Journal of Chromatographic Science Advance Access published June 17, 2015
Journal of Chromatographic Science 2015;1–10
doi:10.1093/chromsci/bmv069

Article

Determination of Levocetirizine in Human Plasma by LC–MS-MS: Validation and Application in a Pharmacokinetic Study

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Received 24 August 2014; revised 3 April 2015

A fast and simple sample cleanup approach for levocetirizine in human was developed using protein precipitation coupled with LC–MS-MS. Samples were treated with 6% trichloroacetic acid in water prior to LC–MS-MS analysis. Chromatographic separation was performed on a reverse phase column with an isocratic mobile phase of acetonitrile and 10 mM ammonium formate pH 3.5 (80:20, v/v) at a flow rate of 1.0 mL/min. The run time was 3.5 min. Mass parameters were optimized to monitor transitions at m/z $[M+H]^+$ 389.0→201.0 for levocetirizine and m/z $[M+H]^+$ 375.3→201.0 for hydroxyzine as internal standard. The lower limit of quantification and the dynamic range were 1.00 and 1.00–500 ng/mL, respectively. Linearity was good for intraday and interday validations ($r^2 \geq 0.995$). The mean recoveries were 59 and 69% for levocetirizine and hydroxyzine, respectively. Matrix effect was acceptable with %CV < 15. Hemolytic effect was negligible. Levocetirizine was stable in human plasma for 27 h at room temperature (25°C), for 16 weeks frozen at −70°C, 4 weeks frozen at −20°C, for 24 h in an autosampler at 15°C and for three freeze/thaw cycles. The validated method was applied in a pharmacokinetic study to determine the concentration of levocetirizine in plasma samples. The study provides a fast and simple bioanalytical method for routine analysis and may be particularly useful for bioequivalence studies.

Introduction

Levocetirizine (2-[2-[4-[(*R*)-(4-chlorophenyl)phenylmethyl]-piperazinyl-1-yl]ethoxy]acetic acid) is the active *R*-enantiomer of racemic cetirizine. It is categorized in the second generation of oral non-sedative antihistamines for the treatment of mild-to-moderate seasonal allergic rhinitis and perennial allergic rhinitis (1, 2) and is also considered as a first-line agent for treatment of chronic idiopathic urticarial (3). Structurally, levocetirizine belongs to the diphenylpiperazine class of compounds with selective H_1-antagonist effects. It has excellent efficacy and a favorable safety profile with low metabolism and an absence of cardiac side effects when coadministered with liver enzyme-inhibiting drugs (4, 5). Levocetirizine shows higher binding affinity for human H_1 histamine receptor by about 30-fold compared with dextrocetirizine, the *S*-enantiomer of racemic cetirizine (6, 7). Oral administration of a levocetirizine 5-mg tablet showed t_{max} at 0.9 h with C_{max} about 270 ng/mL and an elimination half-life of about 8 h (7).

Liquid chromatography-tandem mass spectrometry (LC–MS-MS) is a common technique for determination of drug concentrations in biological matrices because it gives reliable quantitative results and is sensitive and specific. A few analytical methods for determination of levocetirizine in human plasma have been reported (8–10). Morita *et al.* (8) developed an LC–ESI-MS-MS method for determination of levocetirizine in human plasma with a liquid–liquid extraction procedure using cold dichloromethane for sample preparation. This method showed good sensitivity with a lower limit of quantification (LLOQ) of 0.5 ng/mL and a dynamic range of 0.5–500 ng/mL. Gunasakaran *et al.* (9) also described a LC–ESI-MS-MS method for determination of levocetirizine in human plasma with a liquid–liquid extraction procedure using ethyl acetate for sample preparation. This method had an LLOQ of 5.00 ng/mL and a calibration range of 5–600 ng/mL. Kang *et al.* (10) introduced a chiral separation technique by normal phase chromatography with LC–APCI-MS-MS to analyze levocetirizine in plasma using protein precipitation followed by liquid–liquid extraction for sample preparation. This method showed good sensitivity with an LLOQ of 0.5 ng/mL and a linear range of 0.5–300 ng/mL.

Sample cleanup is important in bioanalysis because the complexity of biological matrices can cause inaccuracy, imprecision and ion suppression. Several sample preparation methods have been used for plasma sample cleaning prior to analysis to ensure sensitivity and robustness. These include protein precipitation, liquid–liquid extraction and solid-phase extraction. Among these methods, protein precipitation is usually preferred because it is a fast and simple cleanup procedure, even though it is not a sophisticated method. Complicated sample preparations such as liquid–liquid and solid phase extractions can be used in sample cleaning, but these techniques require skill and time to perform. This may cause unintentional error in the analytical results and is also impractical in a routine assay with numerous samples. Automated sample cleanup is another approach for time reduction in sample preparation, but is not easy to set up.

Although protein precipitation is a fast and simple sample preparation technique, establishment of a suitable protein precipitant at an appropriate concentration is necessary to ensure minimum interference in the remaining sample. There have been several attempts to use conventional protein precipitants such as methanol and acetonitrile, acetonitrile or methanol spiked with a small amount of formic acid and aqueous trichloroacetic acid for sample preparation of levocetirizine for determination in human plasma (8–10). However, such protein precipitants failed to provide a sufficiently clean sample, and recovery was inconsistent for determination of levocetirizine in human plasma. The analytical methods for determination of levocetirizine in human plasma previously reported used liquid–liquid extraction (8, 9) or protein precipitation coupled with

liquid–liquid extraction (10). Sample preparation using protein precipitation alone has not yet been applied for determination of levocetirizine in human plasma.

In the current work, we first report a simple technique of plasma sample preparation with protein precipitation using trichloroacetic acid in acetonitrile. To ensure that protein precipitation provides a sufficiently clean sample with residual interference that insignificantly affects analytical performance, the developed method was tested for matrix and hemolytic effects. In addition, we use hydroxyzine, a structural analog of levocetirizine, as an internal standard with LC conditions capable of eluting levocetirizine and the internal standard within 2 min. The developed sample preparation and LC–MS-MS methods can therefore be considered to be simple, rapid and practical for routine analysis of numerous samples in pharmacokinetic and bioequivalence studies in bioanalytical and clinical laboratories. Because the bioanalytical method presented here has its application in pharmacokinetic and bioequivalence studies, the analytical method validation must be performed as regulated by a government agency such as the FDA prior to its application in such studies. In this manuscript, we have performed bioanalytical method validation strictly in accordance with the US-FDA and EMEA guidelines on bioanalytical method validation (11, 12). The method was fully validated covering all validation parameters listed in the guidance. We also demonstrated the application of the validated method in a pharmacokinetic study to determine the concentration of levocetirizine in plasma samples collected after oral administration of a levocetirizine 5-mg tablet in healthy volunteers.

Experimental

Drugs and chemicals

Levocetirizine dihydrochloride (lot number LCZ-1009012, purity 99.5%) and hydroxyzine dihydrochloride (Fig. 1) (lot number 0000006114, purity 99.0%) were provided by Siam Bheasach Co. Ltd (Bangkok, Thailand). Hydroxyzine dihydrochloride was used as an internal standard (IS). Levocetirizine 5-mg tablets were provided by Siam Bheasach Co. Ltd (Bangkok, Thailand). Trichloroacetic acid was purchased from Merck (Darmstadt, Germany). Formic acid and ammonium formate were purchased from Carlo Erba (Milano, Italy). Acetonitrile and methanol (HPLC grade) were purchased from Scharlau (Barcelona, Spain). All other reagents were of at least analytical grade. Blank human plasma in collection bags containing sodium citrate as an anticoagulant was supplied by the Thai Red Cross Society (Bangkok, Thailand). Na_2EDTA blood collection tubes were used for clinical blood collection in a pharmacokinetic study.

Mass spectrometry and liquid chromatography

Mass spectrometric analysis was performed using an Applied Biosystems/MDS Sciex API4000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. The interface was operated in positive ionization mode. High-purity nitrogen served both as collision-induced dissociation (CAD) gas and curtain gas setting at 6 and 22 psi, respectively. The nebulizer was supplied with ultra-high purity nitrogen, regulated by ion source gas 1 and 2, both at 65 psi. The ion spray temperature (TEM) was maintained at 600°C and the ion spray voltage (ISV) was optimized at a potential of 5500 V.

Optimization of the MS-MS parameters was performed by direct infusion with a 1000 μL syringe connected to a pump with a flow rate of 20 μL/min. Multiple reaction monitoring (MRM) in positive ionization mode was used to monitor transitions at m/z $[M+H]^+$ 389.0→201.0 for levocetirizine and m/z $[M+H]^+$ 375.3→201.0 for hydroxyzine (IS). The collision energy (CE) and collision cell exit potential (CXP) were optimized to achieve the largest response for both levocetirizine and hydroxyzine. The CE values for levocetirizine and hydroxyzine were 30.00 and 27.65 V, respectively. The CXP were set at 19.10 and 11.63 V for levocetirizine and hydroxyzine, respectively. The dwell time for both levocetirizine and hydroxyzine monitoring was set at 200 ms. Acquisition and processing of data were performed using Applied Biosystems/ MDS Sciex Analyst™ software, version 1.4.2.

The liquid chromatography system (Shimadzu, Tokyo, Japan) consisted of a Shimadzu LC-20AD prominence pump, equipped with a Shimadzu DGU-20A3 degasser and a Shimadzu SIL-20AC thermostat autosampler at 15°C. Analytical separation was achieved using an Altima C18 column (150 × 4.6 mm i.d., 5 μm particle size, Grace Associates, Inc., Deerfield, IL, USA) placed in a Shimadzu CTO-20A column oven set at 33°C. Samples were eluted with an isocratic mobile phase of acetonitrile and 10 mM ammonium formate pH 3.5 (80:20, v/v) at a flow rate of 1.0 mL/min. The entire flow was directed into the mass spectrometry source without splitting. The injection volume was 10 μL. The run time was 3.5 min.

Preparation of standard stock solutions

A stock standard solution of levocetirizine was prepared in 50% acetonitrile at a concentration of 50 μg/mL. The stock solution was further diluted in 50% acetonitrile to give appropriate working standard solutions at 10, 20, 100, 500, 1000, 2000, 3500 and 5000 ng/mL for preparation of calibration standards. Another stock solution at the same concentration was diluted with 50%

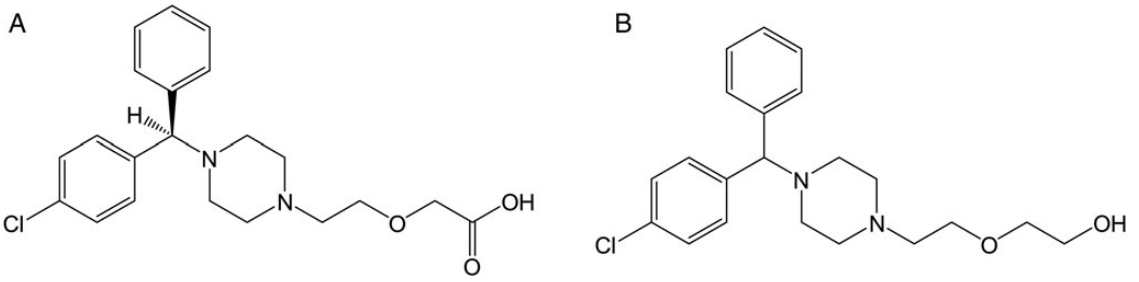

Figure 1. Chemical structures of (A) levocetirizine and (B) hydroxyzine.

acetonitrile to give appropriate working standard solutions at 30, 2500 and 4500 ng/mL for preparation of quality control (QC) samples. An internal standard stock solution of hydroxyzine at 1000 μg/mL was prepared in methanol and further diluted with 50% acetonitrile to obtain a working internal standard solution at 2 μg/mL.

Preparation of calibration standards and QC samples

Eight concentration levels of the calibration standards were prepared by spiking the working standard solutions in blank human plasma to yield final concentrations of 1, 2, 10, 50, 100, 200, 350 and 500 ng/mL. The final concentration at 1.00 ng/mL was used as an LLOQ. Three concentration levels of QC samples were prepared from blank human plasma spiked with the working standard solutions to yield final concentrations of 3, 250 and 450 ng/mL, corresponding to low, medium and high QC concentration levels (LQC, MQC and HQC).

Preparation of clinical blood samples

Blood samples were collected from six adult healthy volunteers who gave informed consent. Blood samples were taken using an indwelling cannula placed in a forearm vein and kept patent with normal saline. Blood samples were collected in Na_2EDTA blood collection tubes and centrifuged for 10 min at 3,200 g. The resulting plasma was transferred into polypropylene tubes and stored at −70°C before analysis.

Preparation of samples prior to LC–MS-MS analysis

Hydroxyzine working IS solution (30 μL) was spiked into 300 μL of each plasma sample (i.e., calibration standards, QC samples and clinical plasma samples). The IS spiked samples were vortexed for 30 s and then treated with 300 μL of 6% trichloroacetic acid (TCA) in water for protein precipitation. The samples were further vortexed for 30 s and centrifuged at 14,000 rpm at 10°C for 10 min. Finally, 10 μL of the supernatant was injected into the LC–MS-MS system.

Method validation

The analytical method was validated in terms of linearity, accuracy, precision, specificity, sensitivity, recovery and stability based on the US-FDA guidance for bioanalytical methods (11). Additionally, matrix effects and impact of hemolysis were also validated (12, 13). The QC sample at 250 ng/mL (MQC) was used to verify the suitability of the chromatographic system before analysis.

Specificity

Specificity was evaluated by screening blank plasma from six different lots spiked with levocetirizine at the LLOQ. The spiked samples were extracted and the presence or absence of interfering peaks at the same retention time of levocetirizine or hydroxyzine IS was examined. The interference of Na_2EDTA was evaluated by comparing the MRM chromatograms of blank plasma with and without Na_2EDTA. The Na_2EDTA-containing blank plasma was prepared by adding blank plasma to an Na_2EDTA blood collection tube and mixed thoroughly before analysis.

Calibration curves and linearity

Calibration curves were constructed by plotting the concentrations of levocetirizine vs. the peak area ratio of the quantifying ion of levocetirizine (m/z $[M+H]^+$ 389.0→201.0) to that of hydroxyzine (m/z $[M+H]^+$ 375.3→201.0). The equation model was obtained by weighted least squares linear regression analysis with a weighting factor of $1/x^2$. The suitability of the equation was confirmed by back-calculating the concentrations of the calibration standards. Linearity was assessed by constructing calibration curves on five consecutive days. The coefficient of determination (r^2) should be >0.99. Concentrations of unknown samples were determined by applying the linear regression equation of the calibration curve to the peak area ratio of the unknown samples.

Accuracy and precision

Intraday accuracy and precision were assessed from five replicates of three QC concentration levels (LQC, MQC and HQC). Interday accuracy and precision were assessed on triplicates for 5-day analysis of the three QC concentration levels. Accuracy of the method is shown as %deviation and was calculated based on the difference between the mean concentration found and concentration added. The %deviation should be within 15% of the actual value. Precision was evaluated based on the percentage coefficient of variation (%CV) of the mean concentration found. The precision determined at each concentration level should not exceed 15% of the CV.

Recovery

According to the US FDA guidance, the term of recovery is related to the extraction efficiency of an analytical method within the limits of variability (11). The efficacy of levocetirizine extraction from human plasma was determined at the LQC, MQC and HQC levels by comparing the peak areas of levocetirizine extracted from spiked plasma samples prepared by spiking levocetirizine in blank plasma with the corresponding concentration of the authentic standard solution prepared by replacing plasma with water and subsequently diluting 6% trichloroacetic acid to mimic the extracted sample. For the IS, the recovery of extract was evaluated using the same procedure. The experiments were performed in five replicates. The extent of recovery is considered to be consistent, precise and reproducible if the %CV of recovery content is within 15%.

Matrix effect

The effect of plasma matrix was determined for levocetirizine and hydroxyzine separately and reported as matrix factors (MFs). The ratio between the MF of levocetirizine and that of hydroxyzine IS is calculated and termed as the IS-normalized MF. Six different lots of blank human plasma were evaluated. The determination was performed at the LQC and HQC levels in triplicate for each lot. The MF of levocetirizine was calculated by comparing the peak area of levocetirizine extracted from samples prepared by spiking levocetirizine in extracted blank plasma with the corresponding concentration of the authentic solution of levocetirizine prepared in 50% acetonitrile. The MF of hydroxyzine IS was determined and calculated in a similar manner. The IS-normalized MF is then calculated by dividing the MF of levocetirizine to that of hydroxyzine. The variability in the matrix

effect measured by coefficient of variation (%CV) of the IS-normalized MF from the six different lots of blank plasma should not exceed 15% (12–14).

Hemolytic effect

For hemolytic effect, the accuracy and precision experiments were performed using the spiked normal plasma for calibration standards and spiked hemolytic plasma for five replicates of each LQC, MQC and HQC samples. The criteria for acceptability of the hemolytic effect data included accuracy within ± 15%deviation from the actual values and precision of less than 15%CV.

Lower limit of quantification

The sensitivity of the method was expressed as LLOQ. Plasma samples spiked with different concentrations of levocetirizine in five replicates were determined and the lowest concentration quantified with precision ≤20%CV and accuracy within ± 20%deviation was set as LLOQ. The peak response of levocetirizine at LLOQ must be at least five times the baseline noise.

Stability

All stability studies of levocetirizine in human plasma were performed in triplicate at the LQC and HQC levels. Freeze–thaw, short-term, long-term and post-preparative stabilities of levocetirizine and hydroxyzine (IS) were tested. The freeze–thaw stability was examined by freezing samples at −70°C and thawing at room temperature (25°C). After three freeze–thaw cycles, the samples were analyzed in comparison with freshly thawed samples at the same concentration. Short-term stability was tested by storage of samples at room temperature (25°C) over 27 h. Long-term stability was tested by storage at −20°C for 4 weeks and at −70°C for 16 weeks. For post-preparative or autosampler stability, the stability of extracted samples was determined in the controlled temperature autosampler (15°C) for 24 h. The level of levocetirizine at any time was determined in comparison with that in corresponding freshly prepared control samples. Samples were considered to be stable when assay values were within ± 15%deviation for accuracy and ± 15%CV for precision.

A pharmacokinetic study

The validated method was used to determine the concentrations of levocetirizine in human plasma samples collected from six healthy volunteers who received a single-dose 5-mg tablet after providing informed consent. The six healthy volunteers included three Thai males and three Thai females. A physical examination including body mass index, pulse, blood pressure and body temperature was a prerequisite for all volunteers. The inclusion criteria for volunteer selection were based on age (20–45 years) and body mass index (18–25 kg/m^2). Vital signs including pulse (60–90 bpm), blood pressure (SBP of 100–135 mmHg and DBP of 60–90 mmHg) and body temperature (36.5–37.5°C) were monitored prior to and during the study. All subjects were in good health, as shown by clinical laboratory screening including serology, hematology and biochemistry tests. None of the volunteers reported a history of allergy to levocetirizine or related derivatives. All subjects abstained from intake of other drugs and alcohol for 2 weeks prior to and throughout the study. Caffeine-containing beverages were not allowed in the 3 days prior to and during the study. All the subjects were informed of the aims and risks in the study and written consent was obtained. The clinical study protocol was submitted to the Local Medical Ethics Committee (Institutional Review Board, Chulalongkorn University, Bangkok, Thailand) for human research ethics approval. The study was approved prior to the study which was carried out in accordance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Blood samples were collected pre-dose and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 6, 9, 12, 24, 36 and 48 h post-dose. Blood samples were collected in Na_2EDTA blood collection tubes. The samples were centrifuged at 4,000 rpm for 10 min and immediately stored at −20 ± 5°C before transfer to storage at −70°C until analysis. A 300-μL aliquot of thawed plasma was spiked with IS and then treated as described in the sample preparation section. The study was performed on three consecutive days. QC samples were distributed among unknown samples in the analytical run to verify the analytical system during analysis. A typical injection sequence for each day was performed in the following order: blank, calibration set, QC set, sample set, QC set, sample set and QC set.

Results

Mass spectrometry

The mass spectrometric parameters optimized in this study provide the highest sensitivity of signal responses for both precursor and product ions of levocetirizine and hydroxyzine. The fragmentation mass spectra of protonated levocetirizine and hydroxyzine species are shown in Figure 2A and B, respectively. The precursor ions of levocetirizine and hydroxyzine were found at m/z $[M + H]^+$ 389.0 and 375.3, respectively. The two compounds produced the same product ion at m/z $[M+H]^+$ 201.0 and this ion was used as the quantifying ion.

Method validation

Specificity

Typical MRM chromatograms of levocetirizine and hydroxyzine (IS) in blank human plasma, blank human plasma spiked with Na_2EDTA, blank human plasma spiked with IS and blank human plasma spiked with levocetirizine at LLOQ (1 ng/mL) are shown in Figure 3A–D, respectively. The retention times of levocetirizine and hydroxyzine IS were about 1.6 and 2.0 min, respectively (Fig. 3D). The analysis of levocetirizine showed no interference peaks from endogenous substances at the retention times of levocetirizine and hydroxyzine (Fig. 3A). The chromatograms of blank plasma containing Na_2EDTA also had no interference peaks at the retention times of levocetirizine and hydroxyzine IS (Fig. 3B).

Calibration curve and linearity

The calibration curve results are shown in Table I. The calibration curve evaluated by determining the best fit of peak area ratios against drug concentration using a weighted-linear least square model with a weighting factor of $1/x^2$ was found to be linear in the concentration range of 1–500 ng/mL ($r^2 > 0.995$, mean

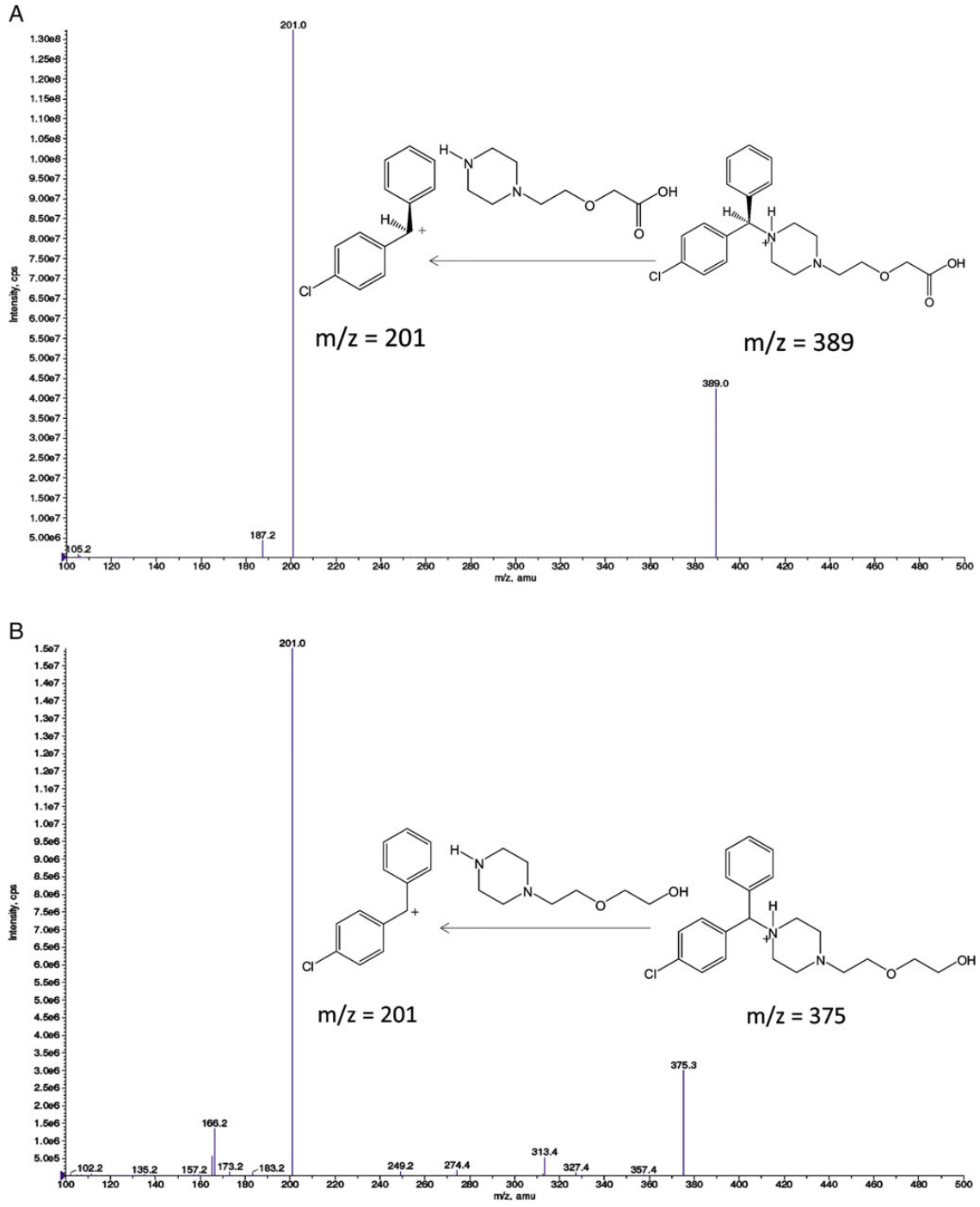

Figure 2. Product ion mass spectra of $[M+H]^+$of (A) levocetirizine and (B) hydroxyzine (IS). This figure is available in black and white in print and in color at *JCS* online.

$r^2 = 0.997$). The %deviation of the mean back-calculated concentration and actual spiked plasma concentration ranged from −6.6 to 6.2% with a %CV of 1.9–6.0%.

Accuracy, precision and recovery

The accuracy and precision data are summarized in Table II. The intraday accuracy and precision showed %deviation ranged from −7.4 to 0.3% with a %CV of 2.5–4.2%. The interday accuracy and precision showed %deviation ranged from −7.1 to −0.5% with a %CV of 2.3–3.7%. Recovery results are shown in Table III. The mean recoveries of extraction of levocetirizine at three QC concentration levels of 3, 250 and 450 ng/mL were 57.8, 64.1 and 55.2%, respectively, with an overall average recovery of 59.0%. The %CV ranged from 8.1 to 13.0%.The mean recovery of hydroxyzine (IS) was 68.8% with a %CV of 9.8%.

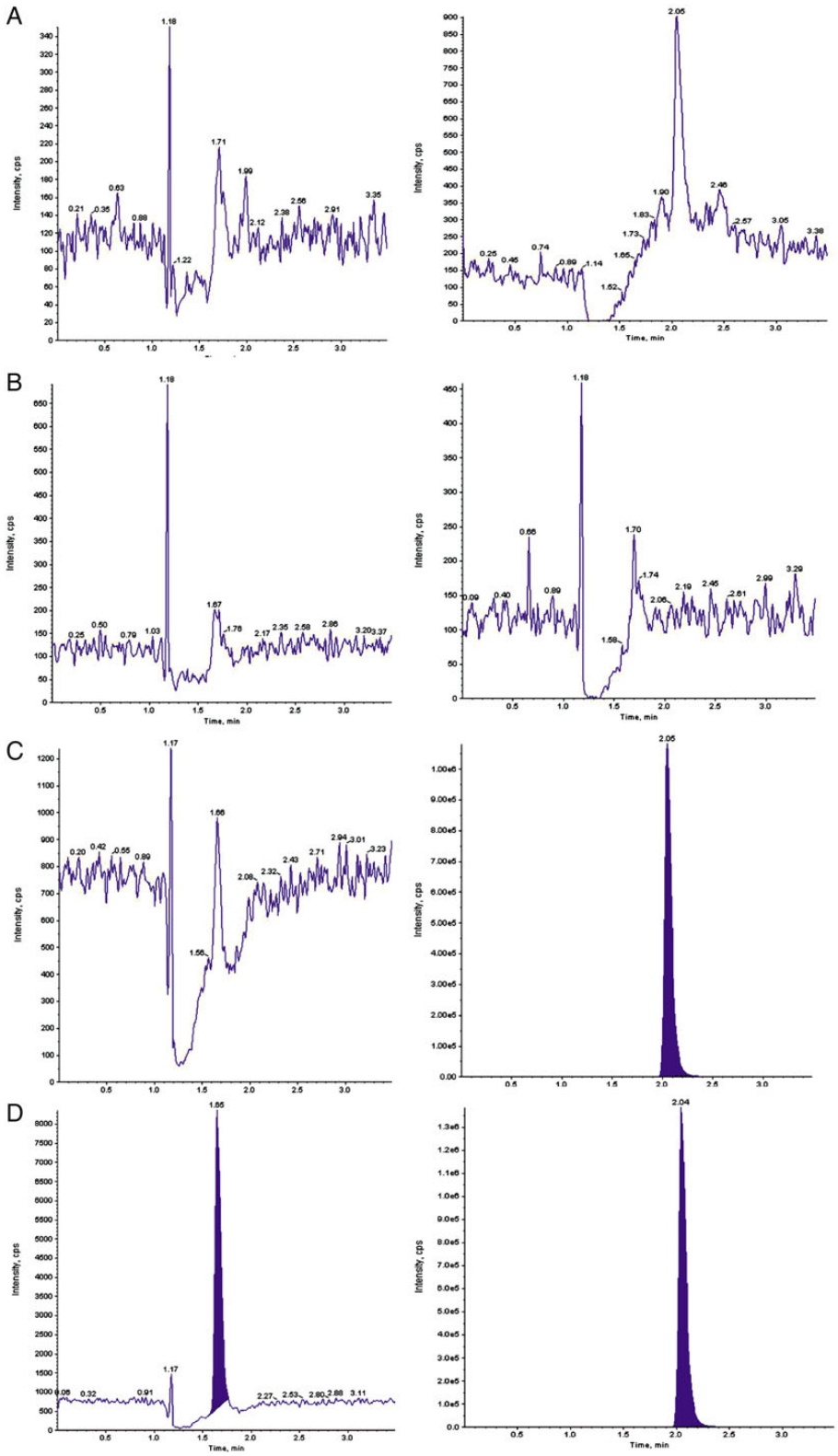

Figure 3. Typical MRM ion chromatograms of levocetirizine (left panel) and hydroxyzine (IS) (right panel) in (A) blank human plasma, (B) blank human plasma with EDTA, (C) blank human plasma spiked with IS and (D) human plasma spiked with levocetirizine at LLOQ (1 ng/mL) and IS. This figure is available in black and white in print and in color at *JCS* online.

Table I
Mean Interday Back-Calculated Standard and Standard Curve Results ($n = 5$)

Concentration added (ng/mL)	Concentration found (ng/mL)					Mean concentration found (ng/mL)	%Deviation	%CV
	Day 1	Day 2	Day 3	Day 4	Day 5			
1	1.00	0.99	0.96	0.96	0.98	0.98	−2.00	1.94
2	1.99	2.05	2.12	2.14	2.06	2.07	3.59	2.85
10	9.74	9.49	11.07	10.09	10.22	10.12	1.22	5.96
50	55.14	51.22	50.86	54.8	53.39	53.08	6.16	3.73
100	101.46	103.11	97.49	98.61	102.11	100.56	0.56	2.38
200	193.06	200.04	197.03	197.1	202.11	197.86	−1.07	1.73
350	352.15	353.17	333.85	346.47	332.93	343.71	−1.80	2.84
500	469.41	484.52	470.77	450.95	458.39	466.81	−6.64	2.75

Table II
Intraday and Interday Accuracy and Precision of Levocetirizine Determination

Concentration added (ng/mL)	Intraday ($n = 5$)			Interday ($n = 15$)		
	Mean concentration found (ng/mL)	Accuracy (%Deviation)	Precision (%CV)	Mean concentration found (ng/mL)	Accuracy (%Deviation)	Precision (%CV)
3	3.01	0.29	2.50	2.99	−0.46	2.29
250	246.60	−1.36	4.19	242.43	−3.03	3.71
450	416.88	−7.36	3.43	418.05	−7.10	3.42

Table III
Recovery of Levocetirizine and Hydroxyzine (IS) in Human Plasma ($n = 5$)

Concentration added (ng/mL)	Mean peak area response		%Mean recovery (%CV)
	Authentic solution	Spiked plasma sample	
Levocetirizine			
3	101,726	58,627	57.8 (8.1)
250	7,696,417	4,934,689	64.1 (9.9)
450	13,405,169	7,380,456	55.2 (13.0)
Hydroxyzine			
180	11,003,786	7,582,818	68.8 (9.8)

Table IV
Matrix Effect of Levocetirizine in Six Lots of Human Plasma ($n = 6$)

Concentration added (ng/mL)	Plasma lot	Matrix factor (%)		IS-normalized MF	%CV
		Levocetirizine	Hydroxyzine (IS)		
3	No. 1	107.3	120.0	89.5	3.9
	No. 2	89.4	107.0	83.5	
	No. 3	82.5	100.3	82.2	
	No. 4	102.4	116.6	87.8	
	No. 5	78.3	95.0	82.4	
	No. 6	81.1	88.3	91.9	
450	No. 1	148.2	118.9	124.8	2.2
	No. 2	139.0	107.4	129.4	
	No. 3	128.4	104.0	123.5	
	No. 4	139.8	109.8	127.4	
	No. 5	115.3	93.8	123.2	
	No. 6	104.4	87.9	118.8	

Table V
Hemolytic Effect of Levocetirizine in Hemolysed Human Plasma ($n = 5$)

Concentration added (ng/mL)	Mean concentration found (ng/mL)	Accuracy (% Deviation)	Precision (% CV)
3	3.13	4.3	3.3
250	247.9	−0.8	3.6
450	436.9	−2.9	1.5

Matrix and hemolytic effects

The effects of plasma matrix on the analysis of levocetirizine are summarized in Table IV. The %CVs of IS-normalized MF were 3.9 and 2.2% for LQC and HQC, respectively. The effects of hemolysis on the analysis of levocetirizine are summarized in Table V. The %deviation ranged from −2.9 to 4.3% with a %CV of 1.5–3.6%.

Lower limit of quantification

A typical chromatogram of an LLOQ sample (1.00 ng/mL) is shown in Figure 3D. The mean signal to noise ratio was 16 (that is, >5, meeting the required criterion). The %deviation ranged from −6.9 to −0.7% with a %CV of 4.1%.

Stability

Stability results are summarized in Table VI. Levocetirizine was stable for at least three freeze–thaw cycles, for at least 27 h in human plasma at room temperature (25°C), for 4 weeks at −20°C, for 16 weeks at −70°C, and for 24 h in the autosampler (15°C).

Application for a pharmacokinetic study

To verify the sensitivity and selectivity of this method in a real situation, the assay was used to determine levocetirizine in human plasma samples collected from six healthy volunteers. A typical mean plasma concentration versus time profile of levocetirizine is illustrated in Figure 4. The areas under the curve from time 0 to t ($AUC_{0\rightarrow t}$) and from time 0 to ∞ ($AUC_{0\rightarrow\infty}$) were 1584.4 ± 307.8 and 1606.5 ± 309.3 ng·h/mL, respectively. The ratio of $AUC_{0\rightarrow t}$ to $AUC_{0\rightarrow\infty}$ was about 98.6%. The observed maximum plasma concentration (C_{max}) was 196.1 ± 36.4 ng/mL and the time to achieve the maximum plasma concentration (t_{max}) was 0.63 ± 0.14 h. The elimination half-life ($t_{1/2}$) of levocetirizine was 7.90 ± 0.82 h. The pharmacokinetic parameters are summarized in Table VII.

Table VI
Stability Results of Levocetirizine in Human Plasma ($n = 3$)

Stability: Storage condition	Initial concentration (ng/mL)	Mean concentration (ng/mL)	% Deviation	% CV
Freeze and thaw stability	3	3.09	2.94	5.49
After 3rd cycle at −70°C	450	493.29	9.62	4.71
Short-term stability	3	3.20	6.69	5.09
Room temperature (25°C) at 27 h	450	466.11	3.58	0.66
Long-term stability	3	3.07	2.46	2.05
4 weeks at −20°C	450	489.20	8.71	2.01
Long-term stability	3	3.13	4.35	3.05
16 weeks at −70°C	450	454.55	1.01	4.96
Post-preparative stability	3	2.91	−3.02	4.75
Auto sampler (15°C) at 24 h	450	429.84	−4.48	3.28

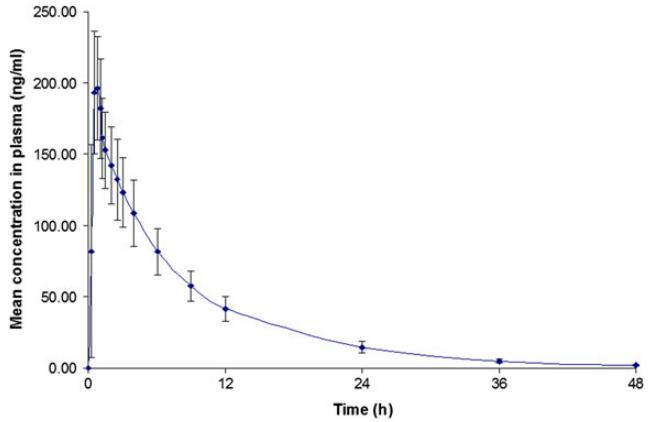

Figure 4. Mean plasma concentration–time profile of six healthy volunteers after oral administration of a single dose of a levocetirizine 5-mg tablet ($n = 6$). This figure is available in black and white in print and in color at *JCS* online.

Table VII
Levocetirizine Pharmacokinetics

Pharmacokinetic parameters	Values
C_{max} (ng/mL)	196.1 ± 36.4
t_{max} (h)	0.63 ± 0.14
$t_{1/2}$ (h)	7.90 ± 0.14
$AUC_{0\rightarrow t}$ (ng/mL/h)	1584.4 ± 307.8
$AUC_{0\rightarrow\infty}$ (ng/mL/h)	1606.5 ± 309.3

Discussion

In this work, a fast LC–MS-MS method for determination of levocetirizine with simple protein precipitation using trichloroacetic acid was developed. We found that trichloroacetic acid added to plasma was sufficient for cleaning and removing plasma proteins in the sample. Trichloroacetic acid also prevented re-precipitation of levocetirizine in the plasma matrix and minimized interference with the chromatogram peaks. Previously, De Jager et al. (14) described the use of acetonitrile as a precipitating solvent for sample preparation for LC–MS-MS determination of cetirizine, a racemic mixture of levocetirizine and dextrocetirizine. Subsequently, Kang et al. (10) attempted to use acetonitrile in determination of levocetirizine in human plasma, but found that protein reprecipitation produced an unclear final solution and interfering residues in the sample. An additional liquid–liquid extraction step following protein precipitation with acetonitrile was introduced to minimize plasma interference (10). However, this method of sample preparation using protein precipitation in conjunction with liquid–liquid extraction is a time-consuming and labor-intensive procedure. Therefore, the use of trichloroacetic acid alone for protein precipitation reported here is a simplification of the sample preparation process. Additionally, a fast and simple technique helps to reduce human error and workload in a routine bioanalysis procedure.

Protein precipitation using conventional protein precipitants such as acetonitrile and methanol is not usually suitable for basic drugs with high protein binding including levocetirizine (10). Trichloroacetic acid has been shown to remove proteins efficiently (15) and an acidic protein precipitant is usually suitable for plasma determination of a basic drug with high protein binding (16, 17). Levocetirizine contains a strongly basic amino group and has high plasma protein binding, and thus trichloroacetic acid is likely to improve the extraction efficiency compared with conventional protein precipitating solvents such as methanol and acetonitrile (16, 17). Unlike conventional protein precipitants, trichloroacetic acid in acetonitrile achieves plasma protein removal and enhances the solubility of the analyte in the precipitating solution. Hydroxyzine is a structurally similar analog of levocetirizine, and thus is appropriate for use as an IS. Hydroxyzine can be metabolized to levocetirizine via oxidation, but this process only occurs in the liver. Therefore, hydroxyzine is not converted to levocetirizine in spiked plasma and can serve as an IS (18).

In addition to protein precipitant optimization based on the chemistry of drug-protein binding and use of a different internal standard, the method is novel with regard to the liquid chromatographic conditions. First, the proposed method uses an aqueous modifier, ammonium formate buffer, which is superior to use of a single acid mobile phase in terms of buffer capacity to ensure a constant pH of the chromatographic system during a sample run. In an attempt at method optimization, use of ammonium formate buffer at pH 3.5 was found to be more appropriate and advantageous compared with the use of formic acid or acetic acid. Consequently, this buffer can compensate for the highly acidic property of the precipitating solvent used in the sample preparation step, resulting in sufficient separation, ruggedness and robustness throughout long-term operation. We note that use of a formic acid solution as mobile phase described by Morita et al. (8) did not give reproducible results after numerous sample

injections with acidic protein precipitants, with the result that liquid–liquid extraction was chosen instead of simple protein precipitation. Secondly, the simple sample preparation method, including a short time for analysis of 3.5 min, is preferable for routine work, and is another strong point of the method. Finally, the use of a composition of 80% organic modifier ensures completeness of volatility during MS analysis, reduces the analysis time, and enhances the sensitivity of detection. Overall, the developed method uses congruent and compatible sample preparation and modifications of chromatographic and mass spectrometric conditions that produce reliable results with a short analysis time.

The Altima C18 analytical column is recommended for operation at pH 2–8, raising a concern of use of highly acidic 6% TCA in the injected sample. However, only a small volume (10 μL) of the sample was injected onto the column. In addition, the injected sample was further diluted during separation in the column with excess mobile phase consisting of acetonitrile and 10 mM ammonium formate pH 3.5 (80:20, v/v). Therefore, the small injection volume and the use of buffer in the mobile phase should significantly raise the pH of the sample to the recommended pH range for the Altima C18 column. During the method validation and pharmacokinetic study, we observed no apparent adverse impacts on the chromatograms after hundreds of injections using the same column, indicating that the use of 6% TCA as a protein precipitant has no significant adverse effect on the column.

The proposed fragmentation mechanism from the precursor ion of $m/z\,[M+H]^+$ 389.0 for levocetirizine and $m/z\,[M+H]^+$ 375.3 for hydroxyzine involves the neutral loss of 2-piperazinyl-1-yl ethoxy acetic acid to give a product ion at $m/z\,[M+H]^+$ 201.0, which contains a resonance-stabilized tropylium ion. The MRM on positive electrospray tandem mass spectrometry in this method was found to be specific for both levocetirizine and hydroxyzine IS analysis. The high percentage of acetonitrile to ammonium formate buffer in the mobile phase coupled with the use of a C18 column provide fast analysis and good separation between the levocetirizine and IS, with retention times of 1.6 and 2.0 min, respectively.

The method developed in this study was fully validated covering all validation parameters, i.e., specificity, linearity, accuracy, precision, recovery, matrix and hemolytic effects. The specificity results indicate that the method is highly selective for levocetirizine analysis with hydroxyzine as an IS. The calibration curve, accuracy and precision data suggest that the method is linear, accurate and precise. The recoveries of both levocetirizine and hydroxyzine were >50% with %CV < 15%, indicating that the recovery of this method was sufficient and reproducible. The %CV of IS-normalized MF < 15% suggest that ion suppression or enhancement from plasma matrix was negligible in the assay. The %deviation and %CV of the hemolytic effect study fell within the acceptance criteria, suggesting that interference from hemoglobin components in plasma had no adverse effects on the accuracy and precision of the assay. The signal-to-noise ratio, %deviation and %CV at 1.00 ng/mL suggest that the levocetirizine analysis at 1.00 ng/mL was sufficiently accurate and precise to set the LLOQ of the calibration curve. The stability results indicate that levocetirizine was stable for application to the routine analysis. In a pharmacokinetic study, the ratio of $AUC_{0\rightarrow t}$ to $AUC_{0\rightarrow\infty}$ was >80%, indicating that the sampling time period and the LLOQ at 1.00 ng/mL were suitable for the study, and the pharmacokinetic parameters i.e., *AUC*, C_{max}, T_{max} and $T_{1/2}$ were successfully determined. Since C_{max} determined from the samples in this study was 196 ± 36 ng/mL, the method can be further revised to truncate the curve range to 1–300 ng/mL for further pharmacokinetics and bioequivalent studies.

Conclusion

A simple, sensitive and rapid LC–MS-MS method was developed and validated for determination of levocetirizine over a concentration range of 1–500 ng/mL in human plasma. Due to the fast and simple extraction technique, the protein precipitation approach was selected and used for the sample cleaning procedure. Protein precipitation by acidic solution is specific and selective for basic compounds with high protein binding and more efficient than with conventional organic solvents such as acetonitrile and methanol. The new method provides not only a sufficiently clean sample but also gives a good sensitivity at an LLOQ of 1.00 ng/mL. This high-throughput approach has significant advantages over those previously reported in terms of analysis time, human error and workload reduction, which are useful in routine analysis. The method was successfully applied in a pharmacokinetic study of levocetirizine. Due to the benefits of the approach, the protein precipitation technique developed in the study should be widely applicable in routine bioequivalence studies in the pharmaceutical industry.

Acknowledgments

The authors thank Dr Ian S. Haworth (University of Southern California) for his suggestions and comments on the manuscript. The authors also thank Mr Sanpeth Limpakomon for assistance with preparation of the manuscript.

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