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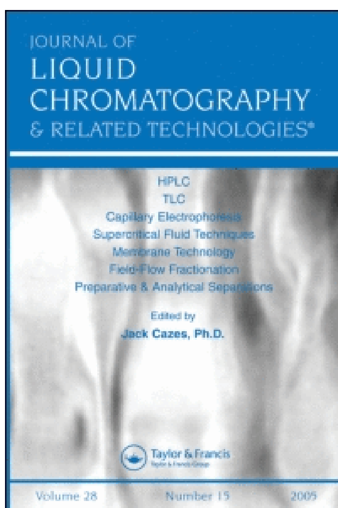

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High-Performance Frontal Analysis/High-Performance Liquid Chromatographic System for the Enantioselective Determination of Unbound Fenoprofen Concentration in Protein Binding Equilibrium

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HIGH-PERFORMANCE FRONTAL ANALYSIS/HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM FOR THE ENANTIOSELECTIVE DETERMINATION OF UNBOUND FENOPROFEN CONCENTRATION IN PROTEIN BINDING EQUILIBRIUM

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ABSTRACT

High-performance frontal analysis (HPFA) and high-performance liquid chromatography (HPLC) were incorporated in an on-line coupled column system for the enantioselective determination of unbound fenoprofen (FP) concentrations in the state of a protein binding equilibrium following direct sample injection. This system consists of a "restricted access" type column for HPFA and a1-acid glycoprotein-immobilized silica column for chiral separation. When a 80-µL portion of a sample solution containing 50 or 90µg/mL FP and 300 or 550 µM human serum albumin (HSA) was directly injected into the HPFA column, FP gave a trapezoidal peak exhibiting a plateau region. The concentration in the plateau range was equal to the unbound FP concentration in the initial sample solution. By the delivery of a 90-µL portion of the eluent in this plateau region into the chiral separation column, the unbound concentrations of FP enantiomers were determined. The results agreed with those obtained by the conventional ultrafiltration-HPLC method. The precision was also confirmed by the within-run and day-to-day reproducibilities (CV<9.95%, n=5). It was found that the binding between FP and HSA is stereoselective, and S-FP binds with HSA more strongly than R-FP.

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INTRODUCTION

It is a common case for an optically active drug that the pharmacological activity, side effect and/or drug disposition are different between the enantiomers [1,2]. This is because the interactions between a drug and biopolymers such as receptors, enzymes and proteins affect the activity and the disposition of the drug, and are highly stereoselective. In plasma, a drug binds, more or less, with proteins such as albumin and α_1-acid glycoprotein. Protein binding is a rapid and reversible interaction, and the concentration of the drug and that of the proteins are in an equilibrium state. Protein binding plays an important role in the pharmacokinetics and pharmacodynamics of the drug [3-5], and is potentially stereoselective [6]. Therefore, the stereoselective investigation on protein binding is important for the pharmacokinetic and pharmacodynamic studies of the drug. For this purpose, the development of a simple analytical method for the determination of unbound concentrations of the drug enantiomers is inevitable.

Fenoprofen (FP) is a non-steroidal anti-inflammatory drug with an asymmetric carbon atom. FP is clinically administered as a racemate, while S-isomer is approximately 35 times as potent as the R-isomer [7]. The irreversible metabolic inversion from R-isomer to S-isomer occurs in vivo like many other 2-arylpropionic acid derivatives [7]. It is known that FP binds very strongly with albumin, and the binding ration of KP in human plasma was more than 99% [8].

Recently, we developed high-performance frontal analysis (HPFA), a chromatographic method to determine unbound drug concentration in the state of protein binding equilibrium [9-13].

HPFA is a simple method because it allows direct sample injection analysis. HPFA does not suffer from protein leakage from and drug adsorption on the filter membrane which cause erroneous results in the conventional ultrafiltration method and dialysis method. In addition, HPFA can be easily incorporated into on-line column switching system. We have developed HPFA-chiral HPLC coupled column system for the enantioselective determination of unbound concentrations of warfarin and ketoprofen [11,13].

In this paper, we developed an on-line HPFA-HPLC system for the direct and stereoselective determination of unbound FP concentrations. This system consists of a "restricted access" type column (Pinkerton column) for HPFA and a chiral HPLC column which contains immobilized α_1-acid glycoprotein as a chiral ligand (Chiral AGP column). The analytical data was compared with those obtained by a conventional ultrafiltration-HPLC method.

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EXPERIMENTAL

Materials

Racemic potassium fenoprofen was supplied by Yamanouchi Pharmaceutical Industries (Tokyo, Japan). Human serum albumin (HSA; fatty acid free) was purchased from Sigma Chemical Company (St.Louis, MO). The concentration of HSA was determined spectrophotometrically by using extinction coefficient $E^{1\%}_{1cm}$=5.31 at 279nm. Pinkerton column (Regis Chemical Co., Morton Grove, IL) and Chiral AGP column (ChromTech AB, Sweden) were purchased from Koken Co. (Tokyo, Japan).

Preparation of Sample Solutions

FP stock solution was made up in methanol. An appropriate volume of the stock solution was put in a 10-mL screw-capped

TABLE I. HPLC Conditions

Subsystem	Condition	
HPFA	Column	Pinkerton column (15cm x 4.6mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH7.4; ionic strength, 0.17)
	Flow rate	1.0 mL/min
	Detection	UV, 240 nm
	Temperature	ambient (25°C)
Chiral separation	Column	Chiral AGP column (10cm x 4.0mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH7.4; ionic strength, 0.02)
	Flow rate	1.0 mL/min
	Detection	UV, 210 nm
	Temperture	30°C controled by water bath

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vial, and methanol was evaporated. An appropriate volume of HSA solution (in phosphate buffer of pH 7.4 and ionic strength of 0.17) was added to the vial to prepare the sample solutions. The sample solutions were kept at 25°C before use.

Apparatus

The HPLC apparatus was as with two pumps TriRotar III (Jasco, Japan) and LC-3A (Shimadzu, Japan), a UV detector SPD-2A (Shimadzu), an integrated data analyzer Chromatopac C-R3A (Shimadzu) and an injector Rheodyne type 7125 injector with a 300µL loop. The HPLC conditions were listed in Table I. A digital polarimeter DIP-360 (Jasco) was used to determine the elution order of FP enantiomers.

Determination of Unbound FP Concentration by Ultrafiltration-HPLC Method

A disposable ultrafiltration kit (Centrifree; Amicon division W.R. Grace & Co., MA) was used as a reference method to

obtain the fraction of unbound FP. The adsorption of FP on the membrane was not observed. One mL portion of the sample solution was applied to the ultrafiltration kit and was centrifuged at 2000 x g at 25°C. To estimate the leakage of HSA through the ultrafiltration membrane, the concentration of HSA in the filtrate was determined by the method of Lowry [14]. The unbound drug concentration (Cf) was corrected according to the following equation: $C_f = (C_{UF} - C_T r)/(1-r)$, where C_{UF} is the concentration of FP in the filtrate, C_T is the total concentration of FP, and r is the leakage fraction of HSA. The leakage was 0.074 ± 0.059%, 0.138 ± 0.096% and 0.146 ± 0.057% for 50μg/mL FP - 300μM HSA solution, 90μg/mL FP - 300μM HSA solution and 90μg/mL FP - 550 μM HSA solution, respectively (n=5).

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RESULTS AND DISCUSSION

Optimization of the Chiral Separation Condition

As shown in Figure 1, the capacity factors of FP enantiomers increased with decreasing pH, while the enantioselectivity was almost constant regardless of pH. The addition of only a few % of organic modifiers such as ethanol and acetonitrile deteriorated the enantioseparation. When the ionic strength of the mobile phase (phosphate buffer without organic modifier, pH7.0) decreased from 0.02 to 0.015 with other conditions kept constant (column temperature, 25°C), the capacity factors of FP enantiomers increased by 1.5-fold (from 16.2 and 20.6 to 26.2 and 32.5), whereas the enantioseparation was not changed. Based on these results, the HPLC condition for the chiral separation was decided as listed in Table I.

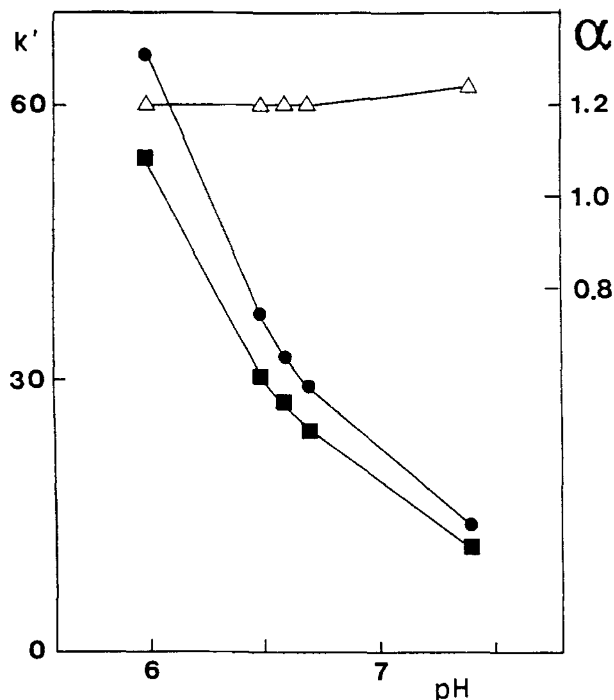

Figure 1. The effect of pH of the mobile phase on the capacity factor (k') and the separation ratio (a) of fenoprofen enantiomers.
HPLC conditions; Column, Chiral AGP (10cm x 4.0mm I.D.). Mobile phase, sodium phosphate buffer (pH7.0, I = 0.02). Flow rate 1.2mL/min. Column temperature, 37°C. Detection, UV210nm.
Abbreviations: ■ capacity factor of the first peak. ● capacity factor of the second peak. △ separation ratio.

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Optimization of HPFA Condition

In HPFA method, drug-protein mixed solution is injected directly to a "restricted access" type HPLC column. With increasing volume of the injected sample, the drug is eluted as a trapezoidal peak with a plateau, and the drug concentration in this plateau region is same with the unbound drug concentration in the sample solution. This is because the binding equilibrium in the

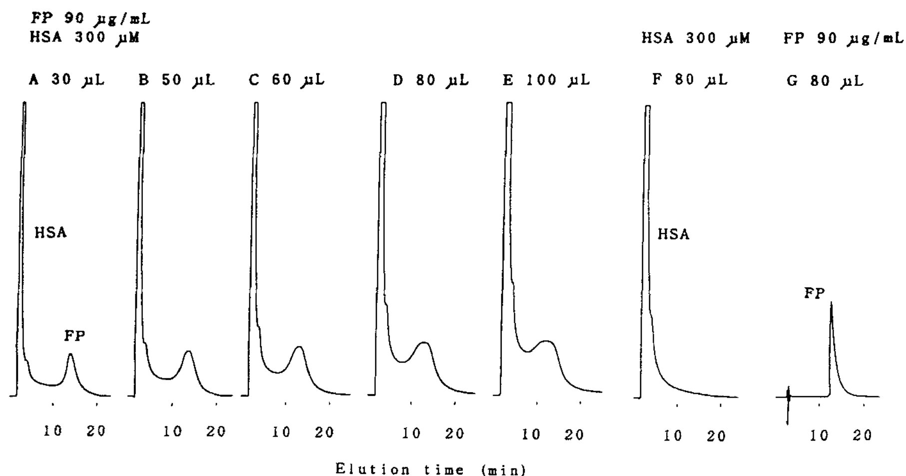

Figure 2. Effect of the injection volume on the chromatogram. HPLC conditions, see table I. Sample, (A-E) 90µg/mL FP and 300µM HSA mixed solution, (F) 300µM HSA, (G) 90µg/mL FP.

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sample solution is reproduced in the mobile phase at the top of the column after the continuous sample loading [10,12]. Therefore, sample volume should be large enough to give the plateau region.

Figure 2 shows the effect of the sample injection volume upon the chromatogram of 90µg/mL FP - 300µM HSA mixed solution. As the injection volume increased, the FP peak reached at the maximum, then became broad and spread toward the protein peak. When more than 80µL of the solution was injected, the peak height reached at a constant level and the plateau region appeared. However, when the same volume of an aqueous FP solution (90µg/mL) without containing HSA was applied under the same conditions, it gave much sharper and narrower peak as found in Figure 2G. Therefore, the peak broadening was obviously at-

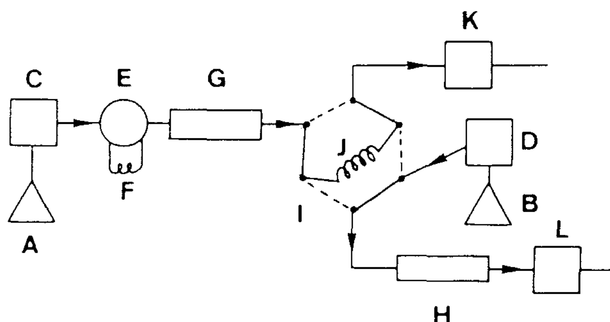

Figure 3. Schematic diagrams of the present HPFA-HPLC system. (A)mobile phase for HPFA; (B)mobile phase for chiral separation; (C,D)pump; (E)sample injector; (F)injector loop; (G)HPFA column; (H)chiral separation column; (I)six-port switching valve; (J)heart-cut loop, (K,L)UV detector;

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tributable to the protein binding of FP in the mobile phase. The injection volume was decided as 80 μL, which was the minimum volume to give the plateau region. FP peak was not completely separated from HSA peak. If a drug is completely separated from a protein peak, the total and unbound drug concentrations can be determined simultaneously from the peak area and from the peak height, respectively [10].

Determination of Unbound FP Enantiomers by the HPFA-HPLC System

The schematic diagram of the coupled column system used in this study is shown in Figure 3. The HPFA column (Pinkerton column) and chiral separation column (Chiral AGP column) were connected in series via a six-port switching valve. When a plateau region of FP peak was eluted, a given portion (90μL) of the eluent for this region passing through the loop (J) was heart-cut and transferred to the chiral column by switching the valve (I) from the solid line to the dashed line to determine the unbound

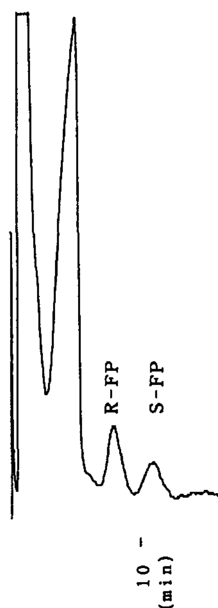

Figure 4. Chiral separation of fenoprofen. HPLC conditions, see Table I.

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concentrations of FP enantiomers. The column switching time was decided at 12 min after sample injection. By this time, HSA was completely size-excluded from Pinkerton column (see Figure 2F). It is interesting to note that the heart-cut volume (90μL) was larger than the injection volume (80μL). In HPFA, the bound drug is released from the protein in the mobile phase and is eluted together with the unbound drug to give the plateau region. Thus, in principle, all the injected drug molecules are included in the plateau region, and the concentration profile of the total drug in the initial sample solution is transformed into the unbound drug concentration profile in the plateau region. As a result, the volume of the plateau region becomes larger than the initial injection volume [13].

TABLE II. Unbound Concentration of FP Enantiomers in HSA Solutions Determined by HPFA-HPLC System and by Ultrafiltration-HPLC Method

Sample		HPFA-HPLC				Ultrafiltration	
		within-run		day-to-day			
FP (μg/mL)	HSA (μM)	R-FP (ng/mL)	S-FP (ng/mL)	R-FP (ng/mL)	S-FP (ng/mL)	R-FP (ng/mL)	S-FP (ng/mL)
50	300	49.6 (3.13%)	38.2 (9.95%)	53.7 (3.99%)	41.2 (4.39%)	63.6 (23.1%)	49.1 (19.1%)
90	300	275 (2.25%)	249 (2.85%)	274 (1.53%)	243 (2.72%)	274 (3.92%)	254 (4.80%)
90	550	47.6 (4.01%)	37.5 (4.83%)	51.4 (5.76%)	39.3 (6.36%)	41.4 (22.5%)	33.0 (28.4%)

The value in the parentheses is S.D.% (n=5).

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Figure 4 shows the chromatogram obtained when 90μg/mL racemic FP and 550μM HSA mixed solution was analyzed by the present HPFA/ HPLC system. FP enantiomers were well separated from each other within 15 min. R-(-)-FP was eluted faster than S-(+)-FP. The concentrations were calculated as 49.1ng/mL and 36.0ng/mL for R- and S-FP, respectively. The detection limit was 4.8 ng/mL (S/N=3). The peaks eluted at 1.0 min and 4.5 min are the system peaks, which also appeared when the buffer eluent of the HPFA was injected.

To prepare calibration lines, the HPFA column was removed from the line, and the injector was directly connected to six-port valve. After the loop (J in Figure 3) was filled with a given concentration of racemic FP standard solution, the valve J was switched from the solid line to the broken line to achieve the chiral separation. The calibration line for each enantiomer

was prepared by plotting the concentration (22.1 - 496 ng/mL) vs. peak area. Each line was in good linearity (R>0.999).

The mixed solutions containing 50 or 90 μg/mL racemic FP and 300 or 550 μM HSA were subjected to the present system. The results of within-run analysis and day-to-day analysis are listed in Table II. The unbound concentrations determined by the present system agreed with those determined by the conventional ultrafiltration-HPLC method. This confirmed the reliability of the present system. The remarkable advantage of the HPFA/HPLC system over the conventional ultrafiltration-HPLC method is the better reproducibility. The CV% of the present system was 1.53-9.95%, while that of the reference method was 3.92-28.4%.

The binding between FP and HSA is known to be very strong [8]. The unbound FP ratio of these samples is 99.4-99.9%. According to the Scatchard-plot analysis with the data set of the within-run analysis listed in Table II, the binding constant (K) between racemic FP and HSA was estimated as 2.44×10^6 M^{-1} and the binding site (n) on one HSA molecule was estimated as 1.46. These values agree with those estimated from the data obtained by the ultrafiltration-HPLC method ($K=2.34 \times 10^6$ M^{-1}, n=1.47). The binding between FP and HSA is stereoselective. As for every sample solution, the unbound concentration of R-FP was 1.1-1.3 times larger than that of S-FP, which indicates that S-FP binds with HSA more strongly than R-FP.

CONCLUSION

The present HPFA/HPLC system enables enantioselective determination of unbound FP concentration under protein binding equilibrium following a direct sample injection. The comparison of the analytical data with those obtained by use of the conven-

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tional ultrafiltration-HPLC method supported the reliability of the present system. The reproducibility of the present system was much better than the reference method. According to the analytical data, it was found that the binding between FP and HSA is stereoselective, and S-FP binds more strongly than R-FP.

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