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On-line determination and resolution of verapamil enantiomers by high-performance liquid chromatography with column switching

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ABSTRACT

High-performance liquid chromatography (HPLC) with column switching was applied to the on-line determination and resolution of the enantiomers of verapamil (VA). This system employs an achiral reversed-phase column coupled to a chiral ovomucoid column via a dilution tube and a trapping column. The reversed-phase column was used to separate VA from the plasma components and impurities, and to determine the total VA concentration using an internal standard method. The eluate containing VA was selectively transferred to the trapping column after suitable dilution with a new mobile phase and VA was concentrated on the trapping column, then passed to the ovomucoid column, where the resolution of enantiomers was performed. It was possible with this system to select independently the optimum mobile phases for both HPLC columns owing to the introduction of the dilution tube and trapping column between them. This method has advantages over the usual HPLC methods in that it is rapid, simple and highly sensitive.

INTRODUCTION

Verapamil (VA), an inhibitor of membrane transport of calcium, is used for the therapy of agina and arrhythmia [1]. VA has a chiral centre (Fig. 1), but is administered as a racemic mixture. However, the two enantiomers of VA differ in pharmacology and pharmacokinetics [2–5]. While methods for the optical resolution of VA are adequate for the analysis of VA standards, they cannot be directly used to determine VA in clinical samples, such as serum, owing to the presence of interfering substances. The conventional methods are also laborious and time consuming, requiring pretreatments

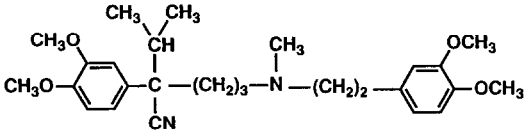

Fig. 1. Structure of verapamil.

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such as fractionation or desalting. Hence, a method is still required to measure the isomers in clinical samples accurately and rapidly.

Recently, Wainer and co-workers [6,7] reported a simple and rapid high-performance liquid chromatographic (HPLC) system for the direct resolution of the stereoisomers of several drugs. This system involved coupling an achiral column to a chiral column by using a column-switching technique. However, similar mobile phases had to be used for the two columns, so that each chromatographic step was not necessarily performed under the optimum conditions, and the peaks of enantiomers were broadened owing to diffusion of the sample during the column-switching procedures.

We have attempted to overcome these problems through the use of an improved column-switching HPLC system. The system was adapted for the determination of VA by coupling an achiral column to an ovomucoid column. The ovomucoid column contains ovomucoid protein as the stationary phase immobilized onto aminopropyl-silica gel [8,9]. The enantiomers of VA can be resolved by using this column with a reversed-phase solvent system. To prevent the mobile phase of the first column from flowing into the second column, we interposed a dilution tube and trapping column between the two columns, whereby the mobile phase of the first column was exchanged completely to the most favourable mobile phase for the second column. The potential usefulness of this technique is demonstrated and discussed.

EXPERIMENTAL

Reagents

Verapamil hydrochloride (VA · HCl) was supplied by Knoll Pharmaceuticals (Ludwigshafen, Germany), sodium 1-pentanesulphonate was purchased from Aldrich (Milwaukee, WI, U.S.A.), *n*-propyl *p*-aminobenzoate was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and dipotassium hydrogenphosphate, potassium dihydrogenphosphate and phosphoric acid of analytical-reagent grade were obtained from Wako (Osaka, Japan). HPLC-grade acetonitrile, ethanol and tetrahydrofuran were used. Distilled water was purified with a Milli-Q system (Millipore).

Apparatus

The HPLC system consisted of three high-pressure pumps (Model LC-9A; Shimadzu, Kyoto, Japan) (P1–P3), a system controller (Shimadzu SCL-6B), two variable-wavelength UV detectors (Shimadzu SPD-6A) (D1, D2) and three six-port switching valves (Rheodyne, Cotati, CA, U.S.A.). The switching diagram is given in Fig. 2 and is explained under *Procedure*. The HPLC columns were a 150 × 4.6 mm I.D. Inertsil ODS-2 column (C1) (Gasukuro Kogyo, Tokyo, Japan) to determine VA, a 10 × 4.0 mm I.D. Ultron ES-OVMG (C2) column as a trapping column and a 150 × 4.6 mm I.D. Ultron ES-OVM (C3) (Shinwa Kako, Kyoto, Japan) column to resolve the enantiomers. The three columns were connected through two Rheodyne switching valves (I and II). Valve I was equipped with a 2-ml sample loop.

Samples

n-Propyl *p*-aminobenzoate was used as an internal standard (I.S.). Sample solutions were prepared by dissolving known amounts of VA and I.S. in water or

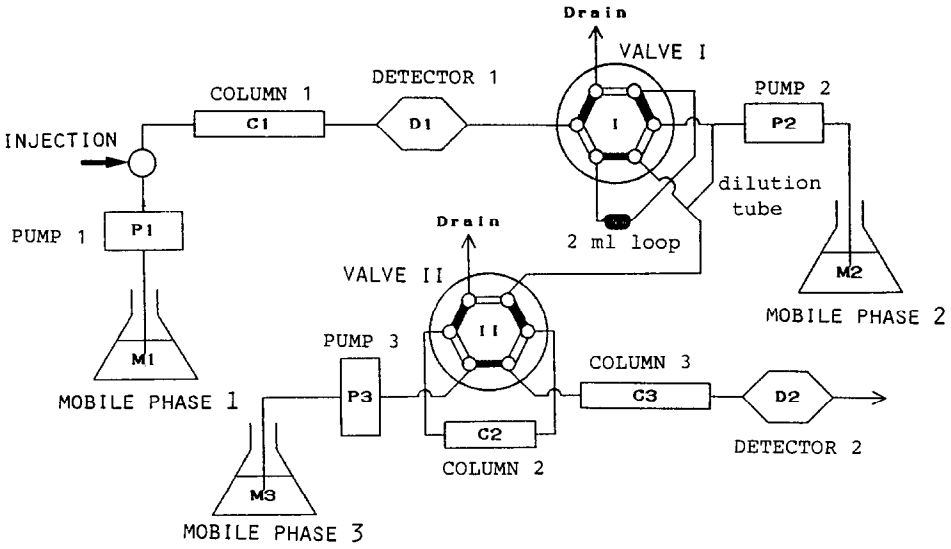

Fig. 2. Schematic diagram of the column-switching system finally developed and used.

human plasma. A 1-ml plasma sample was deproteinized by adding 1 ml of acetonitrile.

HPLC conditions

VA and I.S. were detected by measuring the absorption at 230 nm. Mobile phase 1 (M1) was prepared by mixing 5 m*M* sodium 1-pentanesulphonate with acetonitrile–water (3:7, v/v) and adjusting the pH to 3.0 by adding phosphoric acid. Mobile phase 2 (M2) was composed of dipotassium hydrogenphosphate and potassium dihydrogenphosphate. Mobile phase 3 (M3) consisted of tetrahydrofuran–ethanol–water (1:8:91, v/v/v) containing 20 m*M* potassium dihydrogenphosphate. M1, M2 and M3 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.

Procedure

Samples were injected onto C1 and VA was determined with the aid of the I.S. The eluate containing VA was selectively switched for 2 min into the 2-ml loop via valve I. Then M2 was allowed to flow and wash the above eluate into C2 (trapping column). Finally, by switching valve II, VA was swept by flowing M3 from C2 to C3 (ovomucoid column), where enantiomeric separation was performed.

RESULTS AND DISCUSSION

An HPLC method for the simultaneous determination of VA and its metabolites in plasma has been reported by Kuwada *et al.* [10]. They used an ion-pair chromatographic procedure with a reversed-phase column. In this study, ion-pair

chromatography was performed with a mobile phase consisting of 5 m*M* pentanesulphonic acid in acetonitrile–water (3:7, v/v) as the first HPLC and an I.S. was used to determine the concentration of VA. Next, we examined the on-line resolution of VA enantiomers using a column-switching HPLC system.

First, the eluate containing VA in the 2-ml loop was passed to C3 directly via valve II without using C2 and M2. However, VA was not retained on C3 and was not resolved optically (data not shown), because the eluate contained a large amount of organic solvent (M1) and was too hydrophobic to allow the retention of VA on C3. We therefore developed a system to dilute the eluate in the 2-ml loop by using a dilution tube so that VA could be retained on C3. This attempt was not wholly successful, because diffusion occurred and the peak of VA was greatly broadened at D2 (data not shown). These results suggested that dilution of the eluate in the 2-ml loop followed by a concentration step would be needed in order to perform the on-line determination and resolution of VA. Therefore, the dilution tube was installed to dilute the eluate and C2 was introduced to concentrate the diluted VA.

Effect of dilution ratio

M2 was prepared from 5 m*M* potassium phosphate buffer (pH 7.5). The dilution ratio of the eluate could be varied by using the flow resistivity, which could be changed by altering the length of the diluting tube. Fig. 3 shows the chromatograms obtained with various dilution ratios. The peak of VA was almost undetectable at low dilution, as shown in Fig. 3C, probably because the high hydrophobicity of the eluate meant that VA was not trapped on C2 but passed through it. Moderate dilution allowed VA to be trapped on C2, as shown in Fig. 3B, but did not allow the enantiomeric composition of VA to be determined accurately. However, adequate dilution (1:9) allowed the determination of enantiomers, as shown in Fig. 3A.

Effects of pH and salt concentration of mobile phase 2

VA was not trapped on C2 when M2 was simply water. We therefore examined the conditions of M2. Fig. 4 shows the optical resolution of VA at D2 obtained with various pH values of M2. The peak area of the enantiomers decreased with decreasing pH. The enantiomeric ratios in Fig. 4B and C were different from that in Fig. 4A, and

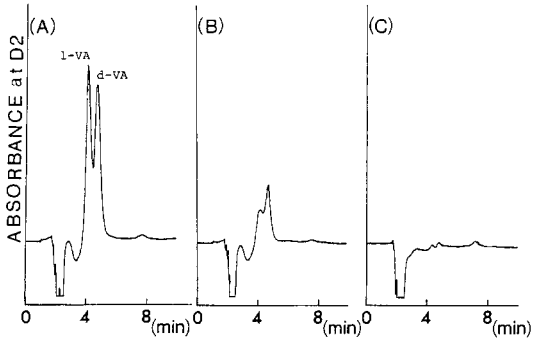

Fig. 3. Effect of dilution ratio. The eluate containing VA was diluted 1:x with M2: (A) $x = 9$; (B) $x = 3$; (C) $x = 0$.

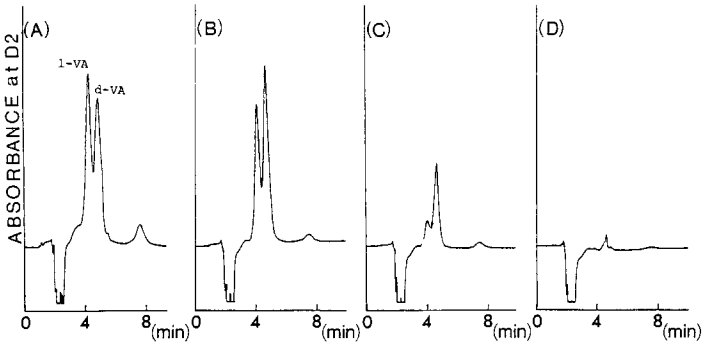

Fig. 4. Effect of pH of mobile phase 2. pH: (A) 7.5; (B) 7.2; (C) 6.9; (D) 6.5.

the peak of VA was almost unobservable in Fig. 4D. These results indicate that the affinity of VA for the ovomucoid column is not sufficiently strong at pH < 7.5, and VA is not completely trapped on C2. This may be the result of decreasing hydrophobicity of VA due to dissociation of the amino group [11]. The ovomucoid column exhibits strong hydrophobic interactions with basic solutes [9].

Fig. 5 shows chromatograms illustrating the effect of salt concentration in M2. The enantiomeric ratio could not be determined accurately with >20 mM salt and the elution of VA was retarded with increasing salt concentration in M2. Retention on the ovomucoid column is known to be affected by the addition of salt [8]. These results show that accurate determination of the enantiomeric ratio requires the use of the optimum pH and optimum salt concentration of M2.

Next, the flow-rate of M2 delivered by pump 2 was examined (data not shown). The retention and resolution of the enantiomers were little affected by changes in flow-rate in the range 1.0–6.0 ml/min.

Reproducibility

Fig. 6 shows chromatograms for the determination with C1 and the enantiomeric resolution with C3, where M2 consisted of 5 mM potassium phosphate buffer

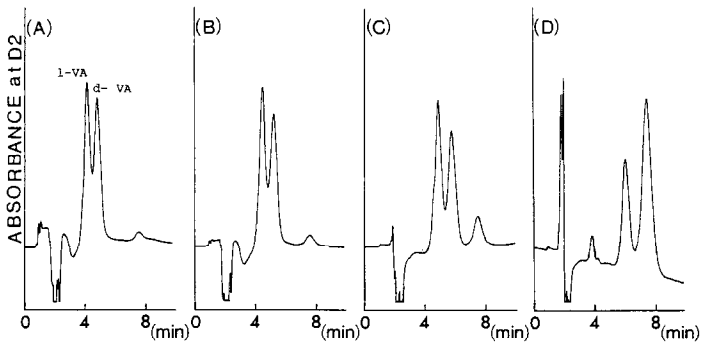

Fig. 5. Effect of salt concentration of mobile phase 2: (A) 5; (B) 10; (C) 20; (D) 50 mM.

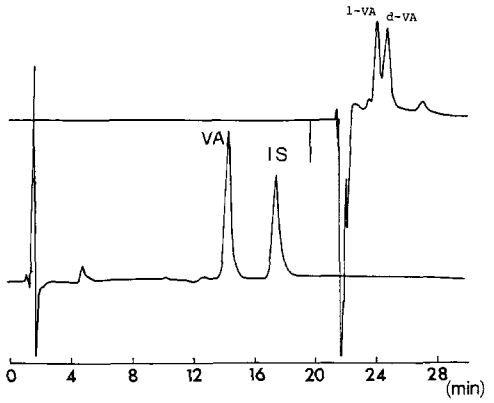

Fig. 6. Representative chromatograms obtained with the achiral reversed-phase column (lower chromatogram) and the ovomucoid column (upper chromatogram).

adjusted to pH 7.5 and the eluate containing VA was diluted 1:9 with M2. Table I indicates the reproducibility of the retention time on C3 and the enantiomeric ratio. The relative standard deviation (R.S.D.) for the retention time of *l*-VA was 1.8% (n = 8) and that of *d*-VA was 2.3% (n = 8). The R.S.D. for the enantiomeric ratio (*l*-VA/*d*-VA) was 2.1% (n = 8). These values are not necessarily satisfactory, but could be improved by further modification of the operating conditions with respect to trapping of sample materials on C2, and by rigorous control of temperature and the use of a reproducible column-switching procedure.

Detection limit

The limit of detection (at D2) for VA was 10 pg at a signal-to-noise ratio of 3. However, when a five-fold greater volume of the same sample concentration (1 ng/ml) was injected onto C1, five-fold larger peaks were observed at D2. The results indicate that this method effectively concentrates samples on C2 and enhances the sensitivity of enantiomer determination.

TABLE I

REPRODUCIBILITY DATA (n = 8)

See text for chromatographic conditions.

Value	Retention time (min)		*l*-VA/*d*-VA peak-area ratio
	l-VA	*d*-VA	
Mean	4.41	5.07	50.3:49.7
R.S.D.	1.78%	2.31%	2.06%

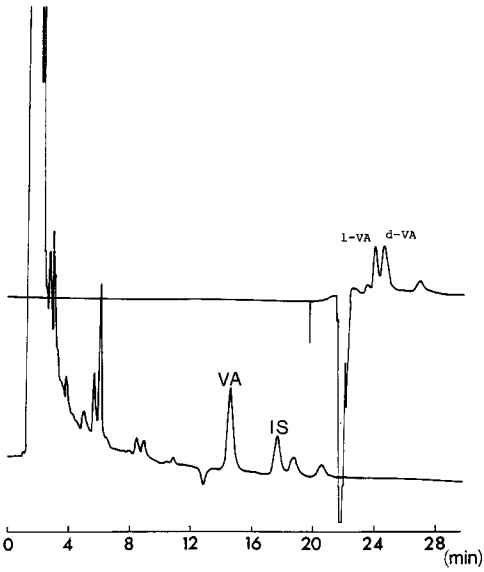

Fig. 7. Representative chromatograms obtained with the achiral reversed-phase column (lower chromatogram) and the ovomucoid column (upper chromatogram) for a plasma sample spiked with VA and internal standard.

CONCLUSION

A column-switching system employing a reversed-phase column coupled to an ovomucoid column via a dilution tube and a trapping column for concentration was developed for the on-line determination and enantiomeric resolution of VA. The use of a dilution tube in conjunction with the trapping column allowed both the achiral and chiral chromatographic steps to be performed under the respective optimum conditions, *i.e.*, each chromatographic step can employ the most favourable mobile phase independently.

This column-switching HPLC method has advantages over conventional HPLC in that it allows the on-line measurement of enantiomers after VA determination without pretreatments such as fractionation, desalting and evaporation, and high sensitivity can be achieved in a relatively short analytical time. Fig. 7 shows the chromatogram obtained by injection of a human plasma sample after deproteinization. VA was separated and determined on the achiral phase, and the enantiomeric measurement was performed on the ovomucoid column.

Applications of this system to pharmacokinetic studies of VA and for clinical purposes will be reported elsewhere.

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