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Short communication

Development of a liquid chromatography/negative-ion electrospray tandem mass spectrometry assay for the determination of cilnidipine in human plasma and its application to a bioequivalence study

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Abstract

A simple method using a one-step liquid–liquid extraction (LLE) with methyl-*t*-butyl ether (MTBE) followed by high-performance liquid chromatography (HPLC) with negative-ion electrospray ionization tandem mass spectrometric (ESI-MS/MS) detection was developed for the determination of cilnidipine in human plasma using benidipine as an internal standard (IS). Acquisition was performed in multiple reaction monitoring (MRM) mode, by monitoring the transitions: *m/z* 491.1 > 121.8 for cilnidipine and *m/z* 504.2 > 122.1 for IS, respectively. Analytes were chromatographed on a CN column by isocratic elution using 10 mM ammonium acetate buffer-methanol (30:70, v/v; adjusted with acetic acid to pH 5.0). Results were linear ($r^2 = 0.99998$) over the studied range (0.1–20 ng/ml) with a total LC–MS/MS analysis time per run of 3 min. The developed method was validated and successfully applied to a cilnidipine bioequivalence study in 24 healthy male volunteers.

Keywords: Cilnidipine; Liquid–liquid extraction; Liquid chromatography/tandem mass spectrometry; Human plasma; Bioequivalence

1. Introduction

Hypertension is an established risk factor for cardiovascular diseases and organ damage, and it may be possible for patients with hypertension or at high cardiovascular risk to be treated with blood pressure-lowering medication to reduce the likelihood of these adverse events and their complications [1]. Several studies have concluded that Ca^{2+} antagonists or a combination of a Ca^{2+} antagonist and an angiotensin blocker improves prognosis in hypertensive patients [2–7]. Dihydropyridine (DHP) Ca^{2+} antagonists are frequently used to treat hypertension, stable angina pectoris and cerebrovascular disease by blocking L-type voltage-gated Ca^{2+} channels [8]. These therapeutic agents relax coronary arteries by peripheral vasodilatation, and decrease left ventricular contraction by reducing the myocardial oxygen demand [8]. Cilnidipine is a novel 1,4-dihydropyridine calcium antagonist with potent inhibitory effects on L- and N-type voltage-dependent calcium channels [9]. Thus, cilnidipine may have clinical advantages with respect to cardiovascular protection. Indeed, it has been found effectively lower blood pressure with less effect on heart rate and sympathetic nerve activity than other Ca^{2+} antagonists in hypertensive patients [10,11].

Previous publications have described HPLC-UV [12,13], LC–MS [14] and LC–MS/MS [15,16] method for the analysis of dihydropyridine drugs in biological samples. However, these methods are not suitable for pharmacokinetics work, because they involve arduous sample preparation and long chromatographic run-times. Moreover, no publication has described the quantitative analysis of cilnidipine using tandem mass spectrometry. The method described in this paper provides a simple and accurate LC–MS/MS method to determine plasma cilni-

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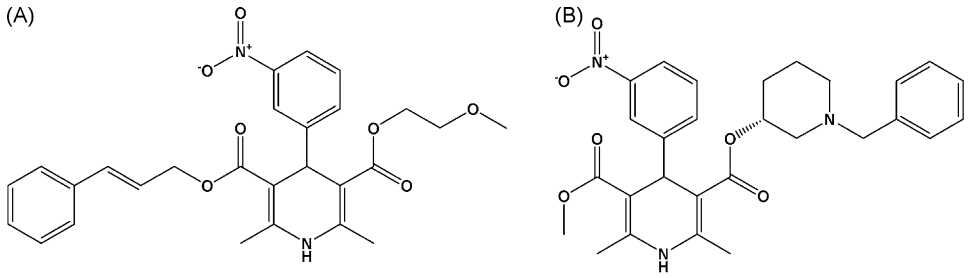

Fig. 1. Structures of (A) cilnidipine and (B) IS.

dipine levels with the ability to analyze samples containing a wide concentrations range (0.1–20 ng/ml). The important benefits of the devised method as compared with the previously described LC–MS method [14] are its lower LLOQ and speed. In order to test the applicability of the devised method, we used it to perform a bioequivalence study in 24 healthy male Korean volunteers administered cilnidipine in tablet form (10 mg).

2. Experimental

2.1. Chemicals and reagents

Cilnidipine and benidipine were purchased from Hanseo Chemical Co. Ltd. (Seoul) and JinHua Hosion Pharma-Chem Tech Co. Ltd. (Zhejiang, China), respectively. The chemical structures of cilnidipine (Fig. 1A) and benidipine (IS) are shown in Fig. 1B. Solvents were of HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q® (Millipore Co., Milford, MA, USA) water purification system was used to produce purified water for HPLC. All other chemicals and solvents were of the highest analytical grades available.

2.2. Stock solutions and working standards

A stock solution of cilnidipine was prepared by dissolving cilnidipine in dimethyl sulfoxide (DMSO), to a concentration of 1.0 mg/ml. This stock solution was diluted appropriately with methanol to obtain working solutions for calibration at 1, 2, 5, 10, 20, 50, 100 and 200 ng/ml of cilnidipine. Calibration curves were prepared by spiking pooled blank plasma with working solutions to final cilnidipine concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml. Quality control (QC) samples (0.1, 1 and 10 ng/ml) were prepared in the same manner. In order to prepare stock solutions (1.0 mg/ml) of the internal standard (IS) benidipine, 10 mg of IS was dissolved in 10 ml of DMSO. This solution was further diluted with methanol to a final concentration of 10 μg/ml. All solutions were stored at −70 °C.

2.3. Sample preparation

All frozen human plasma samples were thawed at ambient temperature. Human plasma samples (0.5 ml) were placed in a 2 ml microtubes and 20 μl of IS solution (10 μg/ml benidipine) was added to each and vortexed for 1 min. Methyl-*t*-butyl-ether (MTBE) was then added (1.2 ml) and samples were then vortexed for 10 min. The tubes were then centrifuged for 10 min at 10,000 rpm. The upper organic phases were then transferred to clean microtubes and evaporated to dryness under N_2 at 40 °C. Dry residues were then dissolved in 0.1 ml of mobile phase, vortexed for 1 min to reconstitute residues, and transferred to 96-well plates.

2.4. Calibration standards and validation

Eight non-zero calibration standards with cilnidipine concentrations ranging from 0.1 to 20 ng/ml were prepared by adding 50 μl of a working solutions and 20 μl of IS solution (10 μg/ml of benidipine) to 450 μl of drug-free human plasma. A calibration curve was then constructed using a blank sample (plasma sample processed without IS), a zero sample (plasma processed with IS) and eight non-zero samples which covered the cilnidipine concentration range 0.1–20 ng/ml (which included the LLOQ (0.1 ng/ml). The limit of detection (LOD) was defined as three times and the LLOQ was defined as ten times the S/N. The calibration curves had correlation coefficients (r^2) of 0.99 or better.

Intra- and inter-day accuracies and precisions were determined by performing five separate analyses per day for 5 days using cilnidipine concentrations of 0.1, 0.5, 1, 5 and 10 ng/ml. Precision and accuracy were within 15% of nominal values, although at the LLOQ these were both 20%. The outcomes of this study were the reliability, reproducibility, and sensitivity of the devised method, and the whole study was conducted in accordance with FDA guidelines [17].

2.5. Ion suppression by matrix constituents and recovery

Blank plasma samples were subjected to LLE and the organic phase layers so obtained were evaporated to dryness. Dry extract then dissolved using analyte working solution and the resulting analysis represented 100% recovery [A]. QC samples spiked with analyte were processed by LLE in a similar manner [B]. Ion suppression was determined by comparing analytical responses to [A] with those of the corresponding analyte calibration solutions [C]. Ion suppression was calculated using $100 \times ([C] - [A])/[C]$, where [A] is the peak area of analyte or IS in post-extraction spiked sample (blank plasma extracted and then spiked with analyte or IS) and [C] is the peak area of indi-

vidual analyte or IS obtained by directly injecting the working solutions. LLE recovery was determined by comparing response to [B] with response to [A]. These experiments were performed at three cilnidipine concentration levels (0.1, 1, and 10 ng/ml) in triplicate. Ion suppression and recovery experiments were also performed using IS in a similar manner.

To assess 'lot-to-lot' matrix variation, five different lots of blank plasma were used to prepare samples at three cilnidipine concentrations (0.1, 1, and 10 μg/ml). The relative standard deviations (RSDs) of the peak area ratios at each cilnidipine concentration versus IS for the five lots were calculated to determine inter-lot matrix variability.

2.6. LC–MS/MS instrument and conditions

A Waters (Watford, UK) 2795 HPLC system and Micromass Quattro Premire triple quadrupole mass spectrometer were used. A 10 μl aliquot of plasma extract was injected into a Capcell Pak (Kyoto, Japan) UG120 CN (50 mm × 2 mm i.d. S-5 μm) analytical column operated at 40 °C. The mobile phase was prepared by mixing methanol with 10 mM ammonium acetate buffer (70:30, v/v; adjusted to pH 5.0 with acetic acid). A flow rate of 0.2 ml/min was found to be adequate for the analysis. The temperature of the autosampler was kept at 4 °C. To optimize mass spectrometry parameters, capillary voltage, cone voltage, and collision energy were varied, and working solutions of cilnidipine (0.1 μg/ml) and IS (0.1 μg/ml) were infused separately into the stainless-steel sample capillary of the electrospray source at a constant flow rate of 200 μl/min, using a 1 ml syringe pump (Hamilton; Bonaduz, Switzerland). Optimum tandem MS parameters, i.e., resolution, ion energy, entrance potential, exit potential, and collision energy were set to maximize product ion intensities (optimal parameters are summarized in Table 1). Two channels were used in negative ion multiple reaction monitoring (MRM) mode to detect cilnidipine and benidipine. Micromass Masslynx 4.0 and Quanlynx were used for data management.

Table 1
LC–MS/MS instrument parameters

Parameters	Value
Source temperature (°C)	150
Desolvation temperature (°C)	350
Desolvation gas flow (l/h)	550
Cone gas flow (l/h)	50
Collision cell gas pressure (mbar)	2.79×10^{-3}
Capillary voltage (kV)	3.11
Extractor (V)	4
RF lens (V)	0
Low mass 1 resolution	13
High mass 1 resolution	13
Ion energy 1 (V)	1
Low mass 2 resolution	13
High mass 2 resolution	13
Ion energy 2 (V)	1
Entrance (V)	2
Exit (V)	2
Multiplier	650
Dwell time (s)	0.5

	Analyte	IS
Molecule ion (*m*/*z*)	491.1	504.2
Product ion (*m*/*z*)	121.8	122.1
Cone energy (V)	37	43
Collision energy (V)	27	28

2.7. Stability test

To test the short- and long-term stabilities of extracted cilnidipine, three QC samples, containing low (0.1 ng/ml), medium (1 ng/ml) and high (10 ng/ml) concentrations, were analyzed after several freeze and thaw cycles by thawing at room temperature for 4 h, and freezing for 24 h. The short-term stabilities of extracted samples were also determined during storage for 12 h at 4 °C, room temperature, and −20 °C. Long-term storage stability at −70 °C was determined after 20 days.

2.8. Application to clinical test

The study population consisted of 24 healthy male Korean volunteers with an average age of 22.7 years and an average weight of 68.0 kg. Each volunteer was administered 10 mg of cilnidipine orally (a Cinalong tablet [Boryung Pharm. Co. Ltd., Korea] or a Cinapin tablet [Seoul Pharm. Co. Ltd., Korea]) using a standard 2 × 2 cross-over model in randomized order. A 1-week washout period was allowed between doses. Blood samples were collected in tubes containing heparin before and

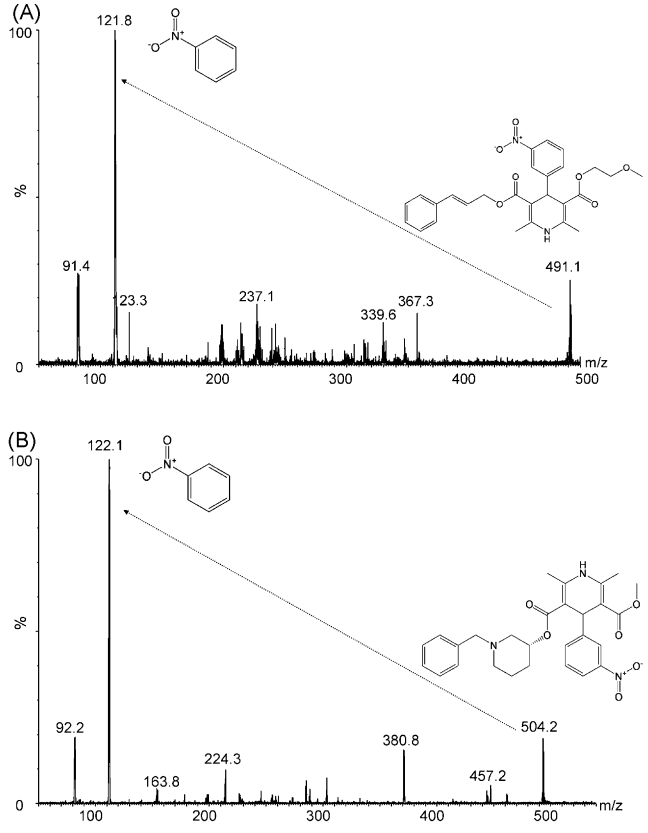

Fig. 2. Product ion spectra of ions of (A) cilnidipine and (B) benidipine.

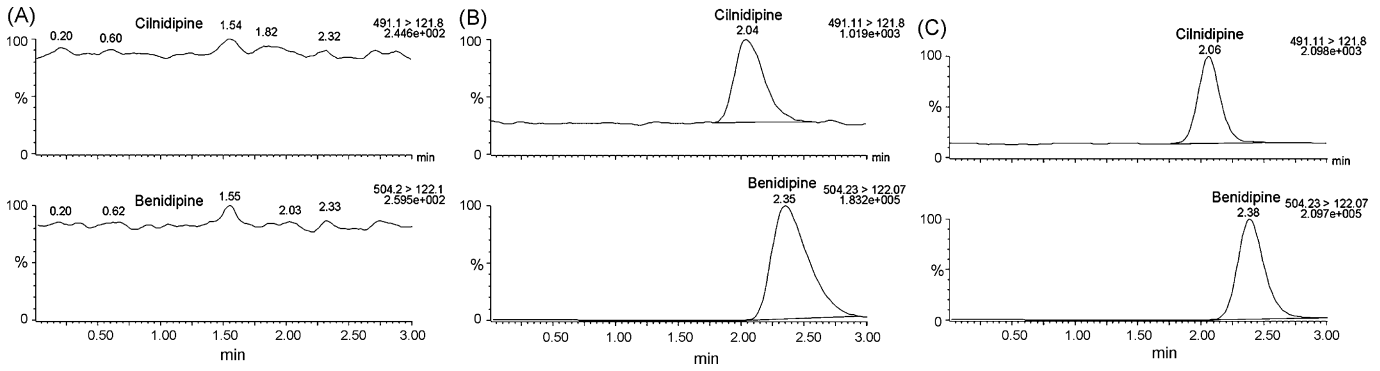

Fig. 3. Chromatogram of (A) blank human plasma, (B) plasma spiked with cilnidipine (1 ng/ml) and IS (10 μg/ml), and (C) sample plasma (measured cilnidipine concentration: 2.4 ng/ml).

after 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h of administration of drug. Blood samples were centrifuged immediately, and plasma samples were frozen at −70 °C until required for LC–MS/MS analysis. This study was approved by the Korean Food and Drug Administration (KFDA) and the Kyung-Hee University Ethical Committee before obtaining written consent from all volunteers.

Finally, C_{max} values (maximum plasma concentrations) and T_{max} times (times to C_{max}) were determined using individual plasma concentration-time profiles. A non-compartmental model for extravascular input, provided in WinNonlin Professional 3.1 software, was used to calculate pharmacokinetics parameters, i.e., $AUC_{12\,h}$ (area under the plasma drug concentration-time curve between 0 and 12 h), extrapolated AUC_{inf} (AUC from 0 to infinity), and $t_{1/2}$ (drug half-life). For bioequivalence analysis, two-way ANOVA (Analysis of Variance) was performed using the K-BE Test 2002 program at a significance level of 0.05.

3. Results and discussion

3.1. MS optimization

The optimization MS operating conditions were carried out for cilnidipine and IS in positive and negative ionization modes, and responses were found to be much higher in negative ionization mode. Therefore, negative ionization mode was selected for the entire study. In addition, preliminary studies showed that acidified solutions had a favorable effect on negative ionization, e.g., the production of negative ions from carboxylic acids can be enhanced by adding weak acid, rather than the expected base [18]. Furthermore, the examined both drugs possess similar ester groups in their structure. Thus, to enhance ion production, ionization efficiencies were examined at five different pH values (3.5, 4.5, 5, 5.5 and 8.5), and pH 5 was chosen because it produced high analyte intensities and low variation (data not shown). The most abundant ion products of cilnidipine and IS were at *m/z* 121.8 (from a precursor ion at *m/z* 491.1) and at *m/z* 122.1 (from a precursor ion at *m/z* 504.2), respectively. Fig. 2 shows the fragmentation patterns for *m/z* 491.1 > 121.8 for cilnidipine and *m/z* 504.2 > 122.1 for IS.

3.2. Method validation

Fig. 3 shows typical chromatograms of a blank plasma sample, a plasma sample spiked with cilnidipine (1 ng/ml) and IS (10 μg/ml), and a plasma sample from a healthy volunteer 2 h after the oral administration of 10 mg cilnidipine (measured cilnidipine concentration in plasma: 2.4 ng/ml). Cilnidipine and IS were well separated from the biological background under the chromatographic conditions used with retention times of 2.0 and 2.4 min, respectively. The total LC–MS/MS analysis time per run was 3 min, and no interference with the constituents from the blank human plasma samples at these retention times. Calibration curves were linear in the studied range from 0.1 to 20 ng/ml, and fitted the equation obtained from eight points was $y = 0.002962(\pm 0.00006)x + 0.000121(\pm 0.00001)$, $r^2 = 0.99998(\pm 0.00001)$. Intra-day accuracies for cilnidipine ranged from 91.1% to 105.7%, and intra-day precisions from 2.2% to 9.8%. Inter-day accuracies ranged from 91.3% to 109.9%, and the inter-day precisions from 2.6% to 4.9% (Table 2). All results were within the ranges for precision (%)

Table 2
Inter-day and intra-day precisions and accuracies for determination of cilnidipine in human plasma ($n = 5$)

Theoretical concentration (ng/ml)	Intra-day			Inter-day		
	Concentration found (ng/ml)	Precision (%)	Accuracy (%)	Concentration found (ng/ml)	Precision (%)	Accuracy (%)
0.1	0.09	8.3	91.1	0.09	5.1	88.9
0.5	0.52	9.4	104.8	0.54	6.1	107.0
1	1.06	9.8	105.7	1.09	4.4	108.8
5	5.14	2.2	102.7	5.16	3.3	103.2
10	10.24	3.2	102.4	10.30	2.4	103.0

Table 3
Recovery of cilnidipine in human plasma using different solvents for extraction (mean ± SD, $n = 3$)

	Analyte concentration (ng/ml)		
	0.1	1	10
n-Hexane (%)	23.7 ± 7.2	18.7 ± 8.0	16.1 ± 8.4
Methyl-*t*-butyl-ether (%)	64.3 ± 4.9	58.1 ± 4.9	57.3 ± 7.7
Butyl acetate (%)	49.4 ± 12.1	59.1 ± 9.1	52.2 ± 10.9
Ethyl acetate (%)	43.2 ± 2.7	54.4 ± 10.7	49.9 ± 8.1

and accuracy (%) specified by the KFDA for bio-analytical applications [17].

Ion suppression of cilnidipine by matrix constituents was investigated by comparing peak areas of cilnidipine working solutions with processed blank samples reconstituted with cilnidipine working solutions. Mean ion suppressions of cilnidipine and benidipine were −0.35 ± 3.1% and 7.14 ± 2.4% in human plasma. The RSDs of lot-to-lot matrix variations at cilnidipine concentrations of 0.1, 1, and 10 μg/ml were 6.5%, 5.5%, and 9.1%, respectively, which suggests that the matrix effect contributed little to inter-source variability. Thus, we conclude that the devised method is both reliable and not susceptible to matrix effect-induce variation.

To decrease the LLOQ, cilnidipine recovery was examined using four different extraction solvents such as *n*-hexane, MTBE, butyl acetate, and ethyl acetate. The average absolute recoveries of cilnidipine at concentrations of 0.1, 1, and 10 ng/ml are shown in Table 3. MTBE was chosen because of its good analyte recoveries and low level of cilnidipine variation; using MTBE the LLOQ was reduced to 0.1 ng/ml. Deviations observed (SD) between recoveries were <20% in the cilnidipine concentration range examined. These results show that the devised method could be applied to determine analyte recoveries by MTBE. The recoveries of cilnidipine at 0.1, 1 and 10 ng/ml were 64.3 ± 4.9%, 58.1 ± 4.9% and 57.3 ± 7.7%, respectively. In addition, the recovery of IS at 400 ng/ml using MTBE was 54.23 ± 9.0%. The extents of recovery of cilnidipine and benidipine were consistent and reproducible, which was satisfactory.

To evaluate cilnidipine stability in human plasma, drug-free plasma samples were spiked at 0.1, 1, and 10 ng/ml. After extraction, samples were arranged in an autosampler and analyzed. No significant deterioration was observed under any of the conditions examined (Table 4).

Table 4
Cilnidipine stability data (mean ± SD, $n = 3$)

Theoretical concentration (ng/ml)	Cilnidipine		
	0.1	1	10
Short-term			
12 h, 4 °C (%)	108.5 ± 3.9	99.8 ± 4.0	93.4 ± 3.2
12 h, room temperature (%)	99.8 ± 9.4	107.2 ± 3.0	98.1 ± 5.0
12 h, −20 °C (%)	91.8 ± 4.6	103.6 ± 9.4	110.0 ± 4.1
Long-term			
20 days, −70 °C (%)	92.4 ± 9.9	97.9 ± 10.4	87.1 ± 4.3
Freeze-thaw stability (%)	103.3 ± 8.5	100.2 ± 7.7	98.7 ± 6.4

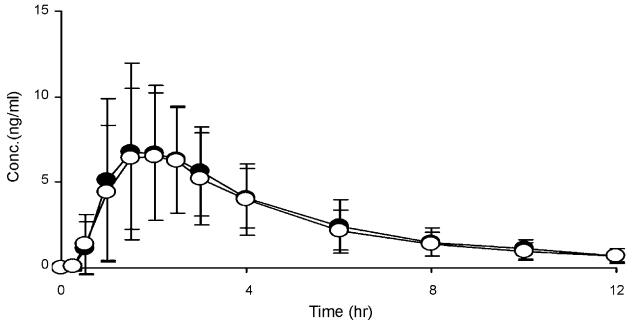

Fig. 4. Mean (±SD) concentration-time pharmacokinetic profiles of cilnidipine from Cinalong tablet (●, reference formulation) and Cinapin tablet (◯, test formulation) in the plasma samples of 24 healthy male volunteers after a single oral administration of 10 mg of cilnidipine.

Table 5
Bioequivalence parameters obtained after oral administration of Cinalong tablet (reference drug) and Cinapin tablet (test drug) at the cilnidipine dose of 10 mg

	Reference drug (mean ± SD)	Test drug (mean ± SD)	90% confidence interval
C_{max} (ng/ml)	9.56 ± 3.8	8.74 ± 3.8	0.8199–1.0123
$AUC_{12\,h}$ (ng/ml h)	33.33 ± 12.4	31.65 ± 13.0	0.8768–1.0171
AUC_{inf} (ng/ml h)	37.65 ± 16.0	35.30 ± 15.0	0.8693–1.0243
T_{max} (h)	2.17 ± 1.1	2.13 ± 1.1	–
$t_{1/2}$ (h)	3.60 ± 2.0	3.45 ± 0.8	–

3.3. Application to clinical test

The proposed method was applied to determine cilnidipine levels in plasma for a bioequivalence study in 24 healthy Korean male volunteers who were orally administered 10 mg of cilnidipine in tablet form. High-throughput sample analysis is of particular importance for studies that require the analysis of large numbers of samples, and the described LLE method of sample preparation is suitable for this purpose. In the present study, 624 clinical samples were divided into two batches, with each batch consisting of a calibration curve, 312 plasma samples from 12 subjects, and QC samples in triplicate. All samples were analyzed within 3 days. Fig. 4 shows the mean plasma concentration-time curves for the two formulations. Pharmacokinetic parameters derived from these curves are presented in Table 5. No significant differences were observed between the two formulations in terms of; C_{max}, $AUC_{12\,h}$, AUC_{inf}, T_{max} or $t_{1/2}$.

4. Conclusions

We describe a simple, rapid, sensitive LC–MS/MS method to determine cilnidipine levels in human plasma. The devised method fully meets KFDA guidelines, has high sensitivity and specificity, and is capable of high sample throughputs.

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