ELSEVIER

Journal of Chromatography B, 768 (2002) 177–188

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Plasma protein binding study of oxybutynin by high-performance frontal analysis

Akimasa Shibukawa[a,*], Nobuko Ishizawa[a], Tomoko Kimura[a], Yuki Sakamoto[a], Kanae Ogita[a], Yuka Matsuo[a], Yukihiro Kuroda[a], Chutima Matayatsuk[a,1], Terumichi Nakagawa[a], Irving W. Wainer[b]

[a]*Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*
[b]*Department of Pharmacology, Georgetown University Medical Center, Washington, DC 20007, USA*

Abstract

Plasma protein binding 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 OXY enantiomers in human plasma, in human serum albumin (HSA) solutions, and in human α_1-acid glycoprotein (AGP) solutions. OXY is bound in human plasma strongly and enantioselectively. The bound drug fraction in human plasma containing 2–10 μM (*R*)- or (*S*)-OXY was higher than 99%, and the unbound fraction of (*R*)-OXY was 1.56 times higher than that of (*S*)-isomer. AGP plays the dominant role in this strong and enantioselective plasma protein binding. The total binding affinities (*nK*) of (*R*)- and (*S*)-OXY to AGP were 6.86×10^6 and 1.53×10^7 M^{-1}, respectively, while the nK values of (*R*)- and (*S*)-OXY to HSA were 2.64×10^4 and 2.19×10^4 M^{-1}, respectively. The binding affinity of OXY to AGP is much higher than that to HSA, and shows high enantioselectivity (*S*/*R* ratio of *nK* values is 2.2). It was found that both enantiomers are bound competitively at the same binding site on an AGP molecule. The binding property between OXY and low density lipoprotein (LDL) was investigated by using the frontal analysis method incorporated in high-performance capillary electrophoresis (HPCE/FA). It was found the binding is non-saturable and non-enantioselective.

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

1. Introduction

Plasma protein binding has a significant effect upon the pharmacokinetic and pharmacodynamic properties of drugs [1–3]. Plasma protein binding comprises variable and complicated binding equilibria. Several plasma proteins such as albumin, α_1-acid glycoprotein (AGP) and lipoproteins possibly are simultaneously involved in the plasma protein binding of a drug, and the overall binding property in plasma is the sum of each protein binding. The concentrations of AGP and lipoproteins in plasma often show person-to-person variation and also change in some disease state. Therefore, both in vivo binding studies using whole plasma and in vitro binding studies using a component of plasma proteins are necessary for the detailed elucidation of

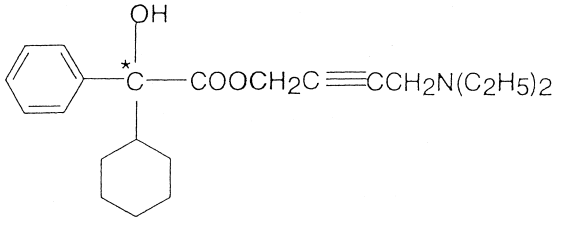
*Corresponding author. Tel.: +81-75-753-4531; fax: +81-75-753-4578.
E-mail address: akimasas@pharm.kyoto-u.ac.jp (A. Shibukawa).
[1]Present address: Faculty of Pharmacy, Mahidol university, Bangkok, 10400 Thailand.

1570-0232/02/$ – see front matter
PII: S0378-4347(01)00497-2

plasma distribution of a drug. In addition, a racemic drug exhibits potential difference in protein binding between the enantiomers, which may result in different pharmacokinetic profiles for the individual enantiomers [4,5].

Oxybutynin (OXY, Fig. 1) is a tertiary amine that mainly acts as a direct smooth muscle relaxant and displays weak antimuscarinic activity [6,7]. OXY is a chiral compound and is clinically used as a racemate. (*R*)-OXY exhibits high antimuscarinic activity relative to the antispasmodic activity, while (*S*)-OXY exhibits relatively weak antimuscarinic activity [8]. OXY is eliminated from the body mainly by hepatic metabolism, and shows low absolute systemic availability, large interindividual variability in the plasma concentrations and apparent absence of intact OXY in urine [9]. Although it is well recognized that plasma protein binding plays an important role in determination of the pharmacokinetic properties of drugs, little is known about the plasma protein binding property of OXY, because of the technical difficulty in the binding analysis. So far, only one paper has reported the bound fractions of OXY in bovine serum albumin (BSA) solution, in human serum albumin (HSA) solution and in dog plasma which were measured by equilibrium dialysis method [10]. However, the binding affinity to other important plasma proteins such as AGP and lipoproteins has not been reported, and the protein binding to human plasma has not been analyzed. In addition, no enantioselective binding study has been reported.

Equilibrium dialysis and ultrafiltration methods have been widely used to determine unbound drug concentrations. However, these conventional methods have problems such as drug adsorption onto the membrane and leakage of bound drug through the membrane. The problems often make it difficult or even impossible to determine the unbound concentrations in the case of highly hydrophobic and strongly bound drugs. To overcome these problems, we developed a novel binding analysis, high-performance frontal analysis (HPFA), to determine the unbound drug concentration in protein binding equilibrium [11–32]. The principle and the feature of this method were recently reviewed [33]. In the present paper, the protein binding of OXY enantiomers in human plasma as well as their binding property to HSA, AGP and lipoprotein were investigated quantitatively and enantioselectively using the HPFA method.



Oxybutynin (OXY)

Fig. 1. Chemical structure of oxybutynin.

2. Experimental

2.1. Materials and apparatus

(*R*)- and (*S*)-OXY were obtained from Sepracor (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, ionic strength 0.17). These mixed solutions and human plasma samples were kept at 37°C before analysis. Develosil 100 Diol 5 was purchased from Nomura Chemicals (Seto, Japan). YMC-Pack ODS-AM was purchased from YMC (Kyoto, Japan). Chemcosorb ODS7 was purchased from Chemco (Osaka, Japan). Chiralcel OJ-R was purchased from Daicel (Himeji, Japan). A Hitachi Himac VP65β (Hitachi Koki, Tokyo, Japan) was used for ultracentrifugation. The capillary P/ACE system 5510 (Beckman Instruments, CA) was used for the binding analysis of lipoproteins.

2.2. On-line HPFA–HPLC system

Fig. 2 shows a schematic diagram of the on-line HPFA–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, LC 9A (Shimadzu, Kyoto, Japan) and model A-30-S (Eldex Lab., San Carlos, CA). UV detector, SPD-6A, (Shimadzu). Injector,

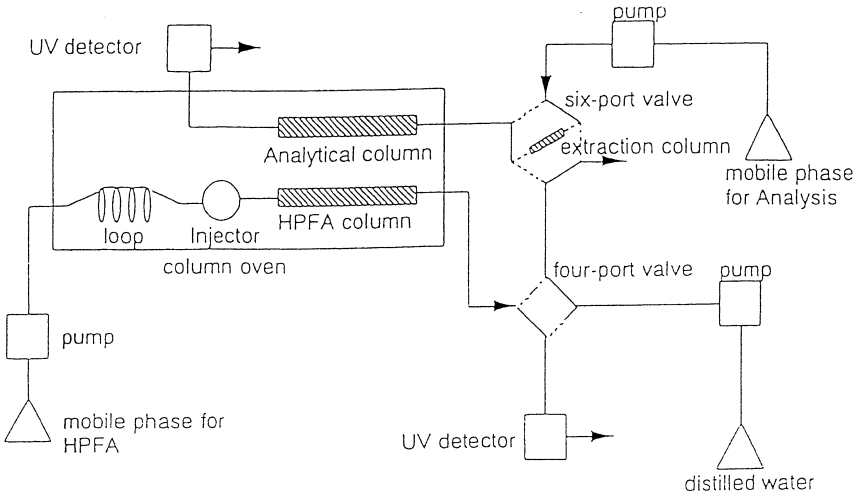


Fig. 2. Schematic diagram of on-line HPFA–HPLC system.

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. A physiological pH (7.4) of sodium phosphate buffer 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 hydrophilic HPFA column (diol–silica column) allowed the elution of hydrophobic OXY enantiomers under the mild mobile phase condition. The ODS column was used to the analyses of samples containing a single enantiomer, while the chiral HPLC column was used for the enantiomer–enantiomer competitive binding study using samples containing both enantiomers.

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

The drug–protein mixed solution was directly injected onto the HPFA column. The drug was eluted out as a zonal peak with a plateau region. In accord with the principle of the frontal analysis, the drug concentration in the plateau region is equal to the unbound drug concentration in the sample solution [32]. Then, a given volume (2 ml for HSA samples and 4 ml for AGP and plasma samples) of the plateau region was transferred into the extraction column by switching the four-port valve ('heart-cut' procedure). The unbound drug was completely trapped on the extraction column. The mobile phase for the analytical column was introduced into the

Table 1
HPFA conditions in Scatchard analysis

Subsystem	Condition	
HPFA	Column	Develosil 100-Diol-5 (5 cm×4.6 mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH 7.4, $I=0.17$)
Extraction	Column	Chemcosorb ODS 7 (1 cm×4.6 mm I.D.)
Analytical HPLC (achiral)	Column	YMC-Pack ODS-AM (15 cm×4.6 mm I.D.)
	Mobile phase	20 m*M* sodium phosphate buffer: MeOH=4:6 (pH 3.0)
Analytical HPLC (chiral)	Column	Chiralcel OJ-R (15 cm×4.6 mm I.D.)
	Mobile phase	Sodium phosphate buffer (pH 6.0, $I=0.05$): CH_3CN=65:35 (pH 6.6)

Flow rate, 1.0 ml/min; column temperature, 37°C; detection, UV 220 nm.

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.

2.4. HPFA profile

It is essential in HPFA that the injection volume should be large enough to obtain the plateau drug zone. Therefore, the suitable injection volume was optimized in advance. Fig. 3 shows the effect of the actual sample injection volume on the HPFA profile of OXY. The upper chromatograms were obtained from different injection volume of 10 μ*M* (*R*)-OXY–30 μ*M* AGP mixed solution. To make the drug elution profile more clearly, the lower chromatograms show the subtraction of the chromatogram of 30 μ*M* AGP solution (without drug) from corresponding upper chromatograms. Although a plateau drug zone was not observed in case of the smaller injection volume (50 μl and 0.25 ml), the plateau region was obtained when 1- or 1.5-ml portion of the sample solution was injected. Further increase in the

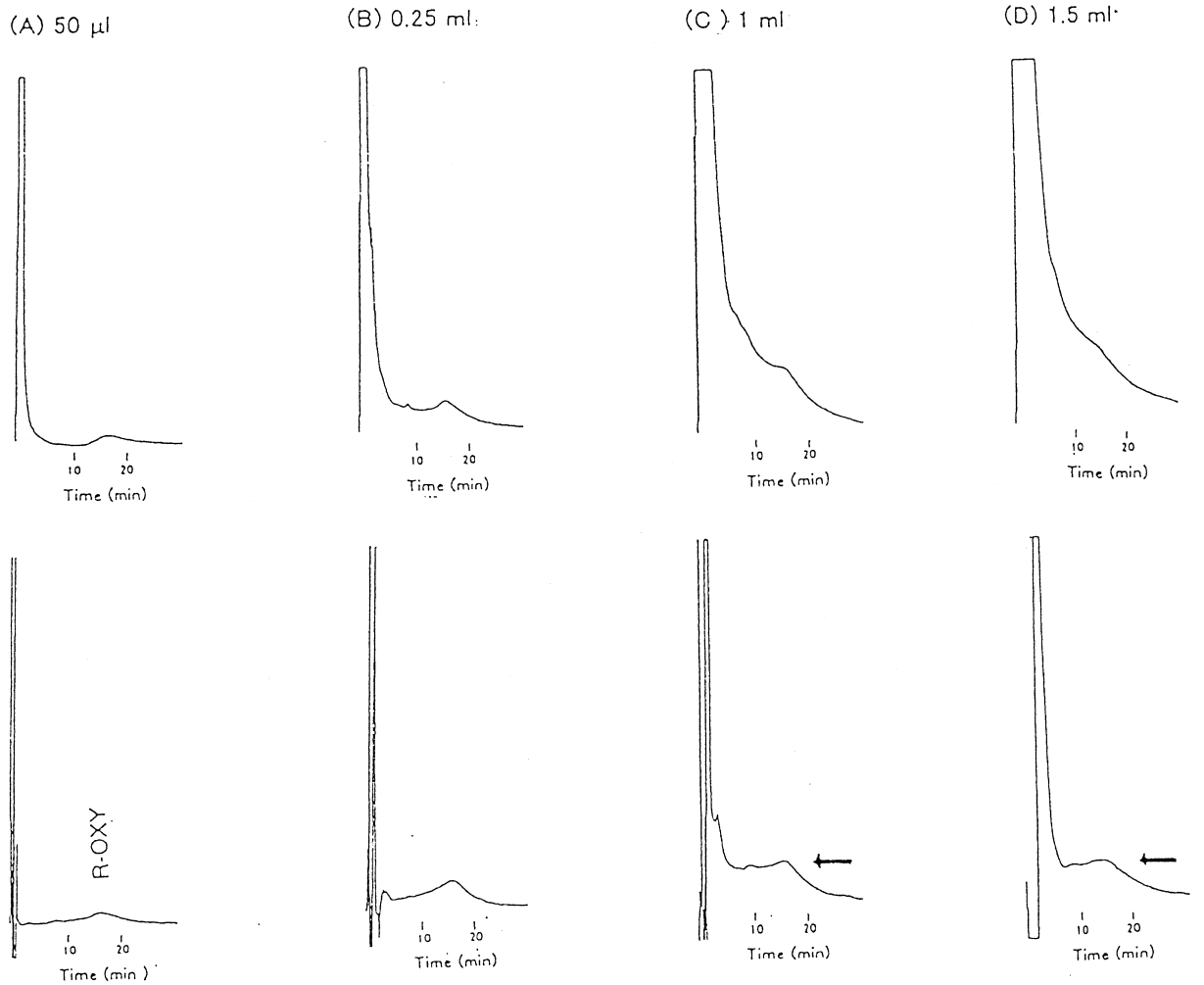


Fig. 3. Chromatograms of (A) 10 μ*M* (*R*)-OXY and 30 μ*M* AGP mixed solution (upper) and the subtraction chromatograms (lower). Actual injection volume: (A) 50 μl, (B) 250 μl, (C) 1 ml and (D) 1.5 ml.

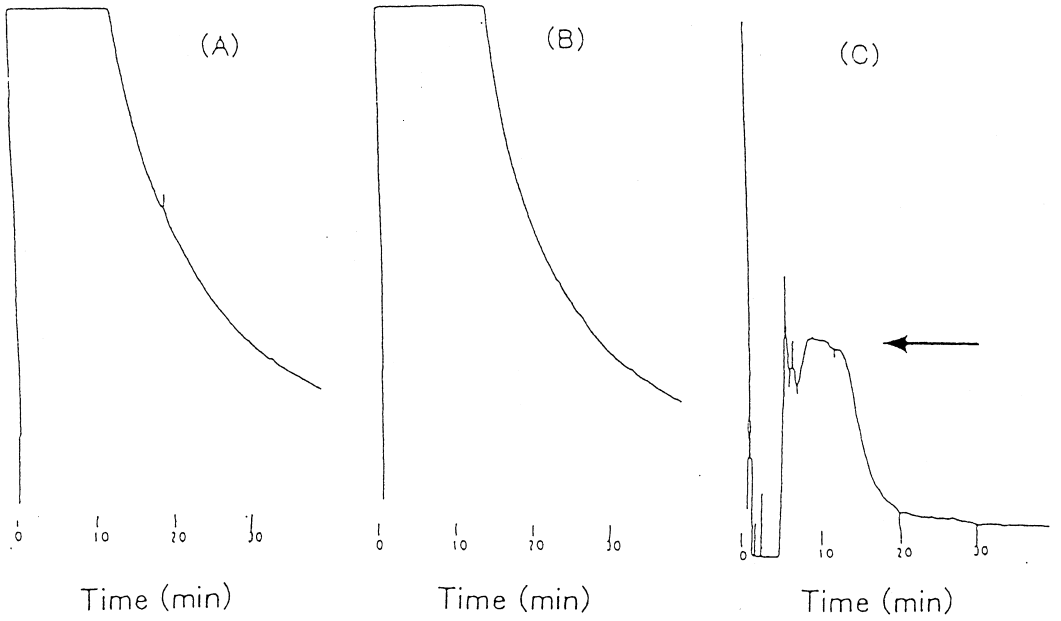


Fig. 4. Chromatograms of (A) 600 μ*M* HSA solution, (B) 10 μ*M* (*S*)-OXY and 600 μ*M* HSA mixed solution and (C) the subtraction chromatogram. Actual injection volume was 1.75 ml.

injection volume did not raise the plateau height level. Fig. 4 shows the chromatogram of 10 μ*M* (*S*)-OXY and 600 μ*M* HSA mixed solution, 600 μ*M* HSA solution and their subtraction chromatogram. As found from the subtraction chromatogram, the plateau region appeared when 1.75-ml portion of the sample solution was injected. In the following studies, the actual injection volume was decided as 1.5 ml for AGP and human plasma samples and 1.75 ml for HSA samples.

A given volume of this plateau zone was 'heart-cut' and was analyzed on the ODS column or the chiral HPLC column. The retention time of OXY on the ODS column was 6.4 min, and the retention times of (*R*)- and (*S*)-OXY on the chiral HPLC column were 11.2 and 12.5 min, respectively (Fig. 5).

2.5. Calibration lines

The calibration lines were prepared as follows. The HPFA column was removed from the on-line system, and the injector loop was changed from 2.5-ml loop to 20-μl loop. After washing the extraction column with distilled water for 30 s, each

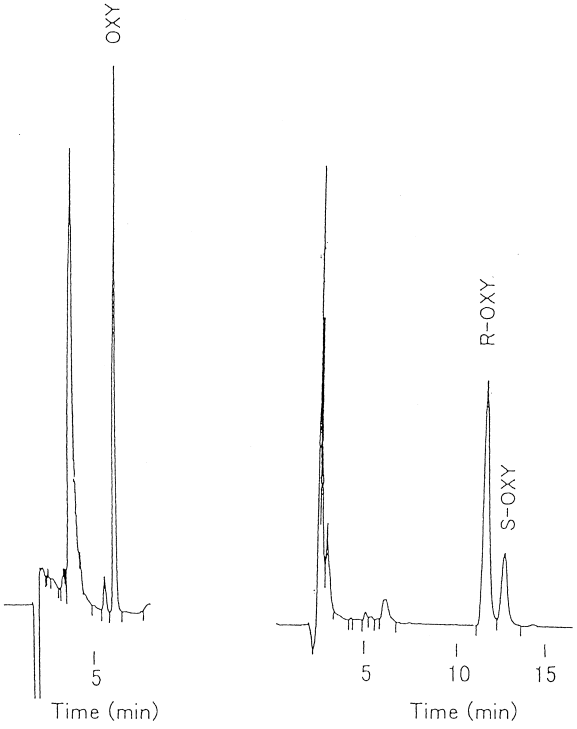


Fig. 5. Typical chromatograms of OXY on the ODS column (left) and the chiral HPLC column (right).

10-μl portion of (*S*)-OXY standard solutions (2.5, 5, 10, 15, 20 and 25 μ*M*) made up in methanol was injected directly into the extraction column. After perfusing the extraction column with water for 30 s, the adsorbed OXY was back-flushed into the analytical column by the column switching procedure. The calibration line was prepared by plotting peak area versus injected drug amount.

$$\text{Area} = 114 \times \text{Amount (pmol)} - 149. \quad \text{RSQ} > 0.999$$

For the analyses of samples containing low total drug concentration (2.5 μ*M*), the standard solutions containing 1.55, 3.88 and 7.75 μ*M* of (*S*)-OXY were used to make the following calibration line.

$$\text{Area} = 115 \times \text{Amount (pmol)} - 259. \quad \text{RSQ} > 0.999$$

The unbound OXY concentrations were then calculated dividing the drug amount (pmol) by the heart-cut volume.

In the enantiomer–enantiomer competitive binding study, the standard solutions containing both enantiomers (20, 40, 60, 80 and 100 μ*M* (*R*)-OXY and 10, 20, 30, 40 and 50 μ*M* (*S*)-OXY) were made up in methanol, and were used in the same way as mentioned above. The following calibration lines were prepared.

(*R*)-OXY, $\text{Area} = 119 \times \text{Amount (pmol)} - 292$. $\text{RSQ} > 0.999$

(*S*)-OXY, $\text{Area} = 123 \times \text{Amount (pmol)} - 285$. $\text{RSQ} > 0.999$

2.6. Preparation of LDL by sequential ultracentrifugation

Human LDL was prepared from plasma of a healthy male volunteer by the sequential ultracentrifugation method [31]. Briefly, human plasma, the density of which was adjusted to 1.006 g/ml by addition of NaBr, was ultracentrifuged for 24 h× 180 000 *g* at 4°C, and the upper fraction was removed. The density of the remainder was adjusted to 1.063 g/ml, and the following ultracentrifugation (20 h×100 000 *g* at 4°C) gave the LDL fraction (upper fraction). The LDL fraction (density, 1.006–1.063 g/ml) was submitted to size-exclusion HPLC. The HPLC condition was as follows. Column, HiLoad Superdex 200 pg (60×2.6 cm I.D., Pharmacia). Mobile phase, sodium phosphate buffer (pH 7.4, $I = 0.17$). Flow rate, 2 ml/min; column temperature, 4°C; detection, UV 254 nm. The isolated LDL fraction was then concentrated on the membrane (Cemtriplus-10, Amicon).

2.7. Determination of lipoprotein concentrations

The concentration of LDL was determined as follows. First, the concentration of apolipoprotein was measured by modified Lowry method in which SDS solutions were used to prevent the interference from lipid components [34]. Bovine serum albumin (fatty acid-free) was used as the standard protein in preparing the calibration line. The concentration of lipoprotein was then calculated assuming that the apolipoprotein content (w/w) and the molecular mass of LDL are 21% and 2.3×10^6 Da, respectively [35].

2.8. Determination of unbound drug concentrations by HPFA–CE method

The sample solution containing 0.870 g/l LDL and 40, 60, 80, 100 or 120 μ*M* (*R*)- or (*S*)-OXY was introduced hydrodynamically (10 s, ca. 80 nl) into the fused-silica capillary (total length, 37 cm, effective length, 30 cm, 75 μm I.D. and 375 μm O.D.), and a positive voltage (+7 kV) was applied to start electrophoresis. The sodium phosphate buffer (pH 7.4, $I = 0.17$) used to prepare the sample solutions was also used as the run buffer. The temperature was set at 25°C, and the current was 70 μA. Detection was achieved by UV-absorption at 200 nm. A series of the standard solutions containing 5, 10, 20 and 30 μ*M* of (*R*)- or (*S*)-OXY were used to prepare calibration lines ($R > 0.992$). After each analysis, the capillary was washed with run buffer for 1 min.

Unbound OXY enantiomers migrated toward the cathodic end (detection side) much faster than the negatively charged lipoproteins and the bound drug. Drug and protein are separated from each other, while the binding condition is kept constant because drug–protein binding is reversible and kinetically rapid. After the unbound drug moved out, bound drug is quickly released to maintain the same

equilibrium condition. As a result, a trapezoidal peak of unbound drug was obtained, and the unbound drug concentration was measured from the plateau height.

3. Results and discussion

3.1. Binding affinity of OXY enantiomers to AGP and HSA

A series of 30 μ*M* AGP solutions containing 2.5–10 μ*M* (*R*)- or (*S*)-OXY were subjected to the on-line HPFA–HPLC system to determine the unbound drug concentrations. Each sample was analyzed in triplicate, the C.V. of the analytical values being less than 12.3%. Fig. 6 shows the Scatchard plots of these results, from which the binding constant (*K*) and the binding site number per one protein molecule (*n*) were estimated according to the Eq. (1),

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

where r and C_u represent the number of drug molecules bound to one protein molecule and the unbound drug concentration, respectively. The correlation coefficient of the plots was 0.90 for (*R*)-isomer and 0.95 for (*S*)-isomer. The contribution of non-specific binding was not observed. The binding constants of (*R*)- and (*S*)-OXY were 1.39×10^7 and 3.02×10^7 M^{-1}, and the number of binding sites of

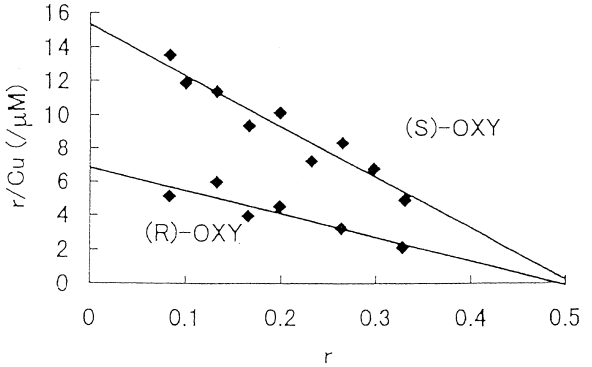


Fig. 6. Scatchard analysis of (*R*)- and (*S*)-OXY to AGP binding. The correlation coefficient was −0.900 and −0.959 for (*R*)- and (*S*)-OXY, respectively.

Table 2
Unbound concentrations of (*R*)- and (*S*)-OXY in 600 μ*M* HSA solutions

C_t (μ*M*)	C_u(*R*) (n*M*)	C_u(*S*) (n*M*)	*S*/*R*
10	611±5.3	723±3.7	1.18
8	486±2.8	571±7.1	1.17
6	364±2.1	424±2.7	1.16
4	234±1.0	280±2.4	1.20
2	113±1.7	138±1.1	1.22

C_t, total drug concentration; C_u, unbound drug concentration.

(*R*)- and (*S*)-OXY were 0.494 and 0.508, respectively.

A series of 600 μ*M* HSA solutions containing 2, 4, 6, 8 and 10 μ*M* (*R*)- or (*S*)-OXY were analyzed, and the results are shown in Table 2. The C.V. of the repeated analyses ($n=3$) were less than 1.55%. The unbound concentration of (*S*)-OXY was about 1.2 times higher than that of (*R*)-isomer. The Scatchard plots of OXY–HSA binding were almost linear as for both enantiomers, which indicates that the OXY–HSA binding is not site-specific. Since *K* and *n* values could not be estimated separately, the total binding affinity (*nK*) was estimated according to the Eq. (2).

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

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

Table 3 compares the total binding affinity of OXY enantiomers to AGP and HSA. The binding affinities of (*R*)- or (*S*)-OXY to AGP are 260 times and 700 times stronger than those to HSA, respectively. A clear enantioselectivity was found in OXY–AGP binding. The binding affinity of (*S*)-OXY to AGP is 2.2 times stronger than that of (*R*)-OXY. Since OXY–HSA binding does not exhibit site-spe-

Table 3
Total binding affinities of (*R*)-OXY (*nK*(*R*)) and of (*S*)-OXY (*nK*(*S*)) with AGP and HSA (pH 7.4, 37°C)

	nK(*R*) (M^{-1})	*nK*(*S*) (M^{-1})	*S*/*R*
AGP	6.86×10^6	1.53×10^7	2.23
HSA	2.64×10^4	2.19×10^4	0.83
AGP/HSA	260	699	

Table 4
Unbound concentrations and bound fractions of OXY enantiomers in human plasma

C_t (μM)	$C_u(R)$ (nM)	$C_u(S)$ (nM)	$C_u(R)/C_u(S)$	Bound (%) (R)	Bound (%) (S)
10	82.5±8.89	55.9±5.69	1.48	99.17±0.089	99.49±0.057
8	64.0±1.90	40.5±3.12	1.58	99.20±0.024	99.49±0.039
6	37.6±2.35	24.1±2.17	1.56	99.37±0.039	99.60±0.036
4	23.6±1.72	13.3±0.78	1.77	99.41±0.043	99.67±0.020
2	8.20±1.45	5.86±0.10	1.40	99.59±0.072	99.71±0.005

Mean±SD ($n=3$). C_t, total drug concentration; C_u, unbound drug concentration.

cific character, high enantioselectivity cannot be expected in this binding. In fact, the enantioselectivity of HSA binding, as shown in Table 3, is weaker than AGP binding.

3.2. Analysis of protein binding in human plasma

Table 4 shows the unbound drug concentrations, the R/S ratio and the bound drug fractions in human plasma containing 2–10 μM (R)- or (S)-OXY. A low concentration (ex. 5.86 nM) of the unbound drug can be determined using an UV detector after the preconcentration of 4-ml portion of the plateau zone. The protein binding in human plasma is strong, and the bound drug fractions were more than 99%. (S)-OXY is bound more strongly than the antipode, and the unbound concentration of (R)-OXY is 1.40–1.77 times higher than that of (S)-OXY. This enantioselectivity is in the same direction as that observed in the AGP solutions.

Table 5 shows the theoretical unbound drug concentrations, the R/S ratio and the bound drug fractions that were calculated using the estimated binding parameters. The plasma concentrations of AGP and HSA used in this calculation were 25 and 600 μM, respectively, and it was assumed that no protein other than AGP and HSA contributes to the plasma protein binding of OXY enantiomers. The theoretical bound drug fractions were almost 99%, which agrees with the observed values. The R/S ratio of the unbound drug concentration was 1.63–2.08, which almost agrees with the observed data. In case that OXY is bound only to HSA, the theoretical bound fractions of (R)- and (S)-OXY should be 94 and 93%, respectively, and the R/S ratio of the unbound drug concentration should be 0.84. These results indicate that, similarly to the cases of many other basic drugs, AGP is the dominant protein responsible for the enantioselective plasma protein binding of OXY. It was also found that the observed unbound drug concentrations were still lower than the theoretical values, which suggests the possible contribution of other plasma proteins such as lipoproteins.

3.3. Enantiomer–enantiomer competitive binding study

When each enantiomer is bound at a completely different binding site on a plasma protein without

Table 5
Theoretical unbound concentrations and bound fractions of OXY enantiomers in human plasma

C_t (μM)	$C_u(R)$ (nM)	$C_u(S)$ (nM)	$C_u(R)/C_u(S)$	Bound (%) (R)	Bound (%) (S)
10	127	77.7	1.63	98.73	99.22
8	82.8	45.6	1.82	98.96	99.43
6	51.3	26.3	1.95	99.15	99.56
4	28.7	14.1	2.03	99.28	99.65
2	12.2	5.89	2.08	99.39	99.71

C_t, total drug concentration; C_u, unbound drug concentration.

any allosteric interaction, the binding of one enantiomer will produce no effect upon the binding property of the other isomer, and, consequently, their unbound concentrations will not change. On the other hand, when both enantiomers are bound at the same binding site, they will compete with each other and the unbound concentrations of both enantiomers will increase. In addition, if an allosteric interaction occurs, the binding of one enantiomer will also affect the binding property of the other. In this case, the changes in their unbound concentrations will not follow a simple competition model. Since OXY is clinically administered as a racemate and AGP is the dominant plasma protein responsible for the binding property of this drug, it is important to investigate whether the OXY enantiomers are bound to AGP independently or competitively with or without allosteric effects.

Two series of sample solutions were analyzed using the HPFA–chiral HPLC system to determine the unbound concentrations of both enantiomers. One series of sample solutions contains (*S*)-OXY (5 μ*M*), AGP (30 μ*M*) and (*R*)-OXY (3, 5 or 7 μ*M*). Another series contains (*R*)-OXY (5 μ*M*), AGP (30 μ*M*) and (*S*)-OXY (3, 5 or 7 μ*M*). The measured unbound drug concentrations were then compared with the theoretical values.

The theoretical values were calculated based on competitive and independent binding models [26]. In the competitive binding model, it is assumed that both enantiomers are bound at the same binding site without any allosteric effect. In the independent binding model, it is assumed that each enantiomer is bound at the different binding site non-competitively without any allosteric effect. In the competitive binding model, the concentration of total binding site (P_t) is the sum of the concentration of unoccupied binding site (P_u) and the bound concentrations of both enantiomers as described in Eq. (3).

$$P_t = nP = P_u + C_b(R) + C_b(S) \quad (3)$$

where n is the number of binding sites per one protein molecule, and P is the total protein concentration. Introducing Eqs. (4) and (5) into Eq. (3), Eq. (6) is obtained.

$$K_R = C_b(R)/P_u C_u(R) \quad (4)$$

$$K_S = C_b(S)/P_u C_u(S) \quad (5)$$

$$\begin{aligned} &K_R(K_R - K_S)C_u(R)^3 + \{K_R K_S C_t(R) - K_S \\ &\quad - K_R K_S(P_t - C_t(R) - C_t(S)) + K_R^2 C_t(S) \\ &\quad + K_R^2(P_t - C_t(R) - C_t(S)) + K_R\}C_u(R)^2 \\ &\quad + \{2K_S C_t(R) + K_R K_S C_t(R)(P_t - C_t(R) - Ct(S)) \\ &\quad - K_R C_t(R)\}C_u(R) - K_S C_t(R)^2 = 0 \end{aligned} \quad (6)$$

where K_R and K_S are the binding constants of (*R*)- and (*S*)-isomer, and $C_u(R)$ and $C_u(S)$ represent the unbound concentrations of (*R*)- and (*S*)-isomer, respectively. Introducing the values of the binding parameters into Eq. (6), $C_u(R)$ can be calculated. The theoretical value of $C_u(S)$ is then obtained from Eq. (7).

$$\begin{aligned} C_u(S) = {} & C_t(R) + C_t(S) - C_u(R) - P_t \\ & + (C_t(R) - C_u(R))/K_R C_u(R) \end{aligned} \quad (7)$$

In the independent binding model, Cu(*R*) and Cu(*S*) are calculated from Eqs. (8) and (9), respectively, where $P_t(R)$ and $P_t(S)$ represent the concentrations of total binding site for (*R*)- and (*S*)-isomer, respectively.

$$\begin{aligned} &K_R C_u(R)^2 + (K_R P_t(R) - K_R C_t(R) + 1)C_u(R) - C_t(R) \\ &\quad = 0 \end{aligned} \quad (8)$$

$$\begin{aligned} &K_S C_u(S)^2 + (K_S P_t(S) - K_S C_t(S) + 1)C_u(S) - C_t(S) \\ &\quad = 0 \end{aligned} \quad (9)$$

Figs. 7 and 8 show the measured and theoretical unbound concentrations. Line 1 shows the theoretical values calculated based on the competitive binding model, and line 2 shows those calculated based on independent binding model. As for both sample sets, the observed unbound concentrations (three data points with SD bars) agree with the competitive binding model, which indicates that both enantiomers are bound at the same binding site competitively, and allosteric effect was not significant.

3.4. Binding with low-density lipoprotein

It is known that plasma lipoproteins also contribute to the plasma protein binding of several basic and neutral hydrophobic drugs [35]. Since OXY is a basic and hydrophobic drug, it is important to

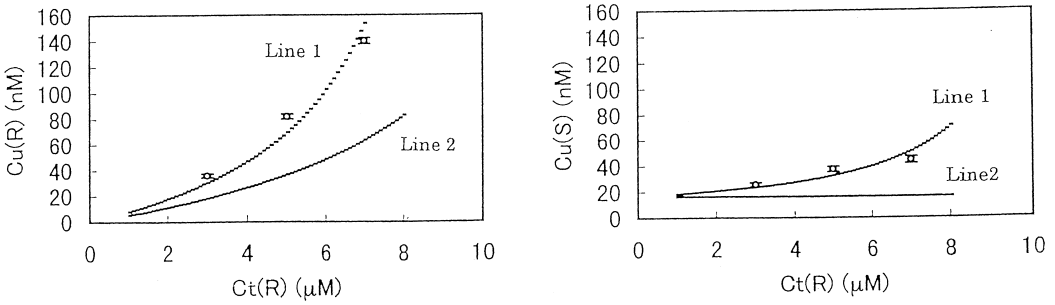


Fig. 7. Observed and calculated unbound concentrations of OXY enantiomers. Samples, 3, 5 or 7 μ*M* (*R*)-OXY+5 μ*M* (*S*)-OXY in 30 μ*M* AGP. Line 1, theoretical value based on the competitive binding model. Line 2, theoretical value based on the independent binding model.

investigate the binding property of OXY to lipoproteins. Plasma lipoproteins are classified into some major subclasses based on the density, such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and chylomicron (CM). Among these, HDL and LDL are the most important drug-transporting proteins because of their higher plasma concentrations than others, and LDL shows much stronger binding affinity than HDL, because of the higher lipid components. Because of less stability and difficulty in the large-scale purification of lipoproteins, a micro-scale binding analysis is desirable for their binding study. In this study, OXY–LDL binding was analyzed using HPFA incorporated in CE system (HPFA/CE), which allowed to determine the unbound drug concentrations with small injection volume (ca. 100 nl).

A series of the mixed solutions containing 40–120 μ*M* (*R*)- or (*S*)-OXY and 0.870 g/l (0.378 μ*M*) human LDL was analyzed. Fig. 9 shows the typical electropherograms, and Table 6 shows the unbound drug concentrations. The C.V. of the repeated analyses ($n=5$) was less than 8.22%. The unbound fractions of OXY enantiomers were almost constant (24.3±1.71%) regardless of the total drug concentration. OXY–LDL binding has unsaturable binding property, suggesting that the partition-like binding to the lipid portion of LDL is dominant in OXY–LDL binding. The total binding affinities of (*R*)- and (*S*)-OXY were estimated as 8.52 (± 0.340)$\times 10^6$ and 8.09 (± 1.05)$\times 10^6$ M^{-1}, respectively, and significant difference in the affinity was not observed between the enantiomers. While the binding affinities to AGP, HSA and whole human plasma were measured at 37°C, the binding affinity to LDL was measured at 25°C. Therefore, the *nK* values at 37°C should be weaker than these values. In addition, the physiological plasma concentration of LDL (1.6–1.9 μ*M*) is much lower than that of AGP (20–30 μ*M*).

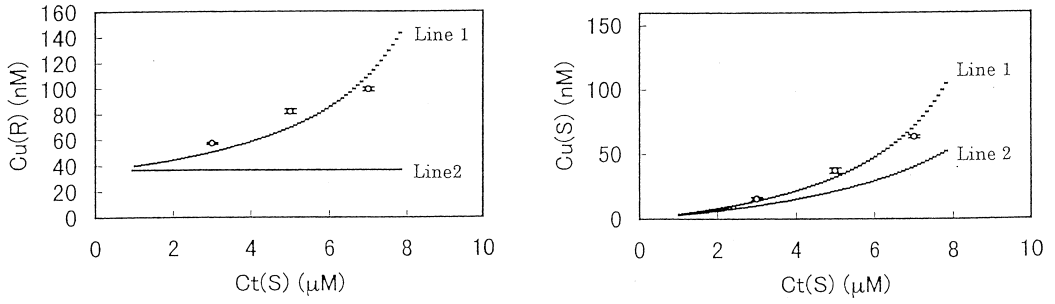


Fig. 8. Observed and calculated unbound concentrations of OXY enantiomers. Samples, 3, 5 or 7 μ*M* (*S*)-OXY+5 μ*M* (*R*)-OXY in 30 μ*M* AGP. Line 1, theoretical value based on the competitive binding model. Line 2, theoretical value based on the independent binding model.

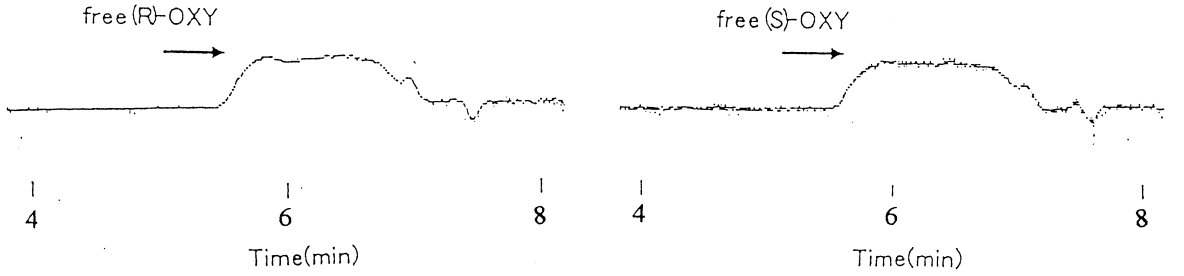


Fig. 9. Typical electropherograms in OXY–LDL binding analysis. Samples, (A) 80 μ*M* (*R*)-OXY and 0.87 g/l LDL, (B) 80 μ*M* (*S*)-OXY and 0.87 g/l LDL.

Table 6
Unbound concentrations and unbound fractions of OXY enantiomers in 0.87 g/l human LDL solutions

C_t (μ*M*)	$C_u(R)$ (μ*M*)	$C_u(S)$ (μ*M*)	$C_u(R)/C_u(S)$	Unbound (%) (*R*)	Unbound (%) (*S*)
120	28.9±0.97	30.0±0.82	0.96	24.0±0.81	25.0±0.68
100	23.2±1.11	21.3±0.52	1.09	23.2±1.11	21.3±0.52
80	18.8±0.38	20.7±0.90	0.91	23.5±0.48	25.9±1.13
60	13.8±0.20	16.5±0.48	0.83	22.9±0.35	27.5±0.82
40	9.91±0.27	9.78±0.48	1.01	24.8±0.67	24.4±1.19

Mean±SD ($n=5$). C_t, total drug concentration C_u, unbound drug concentration.

Therefore, it is considered that the contribution of LDL to the plasma protein binding of OXY is much weaker than AGP.

4. Conclusions

HPFA method incorporated in an on-line HPLC system or in a CE system was applied to the binding study of OXY enantiomers which was never achieved by using any conventional method. AGP plays the most important role in the plasma protein binding of OXY among these three plasma proteins. (*S*)-OXY is bound to AGP more strongly than (*R*)-OXY, and this enantioselectivity is reflected in the enantioselective protein binding of this drug in plasma. OXY enantiomers are bound at the same binding site on AGP molecule competitively without any allosteric effect. The present study demonstrates the applicability of the HPFA method to the quantitative and enantioselective study of strong plasma protein binding which cannot be analyzed using any conventional methods.

References

[1] M.C. Meyer, D.E. Guttman, J. Pharm. Sci. 57 (1968) 895.
[2] J.J. Vallner, J. Pharm. Sci. 66 (1977) 447.
[3] T.C. Kwong, Clin. Chim. Acta 151 (1985) 193.
[4] G.T. Tucker, M.S. Lennard, Pharmacol. Ther. 45 (1989) 309.
[5] T. Noctor, in: I.W. Wainer (Ed.), Drug Stereochemistry, 2nd edition, Marcel Dekker, New York, 1993, Ch. 12.
[6] P.M. Lish, J.A. Labudde, E.L. Peters, S.I. Robbins, Arch. Int. Pharm. Ther. 156 (1965) 467.
[7] G.F. Anderson, D.J. Krelen, Invest. Url. 12 (1975) 317.
[8] E.R. Smith, S.E. Wright, G. Aberg, Y. Fang, J.R. McCullough, Arzenimittelforschung 48 (1998) 1012.
[9] J. Douchamps, F. Derenne, A. Stockis, D. Gangji, M. Juvent, A. Herchuelz, Eur. J. Clin. Pharmacol. 35 (1988) 515.
[10] Y. Shinozaki, M. Nakai, Y. Iwata, T. Kasama, T. Nakahama, Y. Akimoto, G. Urakubo, Iyakuhin Kenkyu 17 (1986) 1063.
[11] A. Shibukawa, T. Nakagawa, N. Nishimura, M. Miyake, H. Tanaka, Chem. Pharm. Bull. 37 (1989) 702.
[12] A. Shibukawa, N. Nishimura, K. Nomura, Y. Kuroda, T. Nakagawa, Chem. Pharm. Bull. 38 (1990) 443.
[13] A. Shibukawa, M. Nagao, Y. Kuroda, T. Nakagawa, Anal. Chem. 62 (1990) 712.
[14] N. Nishimura, A. Shibukawa, T. Nakagawa, Anal. Sci. 6 (1990) 355.
[15] A. Shibukawa, A. Terakita, J. He, T. Nakagawa, J. Pharm. Sci. 81 (1992) 710.

[16] A. Terakita, A. Shibukawa, T. Nakagawa, Anal. Sci. 9 (1993) 229.
[17] A. Shibukawa, M. Nagao, A. Terakita, J. He, T. Nakagawa, J. Liq. Chromatogr. 16 (1993) 903.
[18] A. Shibukawa, C. Nakao, T. Sawada, T. Terakita, N. Morokoshi, T. Nakagawa, J. Pharm. Sci. 83 (1994) 868.
[19] A. Terakita, A. Shibukawa, T. Nakagawa, Anal. Sci. 10 (1994) 11.
[20] A. Shibukawa, M. Kadohara, J. He, M. Nishimura, S. Naito, T. Nakagawa, J. Chromatogr. A 694 (1995) 81.
[21] A. Shibukawa, T. Sawada, C. Nakao, T. Izumi, T. Nakagawa, J. Chromatogr. A 697 (1995) 337.
[22] A. Shibukawa, T. Nakagawa, Anal. Chem. 68 (1996) 447.
[23] J. He, A. Shibukawa, S. Tokunaga, T. Nakagawa, J. Pharm. Sci. 86 (1997) 120.
[24] M.E. Rodriguez Rosas, A. Shibukawa, K. Ueda, T. Nakagawa, J. Pharm. Biomed. Anal. 15 (1997) 1595.
[25] M.E. Rodriguez Rosas, A. Shibukawa, Y. Yoshikawa, Y. Kuroda, T. Nakagawa, Anal. Sci. 15 (1999) 217.
[26] M.E. Rodriguez Rosas, A. Shibukawa, Y. Yoshikawa, Y. Kuroda, T. Nakagawa, Anal. Biochem. 274 (1999) 27.
[27] A. Shibukawa, Y. Yoshimoto, T. Ohara, T. Nakagawa, J. Pharm. Sci. 83 (1994) 616.
[28] T. Ohara, S. Shibukawa, T. Nakagawa, Anal. Chem. 67 (1995) 3520.
[29] H. Shiono, A. Shibukawa, Y. Kuroda, T. Nakagawa, Chirality 9 (1997) 291.
[30] Y. Kuroda, A. Shibukawa, T. Nakagawa, Anal. Biochem. 268 (1999) 9.
[31] N.A.L. Mohamed, Y. Kuroda, A. Shibukawa, T. Nakagawa, S.E. Samia El Gizawy, F. Askal Hassan, M.E.E. Kommos, J. Pharm. Biomed. Anal. 21 (1999) 1037.
[32] N.A.L. Mohamed, Y. Kuroda, A. Shibukawa, T. Nakagawa, S.E. Samia El Gizawy, F. Askal Hassan, M.E.E. Kommos, J. Chromatogr. A 875 (2000) 447.
[33] A. Shibukawa, Y. Kuroda, T. Nakagawa, J. Pharm. Biomed. Anal. 18 (1999) 1047.
[34] M.A.K. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, Anal. Biochem. 81 (1978) 206.
[35] S. Urien, in: J.P. Tillement, E. Lindenlaub (Eds.), Protein Binding and Drug Transport, F.K. Schattauer Verlag, Stuttgart, New York, 1986, p. 63.