the study. Thus the assay procedure for L in plasma samples demonstrated the linearity, precision, and sensitivity needed for the pharmacokinetic studies of this drug.

Conclusion

The objective of this work was to develop a simple, cost-effective, rugged, and a high throughput method for simultaneous estimation of L and its active metabolite DCL in human plasma, especially in the absorption and elimination phase after oral administration of 10 mg formulation. The advantage of using LLE in the present work is due to significant reduction in the labour commonly associated with liquid–liquid extraction technique on account of flash freezing of the aqueous part. The run time per sample analysis of 3.0 min suggests the high throughput of the proposed method. The maximum on-column loading of L and DCL was 125 pg per injection volume of 5 μL. This was considerably less compared to other reported procedures, which helps in extending the lifetime of the column. Moreover, the limit of quantification is low enough to monitor at least five half-lives of L and DCL concentration with good intra- and inter-assay reproducibility (%CV) for the quality controls. From the results of all the validation parameters, the method can be useful for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for the clinical trial samples with desired precision and accuracy.

Figure 7. Mean pharmacokinetic profile of (A) loratadine and (B) descarboethoxyloratadine after oral administration of 10 mg loratadine tablet to 28 healthy subjects.

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