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DETERMINATION OF THE ENANTIOMERS OF VERAPAMIL AND NORVERAPAMIL IN SERUM USING COUPLED ACHIRAL-CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An assay for the plasma concentration of the enantiomers of verapamil and its metabolite norverapamil has been developed using the improved version of the α_1-acid glycoprotein chiral stationary phase (CHIRAL AGP-CSP) coupled to a shielded hydrophobic phase (Hisep) column. The Hisep column was used to separate verapamil and norverapamil from the plasma components and from each other and to quantitate the total verapamil and norverapamil concentrations. The eluents containing verapamil and norverapamil are then selectively transferred to the CHIRAL AGP-CSP where the enantiomers were stereochemically resolved and the enantiomeric composition determined. The system is the first reported for the serum concentration of the enantiomers of verapamil after the clinical administration of the drug and the first to stereochemically resolve and quantitate the enantiomers of norverapamil.

INTRODUCTION

Verapamil (VER) is a calcium channel blocking drug which is used in antianginal therapy. The molecule contains an asymmetric carbon (Fig. 1) and is clinically administered as a racemic mixture of the (+)-*R*- and (−)-(*S*)-enantiomers. Although racemic VER is routinely administered, studies have demonstrated that the enantiomers of VER differ in their pharmacodynamic effects and pharmacokinetic disposition with the (−)-(*S*)-enantiomer being the more pharmacologically active [1–5].

Studies in animals and humans have demonstrated that (−)-*S*-VER is mainly responsible for the negative dromotropic activity on atroventricular conduction [1–3]. For example, studies in the dog have demonstrated that the

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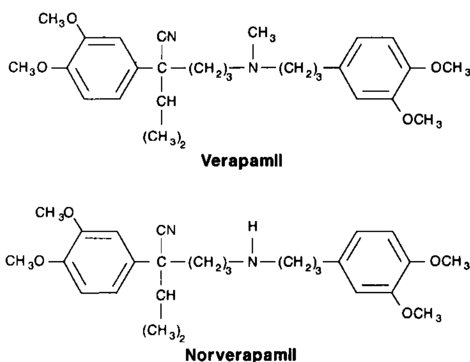

Fig. 1. Structures of verapamil and norverapamil.

(–)-(*S*)-isomer possessed eight to ten times more potent dromotropic effects on the AV nodal conduction than the (+)-(*R*)-enantiomer [1,2]. A similar effect was seen in humans where (–)-(*S*)-VER was eighteen times more potent than (+)-(*R*)-VER in producing 10% PR interval prolongation [3].

The pharmacokinetic disposition of (+)-(*R*)-, (–)-(*S*)- and racemic VER has been studied in humans after intravenous administration [4]. The results of this study demonstrated that the plasma clearance and apparent volume of distribution of (–)-(*S*)-VER was almost twice as high as those of (+)-(*R*)-VER while the values for racemic VER were similar to the averaged values obtained when the isomers were administered separately. In addition, the (+)-(*R*)-VER/(–)-(*S*)-VER plasma concentration ratio was approximately 2. However, the terminal half-lives of the enantiomers were nearly identical.

The pharmacokinetic disposition of the enantiomers of VER has also been studied after oral administration [5]. In this instance, the differences between the pharmacokinetic parameters of the two enantiomers was even greater than those determined after intravenous administration. For example, the apparent oral clearance of (–)-(*S*)-VER was almost five times higher than that of the (+)-(*R*)-isomer and the (+)-(*R*)-VER/(–)-(*S*)-VER plasma concentration ratio was 4.92. In addition, the bioavailability of (+)-(*R*)-VER was 2.5 times greater than that of the (–)-(*S*)-enantiomer. The authors concluded that VER undergoes a stereoselective first-pass biotransformation with preferential elimination of the (–)-(*S*)-enantiomer.

While the pharmacodynamic and pharmacokinetic differences between the enantiomers of VER are well known, this phenomenon has not been extensively studied. This is due to the lack of a universally applicable analytical method for the determination of the plasma concentrations of the enantiomers. The pharmacokinetic studies cited above were conducted using the separate enantiomers (the intravenous study) or a pseudoracemic VER consisting of unlabeled (–)-(*S*)-VER and dideuterated (+)-(*R*)-VER (the oral study). In the latter study, the serum concentrations of each enantiomer were determined using gas chromatography–mass spectrometry. Neither of these approaches are suitable for large-scale studies since they either do not utilize the

clinically administered racemic form of the drug or they involve the use of pseudoracemates which are difficult and costly to prepare.

A solution to this problem has been made possible by the evolution of enantioselective high-performance liquid chromatographic (HPLC) techniques, specifically the development of efficient HPLC chiral stationary phases (CSPs). The chromatographic resolution of the enantiomers of VER has been reported using CSPs based upon α_1-acid glycoprotein (AGP, the Enantiopac CSP) [6] and β-cyclodextrin [7]. In the former case, baseline resolution of (−)-(S)- and (+)-(R)-VER was achieved but the long retention times, poor column efficiency and short column life made analytical applications impossible. In the latter case, baseline resolution of the enantiomers was not achieved which also made this approach unsuitable for clinical studies.

Recently, a new form of the AGP-CSP, CHIRAL AGP, has become commercially available. The CHIRAL AGP-CSP has increased efficiency and column life relative to the Enantiopac CSP and reduced retention times [8,9]. The CHIRAL AGP-CSP is also able to stereochemically resolve (−)-(S)- and (+)-(R)-VER with baseline resolution, Fig. 2A [8,9] as well as the enantiomeric forms of one of the primary metabolites of VER, norverapamil (NORVER, Figs. 1 and 2B).

However, the direct application of this column to studies of VER and NORVER concentrations in serum is not possible since the peaks corresponding to the separate enantiomers overlap when the VER and NORVER are chromatographed together (Fig. 2C). To overcome this problem, we have developed a coupled achiral–chiral chromatographic system.

In this system, an achiral column containing a shielded hydrophobic phase, the Hisep column [10], is used to separate the VER and NORVER from the serum components and from each other. The achiral system is also used to quantitate the total VER and NORVER concentrations. The eluents containing VER and NORVER are then selectively transferred to the CHIRAL AGP-

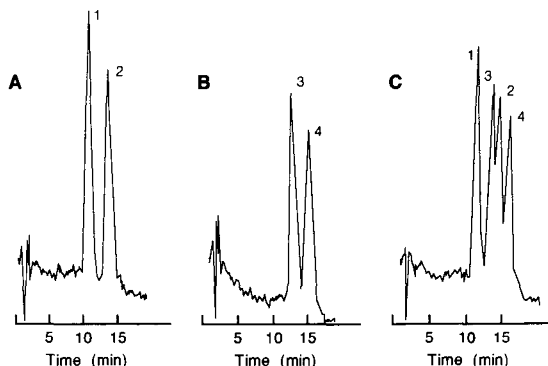

Fig. 2. Representative chromatograms from the chromatography of racemic verapamil and racemic norverapamil on the CHIRAL AGP-CSP. (A) Racemic verapamil; (B) racemic norverapamil; (C) 50:50 mixture of racemic verapamil and racemic norverapamil. Peaks: 1=(+)-(R)-verapamil; 2=(−)-(S)-verapamil; 3=(R)-norverapamil; 4=(S)-norverapamil. See text for chromatographic conditions.

CSP where they are stereochemically resolved and the enantiomeric composition of VER and NORVER determined. This system is accurate, does not require extensive precolumn manipulations and can be automated for use in large-scale clinical studies.

EXPERIMENTAL

Apparatus

The achiral chromatography was performed with a modular liquid chromatograph composed of a Beckman 110B solvent delivery system module pump (Beckman Instruments, Houston, TX, U.S.A.), a Spectra-Physics Spectraflow 980 fluorescence detector (Spectra-Physics, Santa Clara, CA, U.S.A.), a Spectra-Physics SP 4270 computing integrator, a Rheodyne 7125 injection valve (Rainin Instruments, Woburn, MA, U.S.A.) and a 150 mm × 4.6 mm I.D. Hisep HPLC column (Supelco, Bellefonte, PA, U.S.A.).

The enantioselective chromatography was performed with a modular liquid chromatograph composed of a Beckman 110B solvent delivery system module pump (Beckman Instruments), a Spectra-Physics Spectraflow 980 fluorescence detector (Spectra-Physics), A Spectra-Physics SP 4270 computing integrator and a commercially packed CHIRAL AGP-CSP (Advanced Separation Technologies, Whippany, NJ, U.S.A.).

The two systems were connected through a Rheodyne Model 7010 switching valve equipped with a pneumatic actuator and a 500-μl sample loop. When the eluent fraction containing VER was detected, the switching valve was rotated and the eluent flow diverted to the sample loop on the CHIRAL AGP-CSP. After 30 s, the switching valve was rotated and the eluent fraction containing VER injected onto the CHIRAL AGP-CSP. When the eluent fraction containing NORVER was detected, the switching valve was rotated and the eluent flow diverted to the sample loop on the CHIRAL AGP-CSP. After 30 s, the switching valve was rotated and the eluent fraction containing NORVER injected onto the CHIRAL AGP-CSP.

Chemicals

Racemic VER was purchased from Sigma (St. Louis, MO, U.S.A.) and (−)-(*S*)-VER was kindly provided by Dr. J.A. Oppermann (Searle Research and Development, Skokie, IL, U.S.A.). Racemic NORVER was kindly provided by Dr. D.R. Abernethy (Brown University, Providence, RI, U.S.A.) and a sample of the glucuronide of (*R*)-NORVER was provided by Professor W.L. Nelson (University of Washington, Seattle, WA, U.S.A.). The glucuronide was hydrolyzed to (*R*)-NORVER by treatment with hydrochloric acid. The HPLC-grade acetonitrile was purchased from Baxter Healthcare (McGraw Park, IL, U.S.A.) and the other chemicals were reagent grade and used as purchased.

Chromatographic approach

In this system, the achiral column is used to separate the VER and NORVER from the serum components and from each other. The achiral system is also

used to quantitate the total VER and NORVER concentrations. After the determination of total drug concentration, a second sample is chromatographed on the achiral system. The eluents containing VER and NORVER are selectively transferred to the CHIRAL AGP-CSP where they are stereochemically resolved and the enantiomeric composition of VER and NORVER determined.

Chromatographic conditions

The mobile phase used in the achiral chromatography was composed of phosphate buffer (0.03 *M*; pH 5.3)–acetonitrile (95:5, v/v). The chromatography was carried out at ambient temperature at a flow-rate of 1.0 ml/min.

The mobile phase used in the enantioselective chromatography was composed of phosphate buffer (0.03 *M*; pH 5.3)–acetonitrile (96:4, v/v). The chromatography was carried out at ambient temperature at a flow-rate of 1.0 ml/min.

Detection

VER and NORVER were quantitated on both chromatographic systems using a fluorescence detector with an excitation wavelength of 227 nm and no emission filter.

Enantiomeric elution order

The enantiomeric elution order of verapamil and norverapamil were determined by chromatographing unequal mixtures of the two enantiomers.

Sample preparation

To 500 μl of plasma were consecutively added 200 μl of 2 *M* sodium hydroxide and 5.0 ml of diethyl ether. The mixture was vortex-mixed for 1 min and centrifuged (1500 *g*) for 10 min. The aqueous layer was frozen in a dry ice–acetone bath and the ether layer decanted into a clean glass tube. The ether was evaporated to dryness under a stream of nitrogen. The resulting residue was reconstituted in 100 μl of water and injected onto the HPLC system.

Standards

Standard curves. Standard curves were prepared by adding known amounts of racemic VER and racemic NORVER to pooled plasma obtained from the blood bank of St. Jude Children's Research Hospital. The concentrations of the spiked samples were 20, 50, 80, 110, 140 and 170 ng/ml. The standard curves were run in triplicate.

Inter-day and intra-day studies. Plasma spikes for the intra-day and inter-day studies were prepared at concentrations of 50 and 120 ng/ml for both racemic VER and racemic NORVER. The lower concentration reflects the lower limit of detection for the coupled column system.

Extraction efficiency studies. Plasma spikes for the extraction efficiency studies were prepared at concentrations of 50 and 120 ng/ml for both racemic VER and racemic NORVER.

Clinical samples

The clinical samples assayed in this study were from a cross-over study of twelve hypertensive patients who received a sustained release formulation of VER (240 mg) every 12 h for four weeks [11,12]. Blood samples were taken at two-week intervals and 12 h after the last drug dose. The samples were collected in heparinized vacutainers and the plasma was separated and collected. The harvested plasma was frozen at −20°C until analysis.

RESULTS AND DISCUSSION

Achiral chromatography

The results from the chromatography of racemic VER and racemic NORVER on the Hisep column are presented in Table I. The racemic VER and the racemic NORVER were separated on this column with a selectivity (α) of 1.83 and a resolution factor (R_s) of 1.53. The detection limits were 7 ng/ml for racemic VER and 10 ng/ml for racemic NORVER. The signal-to-noise ratio at the lower limit of detection for racemic VER was 6.5 and for racemic NORVER it was 3.3. Representative chromatograms of a 500-μl blank plasma sample and a 500-μl plasma sample spiked with 100 ng each of racemic VER and racemic NORVER are presented in Fig. 3A and B, respectively.

The standard curve for racemic VER was linear over the range investigated. The equation describing the curve was $y = 0.127x - 0.384$ with a correlation coefficient of 0.999. The intra-day and inter-day measurements were carried out with plasma samples spiked with 50 and 120 ng/ml racemic VER. The 50 ng/ml samples represent the lower limit of detection on the coupled column system. The results are presented in Table II. The extraction efficiency averaged 87.2%.

The standard curve for racemic NORVER was linear over the range inves-

TABLE I

CHROMATOGRAPHIC RESULTS

Compound	Hisep column[a]	Chiral AGP-CSP[b]					
	k'^{c}	Direct injection			After switch		
		$k_1'^{d}$	α_{SR}^{e}	R_{SR}^{f}	$k_1'^{d}$	α_{SR}^{e}	R_{SR}^{f}
Verapamil	2.64	6.90	1.32	1.41	6.76	1.29	1.35
Norverapamil	4.82	8.19	1.22	1.28	7.36	1.21	1.16

[a]Chromatographic conditions: mobile phase, phosphate buffer (0.03 *M*; pH 5.3)-acetonitrile (95:5, v/v); flow-rate, 1.0 ml/min; temperature, ambient.
[b]Chromatographic conditions: mobile phase, phosphate buffer (0.03 *M*; pH 5.3)-acetonitrile (96:4, v/v); flow-rate, 1.0 ml/min; temperature, ambient.
[c]Capacity factor.
[d]Capacity factor first-eluted enantiomer.
[e]Stereochemical selectivity.
[f]Stereochemical resolution.

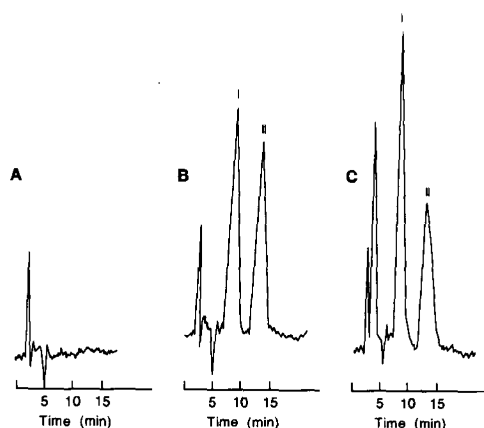

Fig. 3. Representative chromatograms from the chromatography of serum samples on the Hisep HPLC column. (A) Serum blank; (B) serum spiked with 100 ng/ml racemic verapamil and 100 ng/ml racemic norverapamil; (C) serum sample taken after the administration of a 240-mg sustained release dose of racemic verapamil. Peaks: I = racemic verapamil; II = racemic norverapamil. See text for chromatographic conditions.

TABLE II

PRECISION AND ACCURACY STUDIES FOR THE CHROMATOGRAPHY OF VERAPAMIL AND NORVERAPAMIL ON THE HISEP COLUMN

Chromatographic conditions: mobile phase, phosphate buffer (0.03 *M*; pH 5.3)-acetonitrile (95:5, v/v); flow-rate, 1.0 ml/min; temperature, ambient.

Compound	Sample	Spiked concentration (ng/ml)	Mean determined concentration (ng/ml)	Coefficient of variation (%)
Inter-day (n=6)				
Verapamil	A	50.0	47.6	8.0
	B	120.0	118.0	4.4
Norverapamil	A	50.0	55.6	6.1
	B	120.0	121.4	2.9
Intra-day (n=6)				
Verapamil	A	50.0	51.2	3.9
	B	120.0	116.3	4.6
Norverapamil	A	50.0	55.8	7.5
	B	120.0	118.0	6.5

tigated. The equation describing the curve was $y=0.081x-0.983$ with a correlation coefficient of 0.997. The intra-day and inter-day measurements were carried out with plasma samples spiked with 50 and 120 ng/ml racemic NORVER. The 50 ng/ml samples represent the lower limit of the coupled column system. The results are presented in Table II. The extraction efficiency averaged 77.2%.

The chromatogram resulting from the analysis of a plasma sample from one of the subjects in the clinical study is presented in Fig. 3C.

Enantioselective chromatography

The results from the chromatography of racemic VER and racemic NORVER on the CHIRAL AGP-CSP after the compounds had been injected directly onto the column are presented in Table I and Fig. 2A and B, respectively. The elution order of (−)-(*S*)-VER and (+)-(*R*)-VER and the elution order of (*R*)-NORVER and (*S*)-NORVER were determined using unequal mixtures of the resolved enantiomers.

Under the chromatographic conditions employed in this study, (+)-(*R*)-VER eluted first with a capacity factor (k') of 6.76, while the k' for (−)-(*S*)-VER was 9.08. The observed stereochemical selectivity (α_{RS}) was 1.32 and the stereochemical resolution factor (R_{RS}) was 1.41. For NORVER, the (*R*)-enantiomer also eluted first with a k' of 8.19, while the k' for the (*S*)-NORVER was 10.02. The observed α_{RS} was 1.22 and the R_{RS} was 1.28.

A representative chromatogram from analysis of a blank plasma sample on the CHIRAL AGP-CSP coupled to the Hisep column is presented in Fig. 4A. The two peaks in the chromatogram represent the two times that the switching valve was rotated to inject the eluent from the Hisep column onto the CHIRAL AGP-CSP. There were no other peaks observed in the chromatogram.

The results from the chromatography of racemic VER and racemic NORVER after the consecutive switching of the two compounds from the Hisep column to the CHIRAL AGP-CSP are presented in Table I and Fig. 4B. There was very little change in the observed stereochemical selectivity and stereochemical resolution relative to the results obtained when the compounds were injected directly onto the CSP. The α_{RS} for (−)-(*S*)- and (+)-(*R*)-VER after the switch was 1.29 versus 1.32 after direct injection. For (*S*)- and (*R*)-NORVER, the α_{RS} was virtually unchanged, 1.21 after the switch versus 1.22 after direct injection. The calculated R_{RS} values for VER were 1.35 (switch) and 1.41 (direct) and for NORVER 1.16 (switch) and 1.28 (direct).

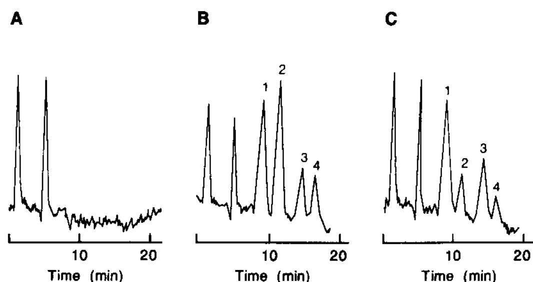

Fig. 4. Representative chromatograms from the chromatography of verapamil and norverapamil on the CHIRAL AGP-CSP coupled to the Hisep HPLC column. (A) Serum blank; (B) serum spiked with 100 ng/ml racemic verapamil and 100 ng/ml racemic norverapamil; (C) serum sample taken after the administration of a 240-mg sustained release dose of racemic verapamil. Peaks: 1 = (+)-(*R*)-verapamil; 2 = (−)-(*S*)-verapamil; 3 = (*R*)-norverapamil; 4 = (*S*)-norverapamil. See text for chromatographic conditions.

The difference between the chromatographic results following the switch and the direct injection is the displacement in time of the (*R*)-NORVER and (*S*)-NORVER peaks in relation to the peaks for the VER enantiomers. In this case, the overlap observed after the direct injection onto the CHIRAL AGP-CSP (see Fig. 2C) has been eliminated. This allows for the direct determination of the enantiomeric composition of both compounds. The coupled column method can quantitate samples containing 25 ng/ml of each isomer of VER and 30 ng/ml of each isomer of NORVER. The observed signal-to-noise ratios were 3.4 for (+)-(*R*)-VER, 3.2 for (−)-(*S*)-VER, 3.1 for (*R*)-NORVER and 2.9 for (*S*)-NORVER.

A representative chromatogram from the analysis of a plasma sample from the clinical study is presented in Fig. 4C.

Since the determination of the enantiomeric composition of the VER and NORVER samples is based upon the relative peak areas of the two pairs of enantiomers, these ratios were determined from duplicate injections at concentrations of 50, 100 and 150 ng/ml. For VER, the average ($n=6$) ratio of the (+)-(*R*)/(−)-(*S*) peak areas was 1.03 ± 0.02 with a coefficient of variation (C.V.) of 0.30%. For NORVER, the average ($n=6$) ratio of the (*R*)/(*S*) peak areas was 1.02 ± 0.03 with a C.V. of 0.49%. Therefore, the relative enantiomeric ratio can be determined directly from the ratio of the peak areas corresponding to the respective enantiomers.

Clinical samples

Six clinical samples were analyzed using the coupled column method and the results are reported in Table III. The samples came from a study which treated hypertensive subjects with a 240-mg sustained release dose of racemic VER every 12 h for four weeks [11,12]. The samples were drawn every two weeks at 12 h after the last dose.

The total concentration of racemic VER ([VER]) and racemic NORVER ([NORVER]) were first determined using the Hisep column. A second analysis was then performed using the coupled Hisep–CHIRAL AGP-CSP system to determine the area percentage of (−)-(*S*)-VER [%(*S*)-VER], (+)-(*R*)-

TABLE III

PLASMA CONCENTRATION OF THE ENANTIOMERS OF VERAPAMIL (VER) AND NORVERAPAMIL (NORVER) FOLLOWING THE ADMINISTRATION OF RACEMIC VERAPAMIL (240 mg, SUSTAINED RELEASE, EVERY 12 h)

Patient No.	(−)-(*S*)-VER (ng/ml)	(+)-(*R*)-VER (ng/ml)	(*S*)-NORVER (ng/ml)	(*R*)-NORVER (ng/ml)
1	24.4	150.2	24.4	86.4
2	15.5	55.1	19.4	43.2
3	25.3	132.9	23.9	75.7
4	20.4	72.4	25.5	65.5
5	94.5	402.7	114.1	266.3
6	34.3	229.3	84.5	253.5

VER [%(*R*)-VER], (*S*)-NORVER [%(*S*)-NORVER] and (*R*)-NORVER [%(*R*)-NORVER]. The total amounts of each isomer were then determined using the following equations:

total (−)-(*S*)-VER= [VER]×%(*S*)-VER
total (+)-(*R*)-VER= [VER]×%(*R*)-VER
total (*S*)-NORVER= [NORVER]×%(*S*)-NORVER
total (*R*)-NORVER= [NORVER]×%(*R*)-NORVER

CONCLUSION

One of the major problems encountered in the application of HPLC CSPs to the analyses of biological samples is the coelution of the enantiomers of the parent compound and metabolite(s). One solution to this problem is the use of achiral–chiral coupled column systems in which the parent compounds and metabolite(s) are separated from interferences from the biological matrix and from each other on the achiral phase and then selectively switched to the CSP for enantiomeric analysis. The assay described in this paper is an example of this approach.

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