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Validation of a sensitive and automated 96-well solid-phase extraction liquid chromatography–tandem mass spectrometry method for the determination of desloratadine and 3-hydroxydesloratadine in human plasma

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Abstract

To support clinical development, a liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method was developed and validated for the determination of desloratadine (descarboethoxyloratadine) and 3-OH desloratadine (3-hydroxydescarboethoxyloratadine) concentrations in human plasma. The method consisted of automated 96-well solid-phase extraction for sample preparation and liquid chromatography/turbo ionspray tandem mass spectrometry for analysis. [$^2H_4$]Desloratadine and [$^2H_4$]3-OH desloratadine were used as internal standards (I.S.). A quadratic regression (weighted 1/concentration^2) gave the best fit for calibration curves over the concentration range of 25–10 000 pg/ml for both desloratadine and 3-OH desloratadine. There was no interference from endogenous components in the blank plasma tested. The accuracy (%bias) at the lower limit of quantitation (LLOQ) was −12.8 and +3.4% for desloratadine and 3-OH desloratadine, respectively. The precision (%CV) for samples at the LLOQ was 15.1 and 10.9% for desloratadine and 3-OH desloratadine, respectively. For quality control samples at 75, 1000 and 7500 pg/ml, the between run %CV was ≤7.5% for desloratadine and ≤6.3% for 3-OH desloratadine. Between run %bias ranged from 4.1 to 8.0% for desloratadine and −11.5 to −4.8% for 3-OH desloratadine. Desloratadine and 3-OH desloratadine were stable in human plasma for 401 days at −22 °C, after five freeze/thaw cycles, up to 24 h at room temperature, and in reconstituted sample extracts (up to 185 h at 5 °C). This LC–MS–MS method for the determination of desloratadine and 3-OH desloratadine in human plasma met regulatory requirements for selectivity, sensitivity, goodness of fit, precision, accuracy and stability.

Keywords: Desloratadine; 3-Hydroxydesloratadine

1. Introduction

High throughput quantitative determination of drugs and their metabolites in pharmaceutical

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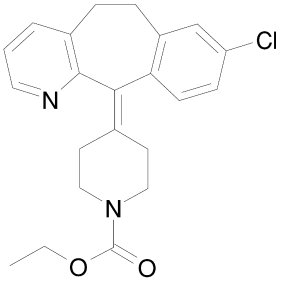
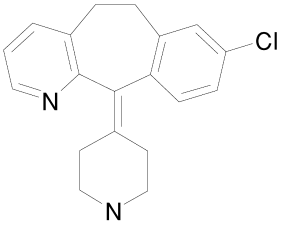
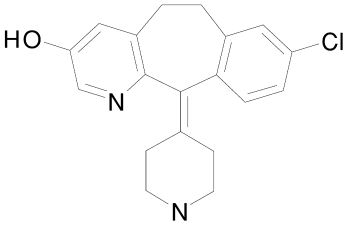
bioanalysis is usually done with liquid chromatography–tandem mass spectrometry (LC–MS–MS) [1–7]. Sample preparation is accomplished using protein precipitation, liquid–liquid extraction or solid-phase extraction with robotic liquid handling systems and 96- or 384-well plates. On-line sample preparation techniques such as turbulent flow chromatography or the Prospekt system for solid-phase extraction have also been used for high throughput bioanalysis [8–10]. The detector of choice is a triple quadrupole mass spectrometer equipped with either an electrospray or an atmospheric pressure chemical ionization ion source.



Loratadine



desloratadine



3-OH desloratadine

Fig. 1. Chemical structures of loratadine, desloratadine and 3-OH desloratadine.

Desloratadine, a major active metabolite of loratadine, is a selective, potent, orally active, peripheral H_1 receptor antagonist while 3-OH desloratadine is a major active metabolite of both desloratadine and loratadine (Fig. 1). Clinical studies have demonstrated that desloratadine 5 mg (as compared to loratadine 10 mg) effectively relieves the signs and symptoms of seasonal allergic rhinitis in patients with this disease [11]. Sutherland et al. have published a manual liquid–liquid extraction LC–MS–MS method for the determination of loratadine and desloratadine with an LLOQ of 0.1 ng/ml [12]. A solid-phase extraction LC–MS–MS method for the determination of desloratadine and 3-OH desloratadine in human plasma with an LLOQ of 25 pg/ml was developed and validated in our laboratory and presented at the 2001 Pharmaceutical Congress of the Americas [13]. In this paper, we describe the development and validation of this method. The validated method has been used to support the desloratadine clinical program. An example of a PK profile from a clinical study is also shown.

As part of the validation, the stability of desloratadine and 3-OH desloratadine in human plasma under conditions expected during typical sample storage, preparation, and analysis, was evaluated. This LC–MS–MS method met acceptance criteria for bioanalytical method validation outlined in the Crystal City guidelines [14].

2. Experimental

2.1. Materials

2.1.1. Test compounds and internal standards

Desloratadine was synthesized by MDS Pharma Services (Montreal, Canada) with a purity of 98.3%. 3-OH Desloratadine with a purity of 99.3% was synthesized at Schering-Plough Research Institute. The internal standards, [$^2H_4$]desloratadine (purity 99.9%) and [$^2H_4$]3-OH desloratadine (purity 100%)

were also synthesized at Schering-Plough Research Institute.

2.1.2. Biological matrix

Human plasma with heparin as the anticoagulant (Biological Specialties Corporation, Colmar, PA, USA) was used for the preparation of calibration standards and quality control (QC) samples. All plasma lots were chromatographically screened for interfering substances prior to use. Plasma blanks were run as part of each validation run to check for interfering substances.

2.1.3. Reagents

Analytical-grade methanol and acetonitrile (EM Science, Gibbstown, NJ, USA), ammonium acetate, ammonium formate, acetic acid, and formic acid (BDH, Toronto, Ontario, Canada), and type 1 water (i.e. ultrapure, organic- and ion-free) were used.

2.2. Methods

2.2.1. Stock solutions

Stock solutions of desloratadine (100 μg/ml) and 3-OH desloratadine (100 μg/ml) in methanol were used to prepare spiking solutions for the calibration standards. Separate stock solutions of desloratadine and 3-OH desloratadine were used to prepare spiking solutions for the QC samples. The calibration standards and QC samples prepared from these stock solutions were used to evaluate the inter- and intra-assay accuracy and precision, and to determine the lower limit of quantitation (LLOQ). Stock solutions of [$^2H_4$]desloratadine and [$^2H_4$]3-OH desloratadine (I.S.) were prepared in methanol to generate an internal standard working solution (ISWS) at a nominal concentration of 4.00 ng/ml in 100 m*M* ammonium acetate pH 6. Stock solutions of the study compounds and I.S. were screened via chromatography and mass spectrometry for interfering substances prior to use.

2.2.2. Calibration standards and quality control samples

Spiking solutions of desloratadine and 3-OH desloratadine were added to plasma to achieve the required concentrations. A set of nine calibration standard plasma pools, ranging from 25 to 10 000 pg/ml for both desloratadine and 3-OH desloratadine, were prepared in triplicate and stored at −22 °C. For stability assessments, calibration standards were freshly prepared. The QC samples were stored at −22 °C until analyzed. Each validation run consisted of calibration standards in triplicate and six replicates of QC samples at low (75 pg/ml), medium (1000 pg/ml), and high (7500 pg/ml) concentrations. In addition, six replicates of QC samples at the lower limit of quantitation (LLOQ, 25 pg/ml) were analyzed in one of the validation runs to determine accuracy and precision. The ability to perform dilution was validated by analyzing QC samples at 7500 and 20 000 pg/ml that were diluted 10-fold.

2.2.3. Extraction procedure

Each plasma sample was thawed at room temperature then vortexed and centrifuged at 1845 *g* for 5 min at room temperature. With the use of an automatic pipette, 500 μl of sample was aliquoted into a titertube, into which 250 μl of [$^2H_4$]desloratadine and [$^2H_4$]3-OH desloratadine ISWS (4.00 ng/ml) was subsequently added. For blanks, 250 μl of 100 m*M* ammonium acetate pH 6.0 was added instead of ISWS. The extraction procedure was carried out using a TOMTEC™ extractor and 3M Empore C_{18} SD 96-well extraction disk plates (3M, Saint Paul, MN, USA). The C_{18} disk plates were conditioned with 1.0 ml of methanol and 1.0 ml of type 1 water. Each sample was loaded onto the plates in three aliquots. Once the sample had completely flowed through the plate, the plate was washed with 1.0 ml of type 1 water. The analytes and their respective I.S. were then eluted into a collection plate using 150 μl of solution B [2 m*M* ammonium acetate, 0.1% acetic acid, 0.1% formic acid in 50:50 (v/v) acetonitrile in methanol]. The C_{18} disk plates were then washed with 150 μl of solution A (2 m*M* ammonium acetate, 0.1% acetic acid, 0.1% formic acid in water). An aliquot of 20 μl was injected into a high-performance liquid chromatograph equipped with a MS–MS detector.

2.3. LC–MS–MS conditions

Desloratadine and 3-OH desloratadine were analyzed using a Perkin-Elmer Sciex (Applied Biosystems/MDS Sciex, Ontario, Canada) API 3000

triple quadrupole mass spectrometer interfaced to a Perkin-Elmer (Wellesley, MA, USA) Series 200 liquid chromatograph. Chromatographic separation was achieved using a Keystone Scientific Inc. (Bellefonte, PA, USA) analytical column (BDS-C_8, 100×2 mm, 5-μm particle size) at room temperature. A Keystone guard column (BDS C_8 20×2 mm, 5-μm particle size) was also used to protect the analytical column. The mobile phase consisted of mobile phase A [(20:80, v/v) acetonitrile/H_2O in 2 m*M* ammonium acetate, 0.1% acetic acid, and 0.1% formic acid] and mobile phase B [(85:15, v/v) methanol/25 m*M* ammonium formate pH 3.5], at a flow-rate of 250 μl/min. The injection volume was 20 μl, and the run time was 5.9 min. A gradient elution (Table 1) was used. Representative retention times for desloratadine, 3-OH desloratadine, [$^2H_4$]desloratadine (I.S.), and [$^2H_4$]3-OH desloratadine (I.S.) were 4.18, 4.02, 4.18, and 4.02 min, respectively.

The PE-Sciex API 3000 mass spectrometer was operated in the positive ion selected reaction monitoring (SRM) mode using turbo ionspray ionization. The ionspray voltage was 4500 V, the declustering potential 40 V and the collision energy 31 V. The following SRM transitions were monitored for the analytes and internal standards: desloratadine, *m*/*z* 311.2 to *m*/*z* 259.1; [$^2H_4$]desloratadine, *m*/*z* 315.2 to *m*/*z* 263.1; 3-OH desloratadine, *m*/*z* 327.2 to *m*/*z* 275.1; [$^2H_4$]3-OH desloratadine, *m*/*z* 331.2 to *m*/*z* 279.1. Dwell time was 150 ms for each transition.

The data were collected using an Apple Quadra computer with PE-SCIEX acquisition software, Sample Control 1.4. Peak integration was done using MacPhase 1.3.1 software (MDS Pharma Services, Montreal, Canada). Regression calculations and QC sample concentrations were determined by inverse-prediction using PhIRST, a software system developed and validated by MDS Pharma Services (Montreal, Canada).

3. Results and discussion

3.1. Sample extraction

The 96-well solid-phase extraction approach was selected for the following two reasons: first, a clean extract was required to minimize any matrix effects so that we could obtain adequate sensitivity at the required LLOQ of 25 pg/ml. Secondly, since this method would be used to support large clinical studies, automation of the extraction procedure was considered as a way to increase sample throughput.

Among the commonly used extraction methods, protein precipitation, solid-phase extraction, and liquid–liquid extraction were all considered during method development. Although protein precipitation was most easily automated in a 96-well format, at an LLOQ of 25 pg/ml the matrix effect was too substantial. Liquid–liquid extraction was thought to potentially provide the cleanest extracts; however, automation in a 96-well format was deemed too difficult, especially with a relatively large sample volume of 0.5 ml. Solid-phase extraction, on the other hand, was not only very easy to automate but also gave relatively clean extracts that allowed us to achieve an adequate signal at the LLOQ.

Initially, samples were eluted with two 150-μl aliquots of solution B resulting in recoveries of approximately 75–80% for desloratadine and 3-OH desloratadine. However, to maintain good chromatographic peak shape for the analytes, the processed samples needed to be evaporated and reconstituted in a solution with high aqueous content (i.e. a mixture of solution A and solution B). To limit the tedious

Table 1
Gradient elution program

Step	Time (min)	Duration (min)	Flow (μl/min)	Mobile Phase A (%)	Mobile Phase B (%)
0	−0.1	0.10	250	100	0
1	0.0	0.30	250	100	0
2	0.3	2.50	250	10	90
3	2.8	1.10	250	10	90
4	3.9	2.00	250	100	0

step of evaporation and reconstitution, solution A was used in the second aliquot resulting in final processed samples with approximately 50% aqueous solution. This resulted in a decrease in recovery of the analytes to approximately 50%. Nevertheless, recoveries were consistent, chromatographic peak shapes were relatively symmetric, and adequate sensitivity was obtained at the LLOQ for both analytes.

3.2. Tandem mass spectrometry

Full scan and product ion mass spectra for desloratadine and 3-OH desloratadine were obtained in the positive ion mode. The full-scan mass spectra of desloratadine and 3-OH desloratadine contained signals for the protonated molecular ions at $m/z = 311.2$ and m/z 327.2, respectively. The product ion mass spectra of desloratadine and 3-OH desloratadine contained predominant fragment ions at $m/z = 259.1$ and m/z 275.1 (Fig. 2). Consequently, the transitions from m/z 311.2 to m/z 259.1 and m/z 327.2 to m/z 275.1 were monitored in the SRM mode for the quantitation of desloratadine and 3-OH desloratadine, respectively. Similarly, for the internal standards, which both contain four deuteriums, the transitions from m/z 315.2 to m/z 263.1 and m/z 331.2 to m/z 279.1 were monitored for [$^2H_4$]desloratadine and [$^2H_4$]3-OH desloratadine, respectively.

3.3. Separation

Apart from the 3-OH desloratadine metabolite, hydroxylated metabolites at other positions were also present in preclinical species. Because these mono-hydroxylated metabolites have the same molecular masses and very similar product ion spectra, they could not be separated by mass to charge ratio. Consequently, to quantify 3-OH desloratadine, a chromatographic system needed to be developed that separated the 3-OH from other potentially interfering mono-hydroxylated metabolites. This was achieved using gradient elution and a 2×100 mm, 5-μm Keystone BDS-C_8 column with a flow-rate of 0.25 ml/min. As shown in Fig. 3, using these chromatographic conditions, 3-OH desloratadine, was separated from three other mono-hydroxylated metabolites.

3.4. Sensitivity

Accuracy (%bias) and precision (%CV) at the LLOQ level were determined in one of the validation runs in which six replicates of LLOQ QC samples (25 pg/ml) for desloratadine and 3-OH desloratadine were processed and analyzed along with other QC samples and standards. The %bias was −12.8 and 3.4% for desloratadine and 3-OH desloratadine, respectively (Table 2). The %CV was 15.1 and 10.9% for desloratadine and 3-OH desloratadine, respectively. Representative chromatograms of a calibration standard at the LLOQ are shown in Fig. 4. For comparison, representative chromatograms of desloratadine, 3-OH desloratadine and the I.S. from an extracted upper limit of quantitation (ULOQ) calibration standard at 10 ng/ml are shown in Fig. 5.

3.5. Selectivity

Blank plasma (20 different lots) did not contain any interference from endogenous components or other sources at the retention time of the analytes and I.S. Representative chromatograms of an extracted blank sample are presented in Fig. 6 demonstrating that there was no interference at the retention times of either desloratadine or 3-OH desloratadine.

3.6. Calibration curve regression

A quadratic regression (weighted 1/concentration^2) gave the best fit for the concentration/detector response relationship for desloratadine and 3-OH desloratadine in human plasma. Coefficients of determination (r^2) for the validation runs were greater than 0.992 for both desloratadine and 3-OH desloratadine.

3.7. Accuracy and precision

For calibration standards, between run accuracy (%bias) ranged from −2.8 to 3.8% and −3.8 to 4.1% for desloratadine and 3-OH desloratadine, respectively. Between run precision (%CV) ranged

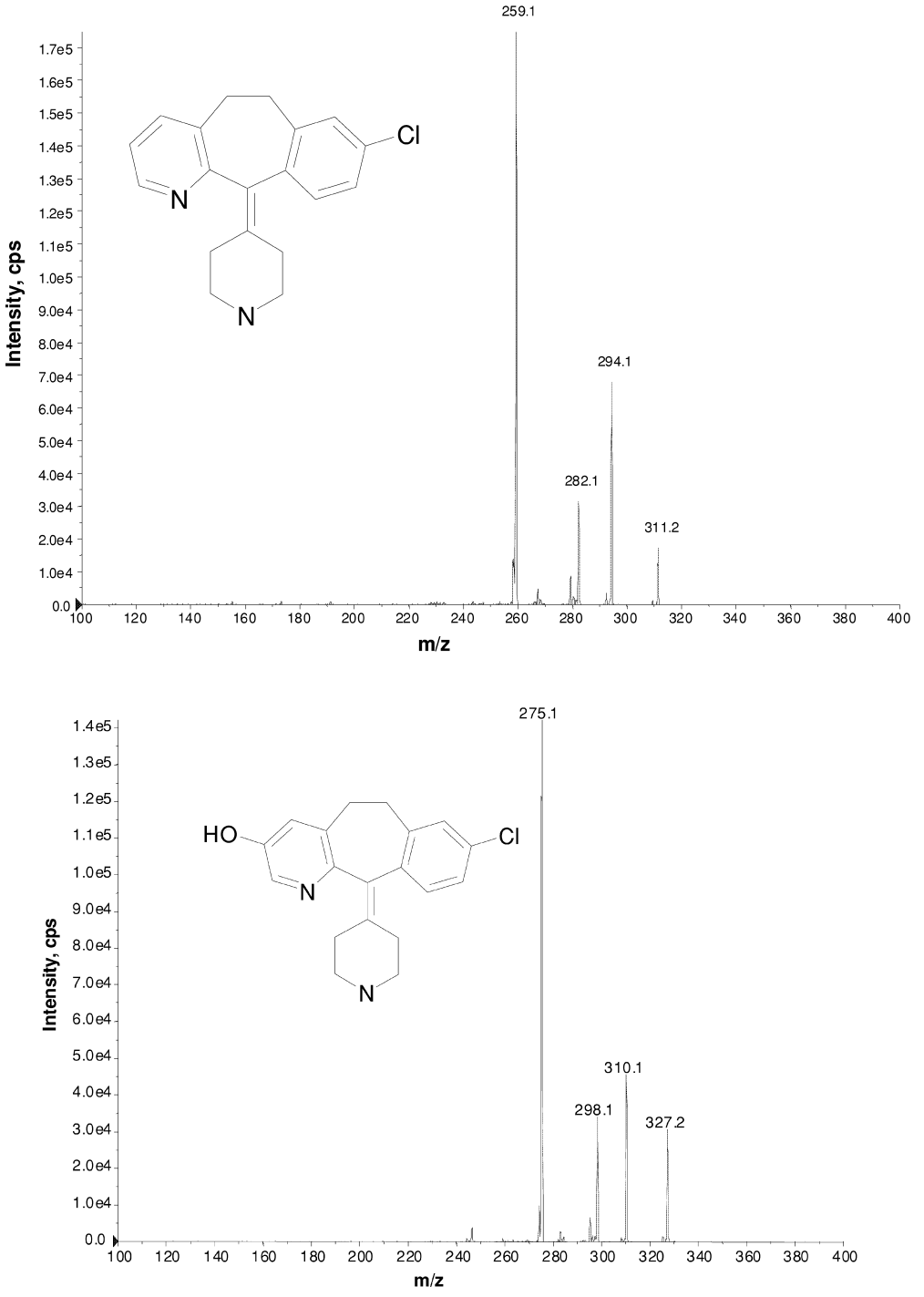


Fig. 2. Product ion mass spectra for (1) desloratadine, (2) 3-OH desloratadine.

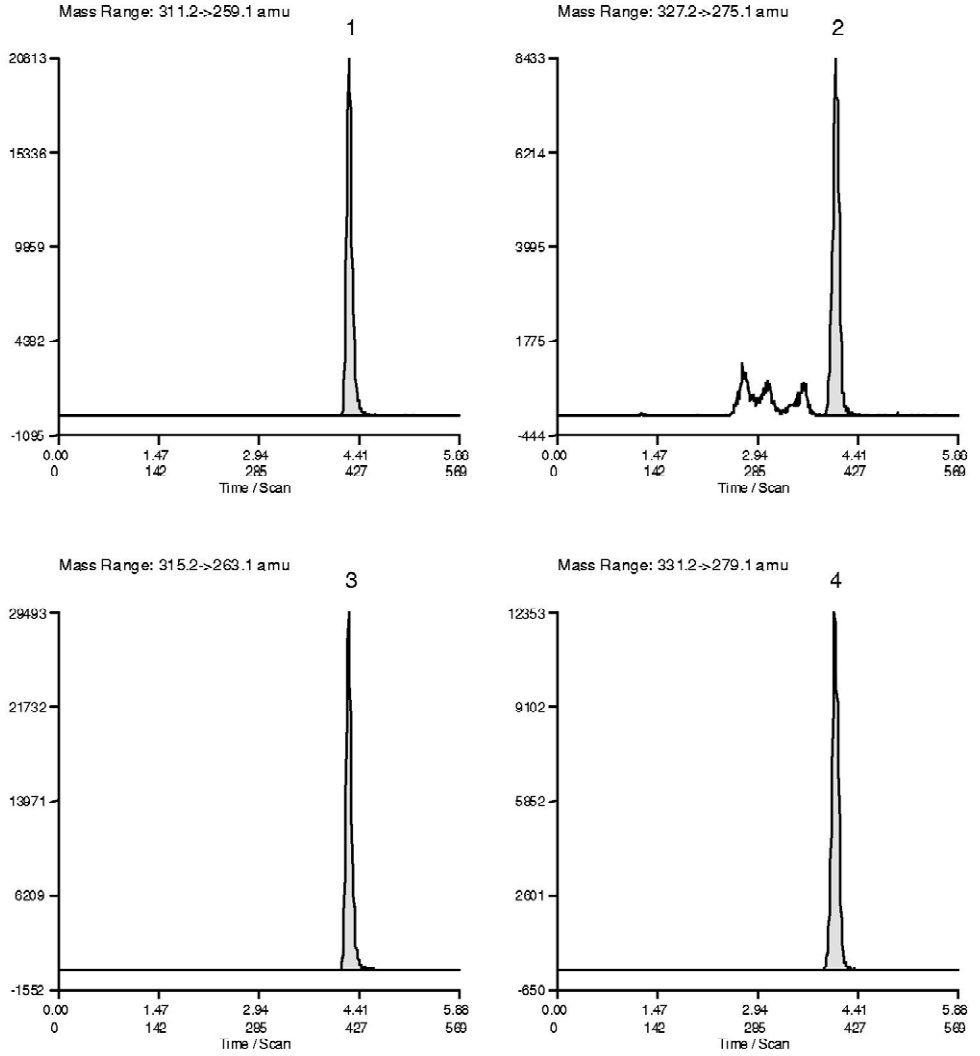


Fig. 3. Selected reaction ion chromatograms for a spiked human plasma sample demonstrating separation of 3-OH desloratadine from three other mono-hydroxylated metabolites: (1) desloratadine, (2) 3-OH desloratadine, (3) [$^2H_4$]desloratadine, and (4) [$^2H_4$]3-OH desloratadine.

Table 2
Quality control sample concentrations of desloratadine and 3-OH desloratadine in human plasma (between-run precision and accuracy)

	Desloratadine QC (pg/ml)				3-OH Desloratadine QC (pg/ml)			
	LLOQ 25[a]	Low 75[a]	Medium 1000[a]	High 7500[a]	LLOQ 25[a]	Low 75[a]	Medium 1000[a]	High 7500[a]
Mean	21.8	77.9	1078	7985	25.9	66.3	952	7092
%CV	15.1	7.5	3.5	2.4	10.9	6.3	3.9	3.2
n	6	18	18	18	6	18	18	18
%Bias[b]	−12.8	4.1	8.0	6.7	3.4	−11.5	−4.8	−5.5

[a] Nominal value (pg/ml).

[b] $\%\ \text{Bias} = \dfrac{(\text{Mean} - \text{Nominal})}{\text{Nominal}} \times 100.$

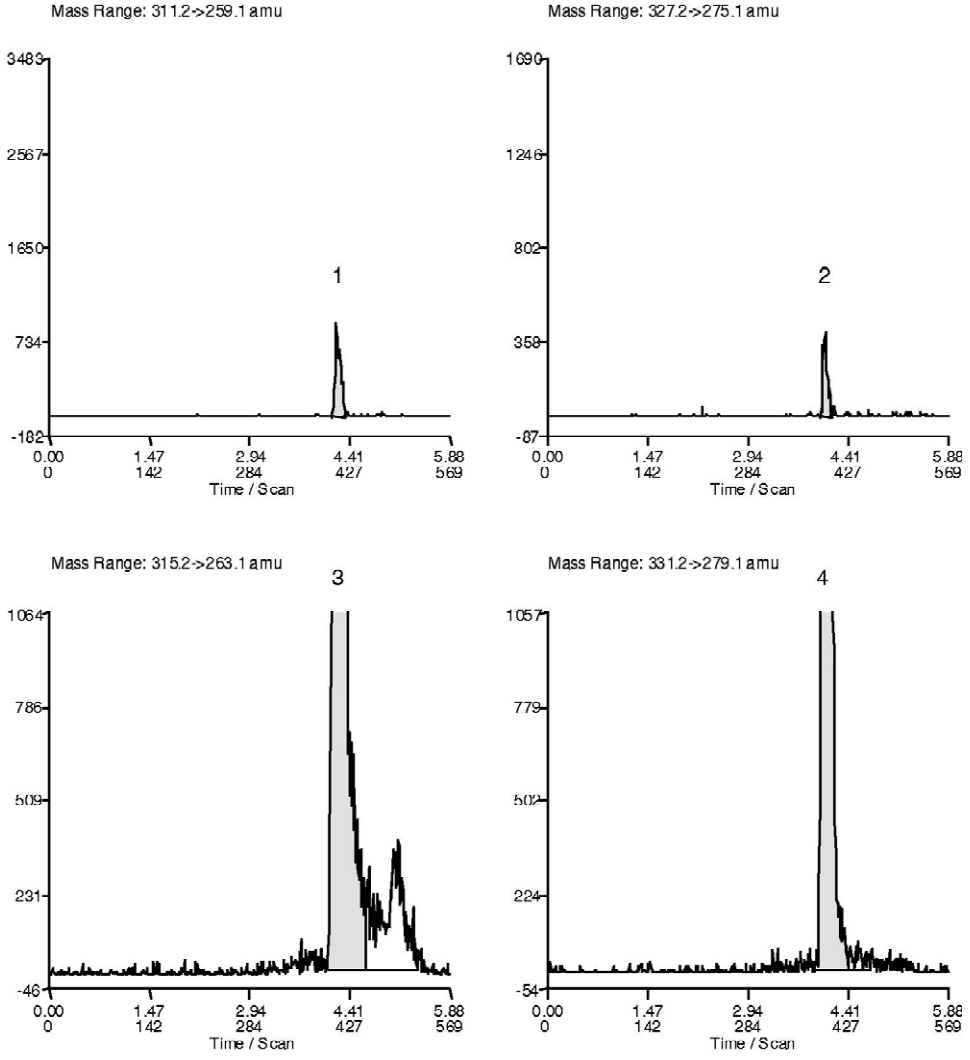


Fig. 4. Selected reaction ion chromatograms for a calibration curve standard at the LLOQ, 25 pg/ml: (1) desloratadine, (2) 3-OH desloratadine, (3) [$^{2}H_{4}$]desloratadine, and (4) [$^{2}H_{4}$]3-OH desloratadine.

from 1.9 to 7.2% and 1.7 to 9.2% for desloratadine and 3-OH desloratadine, respectively.

Between run precision and accuracy results from QC samples for desloratadine and 3-OH desloratadine in human plasma are summarized in Table 2. For desloratadine, between run %bias was 4.1, 8.0 and 6.7% for low, medium and high QC concentrations, respectively. For 3-OH desloratadine, between run %bias was −11.5, −4.8 and −5.5% for low, medium and high QC concentrations, respectively. For desloratadine, between-run %CV was 7.5, 3.5 and 2.4% for low, medium and high QC concentrations, respectively. For 3-OH desloratadine, between run %CV was 6.3, 3.9 and 3.2% for low, medium, and high QC concentrations, respectively.

3.8. Recovery

Recoveries of desloratadine and 3-OH desloratadine from plasma were determined by comparing the peak areas of the QC samples ($n=6$) that were spiked before extraction to the peak areas of

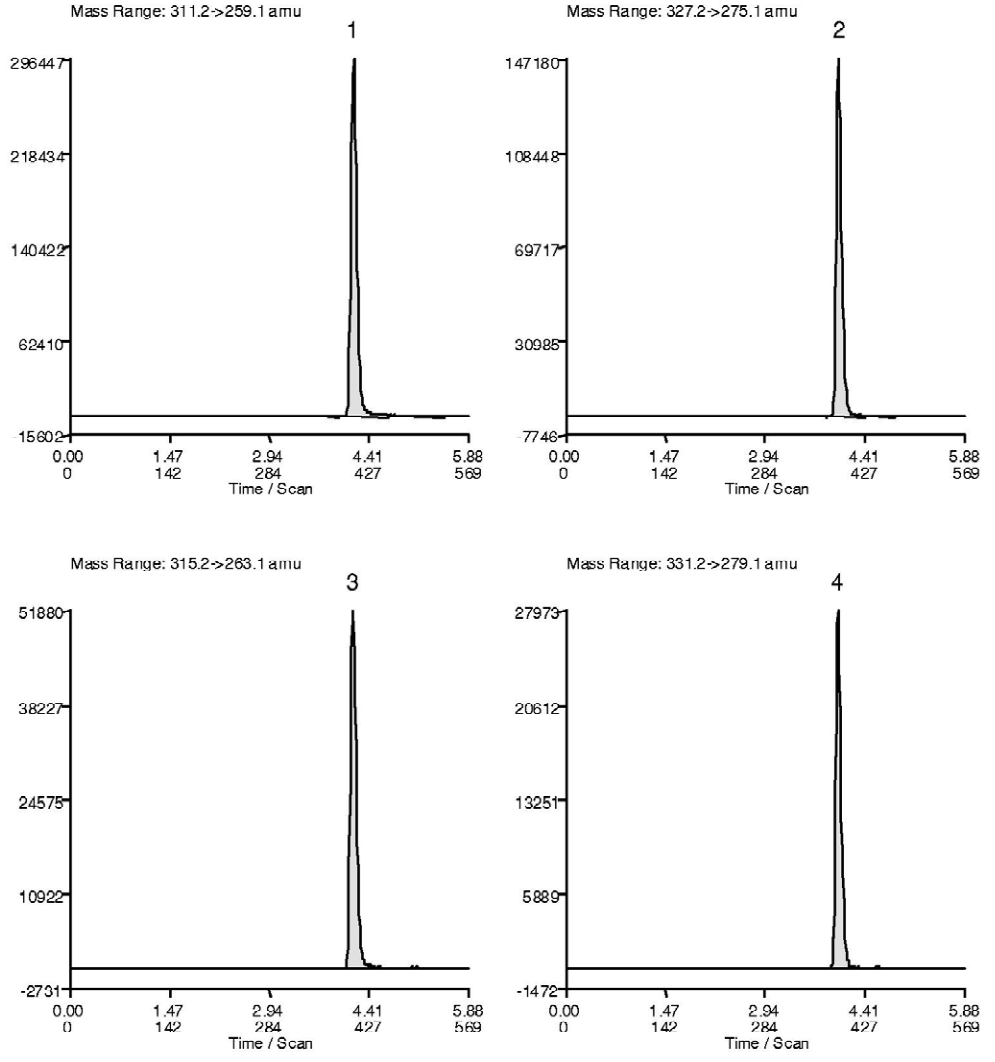


Fig. 5. Selected reaction ion chromatograms for a 10 ng/ml calibration standard in extracted human plasma: (1) desloratadine, (2) 3-OH desloratadine, (3) [$^{2}H_{4}$]desloratadine and (4) [$^{2}H_{4}$]3-OH desloratadine.

QC samples ($n=6$) spiked after extraction. Mean absolute recoveries for the low, medium and high QC levels were 50.0, 47.1, and 45.2% for desloratadine, and 59.7, 55.0, and 59.6%, for 3-OH desloratadine, respectively.

3.9. Integrity of dilution

The effect of dilution on the quantitation of desloratadine and 3-OH desloratadine was determined. For this purpose, a QC sample pool containing desloratadine and 3-OH desloratadine in human plasma was prepared at approximately twice the ULOQ. Six replicates of the high QC (7500 pg/ml) and the QC pool at 20 000 pg/ml were diluted 10-fold with blank plasma and analyzed. The dilution procedure was considered to be valid if the %CV and %bias of diluted QC samples were no greater than 15%. For desloratadine, the %CV and %bias for the high QC samples were 2.7 and 4.7%, respectively. For 3-OH desloratadine, the %CV and %bias for the high QC samples were 4.6 and

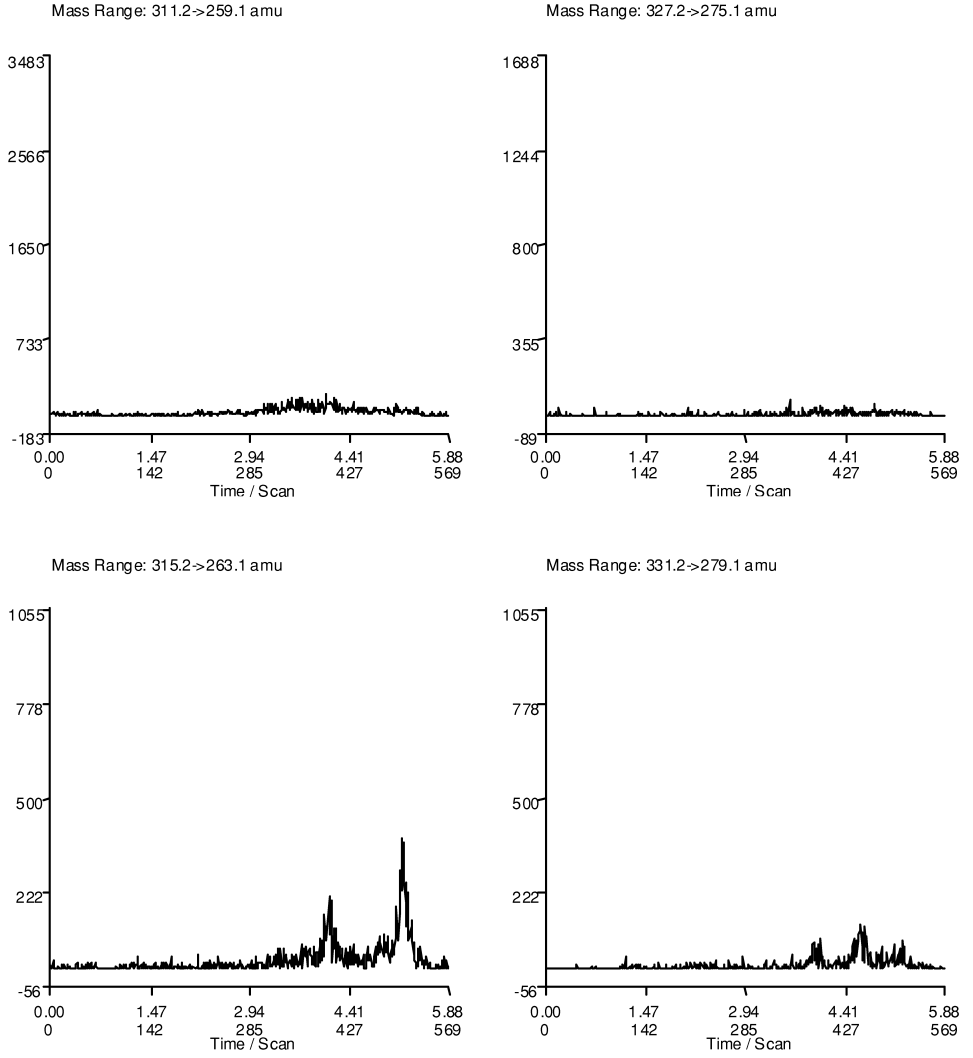


Fig. 6. Selected reaction ion chromatograms for extracted blank human control plasma: (1) desloratadine, (2) 3-OH desloratadine, (3) [$^2H_4$]desloratadine, and (4) [$^2H_4$]3-OH desloratadine.

−9.0%, respectively. For desloratadine, the %CV and %bias for the QC samples at 20 000 pg/ml were 4.5 and 3.1%, respectively. For 3-OH desloratadine, the %CV and %bias for the 20 000 pg/ml QC samples were 3.6 and 3.0%, respectively.

3.10. Stability

Stability assessments were carried out to demonstrate that desloratadine and 3-OH desloratadine were stable under typical sample storage and processing conditions. The stability experiments were carried out using QC samples ($n=6$) at the low, medium and high QC levels. The mean value of the stability QC samples at each level were compared to the mean value obtained from the same QC pool prepared and analyzed fresh (fresh run 1 value). The analytes were considered to be stable if the %CV and %bias (when compared to fresh run 1 values) were no greater than 15%. Using these criteria, desloratadine and 3-OH desloratadine were determined to be stable in human plasma for up to 401 days of

storage at −22 °C, after five freeze/thaw cycles, up to 24 h at room temperature, and in reconstituted sample extracts for 185 h at 5 °C. Desloratadine and 3-OH desloratadine were also determined to be stable in methanolic stock solutions stored at −22 °C for 298 and 274 days, respectively.

3.11. Clinical study application

This validated LC–MS–MS method was successfully applied to clinical studies for the desloratadine development program. A typical time–concentration profile for desloratadine and 3-OH desloratadine following a 5-mg oral dose of desloratadine to a subject in a clinical study is presented in Fig. 7. Both desloratadine and 3-OH desloratadine are slowly eliminated. In the PK profile presented in Fig. 7, the mean half-lives of desloratadine and 3-OH desloratadine are 22 and 32 h, respectively. Thus, to fully characterize PK profiles, both analytes had to be quantified over approximately four half-lives. An LLOQ of 25 pg/ml allowed the detection for most time points, for instance, 47.6 pg/ml of desloratadine at 96 h and 52.3 pg/ml of 3-OH desloratadine at 120 h. Moreover, the ULOQ of 10 ng/ml is well above C_{max} for both desloratadine and 3-OH desloratadine (2.24 and 1.19 ng/ml, respectively for this subject). Therefore, sample dilutions were minimized, enhancing sample throughput.

4. Conclusions

An automated 96-well plate LC–MS–MS method was developed and validated for the determination of desloratadine and 3-OH desloratadine in human plasma over the concentration range of 25–10 000 pg/ml. The method met regulatory requirements for accuracy, precision, sensitivity, selectivity and analyte stability.

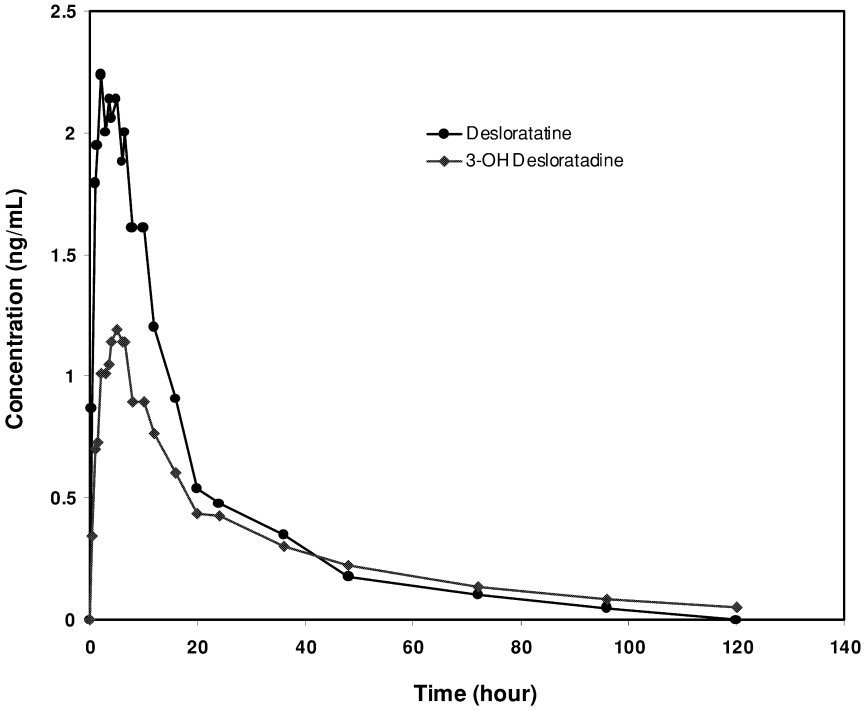


Fig. 7. A typical time–concentration profile of desloratadine and 3-OH desloratadine following a 5-mg oral dose of desloratadine in a clinical study.

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