Glycyrrhizin Binding Site on Human Serum Albumin

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The binding site of glycyrrhizin (GLZ) on human serum albumin was detected through competitive displacement experiments with GLZ and ibuprofen (IBU) (diazepam site), warfarin (WAR), salicylate (SAL) (digitoxin site), or deoxycholic acid (DCA) by means of an ultrafiltration technique. The specific binding of GLZ was subject to competitive inhibition by IBU, WAR, SAL, or DCA (1 or 4 mM). The extent of displacement was in the order of: DCA > IBU > WAR > SAL. Conversely, the specific bindings of WAR and DCA and the low-affinity bindings of IBU and SAL were subject to competitive inhibition by GLZ (1 or 4 mM). The extent of inhibition by GLZ was in the order of DCA > IBU > WAR ≈ SAL. In addition, the low-affinity IBU binding and the specific DCA binding showed mutual competitive inhibition at 4 mM, with almost identical displacements. It was concluded that the specific GLZ binding site on human serum albumin may be located mostly within the low-affinity IBU binding site area (probably the same as the specific DCA binding site area) and partially within the specific WAR binding site area and the low-affinity SAL binding site area.

Keywords: glycyrrhizin; human serum albumin; binding site; ultrafiltration technique; competitive inhibition; ibuprofen; warfarin; salicylate; deoxycholic acid

Introduction

Previously we reported that glycyrrhizin (GLZ) binds to specific and nonspecific binding sites in human serum at concentrations respectively lower and higher than approximately 2 mM.1) It was further found that the binding sites of GLZ in human serum exist mainly on albumin, and more than 99% of the drug binds to albumin over the serum concentration range of 2-60 μg/ml following i.v. administration of 80-200 mg/man.1) If GLZ and other drugs can displace each other from human serum albumin (HSA) binding sites, such displacements may be of clinical importance, assuming that the increase of unbound drug results in a greater pharmacological effect. Many drugs have been shown to bind primarily to one of three distinct binding sites on HSA (diazepam site, warfarin site, or digoxin site).2-8) It has also been shown that bile acids may bind to a novel binding site on HSA which is different from the above three sites, fatty acid, or bilirubin binding sites.9)

The purpose of this study was to identify the binding site of GLZ on HSA through competitive displacement experiments with GLZ and ibuprofen (diazepam site),2,3) warfarin, and salicylate (digoxin site)8) as well as deoxycholic acid.

Experimental

Materials

Monoammonium glycyrrhizinate (GLZ,NH₄L) was kindly supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan); ibuprofen (IBU), warfarin (WAR), sodium salicylate (SAL), and the total bile acids test kit were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Sodium deoxycholate (DCA) and HSA (fatty acid-free, fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were commercial products of analytical grade.

Binding of Ligands to HSA

(1) HSA binding of GLZ in the presence of IBU, WAR, SAL, or DCA was determined by the same method (an ultrafiltration technique) as reported previously for GLZ alone.1) IBU and the drug solutions were adjusted in the range of 7.4-7.5. HSA was dissolved in isotonic phosphate buffer solution (pH 7.4). One milliliter of 4.2% HSA solution containing GLZ-NH₄ (0.24-7.29 mM) and IBU, WAR, SAL, or DCA (1.0 or 4.0 mM each) was applied to a filtration membrane (Amicon Micropartition system, MPS-1, Danvers, MA) after incubation at 37°C for 5 min. Ultrafiltration of samples was accomplished by centrifugation (1000 g) at 37°C for a period (approximately 10 min) sufficient to produce an ultrafiltrate volume of approximately 20% of the initial sample. (2) HSA bindings of IBU (0.5-4.0 mM), WAR, SAL (0.3-4.0 mM each), and DCA (0.2-4.0 mM) in the absence and in the presence of GLZ (1.0 or 4.0 mM) were determined by the above method. (3) HSA bindings of IBU (0.5-4.0 mM) and DCA (2.0-4.0 mM) in each other’s presence (4.0 mM) were also determined by this method. The applied HSA solutions (50 μl each) and their filtrates (200 μl each) were used for the determination of each drug. Adsorption of the drugs on the membrane and the leakage of macromolecules (HSA) were negligible.

Analytical Method

The extraction procedure and high-performance liquid chromatographic (HPLC) method for GLZ in each sample were the same as those described previously.10) as were those for IBU, WAR, and SAL in each sample, except that the volume ratio of the mobile phase, MeOH-0.05 M phosphate buffer solution (pH 2.1), and the ultraviolet wavelength for detection were modified as follows: 3:1 and 220 nm for IBU, 2:1 and 282 nm for WAR, and 1:1 and 304 nm for SAL. DCA was determined using a commercial kit.

Data Analysis

The data analysis was performed using a linear computer.11) Data were weighted with the reciprocals of the molar ratio (r) of the bound drug to the binding protein, HSA, assuming a molecular weight of 60,000.

\[
r = \frac{n_1 K_{C_1}}{1 + n_2 K_{C_1}} \phi C_i
\]

(1)

where \( K_i \) is the association constant corresponding to \( n_i \), the number of specific binding sites, \( C_i \) is the free drug concentration, and \( \phi \) is the linear binding coefficient.

\[
r = \frac{n_1 K_{C_1} + n_2 K_{C_1}}{1 + K_{C_1}}
\]

(2)

where \( K_1 \) and \( K_2 \) are the association constants corresponding to \( n_1 \) and \( n_2 \), the numbers of primary and secondary binding sites, respectively. The binding parameters were calculated using a nonlinear iterative least squares method without parameter constraints.11) The Scatchard plots from the binding data were resolved into two segments to obtain initial estimates of the binding parameters.12)

Results

The GLZ binding site on HSA was detected through competitive displacement of GLZ with IBU, WAR, SAL, or DCA by means of an ultrafiltration technique. Figure 1 shows Scatchard plots of HSA binding data of GLZ in the absence and in the presence of IBU, WAR, SAL, or DCA at a concentration of 1 or 4 mM. The plot of GLZ alone was based on the binding data in the previous paper.1) The specific GLZ binding was reduced by each of the

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other drugs. Fitting of the binding data with Eq. 1 gave converged parameters in all cases. The calculated association constant ($K_i$), the number of binding sites ($n_i$), and the linear binding coefficient ($\phi$) are listed in Table I, together with those for GLZ alone. Similar, $n_i$ values were observed in all cases, but the $K_i$ value was reduced in all cases by the presence of an inhibitor, indicating that the specific GLZ binding was subject to competitive inhibition by the drug examined. The decreases of $K_i$ value by IBU and DCA (ca. 3 and 15 times by IBU and ca. 6 and 35 times by DCA at 1 and 4 mM, respectively) were larger than those by WAR and SAL (ca. 3 and 2 times at both concentrations). The $\phi$ value decreased in the presence of all inhibitors. Figure 2 shows Scatchard plots of the binding data of IBU, WAR, SAL, and DCA in the absence and in the presence of 1 or 4 mM GLZ. The bindings of IBU, WAR, SAL, and DCA were reduced in all cases by the presence of GLZ. Fitting the binding data of WAR and DCA with Eq. 1 and those of IBU and SAL with Eq. 2 gave converged parameters. The calculated values of $n_i$, $K_i$, and $\phi$, the numbers of primary ($n_1$) and secondary ($n_2$) binding sites and the association constants $K_1$ and $K_2$ corresponding to $n_1$ and $n_2$, respectively, are listed in Table II. The $n_i$ values of WAR and DCA alone were almost identical with those in the presence of GLZ, respectively. The $K_i$ value was reduced in all cases by GLZ, but showed a much greater decrease by DCA (ca. 11—20 times) than WAR (ca. 2—3 times). The $\phi$ values tended to decrease in the presence of inhibitors. The values of $n_1$, $n_2$, and $K_1$ of IBU and SAL were almost identical with those in the presence of GLZ, respectively, but the $K_2$ values were decreased by GLZ (ca. 3—9 times and 2.3 times for IBU and SAL, respectively). The results indicate that GLZ competitively inhibits the specific bindings of both WAR and DCA and the low-affinity bindings of both IBU and SAL. As IBU and DCA showed clear inhibitory effects on GLZ binding and their bindings were decreased by GLZ, the bindings of IBU and DCA

### Table I. Binding Parameters of GLZ to HSA in the Absence and in the Presence of Inhibitors Determined by an Ultrafiltration Technique

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitor conc. (mM)</th>
<th>$n_i$</th>
<th>$K_i \times 10^{-5}$ (M$^{-1}$)</th>
<th>$\phi \times 10^{-3}$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLZ alone$^0$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ IBU</td>
<td>1.0</td>
<td>1.95±0.01</td>
<td>3.87±0.94</td>
<td>3.09±0.24</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.68±0.69</td>
<td>0.25±0.20</td>
<td>0.46±0.29</td>
</tr>
<tr>
<td>+ WAR</td>
<td>1.0</td>
<td>2.21±0.11</td>
<td>1.31±0.11</td>
<td>0.63±0.09</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.82±0.20</td>
<td>1.26±0.33</td>
<td>0.58±0.08</td>
</tr>
<tr>
<td>+ SAL</td>
<td>1.0</td>
<td>2.19±0.13</td>
<td>2.14±0.24</td>
<td>0.79±0.11</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.95±0.23</td>
<td>1.92±0.51</td>
<td>0.81±0.09</td>
</tr>
<tr>
<td>+ DCA</td>
<td>1.0</td>
<td>1.73±0.18</td>
<td>0.70±0.18</td>
<td>1.61±0.20</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>2.29±0.38</td>
<td>0.11±0.03</td>
<td>1.17±0.28</td>
</tr>
</tbody>
</table>

The parameters (±S.D.) were calculated according to Eq. 1 by the least squares method. $^a$ The number of specific binding sites. $^b$ The association constant corresponding to $n_i$. $^c$ The linear binding coefficient. $^d$ Reported values. $^1$
in each other’s presence were examined. Figure 3 shows Scatchard plots of the binding data thus obtained (4 mM), together with the plots for each drug alone (Fig. 2). Both drugs showed decreased binding in the other’s presence. Table III shows the binding parameters calculated from Eqs. 1 and 2 for DCA data and IBU data, respectively. The values of $n_1$, $n_2$, and $n_3$, and $K_1$ of IBU alone were almost identical with those in the presence of the other drug. But the $K_2$ value of IBU and the $K_3$ value of DCA decreased in the presence of the other drug, with almost identical extents of decrease (ca. 4.4 times for IBU and ca. 5.4 times for DCA). The results suggest that the low-affinity IBU binding site and specific DCA binding site may be located in an overlapping binding area on HSA. Thus, it was concluded that the specific GLZ binding site on HSA is located largely within the secondary IBU binding site area (probably the same as the specific DCA binding site area) and partially within the specific WAR binding site and secondary SAL binding site area.

**Discussion**

The binding parameters of IBU, WAR, and SAL (Table II) were in reasonable agreement with those reported.\textsuperscript{13–15} The existence of high- and low-affinity DCA binding sites in HSA has been reported.\textsuperscript{16} In this study, DCA binding to HSA showed specific and nonspecific bindings at lower and higher concentrations than approximately 1.5 mM, respectively, but the $n_1$ and $K_1$ values agreed reasonably well with the reported $n_1$ and $K_1$ values,\textsuperscript{16} respectively. As shown in Tables I and II, the $K_2$ and $K_3$ values of each drug alone were in the order of: GLZ > WAR > IBU > SAL. Therefore, the decrease of $K_2$ of GLZ by these inhibitors ought to be in the order of WAR > IBU > SAL; however, the actual extent of the decrease was in the order of IBU > WAR = SAL. The decrease of bindings of IBU, WAR, and SAL by GLZ was in the same order. In addition, an overlapping binding area for IBU and DCA was also suggested. Therefore, the specific GLZ binding site area may be largely common with the low-affinity IBU binding site area in HSA. The value of $\phi$ decreased in all cases in the presence of inhibitor (Tables I and II). This is likely the result of drug-induced conformation change of the albumin molecule.\textsuperscript{8}

It has been reported that the secondary binding site of SAL on HSA may be common with the high-affinity thyroxine site,\textsuperscript{17} which has been classified in the diazepam site group.\textsuperscript{18,19} The primary IBU binding site is also common with the diazepam site.\textsuperscript{2,3} However, GLZ inhibited the low-affinity binding of both IBU and SAL (Table II). Why GLZ did not inhibit the high-affinity IBU binding is not clear.

GLZ has frequently been used in the treatment of chronic hepatitis.\textsuperscript{20} An increase of bile acid concentration (maximum 84.3 $\mu$M) in the serum of patients with chronic hepatitis has been reported.\textsuperscript{21} GLZ and DCA show mutual competitive inhibition of their binding to specific
sites. Therefore, \( C_f \) of GLZ and DCA in the presence of one another was calculated using Eqs. 3 and 4.

\[
C_f = \frac{n_i(p)K_i C_i}{1 + K_i C_i + K_{i1} C_{i1}},
\]

\[
C_{11} = \frac{-X + \sqrt{X^2 + 4YZ}}{2Y}
\]

where subscript \( I \) represents an inhibitor (GLZ or DCA); \( p \) is HSA concentration (0.609 mM, 4.2%); \( C_{tot} \) is total drug concentration. When \( C_b = C_{b1} \), Eq. 4 is obtained from Eq. 3. \( C_f \) was determined by the simulation method of Hanano et al. using a digital computer (NEC PC-9801). Using the parameters for GLZ alone and DCA alone (Tables I and II), the values of \( C_f \) at 60 \( \mu \)g/ml (72.9 \( \mu \)m) GLZ after 200 mg/kg i.v. dosing and at 84.3 \( \mu \)m DCA, each in the presence of the other drug (0—100 \( \mu \)m), were calculated, and found to be in the ranges of 0.168—0.187 and 0.627—0.684 \( \mu \)m, respectively. The increase of \( C_f \) was small, approximately 10% for both drugs, but could be significant in clinical treatment, considering the very high level of binding of GLZ to HSA.

In conclusion, the specific binding site of GLZ on HSA is located mostly within the low-affinity IBU binding site area (probably the same as the specific DCA binding site area), and also takes in parts of the specific WAR binding site area and the low-affinity SAL binding site area.

References and Notes