STUDIES ON PROTEIN BINDING OF CEFAZOLIN AND OTHER ANTIBIOTICS

TATSUNORI SHIMIZU

Department of 3rd Internal Medicine, Sapporo Medical College
Nishi-16 Chome, Minami 1-jo, Chu0-Ku, Sapporo, Japan
(Chief: Prof. SEIGO TATENO)

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The ability of antibiotics to bind to serum proteins was studied. The extent of binding of cefazolin depends on the species of animals used: about 90% with the sera from man, rabbits or rats, and less in degree with the sera from horses, calves or dogs.

In cefazolin, the binding was characterized by its considerably high extent and easy reversibility. Antibiotics which can bind with proteins to a great extent are generally said to cause high total concentrations in the serum when given parenterally, and this applies to cefazolin. In the superposition method and disc method, the presence of serum interfered with the diffusion of cefazolin into agar medium.

The laboratory evaluation of cefazolin was reported in previous papers1-4), and the protein-binding property of this new antibiotic was indicated therein. The present paper reports the results from further investigations verifying the extent of binding to serum proteins, a feature which is somewhat different from that of other antibiotics.

Materials

Human serum (Moni-Torol 1) was obtained from Green Cross Company and other sera were prepared from each animal.

In addition to the cefazolin (Fujisawa Laboratories), the following antibiotics were used for comparison: Ampicillin and cloxazolin from Beecham Laboratories; dicloxacinillin from Bristol Laboratories; cephalexin and cephalothin from Eli Lilly; cephaloridine from Glaxo Laboratories.

Methods

1. Determination of protein binding

The centrifugal ultrafiltration technique5) was used to determine the extent of binding. Solutions of the antibiotics were prepared so as to give various concentrations with 1/15 M SÖRENSEN phosphate buffer. A 0.5-ml aliquot of the antibiotic solution was added to 4.5 ml of serum. The mixture obtained was incubated at 37°C for 1 hour. A reference experiment was performed by use of the buffer in place of serum. Bags for centrifugal ultrafiltration were prepared from Visking cellulose tubing (Visking Company, 8/32 in size). Each of the bags containing 2 ml of the mixture (consisting of an antibiotic solution and serum) or reference mixture (consisting of an antibiotic solution and the buffer solution) was hung in a 15-ml polypropylene tube.

Tubes containing the bag were centrifuged at 1,000×G for about 40 minutes. The antibiotic content of the resultant ultrafiltrate was determined by microbiological assay (paper disc method) using Bacillus subtilis ATCC 6633 as a test organism5).

The extent of binding of an antibiotic to serum was calculated by the equation provided below:

Per cent bound = \( \frac{R - S}{R} \times 100 \)

where S is the antibiotic content in the ultrafiltrate of samples and R is the antibiotic content in the ultrafiltrate of the reference.

2. Superposition method
The author conducted this experiment as mentioned in Torii and Kawakami's paper.

Result

1. Extent of Binding to Serum Proteins from Different Species of Animals

As shown in Table 1, the protein binding extent of antibiotics was studied in 6 species of animals including two additional species of horses and calves. The extent of binding was determined by the previously described method. The amounts of cefazolin bound to the serum protein of humans, rabbits and rats were each around 90% of the initial amount and were comparable to those in dicloxacillin, cloxacillin or cephalothin. On the other hand, the amounts bound to the serum protein of horses (54%), calves (43%), and dogs (54%) were significantly less than in any of the animals listed above.

From these results, the test antibiotics fall into three major classes according to the extent of binding to the human serum: high, moderate or low. In this classification, cefazolin belongs to a group of high extent antibiotics which involves cephalothin, cloxacillin, and dicloxacillin.

Table 1. Extent of binding to serum proteins from different species of animals

<table>
<thead>
<tr>
<th>Antibiotic*</th>
<th>Serum binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Horse</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>54</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>42</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>17</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>5</td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>79</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>70</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>5</td>
</tr>
</tbody>
</table>

* Final concentration: 30 mcg/ml

2. Effect of Concentrations of Antibiotics and Albumin on Protein Binding

(1) Concentration of antibiotics

As has been mentioned, cefazolin highly binds to the serum protein. The author studied the relationship between the firmness of the protein binding and the concentration of the test antibiotics. The test solutions were so prepared as to contain 4% of human albumin and varying concentrations of cefazolin. These solutions were incubated at 37°C for 1 hour to determine the extent of the protein binding by centrifugal ultrafiltration.

Regarding the relationship between the extent of protein binding and cefazolin concentrations,
as much as 90% of the cefazolin was bound to the serum at concentrations of 50 mcg/ml or less, but, at concentration exceeding 50 mcg/ml, the extent decreased with the increase in concentration (Fig. 1). At concentrations up to 50 mcg/ml, which may represent antibiotic concentrations attained in typical clinical use, the amount of cefazolin bound to the serum protein surpassed that of other antibiotics tested. These studies led us to the presumption that the binding site of the human albumin is occupied by cefazolin as the concentration increases.

(2) Albumin concentrations
The relationship between the extent of protein binding and albumin concentration is shown in Fig. 2. The test solutions were so prepared as to contain varying concentrations of human albumin and 30 mcg/ml of cephalothin or cephalexin. These test solutions were treated as mentioned in (1).

In cephalexin, the binding to the serum protein is known to be limited, the amount of bound form was slightly dependent upon the rate of albumin concentration. In cefazolin, the amount of protein-bound form increased greatly with the elevation of the albumin concentration.

3. Effect of Serum on the In Vitro Activity of Antibiotics
In order to clarify the effect of serum on the in vitro antibacterial activity of cefazolin, MICs of test antibiotics were determined in tripticase

Table 2. Effect of serum on the in vitro activity of antibiotics
Test organism; Staph. aureus FDA 209 P
Inoculum size; 10⁴/ml
Medium; Tripticase soy broth

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mcg/ml)</th>
<th>Control</th>
<th>+4% Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefazolin</td>
<td>0.39</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>Cephalexin</td>
<td>3.13</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.2</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>0.78</td>
<td>3.13</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Effect of buffer-dilution on antibiotic-protein binding

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Dilution and % activity recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>48</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>55</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>82</td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>22</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>30</td>
</tr>
</tbody>
</table>

* 0.1 M phosphate buffer

Fig. 3. Effect of serum on diffusion of cefazolin
(1) Superposition method. Incubated after preservation at 5°C for 0, 3 and 18 hours
soy broth with or without 4% human serum albumin. *Staphylococcus aureus* FDA 209 P was used as the test organism at $10^4$ cells per ml of broth. MICs were estimated after incubation at $37^\circ$C for 20 hours.

As shown in Table 2, the MIC of cefazolin in the broth was 0.39 mcg/ml, but increased to 1.56 mcg/ml by the addition of 4% albumin. On the other hand, the MIC values of cephalaxin and cephaloridine were unchanged in the presence of albumin. In spite of the decreased activity, cefazolin is supposed to retain sufficient antibacterial activity against most of the sensitive bacteria.

The MIC of cephalothin was also increased in the presence of 4% albumin similar to the case of cefazolin.

4. Effect of Buffer-Dilution on Antibiotic-Protein Binding

The firmness of antibiotic-protein binding was determined by the following method: Solution of cefazolin or other test antibiotics (200 mcg/ml), was mixed with 9-fold volumes of human serum (Moni-Trol 1). The mixtures were then diluted to 2-, 4-, and 8-fold volumes with 1/15 M phosphate buffer (pH 7.0) to determine the recovery of the antibiotic activity that had been reduced by the serum.

Table 3 shows antibiotic activities in per cent recovered by the dilution. In spite of the markedly decreased activity on adding the serum, cefazolin at the 8-fold dilution, showed sufficient recovery (96%) of its initial antibiotic activity to equal that of cephaloridine. On the other hand, dicloxacillin and oxacillin showed a more markedly decreased activity and an inferior recovery to that of cefazolin or cephaloridine. This fact indicates the reversibility of cefazolin-serum binding.

5. Effect of Serum on Diffusion of Cefazolin

(1) Superposition method

According to superposition method (one-dimentional diffusion method), the length of the
inhibitory zones produced by the standard solutions of cefazolin in phosphate buffer or rabbit serum was compared to study the effect of the serum on the diffusion of antibiotics into the agar medium. Two kinds of standard cefazolin solutions, one in phosphate buffer and the other in the rabbit serum, were superposed respectively upon the agar medium in an assay tube. Each of the agar media was previously inoculated with Streptococcus hemolyticus Strain A-S-8. The inoculated media were preserved at 5°C for 3 or 18 hours, and then incubated at 37°C for 20 hours. The length of the inhibitory zones produced was compared to know the effect of the serum on the diffusion of cefazolin. Inhibitory zones from 18-hour preservation were much larger than those from 3-hour preservation (Fig. 3). This tendency was applicable to the standard solutions from both serum and buffer.

These results indicate that cefazolin in itself is extremely diffusible into the agar medium and that the serum-bound form causes inversely delayed diffusion.

(2) Paper disc method

For a similar purpose, the effect of serum on the diffusion was estimated by the paper disc method. Test solutions were prepared similarly in the buffer and rabbit serum except to contain 50 mcg per ml. Paper discs, 8 mm in diameter and soaked with 25 µl of the cefazolin solution, either in the buffer or serum, were placed on the surface of the agar plate which had been inoculated with Bacillus subtilis ATCC 6633. These discs were removed after contact with the plate for varying intervals, and incubated at 37°C for 20 hours. The diameters of the resulting inhibitory zones were measured. The cefazolin content in the discs was estimated by plotting the diameters against the standard cefazolin curve obtained from the agar plate on which the disc remained intact through the incubation period of 20 hours (Fig. 4).

In discs soaked with the buffer, the antibiotic content and the lapse of time was as follows: The majority of cefazolin, exceeding 90% of the initial amount, was recovered in discs in contact with the agar plate for 90 minutes or more. In cephalexin, however, only 80% of the initial amounts was recovered after an interval as long as 180 minutes. In discs soaked with the serum, the effect of serum on diffusion of cefazolin was so marked as to cause a 60% reduction after contact for 180 minutes. The serum, however, gave little effect on the diffusion of cephalexin.


As has been mentioned, the extent of binding to the serum protein depends on the test species. In this connection, the standard curves of cefazolin in the sera of different animal species were compared with the standard curve in buffer solution. As shown in Fig. 5, the curves in the serum of calves or horses are much closer to the standard than in the serum of man or rabbits. This indicates that the binding to the serum of calves or horses is lower and more reversible than to that in the serum of man or rabbits. The sera of man or rabbits caused a curve discrepant from that of the buffer, horses, or calves. Consequently, the high binding led to a presumable prevention of the bound cefazolin from dissociation and diffusion into the agar medium.

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