Relationship between Lipophilicity and Binding Affinity with Human Serum Albumin for Penicillin and Cepham Antibiotics

Tetsuya TERASAKI, Hiroshi NOUDA, and Akira TSUJI*

Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi 13-1, Kanazawa 920, Japan
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Relationship between structure and binding affinity to human serum albumin (HSA) has been studied for penicillin and cepham antibiotics. For penicillin analogs, a good correlation between the apparent affinity constants, \( K_{\text{app}} \), for HSA binding and the partition coefficient, \( P_{\text{app}} \), determined in isobutyl alcohol–pH 7.4 phosphate buffer system was observed, indicating that the hydrophobic interaction of 6-substituent of penicillins with amino acid of HSA would play an important role for the binding. However, no correlation between the \( K_{\text{app}} \) and \( P_{\text{app}} \) values was observed for cephalothin, cefazolin, cefotetan and ceftriaxone, suggesting the presence of a common binding region in HSA among these cepham antibiotics examined. Significant differences were observed for the \( K_{\text{app}} \) value among cepham having the same 3-substitute of 7-methylthiophenazone in the molecule, i.e., cefazolin, cefotetan, cefoperazone, cefamandole, cefmenoxime, cfemazolone and cefbuperazone, suggesting that 7-substituent of cepham would play an important role for the binding with HSA. Moreover, comparing the binding affinity and the structure of 3-substitute for cepham, all of the analogs having a heterocycle bind strongly with HSA in spite of their low lipophilicity. These observations suggest that an interaction between heterocycle at the position 3 and HSA would contribute to an additional binding force for the binding of cepham antibiotics to HSA.

Keywords — structure binding relationship; cepham antibiotics; human serum albumin; primary binding site; displacement effect; protein binding; \( \beta \)-lactam antibiotics

Introduction

To predict the unbound concentration of \( \beta \)-lactam antibiotics in tissues, we have developed the extracellular fluid model\(^\text{[1-3]}\) by using the in vitro serum protein binding data. Moreover, we have also developed the in vivo extrapolation method to determine directly the unbound drug concentration in the tissue.\(^\text{[4,5]}\) Plasma unbound concentration is one of the determinant factors to affect the in vivo antimicrobial activity after the antibiotic administration. Therefore, the elucidation of the mechanisms of the plasma protein binding provides important insights into (1) the development of new cephalosporin analogs to possess an appropriate binding affinity with human serum albumin (HSA), (2) the choice of proper \( \beta \)-lactam antibiotics to obtain the significant therapeutic effects and (3) the prediction of drug–drug interaction in the plasma protein binding. Although many studies have been performed for the binding of \( \beta \)-lactam antibiotics,\(^\text{[6-10]}\) the binding mechanism and the comparative binding studies on the HSA binding are limited for cepham antibiotics.\(^\text{[11-13]}\)

The purpose of the present study is to examine the binding mechanism of penicillin and cepham antibiotics with HSA by comparing the structure and the binding affinity. The mutual displacement effects have also been performed to examine whether the binding region is the same or not among the analogs on the basis of the kinetic analysis proposed previously.\(^\text{[14]}\)

Materials and Methods

Chemicals — Dicloxacillin, cloxacinil, phenethicillin, phenoxymethylpenicillin and benzylpenicillin (Meiji Seika Kaisha, Ltd., Tokyo), oxacillin and methicillin (Banyu Pharmaceutical Co., Tokyo), piperacillin, cefoperazone and cefbuperazone (Toyama Chemical Co., Toyama), cefpiramide (Sumitomo Chemical and Industrial Co., Osaka and Yamanouchi Pharmaceutical Co., Tokyo), cefotetan (Yamanouchi Pharmaceutical Co., Tokyo), cefazolin, ceftezole and

* To whom correspondence should be addressed.
ceftizoxime (Fujisawa Pharmaceutical Co., Osaka), cefamandole, cephalothin and cephaloridine (Shionogi & Co., Osaka, Japan), cefmenoxime, cefotiam and cepachetrite (Takeda Chemical Industries, Osaka), cefmetazole (Sankyo Co., Tokyo), cefazolin and cephapirin (Bristol Myers Co., Tokyo), cefotaxime (Heochoest Japan Ltd., Kawago), cefoxitin (Japan Merck Banyu Co., Tokyo) and cefuroxime (Japan Glaxo, Tokyo) were generously supplied from the cited companies and used without further purification. HSA (essentially fatty acid-free) was purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. All other reagents and solvents were of reagent grade.

**Albumin Binding Study** — The drug binding study was performed by a centrifugal ultrafiltration method using Microparticulation System MPS-1 (Amicon Co., Danvers, MS., U.S.A.) with a semipermeable membrane, YMT (Amicon Co.) at 37 °C as described previously. HSA was reconstituted at 4.0% (v/w) i.e., 5.8 × 10⁻⁴ M with pH 7.40 Krebs–Ringer bicarbonate buffer (KRB) containing NaCl 88.8 mM, KCl 4.73 mM, CaCl₂ 1.27 mM, MgSO₄ 1.18 mM, KH₂PO₄ 2.50 mM and NaHCO₃ 24.9 mM.

The apparent affinity constant, K_{app}, was estimated from the following equation when the number of binding sites is assumed to be one:

\[ \frac{C_b}{P} / C_t = K_{app} (1 - \frac{C_b}{P}) \]  

(1)

where \( C_b \), \( C_t \) and \( P \) represent the bound and unbound drug concentrations and the HSA concentration, respectively.

For the binding displacement study, unbound drug concentration of inhibitor was predicted by the previous method to maximize the displacement effect at the primary binding site and to minimize at the secondary binding site (See Appendix).

**Determination of Apparent Partition Coefficient** — The apparent partition coefficient was determined at 37 °C by the previous method. Isobutyl alcohol and 55.5 mM phosphate buffer (pH 7.40 ionic strength, 0.15) were used for the

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**Fig. 1. Scatchard Plots of Various β-Lactam Antibiotics Binding with HSA**

Each straight line was constructed by using Eq. 1 with each \( K_{app} \) value given in Table 1. The binding of antibiotics was determined by ultrafiltration at 37 °C. The concentration of albumin was 5.8 × 10⁻⁴ M (4.0%, w/v). The total antibiotic concentrations were in the range from 3 × 10⁻⁵ to 4 × 10⁻³ M. The binding study was performed in the medium of Krebs–Ringer bicarbonate buffer (pH 7.40). Symbols: (A) CPM (□), MDIPC (●), MCIPC (○), CTT (△), CPZ (◇), MPIPC (■), CEZ (○). (B) PEPC (○), CMD (▲), PCV (△), CMX (□), CTZ (●), CMZ (△), CHT (●), CBPZ (■), CFT (◇), CEPR (▲). (C) PCG (○), DMPPC (●), CTX (△), CFX (□), CTM (●), CEC (■), PIPC (○), CXM (▲), CZX (□), CER (●).
Table I. Apparent Affinity Constants for the Binding to Human Serum Albumin, Partition Coefficients between Isobutyl Alcohol and Water and Analytical Conditions for HPLC Determination of β-Lactam Antibiotics

<table>
<thead>
<tr>
<th>No.</th>
<th>Antibiotics</th>
<th>Abbreviation</th>
<th>$K_{app} \times 10^{-3}$ (M$^{-1}$)</th>
<th>$P_{app}$</th>
<th>Wavelength (nm)</th>
<th>Mobile phase (CH$_2$CN v/v%)</th>
<th>II$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dicloxacillin</td>
<td>MDIPC</td>
<td>90.6 $\pm$ 2.6</td>
<td>4.57 $\pm$ 0.74</td>
<td>220</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>Cloxacillin</td>
<td>MCIPC</td>
<td>36.5 $\pm$ 1.1</td>
<td>2.87 $\pm$ 0.35</td>
<td>220</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Oxacillin</td>
<td>MPIPC</td>
<td>19.1 $\pm$ 0.7</td>
<td>2.60 $\pm$ 0.45</td>
<td>220</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Phenethicillin</td>
<td>PEPC</td>
<td>7.05 $\pm$ 0.23</td>
<td>2.15 $\pm$ 0.28</td>
<td>220</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Phenoxymethylpenicillin</td>
<td>PCV</td>
<td>5.50 $\pm$ 0.05</td>
<td>1.42 $\pm$ 0.17</td>
<td>220</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Benzylpenicillin</td>
<td>PCG</td>
<td>1.76 $\pm$ 0.02</td>
<td>1.06 $\pm$ 0.15</td>
<td>220</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>Methicillin</td>
<td>DMPPC</td>
<td>1.25 $\pm$ 0.03</td>
<td>0.517 $\pm$ 0.039</td>
<td>220</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>Piperacillin</td>
<td>PIPC</td>
<td>0.593 $\pm$ 0.019</td>
<td>0.471 $\pm$ 0.042</td>
<td>230</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>Cefpiramide</td>
<td>CPM</td>
<td>94.4 $\pm$ 2.47</td>
<td>0.342 $\pm$ 0.027</td>
<td>240</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>Cefotetan</td>
<td>CTT</td>
<td>24.0 $\pm$ 0.91</td>
<td>0.007 $\pm$ 0.001</td>
<td>290</td>
<td>30</td>
<td>15</td>
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<tr>
<td>11</td>
<td>Cefoperazone</td>
<td>CPZ</td>
<td>21.5 $\pm$ 0.38</td>
<td>0.254 $\pm$ 0.022</td>
<td>230</td>
<td>22</td>
<td>25</td>
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<tr>
<td>12</td>
<td>Cefazolin</td>
<td>CEZ</td>
<td>8.75 $\pm$ 0.37</td>
<td>0.194 $\pm$ 0.016</td>
<td>270</td>
<td>20</td>
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<td>Cefamandole</td>
<td>CMD</td>
<td>6.86 $\pm$ 0.08</td>
<td>0.494 $\pm$ 0.041</td>
<td>270</td>
<td>20</td>
<td>15</td>
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<tr>
<td>14</td>
<td>Cefmenoxime</td>
<td>CMX</td>
<td>5.04 $\pm$ 0.17</td>
<td>0.192 $\pm$ 0.011</td>
<td>260</td>
<td>15</td>
<td>25</td>
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<tr>
<td>15</td>
<td>Ceftezole</td>
<td>CTZ</td>
<td>4.76 $\pm$ 0.02</td>
<td>0.140 $\pm$ 0.009</td>
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<td>25</td>
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<tr>
<td>16</td>
<td>Cefmetazole</td>
<td>CMZ</td>
<td>4.50 $\pm$ 0.09</td>
<td>0.213 $\pm$ 0.017</td>
<td>270</td>
<td>25</td>
<td>15</td>
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<tr>
<td>17</td>
<td>Cefbuperazone</td>
<td>CBPZ</td>
<td>3.02 $\pm$ 0.10</td>
<td>0.078 $\pm$ 0.002</td>
<td>270</td>
<td>15</td>
<td>15</td>
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<tr>
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<td>Cephalothin</td>
<td>CET</td>
<td>4.04 $\pm$ 0.17</td>
<td>0.775 $\pm$ 0.067</td>
<td>240</td>
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<td>15</td>
</tr>
<tr>
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<td>Cefazolin</td>
<td>CFT</td>
<td>1.88 $\pm$ 0.05</td>
<td>0.247$^d$</td>
<td>270</td>
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<td>15</td>
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<tr>
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<td>Cephapirin</td>
<td>CEPR</td>
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<td>0.544 $\pm$ 0.073</td>
<td>260</td>
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<td>15</td>
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<tr>
<td>21</td>
<td>Cefotaxime</td>
<td>CTX</td>
<td>1.17 $\pm$ 0.03</td>
<td>0.183 $\pm$ 0.021</td>
<td>240</td>
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<td>15</td>
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<tr>
<td>22</td>
<td>Cefoxitin</td>
<td>CFX</td>
<td>1.13 $\pm$ 0.04</td>
<td>0.274 $\pm$ 0.008</td>
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<td>15</td>
<td>15</td>
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<td>23</td>
<td>Cefotiam</td>
<td>CMT</td>
<td>0.899 $\pm$ 0.041</td>
<td>0.244 $\pm$ 0.013</td>
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<td>15</td>
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<tr>
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<td>Cephacetrile</td>
<td>CEC</td>
<td>0.854 $\pm$ 0.038</td>
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<td>15</td>
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<tr>
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<td>Cefuroxime</td>
<td>CXM</td>
<td>0.541 $\pm$ 0.017</td>
<td>0.228 $\pm$ 0.012</td>
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<td>10</td>
<td>15</td>
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<tr>
<td>26</td>
<td>Cefitzoxime</td>
<td>CZX</td>
<td>0.342 $\pm$ 0.018</td>
<td>0.106 $\pm$ 0.006</td>
<td>240</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>27</td>
<td>Cephaloridin</td>
<td>CER</td>
<td>0.210 $\pm$ 0.026</td>
<td>0.221 $\pm$ 0.005</td>
<td>240</td>
<td>5</td>
<td>15</td>
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</table>

$^a$ The results represent the mean ± S.D. of 4—6 experiments. $^b$ The values represent the percentage of acetonitrile (v/v) in the mobile phase which was used for the analysis of ultrafiltrate. The water containing 0.01 M ammonium acetate was used. $^c$ The values represent the percentage of acetonitrile (v/v) in the mobile phase which was used for the analysis of the water phase in the study of partition coefficients. The water containing 0.01 M ammonium acetate, 0.01 M tetraethylammonium bromide and 1% (v/v) acetic acid was used. $^d$ The results represent the mean of two experiments.

Organic and the water phases, respectively. The initial antibiotic concentration added in the water phase was $1 \times 10^{-3}$ M.

**Analytical Procedures** — Antibiotic concentration was determined by a high-performance liquid chromatographic (HPLC) assay which was essentially the same as the previously described method $^{(4)}$ except for the mobile phase for the elution. The ultrafiltrate and the water phase were used for the determination of the drug binding and the partition coefficient, respectively. The wavelength of the detection and the contents of the mobile phase for the analysis of ultrafiltrate are summarized in Table I.

**Data Analysis** — The binding parameters were estimated by a least squares regression analysis program, NONLIN$^{(6)}$ using a digital computer at the Information Center, Kanazawa University, Kanazawa, Japan.
Results

Apparent Affinity Constant of $\beta$-Lactam Antibiotics for the HSA Binding

Figure 1 illustrates the Scatchard plots for the binding of $\beta$-lactam antibiotics to HSA. The apparent affinity constants, $K_{app}$, were estimated, assuming that the number of primary binding site is one for $\beta$-lactam antibiotics, and are listed in Table I. The values of $K_{app}$ varied 150-fold among penicillin analogs and 400-fold among cephem antibiotics.

Apparent Partition Coefficient of $\beta$-Lactam Antibiotics

The lipophilic properties were evaluated for $\beta$-lactam antibiotics as the partition coefficient between isobutyl alcohol and pH 7.40 phosphate buffer, $P_{app}$, and are listed in Table I. The values of $P_{app}$ for cephem antibiotics were lower than those of penicillins and varied significantly at 100-fold, i.e., 0.007—0.78, while those of penicillins varied at only 10-fold.

Fig. 2. Relationship between Apparent Affinity Constants for HSA Binding and Apparent Partition Coefficients of $\beta$-Lactam Antibiotics

The drawn line is the relation calculated for penicillins by the linear least-squares regression analysis. Each point represents the mean ± S.D. The numbers next to each point denote the antibiotics which are listed in Table I. Symbols: penicillins (Δ) and cephem antibiotics (●).

Fig. 3. Mutual Displacement Effect of Cephalothin and Cefazolin Binding with HSA

The solid lines are the computer generated best-fit lines by assuming two classes of binding sites. (A) Scatchard plots of the CET binding with HSA in the absence (○) and the presence (●) of CEZ. (B) Scatchard plot of the CEZ binding with HSA in the absence (○) and the presence (●) of CET. The hatched area represents theoretical values calculated for binding of CET (A) and CEZ (B) by assuming competitive displacement effect of CET and CEZ at the primary binding site on HSA. Upper limit and lower limit were calculated from Eqs. A2 and A3 (See Appendix) with unbound concentration range determined for CEZ (from $1.9 \times 10^{-4}$ to $2.1 \times 10^{-4}$ M) and for CET (from $3.5 \times 10^{-4}$ to $4.1 \times 10^{-4}$ M). The concentration of HSA was $5.8 \times 10^{-4}$ M. The total antibiotic concentrations were in the range from $1.1 \times 10^{-4}$ to $3.4 \times 10^{-2}$ M for CET (A) and from $3.8 \times 10^{-5}$ to $1.6 \times 10^{-2}$ M for CEZ (B).
Structure-Binding Relation of \( \beta \)-Lactams

Fig. 4. Displacement Effect of Cefotetan and Cefatrizine on the HSA Binding of Cefazolin

The solid lines are the computer generated best-fit lines by assuming two classes of binding sites. (A) Scatchard plots of the CEZ binding with HSA in the absence (○) and the presence (●) of CTT. (B) Scatchard plots of the CEZ binding with HSA in the absence (○) and the presence (●) of CFT. The hatched area represents theoretical values calculated for binding of CEZ by assuming competitive displacement effect of CTT (A) and CFT (B) at the primary binding site on HSA. Upper limit and lower limit were calculated from Eqs. A2 and A3 (See Appendix) with unbound concentration range determined for CTT (from \( 7.8 \times 10^{-3} \) to \( 9.4 \times 10^{-4} \) M) and for CFT (\( 5.7 \times 10^{-4} \) to \( 6.5 \times 10^{-4} \) M). The concentration of HSA was \( 5.8 \times 10^{-4} \) M. The total concentrations of CEZ was in the range from \( 3.8 \times 10^{-3} \) to \( 1.6 \times 10^{-2} \) M.

Relationship of \( K_{\text{app}} \) and \( P_{\text{app}} \) for \( \beta \)-Lactam Antibiotics

Figure 2 illustrates the double logarithmic plots of \( K_{\text{app}} \) value versus \( P_{\text{app}} \) value. A fairly good correlation was obtained for penicillins \( (r=0.968) \), while no correlation was observed for cephems (Fig. 2).

Displacement Effects on the HSA Bindings of Cephems

The bindings of CET, CEZ, CTT and CFT were kinetically examined at the various antibiotic total concentrations in the range from 3 ×

<table>
<thead>
<tr>
<th>Drug A</th>
<th>Drug B</th>
<th>( n_1 )</th>
<th>( K_1 ) (M(^{-1}))</th>
<th>( n_2 )</th>
<th>( K_2 ) (M(^{-1}))</th>
<th>( K_{1A, b} ) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CET(^{a})</td>
<td>—</td>
<td>1.00 ± 0.06</td>
<td>2590 ± 100</td>
<td>4.54 ± 0.12</td>
<td>259 ± 16</td>
<td>—</td>
</tr>
<tr>
<td>CET</td>
<td>CEZ</td>
<td>1.33 ± 0.17</td>
<td>892 ± 54</td>
<td>4.48 ± 0.25</td>
<td>206 ± 28</td>
<td>860</td>
</tr>
<tr>
<td>CET(^{a})</td>
<td>PCV</td>
<td>1.13 ± 0.23</td>
<td>1140 ± 100</td>
<td>4.17 ± 0.27</td>
<td>237 ± 38</td>
<td>989</td>
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<tr>
<td>CEZ</td>
<td>—</td>
<td>0.80 ± 0.01</td>
<td>10000 ± 175</td>
<td>2.89 ± 0.18</td>
<td>91 ± 6</td>
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</tr>
<tr>
<td>CEZ</td>
<td>CET</td>
<td>0.71 ± 0.01</td>
<td>5610 ± 180</td>
<td>3.07 ± 0.35</td>
<td>85 ± 11</td>
<td>5100</td>
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<tr>
<td>CEZ</td>
<td>CTT</td>
<td>0.73 ± 0.04</td>
<td>3740 ± 361</td>
<td>2.23 ± 0.49</td>
<td>144 ± 41</td>
<td>3700</td>
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<tr>
<td>CEZ</td>
<td>CFT</td>
<td>0.77 ± 0.03</td>
<td>5330 ± 347</td>
<td>1.72 ± 0.28</td>
<td>145 ± 26</td>
<td>5000</td>
</tr>
<tr>
<td>CTT</td>
<td>—</td>
<td>1.09 ± 0.04</td>
<td>19600 ± 905</td>
<td>5.77 ± 0.46</td>
<td>157 ± 14</td>
<td>—</td>
</tr>
<tr>
<td>CFT</td>
<td>—</td>
<td>0.90 ± 0.03</td>
<td>1620 ± 86</td>
<td>7.30 ± 1.12</td>
<td>30 ± 5</td>
<td>—</td>
</tr>
<tr>
<td>PCV(^{a})</td>
<td>—</td>
<td>0.94 ± 0.10</td>
<td>3520 ± 250</td>
<td>5.41 ± 0.40</td>
<td>407 ± 54</td>
<td>—</td>
</tr>
<tr>
<td>PVC(^{a})</td>
<td>CET</td>
<td>1.19 ± 0.39</td>
<td>1690 ± 210</td>
<td>5.76 ± 0.66</td>
<td>295 ± 95</td>
<td>1380</td>
</tr>
</tbody>
</table>

\(^{a}\) The results represent the mean ± S.D. based on the nonlinear least squares fitting of data. \(^{b}\) Theoretical value calculated from the mutual competitive displacement effect using Eq. A5 (see Appendix). \(^{c}\) The values were cited from the previous paper. \(^{d}\)
10^{-5} to 4 \times 10^{-3} \text{ M}. The results are shown in Figs. 3 and 4 as the Scatchard plots. According to Eq. A1 (see Appendix), the numbers of the primary and secondary binding sites, \( n_1 \) and \( n_2 \), and the affinity constants of \( K_1 \) and \( K_2 \) were determined and are listed in Table II.

As demonstrated in Fig. 3A and 3B, the bindings of CET and CEZ were mutually and competitively inhibited respectively by the addition of CEZ at the unbound concentrations observed in the range from 1.9 \times 10^{-4} to 7.1 \times 10^{-4} \text{ M} and by CET at the unbound concentrations observed in the range from 3.5 \times 10^{-4} to 4.1 \times 10^{-4} \text{ M}.

Figure 4A and 4B also demonstrate the Scatchard plots for the displacement effects on CEZ binding by CTT at the unbound concentrations observed in the range from 7.8 \times 10^{-5} to 9.4 \times 10^{-5} \text{ M} and by CFT at the unbound concentrations observed in the range from 5.7 \times 10^{-4} to 6.5 \times 10^{-4} \text{ M}. According to Eq. 4 (see Appendix) for the competitive binding, the various binding parameters were determined and are listed in Table II.

These displacement results in the cephem bindings were well coincided to those predicted by assuming the competitive displacement effects at the primary binding sites.

**Discussion**

A significantly good correlation (Fig. 2) between the apparent affinity constant to HSA, \( K_{app} \), and the apparent isobutyl alcohol–water (pH 7.40) partition coefficient, \( P_{app} \), for penicillins indicates that hydrophobic interaction would be very important for the binding of penicillin analogs to fatty acid free HSA. This observation is in accordance with the previous report obtained for the human serum.\(^{17}\)

Cephem antibiotics bind with HSA in a stronger manner than penicillins, considering the lower lipophilicity of these analogs than those of penicillins (Fig. 2). As shown in Fig. 2, the absence of correlation between the values of \( K_{app} \) and \( P_{app} \) among the structural analogs of cephems suggests at least two possible explanations for the binding of cephem antibiotics, i.e.,

i) the binding region may be different among the analogs and ii) the other binding force rather than the hydrophobic interaction between the side chain of 7-position of cephem antibiotics and HSA may play an important role for the HSA binding.

Mutual competitive displacement effects on HSA binding among CET, CEZ, CTT and CFT shown in Figs. 3 and 4 demonstrate that the primary binding site of these cephems examined is common. It was also reported that CEZ and CPZ have the same binding site on HSA.\(^{18}\) Moreover, based on the kinetic analysis of the displacement effect,\(^{14}\) we have demonstrated recently that CET has the common primary binding site on HSA to that of phenoxyethylpenicillin. Accordingly, the primary binding site of cephem antibiotics may exist in the same region of HSA as that of penicillin analogs, hence, we could rule out the possibility of i).

Comparing the binding affinity and the structure of CPM, CTT, CPZ, CMD, CMX, CMZ and CBPZ which have the same 3-substitute, i.e., \( N \)-methylthiotetrazole, the \( K_{app} \) values were significantly different among these analogs, while the \( P_{app} \) values were very similar to each other (Fig. 2). Thus, the structural difference at the position 7 of cephems would cause the significant effect on the HSA binding, suggesting that an interaction between 7-substitute and the amino acid of HSA would play an important role as a binding force of cephem antibiotics.

One of the major structural differences between cephems and penicillins is that cephem analogs have different kinds of 3-substitute in the molecule. Comparing the binding affinity and the substitution at the position 3 of cephems, all of the analogs having a heterocycle bound strongly with HSA in spite of their low lipophilicity (Fig. 2). These observations suggest that an interaction between the heterocycle at the position 3 and HSA would contribute to the binding of cephems to HSA as an additional binding force. According to our preliminary observations by the modification of amino acid moiety of HSA,\(^{19}\) the tyrosine residue of HSA may interact with the side-chain at the position 3 of cephem antibiotics.

In conclusion, cephem antibiotics are suggested to have the same binding region for the
primary binding site in HSA, which would also be the same region as that of the penicillins. The significant characteristics for the binding of cepham analogs to HSA, i.e., i) strong binding to HSA compared to those of the penicillins in spite of their low lipophilicity and, ii) the absence of the correlation between the lipophilicity and the HSA binding affinity would possibly be ascribed to the presence of an additional binding force between the 3-substitute of cepham antibiotics and the amino acid of HSA. The characterization of binding sites specific for β-lactam antibiotics to HSA is currently being investigated and the detailed results will be reported in a subsequent paper.

Appendix

Assuming that HSA has two independent classes of binding sites, i.e., primary and secondary sites, unbound drug concentration \( C_f \) and bound drug concentration \( C_b \) can be related by the following Langmuir equation.

\[
\frac{C_b}{P} = \frac{n_1 K_1 C_f}{1 + K_1 C_f} + \frac{n_2 K_2 C_f}{1 + K_2 C_f} \tag{A1}
\]

where \( P \) is albumin concentration, \( n \) is the number of binding sites, \( K \) is the affinity constant for the binding and the numbers of subscript, 1 and 2 denote the primary and secondary sites, respectively. When the binding of drug A to HSA is displaced by drug B at the primary site, there would be two different cases for the displacement effect on the drug binding at the secondary site, i.e., either the binding of drug A is displaced by drug B at the secondary site or not.

In the case that the binding of drug A is displaced competitively by drug B at the primary site but not at the secondary site (case I), the following equation can be obtained for the binding of drug A.

\[
\frac{C_{bA}}{P} = \frac{n_1 K_{1A} C_{fA}}{1 + K_{1A} C_{fA} + K_{1B} C_{fB}} + \frac{n_2 K_{2A} C_{fA}}{1 + K_{2A} C_{fA}} \tag{A2}
\]

where the subscripts of A and B denote drugs A and B, respectively.

In the case that the binding of drug A is displaced competitively by drug B at the primary and secondary sites (case II), the following equation can be also obtained for the binding of drug A.

\[
\frac{C_{bA}}{P} = \frac{n_1 K_{1A} C_{fA}}{1 + K_{1A} C_{fA} + K_{1B} C_{fB}} + \frac{n_2 K_{2A} C_{fA}}{1 + K_{2A} C_{fA} + K_{2B} C_{fB}} \tag{A3}
\]

Rearranging of Eq. A3, the following equation can be obtained:

\[
\frac{C_{bA}}{P} = \frac{n_1 K_{1A} C_{fA}}{1 + K_{1A} C_{fA}} + \frac{n_2 K_{2A} C_{fA}}{1 + K_{2A} C_{fA}} \tag{A4}
\]

where

\[
K_{1A} = \frac{K_{1A}}{1 + K_{1B} C_{fB}} \tag{A5}
\]

\[
K_{2A} = \frac{K_{2A}}{1 + K_{2B} C_{fB}} \tag{A6}
\]

\( K_{1A} \) and \( K_{2A} \) represent the apparent affinity constants for the binding of drug A in the presence of drug B at the primary and secondary sites, respectively.

In order to determine the optimal condition for the drug–drug displacement effect at the primary site of human serum albumin, i.e., to maximize the displacement effect at the primary site and to minimize the displacement effect at the secondary site, the same mathematical treatment described previously\(^{14}\) was performed.

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