Column-Switching High-Performance Liquid Chromatography for On-Line Simultaneous Determination and Resolution of Enantiomers of Verapamil and Its Metabolites in Plasma

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An on-line simultaneous assay for the enantiomers of verapamil (VA) and its three metabolites in plasma was developed with column-switching HPLC. This system consists of an ovomucoid protein chiral stationary phase coupled to an achiral reversed-phase column via a dilution tube and a trapping column. The reversed-phase column was used to separate and quantitate VA, its metabolites, and internal standard, without interference from plasma components. Then each of the eluates containing VA and its metabolites was selectively switched into a sampling loop, and the samples were transferred successively to the trapping column after dilution with a new mobile phase. After concentration on the trapping column, each sample was passed to the ovomucoid column, where the enantiomers were resolved and quantitated. The mobile phases for both HPLC columns were independently optimized and the diffusion of the sample during column switching was minimized. This method was shown to be efficient and reliable.

KEY WORDS: high-performance liquid chromatography (HPLC); column switching; on-line determination and enantiomeric resolution; ovomucoid column; verapamil; verapamil metabolites.

INTRODUCTION

Verapamil (VA) is a calcium antagonist with antiarrhythmic, antianginal, and antihypertensive properties (1–3). VA contains an asymmetric carbon and the preparation used clinically is a racemic mixture. *In vitro* experiments and studies in intact animals have demonstrated that the enantiomers of VA differ in their pharmacodynamic effects and pharmacokinetic dispositions, with the (−)-isomer being much more potent than the (+)-isomer (4–7). To investigate the stereoselectivity of VA pharmacokinetics, the enantiomers of VA needed to be resolved. This problem has been solved by the development of efficient high-performance liquid chromatography (HPLC) chiral stationary phases (8–10). However, conventional methods for clinical measurements of the isomers require laborious sample pretreatment. To overcome these problems, Wainer *et al.* have reported a coupled achiral–chiral HPLC system (11–13): the achiral HPLC system is used to determine the total enantiomer content followed by selective switching of the eluate to a chiral column for enantiomeric separation. In their system, however, similar mobile phases have to be used for the two HPLC systems because the solvent used for achiral HPLC could flow into the chiral HPLC system, so that neither chromatography can necessarily be performed under the optimum conditions. Recently, we have developed a new column-switching HPLC system for the analysis of VA (14), which employs a dilution tube and a trapping column between the achiral and the chiral HPLC systems. The eluate from the achiral column is diluted to minimize the influence of the mobile phase of the achiral column, followed by the chiral HPLC after concentration of the diluted eluate on the trapping column. This system allowed both achiral and chiral chromatography steps to be performed under optimum conditions. We have now performed simultaneous determination and optical resolution of VA and its metabolites, each of which has a chiral center (Fig. 1), in plasma by using an improved HPLC system.

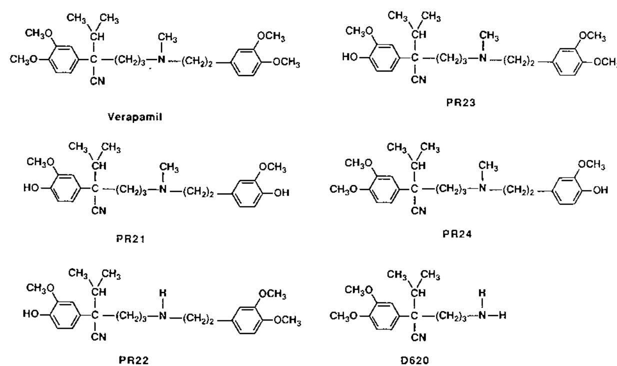

Fig. 1. Chemical structures of verapamil and its metabolites.

EXPERIMENTAL

Reagents

Verapamil and five metabolites (PR21, PR22, PR23, PR24, D620) were synthesized in our laboratories. *n*-Propyl *p*-hydroxybenzoate, the internal standard (IS), was from Kanto Chemical Co., Inc. (Tokyo). Trifluoroacetic acid

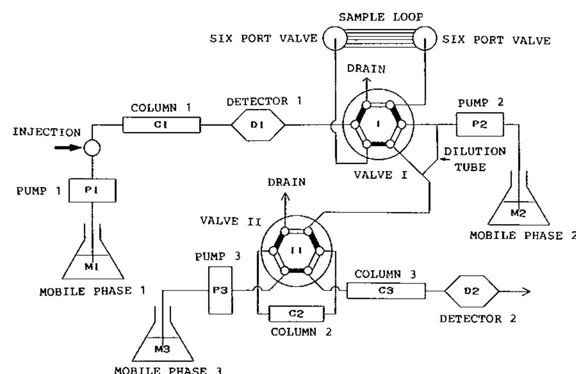

Fig. 2. Schematic diagram of the new column-switching system. Dilution ratio can be varied by changing the length of the dilution tube.

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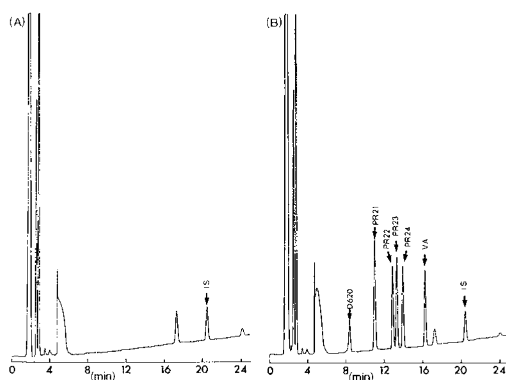

Fig. 3. Representative chromatograms on the achiral reversed-phase column after extraction. (A) Blank plasma; (B) plasma sample spiked with 100 ng/ml each of verapamil and its metabolites. See the text for the chromatographic conditions.

(TFA) of HPLC grade was purchased from Pierce (Illinois). Dipotassium hydrogenphosphate, potassium dihydrogenphosphate, phosphoric acid, ethyl acetate, and sodium hydroxide solution of analytical reagent grade were obtained from Wako Pure Chemicals (Osaka, Japan). HPLC-grade acetonitrile, ethanol and methanol, and distilled water were used.

Apparatus

The HPLC system consisted of four high-pressure pumps (Shimadzu LC-9A, Shimadzu Inc., Kyoto, Japan) [P1 (two pumps), P2, P3], a system controller (Shimadzu SCL-6B), an auto injector (Shimadzu SIL-6B) with a cooling system (WIG-7000A, Ishido Inc., Chiba, Japan), and five six-port switching valves (Rheodyne Inc., CA). The switching diagram is shown in Fig. 2 and is explained in the column-switching procedure section. The HPLC analytical columns were a 150-mm × 4.6-mm-I.D. Inertsil ODS-2 column (C1) (Gasukuro Kogyo Inc., Tokyo) to determine VA and its five metabolites, a 10-mm × 4.0-mm-I.D. Ultron ES-OVMG (C2) as a trapping column, and a 150-mm × 4.6-mm-I.D. Ultron ES-OVM (C3) column (Shinwa Kako Co., Ltd., Kyoto) to resolve their enantiomers. The three columns were connected through two Rheodyne switching valves (I, II). Valve I was equipped with two six port valves for 2-ml sample loops.

Table I. The Extraction Efficiencies and Their Reproducibilities for Verapamil and Its Metabolites from a Plasma Sample

	PAR[a]	SD	CV (%)	Recovery (%)
D620	0.568	0.038	6.67	34.1
PR21	1.588	0.043	5.50	67.1
PR22	1.232	0.055	3.47	68.1
PR23	1.261	0.050	4.33	76.0
PR24	1.259	0.066	4.01	74.5
VA	1.378	0.070	5.07	89.2

[a] Peak area ratio.

Table II. The Equations and the Correlations Describing the Curves of Verapamil and Its Metabolites

D620	$r = 0.9987$	$Y = -1.7922e - 2 + 6.1290e - 3X$
PR21	$r = 0.9978$	$Y = -9.2991e - 2 + 1.5344e - 2X$
PR22	$r = 0.9990$	$Y = -2.5055e - 2 + 1.3962e - 2X$
PR23	$r = 0.9988$	$Y = -2.5145e - 2 + 1.3161e - 2X$
PR24	$r = 0.9988$	$Y = -3.5275e - 2 + 1.3128e - 2X$
VA	$r = 0.9982$	$Y = 1.5881e - 2 + 1.2754e - 2X$

Extraction Procedure for the Recovery Study

Human plasma (1 ml) spiked with known amounts of verapamil and its five metabolites was transferred to a glass screw-capped tube, which contained 0.1 ml of 1 *N* sodium hydroxide solution and 2 ml of ethyl acetate. The mixture was shaken for 15 min and centrifuged at 3000*g* for 5 min. The upper phase was collected in another glass screw-cap tube, which contained 0.5 ml of a 1% phosphoric acid aqueous solution. The mixture was shaken for 15 min and centrifuged at 3000*g* for 5 min. The lower phase was collected and 0.45 ml of it was mixed with 0.05 ml of the internal standard solution (400 ng/ml in methanol), then 150 μl of this solution was injected into the column-switching HPLC system.

HPLC Conditions

Samples were detected by measuring the absorption at 280 nm (D1) in the achiral HPLC (a great rise of baseline was observed at 230 nm owing to a gradient mode) and at 230 nm (D2) in the chiral HPLC. Mobile phase 1 (M1) consisted of acetonitrile/water/TFA (25:75:0.1–50:50:0.1, v/v/v) and the gradient was linear for 25 min. Mobile phase 2 (M2) was composed of 5 m*M* potassium phosphate buffer (pH 7.5). Mobile phase 3 (M3) was prepared by mixing potassium dihydrogenphosphate with ethanol and water. M1, M2, and

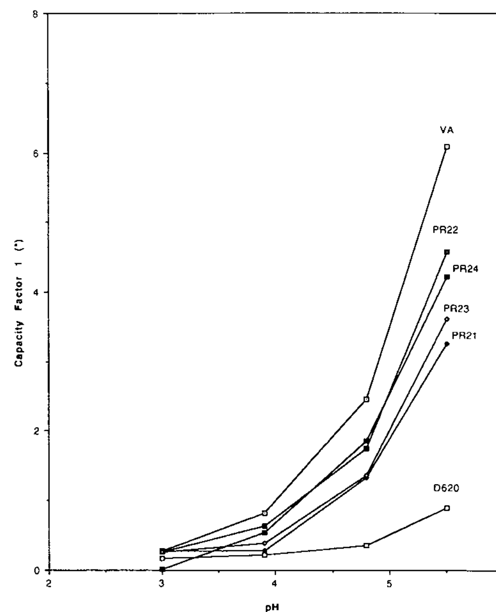

Fig. 4. Effect of pH on the retention for the first-eluted enantiomer. See the text for other conditions.

Table III. Effects of pH on the Separation Factor of Verapamil and Its Metabolites.

	pH				
	3.0	3.9	4.8	5.5	6.2
D620	1	1	1	1	1.28
PR21	1	4.04	4.04	4.92	—
PR22	1	1	1	1	—
PR23	1	2.11	1.91	1.83	—
PR24	1	1.49	1.71	2.03	—
VA	1	1.22	1.37	1.47	—

M3 were thoroughly degassed before use and were delivered by pump 1 at a flow rate of 1.0 ml/min, pump 2 at a flow rate of 4.0 ml/min, and pump 3 at a flow rate of 1.0 ml/min, respectively. All operations were carried out at ambient temperature.

Column-Switching Procedure

Samples were injected onto C1, and VA and the five metabolites were quantified with the aid of the IS. The six eluates containing the compounds were selectively switched into different sample loops by monitoring at D1, via valve I and the six-port valve. Then M2 was allowed to flow and wash the first of the above eluates to C2 (trapping column) for 5 min, via both a sample loop and a dilution tube (flow rate ratio, sample loop/dilution tube = 1/9) and the first eluate was swept by flowing M3 from C2 to C3 (ovomucoid column), where enantiomeric separation was performed, by switching valve II. All procedures were manual operations. In the same way, enantiomeric resolutions were performed successively for the remaining five eluates.

Table IV. Effects of Ethanol Concentration on the Separation Factor of Verapamil and Its Metabolites

	3%	5%	7%	10%	12%
D620	1.23	1	1	1	1
PR21	4.40	4.04	3.70	3.09	3.03
PR22	1.09	1	1	1	1
PR23	2.29	1.91	1.68	1.44	1
PR24	1.96	1.71	1.57	1.40	1
VA	1.46	1.37	1.31	1.19	1

RESULTS AND DISCUSSION

Many HPLC methods for the simultaneous determination of VA and its metabolites in plasma have been reported (15–22). In this study, the separation of VA and its five metabolites from plasma components with adequate sharpness of their peaks was achieved using paired-ion chromatography with a linear gradient from 0.1% TFA in acetonitrile/water (25:75, v/v) to acetonitrile/water (50:50, v/v) over 25 min. An extracted blank plasma sample containing the IS is shown in Fig. 3A. In Fig. 3B a representative chromatogram is shown of a plasma sample spiked with 100 ng/ml each of VA and five metabolites. No interference from endogenous plasma constituents was observed, although there were some unknown background peaks. The extraction efficiencies of the six compounds were assessed by comparing the peak area ratios of chromatograms obtained from plasma extracts with those obtained from standard solutions of each compound at the same injection amount (Table I). The coefficient of variation of this method was about 5% (Table I). Table II provides the six equations describing the plots of peak area ratios as a function of concentration over the concentration range of VA or its six metabolites from 10 to 200 ng/ml. Correlations for this method were good, with $r > 0.99$

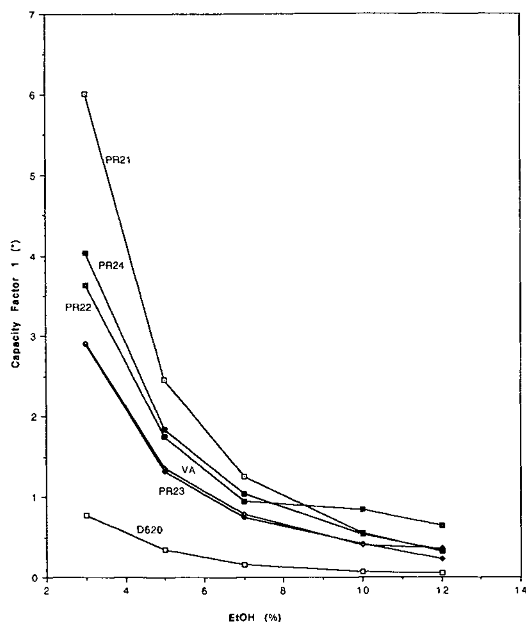

Fig. 5. Effect of ethanol concentration on the retention, for the first-eluted enantiomer. See the text for other conditions.

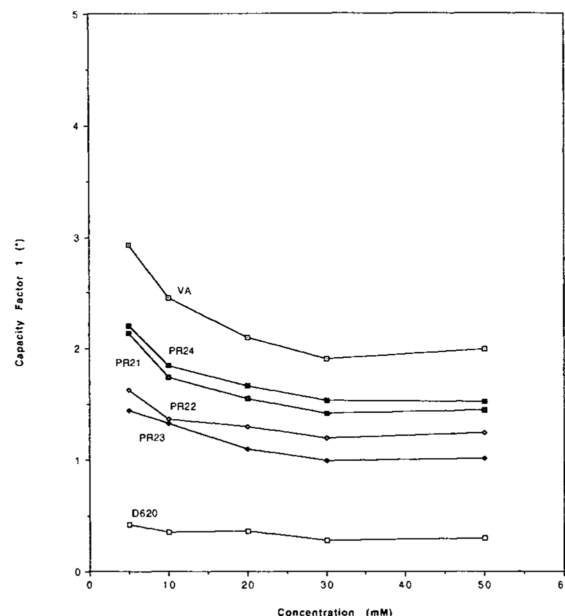

Fig. 6. Salt concentration effect on the retention, for the first-eluted enantiomer. See the text for other conditions.

Table V. Effects of Salt Concentration on the Separation Factor of Verapamil and Its Metabolites

	5 m*M*	10 m*M*	20 m*M*	30 m*M*	50 m*M*
D620	1	1	1	1	1
PR21	4.91	4.04	3.62	3.60	3.44
PR22	1	1	1	1	1
PR23	1.95	1.91	1.87	1.90	1.86
PR24	1.94	1.71	1.56	1.56	1.49
VA	1.45	1.37	1.30	1.31	1.27

for all six compounds. VA and its five metabolites could be determined simultaneously by the present method.

For enantiomer separation, we chose a chiral recognition column, with conjugated ovomucoid as the ligand, developed by Miwa *et al.* (23–25) for the optical resolution of drugs without derivarization. The ovomucoid column (C3) was combined with C2 as a guard column for direct samples injection. Figure 4 shows the effects of the pH of the mobile phase (M3) on the capacity factors (only first-eluted enantiomer) with 10 m*M* potassium phosphate buffer–ethanol (90:10, v/v) and Table III summarizes the influence of the pH of this mobile phase on the separation factor (α). The capacity factors increased with increasing pH value. This may be the result of increasing ionic interaction between ovomucoid and the drugs, as ovomucoid has more negative charges with increasing pH value in this range (pH 4–6) (26) and VA is positively charged (27). However, the separation factors were only slightly improved by increasing the pH value (Table III). The effects of the concentration of ethanol in the mobile phase (10 m*M* potassium phosphate buffer, pH 4.8) on the capacity factors (only first-eluted enantiomer) and separation factors (α) is demonstrated in Fig. 5 and Table IV. With an increase in the concentration of ethanol, the capacity factors and separation factors decreased. This result implies that hydrophobic interactions are involved in the retention of the solute on the ovomucoid column. The effect on the separation factor of the concentration of ethanol is greater than the influence of pH. The capacity factors and separation factor were also influenced by the salt concentration of the mobile phase (Fig. 6 and Table V). VA and its metabolites exhibited a strong interaction with the ovomucoid column at low salt concentration. This result suggests that coulombic interactions may be involved in the interaction between ovomucoid and the compounds, but this interaction seems to be less important for the enantioselectivity.

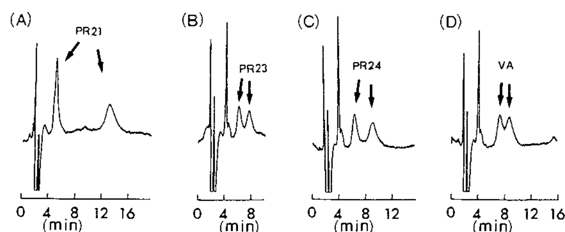

Fig. 7. Representative chromatograms obtained at detector 2 by using the column-switching system. (A) PR21; (B) PR23; (C) PR24; (D) VA resolved optically with C3. See the text for the chromatographic conditions.

Table VI. Comparison of Capacity Factors and Separation Factors After Column Switching and After Direct Injection onto the Ovomucoid Column

	After column switching			Direct injection		
	k_1	k_2	α	k_1	k_2	α
VA	3.05	3.82	1.25	0.55	0.66	1.19
PR21	1.79	6.27	3.50	0.41	1.27	3.09
PR22	2.53	2.53	1	0.85	0.85	1
PR23	2.38	3.07	1.29	0.40	0.57	1.44
PR24	2.79	4.07	1.46	0.54	0.76	1.40
D620	0.71	0.71	1	0.06	0.06	1

VA and its metabolites could be resolved stereochemically by using the ovomucoid column.

Next we performed the on-line determination and enantiomeric resolution of VA and its three metabolites (PR21, PR23, PR24) by using the column-switching HPLC system. VA and its metabolites extracted from plasma were determined by the achiral HPLC, then each of the six compounds was successively trapped on C2 after dilution 1:9 with M2 and, finally, resolved by the chiral HPLC. Representative chromatograms obtained at D2 are presented in Fig. 7. D620 and PR22 were not resolved optically by the mobile phase with 10 m*M* potassium phosphate buffer (pH 4.8)–ethanol (90:10, v/v) as M3. But VA and the other three metabolites (PR21, PR23, PR24) were resolved by using this mobile phase. The coefficient of variation (CV) for the retention time of VA (first eluted) was 1.8% (n = 8) and the CV value for the enantiomeric ratio of a VA standard was 2.1% (n = 8). The capacity factors after column switching and after direct injection onto the ovomucoid column are compared in Table VI. When the column-switching method was used, the capacity factors increased in comparison with direct injection, possibly because the compounds were not eluted until the mobile phase (almost wholly M2) in C2 was replaced with M3 and chiral HPLC attained equilibrium. Further VA and its three metabolites (PR21, PR23, PR24) were not eluted when M3 (10 m*M* potassium phosphate buffer, pH 4.8) contained less than 8% ethanol, although all compounds were eluted with 3% ethanol after direct injection (Table IV). The interaction trapping the compounds on C2 is apparently stronger than that causing optical resolution in the case of direct injection. However, the separation factors (α) were similar between column switching and direct injection (Table V).

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