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Binding study of desethyloxybutynin using high-performance frontal analysis method

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Abstract

Plasma protein binding of *N*-desethyloxybytynin (DEOXY), a major active metabolite of oxybutynin (OXY), was investigated quantitatively and enantioselectively using high-performance frontal analysis (HPFA). An on-line HPLC system which consists of HPFA column, extraction column and analytical column was developed to determine the unbound concentrations of DEOXY enantiomers in human plasma, in human serum albumin (HSA) solutions, and in human α_1-acid glycoprotein (AGP) solutions. DEOXY is bound in human plasma strongly and enantioselectively. The unbound drug fraction in human plasma samples containing 5 μM (*R*)- or (*S*)-DEOXY was 1.19±0.001 and 2.33±0.044%, respectively. AGP plays the dominant role in this strong and enantioselective plasma protein binding of DEOXY. The total binding affinity (*nK*) of (*R*)-DEOXY and (*S*)-DEOXY to AGP was 2.97×10^7 and 1.31×10^7 M^{-1}, respectively, while the *nK* values of (*R*)-DEOXY and (*S*)-DEOXY to HSA were 7.77×10^3 and 8.44×10^3 M^{-1}, respectively. While the *nK* value of (*S*)-DEOXY is weaker than that of (*S*)-OXY (1.53×10^7 M^{-1}), the *nK* value of (*R*)-DEOXY is 4.33 times stronger than that of (*R*)-OXY (6.86×10^6 M^{-1}). This suggests that the elimination of an ethyl group weakens the binding affinity of the (*S*)-isomer because of the decrease in hydrophobicity, while the binding affinity of the (*R*)-isomer is enhanced by the decrease in steric hindrance. The total binding affinity of DEOXY to HSA is much lower than that of DEOXY-AGP binding as well as OXY-HSA binding (2.64×10^4 and 2.19×10^4 M^{-1} for (*R*)-OXY and (*S*)-OXY, respectively). The study on competitive binding between OXY and DEOXY indicated that DEOXY enantiomers and OXY enantiomers are all bound competitively at the same binding site of AGP molecule. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Protein binding; Frontal analysis; Enantiomer separation; Desethyloxybutynin

1. Introduction

The overall protein binding property of a drug in plasma is influenced simultaneously by several proteins such as albumin, α_1-acid glycoprotein (AGP) and lipoproteins, and their binding characters are different from each other [1–4]. In addition, the interindividual variations and disease-depending change in the plasma concentrations of AGP are observed [5–7]. These factors give a complicated

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and dynamic character to plasma protein binding. In case of a racemic drug, the binding affinity is potentially different between the enantiomers, which may cause the difference in their pharmacokinetic properties [4,8]. The enantiomer-enantiomer interaction may also affect the binding equilibrium of both enantiomers. In addition, the competition in protein binding between a drug and its metabolite may occur because of their similarity in chemical structure. These effects further enhance the complexity in plasma protein binding property. Therefore, quantitative and enantioselective binding studies of individual proteins and drug-metabolite interaction are important in developing a racemic drug and its safe and rational use.

N-Desethyloxybytynin (DEOXY, Fig. 1) is a major and active metabolite of oxybutynin (OXY, Fig. 1) which is used in the treatment of urinary incontinence. The plasma concentration–time profile of DEOXY parallels that of OXY, while the maximum plasma concentration (C_{max}) and the area under the concentration–time curve (AUC) value of DEOXY is about 10 times higher than that of the parent drug [9–12]. Both OXY and DEOXY are chiral compounds, and (*R*)-OXY and (*R*)-DEOXY exhibit high antimuscarinic activity relative to the antispasmodic activity, while (*S*)-OXY and (*S*)-DEOXY exhibit relatively weak antimuscarinic activity [12]. Although the important role of plasma protein binding in pharmacokinetic property of drugs is well recognized, the binding property of DEOXY has not been investigated, probably because of the lack of an applicable analytical method.

The analysis of strong protein binding using conventional methods such as ultrafiltration and equilibrium dialysis very often encounters the problems such as difficulty in detecting a low level of unbound drug, undesirable drug adsorption onto membrane and the leakage of bound drug which causes considerable overestimation of unbound drug concentration. Binding analysis using a separation system such as HPLC and capillary electrophoresis has the potentialities to avoid these problems, and several analytical modes have been proposed [13,14]. For example, albumin-immobilized HPLC column was applied to the enantioselective and competitive binding studies of strongly bound drugs [15–19]. High-performance frontal analysis (HPFA) is another chromatographic method suitable for the analysis of strong protein binding. The principle and the features of HPFA method were reported elsewhere [20,21]. In the previous paper, we developed an HPFA-based on-line HPLC system for the sensitive determination of unbound OXY enantiomers, and investigated the enantioselective binding properties [22]. It was found that (i) the binding affinity of OXY enantiomers to AGP is stronger by two orders of magnitude than that to HSA, (ii) (*S*)-OXY is bound to AGP 2.23 times more strongly than (*R*)-OXY, and this enantioselectivity is reflected in the overall human plasma protein binding, (iii) OXY enantiomers are bound to AGP at the same binding site competitively.

In this paper, an on-line HPLC system involving HPFA method is developed for the binding assay of DEOXY enantiomers, and their binding affinities to AGP and HSA were estimated. In addition, drug (OXY)–metabolite (DEOXY) interaction upon AGP binding are investigated.

2. Experimental

2.1. Samples

The enantiomers of (*R*)- and (*S*)-desethyloxybutynin (DEOXY) were obtained from Sepracor

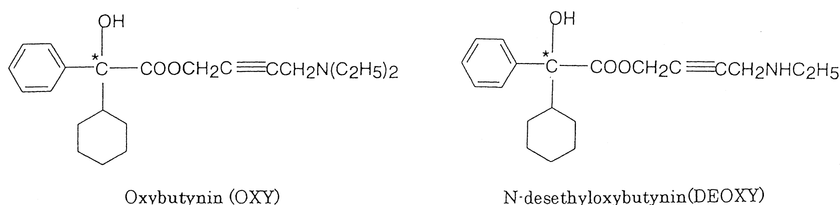

Fig. 1. Chemical structures of oxybutynin (OXY, left) and *N*-desethyloxybutynin (DEOXY, right).

(Newton, MA, USA). HSA (Ca. No. A-3782, fatty acid free) and human AGP (Ca. No. G-9885) were purchased from Sigma (St. Louis, MO, USA). The drug-protein mixed solutions were prepared in sodium phosphate buffer (pH 7.4, $I=0.17$). Develosil 100 Diol 5 was purchased from Nomura Chemicals (Seto, Japan). YMC-Pack ODS-AM was purchased from YMC (Kyoto, Japan). Chemcosorb ODS 7 was purchased from Chemco (Osaka, Japan).

2.2. On-line HPFA/HPLC system

Fig. 2 shows a schematic diagram of the present on-line HPLC system. The HPFA column, the extraction column and the analytical column were connected via a four-port switching valve and a six-port switching valve. The instruments used were as follows: HPLC pumps, HPLC pumps, LC 9A (Shimadzu, Kyoto, Japan) and model A-30-S (Eldex Lab., San Carlos, CA, USA). UV detectors, SPD-6A, (Shimadzu). Injector, Rheodyne Type 8125. Integrated data analyzer, Chromatopac C-R3A and C-R6A (Shimadzu). Column oven, CS-300C (Chromato-Science, Osaka, Japan).

Table 1 lists the HPLC conditions. Sodium phosphate buffer of physiological pH (7.4) was used as the mobile phase for HPFA without addition of any organic modifier so as not to disturb the drug-protein binding equilibrium. The HPFA column (diol-silica column) is hydrophilic so that hydrophobic DEOXY enantiomers were eluted out even under the mild mobile phase condition.

2.3. Determination of unbound drug concentrations by HPFA/HPLC system

The DEOXY–protein mixed solution was directly injected to the HPFA column. DEOXY was eluted out as a zonal peak with a plateau region. In accord with the principle of the frontal analysis, the DEOXY concentration in this plateau region is equal to the unbound concentration in the sample solution. Then, a given volume (2 ml for AGP binding study and 0.5 ml for HSA binding study) of this plateau region was transferred into the extraction column by switching the four-port valve (‘heart-cut’ procedure). The unbound DEOXY was completely trapped on the extraction column. The mobile phase for the

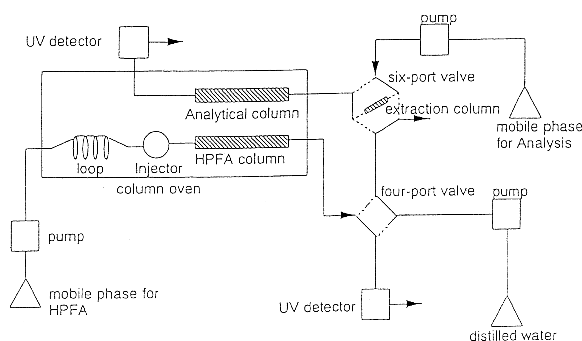

Fig. 2. Schematic diagram of on-line HPFA/HPLC system.

analytical column was introduced into the extraction column by switching the six-port valve, and the trapped unbound drug was completely transferred to the analytical column. The extraction column was washed with distilled water for 30 s. before and after the heart-cut procedure. The HPFA column and the analytical column were kept at 37°C in a column oven. The actual injection volume was 1.5 ml for AGP sample solutions and 0.8 ml for HSA sample solutions.

Table 2 shows the HPLC condition for the competitive study of OXY–DEOXY interaction. OXY was retained more strongly than DEOXY on the diol–silica column. In order to obtain a proper retention of OXY, the length of the HPFA column (inner diameter, 4.6 mm) was reduced from 15 to 10 cm, and the heart-cut time was set at 17.5–19.5 min. Both OXY and DEOXY were eluted as the plateau zone during the heart-cut period. The mobile phase condition is also changed for the simultaneous determinations of OXY and DEOXY as shown in Table 2. The retention times of OXY and DEOXY from ODS column are 15.6 min and 8.8 min, respectively.

2.4. HPFA profile

Fig. 3 shows the frontal analysis profile of 10 μM (S)-DEOXY and 20 μM AGP mixed solution. A clear plateau region is observed. Fig. 4 shows the chromatograms of 10 μM (R)-DEOXY and 600 μM HSA mixed solution (left), 600 μM HSA solution (center) and their subtraction chromatogram (right). A clear plateau zone was observed in the subtraction

Table 1
HPFA conditions for Scatchard analysis

Subsystem	Condition	
HPFA	Column	Develosil 100-Diol-5 (15 cm×4.6 mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH 7.4, $I=0.17$)
	Flow-rate	1.0 ml/min
	Temperature	37°C
Extraction	Column	Develosil ODS 10 (1 cm×4.6 mm I.D.)
Analytical HPLC	Column	YMC-Pack ODS-AM (15 cm×4.6 mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH 6.5, $I=0.04$): CH_3CN=1:1 (v:v), pH 7.3
	Flow-rate	1.0 ml/min
	Temperature	37°C

Detection, UV 220 nm.

chromatogram. A given volume of this plateau zone was 'heart-cut' and was analyzed on the ODS column. The retention time of DEOXY was 5.8 min.

2.5. Calibration lines

The calibration lines were prepared as follows. The HPFA column was removed from the on-line system, and a 5-ml injector loop was replaced by a 20-μl loop which was connected directly to the extraction column. After washing the extraction column with distilled water for 30 s, each 5-μl portion of a series of (*R*)-DEOXY or (*S*)-DEOXY standard solutions (2.5, 5, 10, 15 and 30 μ*M* for AGP binding study, 10, 25, 50, 75, 100, 150 and 200 μ*M* for HSA binding study) made up in methanol was injected. After perfusing the extraction column with water for 30 s, the adsorbed DEOXY was back-flashed into the analytical column by the column switching procedure. The calibration line was prepared by plotting peak area versus injected drug amount. In the study of OXY–DEOXY interaction, each 5 μl of a series of standard solutions containing both (*R*)-OXY+(*R*)-DEOXY or (*S*)-OXY+(*S*)-DEOXY (the concentration of each enantiomer was 10, 20, 40, 60 and 80 μ*M*) made up in methanol was injected. The calibration curves showed good linearity ($r^2>0.994$).

3. Results and discussion

3.1. Binding affinity of DEOXY enantiomers to AGP and HSA

A series of 20 μ*M* AGP sample solutions containing 2, 4, 6, 8 and 10 μ*M* (*R*)-DEOXY or (*S*)-DEOXY were subjected to the on-line HPFA-HPLC analysis to determine the unbound drug concentrations. Each sample was analyzed in triplicate. The

Table 2
HPFA conditions for the study of OXY–DEOXY interaction

Subsystem	Condition	
HPFA	Column	Develosil 100-Diol-5 (10 cm×4.6 mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH 7.4, $I=0.17$)
	Flow-rate	1.0 ml/min
	Temperature	37°C
Extraction	Column	Develosil ODS 10 (1 cm×4.6 mm I.D.)
Analytical HPLC	Column	YMC-Pack ODS-AM (15 cm×4.6 mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH 6.5, $I=0.04$): CH_3CN=65:35 (v:v), pH 5.3
	Flow-rate	1.0 ml/min
	Temperature	37°C

Detection, UV220 nm.

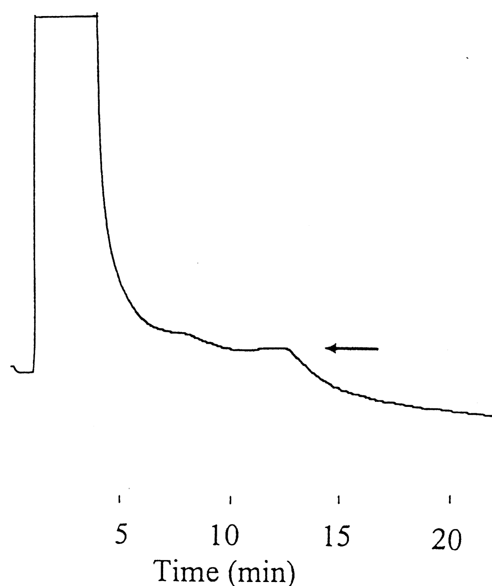

Fig. 3. HPFA profile of 10 μM (S)-DEOXY and 20 μM AGP mixed solution. Injection volume, 1.5 ml. The arrow indicates the plateau heights.

coefficient of variation of the analytical values was less than 5.27%. Fig. 5 shows the Scatchard plots of these results. The binding constant (K) and the binding site number per one protein molecule (n) were then estimated according to Eq. (1),

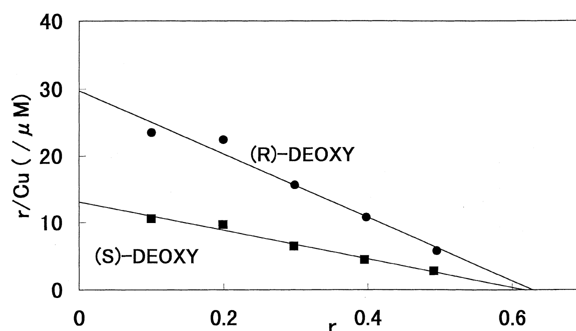

Fig. 5. Scatchard plots of the bindings between AGP and DEOXY enantiomers. The correlation coefficient was −0.947 and −0.988 for (R)-OXY and (S)-OXY, respectively.

$$r/C_u = -Kr + nK \quad (1)$$

where r and C_u represent the bound drug amount per one protein molecule and the unbound drug concentration, respectively, and the correlation coefficients were −0.984 and −0.988 for (R)-isomer and (S)-isomer, respectively. The contribution of non-specific binding was not observed. The binding constants of (R)-DEOXY and (S)-DEOXY were 4.73×10^7 and 2.12×10^7 M^{-1}, and the number of binding sites were 0.628 and 0.617, respectively.

A series of 600 μM HSA solutions containing 8, 10, 12 and 14 μM (R)-DEOXY or 4, 6, 8 and 10 μM

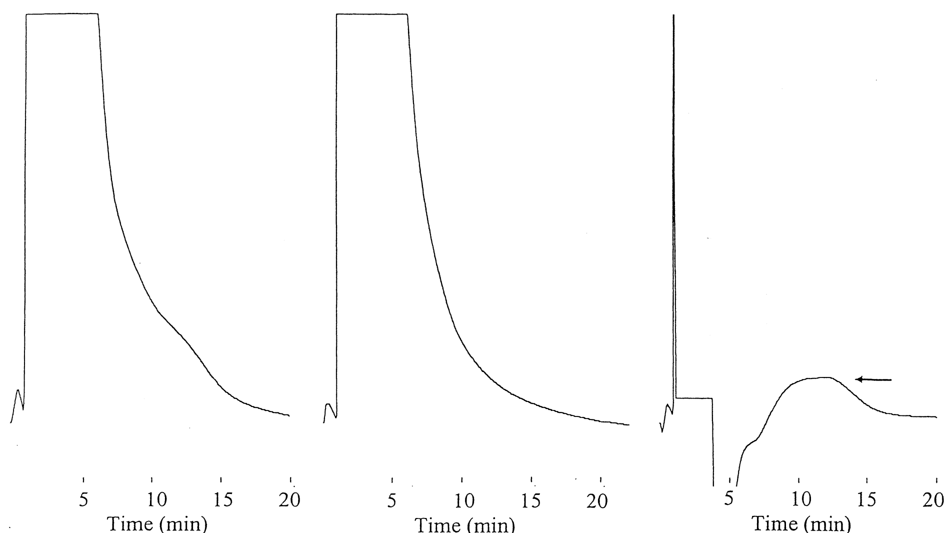

Fig. 4. Chromatograms of 10 μM (R)-DEOXY and 600 μM HSA mixed solution (left), 600 μM HSA solution (center) and their subtraction chromatogram. Injection volume, 0.8 ml. The arrow indicates the plateau heights.

Table 3
Unbound concentrations of (*R*)- and (*S*)-DEOXY in 600 μ*M* HSA

Total conc. (μ*M*)	Unbound conc. (μ*M*)	Unbound fraction (%)
(*R*)-DEOXY		
8	1.39±0.032	17.4±0.27
10	1.82±0.027	18.2±0.39
12	2.14±0.094	17.8±1.14
14	2.42±0.160	17.8±0.78
(*S*)-DEOXY		
4	0.648±0.0228	16.2±0.57
6	1.02±0.033	17.0±0.56
8	1.26±0.016	15.8±0.20
10	1.71±0.045	17.1±0.45

(*S*)-DEOXY were analyzed to estimate the binding affinity to HSA. Table 3 shows the results. The C.V. of the repeated analyses ($n=3$) were less than 4.41%. The unbound fractions of (*R*)-DEOXY and (*S*)-DEOXY were almost constant (17.8±0.35 and 16.5±0.62% for (*R*)- and (*S*)-isomer, respectively), and the Scatchard plots of DEOXY–HSA bindings for both enantiomers were almost linear. This means the DEOXY–HSA binding is not site-specific. Since K and n values could not be estimated separately, the total binding affinity (nK) was calculated according to Eq. (2).

$$nK = C_b/(C_P\, C_u) \qquad (2)$$

where C_b, C_u and C_P represent bound drug concentration, unbound drug concentration and total protein concentration, respectively.

Table 4 lists the total binding affinities of DEOXY enantiomers to AGP and HSA. For comparison, the nK values of OXY enantiomers [22] are also listed. The binding affinities of (*R*)-DEOXY and (*S*)-DEOXY to AGP are 3820 and 1550 times stronger than that to HSA. A clear enantioselectivity was observed in DEOXY–AGP binding. The binding affinity of (*R*)-DEOXY is 2.27 times stronger than that of (*S*)-DEOXY. On the other hand, high enantioselectivity cannot be expected in DEOXY-HSA binding, because DEOXY–HSA binding is relatively weak and does not exhibit site-specific binding character. In fact, as shown in Table 4, the enantioselectivity in this binding is much less significant than AGP binding.

The total binding affinity of DEOXY–HSA binding is smaller than that of OXY–HSA binding, which suggests that the release of an ethyl group from OXY has the effect of reducing the binding affinity to HSA because of the decrease in hydrophobicity. Since the binding between HSA and OXY or DEOXY is not highly enantioselective, it is expected that both enantiomers suffer from this effect in the similar manner. In fact, the decrease in nK value of both enantiomers is almost the same degree. The DEOXY/OXY ratio is 0.294 and 0.385 for (*R*)- and (*S*)-isomer, respectively.

Similarly, the nK value of the binding between AGP and (*S*)-isomer is weakened by the elimination of the ethyl group, although the degree of decrease (DEOXY/OXY ratio of nK value being 0.856) is smaller than that of HSA binding. On the contrary, the total binding affinity of (*R*)-DEOXY is stronger than that of (*R*)-OXY by 4.33 times. According to Kaliszan et al., it is considered that the drug binding site of AGP is like a hydrophobic conical pocket possessing a polar portion which can interact with a positively charged amino group [23]. This polar portion exists in a narrow space of the depths, being apt to suffer from steric hindrance. Therefore, one possible reason for the stronger affinity of (*R*)-DEOXY would be that the elimination of an ethyl group from the positively charged amino group serves to weaken the steric hindrance. By this effect, the binding affinity of (*R*)-DEOXY becomes stronger

Table 4
Total binding affinities (nK) of OXY and DEOXY enantiomers to AGP and HSA (pH 7.4, 37°C)

	(*R*)-OXY[a]	(*S*)-OXY[a]	(*R*)/(*S*)	(*R*)-DEOXY	(*S*)-DEOXY	(*R*)/(*S*)
AGP	$6.86\times10^{6}\ M^{-1}$	$1.53\times10^{7}\ M^{-1}$	0.45	2.97×10^{7} M^{-1}	1.31×10^{7} M^{-1}	2.27
HSA	$2.64\times10^{4}\ M^{-1}$	$2.19\times10^{4}\ M^{-1}$	1.21	7.77×10^{3} M^{-1}	8.44×10^{3} M^{-1}	0.92
AGP/HSA	260	699		3820	1550	

[a] Ref. [22].

than (*S*)-DEOXY, and as a result, the opposite enantioselectivity is observed.

3.2. OXY–DEOXY interaction upon AGP binding

In the case where a drug and its metabolite are also bound strongly to plasma proteins, the study of drug–metabolite interaction upon protein binding is an important issue for the safe and rational use of the drug. As mentioned above, OXY and DEOXY are both bound strongly to AGP. Therefore, the OXY–DEOXY interaction upon AGP binding was investigated.

Two series of sample solutions were analyzed. One series contained 30 μ*M* AGP, 3 μ*M* (*R*)-OXY and different concentrations of (*R*)-DEOXY (1, 3 or 5 μ*M*). Another series contained 30 μ*M* AGP, 3 μ*M* (*S*)-OXY and different concentrations of (*S*)-DEOXY (1, 3 or 5 μ*M*). The measured unbound drug concentrations were then compared with the theoretical values. The theoretical values were calculated based on the competitive binding model as well as on the independent binding model [24].

In the competitive binding model, it is assumed that both enantiomers are bound at the same binding site competitively without any allosteric effect. The unbound concentrations of a drug, $C_u(\mathrm{D})$, and of a metabolite, $C_u(\mathrm{M})$, can be calculated using Eqs. (3) and (4),

$$\begin{aligned} &K_D(K_D - K_M)C_u(\mathrm{D})^3 + \{K_D K_M C_t(\mathrm{D}) \\ &\quad - K_M - K_D K_M(P_t - C_t(\mathrm{D}) - C_t(\mathrm{M})) + K_D^2 C_t(\mathrm{M}) \\ &\quad + K_D^2(P_t - C_t(\mathrm{D}) - C_t(\mathrm{M})) + K_D\}C_u(\mathrm{D})^2 \\ &\quad + \{2K_M C_t(\mathrm{D}) + K_D K_M C_t(\mathrm{D})(P_t - C_t(\mathrm{D}) \\ &\quad - C_t(\mathrm{M})) - K_D C_t(\mathrm{D})\}C_u(\mathrm{D}) \\ &\quad - K_M C_t(\mathrm{D})^2 = 0 \end{aligned} \tag{3}$$

$$\begin{aligned} C_u(\mathrm{M}) = {} & C_t(\mathrm{D}) + C_t(\mathrm{M}) - C_u(\mathrm{D}) - P_t \\ & + (C_t(\mathrm{D}) - C_u(\mathrm{D}))/K_D C_u(\mathrm{D}) \end{aligned} \tag{4}$$

where K_D and K_M are the binding constants of mother drug and metabolite, and $C_u(\mathrm{D})$ and $C_u(\mathrm{M})$ represent the unbound concentrations of mother drug and metabolite, respectively. Introducing the values of the binding parameters into Eq. (3), $C_u(\mathrm{D})$ can be calculated. The theoretical value of $C_u(\mathrm{M})$ is then obtained from Eq. (4). Because the number of binding site on one AGP molecule (n) was slightly different between (*R*)-OXY and (*R*)-DEOXY, the averaged value ($n = 0.561$) was used. In order to keep the nK value constant, the K values were corrected as follows; $K = 1.22 \times 10^7$ and 5.29×10^7 M^{-1} for (*R*)-OXY and (*R*)-DEOXY, respectively. In the same manner, the theoretical unbound concentrations for (*S*)-isomers were calculated using the averaged n value ($n = 0.562$) and the corrected K values ($K = 2.73 \times 10^7$ and 2.33×10^7 M^{-1} for (*S*)-OXY and (*S*)-DEOXY, respectively).

In the independent binding model, it is assumed that each enantiomer is bound at the different binding site independently without any allosteric effect. $C_u(\mathrm{D})$ and $C_u(\mathrm{M})$ are calculated from the Eqs. (5) and (6), respectively, where $P_t(\mathrm{D})$ and $P_t(\mathrm{M})$ represent the concentration of total binding site for mother drug and metabolite, respectively.

$$\begin{aligned} &K_D C_u(\mathrm{D})^2 + (K_D P_t(\mathrm{D}) - K_D C_t(\mathrm{D}) + 1)C_u(\mathrm{D}) \\ &\quad - C_t(\mathrm{D}) = 0 \end{aligned} \tag{5}$$

$$\begin{aligned} &K_M C_u(\mathrm{M})^2 + (K_M P_t(\mathrm{M}) - K_M C_t(\mathrm{M}) + 1)C_u(\mathrm{M}) \\ &\quad - C_t(\mathrm{M}) = 0 \end{aligned} \tag{6}$$

These theoretical equations are essentially the same as those used for the enantiomer–enantiomer interaction upon protein binding [24].

Figs. 6 and 7 show the measured and the theoretical unbound concentrations. Line 1 shows the theoretical values calculated based on the competitive binding model, and line 2 shows those calculated based on the independent binding model. As for both sample sets, the observed unbound concentrations agree with the competitive binding model. In addition, our recent study found that OXY enantiomers are bound at the same binding site on AGP molecule [22]. Therefore, it is considered that DEOXY enantiomers as well as OXY enantiomers are bound at the same site on AGP molecule.

4. Determination of unbound deoxy in human plasma

The present on-line HPLC system was applied to the analysis of human plasma samples. Human

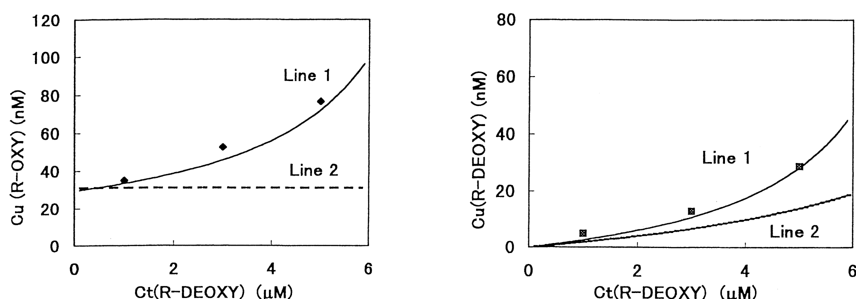

Fig. 6. OXY–DEOXY interaction upon AGP binding. C_t and C_u represent the total drug concentration and unbound drug concentrator, respectively. Sample solutions, 30 μ*M* AGP+3 μ*M* (*R*)-OXY+1, 3 or 5 μ*M* (*R*)-DEOXY.

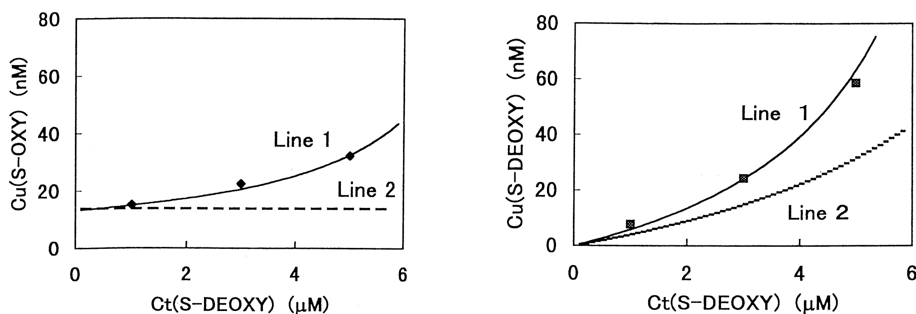

Fig. 7. OXY–DEOXY interaction upon AGP binding. C_t and C_u represent the total drug concentration and unbound drug concentrator, respectively. Sample solutions, 30 μ*M* AGP+3 μ*M* (*S*)-OXY+1, 3 or 5 μ*M* (*S*)-DEOXY.

samples spiked with 5 μ*M* (*R*)-DEOXY or 5 μ*M* (*S*)-DEOXY were analyzed in triplicate. The unbound concentrations thus determined were 60.0±0.40 and 116±2.1 n*M* for (*R*)-DEOXY and (*S*)-DEOXY, respectively. The unbound concentration of the (*S*)-isomer was about 2-fold higher than the (*S*)-isomer. This result indicates that (*S*)-DEOXY is bound more strongly than (*R*)-DEOXY, and this enantioselectivity is in the same direction as in AGP solution.

5. Conclusion

AGP is the major plasma protein responsible for the enantioselectivity in plasma protein binding of DEOXY. DEOXY is bound to AGP more strongly than HSA by three orders of magnitude. The elimination of an ethyl group from OXY gave opposite effects upon protein binding. One effect is to reduce the binding affinity due to the decrease in hydrophobicity. Another effect is to enhance the binding affinity due to the decrease in steric hindrance. The former effect appears in the binding of HSA and (*R*)- or (*S*)-isomer as well as AGP and (*S*)-isomer, while the latter effect comes prominent in case of AGP–(*R*)-DEOXY binding. As a result, the enantioselectivity in the binding to AGP is reversed. This study demonstrates the utility of HPFA method in the quantitative and enantioselective binding study of strong protein binding.

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