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Simultaneous analysis of the enantiomers of verapamil and norverapamil in rat plasma by liquid chromatography-tandem mass spectrometry

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Abstract

An enantioselective micromethod for the simultaneous analysis of verapamil (VER) and norverapamil (NOR) in plasma was developed, validated and applied to the study of the kinetic disposition of VER and NOR after the administration of a single oral dose of racemic-VER to rats. VER, NOR and the internal standard (paroxetine) were extracted from only 100-μL plasma samples using *n*-hexane and the enantiomers were resolved on a Chiralpak AD column using *n*-hexane:isopropanol:ethanol:diethylamine (88:6:6:0.1) as the mobile phase. The analyses were performed in the selected reaction monitoring mode. Transitions 456 > 166 for VER enantiomers, 441 > 166 for NOR enantiomers and 330 > 193 for the internal standard were monitored and the method had a total chromatographic run time of 12 min. The method allows the determination of VER and NOR enantiomers at plasma levels as low as 1.0 ng/mL. Racemic VER hydrochloride (10 mg/kg) was given to male Wistar rats by gavage and blood samples were collected from 0 to 6.0 h ($n = 6$ at each time point). The concentration of (−)-(*S*)-VER was three folds higher than (+)-(*R*)-VER, with an AUC ratio (−)/(+) of 2.66. Oral clearance values were 12.17 and 28.77 L/h/kg for (−)-(*S*)-VER and (+)-(*R*)-VER, respectively. The pharmacokinetic parameters of NOR were not shown to be enantioselective.

Keywords: Verapamil; Enantiomers; Metabolism; Rats; LC–MS/MS

1. Introduction

Verapamil (VER), 2-(3,4-dimethoxyphenyl)-5-[2-(3,4 dimethoxyphenyl)ethyl-methyl-amino]-2-propan-2-yl-pentanenitrile, is a calcium channel-blocking drug and is an effective agent in the treatment of hypertension, supraventricular arrhythmias and angina pectoris. VER is a chiral drug which is administered as a racemic mixture of (+)-(*R*)-VER and (−)-(*S*)-VER. These enantiomers have different pharmacodynamic and pharmacokinetic properties [1–4].

(−)-(*S*)-VER produces negative chronotropic and inotropic effects, delays atrioventricular node conduction and causes systemic and coronary artery vasodilation. In contrast, similar concentrations of (+)-(*R*)-VER produce vasodilation of the arterial and coronary vasculature but are relatively devoid of direct cardiac effects [1]. (−)-(*S*)-VER is 10–20 times more potent than (+)-(*R*)-VER in slowing cardiac A–V conduction velocity in man, dogs and rabbits [2].

The kinetic disposition of VER in rats is enantioselective. VER undergoes an extensive enantioselective first-pass effect after oral administration. The bioavailability of (−)-(*S*)-VER is greater than that of (+)-(*R*)-VER and the clearance of (+)-(*R*)-VER is greater than that of (−)-(*S*)-VER after oral administration. Enantioselectivity in rat plasma is opposite to that observed in human plasma [3]. Plasma protein binding studies have also revealed opposite enantioselectivity in the free fraction in rat ($R > S$) and human ($S > R$) plasma for VER [4]. VER undergoes extensive oxidative metabolism with formation of norverapamil (*N*-demethylation) and D-617 (*N*-dealkylation). These metabolites are subject to further metabolism to form additional secondary metabolites (Fig. 1) [5,6]. CYP3A4,

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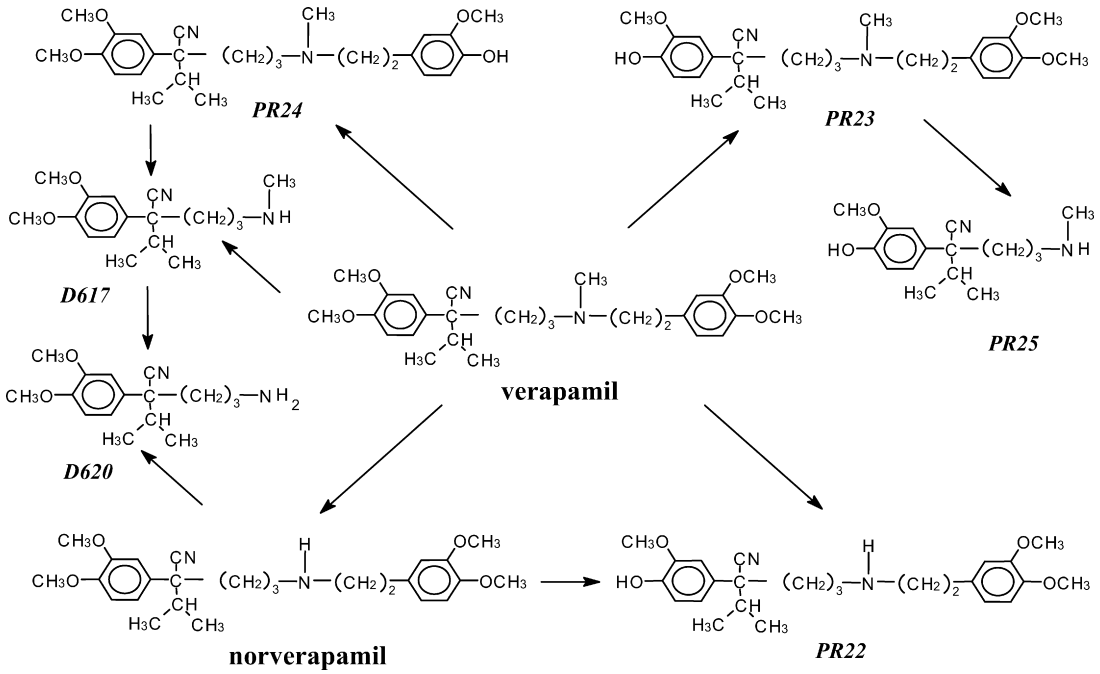

Fig. 1. Metabolism of verapamil [5].

CYP3A5 and CYP2C8 play a major role in VER metabolism [5]. Among these metabolites, norverapamil (NOR) is the most important because it is reported to possess 20% of the coronary vasodilator potency of VER [7]. NOR can be further metabolized by CYPs [5] and is also a chiral compound whose kinetic disposition is enantioselective in humans [7]. VER and NOR are potent inhibitors of P-glycoprotein function [8].

Simultaneous analysis of VER and NOR enantiomers in human plasma or in plasma of experimental animals has been described by few authors using HPLC. The methods initially reported employed achiral separation followed by chiral analysis and fluorescence detection. Chu and Wainer [9] reported the determination of VER and NOR enantiomers in plasma samples using a chiral AGP (α_1-acid glycoprotein) column coupled to a shielded hydrophobic phase (Hisep) column. The Hisep column was used to separate VER and NOR from the plasma components and from each other. The eluents were then selectively transferred to the chiral AGP column where the enantiomers were resolved. Oda et al. [10] developed another achiral–chiral HPLC system coupling an ODS column to a chiral ovomucoid column (Ultron ES-OVM column) via a dilution tube and a trapping column for on-line determination of VER enantiomers. Shibukawa and Wainer [7] described a method for the enantioselective analysis of VER and NOR using a diol silica column (LiChrocart DIOL) and a Chiralpak AD column which were connected in series in this order. Bransteterová and Wainer [11] reported an achiral and chiral HPLC assay of VER and its seven metabolites in serum samples using an achiral reverse-phase NovaPak C18 column coupled to a chiral AGP column. A semipermeable surface C8 pre-column was used for on-line direct analysis of the samples, and SepPak C18 SPE cartridges for off-line SPE preparations.

Stagni and Gillespie [12] reported an improved HPLC method using fluorescence detection for the direct determination of VER and NOR enantiomers in human plasma using a single AGP chiral column. NOR is immediately acetylated to *N*-acetylnorverapamil in the extraction solvent (2% butanol in hexane). *N*-acetylnorverapamil and VER are then separated on a chiral AGP column. Asafu-Adjaye and Shiu [13] reported the determination of VER and NOR enantiomers in urine using a single chiral cellulose-based reverse-phase column (Chiralcel ODR). For sample preparation the authors used a membrane-based solid-phase extraction disk consisting of a thin, particle-loaded membrane inserted into a plastic syringe-like barrel. The particles, which may be C8 or C18 bonded phase, are embedded within a matrix of PTFE fibrils.

Hedeland et al. [14] developed an enantioselective LC–MS-MS method for the simultaneous determination of VER and NOR in human plasma without the need for achiral pre-separation or chemical derivatization. The enantiomers were not well resolved on a chiral AGP column, with the second VER enantiomer eluted interfering with the first NOR enantiomer. However, VER could be separately detected from NOR due to their different molecular masses. The use of LC–MS or LC–MS-MS for the achiral analysis of VER and its metabolites [15] or of VER alone in human plasma [16] has also been reported.

In view of the clinical relevance of including NOR in studies of enantioselective kinetic disposition and metabolism of VER, in the present study we described the development and validation of a micromethod using plasma volumes of only 100 μL, liquid–liquid extraction, a chiral Chiralpak AD column with resolution for the separation of VER and NOR enantiomers, and the use of LC–MS/MS for the simultaneous enantioselective analysis of VER and NOR in rat

plasma, for application in studies of kinetic disposition and metabolism.

2. Methods

2.1. Chemicals and reagents

(±)-Verapamil hydrochloride (99.4%), (+)-(*R*)-verapamil hydrochloride (99.0%) and normethyl-(±)-verapamil hydrochloride (99.0%) (norverapamil) were from Sigma (St. Louis, MO, USA). Paroxetine, used as the internal standard (IS), was kindly provided by Eurofarma Laboratórios Ltda (São Paulo, SP, Brazil). Diethylamine and sodium hydroxide, both analytical grade, were from Merck (Darmstadt, Germany), and *n*-hexane, isopropanol, methanol and isopropanol, all HPLC grade, were from EM Science, Merck (Darmstadt, Germany). The water used during the experiment was obtained with a Milli-Q purification system. The blood samples used for the development and validation of the analytical method were obtained from male Wistar rats weighing 250 ± 10 g. Plasma was obtained by the centrifugation of blood containing sodium heparin as an anticoagulant. The plasma samples were stored at −70 °C until the time for use.

2.2. Standard working solutions

The stock solutions of racemic VER hydrochloride, racemic NOR and the IS, paroxetine, were prepared in methanol at the concentration of 0.2 mg/mL for VER and NOR and 1.0 mg/mL for paroxetine. These solutions were then used to prepare standard solutions at concentrations of 2000, 800, 400, 200, 100, 40, 20 and 8 ng of racemic VER and NOR per mL methanol and 5 ng paroxetine per mL methanol.

2.3. Apparatus

The HPLC apparatus used for the analysis of VER and NOR was a Shimadzu chromatograph (Kyoto, Japan) consisting of a model LC-10 ADVP® pump and a Quattro Micro® mass spectrometry detector (Micromass, Manchester, UK) equipped with an electrospray interface. The analyses were performed in the positive ion mode. The capillary voltage in the ESI probe was 3.0 kV. The source block and desolvation temperatures were set at 100 and 200 °C, respectively. Nitrogen was used as nebulizing gas at 365 L/h and argon was used as collision gas at a pressure of approximately 0.25 Pa. The cone voltage was set at 45 V, and a collision energy of 25 eV was used. Data acquisition and quantitation were performed using the MassLynx software, version 3.5.

MS conditions were optimized by direct infusion of standard solutions (10 μg/mL) prepared in the mobile phase and introduced with an infusion pump at a flow rate of 20 μL/min. The analyses were performed in the selected reaction monitoring mode. Transitions 456 > 166 for VER enantiomers, 441 > 166 for NOR enantiomers and 330 > 193 for the IS were monitored (Fig. 2).

Chromatographic separation of VER and NOR was obtained with a chiral phase Chiralpak AD column (250 mm × 4.6 mm) (Daicel Chemical Industries Ltd., Exton, USA) with 10-μm particles protected by a 4 mm × 4 mm CN Lichrospher 100 guard column with 5-m particles. The mobile phase eluted from the system consisted of *n*-hexane:isopropanol:ethanol:diethylamine (88:6:6:0.1) (v/v/v/v), at a flow rate of 1.35 mL/min and a post-column infusion of a solution of ethanol: 20 mM aqueous ammonium acetate (95:5) and at a flow rate of 0.35 mL/min. To obtain high sensitivity of MS-MS detection, post-column addition of a modifier was required to facilitate the ionization of the analytes. The addition of ammonium acetate solution to the column effluent before the introduction into the electrospray ion source resulted in higher ion intensities.

2.4. Extraction procedure

Plasma aliquots of 100 μL were spiked with 25 μL IS solution, 25 μL 2 mol/L NaOH, and 2 mL hexane. After shaking for 2 min in a mixer-type shaker and centrifugation at 1800 × *g* for 10 min, the organic phases were transferred to conical tubes and evaporated dry with a vacuum evaporation system (RCT90 and RC10.22 model) from Jouan AS (St. Herblain, France) at 30 °C. The residues obtained were reconstituted in 200 μL of the mobile phase and 130 μL aliquots were submitted to chromatographic analysis.

2.5. Validation

The calibration curves were constructed by spiking 0.1-mL aliquots of blank plasma with 25 μL of each standard solution of rac-VER and rac-NOR, in duplicate, with resulting plasma concentrations of 1.0, 2.5, 5.0, 12.5, 25.0, 50.0 and 100.0 ng of each VER and NOR enantiomer/mL. Plots of plasma concentrations versus peak area ratios (VER/IS and NOR/IS) were constructed and the linear regression lines were used for the determination of each enantiomer concentration in plasma samples. The linearity of the method was determined by the analysis of plasma samples spiked with VER and NOR at concentrations of 1.0–250.0 ng of each enantiomer/mL.

The recovery of the extraction procedure was determined by the analysis in triplicate of 0.1-mL plasma aliquots spiked with three rac-VER and rac-NOR concentrations (2.5, 10 and 100 ng of each enantiomer/mL). The samples were submitted to the extraction procedure and the area ratios were compared to those obtained by direct injection of VER, NOR and IS into the mobile phase.

The quantitation limit was obtained by the analysis in quintuplicate of plasma samples spiked with VER and NOR at concentrations as low as 0.5 ng of each enantiomer/mL. The quantitation limit was defined as the lowest plasma concentration of each analyte analyzed with an error of 20% or less.

The precision and accuracy of the method were evaluated by analyzing VER and NOR in plasma samples spiked with three concentrations (1, 10 and 100 ng) of each enantiomer/mL. Aliquots of spiked plasma samples were stored at −70 °C and analyzed in replicate experiments ($n = 10$) using a single cali-

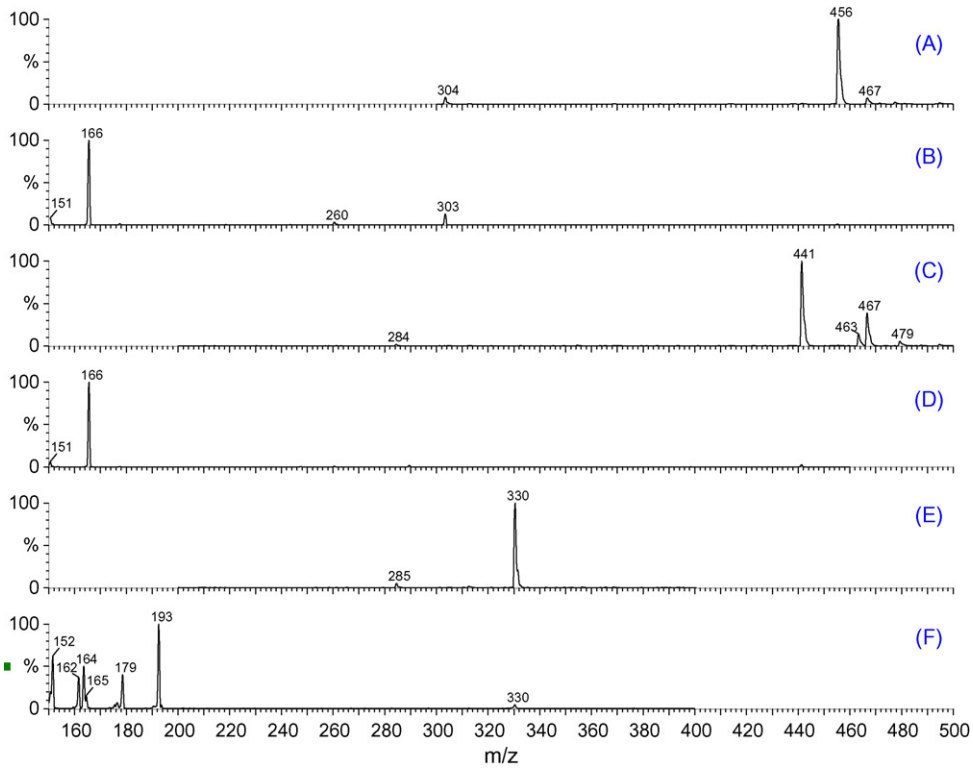

Fig. 2. Full scan mass spectra of the protonated molecular ion and ion product of VER (A and B), NOR (C and D) and paroxetine (E and F).

bration curve for intra-assay evaluation, and in duplicate on five consecutive days for interassay evaluation.

Stability was assured by a long-term study and by three freeze (−20 °C) and thaw (25 °C) cycles at three concentrations (1, 10 and 100 ng of each enantiomer/mL). For long-term study, spiked plasma samples were prepared and an aliquot was assayed on the same day. Another aliquot was kept at −70 °C for 90 days and then assayed. This period corresponds to the maximum time the samples were kept frozen. For freeze–thaw stability, spiked plasma samples were evaluated after three freeze and thaw cycles. The results were compared with those obtained for freshly prepared samples.

2.6. Animal maintenance

The study was conducted on male Wistar rats (250 ± 10 g) provided by the University of São Paulo, Brazil. Three days before the experiments the animals were brought to the local animal room, where temperature and humidity were controlled and the dark–light cycle was set at 12 h. Water and food were offered *ad libitum* until 12 h before the animals received the drug.

The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of Araraquara (São Paulo State University, Brazil) in accordance with international recommendations.

2.7. Dosing and sampling

The method was applied to the investigation of enantioselectivity in the kinetic disposition and metabolism of VER administered in the racemic form in a single dose to rats. After a 12-h fast, the animals received an aqueous solution of rac-VER hydrochloride (10 mg/kg) by gavage. Heparinized blood samples were collected from a caudal vein at times 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0 and 6.0 h after drug administration. Six blood samples were collected for each time point. Three samples were collected from each animal. Plasma samples were stored at −70 °C until analysis.

2.8. Pharmacokinetics

The enantioselective kinetic disposition of VER and NOR was determined using the WinNonlin software, version 4.0 (Pharsight Corp, Mountain View, CA, USA). The pharmacokinetic parameters were obtained by fitting the data to a monocompartmental open model for VER and NOR.

2.9. Statistical analysis

The statistical tests for the calculation of the median and 95% confidence interval (95%CI) were performed using the GraphPad Instat software. The Wilcoxon test was used for the determination of enantiomer ratios different from one for VER and NOR. Statistical significance was set at $p < 0.05$.

3. Results and discussion

The present study describes the simultaneous analysis of VER and NOR enantiomers in 100-μL rat plasma samples employing LC–MS-MS with application to kinetic disposition

Table 1
Matrix effect of VER, NOR and IS in six different lots of rat plasma (mean data)

Nominal concentration (ng/mL)	Matrix effect (%)				
	(−)-(*S*)-VER	(+)-(*R*)-VER	(*S*)-NOR	(*R*)-NOR	IS
					102.5
1.0	107.0	105.9	102.5	100.9	
10.0	95.8	95.7	101.3	99.5	
100.0	102.2	97.4	95.0	95.0	

and metabolism. The study reports for the first time the complete resolution of VER and NOR enantiomers using the chiral Chiralpak AD column, a relevant aspect considering the possibility of adapting the method to studies using HPLC with fluorescence detection. The enantiomers of VER and NOR are eluted from the column within only 12 min, corresponding to almost half the time reported by Hedeland et al. [14].

The elution order of VER enantiomers was obtained by the analysis of pure (+)-(*R*)-VER according to the method developed in the present study. The elution order of NOR enantiomers was obtained by the analysis of individual enantiomers, previously separated and collected from the Chiralpak AD column according to the method described by Shibukawa and Wainer [7].

The matrix effect was evaluated based on direct comparison of the peak areas of VER, NOR and internal standard (IS) injected directly in mobile phase, and spiked post extraction into extracts originating from six different sources of rat plasma. Data showed in Table 1 indicate that the matrix effect for VER, NOR and IS was practically absent.

The analysis of blank plasma samples collected from six different rats did not show interference of endogenous components with VER and NOR eluted from the chiral stationary phase column. Typical chromatograms of standard calibration plasma spiked with VER and NOR and plasma obtained from rats treated with rac-VER are shown in Fig. 3.

The analysis of blank plasma samples collected from six different rats did not show interference of endogenous components with VER and NOR eluted from the chiral stationary phase column. Typical chromatograms of standard calibration plasma spiked with VER and NOR and plasma obtained from rats treated with rac-VER are shown in Fig. 3.

Table 2 summarizes the data obtained in the validation of the method.

The VER and NOR enantiomers were extracted from plasma at pH 10 using hexane as the extracting solvent. The recoveries obtained for both enantiomers of VER and NOR were more than 70% and were independent of the concentration in the 2.5–100.0 ng/mL range. Recoveries close to 80% or 100% for both VER and NOR enantiomers have been reported when 1-mL plasma aliquots are, respectively, extracted with heptane [7] or hexane containing 2% sec-butanol [12]. Borges et al. [16] reported a 95% recovery in the achiral analysis of VER in plasma using diethyl ether as the extracting solvent. VER and NOR enantiomers have also been extracted from plasma samples using C18 [11], end-capped CN- or C2 solid-phase extraction cartridges [15].

The method showed linearity up to 250.0 ng of each enantiomer/mL for both VER and NOR and the correlation coefficients observed were 0.995 or better. The quantitation limit determined by the analysis of only 100 μL plasma was 1.0 ng of each enantiomer/mL for VER and NOR. Hedeland et al. [14] reported quantitation limits of 100 and 120 pg/mL plasma, respectively, for VER and NOR enantiomers using 500-μL plasma volumes. von Richter et al. [15] reported quantitation limits of about 0.5 ng/mL in the achiral analysis of VER and NOR in 1-mL plasma aliquots using LC–MS. Borges et al. [16] reported the achiral analysis of VER, with a quantitation limit of 1.0 ng/mL in the analysis of 200 μL plasma using LC–MS-MS. The methods using fluorescence detection are comparatively less sensitive than those employing LC–MS or LC–MS-MS. The quantitation limits reported in studies using fluorescence detection range from 2.5 to 3.0 ng of each VER enantiomer/mL and from 2.0 to 5.0 ng of each NOR enantiomer/mL [7,11,12].

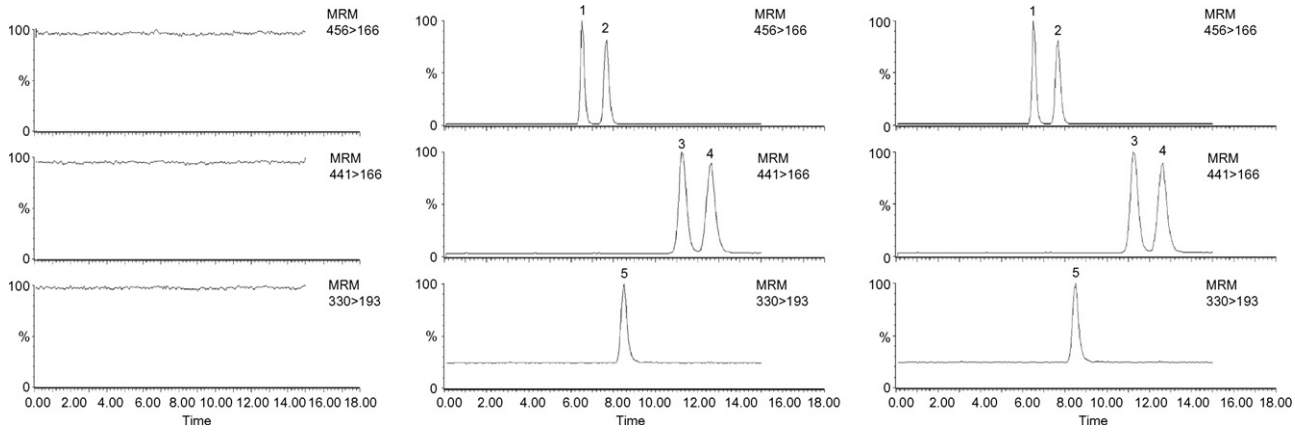

Fig. 3. Chromatograms referring to the analysis of VER and NOR enantiomers in plasma. (Left) Blank plasma, (center) plasma spiked with 100 ng/mL of VER and NOR enantiomers, (right) rat plasma sample collected 2.5 h after the administration of 10 mg/kg of racemic VER hydrochloride. (1) (−)-(*S*)-VER; (2) (+)-(*R*)-VER; (3) (*S*)-NOR; (4) (*R*)-NOR; (5) paroxetine (IS).

Table 2
Validation data

	(−)-(*S*)-VER	(+)-(*R*)-VER	(*S*)-NOR	(*R*)-NOR
Recovery, % ($n = 3$)				
2.5 ng/mL	87.2	86.4	71.3	71.0
10.0 ng/mL	83.8	87.5	74.6	70.5
100.0 ng/mL	90.2	91.3	76.1	73.0
Linearity				
Range (ng/mL)	1.0–250.0	1.0–250.0	1.0–250.0	1.0–250.0
Equation	$y = -0.2005 + 0.0275x$	$y = -0.2221 + 0.0274x$	$y = -0.0545 + 0.0202x$	$y = -0.0691 + 0.0200x$
Coefficient of determination (r)	0.9923	0.9922	0.9995	0.9986
Quantitation limit ($n = 10$)				
Concentration (ng/mL)	1.0	1.0	1.0	1.0
Intra-assay precision (CV %)	6.8	7.5	8.6	8.9
Intra-assay accuracy (%)	13.9	14.1	14.7	14.9
Precision and accuracy				
Intra-assay precision; coefficient of variation (%); $n = 10$				
1.0 ng/mL	6.0	6.4	6.1	10.0
10.0 ng/mL	6.4	5.9	6.3	6.8
100.0 ng/mL	7.0	7.1	5.9	8.0
Interassay precision; coefficient of variation (%); $n = 5$				
1.0 ng/mL	7.6	9.4	8.2	9.4
10.0 ng/mL	8.1	9.4	6.9	8.0
100.0 ng/mL	7.4	7.7	7.2	7.8
Intra-assay accuracy; relative error (%); $n = 10$				
1.0 ng/mL	−10.0	2.0	−6.0	−10.0
10.0 ng/mL	−7.2	−3.6	−2.4	−5.6
100.0 ng/mL	6.7	6.2	8.7	5.4
Interassay accuracy; relative error (%); $n = 5$				
1.0 ng/mL	0.0	6.0	−8.0	−10.0
10.0 ng/mL	0.4	−1.6	1.2	1.6
100.0 ng/mL	13.0	14.2	9.4	4.2

The coefficients of variation obtained in the study of intra- and interassay precision and accuracy were less than 10%, assuring the reproducibility and the repeatability of the results.

Evaluation of stability by long-term test and after three freeze–thaw cycles assured the good condition of the samples, with no variation of more than 10% at any of the concentrations tested (1–100 ng of each enantiomer/mL).

The developed and validated method was applied to the study of enantioselectivity in the kinetic disposition and metabolism of VER administered by gavage in the racemic form in a single 10 mg/kg dose of the hydrochloride salt to rats.

The pharmacokinetics of VER is enantioselective ($p < 0.05$) for the parameters $AUC^{0-\infty}$, Cl/F and C_{max} (Table 3). The data obtained for the Wistar rats investigated here showed higher plasma concentrations of (−)-(*S*)-VER (Fig. 4). The higher $AUC^{0-\infty}$ values obtained for (−)-(*S*)-VER compared to (+)-(*R*)-VER (411.18 vs. 173.81 ng h/mL) may be explained by a lower apparent total clearance (12.17 vs. 28.77 L/h/kg) (Table 3). The (−)/(+) plasma concentration ratio ($AUC^{0-\infty}$) was 2.65. C_{max} was also higher for (−)-(*S*)-VER (111.04 ng/mL) than (+)-(*R*)-VER (33.78 ng/mL). These results agree with Bhatti and Foster [3] who demonstrated that the kinetic disposition of VER is enantioselective in male Sprague-Dawley rats with a (−)/(+) plasma concentration ratio (AUC) close to 2.5. The cited authors also demonstrated that the bioavailability of (−)-(*S*)-VER is greater than that of (+)-(*R*)-VER (7.4 vs. 4.1%, respectively), suggesting that VER undergoes an extensive enantioselective first-pass effect after oral administration to rats. Plasma protein binding studies have revealed enantioselectivity in the free fractions in rat plasma for VER ((−)-(*S*)-VER < (+)-(*R*)-VER; $S/R = 0.61$) [4]. Mehvar and Reynolds [17] demonstrated that enantioselective protein binding is the main determinant of enantioselectivity in the hepatic elimination of VER. Hanada et al. [18] reported that rat plasma concentrations of (−)-(*S*)-VER were about twice those of (+)-(*R*)-VER. However, the cited authors found that the concentration of (+)-(*R*)-VER in lung tissue was higher than that of (−)-(*S*)-VER, the opposite of the plasma result.

The kinetic disposition of NOR was not enantioselective ($p < 0.05$) for the parameters $AUC^{0-\infty}$, C_{max}, t_{max}, $t_{1/2f}$ and $t_{1/2}$ (Table 3). Plasma concentrations versus time curves for the NOR enantiomers showed high variability, whereas pharmacokinetic parameters such as $AUC^{0-\infty}$ and C_{max} only presented a tendency to higher values for the (*S*)-NOR enantiomer. Robinson and Mehvar [4] reported that the free fractions of (*S*)-NOR were lower than (*R*)-NOR in rat plasma and that enantioselectivity was lower for NOR ($S/R = 0.80$) compared with VER ($S/R = 0.61$).

In conclusion, we developed and validated an LC–MS-MS micromethod suitable to study the enantioselective kinetic disposition of VER and NOR. The method is able to quantify

Table 3
Enantioselective kinetic disposition of VER and NOR in plasma of rats treated with a single dose of 10 mg/kg of racemic verapamil hydrochloride (gavage); $n = 6$ for each time

	(−)-(*S*)-VER	(+)-(*R*)-VER	(*S*)-NOR	(*R*)-NOR
C_{max} (ng/mL)	111.04 (53.55–184.78)	33.78* (25.53–47.54)	52.17 (24.36–82.85)	29.19 (16.65–44.47)
t_{max} (h)	1.16 (0.92–1.37)	1.45 (0.96–2.35)	2.05 (1.73–2.63)	2.09 (1.71–2.59)
$t_{1/2a}$ (h)	0.55 (0.26–0.81)	0.70 (0.32–1.07)	0.58 (0.13–1.86)	0.65 (0.40–1.45)
K_a or K_f (h^{-1})	1.45 (0.61–2.80)	1.04 (0.38–2.33)	1.20 (0.89–5.20)	1.07 (0.44–1.30)
$t_{1/2}$ (h)	1.20 (0.36–2.94)	1.57 (0.84–2.72)	1.27 (0.70–1.92)	1.14 (0.57–1.68)
K_{el} (h^{-1})	0.65 (0.26–0.95)	0.44 (0.24–0.72)	0.55 (0.33–0.85)	0.61 (0.34–1.07)
$AUC^{0-\infty}$ (ng h/mL)	411.18 (250.90–601.34)	173.81* (122.40–196.98)	180.04 (84.81–286.60)	71.58 (26.35–110.96)
Cl/F (L/h/kg)	12.17 (7.47–19.48)	28.77* (24.26–41.36)	nd	nd
$AUC^{0-\infty}$ (−)/(+)	2.66 (1.85–3.45)		nd	

nd = not determined.
*$p \leq 0.05$, Wilcoxon test (−)-(*S*)-VER vs. (+)-(*R*)-VER; **$p \leq 0.05$, Wilcoxon test (*S*)-NOR versus (*R*)-NOR.
Data are reported as median (95% CI).

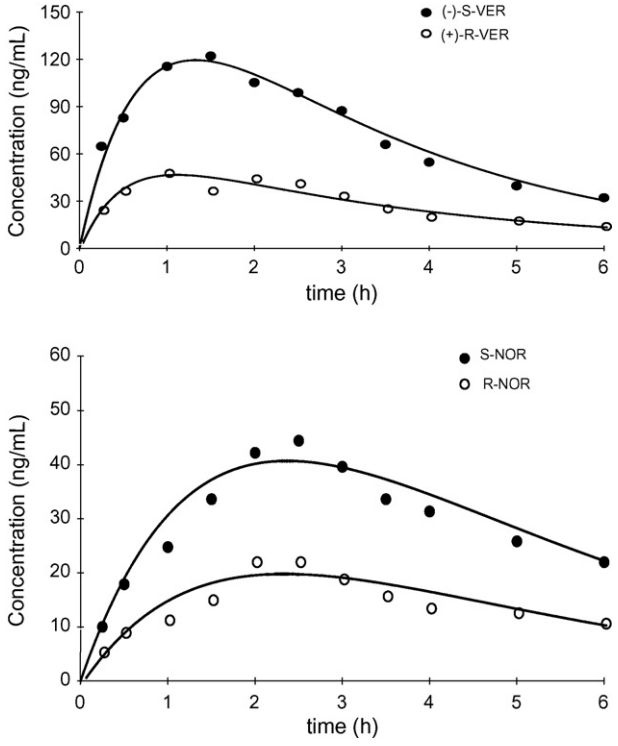

Fig. 4. Plasma concentration versus time curves for (−)-(*S*)-VER and (+)-(*R*)-VER, (*S*)-NOR, and (*R*)-NOR after oral (gavage) administration of a single dose of 10 mg/kg of racemic VER hydrochloride to Wistar rats.

simultaneously plasma concentrations of VER and NOR as low as 1.0 ng/mL employing plasma samples of only 100 μL and resolving all enantiomers on only one chiral column, with a total run time of 12 min. We also reported the enantioselective study of kinetic disposition of VER and NOR in rats.

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