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On-line determination and resolution of the enantiomers of ketoprofen in plasma using coupled achiral–chiral high-performance liquid chromatography

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Abstract: High-performance liquid chromatography (HPLC) using a column-switching technique has been applied to the on-line determination and resolution of the enantiomers of ketoprofen (KPF) as an acidic model compound. The system incorporates a mobile phase conversion stage (LC-2), which has a dilution tube and a trapping column, between the achiral chromatography stage (LC-1) and the chiral chromatography stage (LC-3). KPF in plasma was separated from plasma components and was determined with the aid of an internal standard in LC-1. The eluate containing KPF was selectively transferred to LC-2, where the eluate was adequately diluted with a new mobile phase by using the dilution tube to reduce the influence of the mobile phase from LC-1, and KPF was reconcentrated on the trapping column. Then the KPF enantiomers were resolved in LC-3 after column-switching. This system is accurate and rapid compared with conventional HPLC. Very high sensitivity could be achieved with our system when a microbore ovomucoid column was employed in LC-3. Incorporation of LC-2 allows the most favourable mobile phases for LC-1 and LC-3 to be used independently. This method is greatly superior to usual column-switching HPLC.

Keywords: *Column-switching HPLC; ovomucoid column; on-line determination and resolution; ketoprofen enantiomers; microbore column.*

Introduction

Racemates of chiral drugs are extensively used in therapy. There has been a great deal of interest in recent years in the development of methods for the resolution and quantification of enantiomers, because of the increasing recognition that there are important pharmacodynamic and/or pharmacokinetic differences between the enantiomers of a chiral drug [1, 2]. Resolution and quantification of enantiomers have increasingly come to be performed directly by means of HPLC, by introducing a chiral environment into the chromatographic system [3]. A number of chemically bonded chiral stationary phases (CSPs) are commercially available, but most of them can only be used in the normal-phase mode [4, 5]. This means that the analyte must be transferred from an aqueous to an organic phase before injection, and traces of water might destroy the chiral selectivity [6]. Stable cyclodextrin bonded phases were the first commercial CSPs intended to be used in a reversed-phase mode [7]. Some authors have reported that a protein-conjugated silica gel can be used as a CSP in reversed-phase HPLC. The usefulness of a bovine serum albumin (BSA)-conjugated column to recognize chirality in HPLC has been demonstrated by Allenmark *et al.* [8]. Hermansson has developed a column packed with immobilized α-acid glycoprotein on silica microparticles for liquid chromatographic resolutions of enantiomers of racemic drugs [9]. Miwa *et al.* have developed phases that contain ovomucoid protein as the chiral selector [10]. This column is suitable for separation of enantiomers with widely different structures [11–14]. Although these protein columns can recognize many chiralities, they have a low peak capacity, which implies that there is only a small chance of finding separation conditions where the enantiomers will be sufficiently separated from other compounds such as plasma components. An effective technique for enhancing the peak capacity is the use of a column-switching technique [15]. Wainer and his colleagues have demonstrated the direct

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resolution of stereoisomers using a protein-conjugated CSP coupled to an achiral column [16–18]. They separated the racemic drugs from interfering serum peaks and quantified them using the achiral column and then selectively switched to the CSP for the determination of the enantiomeric composition. However, their chromatographic conditions are very restricted because similar mobile phases need to be used for the two columns. Recently, we have reported a new type of column-switching HPLC in which we employed an achiral reversed-phase column coupled to a chiral ovomucoid column via a dilution tube and a trapping column for on-line determination and resolution of enantiomers of verapamil (a basic compound) [19]. In this system, in order to prevent the mobile phase of the first column from flowing into the second column, a dilution tube and a trapping column were interposed between the two columns, whereby the mobile phase of the first column was exchanged to the optimum mobile phase for the second column. In the present paper, the further application of this system is described using ketoprofen (KPF) as a model acidic compound (Fig. 1).

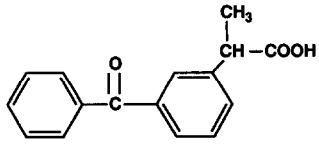

Figure 1
Chemical structure of ketoprofen.

Experimental

Reagents

Ketoprofen (KPF) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Ethylbenzoate was obtained from Wako Pure Chemicals (Osaka, Japan). Potassium dihydrogenphosphate, dipotassium hydrogenphosphate, phosphoric acid and perchloric acid of analytical reagent grade were from Wako Pure Chemicals. HPLC-grade acetonitrile, tetrahydrofuran (THF) and water were used.

Apparatus

The HPLC system consisted of three high-pressure pumps (Shimadzu LC-9A, Shimadzu Inc., Kyoto, Japan) (P1, P2, P3), a system controller (Shimadzu SCL-6B), an auto-injector (Shimadzu SIL-6B), two variable-wavelength UV detectors (Shimadzu SPD-6A) (D1, D2) and three six-port switching valves (Rheodyne Inc., CA, USA). The switching diagram is given in Fig. 2 and is explained in the procedure section. The HPLC analytical columns were a 150 × 4.6 mm i.d. Inertsil ODS-2 column (C1) (GL Sciences Inc., Tokyo, Japan) to determine KPF, a 10 × 4.00 mm i.d. Ultron ES-OVMG (C2) as a trapping column and a 150 × 4.6 mm i.d. Ultron ES-OVM (C3) (Shinwa Kako Co. Ltd, Kyoto) to resolve the enantiomers of KPF. The three columns were connected through two Rheodyne switching valves (I, II). Valve I was equipped with a 1-ml sample loop. A dilution tube was inserted between Valve I and Valve II.

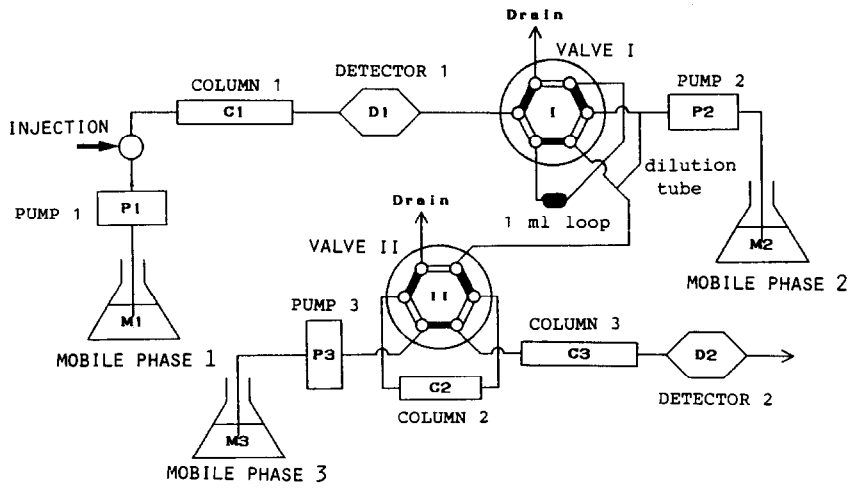

Figure 2
Schematic diagram of the column-switching system.

Samples

Ethylbenzoate was used as an internal standard (IS). Standard samples were prepared by dissolving known amounts of KPF and IS in saline. Plasma samples were human plasma spiked with KPF and IS. Both samples were mixed with the same volume of acetonitrile and the mixtures were centrifuged at 3000 rpm to deproteinize them. A 10 μl portion of the upper layer was injected.

HPLC conditions

KPF and IS were detected at 260 nm. Mobile phase 1 (M1) was composed of acetonitrile–water–perchloric acid (30:70:0.1, v/v/v) and was delivered by P1 at a flow rate of 1.0 ml min^{-1}. Mobile phase 2 (M2) was 1 mM potassium dihydrogenphosphate buffer solution adjusted to pH 4.2 by adding phosphoric acid, and the flow rate was 3.0 ml min^{-1}. Mobile phase 3 (M3) consisted of THF-water (7:93, v/v) containing 20 mM potassium dihydrogenphosphate and was delivered by P3 at a flow rate of 1.0 ml min^{-1}. All operations were carried out at ambient temperature.

Procedure

Samples were injected onto C1 through the injection port and KPF was determined by the internal standard method. The eluate containing KPF was selectively switched for 1 min into the 1 ml loop via Valve I. Then this eluate was diluted by flowing M2 through both the dilution tube and the 1 ml loop to flust out M1, and KPF was trapped on C2. Finally by switching Valve II, KPF was eluted with M3 from C2 to C3, where enantiomeric separation was performed.

Results and Discussion

In this study, the column-switching system consisted of three coupled HPLC units. The first HPLC system (LC-1) was run in the reversed-phase mode to determine KPF in plasma after pretreatment. Figure 3 shows chromatograms of a plasma blank (A) and plasma sample (B). The peaks of plasma components did not interfere with those of KPF and IS, which were separated from each other. A good linear relationship, $r = 0.9996$, was obtained between the concentration of KPF in plasma and the relative peak area, KPF/IS over the concentration range 0.01 to 5 μg ml^{-1}. The recovery of KPF, which was

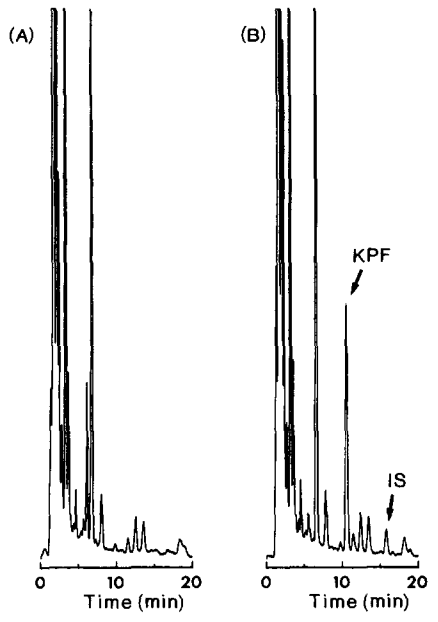

Figure 3
Representative chromatograms on the achiral reversed-phase column of pretreated plasma blank (A), and plasma sample spiked with KPF (400 ng ml^{-1}) and IS (1 μg ml^{-1}) (B). See text for chromatographic conditions.

determined by comparison with the peak area of KPF in standard samples, from the plasma samples was 100.2% at 0.5 μg ml^{-1} and the relative standard deviation (RSD) was 1.4% ($n = 5$).

In the third HPLC system (LC-3), the resolution of KPF enantiomers was performed and the enantiomeric composition was determined. At first, standard samples were directly injected into C3, and the retention behaviour was examined (no column-switching). Figure 4 shows the chromatograms obtained with three kinds of organic solvents to elute the enantio-

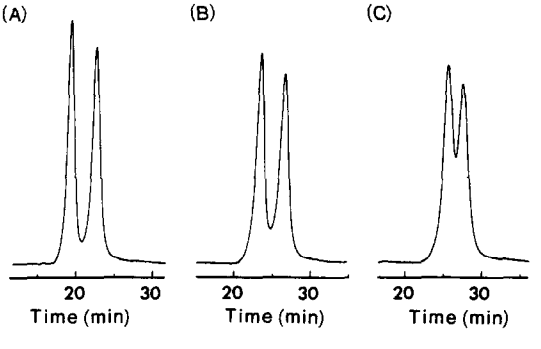

Figure 4
Representative chromatograms on the ovomucoid column of KPF standard sample. Eluted with (A) THF 7%, (B) acetonitrile 10%, (C) ethanol 15%. See text other chromatographic conditions.

mers of KPF. In this study, THF was the best solvent of the three, although the reason for this is not clear. Figure 5 shows the effect of THF concentration on the capacity factor of KPF on C3. The capacity factor was increased with decreasing THF concentration. This is why the ovomucoid column (C3) has the characteristics of a reversed-phase column [10]. The enantiomers of KPF were not resolved at 15% THF in the mobile phase. The relationship between pH value of the mobile phase and the capacity factor of KPF is shown in Fig. 6. The retention of KPF was strongest at around pH 4 in the range pH 3 to pH 6. The reason for this seems to be related to the isoelectric point of the ovomucoid protein and the dissociation constant of KPF. Both factors favour hydrophobic interaction at around pH 4 [10–12]. The enantiomers were not resolved by the mobile phase at pH 6. Figure 7 shows the effect of salt concentration of the mobile phase. The elution of KPF was retarded with decreasing salt concentration, especially at low salt concentration. The retention of KPF on C3 may be related to the electrostatic effect, as in the case of ion-exchange chromatography [12]. The enantiomers of KPF were not resolved when the mobile phase contained no salts, although the separation of the enantiomers was little affected by the salt concentration.

Next, on-line determination and resolution of KPF enantiomers were examined by using column-switching HPLC. KPF was not retained on C3 because of the influence of M1, when LC-1 was directly connected to LC-3. So LC-2 was installed between LC-1 and LC-3. LC-2 was equipped with a dilution tube to reduce the influence of M1, to mix with M2, and to reconcentrate the samples on C2. M2 was pumped into C2 by P2 via both the 1 ml loop and the dilution tube. The dilution ratio was determined by the relative flow-resistivity of the 1 ml loop and the dilution tube. KPF must be trapped on C2, after the eluate containing KPF is diluted by M2, so M2 had the composition which gave the largest capacity factor for KPF on the ovomucoid column and the eluate was diluted 30 times by using the dilution tube. Figure 8(A) shows a chromatogram of the resolution of KPF enantiomers obtained by using the column-switching

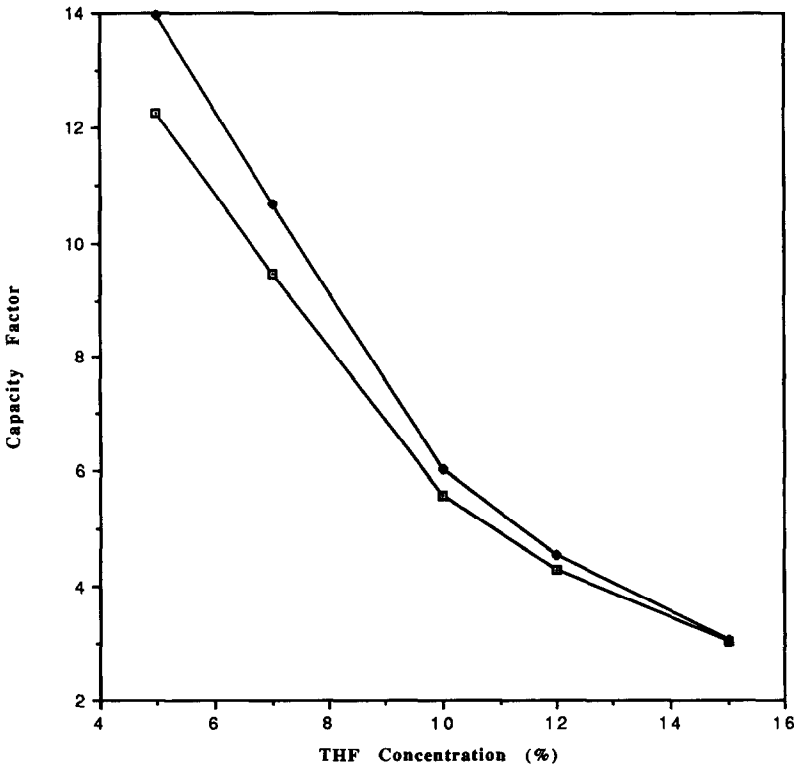

Figure 5
Effect of THF concentration of the mobile phase on the capacity factor for KPF on the ovomucoid column. See text for chromatographic conditions.

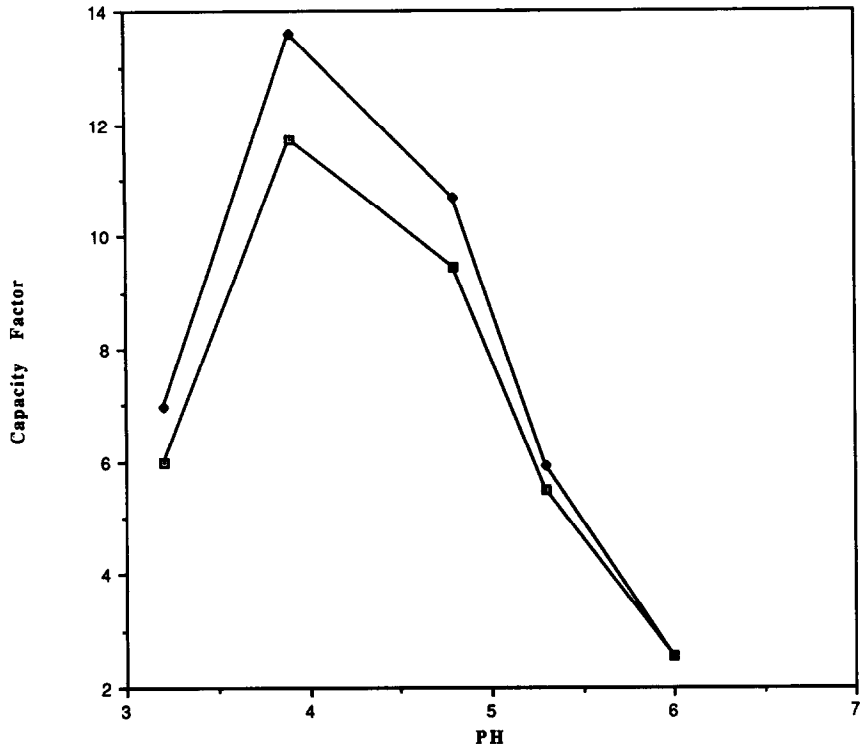

Figure 6
Effect of pH of the mobile phase on the capacity factor for KPF on the ovomucoid column. See text for chromatographic conditions.

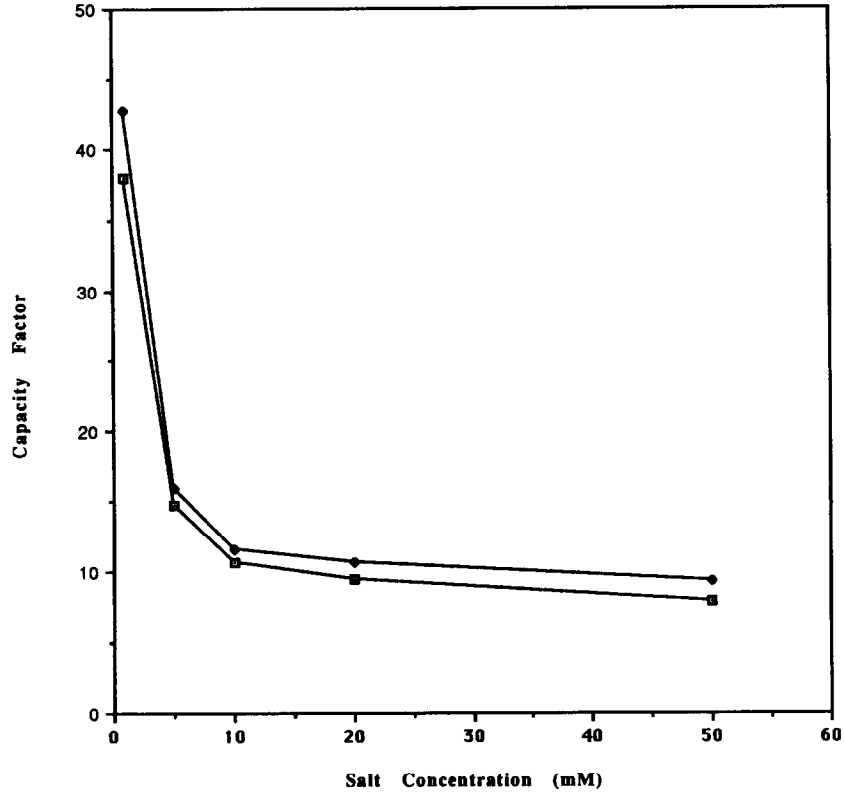

Figure 7
Effect of salt concentration of the mobile phase on the capacity factor for KPF on the ovomucoid column. See text for chromatographic conditions.

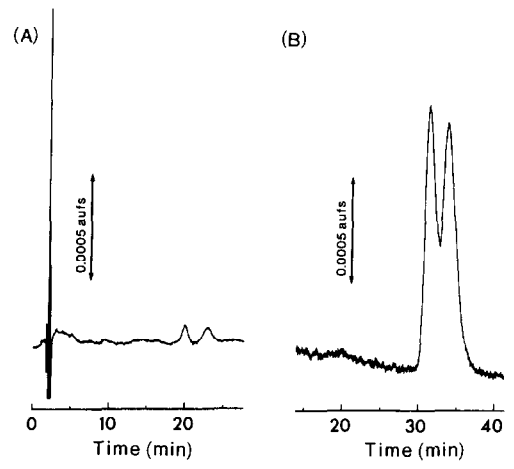

Figure 8
Representative chromatograms of KPF (4 ng was injected into C1) in plasma after column-switching (A) on the conventional ovomucoid column, (B) on the microbore ovomucoid column. See text for chromatographic conditions.

method. KPF was optically well resolved and the detection limit was 0.4 ng at *S/N* 3. Table 1 gives the reproducibility of retention time, the number of theoretical plates and the resolution obtained by this method. The RSD values were good, even though all valve-switching procedures were performed manually. Table 1 also indicates that the resolution of KPF enantiomers by the column-switching method was a little worse than that by the direct injection method (only LC-3). The reason may be the diffusion of KPF on C2, as KPF was trapped after adequate dilution. So it may be necessary to improve the trapping column to trap the compound of interest on the top of the column, in order to minimize the diffusion.

Recently, highly effective microbore packed columns have been developed [20] and the use of an albumin-coated micro-column to resolve enantiomers has been demonstrated by Verzele and co-workers [21]. The advantages of this method are low solvent consumption, high efficiency, and greater mass sensitivity. On the other hand, the main disadvantage is the small injection volume. But, Ishii and co-workers [22] reported that they had been able to overcome this problem by using column-switching HPLC, incorporating a trapping column to concentrate the sample. In our system, C3 was changed to a micro bore ovomucoid column (micro-C3), which was packed with ovomucoid protein conjugated onto aminopropyl silica gel in a 150 × 0.5 mm i.d. stainless steel tube. M3 was delivered by P3 at a flow-rate of 0.02 ml min^{-1}. Thus, LC-2 was used not only as the mobile phase conversion system, but also as the injector into the micro-C3. Figure 8(B) shows the chromatogram obtained by using this column-switching system with the micro-C3. The peak height of KPF was more than 15 times that in Fig. 8(A), although the same sample was injected in each case (KPF: 4 ng). The analytical time was longer than that in Fig. 8(A) because of the time required to change M2 to M3. This improved column-switching system with the micro-C3 showed very high sensitivity, but micro-C3 had poor durability and the enantiomers of KPF were no longer separable after a few injections. The reason for this is not clear, so further research is necessary to improve this system using micro-C3.

Conclusion

A column-switching system employing an achiral column coupled to a chiral column via a dilution tube and a trapping column was developed for on-line determination and enantiomeric resolution of KPF as an acidic model compound. The dilution tube was used to reduce the influence of the mobile phase used in achiral chromatography and the role of the trapping column was to reconcentrate the eluted samples. This system can change the mobile phase used for achiral chromatography to a different mobile phase for chiral chromatography, so that each chromatography can be performed independently under the respective optimum conditions. Very high sensitivity can be achieved by using a microbore ovo-

Table 1
Reproducibility data (five injections). See text for chromatographic conditions

	t_1 (min)	t_2 (min)	N_1	N_2	Rs	N_1*	N_2*	Rs*
Mean	19.95	22.85	2828	2195	1.662	3280	3198	1.847
RSD (%)	0.83	0.84	2.18	3.49	5.07	—	—	—

t_1 or t_2 means the retention time of the first, or the second, eluted enantiomers, respectively.
*Direct injection method.

mucoid column as the chiral column. This technique is potentially very useful for pharmacokinetic studies.

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