[Chem. Pharm. Bull.]
35(2) 734—739 (1987)

Effects of Drug Bindings on the Esterase-like Activity of Human Serum Albumin. VII.¹⁾ Subdivision of R-Type Drugs Inhibiting the Activity towards *p*-Nitrophenyl Acetate

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(Received July 14, 1986)

The subdivision of R-type drugs, which inhibit the reaction of *p*-nitrophenyl acetate with an active site (R site) located near the tyrosine-411 residue of human serum albumin (HSA), is proposed based on the results of kinetic and fluorometric studies. We found two kinds of R-type drugs with regard to the influence on the reaction rate of 3,5-dinitroaspirin (DA) at a site (U site) near the lysine-199 and tryptophan-214 (Trp-214) residues of HSA. One of them (R₂-type drug, *e.g.*, diazepam or medazepam) greatly accelerates the reaction of DA with HSA, and the other (R₁-type drug, *e.g.*, clofibric acid or octanoic acid) does not affect the reaction. The R₂-type drug quenches the fluorescence originating from the Trp-214 residue in the U site of HSA, and the R₁-type drug hardly influences this fluorescence. The acceleration of the reaction was considered to be due to the conformational change of the U site caused by binding of the R₂-type drug to a part of the R site on HSA. Five benzodiazepines, 3 sulfonylureas, and 3 other drugs were classified into R₁-type and R₂-type drugs.

Keywords—human serum albumin; esterase-like activity; drug binding site; classification; benzodiazepine; sulfonylurea; *p*-nitrophenyl acetate; 3,5-dinitroaspirin; fluorescence quenching; conformational change

In the previous papers²⁻⁴⁾ it was reported that human serum albumin (HSA) has esterase-like activity towards *p*-nitrophenyl acetate (NPA)^{2,3)} and 2,4-dinitrophenyl diethyl phosphate (DDP),⁴⁾ and the active site was found to be located close to the tyrosine-411 residue (named the R site³⁾) of the HSA amino acid sequence.⁵⁾ Drugs which inhibit the reaction of NPA with HSA were referred to as R-type drugs.³⁾ Since the R-type drugs were found to correspond to Sudlow's Site II drugs,^{6,7)} the R site is considered to be identical with Site II.

HSA has another reactive site for substituted aspirins (*e.g.*, 3,5-dinitroaspirin, DA), and this site was found to be located near the lysine-199 residue (named the U site) of the sequence.^{8,9)} The single tryptophan-214 (Trp-214) residue is also located in the U site and the binding of a drug to the U site decreased the fluorescence intensity of this residue.^{3,8)} Drugs, which inhibit the reaction of DA with HSA and quench the fluorescence due to the Trp-214 residue, were referred to as U-type drugs. Because the U-type drugs correspond to Sudlow's Site I drugs, the U site appears to be identical with Site I.

Sjöholm *et al.*¹⁰⁾ and Fehske *et al.*¹¹⁾ reported that two drugs, diazepam and warfarin, function as specific markers for two specific drug-binding sites, *i.e.*, the diazepam site and the warfarin site. Diazepam inhibited the reaction of NPA with HSA^{12,13)} and it was, thus, considered to be an R-type drug.¹²⁾ However, diazepam was, unlike other R-type drugs, found to cause acceleration of the reaction with DA and quenching of the fluorescence due to the Trp-214 residue of HSA. In this paper, we describe the basis for the subdivision of the R-type

drugs, and report the dissociation constants of the complex between the R site and various drugs including 5 benzodiazepines, 3 sulfonylureas, and *n*-octanoic acid. The data for clofibril acid and ibuprofen reported previously^{3,8)} are also presented for comparison.

Experimental

Materials and Apparatus—HSA (Sigma Chem. Co., Fraction V, lots 47C-04423, 100F-02061, and 64F-9309) was used after purification by Chen's method.¹⁴⁾ The concentration of HSA was determined from the molar absorptivity ($\epsilon = 3.66 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) at 278 nm, assuming a molecular weight of 69000.^{15,16)} DA^{8,9)} and DDP⁴⁾ were synthesized according to the methods of Zaugg *et al.*¹⁷⁾ and Gulick and Geske,¹⁸⁾ respectively. Diazepam (Takeda Chemical Industries, Ltd.), medazepam (Shionogi and Co., Ltd.), chlordiazepoxide (Takeda Chemical Industries, Ltd.), flunitrazepam (Nippon Roche Co., Ltd.), bromazepam (Nippon Roche Co., Ltd.), acetoexamide (Shionogi and Co., Ltd.), tolbutamide (Yamanouchi Pharmaceutical Co., Ltd.), and chlorpropamide (Ono Pharmaceutical Co., Ltd.) were gifts from the respective manufacturers. All other chemicals were commercial reagent-grade products.

A Hitachi UV-124 spectrophotometer and a Union-Giken RA-401 stopped-flow spectrophotometer were used for the measurements of the reaction rates. A Shimadzu RF-520 spectrofluorophotometer was employed for the measurement of the fluorescence spectra.

Kinetic Runs—The buffer used was pH 7.4, 0.067 M phosphate and the ionic strength was adjusted to 0.2 with NaCl. The reaction temperature was 25 °C. The reactions of substrate ($1.00 \times 10^{-5} \text{ M}$) with HSA ($5.00 \times 10^{-5} \text{ M}$) in the presence and absence of a drug were followed spectrophotometrically by monitoring the appearance of product at an appropriate wavelength. The wavelengths used were 380, 360, and 360 nm for *p*-nitrophenol,³⁾ 2,4-dinitrophenol,⁴⁾ and 3,5-dinitrosalicylic acid,⁸⁾ respectively. The drug concentration was varied as required. The pseudo first-order rate constant (k_{obs}) was determined from a plot of $\log(A_{\infty} - A)$ against time, where A_{∞} and A are the absorbances at completion of the reaction and at time t , respectively.

For experimental convenience, stock solutions of the substrates were prepared in acetonitrile (for NPA and DA) or dioxane (for DDP) so that the reaction solution for the rate measurement always contained 0.5% (v/v) of the organic solvent. Because of the low solubilities of benzodiazepines, 1% (v/v) ethanol was added to the reaction solution. As described previously,¹⁹⁾ the effect of the solvent on the reaction rate with NPA could be interpreted in terms of competitive inhibition of the reactive site on HSA by the solvent. As will be shown later (Figs. 1 and 3), the inhibition (or acceleration) pattern of the reaction caused by the drug was not much affected by the presence of the solvent (1% ethanol). When the concentration of the solvent was fixed for a series of reactions, therefore, the effects of other factors (e.g., the addition of the drug) on the reaction could reasonably be compared, *i.e.*, the solvent effect could be ignored in the comparison.

Fluorescence Measurements—Fluorescence spectra of HSA were measured in the presence and absence of the drug. An excitation wavelength of 300 nm was used for the measurements, since the single Trp-214 residue of HSA was excited at this wavelength.²⁰⁾ The inner filter effect of the drug on the fluorescence intensity was corrected as required.^{21,22)}

Results and Discussion

Effects of Drugs on the Reaction Rates of NPA and DDP with HSA

Figure 1 shows the effects of four drugs on the reaction rate of NPA with HSA. In this figure, k'_{obs} on the ordinate is the rate constant in the presence of drug and the concentrations with subscript 0 on the abscissa show the initial concentrations of drug and HSA. All these drugs inhibit the reaction of NPA with HSA. Figure 1 also illustrates the theoretical inhibition curves assuming different values of the dissociation constant ($K_{\text{I,R}}$) of the complex between the drug and the R site of HSA. The curves were obtained by analog computer simulation of the reaction of NPA with HSA in the presence and absence of the drug. The details of the production of the inhibition curve are described elsewhere.²³⁾ From the inhibition curve giving the best fit to the experimental results, the dissociation constant ($K_{\text{I,R}}$) was estimated, and the values are listed in Table I.

Some of the reciprocal values ($1/K_{\text{I,R}}$, M^{-1}) of $K_{\text{I,R}}$ (M) in Table I are similar to the binding constants found in the literature, and the others are significantly different.^{13,24)} The discrepancies of the parameters presumably arise from the differences of experimental

TABLE I. Dissociation Constants of the Complex between Drug and R site of HSA^{a)}

Drug	$K_{i,R}$ (M)
Medazepam ^{b)}	1×10^{-6}
Diazepam ^{b)}	1×10^{-5}
Chlordiazepoxide ^{b)}	2×10^{-5}
Flunitrazepam ^{b)}	2×10^{-4}
Bromazepam ^{b)}	8×10^{-5}
Acetohexamide	3×10^{-5}
Tolbutamide	6×10^{-5}
Chlorpropamide	8×10^{-5}
<i>n</i> -Octanoic acid	1×10^{-5}
Clofibric acid ^{c)}	5×10^{-6}
Ibuprofen ^{c)}	5×10^{-6}

a) pH 7.4, 0.067 M phosphate buffer ($\mu=0.2$ with NaCl) containing 0.5% (v/v) acetonitrile at 25 °C. b) The organic solvent content was 0.5% (v/v) acetonitrile and 1% (v/v) ethanol. c) The data were reported previously.²³⁾

Fig. 2. Effects of Drugs on the Reaction Rate of DDP with HSA

Δ , tolbutamide; $\blacktriangle$, octanoic acid; $\blacksquare$, medazepam; $\bullet$, diazepam; $\circ$, [DDP]₀ = 1.00×10^{-5} M; [HSA]₀ = 5.00×10^{-5} M; pH 7.4, 0.067 M phosphate buffer ($\mu=0.2$ with NaCl) containing 0.5% (v/v) dioxane and 25 °C; $k_{\text{obs}} = 9.25 \times 10^{-3} \text{ s}^{-1}$; k_{obs} for the case containing 1% ethanol = $7.36 \times 10^{-3} \text{ s}^{-1}$.

Fig. 1. Effects of Drugs on the Reaction Rate of NPA with HSA, and R-Type Inhibition Curves at Different Assumed Dissociation Constants ($K_{i,R}$)

Δ , tolbutamide; $\times$, clofibric acid; $\blacksquare$, medazepam; $\bullet$, diazepam; $\circ$, diazepam without ethanol; curve 1, $K_{i,R} = 1 \times 10^{-3}$ M; 2, 1×10^{-4} M; 3, 6×10^{-5} M; 4, 1×10^{-5} M; 5, 5×10^{-6} M; 6, 1×10^{-6} M; [NPA]₀ = 1.00×10^{-5} M; [HSA]₀ = 5.00×10^{-5} M; pH 7.4, 0.067 M phosphate buffer ($\mu=0.2$ with NaCl) containing 0.5% (v/v) acetonitrile and 25 °C; $k_{\text{obs}} = 6.20 \times 10^{-3} \text{ s}^{-1}$; k_{obs} for the case containing 1% ethanol = $4.95 \times 10^{-3} \text{ s}^{-1}$; data for clofibric acid were reported previously.²³⁾

Fig. 3. Effects of Drugs on the Reaction Rate of DA with HSA

Δ , tolbutamide; $\blacktriangle$, octanoic acid; $\times$, clofibric acid; $\blacksquare$, medazepam; $\bullet$, diazepam; $\circ$, diazepam without ethanol; [DA]₀ = 1.00×10^{-5} M; [HSA]₀ = 5.00×10^{-5} M; pH 7.4, 0.067 M phosphate buffer ($\mu=0.2$ with NaCl) containing 0.5% (v/v) acetonitrile and 25 °C; $k_{\text{obs}} = 2.30 \times 10^{-1} \text{ s}^{-1}$; k_{obs} for the case containing 1% ethanol = $2.17 \times 10^{-1} \text{ s}^{-1}$; data for clofibric acid were reported previously.²³⁾

procedures, such as equilibrium dialysis, ultrafiltration, and spectroscopy, and also from slight differences of the experimental conditions such as buffer components, ionic strength, and source of HSA.

Figure 2 shows the effects of the drugs on the reaction rate of DDP with HSA. All these drugs inhibit the reaction, and the primary binding site for these drugs seems to be the R site.

Influence of Drug Binding on the U Site of HSA

The effects of the drugs on the reaction rate of DA with HSA are represented in Fig. 3. There were two kinds of drugs in terms of the effects on the above reaction, one of which (named R₂-type drugs, e.g., medazepam and diazepam) markedly accelerates the reaction and

the other (named R_1 -type drugs, *e.g.*, octanoic acid and tolbutamide) hardly affects the reaction. In the absence of HSA, medazepam or diazepam does not accelerate the hydrolysis of DA itself. Thus, the acceleration mechanism may be explained by the reaction scheme shown in Chart 1.

Chart 1

Fig. 4. Fluorescence Emission Spectra Excited at 300 nm

$[\text{HSA}]_0 = 1.00 \times 10^{-5} \text{ M}$; $[\text{drug}]_0 = 1.00 \times 10^{-5} \text{ M}$; 1, HSA alone; 2, HSA + octanoic acid; 3, HSA + clofibric acid; 4, HSA + diazepam; 5, HSA + medazepam; 6, 7, 8 and 9, each drug alone; pH 7.4, 0.067 M phosphate buffer ($\mu=0.2$ with NaCl) and 25°C .

Fig. 5. Effect of Drug Concentration on the Fluorescence Intensity

▲, octanoic acid; ×, clofibric acid; ■, medazepam; ●, diazepam; ○, diazepam without ethanol; $[\text{HSA}]_0 = 1.00 \times 10^{-5} \text{ M}$; pH 7.4, 0.067 M phosphate buffer ($\mu=0.2$ with NaCl) and 25°C .

In Chart 1, $\text{DA} \cdot \text{HSA}$ and $\text{HSA} \cdot \text{drug}$ are the complexes between DA (substrate, S) and the U site of HSA and between the drug (inhibitor, I, and/or accelerator) and the R site of HSA, respectively. $K_{S,U}$ and $K_{I,R}$ are the dissociation constants of $\text{DA} \cdot \text{HSA}$ and $\text{HSA} \cdot \text{drug}$, respectively. $\text{DA} \cdot \text{HSA} \cdot \text{drug}$ is the ternary complex. $K'_{S,U}$ and $K'_{I,R}$ are the respective dissociation constants of the ternary complex. The rate constants of the complexes, $\text{DA} \cdot \text{HSA}$ and $\text{DA} \cdot \text{HSA} \cdot \text{drug}$, are represented by $k_{2,U}$ and $k'_{2,U}$, respectively. P and acetyl-HSA are 3,5-dinitrosalicylic acid and acetylated HSA, respectively.⁸⁾ For the acceleration ($(k'_{\text{obs}}/k_{\text{obs}}) > 1$ in Fig. 3) of the reaction of DA with HSA, the following conditions are necessary: $k'_{2,U} > k_{2,U}$ and/or $K_{S,U} > K'_{S,U}$. Since, as reported previously,⁸⁾ $K_{S,U}$ seems to be much smaller than $[\text{HSA}]_0$ (initial concentration of HSA employed, $5 \times 10^{-5} \text{ M}$), $K_{S,U}$ does not contribute to

TABLE II. Subdivision of R-Type Drugs

R ₁	R ₂
Clofibric acid	Medazepam
Ibuprofen	Diazepam
<i>n</i> -Octanoic acid	Chlordiazepoxide
Tolbutamide	Flunitrazepam
Bromazepam	Acetohexamide
Chlorpropamide	

k_{obs} , that is, $k_{\text{obs}} = k_{2,U} \cdot [\text{HSA}]_0 / (K_{S,U} + [\text{HSA}]_0) \approx k_{2,U}$. Even if $K'_{S,U} < K_{S,U}$, therefore, it may not be observable. Consequently, the acceleration is considered to result from $k'_{2,U} > k_{2,U}$ in our present case. The conformational change of the U site induced by the binding of the R₂-type drug to a part of the R site of HSA probably increases the reactivity of the lysine-199 residue in the U site.

The conformational change caused by the R₂-type drug may be supported by the quenching of the fluorescence due to the Trp-214 residue located in the U site of HSA. Figure 4 shows the fluorescence spectral change in the presence of the drug. Figure 5 illustrates the effect of the drug concentration on the fluorescence intensity (corrected for the inner filter effect of the drug).^{21,22)} The strong quenching of the fluorescence by diazepam or medazepam indicates conformational change of the U site. On the other hand, octanoic acid or clofibric acid does not affect the fluorescence intensity of the residue located in the U site of HSA. On the basis of these fluorescence (Fig. 5) and kinetic (Fig. 3) results, we propose the subdivision of the R-type drugs, that is, one group (R₂-type drugs) affects the U site and the other (R₁-type drugs) does not influence the U site.

Concluding Remarks on the Subdivision of R-Type Drugs

Table II summarizes the subdivision of the R-type drugs so far examined. Bruderlein and Bernstein²⁵⁾ suggested that Site I (U site) and Site II (R site) are overlapping. This is consistent with the results on diazepam or medazepam in this study. However, octanoic acid and clofibric acid seem to bind to the R site independently of the U site (see Figs. 1 and 3). On the basis of the finding that there are considerable differences in the displacing effect of bilirubin (another binding site marker) between diazepam and Site II drugs, Maruyama *et al.*²⁶⁾ recently reported that the diazepam site (considered as the R site here) can be regraded as the diazepam site-Site II overlapping area, and they also suggested that the binding of benzodiazepines to the diazepam site may cause limited conformational changes around the bilirubin site. Our results in Table II are similar to those of Maruyama *et al.*²⁶⁾ Sudlow *et al.*⁷⁾ also reported that Site II drugs might be divided into two groups. Since the three dimensional structure of HSA is not known, we can say at present only that there are two kinds of R-type drugs, depending on whether the drug influences the U site or not.

Acknowledgments We wish to thank Mr. S. Masuoka, Mrs. K. Ozeki (née Kamiyama), Miss. H. Hata, and Miss. M. Fukino for their technical assistance. We are also grateful to the cited manufacturers for gifts of the drugs. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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