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Study of the enantioselective binding between BOF-4272 and serum albumins by means of high-performance frontal analysis

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Abstract

High-performance frontal analysis (HPFA) was incorporated in an on-line HPLC system for the study of the enantioselective binding of BOF-4272, a new xanthine oxidase inhibitor, with human, bovine and rat serum albumins. This HPLC system consists of a HPFA column (diol-silica column), an extraction column (C_4 column) and a chiral separation column (β-cyclodextrin immobilized silica column), which were connected in series via two column switching valves. After the direct injection of a solution of 0.5–400 μM racemic BOF-4272 and 550 μM serum albumin onto the HPFA column, BOF-4272 was eluted, under a mild mobile phase condition (phosphate buffer, pH 7.4, ionic strength 0.17), as a zonal peak containing a plateau region. The drug concentration in the plateau region is the same as that for the unbound drug concentration in the sample solution. A given volume of this plateau region was transferred into the extraction column, and subsequently the extracted BOF-4272 was transferred into the chiral separation column to determine the unbound concentration of each enantiomer.

The binding between BOF-4272 and the serum albumins was enantioselective and species dependent. The unbound concentration of the (+)-isomer in rat serum albumin solution was 1.04–1.14 times larger than that of the antipode, while the unbound concentration of the (−)-isomer in bovine serum albumin solution was 1.04–1.16 times larger than that of the antipode. The enantioselectivity of the binding between BOF-4272 and human serum albumin was concentration dependent. When the total concentration of racemic BOF-4272 was low (0.5 μM or 5 μM), the unbound concentration of the (+)-isomer was 1.15 or 1.06 times larger than that of the (−)-isomer. On the contrary, the unbound concentration of the (−)-isomer was 1.05 or 1.34 times larger than the (+)-isomer in case of the higher total drug concentration (50 μM or 400 μM). Based on the Scatchard analysis of the binding between human serum albumin and BOF-4272 enantiomers, it was found that this change is due to the enantiomeric difference in the binding constant (K) and the number of binding site per protein molecule (n); $K = 1.22 \cdot 10^5\ M^{-1}$ and $n = 2.30$ for the (+)-isomer, and $K = 2.32 \cdot 10^5\ M^{-1}$ and $n = 1.30$ for the (−)-isomer.

1. Introduction

Protein binding is a reversible and kinetically rapid interaction between a drug and serum proteins such as albumin or α_1-acid glycoprotein. Protein binding plays an important role in the pharmacokinetics and pharmacodynamics of drugs [1–3]. While unbound drugs can easily transfer from blood into the target organ to exert the pharmaceutical activity or side effect, bound

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drugs pass hardly through the blood cell wall. Some important characteristics of drug disposition, such as hepatic metabolism, renal extraction and volume of distribution, are described as the function of the protein binding fraction (bound/total concentration ratio). Therefore, the protein binding study is important for the effective and safe use of drugs.

Since serum protein is a highly chiral compound, the protein binding of a chiral drug is potentially stereoselective [4]. In addition, serum protein of different animal species sometimes exhibit different binding characteristics, and the understanding of this difference is important for the interspecies scaling in pharmacokinetics of drugs [5]. So far, several papers have reported on the species dependent stereoselectivity of protein binding [6–13]. For example, human serum albumin (HSA) and bovine serum albumin (BSA) are very similar in primary structure [14], but sometimes exhibit different binding characteristics as in the case of the binding with warfarin; HSA binds (*S*)-warfarin more strongly, but BSA binds (*R*)-warfarin more tightly [11]. The serum protein binding of phenoprocoumon, disopyramide and MK-571 (leukotriene D_4 antagonist) in several mammalian species can be classified into three groups with respect to the stereospecificity; those that bind (*R*)-isomer more tightly, those that bind (*S*)-isomer bound more tightly, and those that exhibit no stereoselectivity [10,12,13].

The protein binding study of a highly bound drug often confronts an analytical problem, that is, the difficulty in the determination of low levels of unbound drug concentrations by using conventional analytical methods such as equilibrium dialysis and ultrafiltration followed by HPLC analysis. To overcome this problem, we have developed a high-performance frontal analysis (HPFA) method [15–23]. HPFA uses a special HPLC column which retains a drug of a small molecular size on the stationary phase ligand but excludes macromolecules such as proteins [24,25]. After the drug–protein solution is injected directly and continuously into HPFA column, an equilibrium zone is generated in the column. In this zone, the drug–protein binding equilibrium in the interstices of packing materials is in the same condition as that in the sample solution, and the drug concentration in the mobile phase in the micropores of the packing materials is the same as the unbound drug concentration in the sample solution. After the sample injection, the size-excluded protein is eluted first, and subsequently the drug is eluted as a trapezoidal peak having a plateau region. The concentration in the plateau region is equal to the unbound concentration in the sample solution. Therefore, the unbound drug concentration can be determined from the plateau height or by the heart-cut method of this plateau region followed by the on-line HPLC analysis. This is the principle of the HPFA method, and hence the appearance of the plateau region is essential for this method. The reliability of the HPFA method has been confirmed by comparison with conventional ultrafiltration or by Scatchard analyses using many kinds of drugs (indometacin, salicylate, acetazolamide, diclofenac, carbamazepine, warfarin, ketoprofen and fenoprofen). So far, a variety of 'restricted- access' type HPLC columns such as a Pinkerton column and a Hisep column as well as a polymer-based size-exclusion column have been used as HPFA columns. Our recent paper [23] demonstrated that a diol-silica column is useful for the HPFA of hydrophobic drugs which bind strongly with plasma protein.

BOF-4272 {sodium (±)-8-(3-methoxy-4-phenylsulfinylphenyl)- pyrazolo[1,5-*a*]-1,3,5-triazine-4-olate monohydrate} is a newly synthesized xanthine oxidase inhibitor for the treatment of hyperuricemia and gout (Fig. 1). BOF-4272 has a chiral center at its sulfur atom, and the (−)-isomer shows its pharmaceutical activity. BOF-4272 binds with plasma protein very strongly, and its low unbound concentration and severe adsorption on membranes have been hindering the accurate and precise measurement of its unbound concentration by using conventional methods.

In this paper, an on-line HPLC system which combines a HPFA column and a chiral separation column was developed for the determination of the unbound concentrations of BOF-4272 enantiomers. This system was applied for the enantioselective study on the protein binding

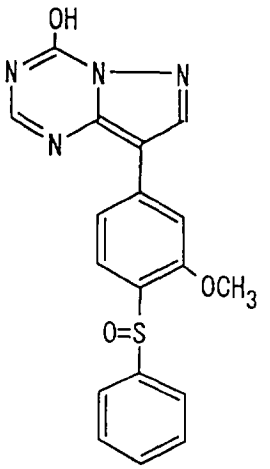

Fig. 1. Structure of BOF-4272.

between BOF-4272 and serum albumins. Interest was focused on the species difference in protein binding.

2. Experimental

2.1. Reagents and materials

Serum albumins (essentially fatty acid free) of human (Cat. No. A-3782), bovine (A-7511) and rat (A-2018) were purchased from Sigma (St.Louis, MO, USA). Racemic BOF-4272 and the enantiomers were obtained from Otsuka Pharmaceutical (Naruto, Japan).

The diol-silica column (Develosil 100Diol5, 30 cm × 8 mm I.D.) was purchased from Nomura (Seto, Japan). β-Cyclodextrin-immobilized silica column (Ultron ED-CD, 15 cm × 6 mm I.D.) was a kind gift from Shinwa (Kyoto, Japan), and the two β-cyclodextrin-immobilized silica columns were connected in tandem. C_4 packing material (Wakosil $5C_4$, Wako, Osaka, Japan) was packed in a stainless-steal column (1 cm × 4 mm I.D.).

2.2. Preparation of sample solution

The stock solutions of BOF-4272 were made up in methanol. An appropriate volume of the stock solution was put in a 10-ml screw-capped glass vial, and the solvent was evaporated by use of a nitrogen gas stream. An appropriate volume of serum albumin solution (in sodium phosphate buffer, pH 7.4, ionic strength, $I = 0.17$) was added to the vial to prepare sample solutions.

2.3. Determination of unbound BOF-4272 enantiomers by the on-line HPLC system

Fig. 2 shows the schematic diagram of the on-line HPLC system. HPFA column F, extraction column G and chiral separation column H were connected via a four-port switching valve I and a six-port switching valve J. Table 1 lists the HPLC conditions.

The BOF-4272–serum albumin solution was injected directly to the diol-silica column from the injector E. BOF-4272 was eluted as a zonal peak with a plateau region. A given volume of the eluent in this plateau region was then heart-cut by switching the valve I, and was transferred into the extraction column G where BOF-4272 was concentrated. The heart-cut volume (1 ml–28 ml) was selected depending on the unbound drug level. By switching the valve J, the extracted drug was desorbed and transferred into the chiral separation column H. The enantiomers were detected at UV 313 nm. The extraction column was washed with water for 1 min before and after the heart-cut procedure. The HPFA

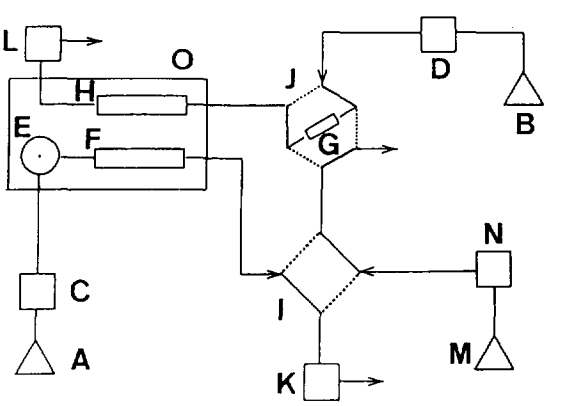

Fig. 2. Schematic diagram of the on-line HPLC system. (A) mobile phase for HPFA; (B) mobile phase for chiral separation; (C,D) pump; (E) sample injector; (F) column for HPFA; (G) column for extraction; (H) column for chiral separation; (I) four-port switching valve; (J) six-port switching valve; (K,L) UV detector; (M) distilled water to wash extraction column; (N) pump; (O) column oven.

Table 1
HPLC conditions of the HPFA–HPLC system

Sub-system		Condition
HPFA	Column	Develosil 100Diol5 (30 cm × 8 mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH 7.4, $I = 0.17$)
	Flow-rate	1.0 ml/min
	Detection	UV 313 nm
	Temperature	37°C
Extraction	Column	Wakosil $5C_4$ (1 cm × 4 mm I.D.)
Chiral HPLC	Column	Ultron ES-CD (15 cm × 6 mm I.D.) two columns were connected in series
	Mobile phase	40 m*M* NaH_2PO_4–MeOH (55:45, v/v), pH 5.6
	Flow-rate	1.0 ml/min
	Detection	UV 313 nm
	Temperature	37°C

column and the chiral separation column were kept at 37°C in a column oven.

The instruments used in this study were as follows: pumps C and D (LC-9A, Shimadzu), pump N (Model A-30-S, Eldex Lab., San Carlos, CA, USA), UV detectors K and L (SPD-6A, Shimadzu), injector E (Rheodyne Type 8125, equipped with 600 μl or 2 ml loop), integrated data analyzer (Chromatopac C-R6A, Shimadzu) and column oven (CS-300C, Chromato-Science, Osaka, Japan).

The injection procedure plays an important role in HPFA. The sample input should ideally be in a rectangular shape in order to create the same drug–protein binding equilibrium zone in the HPFA column as in the sample solution [19]. The sample solution was injected as follows. The injection loop was loaded with 600 μl of the sample solution and then connected to the mobile phase. After 20 s, the injection valve was reswitched, and the loop was detached from the mobile phase flow. Therefore, the actual injection volume was 333 μl. By this injection-reswitching technique, the diffused portion of the sample solution in the injection loop was not introduced into the column, thus preventing the perturbation of the drug–protein binding equilibrium.

Fig. 3 shows the typical time program of valve switching, where the heart-cut time is 32–37 min.

2.4. Calibration lines

The calibration lines were prepared as follows. The diol-silica column was removed from the line, and the injector loop volume was changed from 600 μl to 20 μl. Each 5-μl portion of racemic BOF-4272 standard solutions (5.15, 10.2, 25.3, 51.7, 113, 207 and 354 μ*M*) made up in methanol, was injected directly into the extraction column which had been previously washed with water for 1 min. After perfusing the extraction column with water for 1 min, the adsorbed BOF-4272 was back-flashed into the chiral separation column by the column switching procedure. The calibration line of each enantiomer was prepared by plotting peak area (average of three runs) vs. the amount of each enantiomer injected. The calibration lines were: area = 5.16 × amount (pmole) − 16.2 for the (+)-isomer, and area = 5.20 × amount (pmole) − 15.8 for the (−)-isomer. Each calibration line indicated good linearity (correlation

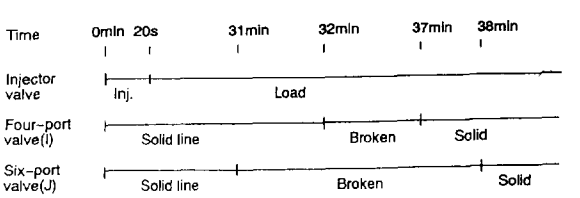

Fig. 3. Typical time program for valve switching.

coefficient, >0.99996). The unbound BOF-4272 concentration was then calculated enantioselectively from the amount of each enantiomer divided by the heart-cut volume.

2.5. Extraction of BOF-4272 on the extraction column

The peak areas of (+)- and (−)-BOF-4272 obtained by injecting a 5-μl portion of 51.7 μM racemic BOF-4272 methanol solution into the extraction column G were 103% and 101% of those obtained by the direct injection of the same volume of the same BOF-4272 solution into the chiral column H. This indicates the complete extraction of BOF-4272 onto the column G and the complete transfer of the extracted BOF-4272 into the chiral separation column.

3. Results and discussion

Fig. 4 shows the elution profile of 50 μM BOF-4272 and 550 μM human serum albumin (HSA) mixed solution. As the injection volume increased from 10 μl to 333 μl, the BOF-4272 peak height reached the maximum level, and trapezoidal drug peak having a plateau region appeared, as shown in Fig. 4C. The plateau height did not increase with the further increase in the injection volume (Fig. 4D). The sample injection volume in HPFA should be large enough to obtain the plateau region of the drug elution profile. Based on this result, the injection volume in the following analyses was 333 μl.

The unique feature of the HPFA method is that the bound drug is not separated from the unbound drug, but is converted into the unbound drug in HPFA column. The eluted amount of the drug is the same as the injected amount, but the concentration of the eluted drug is regulated by the protein binding to be the same as the unbound drug concentration. As a result of this 'regulation effect' [23], the plateau volume becomes larger than the initial injection volume. In Fig. 4C, while the injection volume was 333 μl, as much as ca. 30 ml of the plateau region was observed. As will be mentioned later, the 'regulation effect' is beneficial to detect low levels of unbound drug concentration.

Fig. 5 shows the chiral separation profiles of BOF-4272 on the β-cyclodextrin-immobilized silica column. The sample solutions used in Fig. 5 contained 5 μM racemic BOF-4272 and 550 μM bovine serum albumin (BSA) or rat serum albumin (RSA). The (+)-isomer was eluted faster than the (−)-isomer, and the baseline separation of the enantiomers was achieved within 15 min. Serum albumins did not interfere with the chiral separation, because they were completely excluded before the heart-cut time.

Table 2 lists the unbound concentrations of

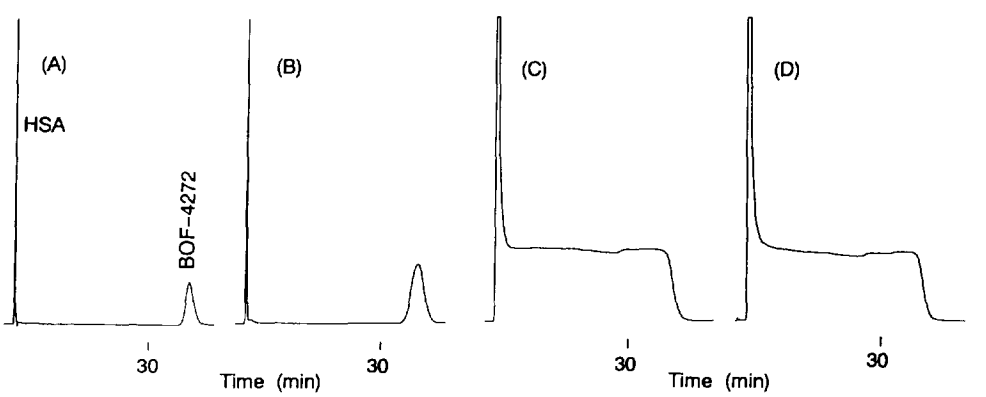

Fig. 4. Elution profile of 50 μM racemic BOF-4272 and 550 μM HSA. Injection volume; (A) 10 μl, (B) 20 μl, (C) 333 μl, (D) 667 μl. HPLC conditions; see Table 1.

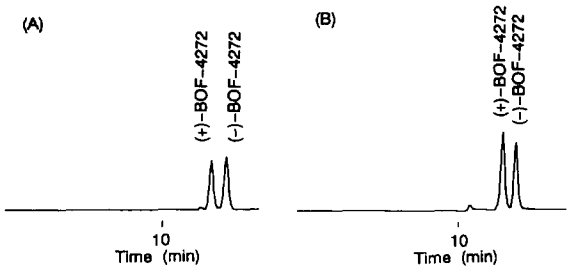

Fig. 5. Chiral separation of BOF-4272 in the heart-cut fraction. Sample solution; (A) 5 μM BOF-4272–550 μM BSA, (B) 5 μM BOF-4272–550 μM RSA. Heart-cut volume, 10 ml. HPLC conditions; see Table 1.

BOF-4272 enantiomers determined by the present on-line HPLC system. The total concentration of racemic BOF-4272 ranges from 0.5 μM to 400 μM. The lowest concentration (0.5 μM) is within the clinical plasma concentration range of BOF-4272. The albumin concentration (550 μM) is its physiological concentration. In the analyses of the samples with the lowest drug concentration (0.5 μM), as much as 28 ml of the plateau region was heart-cut (heart-cut time was from 10 min to 38 min). As a result, low concentrations of unbound enantiomers (1.23–1.52 nM) could be determined by UV detection with good reproducibility (C.V. $< 3.36\%$, $n = 5$). This clearly indicates the usefulness of the 'regulation effect' of the HPFA method for the determination of low levels of unbound drug concentration.

As shown in Table 2, BOF-4272 binds strongly with serum albumins. The unbound fraction was 0.492–1.05% in HSA solutions, 0.44–0.66% in BSA solutions, and 0.532–0.744% in RSA solutions. Comparing the samples containing the same total concentration of BOF-4272 (5 or 50 μM), but different species of serum albumin, the unbound concentrations of both enantiomers were the lowest in BSA solution and were the highest in RSA solution. This means that the protein binding becomes stronger in the order RSA < HSA < BSA.

The binding of BOF-4272 with rat and bovine serum albumins exhibited slight but significant enantioselectivity, and the direction of the selectivity is opposite between these two species. The unbound concentration of the (+)-isomer was 1.04–1.14 times significantly larger ($p < 0.01$) than that of the (−)-isomer in RSA solution. On the other hand, the unbound concentration of the (−)-isomer was 1.04–1.16 times significantly larger ($p < 0.01$) than the antipode in BSA solution.

The binding between BOF-4272 and HSA was also enantioselective, and the selectivity depended on the total drug concentration. In case of the lower total drug concentration (0.5 μM and 5 μM), the unbound concentration of the (+)-isomer was significantly larger ($p < 0.01$) than that of the (−)-isomer. On the other hand, when the total drug concentration was 50 μM,

Table 2
Unbound concentrations (mean ± S.D., $n = 2$) of BOF-4272 enantiomers

Human			Bovine			Rat		
(+)	(−)	(+)/(−)	(+)	(−)	(+)/(−)	(+)	(−)	(+)/(−)
400 μM Racemic BOV-4272 and 550 μM albumin								
1.56 ± 0.091 μM	2.09 ± 0.182 μM	0.748 ± 0.021						
50 μM Racemic BOF-4272 and 550 μM albumin								
170 ± 1.02 nM	178 ± 1.19 nM	0.960 ± 0.003	158 ± 1.01 nM	165 ± 1.06 nM	0.956 ± 0.001	186 ± 0.67 nM	180 ± 0.70 nM	1.04 ± 0.001
5 μM Racemic BOF-4272 and 550 μM albumin								
15.0 ± 0.12 nM	14.1 ± 0.13 nM	1.06 ± 0.002	11.0 ± 0.08 nM	12.8 ± 0.03 nM	0.861 ± 0.01	16.6 ± 0.05 nM	15.4 ± 0.13 nM	1.08 ± 0.01
0.5 μM Racemic BOF-4272 and 550 μM albumin								
1.42 ± 0.025 nM	1.23 ± 0.018 nM	1.15 ± 0.010				1.52 ± 0.051 nM	1.33 ± 0.030 nM	1.14 ± 0.015

the unbound concentration of the (−)-isomer was significantly larger ($p < 0.01$) than the antipode. The enantiomeric difference in the unbound concentration became more prominent for the higher total drug concentration (400 μM).

To elucidate the concentration-dependent enantioselectivity observed in Table 2, Scatchard analyses of the binding between HSA and each enantiomer were performed. Two sets of sample solutions, each containing 550 μM HSA and eight concentrations (0.25–400 μM) of (+)- or (−)-BOF-4272, were analyzed by the present on-line HPLC system, and the Scatchard plots were made as shown in Fig. 6. The correlation coefficient of the line [$r = -0.985$ and -0.990 for (+)-isomer and (−)-isomer, respectively] indicates the good linearity of these plots. The estimated binding parameters were listed in Table 3. The binding constant (K) of the (−)-isomer is almost two times larger than that of the (+)-isomer, whereas the binding site per protein molecule (n) of the (+)-isomer is almost two times larger than that of the (−)-isomer. By using these binding parameters, the enantiomeric unbound concentration ratio [(+)-isomer/(−)-isomer] at the same total drug concentration was calculated from Eq. (1), and was plotted against the total concentration in Fig. 7.

Table 3
Binding parameters for the interaction between HSA and BOF-4272 enantiomers

	$K\ (M^{-1})$	n	R
(+)-BOF-4272	$1.22 \cdot 10^5$	2.30	−0.985
(−)-BOF-4272	$2.32 \cdot 10^5$	1.30	−0.990

Buffer condition: pH 7.4, $I = 0.17$, 37°C.

$$ER = \frac{K_{(-)}(\{[1 + K_{(+)}(n_{(+)}P - C_t)]^2 + 4K_{(+)}C_t\}^{1/2} - 1 - K_{(-)}(n_{(+)}P - C_t))}{K_{(+)}(\{[1 + K_{(-)}(n_{(-)}P - C_t)]^2 + 4K_{(-)}C_t\}^{1/2} - 1 - K_{(-)}(n_{(-)}P - C_t))} \quad (1)$$

where ER is the enantiomeric unbound concentration ratio, C_t is the total concentration of BOF-4272 enantiomer, and P is the protein concentration (550 μM). The subscripts (+) and (−) imply the (+)-isomer and (−)-isomer, respectively. Fig. 7 shows the calculated concentration dependency of the enantioselective BOF-4272–HSA binding, using the enantiomeric unbound concentration ratio as the indication of the enantioselectivity. The effect of mutual enantiomeric displacement is not taken into consideration for convenience. As shown in Fig. 7, when the total drug concentration is low, the calculated (+)/(−) unbound concentration

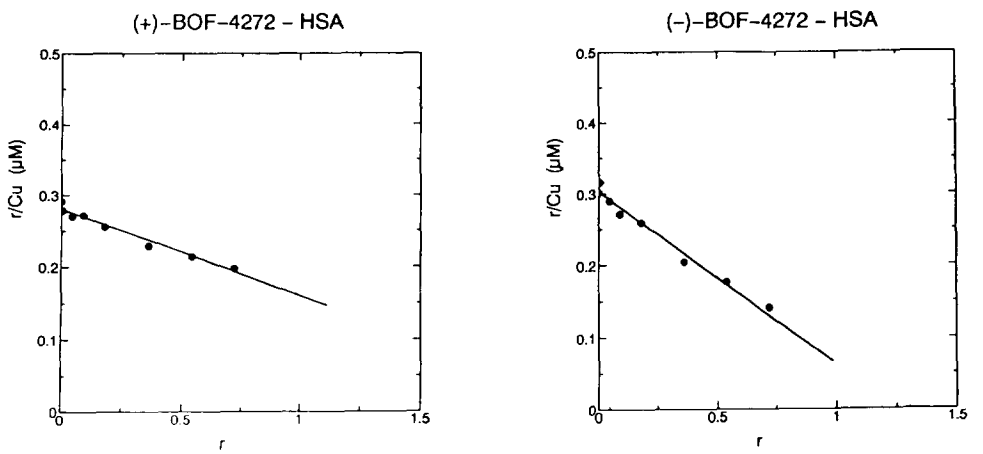

Fig. 6. Scatchard plots of the binding between HSA and BOF-4272 enantiomers. r represents the moles of the bound drug per one mole of HSA. The binding constant (K) and the number of binding sites per protein molecule (n) were estimated by curve fitting using the equation $r/C_u = -Kr + Kn$, where C_u is the unbound drug concentration. The solid lines were the best fitted ones.

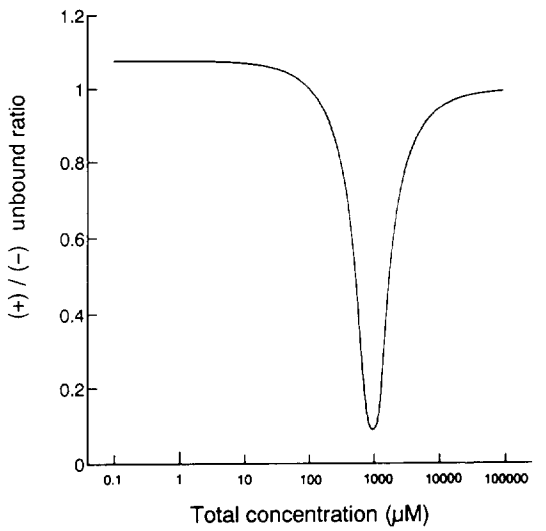

Fig. 7. The relation between the enantiomeric unbound concentration ratio [(+)-isomer/(−)-isomer] of BOF-4272 and the total drug concentration calculated from Eq. 1. The total drug concentration was logarithmically scaled on the abscissa.

ratio is larger than unity, which means that the unbound concentration of the (+)-isomer is larger than that of the antipode. However, with the increase in the total drug concentration, the ratio decreases, and the unbound concentration of the (−)-isomer becomes larger than that of the (+)-isomer. This change agrees with that observed in Table 2. With further increase in the total drug concentration, the enantiomeric unbound concentration ratio reaches the minimum value, and then increases to converge in unity. This concentration dependency can be qualitatively explained as follows. When the total drug concentration is much lower than the protein concentration, plenty of binding sites remain empty. In this condition, the enantiomeric difference in the n value exhibits little effect on the enantioselectivity, and the unbound concentration of the (−)-isomer which has the larger binding constant becomes lower than that of the (+)-isomer. On the contrary, with increasing the drug concentration, the number of the occupied binding sites increases. In this condition, the difference in the n value exhibits more prominent effect on the enantioselectivity than the binding constant, and consequently the unbound concentration of the (+)-isomer which has the larger n value becomes lower than the (−)-isomer. With further increase in the total drug concentration, the unbound fractions of both enantiomers reach unity, and consequently the enantioselectivity diminishes.

A similar concentration dependency was observed in the stereospecific protein binding of disopyramide in cow serum [12], where the binding parameters were different between disopyramide enantiomers in the same fashion as BOF-4272 enantiomers; (*R*)-(−)- disopyramide exhibited the stronger binding constant ($K = 1.3 \cdot 10^5\ M^{-1}$) but smaller binding capacity ($n = 3.7 \cdot 10^{-5}\ M$) than (*S*)-(+)-disopyramide ($K = 0.51 \cdot 10^5\ M^{-1}$, $n = 5.4 \cdot 10^{-5}\ M$). The unbound fraction of (*R*)-(−)-disopyramide was lower than the antipode in case of lower drug concentration (less than 40 μM), and the reverse was true in case of the higher drug concentration (Fig. 2 in Ref. [12]).

For a chiral drug which binds strongly with serum protein, the stereoselectivity of the protein binding may be examined, for the convenience of detection, by using model sample solutions of much higher drug concentration than the therapeutic levels. This approach may be misleading, because the stereoselectivity in the therapeutic levels may be different as is the case for BOF-4272. HPFA serves to avoid this problem by enabling the determination of low levels of stereoselective unbound drug concentrations.

4. Conclusion

The present on-line HPLC system which combines a HPFA column and a chiral separation column allows the simple and easy determination of the concentration of unbound BOF-4272 enantiomers. The 'regulation effect' served to detect as low as 1 nM of unbound BOF-4272 by UV detection. The binding between BOF-4272 with serum albumins was strong and enantioselective. RSA and BSA exhibited different enantioselectivity. The binding between BOF-4272 and HSA exhibited the concentration de-

pendent enantioselectivity based on the enantiomeric difference in the binding parameters.

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