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Simultaneous determination of azelastine and its major metabolite desmethylazelastine in human plasma using high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A selective and sensitive high performance liquid chromatography–tandem mass spectrometric method was developed for the analysis of azelastine and its major metabolite, desmethylazelastine, in human plasma. Azelastine-$^{13}C, d_3$ was used as internal standard. Azelastine, desmethylazelastine and the internal standard were extracted by a liquid–liquid extraction method and separation was performed under isocratic chromatographic condition. An abnormal signal loss issue for desmethylazelastine during method development was investigated and resolved. The developed method was precise and reproducible as shown by good intraday assay and interday assay precision (CV% ≤ 12.8%). The calibration curve was linear over a range of 10.0/10.0–1000/200 pg/mL for azelastine/desmethylazelastine. The method was successfully applied to a pilot bioequivalence study subsequently.

1. Introduction

Azelastine is a second generation histamine antagonist with a broad spectrum of antiallergic and antiasthmatic activities [1]. It has multiple actions that include antihistaminic effects, mast cell stabilizing, and inhibition of inflammatory mediators [2–4]. Azelastine is mostly administrated as nasal spray or ophthalmic eyedrop although it is effective with oral administration. The nasal spray formulation was approved for seasonal allergic treatment in 1996 and nonallergic vasomotor rhinitis in 1999. The eyedrop formulation was approved in 2000 for the treatment of allergic conjunctivitis [2]. Azelastine has a rapid onset of action, only 15 min with the nasal spray and 3 min with the ophthalmic eyedrop [5,6]. It also has protective effect if administrated before exposure to allergen [7]. The effect of azelastine action is also long, for up to 12 h [8].

Azelastine is metabolized in the liver to its major active metabolite desmethylazelastine [9]. The half life of desmethylazelastine is much longer than azelastine and this contributes greatly to the long duration of action [2]. Azelastine is mostly excreted in the feces and the primary form is its major metabolite, desmethylazelastine [10].

Quantitative determination of azelastine and its major metabolite, desmethylazelastine, in plasma was mainly performed by HPLC with fluorometric detection [1,11]. However, fluorometric detection lacks selectivity. Hasegawa et al. applied GC–MS for the analysis of azelastine in dog plasma [12], but the sensitivity was low and the assay range was 10–500 ng/mL which was much higher than the expected human plasma levels [14].

LC–MS/MS is a gold standard for the quantification of drugs in plasma. It offers high selectivity and sensitivity. Heinemann et al. developed a LC–MS/MS method for the investigation of azelastine enantiomeric metabolism in rat [13]. A volume of 3 mL sample was used, and the extraction involved three-step extraction with two different types of solvents followed by back-extraction. The extraction process was long and complicated. Recently, Park et al. developed a method to determine azelastine in human plasma using LC–MS/MS, but the major metabolite was not analyzed [14]. A volume of 1.0 mL plasma sample was needed. Here we report a simple, fast and selective method for the quantitative analysis of azelastine and desmethylazelastine simultaneously in human plasma. Only 0.5 mL sample was required. A straightforward liquid-liquid extraction method was applied and the analytes were analyzed by the LC–ESI-MS/MS in a positive MRM detection mode using a Shimadzu LC-10 AD and an API 4000 mass spectrometer. Good intraday assay and interday assay precision (CV% ≤ 12.8%) and accuracy (≤9.9%) were obtained. A lower limit of quantification (LLOQ) of 10 pg/mL for both azelastine and desmethylazelastine was achieved. The developed and validated method was successfully applied to a pilot bioequivalence study.

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2. Experimental

2.1. Chemicals

Azelastine hydrochloride (99.0%) was provided by MP biomedicals (Solon, OH, USA). Azelastine-^{13}C, d_3 (98.0%) and N-desmethylazelastine (95.0%) were purchased from Toronto Research Chemicals (North York, ON, Canada). Ammonium formate (97%) was bought from Sigma–Aldrich (St. Louis, MO, USA) and buffer concentrate solution (commercial buffer, made from boric acid, potassium chloride, and sodium hydroxide), pH 10, was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methanol and acetonitrile both HPLC grade and formic acid (98%) were all from EMD Chemicals Inc. (Gibbstown, NJ, USA). Deionized water was produced in-house using a Millipore ultrapure water purification system (Billerica, MA, USA). Blank Human K_3EDTA plasma was supplied by Bioreclamation, LLC (Westbury, NY, USA).

2.2. High performance liquid chromatography and mass spectrometry

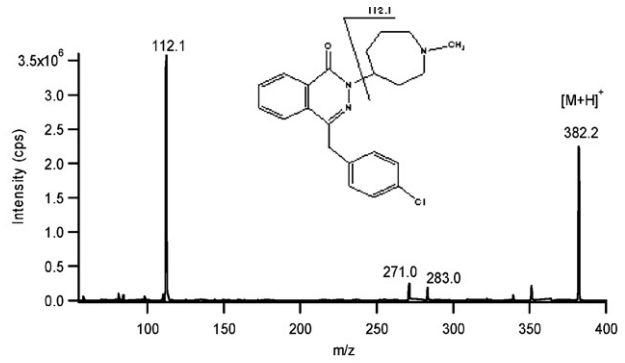
Chromatographic separation was obtained using Shimadzu LC-10AD *vp* LC system (Columbia, MD, USA) with a Phenomonex (Torrance, CA, USA) Synergi Polar-RP column (50 mm × 2.0 mm, 4 μm). The mobile phase consisted of 55% A (10 mM ammonium formate with 0.1% formic acid) and 45% B (acetonitrile) by volume. The flow rate for the mobile phase was set at 0.2 mL/min.

Detection was performed by an AB/Sciex (Concord, ON, Canada) API 4000 triple quadrupole mass spectrometer with a turbo-ion spray interface. Azelastine-^{13}C, d_3 was used as internal standard (IS). The MS/MS transitions (*m*/*z*) in positive ion mode were: azelastine, 382.2 → 112.1; desmethylazelastine, 368.1 → 98.1; azelastine-^{13}C, d_3 (IS), 386.3 → 116.0 (shown in Fig. 1). The fragmentation pathways were also indicated in Fig. 1. The ion source was heated to 650 °C and the spray voltage was set at 2400 V. The nebulizer gas, auxiliary gas, collision-activated dissociation (CAD) gas and curtain gas were applied at 55, 40, 6 and 25 psi, respectively. The collision energy was set at 35 V for azelastine, 33 V for desmethylazelastine and 33 V for azelastine-^{13}C, d_3. The software Analyst 1.4.2 (Sciex, Concord, ON, Canada) was used to control the LC–MS/MS system and acquire the data.

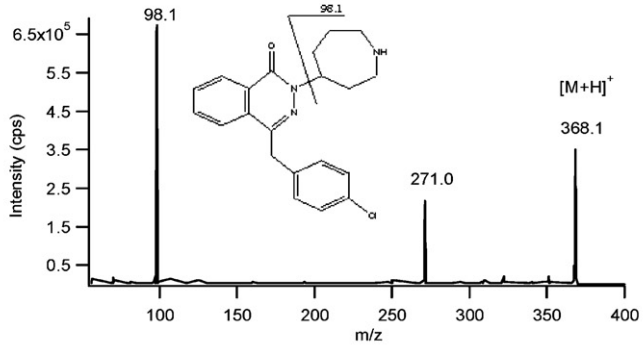
2.3. Preparation of stock solution and standards

Stock solutions of azelastine (1.00 mg/mL), desmethylazelastine (200 μg/mL), and IS (100 μg/mL) were prepared in methanol and stored at −20 °C. Working solutions of azelastine/desmethylazelastine (10,000/2000, 7500/1500, 5000/1000, 1500/750, 500/500, 200/200, 100/100 pg/mL) were prepared by dilution of stock solutions with 50/50 (v/v) deionized water/methanol. The internal standard working solution containing 1.5 ng/mL azelastine-^{13}C, d_3 was diluted from IS stock solution with 50/50 (v/v) deionized water/methanol.

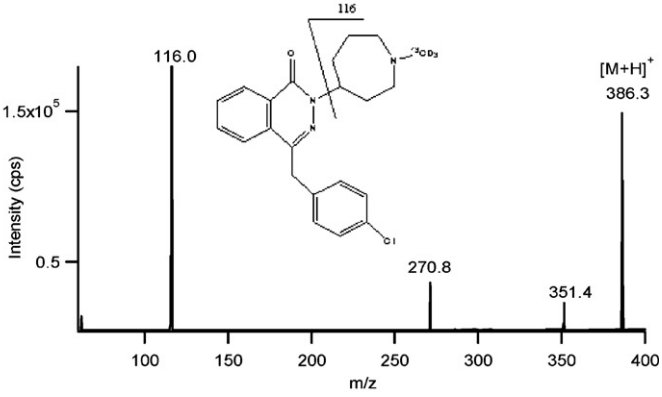
The plasma used for spiking was examined to make sure it was free of interferences with azelastine, desmethylazelastine and IS before preparing the standard calibration curve. A standard curve was prepared daily, in duplicate, at concentrations equivalent to 10/10, 20/20, 50/50, 150/75, 500/100, 750/150 and 1000/200 pg/mL for azelastine/desmethylazelastine by diluting azelastine and desmethylazelastine working solutions with plasma. Quality control (QC) samples were prepared by spiking appropriate amounts of azelastine and desmethylazelastine into the plasma to achieve the concentrations at LLOQ (10/10 pg/mL), lower level (30/30 pg/mL), mid level (550/110 pg/mL) and high level



(a) Product ion mass spectra of $[M+H]^+$ of azelastine



(b) Product ion mass spectra of $[M+H]^+$ of desmethylazelastine



(c) Product ion mass spectra of $[M+H]^+$ of azelastine-^{13}C,d_3

Fig. 1. Product ion mass spectra of $[M+H]^+$ of azelastine (a), desmethylazelastine (b) and azelastine-^{13}C, d_3 (c).

(850/170 pg/mL). The QC samples were stored at −20 °C after their preparation.

2.4. Extraction procedure

Samples were extracted using a liquid–liquid extraction method. A sample volume of 500 μL plasma was used. Briefly, to the polypropylene tubes containing plasma samples, 500 μL of buffer concentrate, pH 10, and 100 μL of IS working solution were added. After the mixture was mixed approximately 10 s on a VX-2500 multi-tube vortexer (VWR Scientific, Bridgeport, NJ), 4 mL of methyl *tert*-butyl ether (MTBE) was added. The tubes were subsequently shaken at high speed for 20 min and then centrifuged at 5833 × *g* for 10 min. The top layer was transferred to a fresh tube and evaporated at 37 ± 5 °C to dryness under a stream of nitrogen. The residue was

reconstituted with 150 μL of mobile phase and mixed well. Only 5 μL was injected into the LC–MS/MS system.

2.5. Method validation

The developed method has been validated for linearity and sensitivity, precision and accuracy, selectivity, recovery, matrix effects, and stability according to US Food and Drug Administration (FDA) bioanalytical method validation guidance [15].

2.5.1. Linearity and sensitivity

Standard calibration curves of seven points that correspond to azelastine/desmethylazelastine concentrations ranged from 10/10 to 1000/200 pg/mL were extracted and analyzed. A blank plasma sample (contains no drugs or IS) and a zero sample (only added IS) were also processed in order to make sure there is no interferences with azelastine, desmethylazelastine and IS. An additional blank sample was extracted as well and used for evaluating carryover if there is any by following the highest concentration sample. The calibration curve was plotted based on the peak area ratio of azelastine/desmethylazelastine to IS versus azelastine/desmethylazelastine concentration. The weighted ($1/x^2$) least squares linear regression analysis was performed for calibration curves.

2.5.2. Precision and accuracy

The intraday assay precision and accuracy were determined by analyzing six replicates of the QC samples at three concentration levels (30/30 pg/mL, 550/110 pg/mL and 850/170 pg/mL) and LLOQ.

The interday assay precision and accuracy were measured by three repeated analyses of the QC samples and LLOQ on three different days. The sample concentrations were determined by standard calibration curve prepared and analyzed on the same day.

2.5.3. Selectivity

Six different lots of blank human K_3EDTA plasma samples from various donors were extracted to assess the selectivity of the method. This is to make sure the absence of interference with azelastine, desmethylazelastine and IS from different plasma sources.

2.5.4. Matrix effects

In ESI-MS, analyte signal may be suppressed or enhanced by the co-eluted endogenous substances. This would affect the reproducibility and accuracy of a method. The matrix effects on the drug and IS should be evaluated. A reference solution containing azelastine, desmethylazelastine and IS was prepared in reconstitution solution, and 150 μL of this solution was mixed with the extracted residual of a blank plasma sample. The MS responses of the mixed solution were compared with those of the neat reference solution to evaluate the matrix effects. In order to confirm the consistency of matrix effects for this method with plasma from various individuals, a low concentration level sample (20 pg/mL for azelastine and 30 pg/mL for desmethylazelastine) from six other different donors were examined. The sample from each lot was extracted in three replicates.

2.5.5. Recovery

Matrix effect factor was considered in order to get the true extraction recovery. Reference QC solutions were prepared in reconstitution solution and mixed with the extracted residual of blank plasma samples to mimic the matrix of extracted samples. The recovery of azelastine, desmethylazelastine and IS was then determined by comparing the peak areas of the extracted QC samples (6 replicates) at three different concentrations (30/30 pg/mL, 550/150 pg/mL, 850/170 pg/mL) with those of reference QC solutions.

2.5.6. Stability

The stability of azelastine and desmethylazelastine in human plasma under different conditions including temperature and time was evaluated. To determine the stability, the stability samples were prepared at two QC concentration levels (low and high). The stability samples (six replicates) were then analyzed with the freshly prepared calibration curves and QC samples.

For freeze–thaw stability evaluation, each cycle includes thawing QC samples unassisted at room temperature until they are completely thawed and refreezing at −20 °C for at least 12 h. For the first cycle, the samples will be frozen for at least 24 h. For bench-top stability determination, QC samples were left on bench at room temperature for a certain time and then analyzed. Processed sample stability was evaluated by leaving the extracted samples in the autosampler at 4 °C for a certain time after the immediate injection. The samples were then reinjected.

3. Results and discussion

3.1. Chromatographic conditions and sample extraction

Different columns were tested with the initial mobile phase consisting of acetonitrile and 5 mM ammonium formate. It was found that the analytes had more retention on phenyl columns and Polar-RP columns than C18 columns. The best retention was obtained with the Polar-RP column. Therefore, a Polar-RP column 5 cm in length was finally chosen for the separation. However, poor peak shape was observed for the analytes due to tailing with the mobile phase used. The pH of the buffer in the mobile phase, 5 mM ammonium formate, was lowered by adding formic acid, and the peak tailing was reduced significantly. The concentration of ammonium formate was then increased to 10 mM, peak shape for the analytes was further improved and the analytes sensitivities remained similar. Ammonium formate of 10 mM with 0.1% formic acid (by volume) and acetonitrile were finally used in the mobile phase.

The plasma samples were extracted using a liquid–liquid extraction method. Different organic solvents including hexane, ethyl estate, dichloromethane and methyl-tert-butyl ether were tested. The highest recovery was achieved with methyl-tert-butyl ether. Various buffer made from 50 mM sodium phosphate, monobasic and dibasic, at pH ca. 4.5, 7.0 and 9.2 and commercially available buffer, buffer concentrate (pH 10), were also examined. The best result was obtained with the buffer concentrate (pH 10). Methyl-tert-butyl ether and buffer concentrate (pH 10) were finally selected for the extraction.

3.2. Abnormal signal loss issue for desmethylazelastine

When the calibration standards were extracted to evaluate the linearity of curves during method development, it was found the linearity was only good for azelastine. For desmethylazelastine, the standard points spread out with abnormal values. An investigation was conducted to find the cause of this issue.

The signals of desmethylazelastine and IS were first examined. It was found that the signal of internal standard was relatively constant throughout all the samples, while for desmethylazelastine many points had unexpected values. The same extracted samples were then re-injected to examine if the abnormal signal resulted from the variation of MS system, but the same results were obtained for the same samples. This suggests that the problem was not caused by the MS and it should result from the extraction steps.

The acetonitrile content in the reconstitution solution and the mixing time of reconstitution solution with the extracted residual were then increased to examine if the issue was caused by the solubility of desmethylazelastine in reconstitution solution. However,

Table 1
The intraday assay precision and accuracy for azelastine and desmethylazelastine ($n = 6$).

Analyte	Nominal concentration (pg/mL)	Mean of calculated concentration (pg/mL)	Accuracy (%)	Precision (CV%)
Azelastine	10	8.96	89.6	7.0
	30	29.51	98.4	2.3
	550	586.9	106.7	1.2
	850	903.6	106.3	2.0
Desmethylazelastine	10	9.35	93.5	7.7
	30	29.79	99.3	6.8
	110	115.8	105.3	7.2
	170	161.4	94.9	7.1

Table 2
The interday assay precision and accuracy for azelastine and desmethylazelastine ($n = 18$).

Analyte	Nominal concentration (pg/mL)	Mean of calculated concentration (pg/mL)	Accuracy (%)	Precision (CV%)
Azelastine	10	9.61	96.1	12.0
	30	30.27	100.8	6.2
	550	587.7	106.9	2.6
	850	905.0	106.5	2.7
Desmethylazelastine	10	10.43	104.3	12.8
	30	32.03	106.8	8.7
	110	119.7	108.8	7.1
	170	166.4	97.9	6.1

the problem still existed. Next, the adsorption of desmethylazelastine to the glass tubes was examined. It was found that the glass tubes had strong adsorption for desmethylazelastine, while no adsorption for azelastine and IS. Individual tube also has different adsorption for desmethylazelastine due to the variation of individual inside wall surface. The different adsorption of individual glass tube and other glassware resulted in the abnormal signal loss issue of desmethylazelastine. The plastic polypropylene tubes were then tested and no adsorption was found for desmethylazelastine. The glass tubes and other glassware were subsequently replaced with plastic ones and excellent results were achieved.

3.3. Method validation

3.3.1. Linearity and sensitivity

The result shows a good linear relationship between the peak area ratio of azelastine/desmethylazelastine to IS and azelastine/desmethylazelastine concentration over the range of 10/10–1000/200 pg/mL. The correlation coefficients for the six

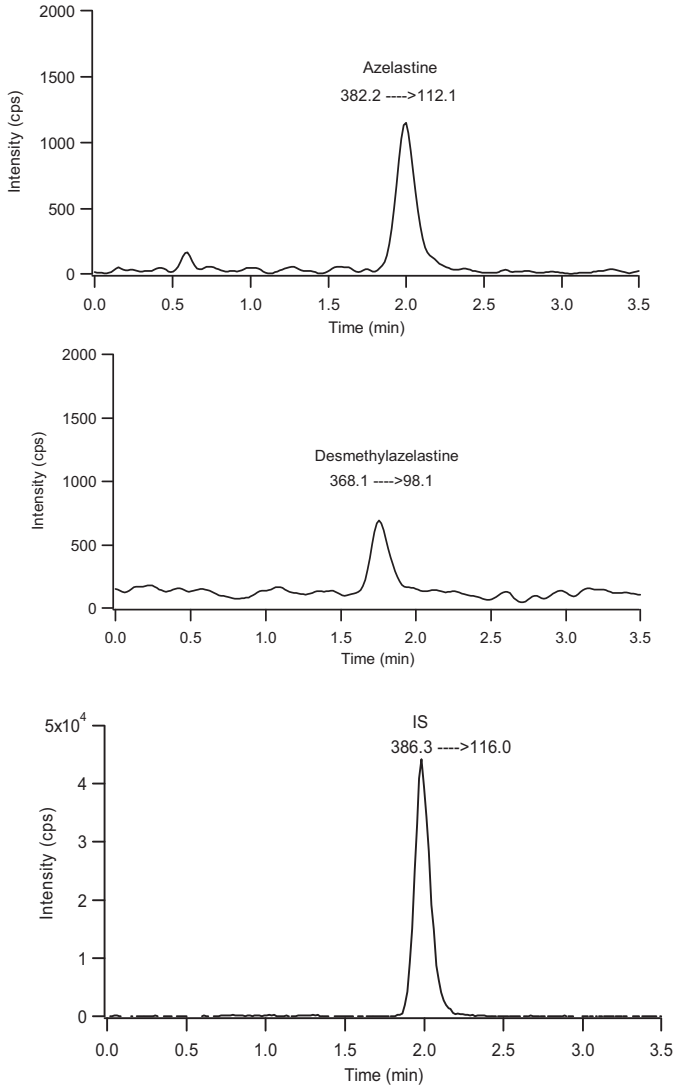


Fig. 2. Chromatogram of an LLOQ.

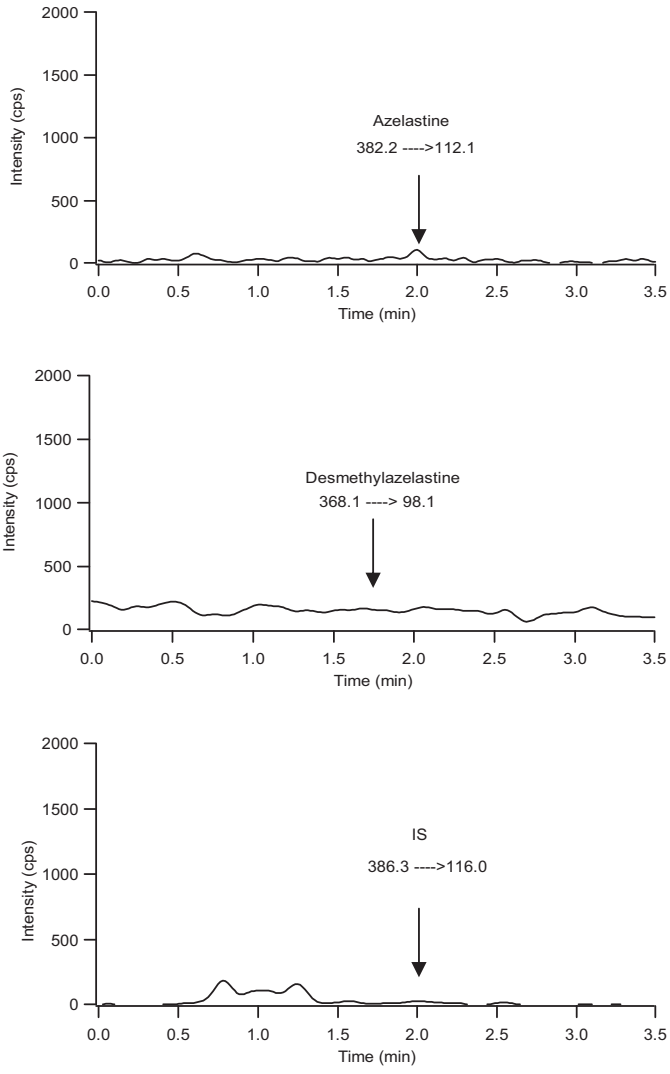


Fig. 3. Chromatogram of an extracted blank plasma sample.

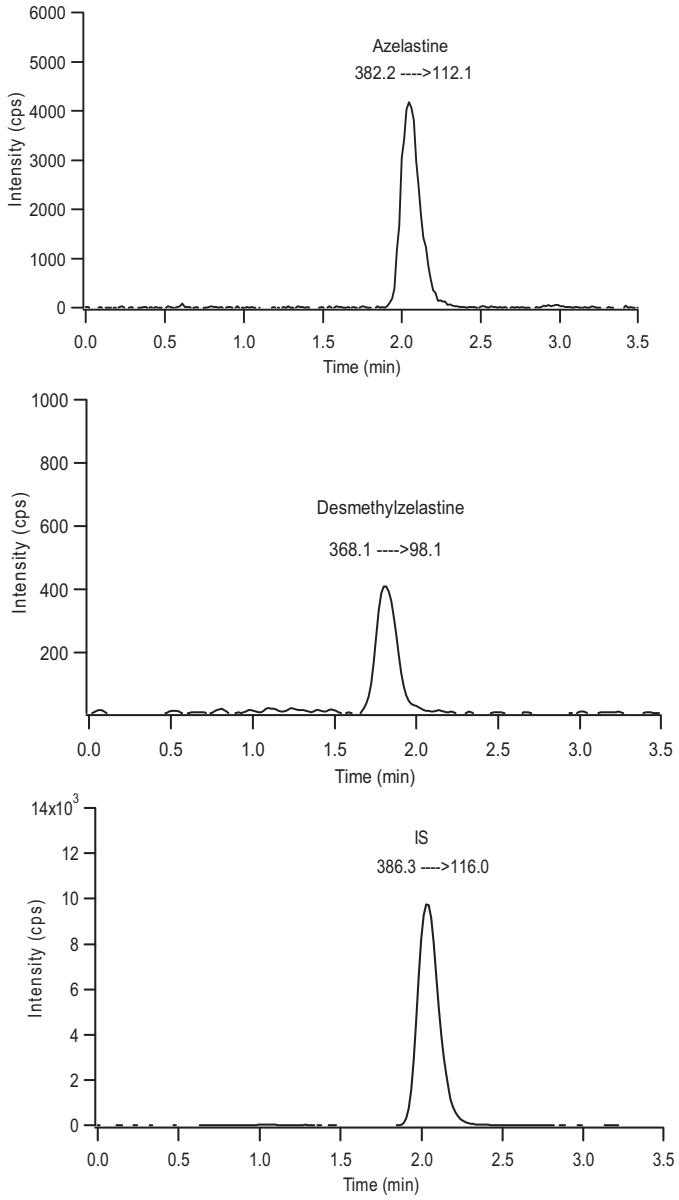


Fig. 4. Chromatogram of an extracted study sample.

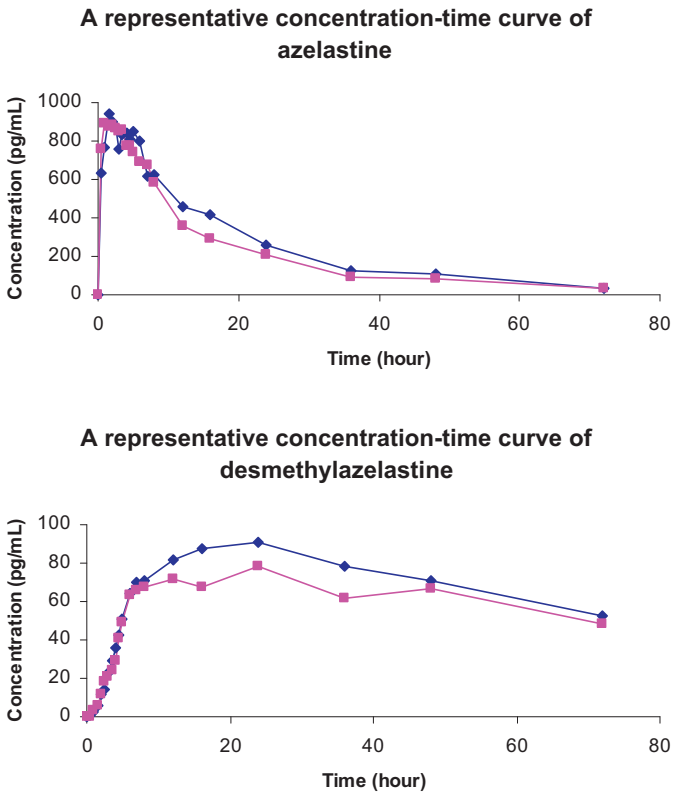


Fig. 5. Representative concentration–time curves of azelastine and desmethylazelastine.

calibration curves were higher than 0.9989 for azelastine and 0.9961 for desmethylazelastine, with an average of 0.9991 for azelastine and 0.9966 for desmethylazelastine. A typical standard calibration curve is described by the weighted least square regression as: $y = 0.00221x - 0.01300$ ($R^2 = 0.9991$) for azelastine and $y = 0.00119x - 0.00230$ ($R^2 = 0.9966$) for desmethylazelastine, where y is the peak area ratio of azelastine/desmethylazelastine to the IS, and x corresponds to the concentration of azelastine/desmethylazelastine added to plasma ranging from 10/10 to 1000/200 pg/mL.

The LLOQ was set at the lowest standard concentration in the calibration curve, 10 pg/mL, for both azelastine and desmethylazelastine. The limit of detection (LOD) was determined to be approximately 2.3 pg/mL for azelastine and 3.3 pg/mL for desmethylazelastine with a signal-to-noise (S/N) ratio of 3. Fig. 2 presents the chromatogram of an extracted LLOQ sample.

The blank plasma sample and zero sample indicated that there were no interferences for azelastine, desmethylazelastine and IS, and the blank plasma sample following the highest standard showed no carryover for azelastine, desmethylazelastine and IS. This ensures the absence of endogenous interference and any impact caused by high concentration samples with the method.

3.3.2. Precision and accuracy

The intraday assay results are presented in Table 1 and the interday assay results are shown in Table 2. The results show high intraday and interday assay precision and accuracy with the method.

3.3.3. Selectivity

The results indicated that the method was selective for azelastine, desmethylazelastine and IS. No endogenous interference was found at the respective retention times of azelastine, desmethylazelastine and IS as illustrated in the chromatogram of an extracted blank plasma sample shown in Fig. 3.

3.3.4. Matrix effects

With a mobile phase of 45/55 (v/v), acetonitrile/10 mM ammonium formate with 0.1% formic acid, the matrix effect was approximately −8%, −16%, −12% for azelastine, desmethylazelastine, and IS, respectively. Therefore, there is no significant matrix effect for drugs and IS with the method. It was also found that the MS responses of azelastine, desmethylazelastine and IS were consistent with the six different plasma sources.

3.3.5. Recovery

The overall extraction recovery was 84.7%, 86.9% and 89.2% for azelastine, desmethylazelastine and IS, respectively. The precision (CV%) at the three QC concentration levels was 4.4% for azelastine and 8.9% for desmethylazelastine.

3.3.6. Stability

Azelastine and desmethylazelastine in human plasma were found to be stable for at least three freeze–thaw cycles, and

azelastine and desmethylazelastine were stable at room temperature for at least 5.3 h. Processed samples were found to be stable in the autosampler at 4 °C for at least 95 h. QC samples stored at −20 °C were stable throughout the time of validation (ca. 2 weeks).

3.4. Application to study

This developed method was successfully applied to analyze plasma samples from healthy volunteers in a nasal spray pilot bioequivalence study. Each volunteer was administered an azelastine hydrochloride nasal spray 0.15% solution and the blood samples were collected over a period of 72 h. K_3EDTA was used as anticoagulant. The blood samples were immediately centrifuged and plasma was separated from blood cells and stored at −20 °C until analysis. A representative chromatogram of a study sample is shown in Fig. 4. An example of plasma concentration–time curves of azelastine and desmethylazelastine from one volunteer is shown in Fig. 5.

4. Conclusion

A highly selective and reproducible LC–MS/MS method was developed and validated for the determination of azelastine and desmethylazelastine concentration in human plasma. The abnormal signal loss during the method development was caused by the absorption of glass materials, and the signal loss issue was solved by replacing the glass volumetric flasks and tubes with polypropylene ones. The method offers a simple and rapid analysis for azelastine and desmethylazelastine, and it is very suitable for pharmacokinetic or bioequivalent studies.

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References

[1] J. Pivonka, F.H. Segelman, C.A. Hartman, W.E. Segl, N. Kucharczyk, R.D. Sofia, J. Chromatogr. 420 (1987) 89.
[2] P.B. Williams, E. Crandall, J.D. Sheppard, Clin. Ophthalmol. 4 (2010) 993.
[3] F. Horak, U.P. Zieglmayer, Expert Rev. Clin. Immunol. 5 (2009) 659.
[4] L. Bielory, K.W. Lien, S. Bigelsen, Drugs 65 (2005) 215.
[5] F. Horak, U.P. Zieglmayer, R. Zieglmayer, Curr. Med. Res. Opin. 22 (2006) 151.
[6] M.H. Friedlaender, J. Harris, N. La Vallee, H. Russell, J. Shilstone, Ophthalmology 107 (2000) 2152.
[7] G. Ciprandi, S. Buscaglia, A. Catrullo, G. Pesce, N. Fiorino, P. Montagna, M. Bagnasco, G.W. Canonica, Clin. Exp. Allergy 27 (1997) 182.
[8] L. Greiff, M. Andersson, C. Svensson, C.G. Persson, Clin. Exp. Allergy 27 (1997) 438.
[9] M. Nakajima, S. Tokudome, N. Shimada, H. Yamazaki, T. Yokoi, Drug Metab. Dispos. 27 (1999) 1381.
[10] H.J. Zechel, N. Brock, D. Lenke, U. Achterrath-Tuckerman, Arzneimittelforschung 31 (1981) 1184.
[11] C.N. Langevin, J. Pivonka, J.K. Wichmann, N. Kucharczyk, R.D. Sofia, Biomed. Chromatogr. 7 (1993) 7.
[12] J. Hasegawa, Y. Tomono, M. Tanaka, T. Fujita, K. Sugiyama, N. Hirose, Arzneim.-Forsch. 31 (1981) 1215.
[13] U. Heinemann, G. Blaschke, N. Knebel, J. Chromatogr. B 793 (2003) 389.
[14] Y.S. Park, S.H. Kim, Y.J. Kim, S.C. Yang, M.H. Lee, L.M. Shaw, J.S. Kang, J. Biomed. Sci. 6 (2010) 120.
[15] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001 (May).