Focussing Review

Application of High-Performance Frontal Analysis to Enantioselective Studies on Drug - Plasma Protein Binding

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Abstract

This review article demonstrates the applicability of high-performance frontal analysis (HPFA) to the enantioselective drug - plasma protein binding study. Semotiadil (R-isomer, Ca-channel blocker) and levosemotiadil (S-isomer, Ca- and Na-channel blocker), an enantiomeric pair of drugs under development, were used as model drugs, and their binding properties to human serum albumin (HSA) and a1-acid glycoprotein (AGP) were investigated. An on-line HPLC system consisting of HPFA column, extraction column and analytical HPLC column was used to determine the unbound concentrations of these enantiomers in the binding equilibrium. The experimental data were subjected to the Scatchard analyses to estimate binding parameters. Semotiadil and levosemotiadil are bound to AGP more strongly than to HSA by 110 times and by 30 times, respectively. Semotiadil is bound to AGP 1.2 times more strongly than levosemotiadil, while levosemotiadil is bound to HSA 3 times more strongly than semotiadil. The enantioselectivity of AGP is reflected in the enantioselective protein binding of these drugs in human plasma. The enantiomer-enantiomer competitive binding study found that both enantiomers are bound at the same site on AGP molecule.

Keywords: semotiadil, levosemotiadil, protein binding, albumin, AGP, high-performance frontal analysis, HPLC.

INTRODUCTION

A drug in plasma is bound to proteins. This binding, called plasma protein binding, is a reversible and kinetically rapid interaction. Unbound drug transfers freely to the target organ, whereas bound drug hardly pass through the blood capillary walls. As a result, unbound drug concentration shows much better correlation with pharmacological activity than the total (bound + unbound) drug concentration. Some important pharmacokinetic properties such as hepatic metabolism rate, renal excretion rate, biomembrane permeation rate and steady-state distribution volume are the function of the unbound drug fraction (unbound / total concentration ratio) [1-3].

Several plasma proteins such as albumin, α_1-acid glycoprotein (AGP) and lipoproteins contribute simultaneously to plasma protein binding of one drug. The binding properties of these plasma proteins are different from each other, and their plasma concentrations may vary depending on gender, age and/or disease state of patients. Because of these complex and dynamic characters, it is necessary to investigate the binding property of individual plasma protein for the comprehensive elucidation of drug distribution in plasma. In case of a racemic drug, the enantioselective plasma protein binding study is inevitable, because usually only one enantiomer shows the pharmacological activity, and plasma protein binding affinity is often different between the enantiomers [4,5].

So far, several methodologies have been proposed for the drug - plasma protein binding study [6,7]. Equilibrium dialysis or ultrafiltration followed by HPLC analysis is the most commonly used to determine unbound drug concentration in plasma protein binding equilibrium. However, these conventional methods are often inapplicable to the analyses of strongly bound drugs because of technical problems such as drug adsorption on the membrane and the leakage of bound drug through membrane. To overcome these problems, we developed high-performance frontal analysis (HPFA) method, a novel binding analysis method using a separation system such as HPLC and capillary electrophoresis. This method has been applied to the highly sensitive analysis of strongly bound drug, enantioselective protein binding study, ultramicro binding study of AGP and lipoproteins [8-29]. This review article deals with the

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application of HPFA method to the enantioselective plasma protein binding study of semotiadil and levosemotiadil, an enantiomeric pair of drugs under development (chemical structure, see Fig.1) [21-23].

Semotiadil (R-isomer) is a calcium antagonist with non-dihydropyridine structure and shows antihypertensive [30,31] and antianginal activities [32,33]. Levosemotiadil (S-isomer) is an antiarrhythmic drug with sodium and calcium channel blocking action [34-39], as well as potassium blocking activity [40]. The optical purity of each enantiomer, determined by the chiral HPLC system, was more than 99%. Both enantiomers are highly bound to plasma proteins, and the unbound drug concentration could not be determined precisely using any conventional methods.

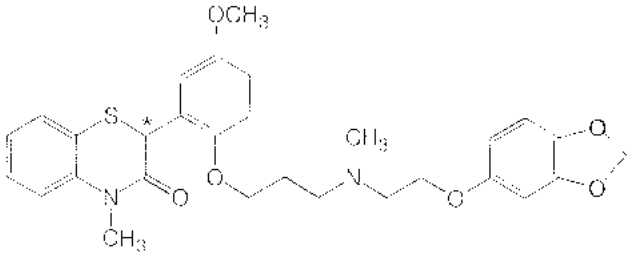

Figure 1. Chemical structure of semotiadil/levosemotiadil.

PRINCIPLE AND FEATURES OF HPFA

1. Principle of HPFA

HPFA method uses a restricted-access type HPLC column [41,42]. This type of columns have the nature to exclude large molecule of plasma protein but to retain a drug of small molecular size mainly in the micropores. Therefore, this type of columns allow a direct injection analysis of plasma samples without deproteinization. If the injection volume is small, the sample solution is diluted with mobile phase, and the bound drug is released from protein. However, if the sample solution is injected continuously, the release of the bound drug in the column is apparently suppressed, and finally an equilibrium zone is generated near the top of the column. In this zone, two different equilibrium states are established simultaneously. One is the chromatographic partition equilibrium inside the micropores, and the other is drug-protein binding equilibrium in the interstices of packed particles (outside the micropores). The drug concentration in the stagnant flow of mobile phase in the micropores is equal to the unbound drug concentration in the bulk mobile phase in the interstices. Since the protein concentration is the same with that in the initial sample solution, the unbound drug concentration in the mobile phase is also equal to that in the initial sample solution. That is, the mobile phase in the interstices is replaced by the sample solution. After the end of sample injection, the drug is separated from the protein. During the separation, the drug- protein binding equilibrium is kept constant, because plasma protein binding is a reversible and kinetically rapid process. Then, the unbound drug zone is generated in the column. This drug zone is eluted as a trapezoidal peak with a plateau region, and the drug concentration in the plateau region is equal to the unbound drug concentration in the initial sample solution. The plateau height and the peak area correspond to the unbound drug concentration and total drug concentration, respectively. This is the principle of HPFA method, and unbound drug concentration is determined from the plateau height or by on-line or off-line analysis of the plateau region.

2. Features of HPFA Method

The features of HPFA method, which were described in detail elsewhere [43], can be summarised as follows,

1) HPFA allows a simple binding analysis following direct sample injection, and does not suffer from undesirable drug adsorption onto membrane nor leakage of the bound drug from the membrane, which are often encountered in the conventional ultrafiltration and equilibrium methods.
2) In HPFA, bound drug is not separated from unbound drug, but is transformed into unbound form. As a result, not only unbound drug concentration but also total drug concentration can be determined simultaneously, as in case of carbamazepine binding analyses [9] .
3) HPFA can be easily incorporated into on-line HPLC system. By coupling HPFA column with a chiral HPLC column, the unbound concentration of a racemic drug can be determined enantioselectively.
4) The detection limit can be markedly improved by coupling HPFA column with a preconcentration column.
5) Frontal analysis achieved in capillary electrophoresis format (HPFA/CE) enables the binding assay using very small sample injection volume (ca. 100nL).

PROTEIN BINDING STUDY OF SEMOTIADIL AND LEVOSEMOTIADIL

1. On-line HPFA/HPLC system

Figure 2 shows the schematic diagram of the on-line HPFA/HPLC system. HPFA column, extraction column and analytical column were connected via four-port and six-port switching valves. The instruments used are as follows: HPLC pumps, LC 6A (Shimadzu, Kyoto, Japan) and Twincle (Jasco, Tokyo, Japan); UV detectors, SPD-2A and SPD-6A, (Shimadzu); injector, Rheodyne Type 8125 equipped with a 5-mL loop; integrated data analyzer, Chromatopac C-R3A and C-R6A (Shimadzu).

Table 1 lists the HPLC conditions. Sodium phosphate buffer (pH 7.4, I=0.17) without adding any organic modifier was used as the mobile phase of HPFA so as not to disturb the drug-protein

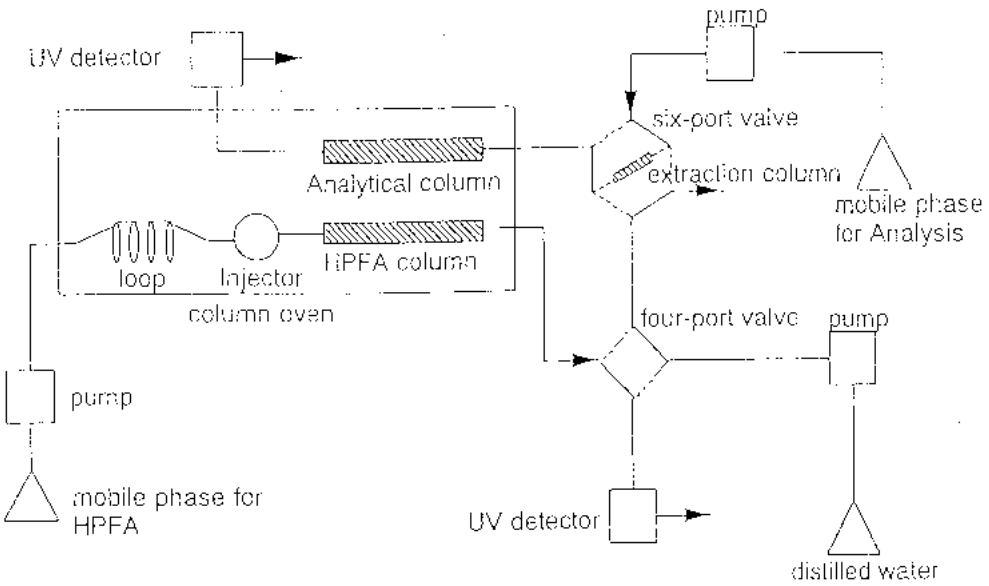

Figure 2. Schematic diagram of on-line HPFA/HPLC system.

Table 1. Condition of on-line HPFA/HPLC system for semotiadil binding study

Subsystem	Condition	
HPFA	Column	Develosil 100 Diol 5 (5cm x 4.6 mm i.d.)
	Mobile phase	Phosphate buffer (pH7.4, I=0.17)*
	Flow rate	1.0 mL/min
xtraction	Column	Wakosil 5C4 (1cm x 4 mm i.d.)
Achiral HPLC	Column	Develosil 300 ODS UG5 (15 cm x 6 mm i.d.)
	Mobile phase	40mM NaH_2PO_4 : MeOH = 4:6 (v:v) *
	Flow rate	1.0 mL/min
Chiral HPLC	Column	Ultron ES-OVM (15 cm x 6 mm i.d.) x 2
	Mobile phase	Phosphate buffer (pH6.5, I=0.05) : MeCN = 8:2 (v:v) *
	Flow rate	1.0 mL/min

Detection, UV 230 nm. Temperature, 37˚C.

* The phosphate buffer (pH7.4, I=0.17) contains 13.5mM NaH_2PO_4 and 52.2mM Na_2HPO_4, and the phosphate buffer (pH6.5, I=0.05) contains 24.0mM NaH_2PO_4 and 8.68mM Na_2HPO_4. Methanol and acetonitrile, both of HPLC grade, were purchased from Wako (Osaka, Japan)..

binding equilibrium. A diol-silica column was used as the HPFA column. This column allows the elution of the hydrophobic drugs under the mild mobile phase condition. HPFA - achiral HPLC system was used when the sample solutions contain only one enantiomer. HPFA - chiral HPLC system was used in the competitive binding study where the sample solutions contain both enantiomers.

2. Determination of unbound drug concentrations

The drug - protein mixed solution was directly injected to the HPFA column. The drug was eluted as a zonal peak with a plateau region, and the drug concentration in the plateau region is equal to the unbound drug concentration in the sample solution. Then, a given volume of the plateau region was transferred into the extraction column by switching the 4-port valve ("heart-cut" procedure). The unbound drug was completely trapped on this column. By switching the 6-port valve, the mobile phase for the analytical column was introduced into the extraction column, and the trapped unbound drug was completely transferred to the analytical column. The area of drug peak (retention time, 3.5 min) was measured at 230 nm. The extraction column was washed with distilled water for 1 min before and 30 s after the heart-cut procedure.

3. Optimization of Sample injection volume

Sample injection volume is a crucial factor in HPFA analyses. If the injection volume is insufficient, the original drug - protein

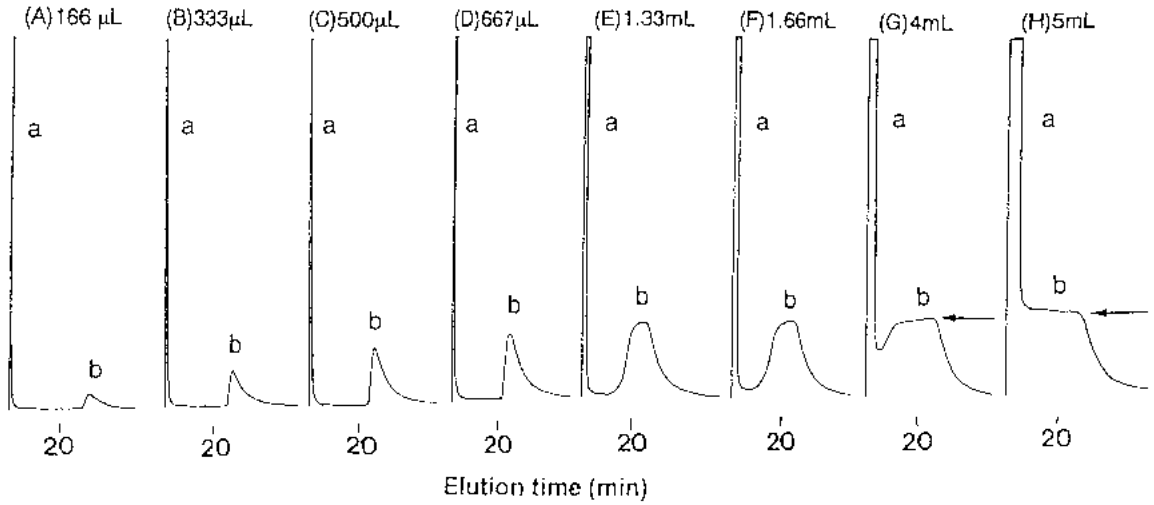

Figure 3. Effect of injection volume upon HPFA profile.
a, HSA. b, semotiadil.
Sample solutions, 80µM semotiadil - 100µM HSA mixed solution.

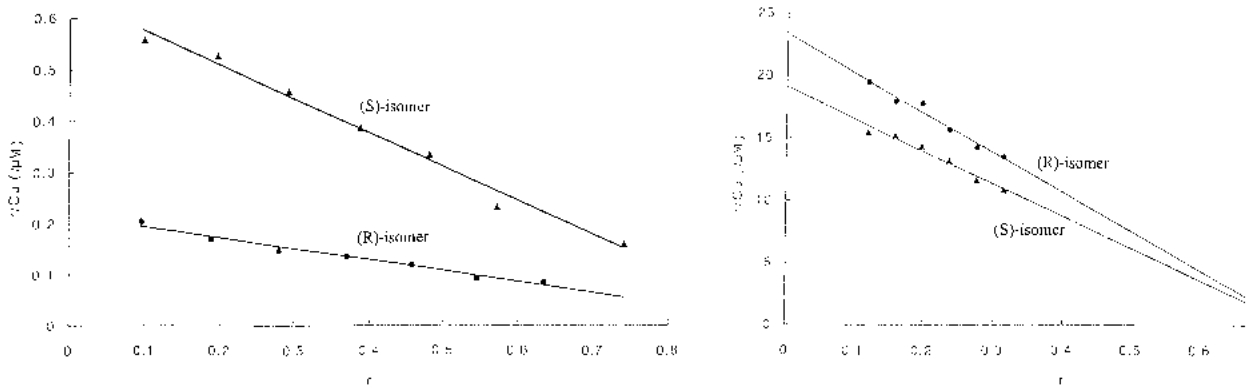

Figure 4. Scatchard plots of semotiadil (R-isomer) and levosemotiadil (S-isomer) to HSA (left) and human AGP (right). Sample solutions, 9.89 - 78.7 mM drug in 100 µM HSA (pH 7.4, I=0.17, 37˚C), and 2.98 - 7.87 mM drug in 25 µM human AGP (pH 7.4, I=0.17, 37˚C). HSA (Ca.No. A-3782, fatty acid free) and human AGP (Ca.No. G-9885) were purchased from Sigma.

binding equilibrium is not reproduced in the column, and the true values of unbound drug concentration cannot be determined. Therefore, it is essential to decide the sufficient injection volume to obtain the plateau region. Figure 3 shows the chromatograms of 80µM semotiadil - 100µM HSA mixed solution which contains the highest unbound drug fraction among the samples used in this binding study. The drug peak height increased with increasing injection volume, and finally reached the maximum level. When a 5-mL portion of the sample solution was injected, a clear plateau peak appeared. Further increase in the injection volume resulted in expansion of the plateau region but did not raise the plateau height. Therefore, the injection volume was decided as 5mL.

It is necessary to prevent the diffusion of the sample solution in the injector loop, because otherwise the binding equilibrium may be disturbed and the plateau region may disappear. The "injector-reswitching technique" is useful to overcome this problem.[12-15] The sample solution was injected as follows. The injector loop was loaded with a 6-mL portion of the sample solution and connected to the mobile phase flow (1mL/min) for 5 min. Therefore, the actual injection volume was 5mL. Due to the above-mentioned procedure, the sample input was regarded as an ideal rectangular shape. In the similar way, the actual injection volume for AGP binding study was optimized as 667µL.

4. Estimation of binding parameters

Figure 4 shows the Scatchard plots of HSA binding and human AGP binding, respectively. The binding parameters were estimated

Table 2. Binding parameters of semotiadil (R-isomer) and levosemotiadil (S-isomer) to HSA and to AGP

	binding to HSA			binding to AGP		
	K (/M)	n	nK (/M)	K (/M)	n	nK (/M)
R-isomer	2.15×10^5	0.99	2.13×10^5	3.17×10^7	0.74	2.34×10^7
S-isomer	6.59×10^5	0.97	6.40×10^5	2.59×10^7	0.74	1.92×10^7

Table 3. Unbound drug concentrations in human plasma

Total drug conc.(μM)	Unbound drug conc.* R-isomer(nM)	S-isomer(nM)	R/S	Bound fraction* R-isomer	S-isomer
1	4.35±0.09	6.11±0.04	0.71	99.57±0.009%	99.39±0.004%
0.5	3.02±0.07	4.32±0.06	0.70	99.40±0.016%	99.14±0.013%

*Mean ± SD (n=5)
Heart-cut volume, 20 mL.

by fitting the experimental data to the following equation

$$r/Cu = -Kr + nK$$

where r, Cu, K and n represent the number of moles of bound drug per one mole of HSA, the unbound drug concentration, binding constant and the number of binding site on one HSA molecule, respectively. Both enantiomers are bound to HSA or human AGP at a single binding site. The correlation coefficient of the lines was -0.97 ~ -0.99, indicating a good agreement of the experimental data with the theoretical equation. The contribution of the binding at non-specific (secondary) binding sites was not observed.

The binding parameters and the total binding affinity (nK) are listed in Table 2. It was found that the total binding affinity of AGP is 110 times for (R)-isomer and 30 times for (S)-isomer stronger than that of HSA. The orientation in chiral discrimination is opposite between HSA and human AGP. Whereas the binding of (S)-isomer to HSA is about three times stronger than that of (R)-isomer, the binding of (R)-isomer to AGP is about 1.2 times stronger than that of (S)-isomer.

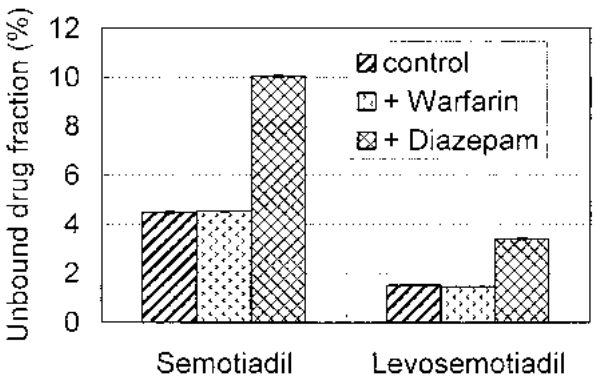

Figure 5. Effect of warfarin and diazepam upon unbound fraction of semotiadil and levosemotiadil in HSA solutions.
Sample solutions, 10μM semotiadil or levosemotiadil + 100 μM HSA with/without 100μM warfarin or 40μM diazepam (pH 7.4, I=0.17, 37˚C). Warfarin (Ca.No. A2250) and diazepam (Ca.No. D0899) were purchased from Sigma.

5. Identification of the binding site on HSA molecule

The warfarin and diazepam binding sites are the two major binding sites on HSA molecule where many drugs are bound. To identify the binding site of semotiadil and levosemotiadil, the change in the unbound drug fraction caused by the addition of warfarin or diazepam was investigated. Figure 5 compares the unbound fraction of semotiadil or levosemotiadil in 100 μM HSA solutions in the absence or presence of 100μM warfarin or 40μM diazepam. The unbound concentrations of both enantiomers were not changed by the addition of warfarin. On the other hand, their unbound fractions were increased more than two-fold by the addition of diazepam. This result suggests that both enantiomers are bound at diazepam site.

6. Enantiomer-enantiomer interaction on AGP binding

The present system was applied to investigate whether both enantiomers are bound at a single binding site on AGP molecule. Figure 6 shows the unbound concentrations of (R)- and (S)-isomers in a series of sample solutions containing 25μM AGP, 3, 5 or 7μM (R)-isomer and 5μM (S)-isomer. Incorporation of the chiral HPLC column into the on-line HPLC system allowed the determination of unbound concentrations of both enantiomers simultaneously. The lines in Figure 6 show the theoretical values calculated assuming the competitive binding (line 1) and the independent binding (line 2).

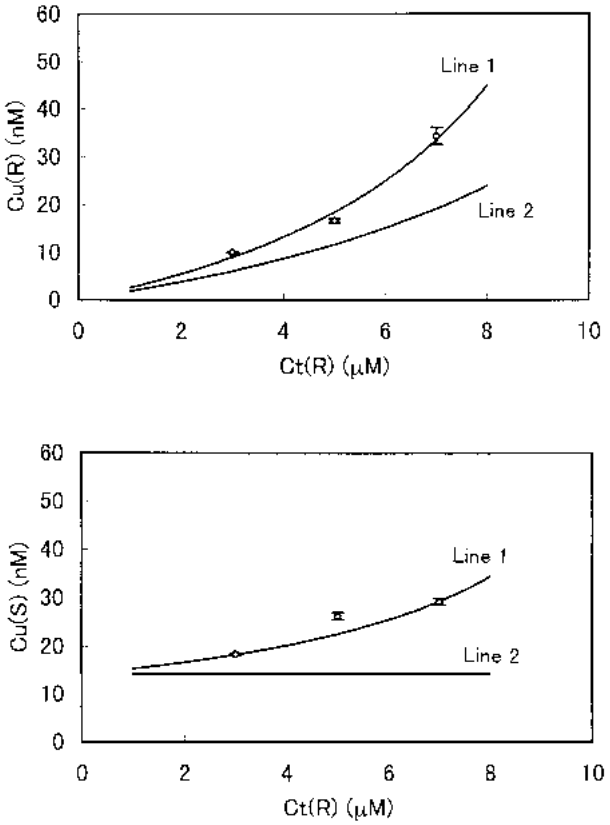

Figure 6. Unbound concentrations of semotiadil (R-isomer) (upper) and levosemotiadil (S-isomer) (lower) in 25μM AGP solutions (pH7.4, I=0.17, 37˚C) containing 3, 5 or 7 μM (R)-isomer and 5mμM (S)-isomer. Line 1, calculated according to competitive binding model. Line 2, calculated according to independent binding model. Ct(R), Cu(R) and Cu(S) represent the total concentration of (R)-isomer, the unbound concentration of (R)-isomer and the unbound concentration of (S)-isomer, respectively.

The observed values are in good agreement with the calculated values based on the competitive binding mode. The agreement indicates that both enantiomers are bound at the same binding site competitively without any kind of allosteric effect.

7. Determination of unbound drug concentrations in human plasma

The present method was applied to the analysis of human plasma samples. Table 3 shows the unbound drug concentrations in human plasma samples. The total drug concentrations are in their therapeutic levels. The unbound concentration as low as 3nM can be determined with good reproducibility using a common UV detector. This is because the unbound drug in as large as 20mL of plateau region was preconcentrated in the present on-line HPLC system. The bound fractions of both enantiomers are more than 99%. Likewise in the AGP solutions, the unbound concentration of R-isomer in human plasma was lower than that of S-isomer. This means that AGP plays a principal role in the enantioselective plasma protein binding.

In conclusion, semotiadil is bound to AGP 1.2 times stronger than levosemotiadil. This enantioselectivity is opposite to that of HSA. Because the affinity of AGP to these enantiomers is 100 or 30 times stronger than HSA, the protein binding in human plasma reflects the enantioselectivity of AGP. Both enantiomers are bound at a single common binding site on AGP molecule competitively. The present study demonstrates the utility of HPFA method in quantitative and enantioselective plasma protein binding study of highly bound drugs.

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