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Enantioselective determination of lercanidipine in human plasma for pharmacokinetic studies by normal-phase liquid chromatography–tandem mass spectrometry

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Abstract

We describe here the first method for the enantioselective analysis of the calcium antagonist lercanidipine in human plasma by high performance liquid chromatography (HPLC) employing tandem mass spectrometric (MS) detection. Routine determination of lercanidipine enantiomers in human plasma in the working range of 0.025–50.0 ng ml^{-1} plasma for each enantiomer with an accuracy and precision less than 15% was possible. Application of the method to a stereospecific study of the pharmacokinetics showed that plasma levels after an oral dose of *rac*-lercanidipine administered to a healthy volunteer were found to be higher for the (*S*)-enantiomer.

Keywords: Pharmacokinetics; Lercanidipine

1. Introduction

Hypertension is one of the major risk factors for coronary artery disease and the most important risk factor for cerebrovascular diseases. By lowering blood pressure, antihypertensive drugs diminish cardiovascular risk in hypertensive patients [1]. The group of dihydropyridine calcium antagonists represents one of the drugs of first choice for monotherapy or combination therapy of hypertension. Calcium antagonists are a group of drugs with different chemical structure, blocking the extracellular calcium-dependent contractions of cardiac and vascular smooth muscles. The calcium antagonists are divided into three subgroups: 1,4-dihydropyridines (DHP), benzothiazepines and phenylalkylamines [2]. Calcium antagonists of DHP

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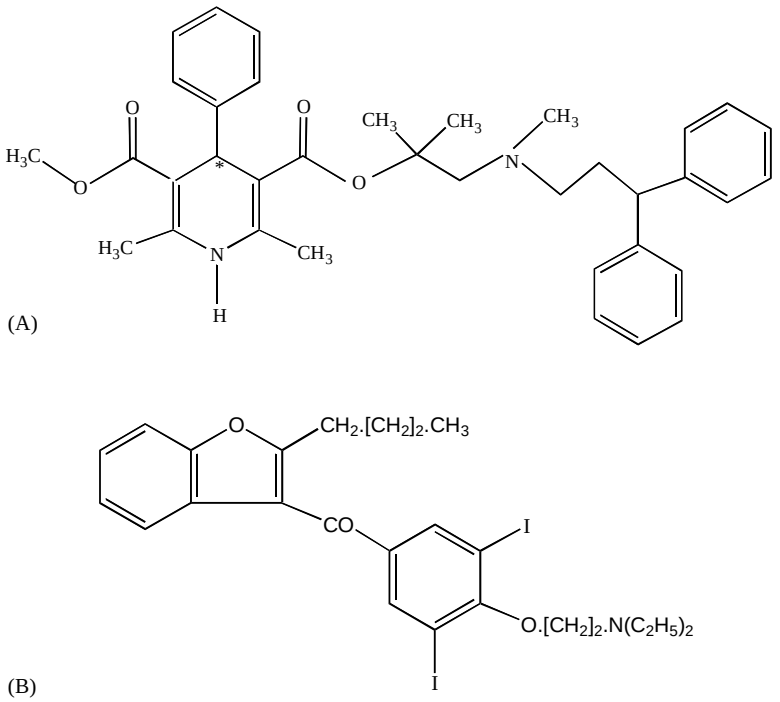

Fig. 1. Chemical structures of lercanidipine (A) and amiodarone (B). (*) denotes the chiral center.

do not affect the atrioventricular conduction system as observed for others subgroups, and are consequently preferred in the treatment of hypertension [3,4].

Lercanidipine hydrochloride, chemically know (±) 3, 5-pyridinedicarboxilic acid, 1,4-dihydro-2,6-dimethyl-4-(3- nitrophenyl)-2-[(3,3-diphenylpropyl)methylamino]- 1,1-dimethyl ethyl methyl ester hydrochloride (Fig. 1A), is a new third-generation synthetic DHP agent developed as an antihypertensive drug. Lercanidipine, also classified as a once-a-day calcium antagonist, is the first to demonstrate a short plasma half-life, on the order of 3 h, with a long duration of action [5,6].

Structurally, the DHP ring of LER has two different ester groups at positions 3 and 5 of the 1,4-DHP ring, leading to the existence of two enantiomers: (*S*)- and (*R*)-LER [2]. Several in vitro and in vivo studies have been performed with the enantiomers of LER. The differences in potency between the two enantiomers were generally observed also in in vivo studies. Studies on blood pressure in normotensive and spontaneously hypertensive rats and in renal hypertensive dogs showed that the overall hypotensive or antihypertensive activity of LER may be ascribed to the (*S*)-enantiomer, since (*R*)-LER did not affect blood pressure at doses much higher than those active for the racemate and (*S*)-enantiomer. The hemodynamic activity confirmed these results, with (*R*)-LER generally showing poor or no activity on peripheral and coronary resistance [7].

The clinical pharmacokinetics of LER was reported by Testa et al. [7] in studies involving the administration of a solution of [^{14}C]-radiolabed LER. A crossover study involving a single administration of either 10 mg of each of the two enantiomers or 20 mg of the racemate was performed. Plasma concentrations were higher for the (*S*)-enantiomer, at all sampling times. No in vivo enantiomer interconversion was observed. Following racemate administration, the C_{max} and AUC of the (*S*)-enantiomer were, on average, 1.2-fold higher than those of the (*R*)-enantiomer [7].

Enantioselective methods for DHP analysis in human plasma for pharmacokinetic studies have been developed based on liquid chromatography (LC) resolution of enantiomers and subsequent gas chromatography (GC) determination of the isolated enantiomers in the LC fractions [8–12]. Commonly, analytical

methods for DHPs in biological samples have been based on GC with electron capture or mass spectrometric (MS) detection [13–16]. There is only one method cited in literature for the enantioselective analysis of LER in biological samples employing thin-layer chromatography and high performance liquid chromatography (HPLC), but the analytical conditions were not reported [17]. Thus, so far there is no sensitive and validated method to quantify LER enantiomers in human plasma samples. An increase in sample throughput for pharmacokinetic studies also requires a reduced analysis time and simplified sample clean-up procedures.

In the last few years, LC coupled to mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS–MS) has been widely used in biomedical fields for both identification and quantitation of drugs in biological fluids at very low concentrations [13]. Although LC–MS has became a common technique, it is usually performed using reverse-phase separations with an aqueous mobile phase. Normal-phase LC–MS with atmospheric pressure ionization sources is still rare (due to the non-polar and flammable nature of the solvents), although the number of examples is increasing.

The present study focuses on the development of a new analytical method based on normal-phase LC–MS–MS for the quantitative determination of trace concentrations of individual enantiomers of LER in human plasma. The method reported here involves sample handling by liquid–liquid extraction (LLE), HPLC resolution of the enantiomers using an amylose based chiral stationary phase and subsequent MS detection in the multiple reaction monitoring (MRM) mode via electrospray ionization using a make-up liquid to the column effluent. The method has been rigorously validated for application to enantioselective pharmacokinetic studies of LER. The validated method was applied to plasma samples from a volunteer treated with 20 mg *rac*-LER and provided the required sensitivity and selectivity for monitoring levels of 0.025 ng ml^{-1} of (*S*)- and (*R*)-LER.

2. Materials and methods

2.1. Chemicals and reagents

Standard racemic lercanidipine hydrochloride (*rac*-LER) was kindly supplied by Recordati Industria Chimia e Farmaceutica S.p.A. (Milan, Italy). Stock standard solutions of *rac*-LER prepared in methanol in the concentration range of 0.002–4.00 μg ml^{-1} were stable for at least 3 months when stored at −20 °C and in the absence of light. Spiked plasma samples were obtained by the addition of known aliquots of these standard solutions to drug-free plasma prior to extraction. The internal standard (IS) solution, amiodarone (Fig. 1B), was prepared in methanol at the concentration of 0.66 μg ml^{-1}.

Hexane, methanol, ethanol and 2-propanol, chromatography grade, were purchased from Merck (Darmstadt, Germany). Analytical grade reagents as sodium hydroxyde pellets (Mallinckrodt Baker Inc., Paris, Kentucky, USA), diethylamine (Merck, Hohenbrunn, Germany) and ammonium acetate (J.T. Baker, Xalostoc, Mexico) were employed. All water was purified in a Milli-Q Plus System (Millipore, Bedford, MA, USA). Argon of 99.9997% purity was used as collision-induced dissociation gas.

2.2. Chromatography

The liquid chromatographic system consisted of an LC10AD pump and a CTO-10AS column oven from Shimadzu (Kyoto, Japan). The chiral column Chiralpak® AD 250 mm × 4.6 mm i.d., 10 μm particle size (Chiral Technologies Inc., Exton, PA, USA) was used for the resolution of the LER enantiomers. A LiChrospher® 100 RP-18 column (4 mm × 4 mm i.d., 5 μm) from Merck (Darmstadt, Germany) was used as guard column. The mobile phase consisted of a mixture of hexane–ethanol–diethylamine (95:5:0.1, v/v/v). The column was kept in an oven set at 30 (±1) °C and a flow-rate of 1.3 ml min^{-1} was used in all experiments.

The make-up liquid was 10 mmol l^{-1} ammonium acetate aqueous solution in ethanol (5:95, v/v). The post-column addition of the make-up liquid was carried out with a Shimadzu LC10AD pump (Kyoto, Japan) and the flow-rate was set at 0.25 ml min^{-1}, before the split. The effluent from the chromatographic column and the make-up liquid were mixed and split by a Micro-splitter valve (Upchurch Scientific, WA, USA) so that the liquid flow-rate into the ion source was about 0.2 ml min^{-1}.

2.3. MS detection conditions

The outlet of the Valco tee connection was attached to the inlet of a Quattro Micro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). Tandem mass spectrometry analysis was carried out in the positive ion mode.

The capillary voltage in the ESI probe was 3.5 kV. The source block and dessolvation temperatures were set at 100 °C and 150 °C, respectively. Nitrogen (N_2) was used as nebulizing gas at 365 l h^{-1} to minimize the risk of accidental ignition of solvent vapors. The argon was used as collision gas at a pressure of approximately 3.5×10^{-3} mbar. The cone voltage was set at 40 V and collision energy of 38.0 and 30.0 eV were used for LER and IS, respectively.

Optimization of MS conditions was obtained by direct infusion of standard solutions (10 μg ml^{-1}) prepared in the mobile phase–make-up liquid (4:1, v/v) and delivered by a syringe pump at a flow-rate of 10 μl min^{-1}. For MRM the ammonium adducts $[M + NH_4]^+$ and their respective product ions were monitored in two functions, 612.40 > 100.10 (0.0–5.0 min) for LER enantiomers and 646.30 > 100.30 (5.0–8.0 min) for the IS. The dwell time was 1.00 s for each mass transition.

A MassLynx (Micromass, Manchester, UK) data sampling system, version 3.5 was used for sample acquisition and quantitation.

2.4. Sample preparation procedure

Human plasma samples from healthy volunteers were supplied by The Blood Center of the University Hospital, Faculty of Medicine of Ribeirão Preto (University of São Paulo, São Paulo, Brazil).

Aliquots of 1.0 ml of human plasma were supplemented with 25 μl 0.66 μg ml^{-1} amiodarone solution and with 50 μl 0.1 mol l^{-1} aqueous sodium hydroxyde solution. LER enantiomers were extracted from plasma samples with a 4.5 ml of hexane–isopropanol (99:1, v/v) by shaking in a vortex for 2 min. After centrifugation at 2000 × *g* for 10 min, the organic phases were collected and evaporated to dryness in a centrifugal evaporator vacuum system (RCT90 and RC10.22 model) from Jouan AS (St. Herblain, France), set at 25 °C. The residues were dissolved in 50 μl hexane–ethanol (95:5, v/v) plus 0.1% (v/v) diethylamine and vortexed for 20 s, and 20 μl were injected into the analytical column. All analytical procedures were carried out under yellow light since several DHPs are photosensitive.

2.5. Method validation

Calibration curves were prepared by analyzing 1.0 ml drug-free plasma samples spiked with a standard *rac*-LER solution in duplicate, resulting in plasma concentrations of 0.025, 0.1, 0.2, 0.5, 1.0, 2.0 and 5 ng ml^{-1} of each enantiomer. Sample preparation and chromatographic conditions were as described before. Plots of plasma concentrations versus peak area ratios, (*S*)- and (*R*)-LER/IS, were constructed and the linear regression lines were used for the determination of enantiomer concentration in plasma samples. Linearity of the method was obtained by analyzing calibration samples in the concentration range of 0.025–50.0 ng ml^{-1} for each LER enantiomer.

The efficiency of the extraction procedure was assessed by analyzing drug-free plasma aliquots of 1.0 ml in triplicate, spiked with five different concentrations of *rac*-LER (0.1–5 ng ml^{-1} for each enantiomer). The samples were submitted to the extraction procedure and the internal standard was added to the extracts. Peak area ratios were compared with those obtained for the analysis of drug-free plasma extracts spiked with IS and *rac*-LER solutions, covering the same range concentration, in duplicate.

The precision and accuracy of the method were evaluated by analyzing the (*S*)- and (*R*)-LER in plasma samples spiked with three concentrations, 0.2, 2.6 and 6.0 ng ml^{-1}, of each enantiomer. Aliquots of spiked plasma samples were stored at −20 °C and analyzed in replicate experiments ($n = 10$) using a single calibration curve for intra-assay evaluation, and in duplicate on five consecutive days for inter-assay evaluation.

The quantitation limit (LOQ) was obtained by the analysis in quintuplicate, of plasma samples spiked with *rac*-LER at concentrations as low as 0.025 μg ml^{-1} of each enantiomer. The LOQ was defined as the lowest plasma concentration of each LER enantiomer analyzed with an error of 20% or lower.

2.6. Clinical study

The method developed was used for the analysis of plasma samples obtained from a healthy volunteer N.B. (a 45-year-old man, 75 kg, 1.67 cm tall) who was informed about the study and gave written consent to participate. After clinical examination and biochemical tests for the confirmation of normal hepatic, renal and cardiac functions the volunteer received two tablets of 10 mg *rac*-LER (Zanidip® 10 mg tablets, Asta Médica, São Paulo, Brazil) after a 12 h fast. Blood samples were collected through an intravenous catheter at times zero, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, and 24 h after *rac*-LER administration. The blood samples were transferred to heparinized tubes (Liquemine® 5000 IU, Roche, Rio de Janeiro, Brazil) and centrifuged at 1800 × *g* for 10 min. The plasma samples were separated and stored at −20 °C until chromatographic analysis.

The enantioselective kinetic disposition of LER was determined based on an open bicompartmental model. The distribution ($t_{1/2}\alpha$) half-life was determined after correction of the respective phase by the residue method. The elimination half-life ($t_{1/2}\beta$) was directly determined by the graphic method (log *c* versus *t*). The distribution (α) and elimination (β) rate constants were calculated using the $0.693/t_{1/2}$ equation. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly calculated from the plasma concentrations of the enantiomers obtained. The area under the plasma concentration time curve ($AUC^{0-\infty}$) was calculated by the trapezoidal method and extrapolated to infinity using the final plasma concentration divided by the slope of the elimination phase [8].

3. Results and discussion

3.1. Method optimization

Several chiral stationary phases were evaluated to discriminate the LER enantiomers. In the preliminary experiments for LER resolution were em-

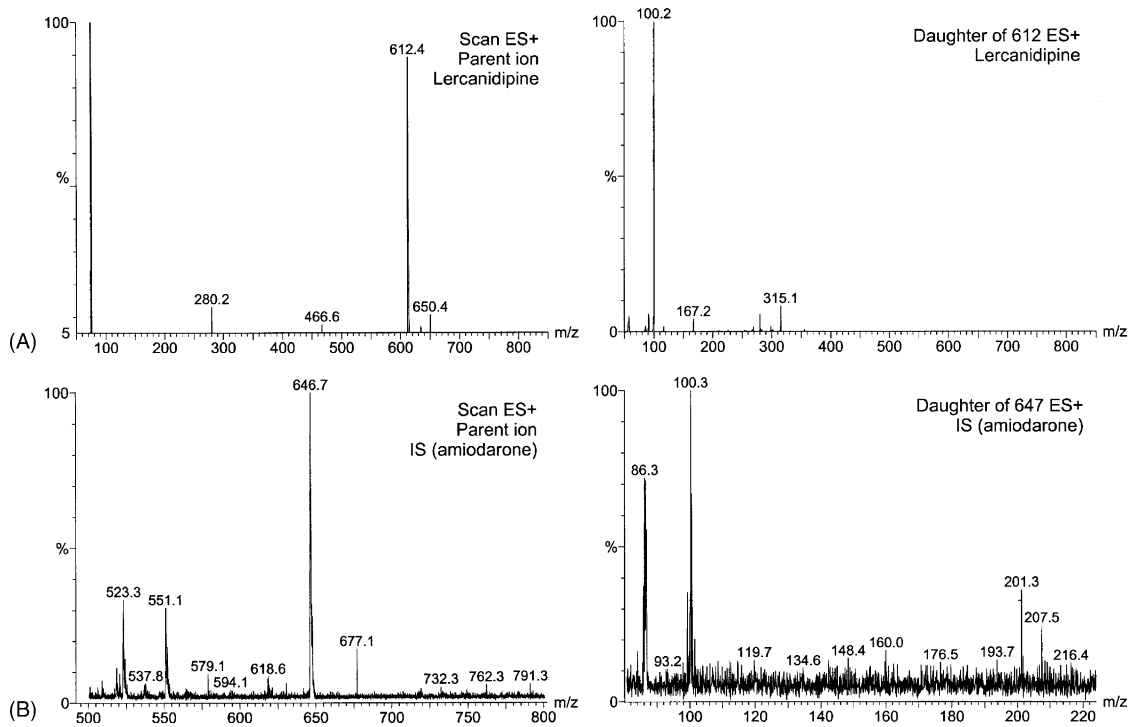

Fig. 2. Mass spectra from infusion experiments with lercanidipine (A) and IS (B) carried out on the mobile phase plus ammonium acetate as an additive.

ployed cellulose-based columns (Chiralcel® OD-H, 150 mm × 4.6 i.d., 5 μm particle size, Chiralcel® OD-R, 250 mm × 4.6 mm i.d., 10 μm particle size, and Chiralcel® OJ, 250 mm × 4.6 i.d., 10 μm particle size), and amylose derivatives (Chiralpak® AS, 250 mm × 4.6 mm i.d., 10 μm particle size, and Chiralpak® AD, 250 mm × 4.6 mm i.d., 10 μm particle size), but in the employed conditions was not obtained enough resolution. The best chromatographic separation for the LER enantiomers was achieved in the Chiralpak® AD, which was operated under normal-phase conditions, using a mixture of hexane–ethanol (95:5, v/v) plus 0.1% (v/v) diethylamine. However, this LC mobile phase is not compatible with electrospray ionization. Under these conditions, LER enantiomers could not be detected. To obtain high sensitivity of MS detection, post-column addition of a modifier was required to facilitate the ionization of the analytes. The addition of ammonium acetate (10 mmol l^{-1}) to the chromatographic column effluent before the introduction into the electrospray ion source resulted in higher ion intensities, as already observed by some authors [18–20]. Fig. 2 shows full scan and product ions scan spectra for LER and IS produced by ESI–MS–MS under the conditions described in Section 2.3. The mass spectra were the same for both LER enantiomers.

The LLE method was applied to isolate LER from the human plasma matrix. There are no reported procedures for the extraction of LER from plasma samples. Clinical pharmacokinetic studies have been described using [^{14}C]-radiolabed LER [7]. The optimization of the recovery of LER ennatiomers from human plasma was achieved by preliminary tests using toluene, diisopropyl ether, methyl t-butyl ether and a mixture of hexane–isopropanol (99:1, v/v) as extractor solvents. Absolute recoveries up to 70% were obtained for both LER enantiomers employing

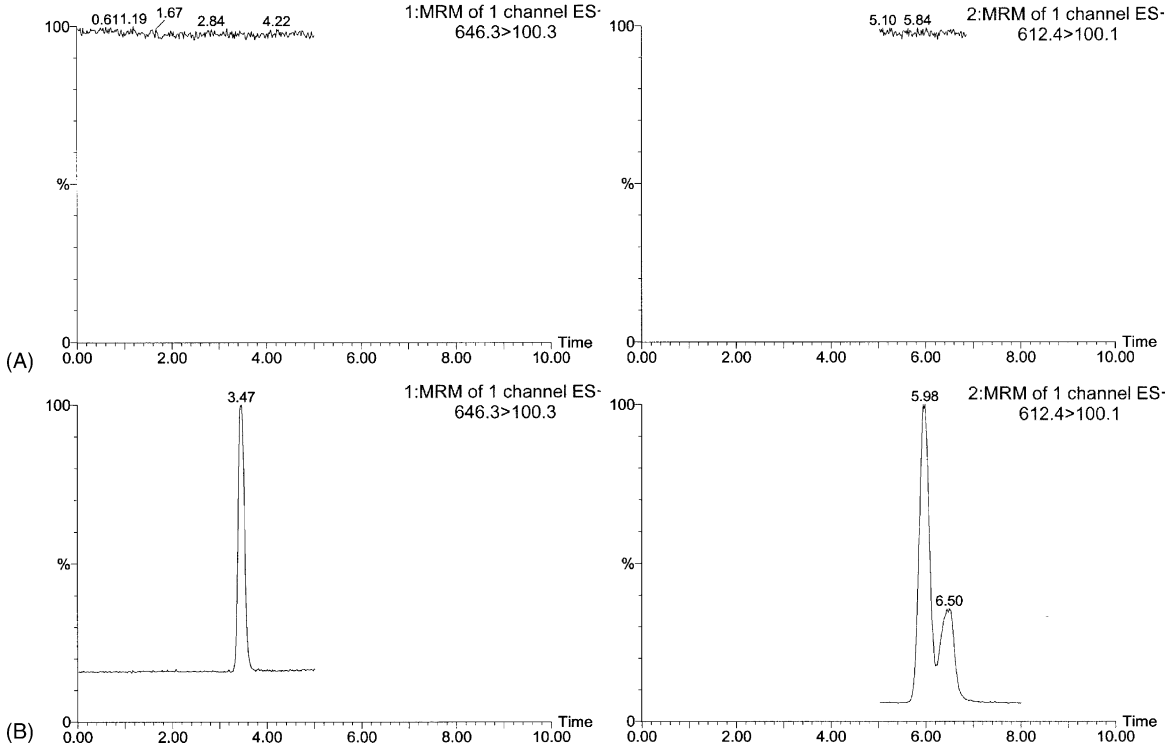

Fig. 3. Chromatograms for the enantioselective analysis of LER in human plasma samples. A drug-free human plasma sample (A), and a plasma sample from a healthy volunteer collected 2.5 h after administration of a 20 mg oral dose of *rac*-LER (B). Column: Chiralpak® AD, 250 mm × 4.6 mm i.d., 10 μm particle size, with hexane–ethanol–diethylamine (95:5:0.1, v/v/v) as mobile phase. The plasma concentrations of (*S*)- and (*R*)-LER were 1.08 and 0.36 ng ml^{-1}, respectively. Other conditions are as described in Sections 2.2, 2.3 and 2.4.

toluene or hexane–isopropanol (99:1, v/v) under alkalinized conditions (pH set at 9.3 ± 2). Although toluene is the solvent most frequently used for DHP extraction from plasma samples [8,18], a mixture of hexane–isopropanol (99:1, v/v) was selected due to shorter time sample handling. To exclude the possibility of racemization, plasma samples spiked with (*S*)- or (*R*)-LER were processed separately according to the assay procedure and analyzed. No formation of the other enantiomer was observed. Typical chromatograms of drug-free human plasma and a plasma sample from a healthy volunteer collected 2.5 h after the administration of a 20 mg oral dose of rac-LER in Fig. 3 show that LER enatiomers were clearly separated without interference from endogenous compounds from the matrix. Under these conditions, the elution order was IS followed by (*S*)- and (*R*)-LER. The elution order of LER enantiomers was established according to Testa et al. [7].

3.2. Method validation

The optimized method was validated by assessment of recovery, linearity, quantitation limit, precision, and accuracy. Coefficients of variation and relative errors of less than 15% were considered acceptable, except for the quantitation limit, whose values were extended to 20%, as recommended by Shah et al. [21] and Bressole et al. [22] for the analysis of biological samples for pharmacokinetic studies. The results of these series of experiments are shown in Table 1.

Table 1
Confidence limits of the analysis of lercanidipine enantiomers in human plasma

	(*S*)-Lercanidipine	(*R*)-Lercanidipine
Recovery, % ($n = 3$)		
0.2 ng ml^{-1}	65.4 (CV, 12.3%)	61.0 (CV, 11.5%)
0.5 ng ml^{-1}	71.7 (CV, 13.3%)	78.7 (CV, 9.6%)
5.0 ng ml^{-1}	70.3 (CV, 3.9%)	69.1 (CV, 4.4%)
Linearity		
Range (ng ml^{-1})	0.025–50.0	0.025–50.0
Equation	$y = 2.2603 + 5.6784x$	$y = 2.6651 + 5.3926x$
Determination coefficient (r)	0.998	0.997
Quantitation limit ($n = 5$)		
Concentration (ng ml^{-1})	0.025	0.025
Intra-assay precision (CV, %)	9.0	9.3
Intra-assay accuracy (%)	−8.1	−8.3
Analytical precision and accuracy		
Intra-assay precision: coefficient of variation (%), $n = 10$		
0.2 ng ml^{-1}	5.3	7.1
2.6 ng ml^{-1}	11.3	11.8
6.0 ng ml^{-1}	13.9	12.8
Inter-assay precision: coefficient of variation (%), $n = 5$		
0.2 ng ml^{-1}	12.9	10.9
2.6 ng ml^{-1}	14.0	14.9
6.0 ng ml^{-1}	11.2	12.6
Intra-assay accuracy: relative error (%), $n = 10$		
0.2 ng ml^{-1}	0.6	0.7
2.6 ng ml^{-1}	7.8	7.5
6.0 ng ml^{-1}	14.8	12.8
Inter-assay accuracy: relative error (%), $n = 5$		
0.2 ng ml^{-1}	−3.2	1.0
2.6 ng ml^{-1}	−8.6	−5.7
6.0 ng ml^{-1}	−14.8	−14.0

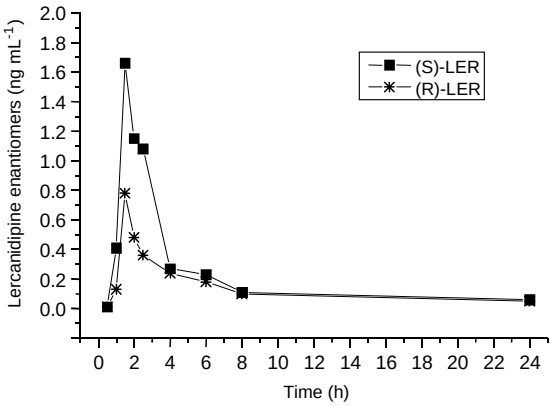

Fig. 4. Plasma concentration–time curves for (*S*)- and (*R*)-LER from a healthy volunteer who received a single dose of 20 mg of racemic LER (Zanidip® 10 mg tablets, Asta Médica, São Paulo, Brazil).

Under the described conditions, LOQ was 0.025 ng ml^{-1} for each LER enantiomer with acceptable precision and accuracy. MS–MS detection proved to be a system of high specificity, precision and accuracy for LER enantiomers analysis.

3.3. Clinical study

The validated method was applied to the stereoselective analysis of LER in plasma samples obtained from a healthy volunteer who received a single oral dose of 20 mg of *rac*-LER (Zanidip® 10 mg tablets, Asta Médica, São Paulo, Brazil). The concentrations of LER enantiomers in plasma samples were determined as described in Section 2.4. Fig. 4 shows the plasma concentration–time curves for (*R*)- and (*S*)-LER. The plasma concentration of both enantiomers could be quantified up to 24 h after the administration of a single dose of *rac*-LER (Fig. 4). It can be observed that the levels of (*S*)-LER were always higher than those of (*R*)-LER. AUC$^{0-\infty}$ for (*S*)-LER was 1.7-fold higher than that for (*R*)-LER (Table 2). These results agree with those obtained in previous studies using [^{14}C]-labeled LER and confirm the stereoselective disposition of LER [7].

Table 2
Enantioselective kinetic disposition of lercanidipine in a healthy volunteer

	(*S*)-Lercanidipine	(*R*)-Lercanidipine
C_{max} (ng ml^{-1})	1.66	0.78
t_{max} (h)	1.5	1.5
AUC$^{0-\infty}$ (ng h ml^{-1})	7.55	4.27
$t_{1/2}\alpha$ (h)	0.5	0.6
α (h^{-1})	1.39	1.16
$t_{1/2}\beta$ (h)	9.5	10.0
β (h^{-1})	0.07	0.07
AUC$^{0-\infty}$ (*S*)/(*R*)	1.7	

4. Conclusion

In the present study, LER enatiomers could be analyzed in human plasma samples within less than 8 min (Fig. 3) using LC–MS–MS. Normal-phase LC provides individual LER enantiomers separation and MS provides the high specificity and sensitivity required for the determination of these compounds in complex human plasma samples. The proposed method was successfully validated and LER enantiomers could be measured in plasma with acceptable accuracy and precision.

The method proved to be adequate for enantioselective pharmacokinetic studies after a single oral administration of *rac*-LER to a healthy volunteer.

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