

## ANALYSIS

STEREOSELECTIVE CZE METHOD FOR ANALYSIS OF VERAPAMIL  
AND NORVERAPAMIL IN HUMAN PLASMA

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**Abstract:** A stereospecific capillary zone electrophoresis (CZE) method was developed for the determination of verapamil (VER) and its main metabolite norverapamil (NOR) in human plasma. Optimal temperature, cyclodextrin selectors (CDs), pH of background electrolyte (BGE) and voltage were established to obtain complete separation of the chiral analytes and clopidogrel internal standard (I.S.) during one analytical run. Successful resolution of analytes was obtained in silica capillary filled with BGE consisting of heptakis 2,3,6-tri-O-methyl- $\beta$ -CD in phosphate buffer, pH 2.5 at 15°C of capillary temperature. The calculated electrophoretic parameters of the analytes were as follows: apparent electrophoretic mobility, for VER enantiomers:  $\mu_{\text{ap}(+)-\text{R}} = 1.11 \times 10^{-4}$ ,  $\mu_{\text{ap}(-)-\text{S}} = 1.06 \times 10^{-4}$  cm<sup>2</sup>/Vs and for NOR enantiomers:  $\mu_{\text{ap}(+)-\text{R}} = 1.09 \times 10^{-4}$ ,  $\mu_{\text{ap}(-)-\text{S}} = 1.04 \times 10^{-4}$  cm<sup>2</sup>/Vs, resolution factors,  $R_s = 5.4$ -6.6. Liquid extraction was applied for isolation of the analytes. The calibration curves were linear in the range 0.25-10  $\mu\text{g/mL}$  for VER and NOR enantiomer concentrations. The validation parameters were also established. The precision and accuracy of intra- and inter-day analysis were less than 15%. The lower limit of detection and limit of quantification for single enantiomers were 0.1 and 0.2  $\mu\text{g/mL}$ , respectively. Recovery of the enantiomers from plasma was in the 91-103% range. To evaluate analytical applicability of the proposed method, plasma sample from patient suffering from arterial hypertension treated with 80 mg of commercial tablets was analyzed.

**Key words:** stereoisomers, chiral selector, metabolite, liquid extraction, validation

Verapamil, 2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl]-(methylamino)-2-prop-2-ylpentanenitrile (VER), is a calcium blocker widely applied in a variety of cardiovascular disorders. Although this compound is well absorbed orally, bioavailability is only 10-20%, due to an extensive hepatic first-pass effect. In humans, verapamil is metabolized to more than six metabolites, which are excreted in the urine. The major metabolite – norverapamil (NOR) has 20% of the coronary vasodilator potency of verapamil (1, 2). It is a chiral drug; however, it is used as a racemic mixture. The stereoselective properties of verapamil caused by a stereogenic center within its molecular structure indicate the existence of two enantiomers of verapamil, which differ in their pharmacology and pharmacokinetics. The pharmacological properties are up to 20 times more potent for (-)-S-verapamil than (+)-R-verapamil. After intravenous administration, plasma clearance and apparent volume of distribu-

tion of (-)-S-verapamil are almost twice as high as those of (+)-R-verapamil. After oral administration (-)-S-verapamil undergoes extensive first-pass metabolism, resulting in predominance of the (+)-R-verapamil in plasma, and the protein binding is enantioselective with the free fraction of (-)-S-verapamil greater than that of (+)-R-verapamil (3, 4).

Several analytical HPLC methods with fluorescence detection (1, 2, 4-7) have been developed for quantitative determination of racemic verapamil in plasma or serum samples. Experimental studies have shown that various ranges of calibration curves for VER and its metabolite (NOR) were obtained. Rambla-Alegre et al. (1) reported the HPLC-FLD method with linearity of 100-2000 ng/mL of VER. The procedure developed by Jhee et al. (5) reported 10-250 ng/mL range of VER concentration. Furthermore, HPLC assay with spectrophotometric detection linear in the range of 0.5-10  $\mu\text{g/mL}$  verapamil concentration have been also described (8).

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Resolution of the analytes using HPLC methods were achieved even after 37 min (4), it means they are time-consuming. GC method with nitrogen-phosphorus detection was also applied for chiral analysis of VER (9). Capillary electrophoresis (CE) is a promising alternative. To the best of our knowledge, in the literature only a one validated CE methods for the determination of VER enantiomers and its active metabolite in plasma have been reported so far (10). The advantages of HPCE method compared with HPLC are: reduction of amount of reagents, high efficiency, small volume of injected samples and short analysis time.

Taking into consideration above advantages, the aim of this work was to develop and to validate a rapid CE method for the determination of VER and NOR enantiomers in human plasma with possibility to apply it for pharmacokinetic studies.

## MATERIALS AND METHODS

### Chemicals and reagents

Verapamil hydrochloride, norverapamil hydrochloride, heptakis 2,3,6-tri-O-methyl- $\beta$ -CD (TM $\beta$ CD), potassium dihydrogen phosphate and sodium dihydrogen phosphate dihydrate were from Sigma (St. Louis, MO, USA). Clopidogrel bisulfate (internal standard I.S.) was a generous gift of Pharmaceutical Research Institute (Warszawa, Poland). Chiral selectors  $\beta$ -CD and  $\gamma$ -CD were kindly supplied by Hewlett Packard, Hungary. Solutions: 1.0 M and 0.1 M NaOH, 50 mM phosphate buffer of pH 2.5 for HPCE and water for HPCE were from Agilent Technologies, Waldbronn, Germany, whereas 85% orthophosphoric acid was from POCh Gliwice, Poland. Methanol, propanol, hexane (gradient grade) were from Merck Darmstadt, Germany. Deionized water was always used to prepare solutions for rinsing of capillaries, Simplicity UV (Millipore, Billerica, MA, USA).

### Electrophoretic conditions

The automated analyses were conducted using an Agilent model <sup>3</sup>BCE apparatus with an UV detector (Agilent Technologies, Waldbronn, Germany). The separation was performed in a fused silica capillary, 33 cm  $\times$  50  $\mu$ m i.d., 24.5 cm to the detector. Analytes were detected at a wavelength of  $\lambda = 200$  nm. The automated CE instrument was controlled by ChemStation software. The capillary temperature was maintained at 15-30°C by a thermostatic system. The separations were performed at 15-25 kV. The samples were injected at 50  $\times$  5 mbar·s (4.6 nL injected volume). The volume of sample loaded to

the capillary was calculated using the Hagen-Poiseuille equation (11). The BGE (background electrolyte) used for the study to optimize electrophoretic resolution of VER, NOR and I.S. was composed of commercially available phosphate buffer 2.5 or was prepared by mixing appropriate volumes of aqueous solutions of Na<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> (pH 2, pH 5). CDs solutions (2%) were prepared by dissolving 40 mg CDs in 2 mL of phosphate buffer. The 3.5% TM $\beta$ CD solution was prepared by dissolving 70 mg in 2 mL of phosphate buffer. All sample solutions were filtered through 0.45  $\mu$ m filter before injection into the capillary. The capillary was conditioned for 10 min each with 1.0 M and 0.1 M sodium hydroxide, deionized water and 0.05 M phosphate buffer. Before each analysis, the capillary was washed with 0.1 M sodium hydroxide, deionized water and 0.05 M phosphate buffer containing chiral selector for 1, 2 and 2 min, respectively.

### CE calculations

CE parameters such as apparent electrophoretic mobilities  $\mu_{ap}$ , resolution factor ( $R_s$ ), and relative migration times ( $t_{s,R}$ ) were calculated for each enantiomer according to equations given in the literature (11, 12).

### Sample preparation

Standard solutions of 5.0, 8.0, 20, 40, 80 and 100  $\mu$ g/mL rac-VER, rac-NOR hydrochloride and 50  $\mu$ g/mL clopidogrel (I.S.) were prepared in methanol. Then, a volume of 50  $\mu$ L of each of the solutions was added to 0.5 mL of blank plasma. The resulting analytes concentrations in plasma were 0.25-10  $\mu$ g/mL of each verapamil enantiomer and 5  $\mu$ g/mL of I.S. To 0.5 mL of plasma samples in a screw-capped glass centrifuge tube a volume of 0.5 mL of 0.1 M sodium hydroxide and 2.5 mL of hexane-isopropanol mixture (90/10, v/v) were added. Analytes were extracted into the organic layer by vortex-mixing for 10 min. After centrifugation for 10 min, the hexane layer was decanted into a clean glass tube and evaporated to dryness at 40°C under nitrogen stream. The resulting residue was reconstituted in 100  $\mu$ L of methanol and 4.9 nL aliquot was injected into capillary.

### Validation parameters

#### Selectivity

The control blank samples of human plasma extracted according the procedure described above were analyzed to determine the extent to which endogenous components may contribute to interfere with retention time of the drug.

#### Linearity range of calibration curves

The linearity of the method was estimated as a relationship of the ratio of the peak area of VER and NOR enantiomers to peak of internal standard (I.S.) and the concentrations of the analytes in plasma samples covering the range 0.25-10  $\mu\text{g/mL}$ . The linearity of the standard curves was evaluated using linear regression analysis. The correlation coefficient  $r$  was calculated.

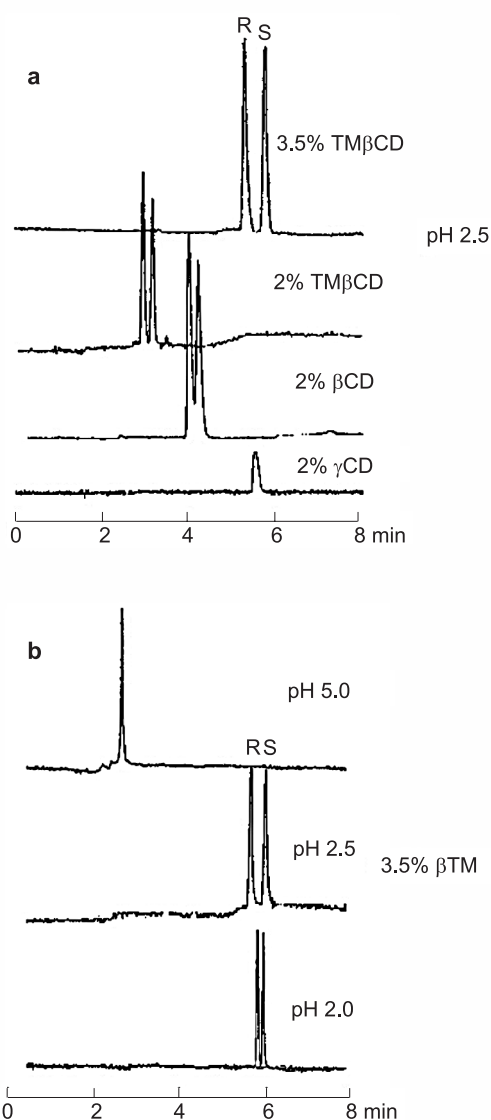


Figure 1. Effect of cyclodextrins (a) and pH (b) on verapamil enantiomers separation. CE conditions: fused-silica capillary: 33 cm  $\times$  50  $\mu\text{m}$  i.d., temperature 25°C, voltage 25 kV, current 18-22  $\mu\text{A}$ , UV detection absorbance at 200 nm

#### Limit of detection and limit of quantitation

The limit of detection (LOD) for enantiomers was determined as the concentration of drug giving a signal to noise ratio greater than 3. The lower limit of quantitation (LOQ) was determined as the minimum concentration that can be accurately and precisely quantified.

#### Precision and accuracy

Intra-day precision of the method in plasma, expressed as % C.V., has been estimated for the following concentrations: 2.0, 4.0 and 5.0  $\mu\text{g/mL}$  of each enantiomer. Inter-day precision was estimated for all enantiomer concentrations within the calibration curve range. Accuracy was estimated for the same ranges of enantiomer concentrations as for evaluation of precision of the method. It was expressed by the percent difference between the mean determined concentration and the nominal concentration.

#### Recovery

The recovery of each VER and NOR enantiomers was calculated at the level of 1.0 and 4.0  $\mu\text{g/mL}$ . These results were obtained by comparing the averaged peak areas of the analytes extracted from plasma with the averaged peak areas of non-extracted standards injected at the same concentrations.

#### Applicability of the method

To evaluate analytical applicability of the proposed method, Staveran tablets containing 80 mg of VER were administered to hospital patients suffering from arterial hypertension. Plasma samples were collected one hour after administration and immediately centrifuged and stored at -20°C until the analysis.

## RESULTS AND DISCUSSION

#### Method development for enantiomeric separation

The first step was to select the type of cyclodextrin (CD) which have been widely used as additive in the background electrolyte to induce the separation of enantiomers. CDs are cyclic oligosaccharides that resemble a truncated cone. The interior of the cavity is relatively hydrophobic while its external surface is hydrophilic. In this study, two types of CDs:  $\beta$  and  $\gamma$  were tested. Different concentrations of CDs were also tried. The separation of VER enantiomers results in a difference of stability of cyclodextrin complex with (+)-R and (-)-S enantiomers. Most probably the (+)-R creates more sta-

ble complex with TM $\beta$ CD than the (-)-S enantiomer, and therefore the (+)-R migrates earlier than its antipode. The best separation for VER enantiomers was obtained using 3.5% TM $\beta$ CD while lack of separation was observed for  $\gamma$ CD and  $\beta$ CD (Fig. 1a).

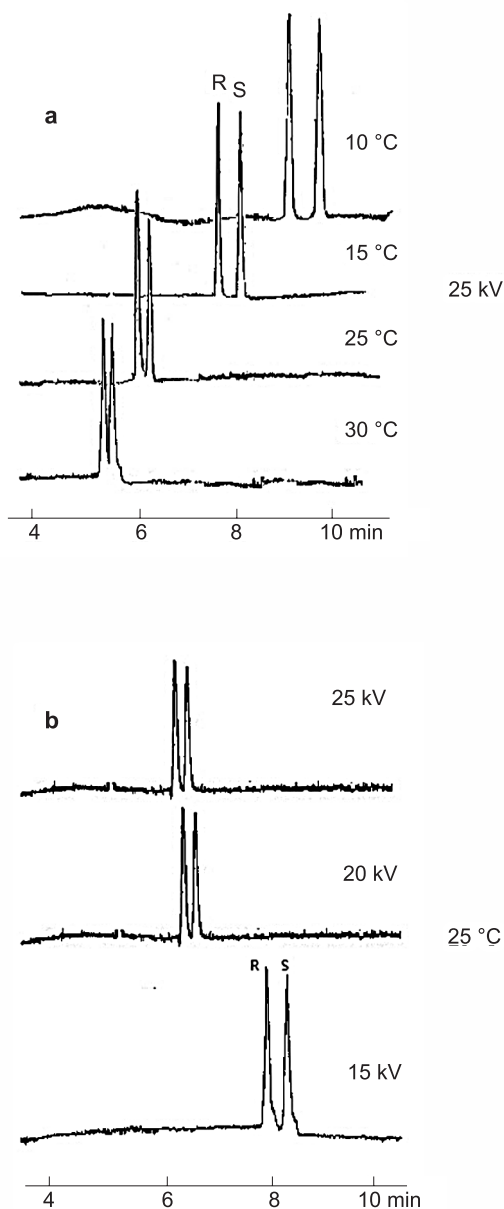


Figure 2. Effect of temperature (a) and applied voltage (b) on verapamil enantiomers separation. CE conditions: phosphate buffer pH 2.5 with 3.5% of TM $\beta$ CD, fused-silica capillary: 33 cm  $\times$  50  $\mu$ m i.d., current 18–22  $\mu$ A, UV detection absorbance at 200 nm

A variation of the buffer pH was carried out from pH 2.0 up to pH 5.0. The pH of the buffer can affect the ionic nature of the analyte, the magnitude of electrosmotic flow and the charged nature of CD. Under acidic conditions, VER is positively charged and migrates toward the cathode. A good resolution was achieved at pH 2.5. Migration times were similar at pH 2.0 and 2.5. At pH 5, migration time was reduced and resolution between the enantiomers

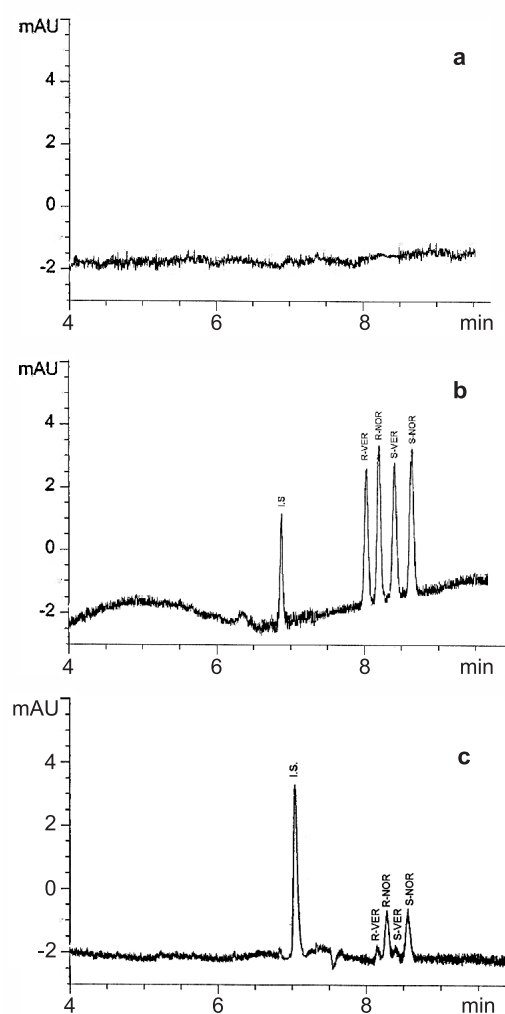


Figure 3. Electropherograms of VER and NOR enantiomers of human plasma samples: a – blank plasma, b – blank plasma spiked with verapamil R,S-enantiomers 5.0  $\mu$ g/mL, norverapamil R,S-enantiomers 5.0  $\mu$ g/mL and clopidogrel (I.S.), 5.0  $\mu$ g/mL, c – plasma sample collected from patient at 1 h elapsed from administration of 80 mg Staveran tablet. BGE - phosphate buffer pH 2.5 with 3.5% of TM $\beta$ CD, fused-silica capillary: 33 cm  $\times$  50  $\mu$ m i.d., temperature 15 $^{\circ}$ C, voltage 15 kV, current 18–22  $\mu$ A, UV detection absorbance at 200 nm

Table 1. Electrophoretic parameters of VER and NOR enantiomers from plasma.

Enantiomer	$t_{\text{migr}}$ (min)	$\mu_{\text{ap}}$ (cm <sup>2</sup> /Vs)	$R_s$	$t_{\text{R,S}}$ (min)
VER (+)-R	8.00	$1.11 \times 10^{-4}$	5.38	1.17
(-)-S	8.45	$1.06 \times 10^{-4}$		1.22
NOR (+)-R	8.20	$1.09 \times 10^{-4}$	6.57	1.19
(-)-S	8.65	$1.04 \times 10^{-4}$		1.25

Table 2. Calibration curve equations for VER and NOR enantiomers.

Compound ( $\mu\text{g/mL}$ )	Enantiomer	Calibration curve equation	Correlation coefficient, r
Verapamil	(+)-R	$P_{(+)-\text{R-VER}}/P_{\text{CLP}} = 0.3278 \cdot C_{(+)-\text{R-VER}}$	0.997
	(-)-S	$P_{(-)-\text{S-VER}}/P_{\text{CLP}} = 0.3426 \cdot C_{(-)-\text{S-VER}}$	0.996
Norverapamil	(+)-R	$P_{(+)-\text{R-NOR}}/P_{\text{CLP}} = 0.3475 \cdot C_{(+)-\text{R-NOR}}$	0.992
	(-)-S	$P_{(-)-\text{S-VER}}/P_{\text{CLP}} = 0.3316 \cdot C_{(-)-\text{S-NOR}}$	0.996

n=3

Table 3. Validation parameters.

Parameter	VER		NOR	
	(+)-R	(-)-S	(+)-R	(-)-S
LOD ( $\mu\text{g/mL}$ )	0.10	0.10	0.10	0.10
LOQ ( $\mu\text{g/mL}$ )	0.20	0.20	0.20	0.20
Range of calibration curve ( $\mu\text{g/mL}$ )	0.25–10.0		0.25–10.0	
Precision (% CV)	0.77–3.30	0.63–11.09	1.15–9.53	1.19–9.96
Accuracy (%)	0.67–4.06	2.45–11.65	1.83–11.51	3.92–7.95

n = 3

was not achieved (Fig. 1b). With the choice of TM $\beta$ CD as a chiral selector and optimal phosphate buffer with pH 2.5 other important experimental parameters were also investigated. Different capillary temperatures 10–30°C were tried. In our study, migration times and resolution decreased with increasing temperature because of the lower viscosity of the buffer, which was leading to higher diffusion rates. The highest resolution was obtained at 10°C, however, the analysis time was significantly prolonged. Finally, the temperature of 15°C was chosen (Fig. 2a). Different voltages (15–25 kV) were applied. A voltage of 15 kV was suitable for this BGE (Fig 2b). At high voltage - 25 kV, enantioresolution was not sufficient to determine separation

of VER and NOR enantiomers in one analytical run. A fundamental electrophoretic phenomenon occurring in CE is electroosmotic flow (EOF), which essentially is an electrical field-driven bulk solution flow from the anode to the cathode. In this study, EOF was not noticeable due to its high dependence on buffer pH: i.e., it increases with raising pH and reaches plateau at about 8, but is not significant below pH 4 (12). For this reason only apparent electrophoretic mobility was calculated. Resolution coefficient,  $R_s$  between VER and NOR enantiomers was more than 5 and has confirmed complete resolution of all enantiomers. Electrophoretic parameters of VER and NOR enantiomers are presented in Table 1.

In summary, the most optimal conditions for separation of VER, NOR enantiomers and I.S. in one analytical run in short time of 9 min were established at buffer's pH of 2.5 containing 3.5% TM $\beta$ CD, temperature 15°C and applied voltage 15 kV.

### Validation of the method

#### Selectivity

Comparison of electropherogram of extracted blank samples of plasma (Fig. 3a) with electropherogram of spiked sample with VER enantiomers and I.S. (Fig. 3b) or electropherogram prepared from sample received from patient following the administration of rac-VER (Fig. 3c) show separation from each other peaks of the analytes. No peak, which could originate from endogenous compounds of the plasma, interfered with VER enantiomers or I.S.

#### Linearity range of calibration curves

Standard curves were linear ( $r = 0.992-0.997$ ) in the concentrations range 0.25-10.0  $\mu\text{g/mL}$  of each of VER and NOR enantiomers. Intercepts were insignificantly different from zero, therefore, they are not reported. The resulting equations of standard curves and correlation coefficient are presented in Table 2. The equations of the standard curves for the chiral analytes have been applied for quantification of the analytes in patient plasma following oral administration of rac-VER in tablet.

#### LOD and LOQ

The lower limit of detection at an S/N baseline ratio 3:1 for VER and NOR enantiomers was 0.1  $\mu\text{g/mL}$ . The limit of quantitation of 0.2  $\mu\text{g/mL}$  for the analytes was supported by coefficient of variation and accuracy both below 15%. The obtained yield corresponded to the literature data where UV detection was applied. The minimum quantifiable level of analyte was 0.25  $\mu\text{g/mL}$  (8). In enantioselective CE procedure elaborated by Dethy et al. (10) LOQ was equal to 2.5 ng/mL. A strangely low value of LOQ was reached in spite of use the same UV detection.

#### Precision and accuracy

Precision and accuracy for this method was controlled by calculating the intra- and inter-batch at prepared concentrations. The assay precision values expressed as the % C.V. were in the range of 0.63-13.30%. Accuracy at the analyzed enantiomer concentrations was in the range of 0.67-11.65% (Table 3). These data indicated reproducible results, and that the assay was accurate and reliable.

#### Recovery

In the assay, liquid-liquid extraction was used to clean-up the samples and as the concentration step before CE analysis. The absolute recovery of the VER and NOR enantiomers from plasma at concentration 1.0 and 4.0  $\mu\text{g/mL}$  was relatively high. It has ranged from 91 to 103%. The obtained yield corresponded to the literature data which showed recoveries between 91-100% (10).

#### Applicability of the method

The applicability of the method developed to determine VER and NOR enantiomers was verified by analysis of serum from hospital patient suffering from arterial hypertension. One hour after oral administration of 80 mg of Staveran tablet, the plasma sample was analyzed (Fig. 3c). Determined concentrations of R,S VER and R,S NOR enantiomers were 0.25, 0.22, 0.66 and 0.70  $\mu\text{g/mL}$ , respectively. The data prove the suitability of the proposed method for clinical studies of VER and its active metabolite NOR enantiomers.

### CONCLUSION

A rapid and simple capillary electrophoresis assay was developed for analysis of VER and NOR enantiomers in human plasma. Optimal CE conditions were established to obtain the complete separation of enantiomers. Parameters of validation demonstrate linearity, good precision, accuracy and recovery, which proves the reliability of the proposed method. The developed method could be used for pharmacokinetic studies of the enantiomers of VER and NOR.

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