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Journal of Chromatography B, 803 (2004) 201–207
JOURNAL OF CHROMATOGRAPHY B
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Selective and rapid liquid chromatography-mass spectrometry method for the determination of lercanidipine in human plasma

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Received 18 September 2003; received in revised form 24 November 2003; accepted 23 December 2003

Abstract

A specific liquid chromatography–mass spectrometric (LC–MS/MS) assay was developed and validated for the determination of lercanidipine, a dihydropyridine calcium channel blocker, in human plasma. Lercanidipine *R-D3* was used as internal standard (IS). The drug was extracted from plasma using liquid–liquid extraction technique utilizing hexane: ethyl acetate as extraction solvent. The samples were analyzed using a prepacked Thermo Hypersil C_8 column and a mobile phase composed of a mixture of aqueous acetic acid and triethylamine in methanol. An ion trap mass spectrometer equipped with electrospray ionization (ESI) source operating in the positive ion mode was used to develop and validate the method. The method was proved to be sensitive and specific by testing six different human plasma batches. Linearity was established for the concentration ranges of 0.1–16 ng/ml with a regression factor of 0.9996. The lower limit of quantitation was identifiable and reproducible at 0.1 ng/ml with a precision of 7.2%.
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Keywords: Lercanidipine

1. Introduction

Lercanidipine, (±)2-[(3,3-diphenylpropyl)methylamino]-1,1-dimethylethyl methyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate monohydrochloride (Fig. 1), is a dihydropyridine calcium channel blocker used in the treatment of hypertension [1]. It is derived from a group of new 4-aryl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acids dialkyl esters with different lipophilic amino-alkyl groups in one of the two ester-groups. In order to improve the activity duration, by increasing the overall lipophilicity, structural variations at the aryl group at position 4 of the 1,4-dihydropyridine and at the non-basic alkyl-ester were introduced. Structural variations in the length and branching of the alkyl group linking the amino group to the dihydropyridine nucleus have been introduced. The presence of a 3,3-diphenyl propylmethylamino-2-methyl-2-propyl chain improves the lipophilic properties of this drug. Lercanidipine is administered orally as lercanidipine hydrochloride racemate tablets.

Only one method of analysis has been cited for lercanidipine determination in human plasma [2]. This citation refers to an HPLC/UV method with a limit of quantitation of 0.5 ng/ml. Addition to this reference, in a pharmacokinetic paper [3], the authors mentioned the use of a TLC-radiometric (^{14}C) technique for lercanidipine determination in human plasma. Unfortunately, no further details on the method's procedures or results were presented [3]. Other calcium antagonists of the same group, e.g. Amlodipine, are usually determined in plasma by HPLC (fluorescence or ECD) and by LC–MS methods [4–7].

The aim of the present paper was to develop and validate a sensitive, robust and reproducible reversed-phase LC–MS/MS method for lercanidipine determination in human plasma and at the same time to increase dramatically sample throughput. Although lercanidipine exists in *R*,*S* enantiomer forms, nevertheless, and adhering to the FDA guidance for bioavailability and bioequivalence studies [8], only the racemic form of this drug was monitored in this study. The method was to have a limit of reliable quantification of at least 0.10 ng/ml, in order to support clinical studies employing a single dose of 20 mg (2 × 10 mg) lercanidipine tablets. At the same time, it was expected that

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doi:10.1016/j.jchromb.2003.12.019

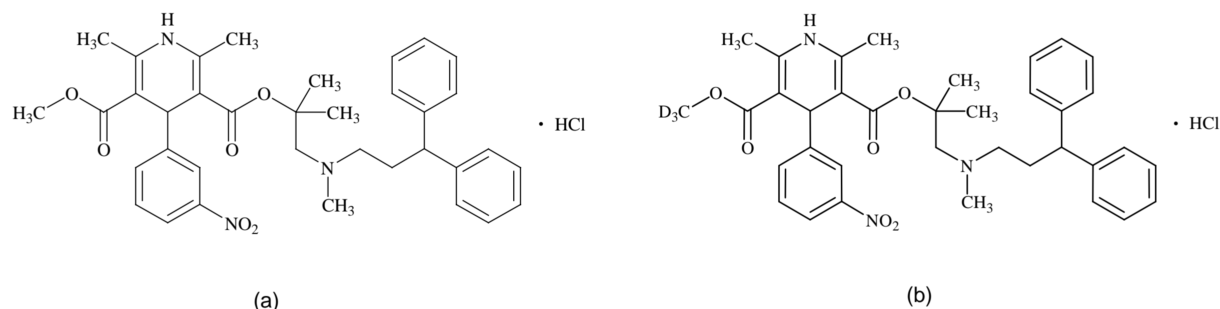

Fig. 1. Chemical structures of lercanidipine (a) and lercanidipine *R-D3* (internal standard, b).

this method would be efficient in analyzing large number of plasma samples obtained from clinical (e.g. bioequivalence) studies.

2. Experimental

2.1. Reagents

Lercanidipine and 5-trideutero-lercanidipine *R-D3* (Fig. 1) were donated by Recordati Industria Chimica e Farmaceutica S.p.A. (Milan, Italy). The following chemicals and reagents were used: HPLC grade methanol, acetic acid and ethyl acetate (Acros, Belgium), sodium hydroxide, triethylamine and sulfuric acid (BDH laboratories, UK), acetone, hexane and ammonia (Scharlau, Spain). A Milli-Q® (Millipore, France) water purification system was used to obtain the purified water for the HPLC analysis. Lithium heparin plasma (six different batches) of healthy volunteers was obtained from Jordan University Hospital (Amman, Jordan).

2.2. Preparation of stock solutions

All stock solutions were prepared weekly under the light of a sodium lamp to prevent degradation. Amounts equivalent to 10.0 mg of lercanidipine (using lercanidipine hydrochloride standard powder) were dissolved in 100 ml methanol to produce a concentration of 100.0 μg/ml and stored at −70 °C. Working solutions of lercanidipine were prepared daily in methanol by appropriate dilution at 5.0, 25.0, 50.0, 250.0, 500.0 and 800.0 ng/ml. The internal standard stock solution was prepared by dissolving amounts of IS equivalent to 10.0 mg (using lercanidipine *R-D3* hydrochloride standard powder) in 100 ml methanol to produce a concentration of 100.0 μg/ml. This solution was stored at −70 °C.

2.3. Calibration curves

Calibration curves were prepared by spiking different samples of 1 ml plasma each with 20 μl of one of the abovementioned working solutions to produce the calibration curve points equivalent to 0.1, 0.5, 1.0, 5.0, 10.0 and 16.0 ng/ml of lercanidipine. Each sample contained also 8.0 ng/ml of internal standard. Single blank plasma samples used in each run were prepared containing 8.0 ng/ml of internal standard only. In each run, a plasma double blank sample (no IS) was also analyzed. Neither the double blank sample nor the single blank one were used to construct the calibration function. Calibration curves were run daily together with quality control (QC) samples.

2.4. Quality control samples

Quality control samples were prepared at three levels: low level (three times the lower limit of quantitation, LLOQ), middle level and a high level (80% of the upper limit of quantitation limit, ULOQ). QCs were prepared daily by spiking different samples of 1 ml plasma each with 20 μl of the corresponding standard solution to produce a final concentration equivalent to 0.3, 8.0, and 12.0 ng/ml of lercanidipine and 8.0 ng/ml of internal standard.

2.5. Quality control samples for dilution process

Samples with drug concentrations above the upper limit of quantitation (ULOQ) found during routine analyses, were to be diluted properly in human plasma in order to bring concentrations down into the calibration curves linear range. One ml of human plasma were placed in each tube and spiked with 20 μl from the freshly prepared standard solutions (800.0, 1000.0 and 1500.0 ng/ml). Accordingly, the plasma samples contained a final concentration of lercanidipine equivalent to 16.0, 20.0 and 30.0 ng/ml, respectively.

2.6. Extraction

All QC, calibration curve, single blank and double blank plasma samples were extracted using a liquid–liquid extraction technique. The extraction procedure was done under sodium lamp light to avoid possible drug degradation. Four milliliters of acetone were added to each plasma sample, vortexed for 30 s and centrifuged for 3 min at 3200 × *g* (Eppendorf 5810R, Germany). The supernatants were transferred to different extraction tubes, where 1 ml of 1.0 M

NaOH and 8 ml hexane were added to each tube. Samples then were shaken for 20 min and centrifuged for 5 min at $3200 \times g$. Again, the supernatants were transferred to different extraction test tubes. To these last tubes, 1.5 ml 0.2 M H_2SO_4 were added, shaken for 20 min and centrifuged for 5 min at $3200 \times g$. Samples were then frozen at −70 °C for 20 min, after which the organic layer was discarded. Seven milliliters of hexane and 0.75 ml of 1.0 M NH_4OH:ethyl acetate (80:20) were added, shaken for 20 min and centrifuged for 8 min at $3200 \times g$. Samples were frozen again at −70 °C for 20 min, afterwards, the organic layer was transferred to evaporating tube. The organic layer was evaporated under nitrogen stream at 40 °C. Finally, samples' residues were reconstituted with 100 μl of 0.8% acetic acid in methanol:H_2O (1:1). Samples were transferred to the prelabeled HPLC vials from which 60 μl were injected into the HPLC column.

QC samples prepared in Section 2.5, and those of volunteers' routine analysis with concentrations higher than the ULOQ of the calibration curve, were diluted with the same plasma matrix in order to be properly calculated applying the calibration curves ranges. Therefore, 0.5 ml of the plasma sample were diluted with a complementary volume of blank plasma (0.5 ml) and the resultant 1 ml of the diluted sample was spiked with 20 μl from the prepared IS working solution (400.0 ng/ml) and processed as described above.

2.7. HPLC conditions

Chromatography for separation and determination of the drug was carried out by applying the samples to a prepacked Thermo Hypersil C_8 3 μm, 150 mm × 4.6 mm (Thermo Hypersil, UK), using a Waters 717 autosampler and 515 isocratic pump (Milford, MA, USA). The analytical column was protected by a 4 mm × 2.0 mm i.d. Phenomenex C_{18} guard column (Phenomenex, USA). The combination of the mobile phase, prepared by mixing aqueous acetic acid (0.5%) and triethylamine (0.5%) in methanol and a flow rate of 0.80 ml/min was found to be adequate for the samples analysis. Separations were performed at room temperature (22 ± 2 °C) however samples were kept in the autosampler at 4 °C. The total chromatographic run time was 10 min although shorter run time (7 min) was applied during routine analyses.

2.8. MS/MS conditions

Drug monitoring and quantitation were achieved using a Finnigan LCQ^{DUO} quadrupole ion trap mass spectrometer (Finnigan ThermoQuest, USA) set at unit resolution in the multiple reaction monitoring mode. Electrospray ionization was used for ion production employing an ESI source (Finnigan). The analyses were run by XCALIBUR 1.2 software.

Operating conditions for the ESI source, used in the positive ionization mode, were optimized by constantly adding lercanidipine in methanol (100 μg/ml) to the HPLC flow by a syringe pump via a T-connector in the infusion mode. The signal was optimized on the total ion current in MS mode, producing a transfer capillary temperature of 200 °C, a spray voltage of 7.5 kV, and a sheath gas flow of 70 units (units refer to arbitrary values set by the LCQ software). At the same time, the selection of ions and the collision voltages were optimized using LCQ software. In the MS/MS experiments, the protonated precursor $[M+1]^+$ of lercanidipine (*m/z* 612) and the IS (*m/z* 615) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 53%. The mass spectra resulting from these fragmentations were acquired in the SRM mode at *m/z* 280/298 for lercanidipine and *m/z* 280/298 for IS. These product ions, *m/z* 280/298 for lercanidipine and *m/z* 280/298 for the IS, were extracted for quantification.

The detection in MS/MS technique is highly specific and sensitive, nevertheless, endogenous substances can exist in much higher concentration than the analytes of interest and may co-elute with those affecting the ionization of the analytes leading to high imprecision and loss of sensitivity [9]. In order to determine ion suppression matrix effect profiles, analytes were infused into the mobile phase through a T-connection between the column and the interface while injecting the extracted blank plasma samples. The purpose of this post-column infusion with the analytes is to raise the background level so the suppression matrix will appear as negative peaks.

2.9. Data treatment

The linearity of lercanidipine method determination in human plasma was tested for a range of concentrations from 0.10 to 16.0 ng/ml. Calibration curves were prepared by determining the best-fit of peak area ratios (peak area analyte/peak area internal standard) versus concentrations, and fitted to the equation $y = bx + a$ by least-squares regression. Different runs have been made and the best fit was obtained by using $1/x$ weighing.

3. Results and discussion

3.1. Separation and specificity

ESI is a "gentle" ionization technique that produces high mass-to-charge $[M+1]^+$ precursor ions with minimal fragmentation of the analyte. Lercanidipine and IS gave protonated precursor $[M+1]^+$ in the MS mode. The major ions observed were *m/z* 612 for lercanidipine (Fig. 2) and *m/z* 615 for the IS (Fig. 3). The most intense product ions observed in the MS/MS spectra were *m/z* 280/298 for lercanidipine and *m/z* 280/298 for the IS. The corresponding spectra of lercanidipine and the IS are shown in Figs. 2 and 3, respectively.

The combination of HPLC (under the isocratic conditions described) with ESI-MS/MS leads to short retention times and yields both high selectivity and sensitivity. The SRM

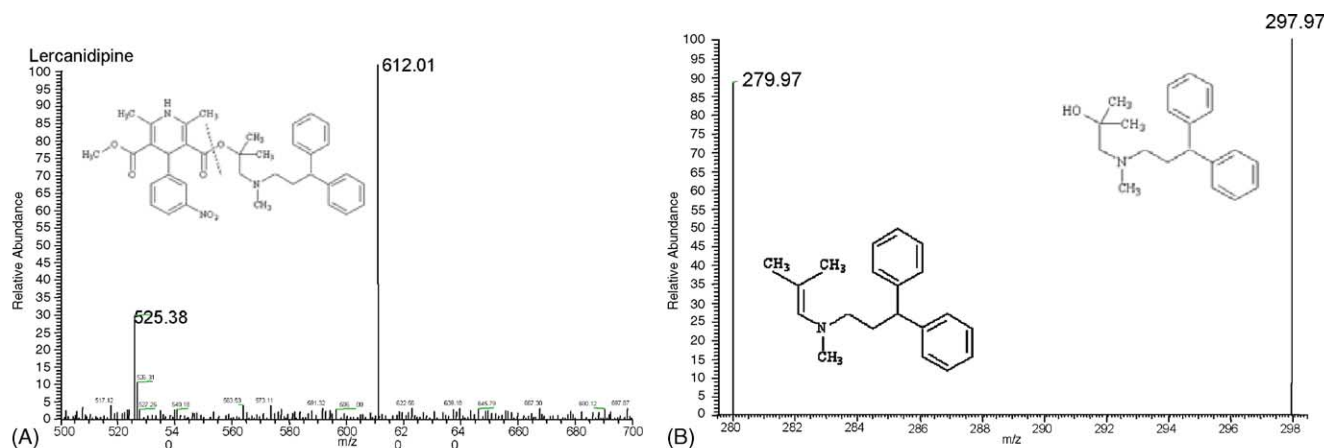

Fig. 2. Positive ion electrospray mass spectrum (A) and product ion mass spectrum (B) used in SRM for lercanidipine determination.

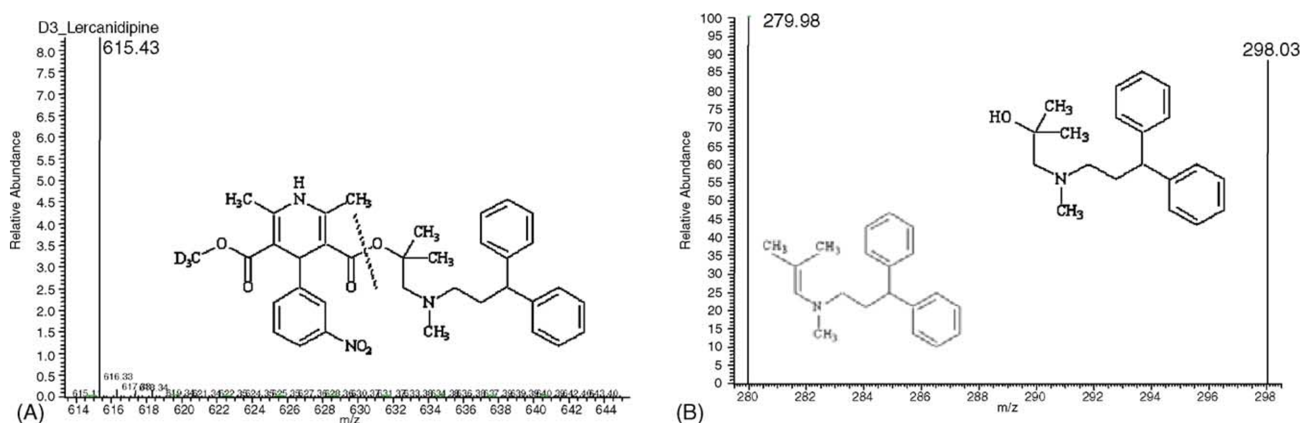

Fig. 3. Positive ion electrospray mass spectrum (A) and product ion mass spectrum (B) used in SRM for *R-D3* lercanidipine determination.

chromatograms obtained from an extracted double blank and 0.3 ng/ml spiked plasma samples are depicted in Fig. 4. As shown, the retention times of lercanidipine and the IS were 5.3 and 5.3 min, respectively. No interferences of the analytes were observed because of the high selectivity of the MS/MS technique. Fig. 4 shows also an HPLC chromatogram of a double blank plasma sample indicating no endogenous peaks at the retention times (t_R) of lercanidipine or internal standard (lercanidipine *R-D3*). No ion suppression effects were observed.

The SRM chromatograms obtained from an extracted plasma sample of a healthy volunteer who participated in a bioequivalence study conducted on 36 volunteers, is depicted in Fig. 5, where lercanidipine was unambiguously identified and was quantified as 7.08 ng/ml.

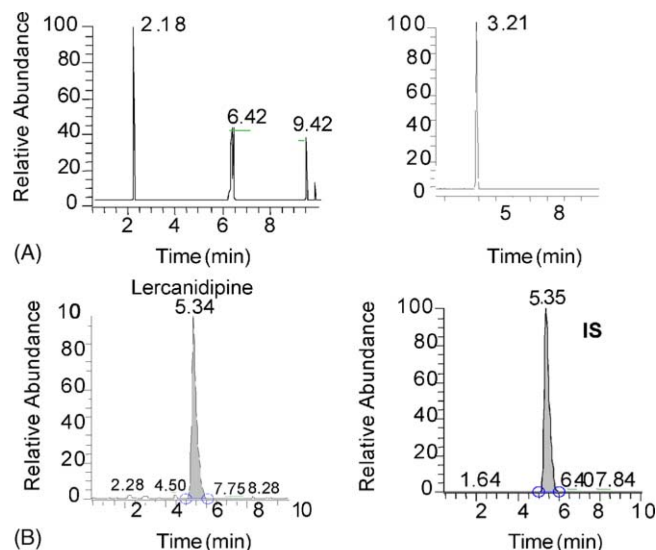

Fig. 4. LC–MS/MS chromatograms showing: (A) a double blank human plasma sample and (B) human plasma sample spiked with 0.3 ng/ml lercanidipine and 8.0 ng/ml lercanidipine *R-D3* (internal standard). The drug and the IS were monitored at *m/z* 612, 615 (parent), respectively, and 280/298 (daughters).

3.2. Method validation

In our laboratory, samples analysis is always carried out in a GLP-compliant manner and therefore the LC–MS/MS methods need to be validated according to currently accepted US Food and Drug Administration (FDA) bioanalytical

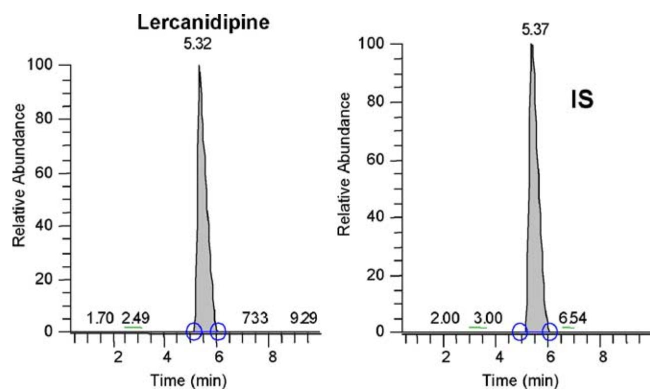

Fig. 5. LC–MS/MS chromatograms showing volunteer's plasma sample after the administration of an oral single dose of 20 mg (2 × 10 mg) lercanidipine tablets. The sample's concentration was found to be 7.08 ng/ml.

method validation guidance [10]. The following parameters were considered:

To test the specificity, six batches of human plasma were tested. Each double blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectrometric conditions and compared with those obtained with an aqueous solution of the analyte at a concentration near to the LLOQ. No significant interference at the retention time of the drug or internal standard were found, as illustrated in the chromatograms presented in Fig. 4A.

In order to test possible matrix effects, five different (1 ml) blank plasma samples were extracted and processed as abovementioned. Afterward, each one of the dried samples was spiked with lercanidipine at the middle QC level (8.0 ng/ml). The samples were vortexed and 60 μl were injected into the HPLC column. The matrix effect was calculated by comparing peak areas obtained for these samples with the unextracted pure authentic standard solution peak areas at the middle QC level (8.0 ng/ml). Compared to the absolute recovery at the same middle QC level (5.0 ng/ml), the results revealed 4.66% matrix effect.

Linearity was tested for the concentration ranges of 0.1–16 ng/ml, employing standard calibration curves of at least six points (non-zero standards). In addition, a double blank (none-spiked sample) and single blank plasma samples (only spiked with IS) were also analyzed to confirm absence of interferences. These two samples were not used to construct the calibration function. The method exhibited a good linear response for the range of concentrations from 0.1 to 16 ng/ml. Correlation coefficients ranged from 0.9995 to 0.9998 (CV 0.01%), while the calculated accuracy, at the lower point of the calibration curve, ranged from 98.50 (CV 6.55%) to 101.54 (CV 9.97%). The obtained results were within the acceptance criteria of no more than 20% deviation at LLOQ and no more than 15% deviation for standards above this point (LLOQ). The acceptance criterion for correlation coefficient was 0.998 or more, otherwise the calibration curve should be rejected.

Table 1
Inter-day accuracy, precision and relative error for lercanidipine determination in human plasma samples

Analyzed on Day	Lercanidipine concentration in human plasma		
	QC sample 0.3 ng/ml	QC sample 8.0 ng/ml	QC sample 12.0 ng/ml
Day 1	0.3	8.2	12.2
	0.3	8.7	11.8
	0.3	8.4	12.1
	0.3	9.1	11.4
	0.3	8.6	10.8
Day 2	0.3	7.1	10.5
	0.3	7.2	11.3
	0.3	7.0	10.8
	0.3	7.6	10.7
	0.3	7.4	12.4
Day 3	0.3	8.0	12.3
	0.3	7.6	11.0
	0.3	7.6	11.9
	0.3	8.2	12.0
	0.3	7.5	12.6
Mean	0.3	7.9	11.6
S.D.	0.0	0.7	0.7
Precision as CV (%)	3.8	8.2	6.1
Accuracy (%)	101.0	98.5	96.5
RE (%)	1.0	1.5	3.5

The intra-day precision and accuracy of the assay was measured by analyzing five spiked samples of lercanidipine at each QC level (0.3, 8.0 and 12.0 ng/ml). Intra-day accuracy of the method for lercanidipine ranged from 97.11 to 107.79%, while the intra-day precision (CV) ranged from 2.04 to 4.85% at the QC levels. The acceptance criteria for precision and accuracy deviation values was ±15% of the actual values.

The inter-day precision and accuracy was determined over 3 days by analyzing 45 QC samples. Data for the inter-day precision and accuracy are presented in Table 1. These results were within the acceptance criteria for precision and accuracy which establish the deviation values should be within ±15% of the actual values.

The accuracy and precision of the QC samples used for dilution were evaluated, giving satisfactory results as reflected in Table 2. The accuracy and precision ranged from 94.47 to 95.39% and from 5.67 to 7.21%, respectively (Table 2).

The absolute recoveries were evaluated for both lercanidipine and IS by comparing peak areas of the extracted samples with the unextracted pure authentic standard solutions peak areas at three QC levels (0.3, 8.0 and 12.0 ng/ml). Results ranged from 59.63% (CV 16.47%) to 70.92% (CV 4.37%) at the three QC levels. Absolute analytical recovery of internal standard (lercanidipine *R-D3*) was 70.23% (2.76%).

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the

Table 2
Accuracy, precision and relative error for quality control samples prepared for dilution process of lercanidipine

	Lercanidipine concentration in human plasma (ng/ml)					
	After dilution	Before dilution	After dilution	Before dilution	After dilution	Before dilution
Quantitated concentration (ng/ml) (dilution factor = 2)	8.0	16.0	10.0	20.0	15.0	30.0
Day 1	8.0	15.9	9.7	19.3	15.9	31.8
	7.5	15.0	9.8	19.5	15.2	30.3
	7.7	15.4	9.9	19.9	14.8	29.7
	8.7	17.4	9.8	19.5	14.7	29.5
	7.6	15.2	10.0	19.9	15.0	30.0
Day 2	7.8	15.7	9.4	18.9	14.7	29.5
	7.6	15.3	10.0	20.1	15.4	30.8
	8.0	16.0	10.2	20.5	14.7	29.4
	8.1	16.2	8.9	17.8	15.2	30.4
	7.9	15.7	9.8	19.5	13.8	27.5
Day 3	7.0	14.0	8.8	17.7	13.0	26.0
	6.9	13.7	8.7	17.5	13.2	26.4
	7.1	14.3	9.0	18.0	12.9	25.8
	7.0	14.0	9.1	18.1	13.1	26.3
	7.0	14.1	8.6	17.2	13.0	25.9
Mean	7.6	15.2	9.4	18.9	14.3	28.6
S.D.	0.5	1.0	0.5	1.1	1.0	2.1
Precision as CV (%)	6.8		5.7		7.2	
Accuracy (%)	94.9		94.5		95.4	
RE (%)	5.1		5.5		4.6	

lower limit of quantitation. The lower limit of quantitation for lercanidipine was proved to be 0.1 ng/ml, with 98.20% accuracy and 7.22% precision. The LLOQ met the following criteria: LLOQ response was more than five times the response of the double blank and the LLOQ response was identifiable, discrete and reproducible with precision of 20% and 80–120% accuracy.

The stability of the analytes in human plasma under different temperature and timing conditions, as well as the stability of the analytes in stock solution, was evaluated as follows:

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during the routine sample preparation (around 6 h). Samples were extracted and analyzed as abovementioned. Results are given below in Table 3. Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs.

The post-preparative stability of QC samples kept in the autosampler for 24 h at 4 °C, was also assessed. The mean recoveries of the low, mid and high QC levels were above 95%. The results indicate that lercanidipine and internal standard can remain at the autosampler temperature (4 °C) for at least 24 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The data that represent the stability of lercanidipine plasma samples at three QC levels over three cycles of freeze (−70 °C) and thawing (room temperature) are given

Table 3
Data showing short-term stability of lercanidipine in human plasma

Run	Low QC (0.3 ng/ml) recovery (%)	Middle QC (8.0 ng/ml) recovery (%)	High QC (12.0 ng/ml) recovery (%)
1	89.74	83.21	110.74
2	93.95	87.54	106.71
3	117.98	83.69	103.90
4	95.73	87.72	104.05
5	95.36	92.75	104.24
Mean	98.55	86.98	105.93
S.D.	11.12	3.85	2.93
CV (%)	11.28	4.42	2.76

Table 4
Data showing freeze and thaw stability of lercanidipine in human plasma

Run	Low QC (0.3 ng/ml) recovery (%)	Middle QC (8.0 ng/ml) recovery (%)	High QC (12.0 ng/ml) recovery (%)
1	94.87	81.72	106.71
2	92.53	85.66	113.45
3	103.00	96.79	104.30
4	103.05	96.55	114.18
5	80.50	95.31	107.64
Mean	94.79	91.20	109.25
S.D.	9.29	7.02	4.34
CV (%)	9.80	7.70	3.97

Table 5
Data showing long-term stability of lercanidipine in human plasma

Run	Low QC (0.3 ng/ml) recovery (%)	Middle QC (8.0 ng/ml) recovery (%)	High QC (12.0 ng/ml) recovery (%)
1	91.33	84.97	101.70
2	90.01	85.28	99.61
3	88.02	83.08	98.14
4	95.63	82.98	99.95
5	94.97	99.16	98.73
Mean	91.99	87.09	99.63
S.D.	3.25	6.82	1.36
CV (%)	3.53	7.84	1.36

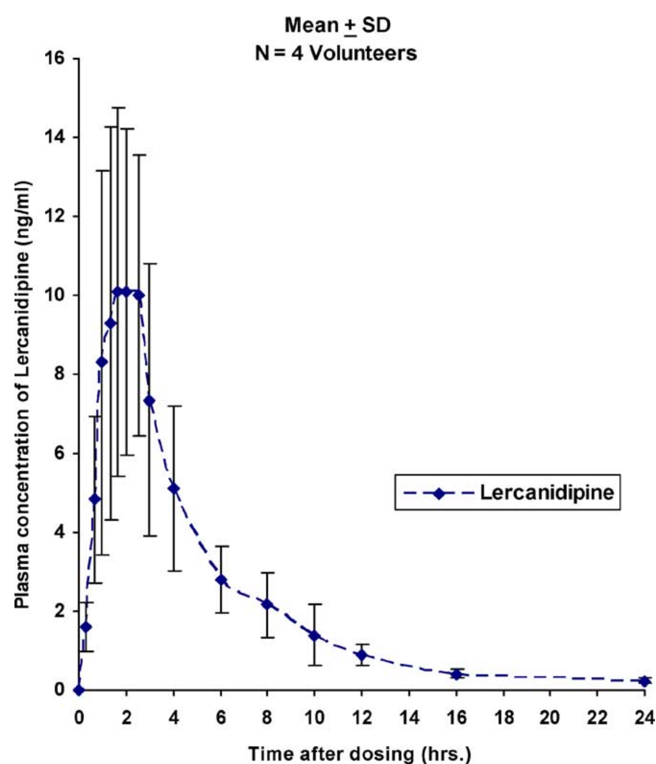

Fig. 6. Representative data showing mean plasma concentration–time profiles of four healthy volunteers after the administration of an oral single dose of 20 mg (2 × 10 mg) lercanidipine tablets. The error bars represents ±S.D.

in Table 4. The performed tests indicate that the analyte is stable in human plasma for three cycles of freeze and thaw, when stored at −70 °C and thawed to room temperature.

Table 5 summarizes also the long-term stability data of lercanidipine in plasma samples stored for a period of 8 weeks at −70 °C. The stability study of lercanidipine in human plasma showed reliable stability behavior as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicated that storage of lercanidipine plasma samples at −70 °C is adequate, and no stability-related problems would be expected during the samples routine analysis for bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 12 h in absence of light. The stability was also tested and established after 1 week of preparation and storage at 4 °C protected from light. The recoveries for lercanidipine were 97.02% (CV 7.42%) and 94.52% (CV 0.77%) at 12 h and 1 week, respectively. Lercanidipine *R-D3* recoveries were 99.72% (CV 1.30%) and 95.59% (CV 4.59%) at 12 h and 1 week, respectively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily and weekly use. Working solutions and serial dilution standard solutions were prepared freshly just before spiking for both the calibration curve and the QC samples and were not allowed to stand for a period of time more than that needed to complete spiking of plasma samples.

4. Application

This specific and sensitive method was applied to analyze plasma samples obtained after the administration of a single dose of 20 mg (2 × 10 mg) lercanidipine tablets to 36 healthy volunteers in a bioequivalence study. The mean plasma concentration–time profile of four volunteers is represented in Fig. 6.

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