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Journal of Chromatography B, 862 (2008) 132–139
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of levocetirizine in human plasma by liquid chromatography–electrospray tandem mass spectrometry: Application to a bioequivalence study

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Received 13 June 2007; accepted 25 November 2007
Available online 4 December 2007

Abstract

We describe a liquid chromatography–tandem mass spectrometric method (LC–MS/MS) for levocetirizine quantification (I) in human plasma. Sample preparation was made using a fexofenadine (II) addition as internal standard (IS), liquid–liquid extraction using cold dichloromethane, and dissolving the final extract in acetonitrile. I and II (IS) were injected in a C18 column and the mobile phase composed of acetonitrile:water:formic acid (80.00:19.90:0.10, v/v/v) and monitored using positive electrospray source with tandem mass spectrometry analyses. The selected reaction monitoring (SRM) was set using precursor ion and product ion combinations of m/z 389 > 201 for I and m/z 502 > 467 for II. The limit of quantification and the dynamic range achieved were 0.5 ng/mL and 0.5–500.0 ng/mL. Validation results on linearity, specificity, accuracy, precision and stability, as well as its application to the analysis of plasma samples taken up to 48 h after oral administration of 5 mg of levocetirizine dichloridrate in healthy volunteers demonstrate its applicability to bioavailability studies.

Keywords: Mass spectrometry; Bioavailability; Levocetirizine

1. Introduction

Levocetirizine (CAS—130018-77-8) (I), 2-[2-[4-[(*R*)-(4-chlorophenyl)-phenyl-methyl] piperazinyl-1-yl]ethoxy] acetic acid, the *R*-enantiomer of racemic cetirizine, is a selective, potent, H_1-antihistamine compound indicated for the treatment of allergic rhinitis and chronic idiopathic urticaria [1]. The recommended dosing of levocetirizine is 5 mg per day. It has a rapid onset, achieving maximum plasma concentration (t_{max}) in 0.9 h [2], with peak serum levels (C_{max}) of approximately 270 ng/mL [1]. In the plasma, 91% of the drug is bound to proteins and its volume of distribution (V_d) is small (0.4 L/kg). The drug undergoes minimal metabolism, which increases the bioavailability and its half-life of elimination time is 8 h. Levocetirizine is generally well tolerated in adults, adolescents and children with allergic conditions [2].

The use of LC coupled with electrospray tandem mass spectrometry has become the first choice in bioavailability studies owing to the fast, sensitive, and reliable results generated by its use [3]. The state-of-the-art instruments use the two powerful techniques to separate interference and MS/MS to analyze with selectivity and sensitivity to give very reliable results of concentrations in biological fluids.

Other techniques have been previously used to determine cetirizine in human plasma. These methods include gas chromatography (GC) [4], high-performance liquid chromatography (HPLC) with UV detection [5,6] and HPLC-MS/MS [7,8]. The HPLC–UV method has higher limit of quantification (10.0 ng/mL) than others. The LC–MS/MS method described by Jager et al. has a low limit of quantification (0.5 ng/mL) but, the retention time is 1.87 min. In the methods mentioned above,

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doi:10.1016/j.jchromb.2007.11.028

the extraction used to prepare the sample is protein precipitation, which could lead to high level of interferences.

This article describes LC–MS/MS method with sensitivity as good as the previous one (0.5 ng/mL), faster than articles previously reported (retention time: 0.91 min) that uses liquid–liquid extraction for levocetirizine quantification in human plasma and reports its application for bioavailability study.

2. Experimental

2.1. Materials and reagents

Levocetirizine (lot number 0281105) was obtained from Symed Labs Limited (São Paulo, SP) and fexofenadine (lot number FEX0503020) was obtained from Ind. Swift Laboratories Limited (Rio De Janeiro, RJ). Acetonitrile, methanol (HPLC grade) and NaCl were purchased from J.T. Baker (Deventer, Netherlands). Dichloromethane and formic acid were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Analyses conditions and instrumentation

Elution was performed using acetonitrile:water:formic acid (80.00:19.90:0.10, v/v/v) at the mobile phase, at a flow-rate of 0.2 mL/min, the injection volume was 5 μl and total run time was set for 2.0 min. The samples were injected into an Alliance HT 2795 (Waters, Milford, MA, USA) at room temperature, and the stationary phase was a Polaris C_{18} (particle size, 3 μm; 50 mm × 2.0 mm, i.d.) column (Varian, Palo Alto, CA, USA). Mass spectrometric analysis was performed using a Quattro Premier XE (Waters, Milford, MA, USA) tandem mass spectrometry, equipped with an electrospray (ESI) source. The temperatures of desolvation gas and source block were 350 °C and 100 °C, respectively. The electrospray source was operated in the positive ionization mode (ESI+) at 3 kV, and selected reaction monitoring mode (SRM), *m/z* 389 > 201 and *m/z* 502 > 467 were used for quantification of levocetirizine and fexofenadine, respectively. Cone voltage, collision energy and collision gas pressure (argon) were 25.0 V, 12.0 V, and 2.75×10^{-3} mbar, respectively for levocetirizine, and 30.0 V, 20.0 V, and 2.75×10^{-3} mbar for fexofenadine, respectively.

2.3. Standards and quality control samples preparation

A stock solution of levocetirizine (100 μg/mL) was prepared in Milli-Q water. An aliquot of this solution was added to a blank plasma obtaining a final concentration of 2 μg/mL. This solution was used to spike blank plasma in order to obtain calibration standards of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 and 500.0 ng/mL and three levels of quality controls (QCs) fixed at 1.50, 200.00 and 400.00 ng/mL (low, medium and high). The spiked plasma was stored at −70 °C. Stock solutions of fexofenadine (internal standard) were prepared by dissolving the drug in methanol to a final concentration of 100 μg/mL. This solution was diluted with methanol to get a final concentration of 2 μg/mL.

2.4. Sample extraction

Twenty-five microliter of internal standard solution (fexofenadine, 2 μg/mL) was added to 200 μL plasma and mixed for 1 min. Then, 50 μL of saturated NaCl and 1000 μL of cold dichloromethane were added and the contents were mixed again for 5 min. The tube was centrifuged at 16,100 × *g*, for 5 min at 4 °C and the lower organic phase (700 μL) was transferred to another tube and evaporated to dryness under an air stream at room temperature. The residue was dissolved in 200 μL of acetonitrile and mixed for 5 min. The sample was then transferred to the glass autosampler vial and 5 μL was injected into the chromatographic system.

2.5. Recovery

The efficacy of levocetirizine extraction from human plasma was measured analyzing three levels of quality control samples (QCs). The drug recovery was determined by comparing peak areas obtained from the spiked plasma samples (QCs) after extraction and reconstitution to the standard solution at the same concentration of the spiked plasma samples. The recovery of IS was also evaluated using the same procedure. The recovery of analyte and IS should be consistent, precise, reproducible and higher than 50% according to our protocol of validation.

2.6. Limit of quantification

The limit of quantification (LOQ) was defined as the lowest concentration of the analyte that could be determined with precision and accuracy under the stated experimental conditions. Biological samples spiked with levocetirizine were injected in decreasing concentrations until the lowest concentration quantified with precision and accuracy, expressed by relative standard deviation (R.S.D.) lower than 20%. The experimental LOQ obtained was 0.5 ng/mL.

2.7. Analytical curves

The analytical curves were constructed using concentration values ranging from 0.5 to 500.0 ng/mL of levocetirizine in human plasma. Linear analytical curves were obtained by weighted linear regression (weighing factor: $1/x$), the ratio of levocetirizine peak area to fexofenadine peak area was plotted versus the ratio of levocetirizine concentration to that of the internal standard, in ng/mL. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards. For calibration curves, the correlation coefficient (r) should be higher than 0.98.

2.8. Linearity, precision and accuracy

The linearity was evaluated by analyses of three calibration curve batches. The acceptance criteria were that the coefficient

of variation (CV) and accuracy must not exceed 20% for LOQ and 15% for other standards.

Four different concentrations (0.50, 1.50, 200.00 and 400.00 ng/mL) of QC samples in five replicates were analyzed in three different batches in order to determine intra- and inter-batch precision and accuracy. The accuracy of the method was shown as relative error (RE) and calculated based on the difference between the mean calculated and nominal concentrations; whereas, precision was evaluated by calculating the within- and between-run CV. The acceptance criteria for each quality control were that the CV accuracy must not exceed 15% and 20% for the LOQ.

2.9. Freezing and thawing stabilities

Freezing and thawing stabilities for levocetirizine in plasma samples were evaluated after three cycles and the analytical process with control concentrations in four plasma batches. Samples were frozen at −70 °C in three cycles of 24, 36 and 48 h. Autosampler stability was studied over a 16-h storage period in the autosampler tray with quality control concentrations. The acceptance criteria for each quality control were that the CV and accuracy must not exceed 15%.

2.10. Bioequivalence study

Thirty-five adult volunteers, aged between 18 and 50 years with an index of corporal mass between 19 and 28.5 kg/m^2, were selected for the study after assessment of their health status by clinical evaluation (physical examination, electrocardiogram) and the following laboratory tests: albumin, alkaline phosphatase, AST (aspartate aminotransferase), ALT (alanine aminotransferase), blood glucose, creatinine, total bilirubin and total protein, triglyceride, total cholesterol, hemoglobin, hematocrit, total and differential white blood cells count, routine urinalysis and negative sorology for HIV, HBV and HCV. All the subjects gave written informed consent and the Assertiva Ethics Committee approved the clinical protocol.

The study was a single-dose, two-way randomized crossover design with 7 days washout period between doses.

During each period, the volunteers were hospitalized at 7:30 p.m. and had a supper before 9:00 p.m., after an overnight fast they received (at ∼7:00 a.m.) a single-dose of levocetirizine dichloridrate (5 mg of either formulation). Water (200 mL) was given immediately after the drug administration and the volunteers then fasted for 4 h, after which period a standard lunch was served. After 7 h, they were served a snack. After 12 and 14 h were served meals. No other food was permitted during the "in house" period and liquid consumption was allowed *ad libitum* 2 h after levocetirizine dichloridrate administration (with the exception of xanthine-containing drinks, including tea, coffee and cola). At 0, 1, 2, 4, 6, 8, 10, 12, 16, 24 and 36 h after the dose administration, systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded. The hospitalization period was 36 h. The volunteer came to the unit to supply blood samples at 48.0 h post-dosing. The following formulations were employed: levocetirizine dichloridrate (test formulation) and Zyxem® (standard reference formulation from Farmalab Ltda).

Blood samples (8 mL) from a suitable antecubital vein were collected by indwelling catheter into heparin-containing tubes at 0, 0.25, 0.5, 0.66, 0.83, 1.0, 1.16, 1.33, 1.5, 1.75, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0 h and 48.0 h post-dosing. The blood samples were centrifuged at ∼2000 × *g* for 10 min at 4 °C and the plasma was stored at −70 °C until assayed for levocetirizine content.

2.11. Pharmacokinetics and statistical analysis

Pharmacokinetic parameters were calculated from plasma levels applying a non-compartmental method using SAS 9.1.3 software (SAS Institute Inc., Cary, NC, USA). Following Food and Drug Administration (F.D.A.) guideline [9], blood samples were taken from each subject ($n = 35$) considering a period of 3–5 times the terminal elimination half-life ($t_{1/2}$). C_{max} and T_{max} values were determined directly from plasma levocetirizine concentration–time profiles. The area under the concentration–time curve (AUC_{0-t}) was obtained by the trapezoidal method. The total area under the curve ($AUC_{0-\infty}$) was calculated up to the last measured concentration and extrapolations were obtained using the last measured concentration and the terminal elimination rate constant (K_e). The terminal elimination rate constant, K_e, was estimated from the slope of the terminal exponential phase of the plasma of levocetirizine concentration–time curve (by means of the linear regression method). The terminal elimination half-life, $t_{1/2}$, was then calculated as $0.693/K_e$. Results were reported as mean ± standard deviation through out the paper. Regarding AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}, bioequivalence was assessed by means of an analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratios test/reference (logarithmically transformed data). Bioequivalence was considered when the ratio of averages of log-transformed data were within 80–125% for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}.

3. Results and discussion

3.1. Method development

Mass spectrometric detection coupled with liquid chromatography has been known as the state-of-the-art technique to perform bioanalytical analysis with maximum selectivity and sensitivity. The MS tuning was carried out by direct infusion of solutions of both levocetirizine and fexofenadine (IS) into the ESI source of the mass spectrometer.

The critical parameters in the ESI-MS/MS equipment include ionization in the solution (mobile phase), flow rate, needle (ESI) voltage and polarity (charge separation), drying gases (ion evaporation model and charged residue model) [10], and ion transmission. In our case, the formation of protonated ionic levocetirizine and fexofenadine (IS) molecules was observed (Fig. 1).

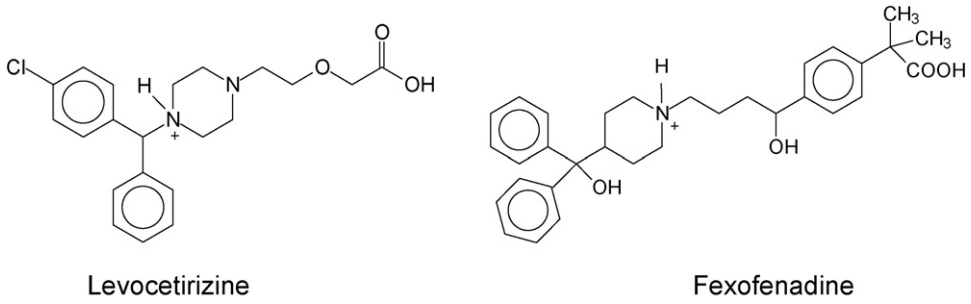

Fig. 1. Chemical structures of levocetirizine and fexofenadine (IS).

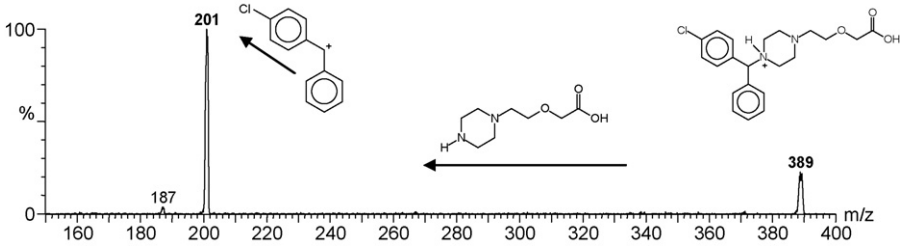

Fig. 2. CAD mass spectra of the levocetirizine protonated molecule.

A collisionally activated dissociation (CAD) product ion spectrum for levocetirizine yielded high-abundance fragment ions of *m/z* 201 (Fig. 2).

The proposed dissociation mechanism for the protonated levocetirizine of *m/z* 389, a protonated 2-piperazinyl-1-yl]ethoxy]acetic acid loss forming the product ion of *m/z* 201 that is a tropilium ion stabilized by resonance.

A CAD product ion spectrum for internal standard fexofenadine yielded high-abundance fragment ions of *m/z* 484 and *m/z* 467 (Fig. 3).

The proposed dissociation mechanism for the protonated fexofenadine of *m/z* 502, corresponds to two water losses in tandem forming more stabilized ions (resonance stabilization).

After the SRM channels were tuned, the mobile-phase was changed from an organic phase to a more aqueous phase with acid dopant to obtain a fast and selective LC method. The better signal was obtained using acetonitrile:water:formic acid (80.00:19.90:0.10, v/v/v) that was tested using three triplicate curves with five low quality controls, five medium quality controls and five high quality controls.

3.2. Specificity

The analysis of levocetirizine and fexofenadine using a SRM function was highly selective, with no interfering compounds or significative ion suppression from endogenous substances observed at the retention times for levocetirizine and fexofenadine; as shown in Fig. 4a and b. The chromatographic run was executed using a short (50 mm) Polaris column, which is convenient for running a high throughput of samples. There was no chromatographic separation, owing to the high degree of similarity shared by the two structures, with the adjusted retention time being as short as 1.4 min, in order to increase the analytical capability. Chromatograms obtained from plasma spiked

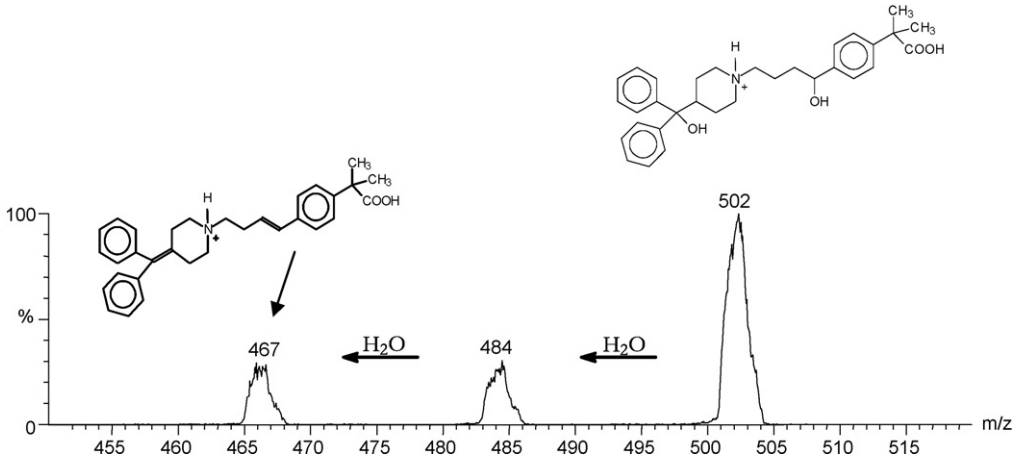

Fig. 3. CAD mass spectra of the fexofenadine protonated molecule.

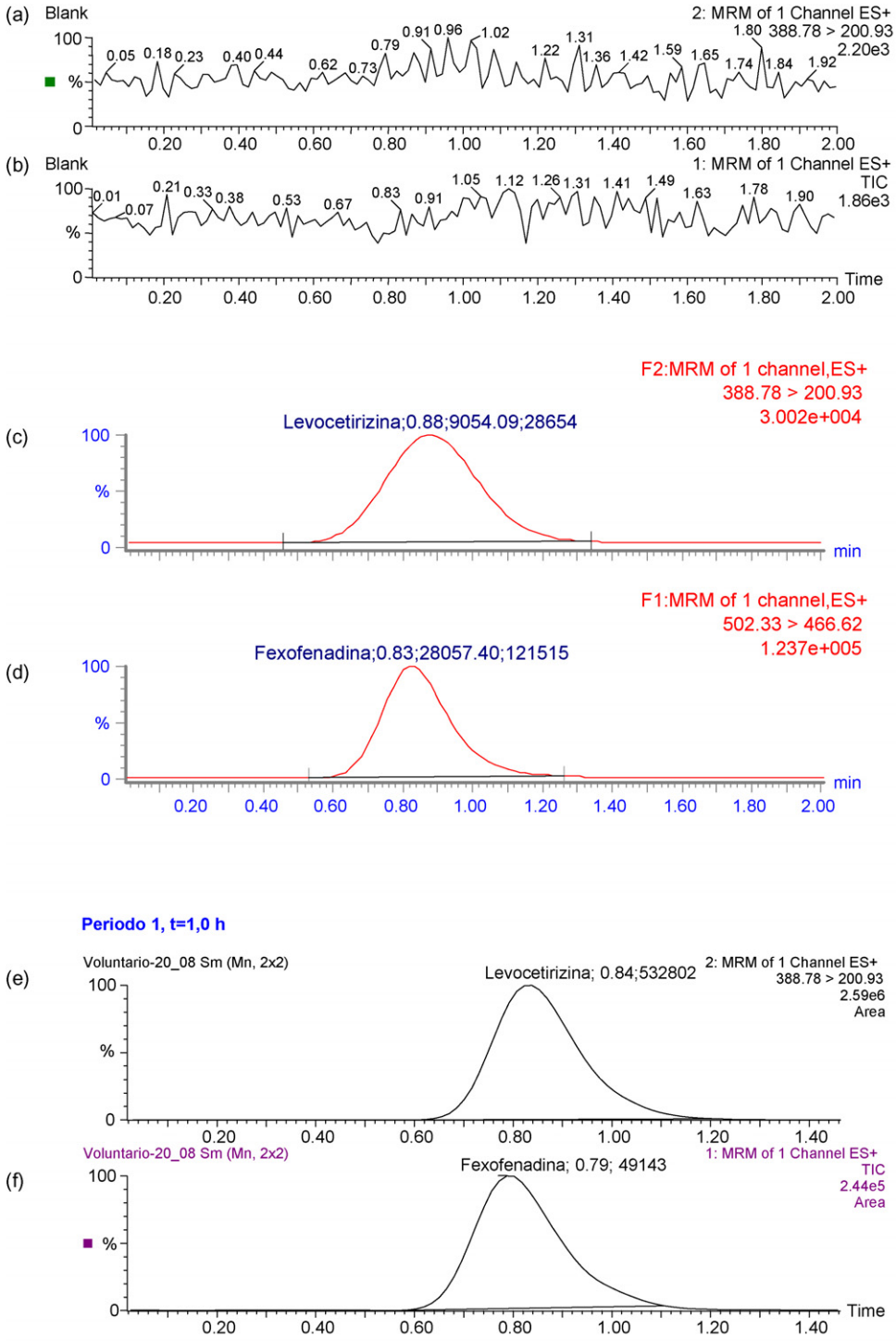

Fig. 4. Representative SRM chromatograms of levocetirizine in human plasma: (a) levocetirizine and (b) fexofenadine blank human plasma, (c) levocetirizine and (d) fexofenadine-spiked human plasma containing 0.50 ng/mL levocetirizine and internal standard, (e) levocetirizine and (f) fexofenadine from a volunteer 1 h after administration of 5 m of levocetirizine.

with levocetirizine (0.5 ng/mL) and fexofenadine (2 μg/mL) are shown in Fig. 4c and d. Levocetirizine and fexofenadine from a volunteer 1 h after administration of 5 mg of levocetirizine are shown in Fig. 4e and f.

The matrix effect was evaluated directly by extracting blank plasma and then spiking with the analyte at the LOQ concentration. There was no difference observed between the signal for the solution and the spiked extract at the LOQ concentration.

3.3. Linearity, precision and accuracy

Calibration curves were plotted as the peak area ratio (drug/IS) versus drug concentration. The assay was linear in the

Table 1
Calibration curves from one batch of the validation section

Spiking plasma concentration (ng/mL)	Concentration measured (mean) (ng/mL)	R.S.D.[a] (%) ($n=3$)	Relative error[b]
0.50	0.50	0	0
1.00	1.03	5.82	3.00
5.00	5.43	6.99	8.60
10.00	9.83	2.13	−1.70
50.00	49.13	3.46	−1.74
100.00	96.43	1.10	−3.57
500.00	487.17	0.94	−2.56

Relative error of the mean is calculated as. Typical calibration curve for levocetirizine: $y=0.0613675x+0.0120891$ (correlation coefficient, $r=0.997608$), y: concentration in ng/mL, x: ratios of levocetirizine peak area to that of IS.
[a] Standard deviation/mean concentration measured × 100.
[b] ((Mean concentration measured − spiked plasma concentration)/spiked plasma concentration) × 100.

concentration range of 0.5–500.0 ng/mL. The R.S.D. were less than 7%. The relative error (RE) of the mean of the measured concentrations ranged from 8.60% to −3.57%. The determination coefficients (R^2) were greater than 0.997 for all curves (Table 1). Precision and accuracy for this method were controlled by calculating the intra-batch and inter-batch variation at three concentrations (1.50, 200.00 and 400.00 ng/mL) of QC samples in five replicates. As shown in Table 2, the intra-batch R.S.D.s and REs were less than 6%. These results indicate that the method is reliable and reproducible within its analytical range.

3.4. Freezing and thawing stabilities

The results of the freeze–thaw stability studies are shown in Table 3. Quantification of the analyte in plasma subjected to a number of freeze–thaw (−70 °C to room temperature) cycles showed that the analyte is stable after three cycles. No analyte degradation had taken place over a 16-h storage period in the autosampler tray with the final concentrations of levocetirizine ranging from 88.89% to 104.42% of the theoretical values. In addition, the long-term stability of levocetirizine in QC samples after 68 days of storage at −70 °C was also evaluated. The concentrations ranged from 97.33% to 104.25% of the theoretical values. Levocetirizine was therefore stable in human plasma for at least 68 days at −70 °C.

3.5. Recovery

There were tested a variety of extractions, ethyl acetate, hexane, chloroform, otherwise the best recovery was achieved for the liquid–liquid extraction with dichloromethane. The recovery was calculated by comparing the peak area ratios of levocetirizine in plasma samples with the peak area ratios of solvent samples, was estimated at control levels of levocetirizine. The recovery of levocetirizine, determined at three different concentrations (1.50, 200.00 and 400.00 ng/mL), were 82.97%, 85.78% and 95.04%, respectively; the overall average recovery was 87.93%.

The recovery of IS was also tested using the same methodology the mean recovery of fexofenadine was 103.44%.

3.6. Application to biological samples

The proposed analytical method was applied to a bioequivalence study of two levocetirizine formulations. This randomized crossover study was conducted with 35 subjects after a single oral dose (5 mg) of the drug. Typical plasma concentration versus time profiles is shown in Fig. 4. Plasma concentrations of levocetirizine were in the standard curve range and remained above the 0.5 ng/mL (quantification limit for the entire sampling period). The observed maximum plasma concentration (C_{max}) was 228.11 ± 60.55 ng/mL for the reference and 226.91 ± 61.76 ng/mL for the test. Time to achieve

Table 2
Precision and accuracy (analysis with spiking plasma samples at three different concentrations)

Spiking plasma concentration (ng/mL)	Within-run			Between-run		
	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D.[a] (%) ($n=5$)	Relative error[b] (%)	Concentration measured (mean ± S.D.) (ng/mL)	RSD[a] (%) ($n=15$)	Relative error[b] (%)
1.50	1.48	2.70	−1.33	1.47	4.76	−2.00
200.00	200.66	5.66	0.33	199.27	7.14	−0.36
400.00	395.30	4.53	−1.17	391.34	3.77	−2.16

[a] (Standard deviation/mean concentration measured) × 100.
[b] ((Mean concentration measured − spiked plasma concentration)/spiked plasmaconcentration) × 100.

Table 3
Freeze and thaw stabilities of the samples

Sample concentration (ng/mL)	Initial (0 h)		Cycle 1 (24 h)		Cycle 2 (36 h)		Cycle 3 (48 h)	
	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D.[a] (%) ($n = 5$)	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D.[a] (%) ($n = 5$)	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D.[a] (%) ($n = 5$)	Concentration measured (mean ± S.D.) (ng/mL)	R.S.D.[a] (%) ($n = 5$)
1.50	1.46 ± 0.05	3.42	1.44 ± 0.13	9.02	1.60 ± 0.17	10.62	1.58 ± 0.11	6.96
200.00	208.50 ± 6.93	3.32	218.16 ± 7.37	3.37	217.54 ± 7.79	3.58	222.06 ± 4.16	1.87
400.00	412.66 ± 19.15	4.64	423.52 ± 9.89	2.33	428.18 ± 17.73	4.14	422.64 ± 40.67	9.61

[a] (Standard deviation/mean concentration measured) × 100.

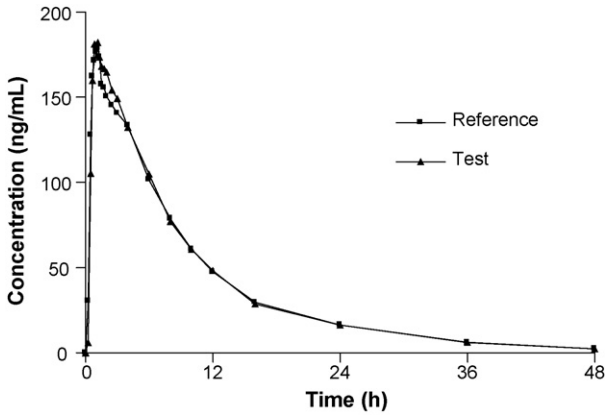

Fig. 5. Plasma concentration–time curves of levocetirizine test and reference formulations administrated to 35 volunteers.

maximum plasma concentration (T_{max}) was 0.83 ± 3.50 h for the reference and 0.83 ± 2.34 h for the test. The value of area under the curve from time 0 to the last sampling time (AUC_{0-t}) was 1743.71 ± 492.24 ng h/mL for the reference and 1752.91 ± 447.02 ng h/mL for the test, and area under the curve from 0 to ∞ ($AUC_{0-\infty}$) was 1775.40 ± 507.29 ng h/mL for the reference and 1785.85 ± 461.27 ng h/mL for the test.

The elimination half-life ($t_{1/2}$) was 8.07 ± 5.86 h for the reference and 8.99 ± 4.67 h for the test. The pharmacokinetic data obtained were similar to those reported by Passalacqua and Canonica [2]. In addition, the mean ratio of $AUC_{0-t}/AUC_{0-\infty}$ was higher than 80% in accordance with the Food and Drug Administration Bioequivalence Guideline [11].

The ratio of test/reference (T/R) and 90% percent confidence intervals (90 CIs) for overall analysis were comprised within the previously stipulated range (80–125%).

The ratio T/R and 90 CIs (in parenthesis) was 99.43% (94.29–104.86%) for C_{max}, 101.11% (97.42–104.93%) for AUC_{0-t} and 101.17% (97.49–104.99%) for $AUC_{0-\infty}$. Therefore, the results demonstrated the bioequivalence of the two formulations of levocetirizine (Fig. 5).

4. Conclusion

In conclusion, the use of LC–MS/MS allows an accurate, precise and reliable measurement of levocetirizine concentrations in human plasma for up to 48 h after a single oral dose of 5 mg to healthy volunteers. The described method has proven to be fast and robust, with each sample requiring less than 3 min analysis time. The sensitivity of the assay is sufficient to follow accurately the pharmacokinetics of this drug.

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