Simultaneous Determination of Levocetirizine Dihydrochloride and Montelukast Sodium in Human Plasma by LC–MS/MS: Development, Validation, and Application to a Human Pharmacokinetic Study

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Objectives: A simple, rapid, selective, and sensitive high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of levocetirizine dihydrochloride and montelukast sodium in human plasma using fexofenadine hydrochloride as an internal standard.

Method: Liquid–liquid extraction of both drugs and internal standard from plasma into ethyl acetate was used for sample preparation and analysis. Separation of both drugs and internal standard was achieved on an Inertsil ODS-3 (4.6 mm × 50 cm, dp 5 μm, particle size) column using an isocratic mobile phase of acetonitrile and 10 mM ammonium formate adjusted to pH 8 with 50 μL ammonium hydroxide in composition of 73:27 (v/v) at a flow rate of 0.7 mL/min. The LC–MS/MS was operated under the multiple reaction monitoring mode (MRM) using an electrospray ionization technique. Mass parameters were optimized to monitor transitions at m/z $[M + H]^+$ 389.0 → 200.8 for levocetirizine dihydrochloride, m/z $[M + H]^+$ 586.2 → 422.2 for montelukast sodium, and m/z $[M + H]^+$ 502.2 → 466.0 for fexofenadine hydrochloride.

Results: The method was found to be linear in the range of 1–500 ng/mL for both drugs. The intra-day and inter-day precision were in the range of 0.96–1.92% and 1.03–1.55%, respectively. Matrix effect was acceptable with %RSD < 15.

Conclusion: The proposed method was validated and successfully applied for a pharmacokinetic study of both drugs in human plasma after oral administration of their pharmaceutical preparation.

Keywords: levocetirizine dihydrochloride, montelukast sodium, LC–MS/MS, validation, human plasma, pharmacokinetics

Introduction

Levocetirizine (LCZ) is chemically described as 2-[2-[4-[(*R*)-(4-chlorophenyl)-phenyl-methyl]piperazin-1-yl]ethoxy]acetic acid [1]. It is the active (*R*)-enantiomer of racemic cetirizine and an orally active, potent, selective, and long acting H1-histamine receptor antagonist with no anticholinergic activity. It is a third-generation non-sedative antihistamine used for the treatment of allergic rhinitis and chronic idiopathic urticarial [2]. LCZ has the advantages of higher efficacy, less side effects, and longer duration over other antihistamines and has begun to replace cetirizine in clinical therapy stepwise [3]. Oral administration of a 5-mg LCZ tablet showed t_{max} at 0.9 h with C_{max} of about 270 ng/mL and an elimination half-life of about 8 h [4]. Literature survey revealed that LCZ has been reported to be determined by ultraviolet (UV) spectrophotometry [3, 5], liquid chromatography–tandem mass spectrometry (LC–MS/MS) in human plasma [6], high-performance liquid chromatography (HPLC) [7, 8], and thin-layer chromatography (TLC) [9]. LCZ was also assayed in combination with other drugs in pharmaceutical dosage form by UV spectrophotometry [10], reversed-phase HPLC (RP-HPLC) [11–13], and high-performance thin-layer chromatography (HPTLC) [14]. Montelukast (MLK) is described chemically as 2-[1-[[1(*R*)-1-[3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-[hydroxypropan-2-yl]phenyl]propyl]sulfonylmethyl]cyclopropyl]acetic acid [15]. MLK is a selective and orally active leukotriene receptor antagonist that inhibits cysteinyl leukotriene receptors in the lungs and bronchial

tubes. It is used for the treatment of asthma and to relieve symptoms of seasonal allergies. It has been demonstrated by recent studies that the treatment of allergic rhinitis with concomitant administration of an antileukotriene (MLK) and an antihistamine (LCZ) shows significantly better symptom relief compared with the modest improvement of rhinitis symptomatically with each of the treatments alone [16–21], usually taken once daily in the evening for prevention of asthma or allergy symptoms. The maximum plasma concentrations of MLK were achieved within 3 h after oral administration of 10 mg tablet with an elimination half-life of 2.7 to 5.5 h [22]. MLK has been reported to be determined by UV Spectrophotometry [23], LC–MS/MS in human plasma [22, 24], HPLC [25], HPTLC [26], and fluorimetry [27]. MLK was determined in combination with other drugs in pharmaceutical dosage form by UV spectrophotometry [28], LC–MS/MS [29], RP-HPLC [30], and HPTLC [31, 32]. HPLC and HPTLC methods have been reported for simultaneous estimation of LCZ and MLK in pharmaceutical dosage forms [21, 33–36]. However, there is no LC–MS/MS method available for simultaneous estimation of both drugs in human plasma. Therefore, an attempt was made to develop a new, rapid, and sensitive method for the determination of LCZ and MLK in human plasma and evaluate the pharmacokinetics of both drugs after an oral administration to their pharmaceutical preparation.

Experimental

Apparatus and Conditions. Mass spectrometric analysis was performed using an Agilent 1200 series (Agilent

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Technologies, Deutschland GmbH, Waldbronn, Germany) equipped with a quaternary pump (G1311A), an auto sampler (G1329A), a vacuum degasser (G1322A), and a thermostatic column compartment (G1316A) or a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. The interface was operated in the positive ionization mode with drying gas temperature (N_2) maintained at 300 °C, nebulizer pressure at 50 psi, and ion spray voltage potential at 4000 V. Low-purity nitrogen served as collision-induced dissociation (CAD) gas. The collision energy (CE) and accelerated cell collision (Acc) energy were optimized to achieve the largest response for LCZ, MLK, and IS. The CE values were 10, 25, and 25 eV, while the optimized fragmentor voltage was set at 135, 200, and 135 V for LCZ, MLK, and IS, respectively. The Acc energy was set at 7 eV for all drugs. The dwell time for both drugs and IS monitoring was set at 200 ms. Detection of ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the precursor/product ion pairs at m/z $[M + H]^+ 389.0 \rightarrow 200.8$, m/z $[M + H]^+ 586.2 \rightarrow 422.2$, and m/z $[M + H]^+ 502.2 \rightarrow 466.0$ for LCZ, MLK, and IS, respectively. Acquisition and processing of data were performed using MassHunter software.

Chromatographic separation was carried on an Inertsil ODS-3 (4.6 mm $\times$ 50 cm, dp 5 μ m) column at ambient temperature. Samples were eluted with an isocratic mobile phase consisting of acetonitrile and 10 mM ammonium formate adjusted to pH 8 with 50 μ L ammonium hydroxide (73:27, v/v) at a flow rate of 0.7 mL/min. The injection volume was 5 μ L, and the run time was 2.8 min.

Reagents. Pure samples of LCZ (batch no. LCZ-1304009) and MLK (batch no. MK-0180513) were kindly supplied by EGY Pharm (Egypt). Its purity was found to be 99.78% or 99.30%, respectively, as stated by the supplier. Fexofenadine hydrochloride (IS) (batch no. FXD-2020) pure sample was kindly supplied by Organo Pharma (Egypt). Its purity was found to be 99.30%, as stated by the supplier. Market samples, Montair-LC[®] tablets (batch no. D3184-8), are labeled to contain 5 mg of LCZ and 10 mg of MLK (Cipla, India).

All reagents used were of analytical grade, and solvents were of spectroscopic grade. Ultra-pure grade water used throughout the study was prepared from deionized water, PURELAB Prima, VWS Ltd. (ELGA Lab Water Global Operation Centre, High Wycombe, HP14 3BY, Bucks, UK).

– Methanol and acetonitrile HPLC grade (Sigma-Aldrich, Germany).

– Ammonium formate (Sigma-Aldrich, Germany).

– Ammonium hydroxide (Sigma-Aldrich, Germany).

– Ethyl acetate (Sigma-Aldrich, Germany).

– Blank (drug-free) human plasma was obtained from the Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt.

Preparation of Standard Solutions and Calibration Standards. (a) Primary stock solutions of the drugs and IS (0.2 mg/mL) were prepared by dissolving 20 mg of LCZ, MLK, or fexofenadine in 100 mL methanol. The primary stock solutions of the drugs were further diluted in methanol to produce calibration standards at 1, 5, 10, 20, 50, 100, 200, 300, and 500 ng/mL for preparation of calibration curves. Another set of stock solutions of the drugs were diluted with methanol to give appropriate calibration standards at 3, 250, and 400 ng/mL for preparation of quality control (QC) samples. A working internal standard solution of (2000 ng/mL) was prepared in methanol.

(b) The plasma calibration curves were prepared at concentrations of 1, 5, 10, 20, 50, 100, 200, 300, and 500 ng/mL by spiking 450 μ L of human blank plasma with 50 μ L of working standard of each drug (to make up to 500- μ L total

sample volume), and then 50 μ L IS was added for every sample at an appropriate concentration. The final concentration at 1 ng/mL was used as lower limit of quantification (LLOQ). Three concentration levels of quality control (QC) samples were prepared from blank human plasma spiked with the working standard solutions to yield final concentrations of 3, 250, and 400 ng/mL, corresponding to low, medium and high QC (LQC, MQC, and HQC) concentration levels, respectively.

Sample Preparation. Fexofenadine IS solution (50 μ L) was spiked into 500 μ L of each plasma sample. Three milliliters of ethyl acetate were added, and the mixture was vortexed for 10 min at 3000 rpm by Vortex (VWR VV3 S540 International West Charter, USA). The organic layer was separated and evaporated to dryness under vacuum at 60 °C using infrared (IR) vacuum concentrator (model no. NB-503CIR, N-BIOTEK, Korea). The residue was reconstituted in 200 μ L of the mobile phase, and 5 μ L was then injected into the LC–MS/MS system.

Specificity. Specificity of the proposed method was evaluated by screening 6 different lots of blank plasma spiked with LCZ and MLK at the LLOQ. The spiked samples were extracted, and the presence or absence of interfering peaks at the same retention time of LCZ, MLK, and IS was examined.

Stability. The stability studies of LCZ and MLK in human plasma were performed in triplicate at the LQC and HQC (3 and 400 ng/mL) levels. Freeze–thaw, auto-sampler, short-term, and long-term stabilities of LCZ, MLK, and IS were tested. The freeze–thaw stability was examined by freezing samples at –80 °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. Meanwhile, auto-sampler stability was tested by storage of samples in an auto sampler at room temperature (25°C) for 6 h. Short-term stability was tested by storage of samples at room temperature (25°C) for 12 h. Long-term stability was evaluated after samples storage at –80 °C for 30 days. The level of LCZ, MLK, or IS was determined at different times in comparison with that of the corresponding freshly prepared control samples.

Preparation of Clinical Blood Samples. Blood samples were collected from 6 adult healthy fasted male volunteers, who received single oral dose of Montair-LC[®] tablets. The independent ethics committee approved the protocol, and the patient provided written informed consent. 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/m²). 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. Blood samples were taken using an indwelling cannula placed in a forearm vein and kept patent with normal saline. Blood samples were collected pre-dose, 5 min, 10 min, and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 10, 12, 24, and 48-h post dose in heparin blood collection tubes. The samples were centrifuged for 10 min at 3000 rpm and stored at –80 °C until analysis.

Results and Discussion

The main objective of our research work was to develop a simple, sensitive, and high-throughput LC–MS/MS method for simultaneous determination of LCZ and MLK in human plasma, which has a simple extraction procedure from small volumes of plasma, high recoveries, and a short run time.

LC–MS/MS Parameters. The mass spectrometric parameters optimized in this study provide the highest sensitivity of signal responses for both precursor and product ions of LCZ, MLK, and IS. From the full-scan mass spectra of LCZ, MLK, and IS, the $[M + H]^+$ ion with m/z 389.0, 586.2, and 502.2, respectively, was selected as the precursor ion. Under the product ion scan mode, the most abundant fragments were recorded at m/z 200.8, 422.2, and 466.0, respectively. Therefore, quantitation was performed using the MRM mode with target ions at m/z $[M + H]^+ 389.0 \rightarrow 200.8$ for LCZ, m/z $[M + H]^+ 586.2 \rightarrow 422.2$ for MLK, and m/z $[M + H]^+ 502.2 \rightarrow 466.0$ for IS, respectively (Figure 1). The MRM on positive electrospray tandem mass spectrometry in this method was found to be specific for LCZ, MLK, and IS.

LC Conditions. Initially to estimate LCZ and MLK, several mixture of solvent in different ratios, including acetonitrile or methanol with different buffers such as formic acid, ammonium acetate, and ammonium formate, were investigated and compared to identify the optimal mobile phase. Later, acetonitrile was chosen because it generated a shorter retention time and a better peak shape. The best signal

and good ionization was achieved at ambient temperature on Inertsil ODS-3 column using acetonitrile and 10 mM ammonium formate adjusted to pH 8 with 50 μ L ammonium hydroxide (73:27, v/v) as the mobile phase at a flow rate of 0.7 mL/min, and the injection volume was 5 μ L. The retention times of LCZ, MLK, and IS under these conditions were 0.908, 2.327, and 0.798 min, respectively. Fexofenadine was selected as the internal standard due to its similar extraction and ionization efficiency to LCZ and MLK.

Extraction Procedure. Various organic solvents including methanol, acetonitrile, acetone, ethanol, or a mixture of these solvent at various proportions were investigated for the extraction of LCZ, MLK, and IS from plasma samples. Ethyl acetate provided the best recoveries with minimum endogenous interferences and was chosen as the extraction solvent.

Method Validation. The developed method was validated for linearity, sensitivity, accuracy, precision, specificity, recovery, and stability according to the Food and Drug Administration (FDA) guidelines for bioanalytical method validation and other related guidelines [37]. Additionally, the matrix effect was also

Figure 1. Chemical structures and product ion mass spectra of (a) LCZ, (b) MLK and (c) IS

validated. The QC sample at 250 ng/mL (MQC) was used to verify the suitability of the chromatographic system before analysis.

Calibration Curve, Linearity, and Sensitivity. The plasma calibration curves were prepared using 9 calibrators each in triplicate (1–500 ng/mL for each drug). The standard curve had a reliable reproducibility over the standard concentrations for both the drugs across the calibration range. Calibration curves were constructed by plotting the peak area ratios (peak area of the LCZ or MLK/peak area IS) against the concentrations of LCZ or MLK, respectively. 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. The coefficient of determination (r^2) was 0.9974 or 0.9975 for LCZ or MLK. The lowest concentration quantified with precision $\leq 20\%$ RSD was taken as LLOQ and was found to be 1 ng/mL for both drugs; the peak response of LCZ and MLK at LLOQ must be at least five times the baseline noise. Moreover, the accuracy between 80 and 120% was estimated in the process of calibration curve construction as shown in Table 1.

Accuracy and Precision. The accuracy and precision of the proposed method were determined using 3 different QC concentrations (LQC, MQC, and HQC) in addition to LLOQ, each in triplicate, within one day for intra-day and three different days for inter-day. Table 2 revealed that the obtained results were in agreement with the acceptable limits for the validation of bioanalytical methods suggesting that this assay was reproducible and reliable.

Specificity. Although the detection in MS/MS technique is highly specific and sensitive, nevertheless, interference arising from endogenous substances can exist in much higher concentration than the drugs of interest and may co-elute with those affecting the ionization of the drugs leading to high imprecision and loss of sensitivity. The developed LC–MS/MS method demonstrated good specificity as the extracted ion chromatograms showed that there were no interferences at the same retention time of LCZ or MLK (0.908 or 2.327 min) and IS (0.798 min). Representative multiple reaction monitoring (MRM) chromatograms of blank plasma, blank plasma spiked with LCZ, MLK, or IS, and extracted plasma samples are shown in Figure 2. These results supported the high specificity and selectivity of this method.

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 [38]. Recoveries of LCZ and MLK were determined at LQC, MQC, and HQC levels (3, 250, and 400 ng/mL) by comparing the peak areas of each drug extracted from spiked plasma samples with the corresponding concentration of the authentic standard solution. The recovery of the IS was determined using the same procedure at a single concentration of 2000 ng/mL. The

Table 2. Within-day and between-day accuracy and precision for the determination of LCZ and MLK ($n = 3$)

Spiked conc. (ng/mL)	Within-day			Between-day		
	Conc. measured (ng/mL)	Accuracy (%R)	%RSD	Conc. measured (ng/mL)	Accuracy (%R)	%RSD
LCZ						
1	0.99	99.00	1.71	0.98	98.00	1.21
3	2.95	98.33	1.52	2.91	97.00	1.55
250	247.10	98.84	1.92	243.76	97.50	1.03
400	404.31	101.08	1.23	389.08	97.27	1.44
MLK						
1	0.98	98.00	1.33	0.99	99.00	1.57
3	2.97	99.00	1.72	2.95	98.33	1.32
250	245.56	98.22	1.83	255.23	102.09	1.37
400	406.63	101.66	0.96	410.16	102.54	1.07

recoveries of both drugs and IS were $>50\%$ with $\%RSD < 15\%$, indicating that the recovery of this method was sufficient and reproducible as shown in Table 3.

Matrix Effect. The effect of plasma matrix was determined for LCZ, MLK, and IS separately and reported as matrix factor (MF). The ratio between the MF of LCZ and MLK and that of fexofenadine (IS) is calculated and termed as the IS-normalized MF [39, 40]. Six different lots of blank human plasma were evaluated, and the determination was performed at the LQC and HQC levels in triplicate for each lot. The MFs of LCZ and MLK were calculated by comparing the peak area of both drugs extracted from samples prepared by spiking LCZ and MLK in extracted blank plasma with the corresponding concentration of the authentic solution of LCZ and MLK prepared in methanol. The MF of fexofenadine (IS) was determined and calculated in a similar manner. The IS-normalized MF is then calculated by dividing the MF of LCZ and MLK to that of fexofenadine (IS) as shown in Table 4.

Stability. Samples were considered to be stable when assay values were within $\pm 15\%$ deviation for accuracy and $\pm 15\%$ RSD for precision, except for LLOQ, where it should not exceed 20% of RSD [33]. The stability of LCZ and MLK in human plasma was investigated by analysis of two levels of QC samples (3 and 400 ng/mL) after 3 freeze–thaw cycles (-80°C to 25°C), stored at 25°C for 6 or 12 h (auto-sampler or short-term stability) and stored at -80°C for 30 days (long-term stability) as shown in Table 5, and revealed that LCZ and MLK were stable for three freeze–thaw cycles, 12 h in human plasma at room temperature (25°C) and 30 days at -80°C .

Application to Pharmacokinetic Study. The validated method was used for determination of LCZ and MLK concentrations in human plasma samples collected from 6 healthy male volunteers who received a single oral dose of Montair-LC[®] tablets containing 5 mg of LCZ and 10 mg of MLK after providing informed consent. The resulting mean plasma concentration–time profiles of LCZ and MLK are

Table 1. Mean inter-day back-calculated standard curve results for LCZ and MLK ($n = 3$)

Added conc. (ng/mL)	LCZ						MLK					
	Found conc. (ng/mL)			Conc. measured (ng/mL)	SD	%RSD	Found conc. (ng/mL)			Conc. measured (ng/mL)	SD	% RSD
	Day 1	Day 2	Day 3				Day 1	Day 2	Day 3			
1	0.99	0.98	1.00	0.99	0.02	2.02	0.99	0.98	1.00	0.99	0.02	2.02
5	4.94	4.85	4.92	4.90	0.09	1.84	5.07	4.95	5.10	5.04	0.09	1.79
10	10.61	10.22	10.34	10.39	0.14	1.35	10.02	10.34	10.92	10.43	0.23	2.21
20	20.31	20.15	20.41	20.29	0.36	1.77	20.35	20.55	20.28	20.40	0.26	1.27
50	49.54	50.23	49.32	49.70	0.95	1.91	48.71	50.03	49.94	49.56	0.56	1.13
100	98.40	98.62	99.13	98.72	1.24	1.26	99.39	99.12	100.45	99.65	1.59	1.60
200	202.21	201.45	200.67	201.44	1.70	0.84	196.00	198.75	199.15	197.97	1.26	0.64
300	297.57	298.91	300.50	299.00	1.94	0.65	295.86	297.21	299.78	297.62	1.83	0.61
500	510.06	508.12	505.32	507.83	0.67	0.13	512.47	520.12	500.90	511.16	0.93	0.18

Figure 2. Representative MRM chromatogram of LCZ (left panel), MLK (middle panel), and fexofenadine (IS) (right panel) in (a) blank human plasma, (b) blank human plasma spiked with IS, (c) human plasma spiked with LCZ, MLK at LLOQ (1 ng/mL), and IS, and (d) human plasma spiked with LCZ, MLK (200 ng/mL), and IS

Table 3. Recovery of LCZ, MLK and fexofenadine (IS) in human plasma ($n = 3$)

Conc. (ng/mL)	Mean peak area		Recovery %	% RSD
	Solvent sample	Spiked plasma sample		
LCZ				
3	100791	75701	75.11	6.52
250	7213801	5498411	76.22	8.11
400	12181012	9740788	79.97	11.13
MLK				
3	6945	5683	81.83	5.20
250	463278	364678	78.72	7.96
400	861130	615293	71.45	9.82
IS				
2000	18156287	13025063	71.74	11.22

shown in Figure 3. The calculated pharmacokinetic parameters were the maximum plasma concentration ($C_{\max}$), the time to achieve maximum plasma concentration ($t_{\max}$), the area under the curve from 0 to 48 h (AUC_{0-48}) and from 0 to ∞ ($AUC_{0-\infty}$), the elimination half-life ($t_{1/2}$), and the elimination rate constant (K_{el}). The mean value of $C_{\max}$ was found to be 187.68 or 315.82 ng/mL and $t_{\max}$ was reached after 0.5 or 2.7 h for LCZ or MLK, respectively. Also, mean AUC_{0-48} and $AUC_{0-\infty}$ were found to be 2052.89 or 2898.09 and 2132.29 or 2877.35 ng·hr/mL, while $t_{1/2}$ was 9.55 or 9.50 h, respectively. The ratio of AUC_{0-48} to $AUC_{0-\infty}$ was $> 80\%$ for both drugs, indicating that the sampling time period and the LLOQ at 1.00 ng/mL were suitable for the study; the main pharmacokinetic parameters were summarized in Table 6.

Table 4. Matrix effect of LCZ and MLK in human plasma ($n = 6$)

Spiked conc. (ng/mL)	Plasma lot	LCZ				MLK			
		Matrix factor (%)		IS-normalized MF	%RSD	Matrix factor (%)		IS-normalized MF	%RSD
		Drug	IS			Drug	IS		
3	No.1	96.70	99.10	97.58	5.00	108.10	99.10	109.08	7.00
	No.2	88.10	97.90	89.99		102.10	97.90	104.29	
	No.3	109.60	110.70	99.00		107.70	101.70	105.90	
	No.4	105.80	108.10	97.87		125.40	108.10	116.00	
	No.5	102.60	99.00	103.64		95.00	99.00	95.96	
	No.6	100.30	96.80	103.62		101.50	96.80	104.86	
400	No.1	97.10	97.50	99.59	7.20	94.20	100.40	93.82	3.90
	No.2	96.60	99.10	97.48		92.20	96.80	95.25	
	No.3	90.70	113.30	80.05		93.40	103.60	90.15	
	No.4	89.00	100.50	88.56		93.30	106.10	87.94	
	No.5	106.60	96.50	110.47		99.67	100.30	99.37	
	No.6	112.90	97.80	115.44		98.56	97.30	101.29	

Table 5. Stability results of LCZ and MLK in human plasma ($n = 3$)

Stability: storage condition	Spiked plasma conc. (ng/mL)	LCZ			MLK		
		Conc. measured (ng/mL)	SD	%RSD	Conc. measured (ng/mL)	SD	%RSD
Freeze and thaw stability	3	2.91	0.18	6.19	3.13	0.23	7.35
After 3 rd cycle from -80 to 25 °C	400	391.03	1.47	0.38	390.24	1.73	0.44
Auto sampler stability at 25 °C for 6 h	3	2.94	0.21	7.14	3.09	0.21	6.80
	400	428.95	2.11	0.49	441.35	1.91	0.43
Short term stability at 25 °C for 12 h	3	2.89	0.10	3.46	2.83	0.37	13.07
	400	410.96	1.41	0.34	381.85	1.51	0.40
Long term stability at -80 °C for 30 days	3	3.10	0.17	5.48	3.07	0.18	5.86
	400	479.61	3.54	0.74	462.56	5.75	1.24

Figure 3. Mean plasma concentration–time profile of 6 healthy volunteers after oral administration of a single dose of Montair-LC[®] tablets: (a) 5 mg LCZ and (b) 10 mg MLK ($n = 6$)

Conclusion

A simple, sensitive, accurate, and reproducible LC–MS/MS method was developed and validated for the simultaneous

determination of LCZ and MLK in human plasma. This LC–MS/MS method was successfully applied to pharmacokinetic studies on 6 healthy fasted male volunteers after an oral administration of Montair-LC[®] tablets containing 5 mg of LCZ

Table 6. Pharmacokinetics of LCZ and MLK ($n = 6$)

Parameter	LCZ	MLK
$C_{\max}$ (ng/mL)	187.68 ± 19.90	315.82 ± 11.98
$t_{\max}$ (h)	0.50 ± 0.09	2.70 ± 0.47
AUC _{0–48} (ng·h/mL)	2052.84 ± 253.49	2808.09 ± 131.18
AUC _{0–∞} (ng·h/mL)	2132.29 ± 288.46	2877.35 ± 142.08
K_{el} (h ^{−1})	0.074 ± 0.013	0.091 ± 0.05
$t_{1/2}$ (h)	9.55 ± 1.61	9.50 ± 4.69

and 10 mg of MLK. The advantages of the developed method include simple sample preparation procedure with a commercially available IS, short analysis time (2 min per sample), and high sensitivity (LLOQ = 1 ng/mL).

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