Determination of Ketoprofen Enantiomers in Plasma by Solid Phase Extraction and Column Switching High Performance Liquid Chromatography

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Protein-coated cyanopropyl silica gel packed in a precolumn was successfully used as an adsorbent for ketoprofen in blood plasma. After column switching, ketoprofen collected in the precolumn was transferred into an ovomucoid column, and the chiral separation was carried out by a mobile phase of 0.1 M phosphate solution (pH 3.2) containing 20% methanol. The separation factor was 1.32, and the resolution was 1.30. The recovery by direct injection of plasma samples was approximately 100%, with a relative standard deviation of about 5% between days ($n=6$). The calibration graph of each enantiomer was a straight line up to 1 nmol; each detection limit was 5 pmol ($S/N=5$).

Keywords Column switching high performance liquid chromatography, solid phase extraction, ketoprofen enantiomer, blood plasma sample, direct injection, on-line deproteinization

The column switching high performance liquid chromatography (HPLC) coupled with solid phase extraction in a precolumn was a useful technique for analyses of drugs or biological constituents in complex matrices.^{1,2} Recently, we employed this technique for chiral separation and determination of propranolol enantiomers in blood or tissues.³ A chiral stationary phase using a biopolymer such as bovine serum albumin (BSA)⁴, α_1 -acid glycoprotein⁵ or ovomucoid (OVM)⁶ was recommended for a sample in aqueous solution. An aqueous mobile phase containing an organic solvent could be used for the chiral separation. It is essential that the analyte collected in a precolumn be eluted and transferred perfectly into the chiral column and separated by the same mobile phase. Choice of a precolumn packing was also necessary in order to ensure the quantitative recovery and perfect elution.

Ketoprofen (KP), *R,S*-2-(3-benzoylphenyl)propionic acid, is a clinically important non-steroidal anti-inflammatory drug;⁷ it possesses a chiral center in the propionic acid moiety. Commercially, KP can be obtained in the racemic form. An *in vitro* experiment proved that the *S*(+) enantiomer exhibited pharmacological effects, but *R*(-) was inactive. An *in vivo* experiment showed that the chiral inversion took place from *R*(-) to *S*(+), the degree of inversion being varied from one animal species to another.⁸ Therefore, the stereoselective analysis attracts attention in relation to the pharmacokinetic studies.

We developed a method of column switching HPLC with direct injection of plasma samples for chiral

separation of KP by using a protein-coated cyanopropyl silica precolumn for solid phase extraction and an OVM-bonded silica column for chiral separation. We applied this method to study the time course of elimination of KP enantiomers in rat plasma. The details are presented in this paper.

Experimental

Materials

Racemic KP was obtained from Sigma (St. Louis, MO, USA). Human plasma was purchased from Cappel (West Chester, PA, USA). All other chemicals and solvents were of analytical reagent grade, and were used without further purification.

HPLC

A short precolumn (1 cm×4 mm i.d.) was packed with cyanopropyl silica gel (Nucleosil 5CN, Macherey-Nagel, Düren, Germany). The external surface of the porous gel was coated with plasma proteins by the same procedure as reported in the previous paper.⁹ A guard column (1 cm×4 mm i.d.) packed with aminopropyl silica gel (Develosil NH₂-5, particle size 5 μ m, Nomura Chemical Co., Ltd., Seto, Japan) was attached to the precolumn in order to protect the precolumn from interfering endogenous constituents in blood. An OVM-bonded silica column (Ultron ES-OVM, 15 cm×4.6 mm i.d., Shinwa Kako Co., Kyoto, Japan) was used for the chiral separation. A guard column (Ultron ES-OVM G, 1 cm×4 mm i.d., Shinwa Kako Co.) was attached to

the OVM column.

A Waters Model 501A (Waters, Division of Millipore, Bedford, MA, USA) was used for the HPLC pump. The flow rate was 0.8 ml/min throughout the experiment. Detection was carried out at 265 nm with a Waters Model 484 Tunable Absorbance Detector. The column switching device and other pieces of apparatus were the same as reported in the previous paper.^{10,11}

Results and Discussion

Enantiomer resolution

The chiral resolution of KP in an OVM column was remarkably influenced by electrolyte concentration, pH and organic modifiers in the mobile phase.

In the absence of electrolyte, KP was not eluted over 1 h even by the presence of 15% ethanol or 20% methanol. The elution and enantiomer resolution were dramatically improved by the addition of electrolyte (phosphate, 50–200 mM). Figure 1(A) shows the effect of phosphate concentration on the capacity factor, k' . In this case, phosphate concentration as high as 50 mM was preferable.

The value of the separation factor, α , increased gradually with decrease of pH from 6.5 to 3.0 in solution of 50 mM phosphate containing 10% ethanol. The value of k' showed a maximum at about pH 4.0 (Fig. 1(B)). It is noteworthy that the isoelectric point of OVM is 4.3 and pK_a of KP is 3.9. Therefore, it seems

Fig. 1 Effect of phosphate concentration (A) and pH (B) on k' value. ●, $S(+)$ KP; ○, $R(-)$ KP.

Table 1 Effect of organic modifiers

Organic modifier ^a		k' ^b	α
Methanol	(15–25%)	13.4–4.0	1.4–1.2
Ethanol	(10–15%)	12.5–6.1	1.3–1.1
1-Propanol	(5–15%)	9.4–2.1	1.1–1.0
2-Propanol	(7.5–15%)	9.6–3.0	1.1–1.0
Acetonitrile	(10–15%)	8.6–2.5	1.1–1.0
Tetrahydrofuran	(5–10%)	7.7–3.8	1.1–1.0

a. Addition to 50 mM phosphate solution (pH 3.2).

b. $S(+)$ KP.

that the k' value increases at about pH 4.0 owing to the interaction between KP anion and positively charged OVM protein.

When the carbon number of primary normal alcohols was increased from 1 to 3, both k' and α were decreased. Among the organic modifiers investigated, 20% methanol was most preferable, as shown in Table 1.

The value of k' decreased with increase of temperature from 15° to 40°C. In this connection, 20°C was most preferable.

When a mobile phase of 0.1 M phosphate solution (pH 3.2) containing 20% methanol was used, the retention time was 9.6 min for $S(+)$ and 12.0 min for $R(-)$, and chiral separation was satisfactory ($\alpha=1.32$ and $R_s=1.30$) under the column temperature at 20°C. The free energy difference in the chiral resolution was estimated to be as small as -163 cal/mol from the separation factor.

Solid phase extraction

C-18 bonded silica gel strongly adsorbed KP, so that the elution was difficult by using the mobile phase suitable for the chiral separation. C-8 bonded silica gel, a hydrophobically interacting adsorbent gel (Butyl Toyopearl 650-M) or an anion exchange resin showed low recovery or inadequate elution for KP. Only mildly adsorbing cyanopropyl silica was suitable to elute out with an eluant containing a low concentration of organic modifiers.

Figure 2(A) shows the chromatogram of standard aqueous KP injected on a column (1 cm×4 mm i.d.) packed with protein-coated cyanopropyl silica gel and eluted by 0.1 M phosphate solution (pH 3.2). When the same quantity of KP spiked in blood plasma was injected, the KP peak was slightly broadened (Fig. 2(B)). The broadening may be caused by the pH shift of the eluant due to plasma proteins. When the plasma sample was acidified (pH 3–4) with the same volume of 0.1 M phosphoric acid, peak broadening was no longer observed (Fig. 2(C)).

Plasma proteins in the sample were not adsorbed on

Fig. 2 Elution profile of 2 nmol KP from precolumn. Samples: (A) standard aqueous sample, (B) standard spiked in plasma and (C) the plasma sample acidified with phosphoric acid.

Table 2 Recovery of KP enantiomers in plasma

Concentration/ μ M	Recovery, %	
	S(+)	R(-)
1	100.5	98.5
10	101.1	101.3
50	99.5	97.5

the protein-coated cyanopropyl silica, and were eluted at the void volume. The recovery of the eluted proteins from the plasma sample was estimated to be 95–102% by the Commassie Brilliant Blue method. Plasma proteins coated on the gel were not eluted until 40% methanol. When the mobile phase of 0.1 M phosphate solution (pH 3.2) containing 20% methanol was used, the recovery of KP in plasma samples was approximately 100% (Table 2), regardless of the high protein binding ratio of KP. The protein binding ratio is reported to be over 90% at the therapeutic concentration of 5–12 μ g/ml.¹² Deterioration of the precolumn by some endogenous constituents was avoided by a short guard column (1 cm $\times$ 4 mm i.d.) packed with aminopropyl silica gel.

Column switching HPLC

A mobile phase of 0.1 M phosphate solution (pH 3.2) was delivered to the precolumn. A plasma sample (20 μ l) diluted and acidified by the same volume of 0.1 M phosphoric acid was injected onto the precolumn at room temperature. Proteins and hydrophilic components in plasma were eluted and discarded. In 5 min after injection, the precolumn was connected to the OVM column in stream. The OVM column was previously equilibrated with 0.1 M phosphate solution (pH 3.2) containing 20% methanol, and controlled the temperature at 20°C. Then, by a mobile phase of 0.1 M phosphate solution (pH 3.2) containing 20% methanol, KP collected in the precolumn was eluted, transferred into the OVM column, and KP enantiomers were separated in the OVM column by the same mobile phase. KP enantiomers were detected spectrophotometrically at 265 nm. During the separation on the OVM column, the precolumn was rinsed with 0.1 M phosphate solution (pH 7.0) containing 20% acetonitrile for 3 min, water for 1 min, and then absolute methanol for 3 min.

Analysis of KP in blood plasma sample

The typical elution profiles of KP in a plasma sample at the initial use of an OVM column and that after 200 times injection are shown in Figs. 3(A) and 3(B), respectively. The resolution was still good after 200 times use, though the shapes of artifact peaks were changed. The calibration graph showed a straight line in the concentration range from 5 pmol to 1 nmol of each enantiomers. The recovery data were estimated by comparing the elution peak height of an aqueous

Fig. 3 Elution profile of KP enantiomers in plasma obtained with (A) new OVM column and (B) the column after 200 times injection. Sample: human plasma (20 μ l) spiked with 100 pmol racemic KP after acidification with phosphoric acid. Eluent: 0.1 M phosphate solution (pH 3.2) containing 20% methanol.

Table 3 Reproducibility by the present method

		RSD, %	
		S(+)	R(-)
Within day	(n=10)	1.5	1.7
Between days	(n=6)	4.5	5.2

Sample: 20 μ l of plasma spiked with 100 pmol of racemic ketoprofen.

Fig. 4 Concentration of KP enantiomers in blood plasma of a rat after oral administration. ●, S(+) KP; ○, R(-) KP.

standard sample and plasma sample spiked with KP (Table 2). The reproducibility was expressed by the relative standard deviation (RSD) of successive injections (Table 3). The OVM column was rinsed with 30% aqueous 2-propanol solution every day after use; thus, the OVM column could be used over 200 times for plasma samples.

Racemic KP was dissolved in 0.04 M phosphate buffer (pH 7.4), and administered orally to a Wistar rat (dose, 2.5 mg/kg). The plasma concentration of KP

enantiomers after administration is shown in Fig. 4. *R*(−) KP eliminated fairly quickly, and in 3 h it mostly disappeared, while *S*(+) KP showed a maximum blood concentration at about 3 h after administration. A coupled effect of the metabolic chiral inversion and elimination pathway seems to take place in this case.

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