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Simultaneous direct determination of the enantiomers of verapamil and norverapamil in plasma using a derivatized amylose high-performance liquid chromatographic chiral stationary phase

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ABSTRACT

Chiralpak AD is a commercially available high-performance liquid chromatographic column containing a chiral stationary phase composed of 3,5-dimethylphenylcarbamate-derivatized amylose coated on silica. This column was applied to the assay for the plasma concentrations of the enantiomers of verapamil and its major metabolite norverapamil. After the extraction from plasma, the analytes were separated on a diol silica column (LiChrocart DIOL) and Chiralpak AD column which were connected in series in this order and detected using a fluorescence detector (excitation at 272 nm, emission at 317 nm). The enantiomers of verapamil and norverapamil were separated from each other and other metabolites using a mobile phase of hexane–isopropanol–ethanol (85:7.5:7.5, v/v/v) containing 1.0% triethylamine. The calibration curves were linear ($R > 0.9989$) in the plasma concentration range 2.5–100 ng/ml for verapamil enantiomers and 5.0–100 ng/ml for norverapamil enantiomers. The intra-day and inter-day reproducibility tests showed good reproducibilities; coefficients of variation of each enantiomer were less than 14.9% at the lowest concentration and less than 2.0% at the highest concentration. The effect of organic modifier content and column temperature upon the retention and the enantioseparation were also discussed.

INTRODUCTION

Verapamil (VER) is a calcium channel-blocking drug and is widely used in the therapy of hypertension, supraventricular arrhymthmias and angina pectoris [1]. VER is a chiral compound which is administered as a racemic mixture of (*R*)-VER and (*S*)-VER. However, the enantiomers show different pharmacodynamic and pharmacokinetic properties. For example, (*S*)-VER is ten to twenty times more potent than (*R*)-VER [2]; after intravenous administration, the plasma clearance and apparent volume of distribution of (*S*)-VER are almost twice as high as those of (*R*)-VER [3]; after oral administration, (*S*)-VER undergoes extensive first-pass metabolism, resulting in the predominance of (*R*)-VER in plasma [4]; the protein binding is enantioselective, and the free fraction of (*S*)-VER is larger than that of (*R*)-VER [5]. The major metabolic routes of VER are N-demethylation and N-dealkylation to form three major metabolites: norverapamil (NOR), D617 and D620 (Fig. 1). Among these metabolites, NOR is the most important metabolite because it is reported to possess 20% of the coronary vasodilator potency of VER [6]. NOR is also chiral, and its pharmacokinetic disposition is enantioselective [7].

While the stereoselective pharmacokinetics and pharmcodynamics of VER and NOR are important, these parameters have not been investigated extensively because of the lack of a simple

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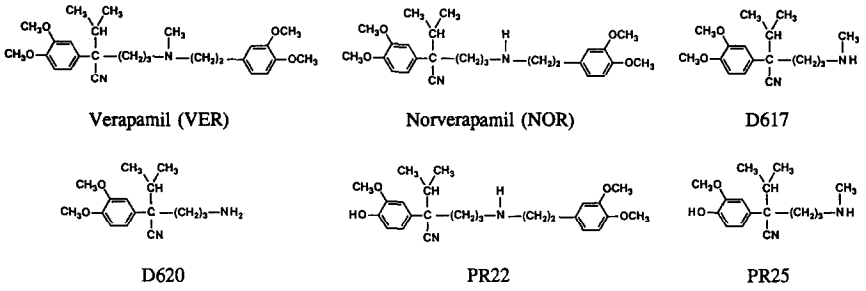

Fig. 1. Structures of verapamil and its metabolites.

analytical method. The pharmacokinetic studies cited above were carried out using the separate VER enantiomers [3,5] or a pseudoracemate [4,5,7]. In the latter approach, the pseudoracemate consisted of dideuterated (*R*)-VER and unlabeled (*S*)-VER or *vice versa*, and the serum levels were determined by gas chromatography–mass spectrometry. However, these approaches can hardly be applicable to large-scale clinical studies.

Recently, high-performance liquid chromatographic (HPLC) assays have been reported for the determination of the serum concentrations of VER and NOR enantiomers. Chu and Wainer [8] developed an achiral–chiral HPLC method where VER and NOR were initially separated from each other and from plasma components on an achiral stationary phase (a reversed-phase Hisep column). The eluent fractions containing VER and NOR were then selectively transferred via an on-line column switching valve to a column containing a chiral stationary phase based upon α_1-acid glycoprotein (Chiral AGP column) for the enantioseparations. Oda *et al.* [9] developed another achiral–chiral HPLC system for the determination of VER enantiomers which coupled an ODS column and a column containing a chiral stationary phase based upon immobilized ovomucoid (the Ultron ES-OVM column). In both of these studies, the reversed-phase (achiral) columns were incorporated into the systems to compensate for the poor selectivity of the protein-coated chiral stationary phases which were unable to separate VER from its metabolites. Although these systems can be used in pharmacokinetic studies of VER and NOR enantiomers, they are not easy to apply and are time-consuming.

This paper reports a novel assay for the simultaneous determination of VER and NOR enantiomers in plasma using a diol silica column and a chiral HPLC column composed of 3,5-dimethylphenylcarbamate-derivatized amylose coated on aminopropyl silica (Chiralpak AD) [10] which were connected in series. The present assay is quite simple because it does not use a column-switching system.

EXPERIMENTAL

Chemicals

Racemic verapamil hydrochloride was purchased from Sigma (St. Louis, MO, USA). (*R*)-VER, racemic NOR and (*R*)-NOR were received from G. D. Searle (Chicago, IL, USA) as hydrochloride salts. Racemic D617, D620, PR22 and PR25 were kindly supplied by Dr. Darrell Abernethy, Brown University (Providence, RI, USA) as hydrochlorides. The structures of these compounds are shown in Fig. 1. (+)-Glaucine, the internal standard, was purchased from Aldrich (Milwaukee, WI, USA). Hexane and isopropanol of HPLC grade were purchased from Anachemia (Montreal, Canada). Triethylamine reagent was purchased from A & C American Chemicals (Montreal, Canada). All reagents were used without further purification.

Apparatus

The modular liquid chromatograph consisted of a Beckman 110B solvent delivery system modular pump (Beckman Instruments, Houston, TX, USA), a Spectra-Physics SP8880 autosampler (Spectra-Physics, Santa Clara, CA, USA), a Waters 470 fluorescence detector (Millipore, Mil-

ford, MA, USA), a Haake G column temperature controller (Haake, Berlin, Germany), a Shimadzu C-R6A integrator (Shimadzu, Kyoto, Japan), a 50 mm × 4.0 mm I.D. LiChrocart DIOL column (5 μm, Merck, Darmstadt, Germany) and a 250 mm × 4.6 mm I.D. Chiralpak AD column (10 μm, Daicel Chemical Industries, Tokyo, Japan).

Detection

The excitation and the emission wavelengths of the fluorescence detector were set at 272 and 317 nm, respectively, with the bandwidth of 18 nm.

Enantiomeric elution order

The enantiomeric elution orders of (*R*)- and (*S*)-VER and (*R*)- and (*S*)-NOR were determined using mixtures containing an excess of the (*R*)-isomers of VER and NOR.

Sample preparation

To 1.0 ml of plasma in a screw-capped glass centrifuge tube were added 50.0 μl of 400 ng/ml (+)-glaucine spiking solution, 100 μl of 2 *M* sodium hydroxide solution, 1.0 ml of sodium phosphate buffer (pH 7.0, ionic strength 0.1) and 6 ml of heptane. After vortex-mixing for 1 min and centrifugation for 10 min at 1500 *g*, the aqueous layer was frozen in a dry ice–acetone bath and the heptane layer decanted into a clean glass tube. The heptane was evaporated to dryness in a vacuum centrifuge at 60°C, the resulting residue reconstituted in 250 μl of the mobile phase, and the 100-μl portion injected into the HPLC system.

Standard curves

Spiked plasma samples were prepared by adding known amounts of racemic VER and racemic NOR to pooled plasma obtained from the blood bank of Royal Victoria Hospital (Montreal, Canada). The concentrations of the spiked samples were 5.0, 20.0, 50.0, 100, 150 and 200 ng/ml for racemic VER and 10.0, 20.0, 50.0, 100, 150 and 200 ng/ml for racemic NOR. The standard curves were run in duplicate.

Intra-day and inter-day studies

Plasma spikes for the intra-day and inter-day studies were prepared at concentrations of 5.0 ng/ml racemic VER and 10.0 ng/ml racemic NOR (low standard) and of 200 ng/ml racemic VER and 200 ng/ml racemic NOR (high standard). The samples had been stored at −20°C before analysis.

Extraction efficiency study

Plasma spikes for the extraction efficiency study were prepared at concentrations of 20 and 200 ng/ml for both racemic VER and racemic NOR.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The Chiralpak AD column contains 3,5-dimethylphenylcarbamate derivatives of amylose coated on 10-μm, 100-Å aminopropyl silica [10]. The solvents which dissolve and/or swell the amylose derivative should not be used with the column, and hexane–isopropanol and hexane–ethanol mixtures are recommended as the suitable mobile phases by the manufacturer. The addition of triethylamine (less than 1.0%) into the mobile phase is also recommended in case the basic sample shows a considerable tailing of the elution peak. Within these recommended condi-

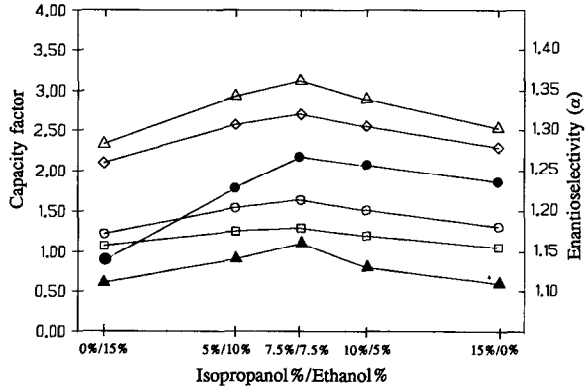

Fig. 2. Effect of the organic solvent content in mobile phase on the retention and enantioseparation of verapamil (VER) and norverapamil (NOR). HPLC conditions: stationary phase, Chiralpak AD (25 cm × 4.6 mm I.D.); mobile phase, mixture of 85% hexane, 0–15% isopropanol and 0–15% ethanol containing 0.4% triethylamine; column temperature, 30°C; flow-rate, 1.5 ml/min; injection volume, 100 μl. Symbols for capacity factor: ○ = (*R*)-VER; □ = (*S*)-VER; △ = (*R*)-NOR, ◇ = (*S*)-NOR; symbols for enantioselectivity (α): ● = VER; ▲ = NOR.

tions, the retention behaviors and the enantioseparations of VER and NOR on this chiral column were investigated in order to optimize the mobile phase conditions.

Fig. 2 shows the effects of isopropanol and ethanol content in the mobile phase on the capacity factors and the enantioselectivity (α) of the VER and NOR enantiomers, where the content of hexane was kept constant (85%). Unlike the protein-based chiral stationary phases, there was no overlap between the VER and NOR peaks under all mobile phase conditions investigated (Fig. 2).

The magnitude of the enantioseparations depended strongly upon the structure of the organic modifier (Fig. 2). By changing the mobile phase from hexane–ethanol (85:15, v/v) to hexane–isopropanol (85:15, v/v) the enantioselectivity of VER enantiomers improved from 1.14 to 1.24, while that of NOR enantiomers did not change (α = 1.11). These results are consistant with previous observations of the effect of the steric structure of alcoholic mobile phase modifiers on enantioselectivity and retention on a related chiral stationary phase based upon cellulose tribenzoate [11,12].

A ternary hexane–isopropanol–ethanol mixture further improved the enantioselectivity of both VER and NOR. The maximum α values (α = 1.27 for VER and α = 1.16 for NOR) as well as the longest retention were obtained when the contents of isopropanol and ethanol were the same (7.5%). When the hexane content was increased from 85 to 90% keeping the isopropanol-to-ethanol ratio 1, the capcity factors increased by 1.55–1.75 times, but the separation factors between enantiomers scarcely changed.

The addition of triethylamine in the mobile phase was necessary to suppress the peak tailing and to obtain maximum enantiomeric resolution. An increase in triethylamine concentration resulted in improved enantiomeric resolution; for example, the R_s values improved from 1.63 to 1.76 for VER and from 1.18 to 1.24 for NOR by increasing the triethylamine content from 0.4 to 1.0%.

The effect of temperature on the retention of the VER and NOR enantiomers is depicted in the Van 't Hoff plot presented in Fig. 3. The capacity factor of each enantiomer increased logarithmically with increasing the reciprocal of the column temperature (20, 25, 30 and 35°C). The enantioselectivity was almost constant within this temperature range (α = 1.25–1.29 for VER and α = 1.15–1.17 for NOR), which suggests that the enantioseparation mechanism is consistent under the temperature range investigated. When the column temperature was 35°C and more, (*S*)-VER was eluted so fast that the peak overlapped with the dip of the system peak, which interfered the determination at a low concentration. This is expected but would be regulated with the polarity of the mobile phase.

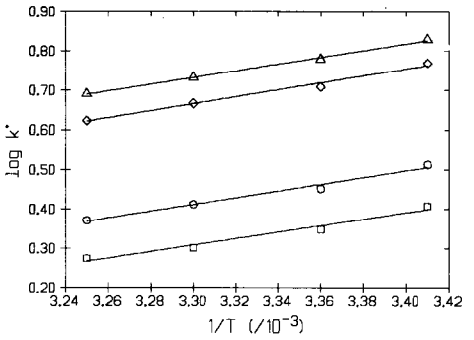

Fig. 3. Effect of the column temperature on the retention of verapamil (VER) and norverapamil (NOR) enantiomers. HPLC conditions: stationary phase, Chiralpak AD (25 cm × 4 mm I.D.); mobile phase, hexane–isopropanol–ethanol (90:5:5, v/v/v) containing 0.4% triethylamine; column temperature, 20, 25, 30 and 35°C; flow-rate, 1.5 ml/min; injection volume, 100 μl. Symbols: ○ = (*R*)-VER; □ = (*S*)-VER; △ = (*R*)-NOR; ◇ = (*S*)-NOR.

Based on these results, the optimum mobile phase conditions were determined as hexane–isopropanol–ethanol (85:7.5:7.5, v/v/v) containing 1.0% triethylamine. The column temperature was kept at 30°C, and the flow-rate was 1.0 ml/min. Fig. 4 shows the chromatograms of spiked plasma samples containing (A) 200 ng/ml racemic VER and 200 ng/ml racemic NOR, (B) 5.0 ng/ml racemic VER and 10.0 ng/ml racemic NOR and (C) blank plasma. No significant interference of plasma components was observed. For both VER and NOR, the (*S*)-isomer was eluted faster than the (*R*)-isomer.

The elution profiles of the other metabolites (D617, D620 and two minor metabolites PR22 and PR25 which were the O-demethylated forms of NOR and D617, respectively) were also investigated using these chromatographic conditions.

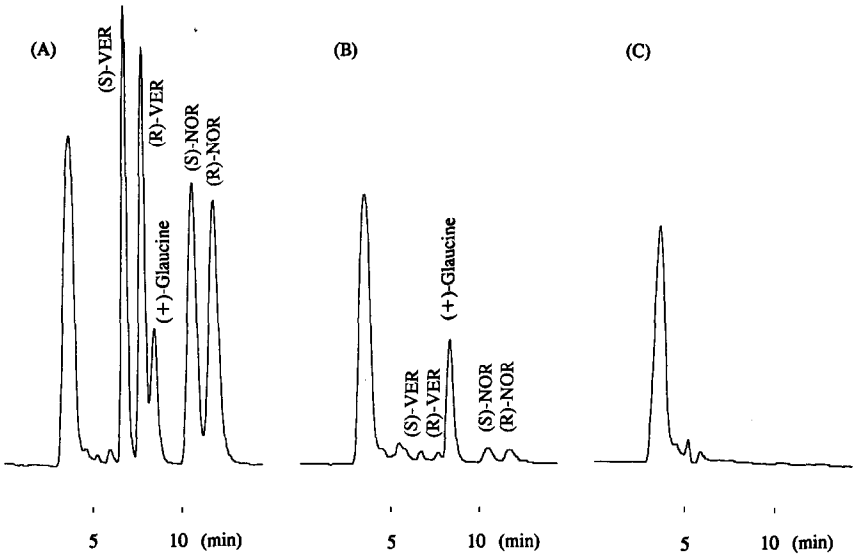

Fig. 4. Typical chromatograms of (A and B) verapamil (VER) and norverapamil (NOR) enantiomers in plasma and (C) plasma blank. HPLC conditions: stationary phase, Chiralpak AD (25 cm × 4.6 mm I.D.); mobile phase, hexane–isopropanol–ethanol (85:7.5:7.5, v/v/v) containing 1.0% triethylamine; column temperature, 30°C; flow-rate, 1.0 ml/min. Concentration in sample: (A) 200 ng/ml racemic VER and 200 ng/ml racemic NOR in human plasma; (B) 5.0 ng/ml racemic VER and 10.0 ng/ml of racemic NOR in human plasma. The extraction procedure was the same as described in the Experimental section except that the concentration of (+)-glaucine spiking solution was 200 ng/ml. Injection volume, 100 μl. Detection: excitation wavelength, 272 nm, emission wavelength, 317 nm.

The enantioseparations of these metabolites were not achieved except for PR22, and the peaks of D617 and PR25 overlapped with those of VER enantiomers and NOR enantiomers, respectively. Since the enantiomers of the compounds that possess two benzene rings (VER, NOR and PR22) were stereochemically resolved but those of the compounds containing only one benzene

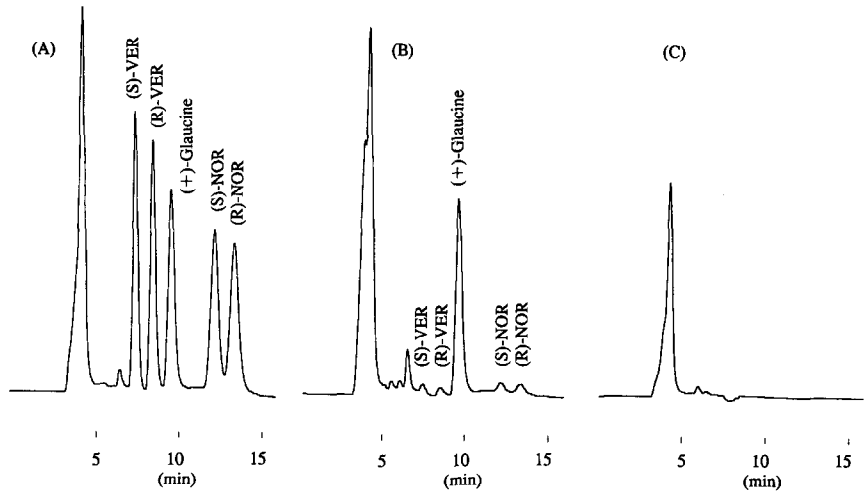

Fig. 5. Typical chromatograms of (A and B) verapamil (VER) and norverapamil (NOR) enantiomers in plasma and (C) plasma blank. HPLC conditions: stationary phase, LiChrocart DIOL (5 cm × 4.0 mm I.D.) plus chiralpak AD (25 cm × 4.6 mm I.D.); mobile phase, hexane–isopropanol–ethanol (85:7.5:7.5, v/v/v) containing 1.0% triethylamine; column temperature, 30°C; flow-rate, 1.0 ml/min. Concentration in sample: (A) 200 ng/ml racemic VER and 200 ng/ml racemic NOR in human plasma; (B) 5.0 ng/ml racemic VER and 10.0 ng/ml racemic NOR in human plasma. The extraction procedure was the same as described in the Experimental section. Injection volume, 100 μl. Detection: excitation wavelength, 272 nm; emission wavelength, 317 nm.

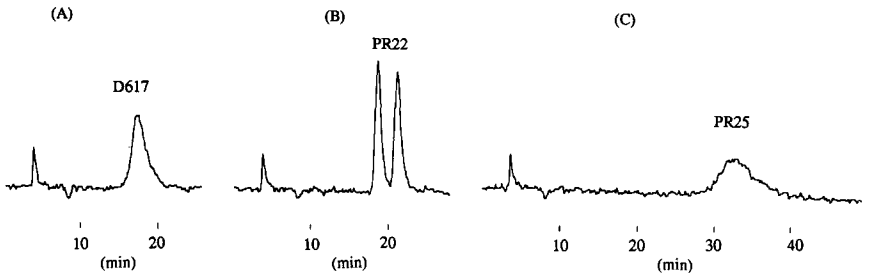

Fig. 6. Chromatograms of the metabolites of verapamil. HPLC conditions were the same as those in Fig. 5.

ring (D617 and PR25) were not, the existence of two benzene rings in one molecule seems to be essential for the enantioseparation of VER analogues.

In order to separate VER and NOR enantiomers from these metabolites, a diol silica column (LiChrocart DIOL, 5 cm × 4.0 mm I.D.) was directly connected in front of the chiral column. The column temperature for the diol silica column was left ambient (*ca.* 22°C), while that for the chiral column was kept at 30°C. Fig. 5 shows the chromatograms of spiked plasma samples containing (A) 200 ng/ml racemic VER and 200 ng/ml racemic NOR, (B) 5.0 ng/ml racemic VER and 10.0 ng/ml racemic NOR and (C) blank plasma. No significant interference of plasma components was observed. Fig. 6 shows the chromatograms of 800 ng/ml D617 (A), PR22 (B) and PR25 (C) resolved in the mobile phase. The retention times of VER and its metabolites before and after connecting the diol silica column were compared in Table I. Because D617 and PR25 are more hydrophilic and therefore were retained more strongly on the normal-phase diol silica column than VER and NOR, the increase in the retention times of D617 and PR25 (9.6 and 22.1 min, respectively) were much larger than those of VER enantiomers and NOR enantiomers (0.7 and 1.7 min, respectively). Consequently, VER and NOR were completely separated from these metabolites. The peaks of PR22 enantiomers did not overlap with those of VER and NOR enantiomers. D620 was eluted very broadly during 5–40 min. The peak was so broad that no significant peak was recognized even after 800 ng/ml D620 resolved in the mobile phase was injected. Since this concentration is considered to be higher than the clinical level, practically there will be no interference by D620 in the clinical assay of VER and NOR in plasma. The use of the diol silica column has the further advantage that it enables the baseline separation between (*R*)-VER and (+)-glaucine. Although baseline resolution was not achieved between the NOR enantiomers ($\alpha = 1.16$, $R_s = 1.24$), it did not influence the determinations over the concentration range investigated.

TABLE I

RETENTION TIMES OF VERAPAMIL, ITS METABOLITES AND (+)-GLAUCINE

HPLC conditions: stationary phase, Chiralpak AD (25 cm × 4.6 mm I.D.) with (B) or without (A) LiChrocart DIOL (5 cm × 4.0 mm I.D.); mobile phase, hexane–isopropanol–ethanol (85:7.5:7.5, v/v/v) containing 1.0% triethylamine; flow-rate, 1.0 ml/min; excitation, 272 nm; emission, 317 nm; injection volume, 100 µl.

Compound	Retention time (min)	
	A	B
(*R*)-Verpamil	7.7	8.4
(*S*)-Verapamil	6.7	7.4
(*R*)-Norverapamil	11.7	13.4
(*S*)-Norverapamil	10.5	12.2
D617	7.4	17.0
PR22	15.5	18.6
	18.2	21.2
PR25	10.7	32.8
(+)-Glaucine	8.4	9.5

Results of the assay validation in plasma

The calibration curves for all enantiomers were linear over the range investigated. The equation describing the line was $y = 59.3\,x - 0.588$, $y = 54.3\,x - 0.406$, $y = 97.1\,x - 0.931$ and $y =$

TABLE II

INTRA-DAY AND INTER-DAY REPRODUCIBILITIES OF VERAPAMIL (VER) AND NORVERAPAMIL (NOR) ENANTIOMERS

Compound	Spiked concentration (ng/ml)	Intra-day (n = 10)		Inter-day (n = 15)	
		Mean determined concentration (ng/ml)	C.V. (%)	Mean determined concentration (ng/ml)	C.V. (%)
At low concentration					
(*R*)-VER	2.50	2.86	10.7	2.67	13.1
(*S*)-VER	2.50	2.76	14.9	2.70	14.0
(*R*)-NOR	5.00	4.80	11.3	5.33	13.8
(*S*)-NOR	5.00	5.51	10.3	5.04	13.8
At high concentration					
(*R*)-VER	100	102	1.40	103	1.95
(*S*)-VER	100	103	1.51	104	1.98
(*R*)-NOR	100	102	1.59	101	2.04
(*S*)-NOR	100	102	1.65	101	1.92

91.8 x − 1.19 for (*R*)-VER, (*S*)-VER, (*R*)-NOR and (*S*)-NOR, respectively, where x represents the peak height and y the concentration. The correlation coefficient for each line was more than 0.9989. The observed signal-to-noise ratios at the lowest concentration (2.5 ng/ml for each VER enantiomer and 5.0 ng/ml for each NOR enantiomer) were 4.74, 5.49, 5.41 and 5.79 for (*R*)-VER, (*S*)-VER (*R*)-NOR and (*S*)-NOR, respectively.

The intra-day (n = 10) and inter-day (n = 15) measurements were carried out with plasma samples spiked with 5.0 ng/ml racemic VER and 10.0 ng/ml racemic NOR (low standard) and those spiked with 200 ng/ml racemic VER and 200 ng/ml racemic NOR (high standard). The results listed in Table II showed good reproducibility; the coefficients of variation (C.V.) were 2.0% or less for the high standard and 14.9% or less for the low standard. The deviation of the mean determined value from the spiked concentration of each enantiomer was 3.8% or less for the high standard and 14.4% or less for the low standard.

The extraction recoveries of (*R*)-VER, (*S*)-VER, (*R*)-NOR and (*S*)-NOR were 88.4 ± 5.4, 87.7 ± 5.6, 84.6 ± 5.8 and 84.6 ± 5.9 at 100 ng/ml (n = 10) and 84.3 ± 0.8, 85.2 ± 2.2, 89.8 ± 0.3 and 89.2 ± 1.3% at 10 ng/ml (n = 2), respectively. The extraction recovery of (+)-glaucine was 89.9 ± 4.8% (n = 10). No significant deterioration in the efficiency of the chiral column was observed after more than two hundred injections of plasma extract. The retention time of D617 decreased from 17.0 to 15.8 min after about one hundred sample injections. However, the retention time recovered to the initial level after washing the diol silica column with 10 ml each of the following series of solvents: ethanol, water, ethanol, isopropanol.

Fig. 7 shows the chromatogram of the plasma extract collected 24 h after steady-state oral administration of 240 mg of racemic VER to a healthy volunteer in sustained-release formulation. No interference of the metabolites and en-

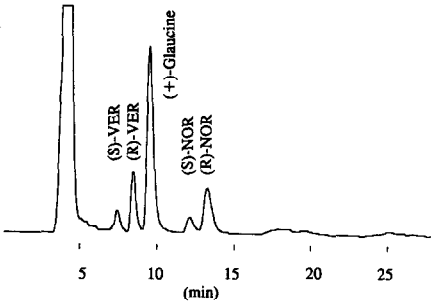

Fig. 7. Chromatogram of plasma from a healthy volunteer after chronic administration of 240 mg of sustained-release verapamil every 12 h. HPLC conditions were the same as those in Fig. 5.

dogenous plasma components was observed. The concentrations of (*R*)-VER, (*S*)-VER, (*R*)-NOR and (*S*)-NOR were determined as 19.0, 5.95, 23.0 and 7.29 ng/ml, respectively. For both VER and NOR, the concentration of (*R*)-isomer was about three times larger than that of (*S*)-isomer. This agrees with the literature which reported the predominance of the (*R*)-isomer in plasma [4,7].

CONCLUSIONS

The present assay is the first HPLC assay which allows simultaneous determinations of the enantiomers of VER and NOR in plasma samples without using a column-switching technique. The present method was validated by the intra-day and inter-day reproducibility tests. The combined use of the diol silica and Chiralpak AD columns will be beneficial to the large-scale pharmacokinetic and pharmacodynamic studies where VER is administered as a racemic mixture.

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