
Note

The Effect of Ethanol on the Hydrolysis of Ester-type Drugs by Human Serum Albumin

Akitoshi Tatsumi,* a Sachiyo Inoue,a Tsuneo Hamaguchi,a and Seigo Iwakawa b

a Educational Center for Clinical Pharmacy, Kobe Pharmaceutical University; 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan: and bDepartment of Pharmaceutics, Kobe Pharmaceutical University; 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan.

*To whom correspondence should be addressed. e-mail: atatsumi@kobepharma-u.ac.jp
Summary

Human serum albumin (HSA) has two major ligand-binding sites, sites I and II, and hydrolyzes compounds at both sites. Although the hydrolytic interaction of ester-type drugs with other drugs by HSA has been reported, there are only a few studies concerning the effect of pharmaceutical excipients on the hydrolysis of ester-type drugs by HSA. In the present study, we investigated the effect of ethanol (2 vol%; 345 mM) on the hydrolysis of aspirin, p-nitrophenyl acetate, and olmesartan medoxomil, which are ester-type drugs, with 4 different lots of HSA preparations. The hydrolysis activities of HSA toward aspirin, p-nitrophenyl acetate, and olmesartan medoxomil were measured from the pseudo-first-order degradation rate constant ($k_{\text{obs}}$) of salicylic acid, p-nitrophenol, and olmesartan, respectively, which are the HSA-hydrolyzed products. Ethanol inhibited hydrolysis of aspirin by HSA containing low levels of fatty acids, but not by fatty acid-free HSA. Ethanol inhibited hydrolysis of p-nitrophenyl acetate by both fatty acid-free HSA and HSA containing low levels of fatty acids. In contrast, the hydrolysis of olmesartan medoxomil by HSA was insignificantly inhibited by ethanol, but inhibited not only by warfarin and indomethacin but also by naproxen, which are site I binding drugs and a site II binding drug, respectively. These results suggest that the inhibitory action of ethanol on the hydrolysis of ester-type drugs by HSA differs between site I binding drugs and site II binding drugs.

Key words  human serum albumin; ethanol; hydrolysis; olmesartan medoxomil; aspirin; drug interaction
INTRODUCTION

Human serum albumin (HSA) is a monomeric, 585-residue protein, and contains three structurally similar $\alpha$-helical domains (I-III); each domain is divided into subdomains A and B, which contain six and four $\alpha$-helices, respectively.\textsuperscript{1,2)} Crystallographic studies identified drug-binding sites I and II, which are the two major ligand-binding sites in subdomains IIA and IIIA, respectively, in the HSA molecule.\textsuperscript{2,3)}

HSA is the most abundant protein in blood plasma and serves as a transport and depot protein for numerous endogenous and exogenous compounds. This multifunctional protein possesses an enzymatic property (esterase activity). Aspirin,\textsuperscript{4)} sulbenicillin,\textsuperscript{5)} and diflunisal glucuronide\textsuperscript{6)} are hydrolyzed in site I, and $p$-nitrophenyl acetate,\textsuperscript{7,8,9,10)} several $N$-carbobenzoxy-D(L)-alanine $p$-nitrophenyl esters,\textsuperscript{11)} and carprofen glucuronide\textsuperscript{12)} are hydrolyzed in site II (Fig. 1). Olmesartan medoxomil is also hydrolyzed in both site I and site II (Fig. 1).\textsuperscript{13)}

Ethanol is widely used as a pharmaceutical excipient for the solubilization of many hydrophobic drugs. Various drugs can pharmacokinetically or pharmacodynamically interact with alcohol.\textsuperscript{14)} Ha et al.\textsuperscript{15)} reported that more than 2 vol$\%$ ethanol change the conformation of HSA and the binding of warfarin to HSA. Michnik and Drzazga\textsuperscript{16)} studied that the thermal stability of HSA was changed by more than 2 vol$\%$ ethanol. Preparations for intravenous injection for practically insoluble drugs, such as paclitaxel and phenytoin, contain 5-10 vol$\%$ ethanol and the preparation is injected into the bloodstream directly. The conformation of HSA may be altered by the high concentration of ethanol just after the intravenous injection and the binding of drugs to HSA would be changed, although it has not been studied whether the conformation change of HSA molecule by the high concentration of ethanol is reversible or irreversible. Previously, we suggested that ethanol (2.9 vol$\%$; 500 mM) changes the binding of warfarin enantiomer to HSA stereoselectively, and ethanol decreases the dissociation constant of $S$-warfarin but increase the dissociation constant of $R$-warfarin.\textsuperscript{17)} We also suggested that ethanol of 0.1 vol$\%$ or higher inhibits $S$-warfarin metabolism by recombinant human CYP2C9.1 and 3
vol% ethanol inhibits diclofenac metabolism by the CYP2C9.1. However, studies on the effect of ethanol on the hydrolysis of ester-type drugs by HSA have been limited. We previously reported the effect of warfarin and naproxen on the hydrolysis of aspirin and p-nitrophenyl acetate by HSA.

In the present study, we investigated the effect of ethanol (2 vol%; 345 mM) on the hydrolysis of aspirin, p-nitrophenyl acetate, and olmesartan medoxomil from 4 different lots of HSA preparations. We also studied the effects of warfarin, indomethacin, and naproxen, which are site I and site II binding drugs, on the hydrolysis of olmesartan medoxomil from 4 different lots of HSA preparations.

MATERIALS AND METHODS

Materials  HSA (A3782), olmesartan medoxomil, olmesartan, and acetonitrile for HPLC were purchased from Sigma-Aldrich (MO, U.S.A.). Four different lots (113K7601, 085K7541, 090M7001V, and SLBD7204V) of HSA (A3782) were used. Aspirin, sodium salicylate, p-nitrophenyl acetate, p-nitrophenol, ethanol, warfarin sodium, indomethacin, naproxen, and o-toluic acid were purchased from Wako Pure Chemical Ind. (Osaka, Japan). All other chemicals and solvents were of analytical grade or higher.

Hydrolysis Activity Measurement  Hydrolysis activity to aspirin and olmesartan medoxomil was measured by modifying the method of Ma et al. HSA (final concentration: 200 µM) with or without 2% ethanol, warfarin, indomethacin, or naproxen (200 µM each) in 67 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 37°C for 5 min, and aspirin (100 µM) or olmesartan medoxomil (40 µM) was then added to the pre-incubated mixture at 37°C. The total incubation volume was 0.13 mL. The incubation, which proceeded for 1, 2, 3, or 4 h for aspirin and 2, 3, 4, or 5 min for olmesartan medoxomil, was terminated by the addition of 0.4 mL of ice-cold acetonitrile for aspirin or ice-cold ethanol for olmesartan medoxomil, and then 0.1 mL of 0.1 M sodium hydroxide was added to adjust the pH to 7.4.
phosphate buffer (pH 2.0) containing 62.5 µM o-toluic acid for aspirin, 2.5 mM o-toluic acid for olmesartan medoxomil for the inhibition study by indomethacin, or 200 µM o-toluic acid for olmesartan medoxomil as an internal standard was added to the incubation mixture. The mixture was centrifuged at 18,620 × g at 4°C for 5 min. After centrifugation, the supernatant was used in the analysis of salicylic acid (hydrolysis product of aspirin) or olmesartan (hydrolysis product of olmesartan medoxomil) by an HPLC system. These procedures were performed in duplicate. The pseudo-first-order degradation rate constant (k_{obs}) of salicylic acid or olmesartan was obtained by a linear regression from the semi-logarithmic plot of the decay of the concentration of aspirin or olmesartan medoxomil subtracted from that in the buffer control versus time.

Hydrolysis activity to p-nitrophenyl acetate was measured by modifying the method of Ikeda et al. HSA (50 µM) with or without 2% ethanol in 67 mM potassium phosphate buffer (pH 7.4) was preincubated at 25°C for 5 min, and 10 µM p-nitrophenyl acetate was then added to the preincubated mixture at 25°C. The reaction of p-nitrophenyl acetate with HSA was followed by spectrophotometrically monitoring the appearance of p-nitrophenol at 400 nm. k_{obs} was obtained by linear regression from the semi-logarithmic plot of the decay of the concentration of p-nitrophenyl acetate versus time. Because the pseudo-first-order degradation rate constant of p-nitrophenol is significantly larger than that of the buffer control, that of the buffer control was ignored.

**HPLC Analysis** HPLC analysis of salicylic acid and olmesartan was performed according to the method of Fogel et al. and Ma et al., respectively, with some modifications. The HPLC system consisted of a Shimadzu SCL-10Avp system controller, Shimadzu LC-10ADvp pump, Shimadzu SIL-10ADvp auto injector, Shimadzu SPD-10Avp variable wavelength UV detector, and Shimadzu RF-10AXL fluorescence detector (Kyoto, Japan). A column of Wakopak Wakosil 5C18 AR (4.6 mm I.D. 250 mm; Wako Pure Chemical Industries; Osaka, Japan) was used as the stationary phase. The column temperature was maintained at 40°C. The injected volume was 25 µL. The mobile phase consisted of 0.1 M sodium dihydrogen phosphate pH 2.0/acetoniitrile (69/31). The
flow rate was 0.5 mL/min. The effluent was monitored at 295 nm for salicylic acid and 254 nm for olmesartan with a UV detector, and at an excitation wavelength of 260 nm and emission wavelength of 370 nm with an indomethacin fluorescence detector for olmesartan medoxomil.

**Statistical Analysis** Data are expressed as mean values and the standard deviation. The Student’s *t*-test was used to compare data obtained in 2 groups. Multiple comparisons for the data obtained in 5 groups were performed by ANOVA followed by the Dunnett’s pairwise *t*-test. Significance was defined as *p*<0.05.

RESULTS

The effects of ethanol on the hydrolysis of aspirin by HSA from 4 different lot preparations are summarized in Table 1. Ethanol inhibited the hydrolysis of aspirin by HSA from 090M7001V and SLBD7204V, which decreased by 22% and 35%, respectively, but ethanol did not affect the hydrolysis of aspirin by HSA from 113K7601 and 085K7541.

The effects of ethanol on the hydrolysis of *p*-nitrophenyl acetate by HSA from 4 different lots are summarized in Table 2. Ethanol significantly inhibited hydrolysis of *p*-nitrophenyl acetate by HSA in all lots. Although ethanol inhibited the hydrolysis by HSA from 113K7601 by 15%, ethanol inhibited hydrolysis by HSA from the other 3 lots by about 50%.

The effects of ethanol, warfarin, indomethacin, and naproxen on the hydrolysis of olmesartan medoxomil by HSA from 4 different lots are summarized in Table 3. Ethanol did not inhibit hydrolysis of olmesartan medoxomil by HSA from all lots. Hydrolysis of olmesartan medoxomil by HSA in each lot were similarly decreased 33-43% by the addition of warfarin and 34-49% by the addition of indomethacin. In contrast, hydrolysis of olmesartan medoxomil by HSA from each lot were decreased by the addition of naproxen from 35 to 72%.
DISCUSSION

The effect of ethanol on the hydrolysis of ester-type drugs by HSA from 4 different manufacturing lots was investigated. Ethanol inhibited hydrolysis of aspirin by HSA from 090M7001V and SLBD7204V, but did not affect hydrolysis by HSA from 113K7601 and 085K7541. Previously, we detected low levels of fatty acids in HSA from 090M7001V and SLBD7204V, and fatty acids were not detected in HSA from 113K7601 and 085K7541.19) These results were also similar to the previous report that hydrolysis of aspirin by HSA containing low levels of fatty acids were noticeably inhibited by warfarin, indomethacin, and naproxen.19) It is reported that Lys-199, His-242, and Arg-257 are important for the esterase activity to aspirin and the enzymatic activity is facilitated by the simultaneous presence of a deprotonated Lys-199 and a protonated Lys-195.21, 22) Fatty acid binding to HSA induces conformational changes, which is changes of surrounding environment by the movement of Tyr-150 and Arg-257 position on drug pocket of Site I, and increases binding of warfarin and aspirin to HSA.23, 24, 25, 26) Ethanol displace a fatty acid with binding to hydrophobic fatty acid binding sites on bovine serum albumin which is 76% sequence homologous with HSA.27) On the other hand, ethanol have a significant effect on the fluorescence intensity of HSA-bound warfarin indicating structural changes in the binding site.15) It is considered that the enhanced hydrolysis of aspirin by HSA containing low levels of fatty acids was inhibited by the displacement of fatty acid and changes of surrounding environment on site I in the presence of ethanol.

Ethanol inhibited hydrolysis of p-nitrophenyl acetate by both fatty acid-free HSA and HSA containing low levels of fatty acids. The hydrolysis activity of HSA to p-nitrophenyl acetate is species dependent (human > bovine > rabbit > dog > rat > horse), although Tyr-411 and Arg-410 in site II are perfectly preserved in all these species.28) Kragh-Hansen indicated that the species dependent of hydrolysis activity is differences in the microenvironment of albumin molecule.29) Ethanol induces conformational changes in the surrounding environment at the binding site of HSA.15, 30) We speculate that the effect of ethanol on the hydrolysis of p-nitrophenyl acetate is induced by the changes of

---

Biological and Pharmaceutical Bulletin Advance Publication
microenvironment and conformation in the esterase-like hydrolysis site.

In contrast, ethanol insignificantly inhibited hydrolysis of olmesartan medoxomil by HSA. The hydrolysis of olmesartan medoxomil by HSA was inhibited by warfarin, indomethacin, and naproxen similar to the effects toward aspirin and p-nitrophenyl acetate in our previous study\textsuperscript{10}). These results were similar to the paper reported by Ma \textit{et al.}\textsuperscript{13}) which indicated that the hydrolysis of olmesartan medoxomil by HSA was inhibited by warfarin and ibuprofen (site II ligand). Although the hydrolysis of olmesartan medoxomil was inhibited by both site I and II high affinity drugs, the hydrolysis of olmesartan medoxomil was not inhibited by ethanol. The $k_{\text{obs}}$ among aspirin, p-nitrophenyl acetate, and olmesartan medoxomil showed poor relationship in the control samples from 4 different lots of HSA preparations. These findings suggested that the effect of ethanol on the hydrolysis of ester-type drugs by HSA is substrate specific and each esterase-like hydrolysis site would be independent to other hydrolysis sites. Ma \textit{et al.}\textsuperscript{13}) speculated that importance of Glu-292 as the catalytic residue for olmesartan medoxomil, as well as Lys-199 as the principal catalytic residue in site I. In contrast, the residues of Lys-195, Lys-199, His-242, and Arg-257 are important for the catalytic residue for aspirin. They also indicated that the hydrolytic mechanism of olmesartan medoxomil is almost the same as one of p-nitrophenyl acetate although instead of Arg-410 for p-nitrophenyl acetate, Lys-414 is used to create an oxyanion hole along with Tyr-411 as chief catalytic residue. The conformational and microenvironment changes in these catalytic residues by ethanol may be differences in the hydrolytic activity between olmesartan medoxomil and aspirin or p-nitrophenyl acetate.

The present study demonstrated that the effect of ethanol on the hydrolysis of ester-type drugs by HSA varies depending on the drug structure and the conformation of the HSA molecule. Our results also suggest that the inhibitory action of ethanol on hydrolysis of site I binding ester-type drugs by HSA is different from that of site II binding ester-type drugs. Ethanol may affect the pharmacokinetics of drugs that are hydrolyzed by HSA. Further studies are needed in order to demonstrate the microenvironment and conformation changes of hydrolysis site on the HSA molecule by ethanol.
Conflict of Interest  The authors declare no conflict of interest.
REFERENCES


20) Fogel J, Epstein P, Chen P. Simultaneous high-performance liquid chromatography


30) Lin SY, Wei YS, Li MJ, Wang SL. Effect of ethanol or/and captopril on the
Figure legend

Fig. 1. Hydrolysis of Aspirin, \( p \)-Nitrophenyl Acetate, and Olmesartan Medoxomil by Human Serum Albumin

Abbreviation: HSA, human serum albumin
Fig. 1
Table 1. The Effect of Ethanol on the Hydrolysis of Aspirin by Human Serum Albumin from 4 Different Lot Preparations

<table>
<thead>
<tr>
<th>Manufacturing lots of HSA</th>
<th>$k_{\text{obs}}$ for aspirin ($10^{-6} \text{s}^{-1}$)</th>
<th>Student’s $t$-test results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With ethanol</td>
</tr>
<tr>
<td>113K7601$^a$</td>
<td>$7.4 \pm 0.1$</td>
<td>$6.4 \pm 1.0$</td>
</tr>
<tr>
<td>085K7541$^a$</td>
<td>$10.3 \pm 2.4$</td>
<td>$10.6 \pm 1.3$</td>
</tr>
<tr>
<td>090M7001V$^b$</td>
<td>$12.1 \pm 1.3$</td>
<td>$9.4 \pm 0.7$</td>
</tr>
<tr>
<td>SLBD7204V$^b$</td>
<td>$12.6 \pm 1.7$</td>
<td>$8.2 \pm 0.3$</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n=3). HSA concentration: 200 µM. Aspirin concentration: 100 µM. Ethanol concentration: 2%. NS: not significant. $^a$: not detected fatty acids, $^b$: detected low levels of fatty acids by NEFA C-Test Wako.$^{19}$
Table 2. The Effect of Ethanol on the Hydrolysis of \( p \)-Nitrophenyl Acetate by Human Serum Albumin from 4 Different Lot Preparations

<table>
<thead>
<tr>
<th>Manufacturing lots of HSA</th>
<th>( k_{obs} ) for ( p )-nitrophenyl acetate (( 10^{-3})s(^{-1}))</th>
<th>Student’s ( t )-test results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With ethanol</td>
</tr>
<tr>
<td>113K7601</td>
<td>4.1 ± 0.2</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>085K7541</td>
<td>22.1 ± 0.6</td>
<td>11.9 ± 0.1</td>
</tr>
<tr>
<td>090M7001V</td>
<td>17.1 ± 0.3</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td>SLBD7204V</td>
<td>20.6 ± 0.4</td>
<td>10.6 ± 0.4</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n=3). HSA concentration: 50 µM. \( p \)-Nitrophenyl acetate concentration: 10 µM. Ethanol concentration: 2%.
Table 3. The Effect of Ethanol, Warfarin, Indomethacin, and Naproxen on the Hydrolysis of Olmesartan Medoxomil by Human Serum Albumin from 4 Different Lot Preparations

<table>
<thead>
<tr>
<th>Manufacturing lots of HSA</th>
<th>$k_{obs}$ for olmesartan medoxomil ($10^{-3} \text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>113K7601</td>
<td>1.28 ± 0.11</td>
</tr>
<tr>
<td>085K7541</td>
<td>1.01 ± 0.02</td>
</tr>
<tr>
<td>090M7001V</td>
<td>1.16 ± 0.07</td>
</tr>
<tr>
<td>SLBD7204V</td>
<td>1.32 ± 0.22</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n=3). HSA concentration: 200 µM. Olmesartan Medoxomil concentration: 40 µM. Ethanol concentration: 2%. Warfarin, indomethacin, and naproxen concentration: 200 µM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different from the control (Dunnett’s pairwise multiple comparison $t$-test).