Effect of Protein Binding on Serum Bactericidal Activities of Ceftazidime and Cefoperazone in Healthy Volunteers

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The effect of protein binding on antibiotic efficacy is controversial. The pharmacologic effect of an antibiotic is believed to be related to its unbound concentration at the site of infection. It is unknown whether antibiotics with a low degree of serum protein binding are clinically superior to antibiotics that are highly protein bound. In a randomized, crossover investigation, the serum bactericidal activities of a single dose of ceftazidime (30 mg/kg) and cefoperazone (30 mg/kg) were studied in six healthy volunteers against three clinical isolates of *Pseudomonas aeruginosa* for which both antibiotics had similar MICs and MBCs. Serum samples were collected over 12 h. The total and unbound antibiotic concentrations were determined by high-pressure liquid chromatography. Mean peak total concentrations of ceftazidime and cefoperazone in serum were 101.7 ± 18.6 and 264.1 ± 149.6 μg/ml, respectively. Due to its lower protein binding (21 ± 6%), ceftazidime had significantly higher unbound concentrations in serum than did the highly bound cefoperazone (91.5 ± 2%). Mean peak unbound concentrations were 78.5 ± 12.5 and 24.2 ± 17.8 μg/ml for ceftazidime and cefoperazone, respectively. The unbound concentration of ceftazidime at each sampling time was higher than that of cefoperazone. Although total concentrations were consistently higher than the MICs, serum containing cefoperazone showed minimal bactericidal activity against the isolates. In contrast, despite lower total concentrations, ceftazidime had greater antibacterial activity than cefoperazone. Serum bactericidal activity was more closely related to unbound rather than total antibiotic concentrations. Our data support the concept that only the unbound drug is microbiologically active.

The clinical significance of serum protein binding on the distribution, elimination, and microbiological activity of antibiotics is controversial (2, 4, 7, 11, 14–16, 18, 20, 21, 24, 26–28). Pharmacological action depends on the capability of a drug to bind to its target receptors in tissue. The unbound drug rather than the protein-bound drug is presumed to be biologically active. The extent of serum protein binding is a function of the affinity between the drug and the protein, the concentrations of protein and drug in the serum, and the number of binding sites on the protein (23).

Chambers et al. reported treatment failures with cefonicid in patients with endocarditis due to *Staphylococcus aureus* (6). The MIC determined in broth diluent for the clinical isolates was well below achievable cefonicid concentrations in serum. However, little serum bactericidal activity was observed, and breakthrough bacteremia occurred in three of the four patients treated. The authors suggest that the failure was probably due to the high degree of protein binding of cefonicid (up to 98%) and, therefore, the small amount of unbound drug present in the serum.

In the presence of serum, highly protein bound antibiotics generally have less antimicrobial activity in vitro; this is related to the reduced amount of unbound antibiotic (12, 13, 19, 22). On the other hand, the diluent used in the determination of the MIC is broth, which contains no serum proteins. Highly protein bound antibiotics might therefore have insufficient bactericidal activity despite impressive

MICs or MBCs. The serum bactericidal activity (SBA) test, which measures the bactericidal activity of serum against infecting organisms, may be a better measure of antibiotic activity.

MATERIALS AND METHODS

Clinical study. Six healthy adult male volunteers (26 to 39 years; mean body weight, 77.8 kg) were recruited. The study was approved by the Committee on Human Research of the University of California, San Francisco. Written informed consent was obtained from the subjects before their enrollment into this randomized, crossover study. Each subject received a single dose of cefoperazone (Roerig Pharmaceuticals; lot no. 62027, 30 mg/kg) and ceftazidime (Glaxo Inc.; lot no. B5166EA, 30 mg/kg) separated by a 1-week interval. Both antibiotics were administered over 30 min in 50 ml of 5% dextrose by a Harvard infusion pump. Venous blood (10 ml) was collected at 0 (baseline), 1, 2, 4, 8, and 12 h after the start of the drug infusion. Samples were allowed to clot and centrifuged, and the serum was harvested and frozen at −70°C until assayed.

High-pressure liquid chromatography analysis. Serum concentrations of ceftazidime and cefoperazone were determined by high-pressure liquid chromatography performed with a Waters C-18 column. For determination of ceftazidime concentrations, the mobile phase consisted of 10% acetonitrile and 0.5% glacial acetic acid. The pH of the solution was adjusted to 4.0 with sodium hydroxide. The internal standard used was hydrochlorothiazide. For determination of cefoperazone concentrations, the mobile phase consisted of 30% acetonitrile, 0.1% orthophosphoric acid, and 0.03% tetramethylammonium chloride solution. The

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TABLE 1. Pharmacokinetic parameters of ceftazidime and cefoperazone in six healthy subjects[a]

Parameter (unit)	Ceftazidime	Cefoperazone
Peak total concn[b] (μg/ml)	101.7 (18.6)	264.1 (149.6)
Peak free concn[b] (μg/ml)	78.5 (12.5)	24.2 (17.8)
Half-life (h)	2.2 (0.8)	2.2 (0.5)
Protein binding (%)	21.0 (6.0)	91.5 (2.0)
AUC_{total} (mg · h/liter)	234.3 (31.4)	496.1 (207.1)
AUC_{free} (mg · h/liter)	185.0 (26.5)	43.9 (26.2)
Total clearance (liter/h)	10.1 (1.3)	5.4 (2.2)
Unbound clearance (liter/h)	12.7 (1.5)	70.6 (39.2)
Vol of distribution at steady state (liter)	26.3 (5.4)	12.7 (5.8)

[a] Results are presented as means, with standard deviations within parentheses.

[b] Thirty minutes after the end of infusion.

internal standard used was ticarcillin. For both assays, the mobile phase was filtered through a Millipore membrane filter before use. Column elution was carried out with a flow rate of 1 ml/min and a pressure of 1,500 lb/in². The effluent was monitored by UV absorbance at 254 nm. Standards of known ceftazidime or cefoperazone concentrations were made up in pooled human serum to give concentrations ranging from 0.5 to 50 and from 0.5 to 100 μg/ml, respectively. Serum protein precipitation was performed by mixing the sample with acetonitrile containing the internal standard. The supernatant (15 μl) was injected directly onto the column. The sensitivity limit for both assays was 0.5 μg/ml. Reproducibility measurements yielded interday and intraday variability of less than 10%.

Pharmacokinetic analysis. The pharmacokinetic parameters of both antibiotics were estimated by noncompartmental methods. The area under the serum concentration-time curve (AUC) for total and unbound antibiotic (AUC_{total} and $AUC_{unbound}$, respectively) was determined by the log trapezoidal rule and extrapolated to infinity by dividing the last measured serum concentration value by k_{el}. k_{el} is the terminal elimination rate constant estimated by using at least the last three data points on the terminal log-linear phase of the serum concentration-time curve. Half-life was calculated by dividing the natural logarithm of 2 by k_{el}. The total and unbound clearances were calculated as dose/AUC_{total} and dose/$AUC_{unbound}$, respectively. Volume of distribution at steady state was determined by the equation V_{ss} = dose $(AUMC)/(AUC)^2$, where AUMC is the area under the first moment of the serum concentration-time curve. A correction was made for the infusion by subtracting $[(t/2) \times (dose/AUC)]$ from the V_{ss} values, where t is the duration of the infusion.

SBA. Three different isolates of *Pseudomonas aeruginosa* for which ceftazidime and cefoperazone MICs and MBCs were similar were selected from clinical specimens at the University of California Hospitals. The MIC of ceftazidime for all three isolates was 2 μg/ml, in contrast to 4 to 8 μg/ml for cefoperazone. The MBC of ceftazidime for the same isolates was 8 to 16 μg/ml, compared with 16 μg/ml for all isolates for cefoperazone. The bactericidal activity of serum samples at each collection time was determined in triplicate by a microdilution technique (17). The serum bacteriostatic activity was defined as the highest dilution without visible turbidity after an 18 to 24-h incubation period at 35°C. A 10-μl sample from each well showing no visible growth was subcultured onto antibiotic-free blood-agar medium and incubated at 35°C for 18 to 24 h. From the number of colonies

that grew, the volume of sample subcultured, and the size of the initial inoculum, the fraction of the initial inoculum that was killed was calculated. The greatest dilution of a serum sample that kills ≥99.9% of the initial inoculum was defined as the SBA. The MICs and MBCs were determined in a similar fashion: standard solutions of the antibiotics were used instead of test sera, and the dilution step was made in supplemented Mueller-Hinton broth instead of normal human serum.

Pharmacodynamic analysis. To quantify the bactericidal activity of the two antibiotics, the total area under the SBA curve (AUBC) was calculated by plotting the reciprocal of the bactericidal titer values versus time and applying the trapezoidal rule from 0 to 12 h (3, 8).

Protein binding. The extent of protein binding of ceftazidime and cefoperazone in serum at 1.0, 4.0, and 12.0 h after antibiotic administration was determined by the ultrafiltration technique with Amicon (Amicon Corp., Lexington, Mass.) tubes with a molecular weight exclusion of 50,000. Different factors that may affect protein binding, e.g., antibiotic concentration, binding to filter membrane, pH, and temperature, were evaluated and found not to affect the results. After ultrafiltration, the unbound concentrations of the two antibiotics were determined by high-pressure liquid chromatography.

Statistical analysis. The mean AUBC determined for each isolate of *P. aeruginosa* was tested by using the paired *t* test to determine significant differences ($P < 0.05$) between the two drugs. The relationship between AUBC and either AUC_{total} or $AUC_{unbound}$ of the two antibiotics was analyzed by linear regression analysis.

RESULTS

Pharmacokinetic properties of ceftazidime and cefoperazone. The mean pharmacokinetic parameters of ceftazidime and cefoperazone are presented in Table 1. Figure 1 shows the mean total and unbound serum concentration-versus-time curves for the two antibiotics. The total concentration declined biexponentially with a terminal half-life of 2.2 h for both antibiotics. For cefoperazone and ceftazidime, respectively, the mean total concentrations in serum 30 min after the end of the infusion were 264.1 ± 149.6 and 101.7 ± 18.6 μg/ml, and the mean total concentrations in serum 8 h after the dose were 7.5 ± 3.8 and 4.2 ± 1.9 μg/ml.

The mean protein binding of ceftazidime and cefoperazone was 21.0 ± 6.0% and 91.5 ± 2.0%, respectively. At each sampling time, the unbound concentration of ceftazidime was higher than that for cefoperazone. The mean unbound concentration of ceftazidime 30 min after the end of infusion was 78.5 μg/ml, compared with 24.2 μg/ml for cefoperazone. At 8 h after the dose, the mean unbound concentrations of ceftazidime and cefoperazone were 3.3 ± 1.7 and 0.7 ± 0.2 μg/ml, respectively.

There was a significant difference in the clearance of unbound drug: 70.6 liters/h for cefoperazone and 12.7 liters/h for ceftazidime. Conversely, less difference in clearance of total drug was observed: 10.1 liters/h for ceftazidime and 5.4 liters/h for cefoperazone. The difference in protein binding between the two antibiotics is also reflected in the difference in volume of distribution at steady state. The highly bound cefoperazone would therefore be expected to be less available for distribution to peripheral compartments.

SBAs of ceftazidime and cefoperazone. Both ceftazidime and cefoperazone showed similar activity (MICs and MBCs) against the three clinical isolates of *P. aeruginosa*. Never-

theless, the SBAs of ceftazidime and cefoperazone against the same clinical isolates varied markedly. Serum taken before administration of antibiotic was not bactericidal against any isolates. There was minimal SBA observed with cefoperazone against all the three isolates, even at peak serum concentrations. In contrast, the SBA of ceftazidime against the same three isolates was ≥1:8 in approximately 60% of samples at peak serum concentration. The duration of detectable SBA was also longer for ceftazidime after dosage administration. Mean unbound ceftazidime concentrations were above both the MIC and MBC for the three clinical isolates for 4 to 6 h as compared with less than 1 h for cefoperazone.

The reciprocals of the mean serum bactericidal titer-versus-time curves for ceftazidime and cefoperazone for the three isolates of *P. aeruginosa* are shown in Fig. 2. As a result of the minimal bactericidal activity, the AUBC of cefoperazone was negligible for all three clinical isolates. The AUBC for ceftazidime against each strain of *P. aeruginosa* was significantly greater than that of cefoperazone ($P < 0.05$). In plotting $AUC_{unbound}$ versus AUBC, greater bactericidal activity was observed with ceftazidime compared with cefoperazone (Fig. 3). Ceftazidime had greater killing activity as a result of higher $AUC_{unbound}$. The minimal $AUC_{unbound}$ achieved with comparable dosage of cefoperazone was associated with much less killing activity. There
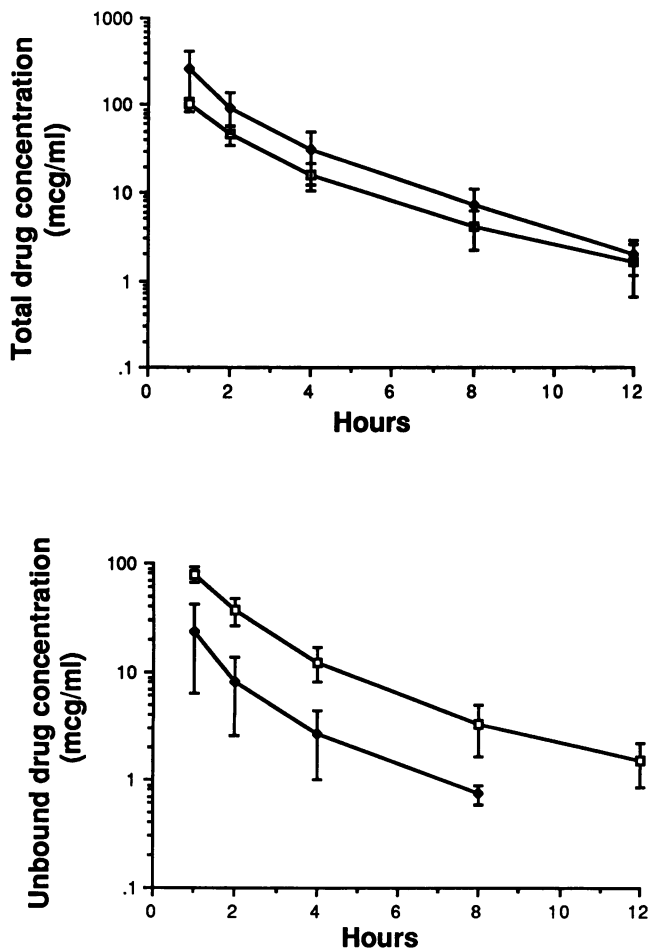

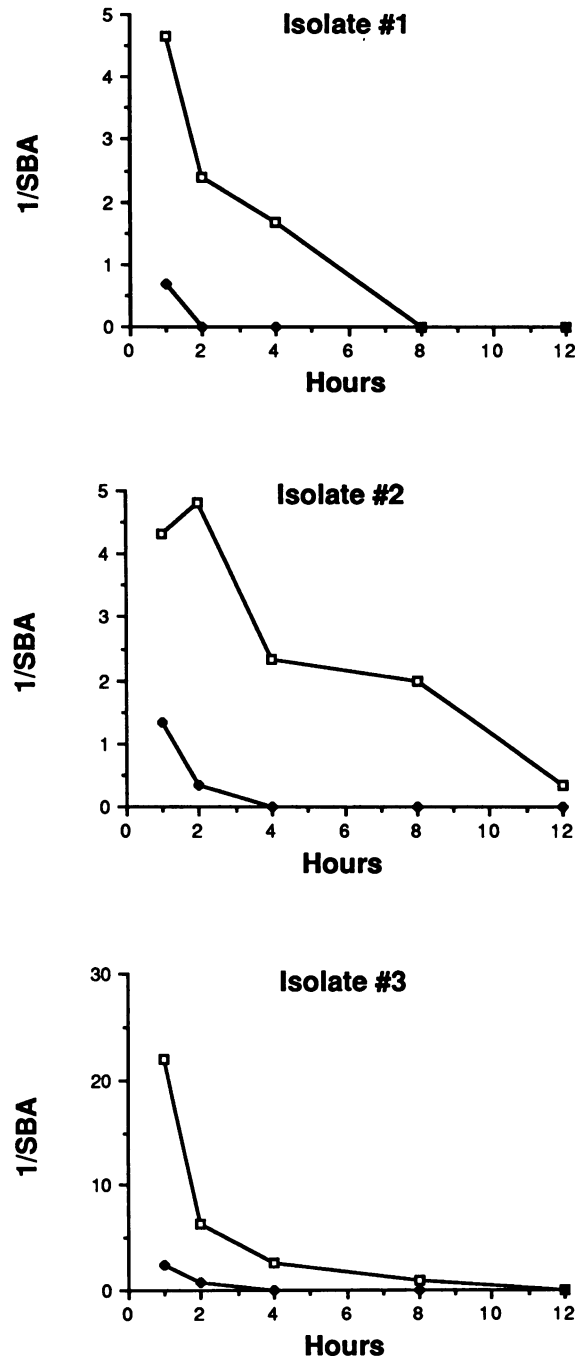FIG. 1. Mean total and unbound serum concentration-versus-time curves for ceftazidime (□) and cefoperazone (◆).

FIG. 2. Mean AUBCs for ceftazidime (□) and cefoperazone (◆) for three isolates of *P. aeruginosa*.

was no positive correlation between AUBC and the AUC_{total} of the two antibiotics.

DISCUSSION

A common method for estimation of potential efficacy of an antibiotic is to compare the MICs for organisms with the achievable antibiotic concentration. The organism is generally considered susceptible if the antibiotic concentration is substantially higher than the concentration required to inhibit the growth of the bacteria. However, the results may be
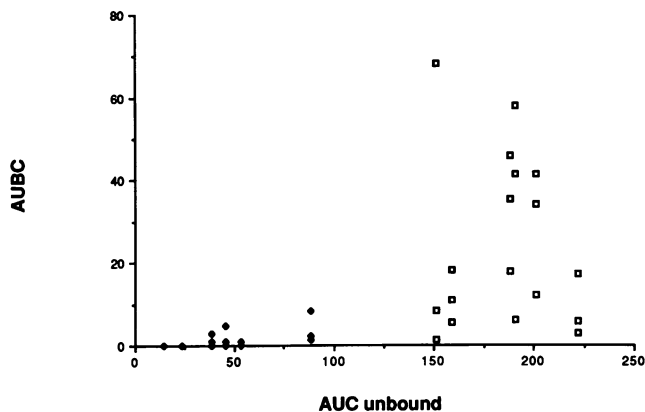
FIG. 3. Relationship of protein binding ($AUC_{unbound}$) with bactericidal activity (AUBC) for ceftazidime (□) and cefoperazone (◆).

varied by changing the test conditions, e.g., inoculum size and test medium. Also, this in vitro test does not take into consideration other important factors that might affect the pharmacologic response, such as host defense, the effect of concomitant antibiotics, postantibiotic effect, and pharmacokinetic properties of the antibiotics. Furthermore, the diluent used in the in vitro test is broth and contains no serum protein. Highly protein bound antibiotics may have minimal bactericidal activity despite impressive MICs or MBCs (10). The treatment failures with cefonicid (6) are examples of the limitation of MICs for predicting efficacy of antibiotic treatment, especially for highly protein bound drugs. Measurement of the bactericidal activity of serum against the infecting microorganism, therefore, may be a better test to predict antibacterial effect.

No postantibiotic effect has been demonstrated when *P. aeruginosa* is exposed to ceftazidime and cefoperazone, and regrowth occurs almost immediately when the drug concentrations fall below the MIC (5, 9). Anderson et al. reported breakthrough bacteremia in 12 of 19 patients treated with gentamicin, cephalexin, and ampicillin (1); the antibiotic concentrations in all 12 patients were below the MIC for the infecting organisms, which were demonstrated microbiologically to be susceptible to the administered antibiotics. Hence, experimental and clinical experience suggests that maintaining the concentration of beta-lactam antibiotics above the MICs for infecting organisms is beneficial in the treatment of gram-negative bacterial infections.

Despite similar MICs and MBCs, the SBAs of ceftazidime and cefoperazone against three clinical isolates of *P. aeruginosa*, varied markedly. This variation in SBA could not be accounted for on the basis of pharmacokinetic differences between the two antibiotics. Both ceftazidime and cefoperazone were administered at the same dose of 30 mg/kg. The resulting total serum concentration of cefoperazone was 2.5 times that of ceftazidime. The mean serum half-life for both drugs was 2.2 h. Therefore, neither the magnitude nor the duration of total serum concentrations could be correlated with the variation of SBAs.

Based on total drug concentration, both antibiotics would be expected to have effective antimicrobial activity for 6 to 8 h (Fig. 1). However, when unbound concentrations of the two antibiotics were compared, cefoperazone did not provide adequate killing and showed no SBA against the three isolates in more than 85% of the serum samples. The unbound serum concentration of ceftazidime was above the MICs and MBCs for a significantly longer time (4 to 6 h) than

that of cefoperazone (less than 1 h) in our subjects. In general, both antibiotics were effective only when the MICs and MBCs for the isolates were exceeded by unbound drug concentration. When total drug concentrations were above but unbound drug concentrations below MICs and MBCs, minimal bactericidal activity was observed. Our data support the concept that unbound drug is the active component of an antibiotic against microorganisms; therefore, it may be important to maintain the unbound beta-lactam concentrations above the MICs and MBCs for microorganisms at all times.

We have utilized the AUBC method to compare the bactericidal activity of the two antibiotics. This method is potentially a more clinically relevant measure of bactericidal activity of different antibiotics than the common approach of comparing attainable concentrations in serum to MICs or MBCs (3, 8). Because of the lack of bactericidal activity for all three isolates of *P. aeruginosa*, the AUBC for cefoperazone was significantly less than that of ceftazidime (Fig. 2).

Our SBA results agree with the findings of other investigators (2, 15, 16) that serum protein binding may have an important influence on therapeutic efficacy. Although no determination of unbound drug concentrations was carried out in the study by Van Laethem et al. (25), the significant difference in the SBA could also be due to the much lower protein binding of ceftazidime versus that of cefoperazone. With other factors being equal, an antibiotic that is highly protein bound may be less efficacious than one with a low degree of binding. This phenomenon would appear to be relevant with organisms for which the MICs are in excess of achievable unbound antibiotic concentrations. The presence of therapeutic unbound antibiotic concentrations may play a critical role in the outcome of deep-seated infections such as endocarditis. Although this study demonstrated the microbiologic importance of unbound antibiotic concentrations, additional clinical investigations are warranted to determine the relevance of protein binding in the treatment of infection.

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