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Direct determination of the enantiomeric ratio of verapamil, its major metabolite norverapamil and gallopamil in plasma by chiral high-performance liquid chromatography☆

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ABSTRACT

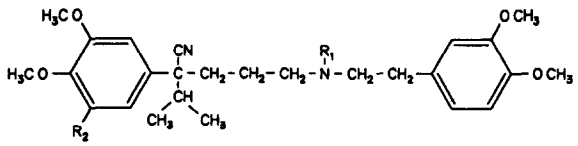
Two methods for the determination of the enantiomeric ratio of verapamil in plasma by high-performance liquid chromatography have been developed. On an α_1-acid glycoprotein chiral stationary phase (Chiral-AGP) verapamil was separated after the acetylation of the main metabolite norverapamil, which interferes with the resolution of verapamil. On an amylose tris-3,5-dimethylphenylcarbamate column (Chiralpak AD) verapamil and norverapamil were determined simultaneously without prior derivatization. Gallopamil was separated on both columns under similar conditions.

INTRODUCTION

The chiral calcium antagonist verapamil (Fig. 1) is used in therapy as its racemate, although the enantiomers differ in their bioavailability [1] as well as in their pharmacokinetic [2] and pharmacological effects [3]. The pharmacologically more potent *S*-(−)-verapamil is preferentially eliminated.

The calcium antagonist gallopamil (Fig. 1), a methoxy derivative of verapamil, is also administered as its racemate. Like *S*-(−)-verapamil, *S*-(−)-gallopamil is the pharmacologically more potent enantiomer [4]. Whereas the first-pass metabolism of verapamil is stereoselective that of gallopamil is not [4]. For these studies pure enantiomers or pseudoracemates were administered. For studies in which the enantiomeric ratio of clinically administered racemates in body fluids is analysed simple assays are required.

Verapamil can be separated on the Chiral AGP column [5]. It is not possible, however, to determine the ratio of the enantiomers in plasma, because the peaks of verapamil and its main metabolite norverapamil overlap. Chu and Wainer [6] reported a coupled achiral–chiral high-performance liquid chromatographic (HPLC) pro-



	R_1	R_2
verapamil	$-CH_3$	$-H$
norverapamil	$-H$	$-H$
gallopamil	$-CH_3$	$-OCH_3$

Fig. 1. Structures of verapamil, norverapamil and gallopamil.

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cedure to solve this problem. Recently, a second coupled achiral–chiral HPLC procedure, using a chiral ovomucoid column, was published [7].

In order to reduce the cost of the apparatus, we developed a new method to determine the enantiomeric ratio of verapamil on the Chiral-AGP column. Norverapamil, a secondary amine, was acetylated to its N-acetyl derivative [8], which elutes earlier, while the tertiary amine verapamil was not affected. Other known metabolites, such as D 617 and D 620, do not interfere either before or after acetylation.

Furthermore, this paper describes a direct method for the simultaneous determination of the enantiomeric ratios where the peaks of the enantiomers of verapamil and its metabolite norverapamil do not overlap. This assay was also used to determine gallopamil in plasma.

EXPERIMENTAL

Chemicals

Verapamil hydrochloride, norverapamil hydrochloride, gallopamil hydrochloride, *R*-(+)-gallopamil hydrochloride and *S*-(−)-gallopamil hydrochloride were obtained from Knoll (Ludwigshafen, Germany). *R*-(+)-Verapamil hydrochloride and *S*-(−)-verapamil hydrochloride were obtained as published [9]. Acetonitrile, methanol, 2-propanol and *n*-hexane were LiChrosolv reagents from Merck (Darmstadt, Germany). The other chemicals used were of analytical grade.

Apparatus

The HPLC system consisted of an L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany) with a Spectroflow 980 fluorescence detector (Applied Biosystems, Weiterstadt, Germany) and a D-2000 Chromato-Integrator (Merck-Hitachi).

The chiral columns were a 100 mm × 4 mm I.D. Chiral-AGP column with a 10 mm × 3 mm I.D. Chiral-AGP guard column (ChromTech, Norsborg, Sweden) and a 250 mm × 4.6 mm I.D. Chiralpak AD column with a 50 mm × 4.6 mm I.D. Chiralpak AD guard column (Baker, Gross-Gerau, Germany). For monitoring of the acetylation of norverapamil, a LiChrospher 60 RP-select B column, 5 μm, 125 mm × 4.0 mm I.D, with a LiChrospher 60 CN precolumn, 10 μm, 30 mm × 4.0 mm I.D., (both Merck), were used.

Chromatographic conditions

The detector was set at an excitation wavelength of 223 nm without using an emission filter. The mobile phase used for the Chiral-AGP column consisted of 0.01 *M* phosphate buffer (pH 7.0)–acetonitrile (90:10, v/v) [5] at a flow-rate of 0.9 ml/min. *n*-Hexane–2-propanol (90:10, v/v) with 0.1% diethylamine at a flow-rate of 1.0 ml/min was used as the mobile phase for the Chiralpak AD column. Methanol–0.1 *M* ammonium acetate (55:45, v/v) [10] at a flow-rate of 1.0 ml/min was used for the monitoring of the acetylation on the LiChrospher RP-select B column. The retention times were 6.0 min for norverapamil, 15.4 min for N-acetylnorverapamil and 10.7 min for verapamil.

Sample preparation

For the determination of verapamil on the Chiral-AGP column, 200 μl of 2 *M* sodium hydroxide and 4.0 ml of diethyl ether were added to 1 ml of plasma. The sample was vortexed for 30 s, mixed for 10 min and centrifuged for 10 min at 2500 *g*. The organic phase was transferred to a clean tube, and 200 μl of an acetic anhydride solution (2.5 μl of acetic anhydride in 200 μl of anhydrous diethyl ether) were added. The tube was vortex-mixed for 10 s and kept at room temperature for 10 min. Evaporation of the organic phase to dryness under a stream of nitrogen yielded a residue, which was rinsed from the walls of the flask with 1.0 ml of diethyl ether. This solution was evaporated carefully to dryness again. The resulting residue was dissolved in 100 μl of the mobile phase. A 50-μl aliquot was injected into the column. Previous experiments had confirmed that the N-acetylation of norverapamil under these conditions proceeded completely in the range 20–500 ng of norverapamil hydrochloride per ml of plasma. The concentration of the tertiary base verapamil was not affected by this

reaction step. Additionally, the complete acetylation of norverapamil was proved by chromatography on the Chiral-AGP column: after acetylation, the peaks of the norverapamil enantiomers (17.6 and 20.3 min) were shifted in the chromatogram to much lower retention times (*ca.* 5.2 and 6.1 min) with partial resolution of the N-acetylnorverapamil enantiomers.

For the determination of verapamil, norverapamil and gallopamil on the Chiralpak AD column, 1 ml of 0.9% sodium chloride solution, 120 μl of 2 *M* sodium hydroxide and 5.0 ml of *n*-hexane were added to 1 ml of plasma. After vortex-mixing for 30 s the sample was mixed for 10 min and centrifuged for 20 min at 2500 *g*. The organic phase was evaporated to dryness. After repeating the extraction procedure with 4.0 ml of *n*-hexane, the residue was rinsed from the walls with 1.0 ml of *n*-hexane and evaporated carefully to dryness. This residue was dissolved in 50 μl of 2-propanol. A 25-μl sample was injected into the column.

Enantiomeric elution order

The elution order of verapamil and gallopamil enantiomers was determined by chromatography of the pure enantiomers. In order to determine the elution order of norverapamil, both enantiomers of verapamil were incubated separately with rat liver microsomes from male Sprague–Dawley rats as published [11,12]. The extraction procedure was similar to that used for the Chiralpak AD column. Furthermore, plasma samples from patients who received *R*-(+)-verapamil were determined.

Calibration and reproducibility of chiral analysis

For calibration on the Chiralpak AD column, pure enantiomers of verapamil were combined to obtain four mixtures of known enantiomeric ratios in the range from 4:1 to 1:1 (*R:S*). From each stock solution, concentrations (50, 100, 250 and 500 ng of verapamil hydrochloride per ml of plasma) were prepared and analysed. The results are presented in Table I. Similar experiments were performed for the Chiral-AGP column. Additionally, plasma was spiked with different concentrations of racemic verapamil hydrochloride (20–500 ng/ml of plasma), racemic norverapamil hydrochloride (20–500 ng/ml of plasma) and racemic gallopamil hydrochloride (30–200 ng/ml of plasma), respectively, and analysed on different days for enantiomeric ratios. Each concentration was analysed two to four times.

TABLE I

CALIBRATION AND REPRODUCIBILITY OF THE DETERMINATION OF VERAPAMIL ENANTIOMERS AFTER EXTRACTION

Content of *R*-(+)-verapamil (%)	*R*-(+)-Verapamil analysed (mean ± S.D., $n = 4$) (%)
80	78.6 ± 2.4
70	70.2 ± 3.2
60	61.6 ± 1.2
50	51.5 ± 1.1

RESULTS AND DISCUSSION

The results from the chromatography of verapamil, norverapamil and gallopamil on the Chiral-AGP column and on the Chiralpak AD column are presented in Table II. The direct separation of verapamil in plasma samples on a Chiral-AGP column was achieved after acetylation of norverapamil to its N-acetyl derivative. Gallopamil was resolved on the Chiral-AGP column under similar conditions.

The separation of verapamil on a Chiralpak AD column has been reported previously. *n*-Hexane–2-propanol (90:10. v/v) was used as the mobile phase [13]. By addition of diethylamine, suppression of the peak tailing and complete separation of the metabolite norverapamil were achieved. Thus, verapamil and norverapamil enantiomers could be determined simultaneously in plasma samples. Other known metabolites do not overlap with the peaks of verapamil and norverapamil. On the Chiralpak AD column, plasma samples of gallopamil enantiomers were also determined.

For samples that were analysed on the Chiralpak AD column, the apolar solvent *n*-hexane was

TABLE II

SEPARATION OF RACEMATES

Compound	Column	Capacity factor		Selectivity (α)	Resolution factor (R)
		k'_1 (first peak)	k'_2 (second peak)		
Verapamil	Chiral-AGP	9.09	12.83	1.30	2.15
Gallopamil	Chiral-AGP	8.72	14.43	1.65	2.77
Verapamil	Chiralpak AD	1.61	2.08	1.29	2.36
Norverapamil	Chiralpak AD	4.03	4.51	1.12	1.42
Gallopamil	Chiralpak AD	1.79	2.49	1.39	2.82

chosen in order to obtain extracts without residual water, which could damage the column.

Enantiomeric elution order

The elution order of verapamil and gallopamil was determined after injection of the pure enantiomers. On the Chiral-AGP column a reversed elution order was obtained.

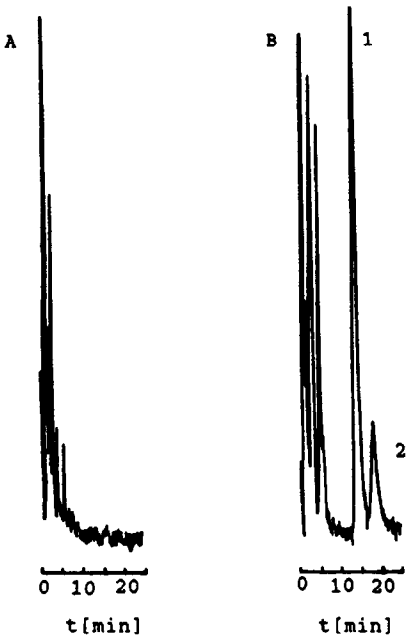

Fig. 2. Chromatograms of (A) blank plasma and (B) a plasma sample after oral administration of 240 mg of verapamil hydrochloride. Peaks: 1 = *R*-(+)-verapamil; 2 = *S*-(−)-verapamil. Chromatographic conditions: column, Chiral-AGP column with Chiral-AGP guard column; mobile phase, 0.01 *M* phosphate buffer (pH 7.0)–acetonitrile (90:10, v/v); flow-rate, 0.9 ml/min.

To determine the elution order of norverapamil enantiomers, which were not available, verapamil enantiomers were incubated with rat liver microsomes, extracted and chromatographed on the Chiralpak AD column. The first norverapamil peak originated from *S*-(−)-verapamil, the second norverapamil peak from *R*-(+)-verapamil. Plasma samples obtained after oral administration of *R*-(+)-verapamil confirmed these results. On the Chiral-AGP column the reversed elution order was obtained. Because inversion of enantiomers is unlikely to occur during N-demethylation it is concluded that *R*-norverapamil elutes first on a Chiral-AGP column and *S*-norverapamil elutes first on a Chiralpak AD column.

Selectivity

Chromatograms of drug-free plasma samples and of samples after oral administration of verapamil and of (R/[$^2H_2$]S) pseudoracemic gallopamil, respectively, are shown in Fig. 2 (Chiral-AGP column) and in Fig. 3 (Chiralpak AD column). No interfering plasma components were observed.

Recovery, calibration and reproducibility

The recoveries after extraction with *n*-hexane were 65.6 ± 9.5% for verapamil, 63.0 ± 8.4% for gallopamil and 54.1 ± 3.6% for norverapamil. For calibration of the assays, pure enantiomers of verapamil were combined in order to obtain mixtures with known enantiomeric ratios. Plasma was spiked with these mixtures and determined as described above. Linear cali-

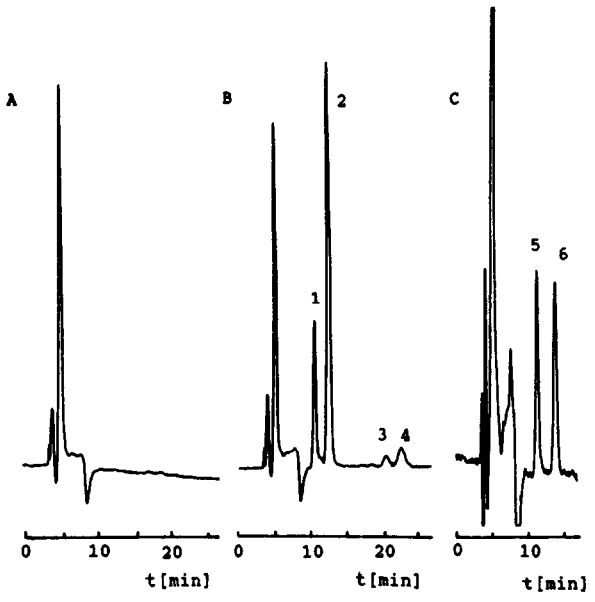

Fig. 3. Chromatograms of (A) blank plasma, (B) a plasma sample after oral administration of 480 mg of verapamil hydrochloride and (C) a plasma sample after oral adminstration of 50 mg of (*R*/[$^2H_2$]*S*) pseudoracemic gallopamil hydrochloride. Peaks: 1 = *S*-(−)-verapamil; 2 = *R*-(+)-verapamil; 3 = *S*-norverapamil; 4 = *R*-norverapamil; 5 = *S*-(−)-gallopamil; 6 = *R*-(+)-gallopamil. Chromatographic conditions: column, Chiralpak AD column with Chiralpak AD guard column; mobile phase, *n*-hexane–2-propanol–diethylamine, (90:10:0.1, v/v); flow-rate, 1.0 ml/min.

bration curves for each enantiomer on both columns were obtained. Data for the Chiralpak AD column are shown in Table I. The correlation coefficients were of the order of 0.982.

To determine the reproducibility, plasma was spiked with different concentrations of the racemates of verapamil, norverapamil and gallopamil, respectively, and analysed as described above. Table III summarizes the good reproducibility data of the enantiomeric ratios.

In preliminary experiments, plasma levels of both enantiomers were determined directly on the Chiralpak AD column by using an analogue of verapamil as the internal standard. However, assays were more accurate when enantiomer concentrations were calculated from the enantiomeric ratio and the total concentration was determined more precisely on a reversed-phase column.

Limit of quantification

The limit of quantification for the racemates on both chiral stationary phases was 20 ng/ml verapamil hydrochloride, 50 ng/ml norverapamil hydrochloride and 30 ng/ml gallopamil hydrochloride at a signal-to-noise ratio of 5:1. At least for verapamil and norverapamil, these limits are sufficient for pharmacokinetic studies.

Other metabolites of verapamil, such as D 617 and D 620 [14], are not eluted from the Chiralpak AD column and therefore do not interfere with the determination. The phenolic metabolites PR 23 and PR 24 [14] were not found to be present in plasma after oral administration of verapamil. All four compounds had been available as racemates. D 617 and D 620 could also be separated into their enantiomers on the Chiral-AGP column, and PR 23 and PR 24 on both columns.

TABLE III

REPRODUCIBILITY DATA

Values in parentheses are coefficients of variation (%).

Racemate	Column	*n*	Mean determined enantiomeric ratio (%)	
			R	*S*
Verapamil	Chiral-AGP	10	48.9 (3.8)	51.1 (3.6)
Verapamil	Chiralpak AD	24	48.6 (5.2)	51.4 (4.9)
Norverapamil	Chiralpak AD	20	51.0 (4.5)	49.0 (4.7)
Gallopamil	Chiralpak AD	21	50.2 (7.4)	49.8 (7.5)

CONCLUSION

The methods described here are selective, convenient and suitable for the analysis of the enantiomeric ratios of verapamil, norverapamil and gallopamil in plasma samples.

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