Prediction of Cefazolin Penetration into High- and Low-Protein-Containing Extravascular Fluid: New Method for Performing Simultaneous Studies

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Received for publication 21 March 1978

The effect of extravascular fluid protein concentration and drug-protein binding was studied in rabbits with subcutaneously implanted Visking chambers. Chambers containing serum or saline were simultaneously studied in three animals by injecting cefazolin (30 mg/kg) intramuscularly every 4 h for four doses. Five additional animals were then studied for an 8-h period while receiving two doses of cefazolin. Peak and trough serum and chamber fluid were assayed for cefazolin concentration. The three-animal experiment demonstrated that equilibrium in the subcutaneous chambers was reached after two intramuscular injections and that the extravascular fluid levels were stable in comparison to the wide fluctuation of the serum levels. The five-animal experiment demonstrated that after two doses the serum-filled chambers contained 12.9 μg of cefazolin per ml, whereas saline-filled chambers contained 1.8 μg of cefazolin per ml. Ratios of chamber to peak serum for the second study were 12.4% for serum chambers and 1.7% for saline chambers. The cefazolin concentration in high- and low-protein extravascular fluid in both studies could be predicted from the logarithmic mean of the peak and trough serum cefazolin concentration and the antibiotic binding to rabbit serum and extravascular fluid proteins. These observations explain the previously published apparent discrepancies seen in studies of extravascular penetration of highly protein-bound antibiotics such as cefazolin.

The distribution of antibacterial agents to body tissue spaces is an important factor in chemotherapeutic effectiveness. Extravascular fluid penetration of cefazolin, a cephalosporin antibiotic with high serum protein binding (9), has been studied in both human (11) and animal (1, 3, 4, 8) models of drug distribution. In spite of these studies, the interpretation of tissue fluid penetration appears to be poorly understood, as evidenced by the reports of poor cefazolin extravascular penetration in one study (11) and good penetration in others (1, 3, 4, 8).

The purposes of this report are: (i) to describe a new rabbit model for extravascular drug penetration which enables simultaneous evaluation of high- and low-protein-containing fluid, (ii) to demonstrate the effect of extravascular fluid protein on antibiotic levels, and (iii) to illustrate the need to know drug-protein binding in both serum and extravascular fluid to interpret extravascular fluid penetration studies.

(This paper was presented in part at the 18th Interscience Conference on Antimicrobial

Agents and Chemotherapy, Atlanta, Ga., 1-4 October 1978.)

MATERIALS AND METHODS

Animal model. Experiments were performed with 2.3- to 2.7-kg albino rabbits. Tissue fluid chambers were a modification of the regenerated cellulose chambers used by Gladstone and Glencross for study of staphylococcal growth and toxin production (5). The chambers are shown in Fig. 1. Visking tubing (Union Carbide Corp., Chicago, Ill.) 0.8 by 15 cm long (measurement on flat tubing) was rinsed in sterile water and double tied at one end. The open end was placed over the tapered end of a cork through which tubing (the needle previously removed) from a 21-gauge Butterfly intermittent infusion set (Abbott Hospitals, Inc., North Chicago, Ill.) had been passed. The Visking tubing was secured to the cork with 2-0 Deknatel black braided-silk surgical suture (Deknatel Inc., Queens Village, N.Y.), and the entire chamber was placed in sterile water until ready for implantation.

Rabbits were anesthetized with intramuscular ketamine hydrochloride, their backs were shaved, and the skin was swabbed with providone-iodine followed by absolute alcohol. A 7-cm incision was made across the

FIG. 1. Schematic drawing of a subcutaneous Visking chamber before implantation. The Butterfly catheter has a soft rubber entrance port through which the system can be entered with a small-gauge needle.

shoulders or lower back, and the subcutaneous space was bluntly dissected. Four chambers were implanted subcutaneously parallel to the spinal axis, and the incision was closed with nonabsorbable silk sutures. Rabbits were rested for 5 to 6 days; then the injection port of the Butterfly infusion set was palpated, and a 25-gauge needle attached to a 3-ml syringe was percutaneously passed through the injection port to fill the chambers with fluid. By using this technique, two of the subcutaneous chambers in each animal were filled with 2 ml of pooled rabbit serum and two were filled with 2 ml of sterile saline immediately before antibiotic studies. This model provides both high- and low-protein fluid-filled chambers in contact with the interstitial space of a single animal.

Chamber studies. Initially, three rabbits, each with two serum- and two saline-filled chambers, were given intramuscular cefazolin (30 mg/kg) every 4 h for four doses. After each injection, blood, one serum- and one saline-filled chamber, from each rabbit was sampled (0.2 ml) at 0.5, 1, 2, 3, and 4 h. Subsequently, five rabbits, each with two serum-filled chambers and two saline-filled chambers, were injected intramuscularly with 30 mg of cefazolin per kg of body weight at 0 and 4 h. Serum samples were measured at 0.5, 4, 4.5, and 8 h to evaluate the peak and trough drug concentrations. Samples (0.5 ml) of serum or saline were removed from all four subcutaneous chambers in each rabbit by percutaneous aspiration through the soft rubber injection port of the Butterfly infusion set with a 25-gauge needle and a 1-ml syringe. All samples were taken from each rabbit in less than 5 min. To simplify this experiment, chamber samples were taken at 4, 4.5, 8, and 24 h to measure cefazolin levels and drug persistence at 24 h (4). Protein content of serum- and saline-filled chamber fluid was measured at 0 and 24 h. A cross-section biopsy of a subcutaneous chamber was performed at 7 days to determine cellular reaction to the regenerated cellulose bag.

Assays. Cefazolin was assayed in triplicate by a microbiological disk-plate method with 24-h incubation at 37°C (6, 10). Triplicate assays provide results with less than 10% error (6). Assays were performed with *Staphylococcus aureus* ATCC 6538P on antibiotic medium no. 1 (Difco Laboratories, Detroit, Mich.) at pH 6.6. Serum samples were assayed with a serum standard curve, and saline samples were assayed with a saline standard curve. Preliminary studies had demonstrated that saline standard curves and those containing 1.6 µg of rabbit serum protein per ml in saline were identical for cefazolin.

Protein concentrations were determined by the method of Lowry et al. (7), and albumin content was

determined by cellulose acetate serum protein electrophoresis (Beckman microzone electrophoresis, Beckman Instruments, Inc., Palo Alto, Calif.).

Protein binding. Serum protein binding was determined by ultracentrifugation (4, 9). Four samples were also diluted with saline to contain 1.1 mg of protein per ml. This dilution was then mixed with cefazolin to obtain a concentration of 6.0 µg/ml, and 8-ml samples were centrifuged at $295,000 \times g$ for 3 h at 30 to 35°C. The top 0.5 ml of each centrifuged sample was removed and assayed, along with each starting sample, for cefazolin concentration to determine protein binding. Assay standard curves for diluted serum studies were prepared in saline. Serum standard curves were used to assay whole-serum samples, and serum ultrafiltrate was used to prepare standard curves to assay the top 0.5 ml of each centrifuged serum sample (9). Antibiotic-protein binding in the diluted serum was necessary to approximate the antibiotic-protein binding in the subcutaneous saline-filled chambers.

Calculations. The log mean serum concentration used to predict antibiotic concentrations within the subcutaneous chambers was obtained by graphically determining the midpoint of the mean serum peak and trough levels of cefazolin on a semilogarithmic graph (D. N. Gerding, L. R. Peterson, J. K. Salomonson, W. H. Hall, and E. A. Schierl, *J. Infect. Dis.*, in press). This number represents the logarithmic mean of the peak and trough serum concentration and is a mathematical approximation of the antibiotic concentration which must be maintained by a constant antibiotic infusion to give the same area under the curve as that obtained by intermittent drug administration. Prediction of cefazolin concentration in chamber fluid was based on the assumption that unbound, or free, antibiotic diffuses readily and at equilibrium the free drug concentrations in serum and chamber fluid are identical. Total (free plus protein-bound) chamber fluid concentration at equilibrium ($[C_c]$) is determined by:

$$[C_c] = [C_s] \cdot \frac{F_s}{F_c} \quad \text{Equation 1}$$

where $[C_s]$ = log mean serum concentration, F_s = free fraction of cefazolin in serum (percent free in serum per 100), and F_c = free fraction of cefazolin in chamber fluid (percent free in chamber fluid per 100). It is important to note that as the protein binding in the chamber fluid decreases the free fraction in chamber fluid (F_c) rises and the total equilibrium antibiotic concentration ($[C_c]$) in chamber fluid falls.

RESULTS

Chamber analysis. Serum-containing chambers had a protein concentration of 64.8 mg/ml at 0 h and 62.0 mg/ml at 24 h. Saline-containing chambers had no protein at 0 h and 1.1 mg/ml at 24 h. Electrophoresis revealed the main protein component of the 24-h saline samples to be albumin. Greater than 90% of fluid placed in both chamber types was recovered at 24 h.

Biopsy of a cross section through a Visking

chamber and surrounding tissue at 7 days after implantation revealed the cellulose membrane to be encapsulated by fibrous connective tissue containing occasional mononuclear and neutrophil leukocytes. This was interpreted as representing mild acute and chronic inflammation in the fibrous tissue surrounding the Visking chamber. Subcutaneous chambers remained usable for 14 days. After 14 days increasing numbers of leukocytes became attracted to the area of implantation, and by 6 weeks the chambers were localized to a subcutaneous abscess. Rabbits whose chambers had been removed at the end of 2 weeks demonstrated no subcutaneous adhesions and could be reimplanted with new chambers without difficulty.

Protein binding. Cefazolin binding to rabbit serum proteins was found to be 95% as previ-

ously reported (4). Dilution of rabbit serum revealed cefazolin to be $36.9 \pm 4.9\%$ bound at 1.1 mg of protein per ml.

Cefazolin penetration. Results of the experiments are presented in Fig. 2 and 3. It can be readily seen that the protein concentration, and therefore the protein binding, of the chamber fluid markedly affected the degree of apparent cefazolin penetration in both experiments. The reason for the effect of protein binding on cefazolin penetration is that protein binding in a tissue can markedly affect the total drug concentration achieved. Also, it is evident from Fig. 2 that equilibrium was reached in the subcutaneous chambers after the second intramuscular cefazolin dose.

Penetration kinetics. The ranges of log mean total and log mean free serum concentra-

FIG. 2. Semilogarithmic plot of experiment 1 with cefazolin concentrations in serum and subcutaneous Visking chambers containing serum or saline. Serum concentrations represent the mean of three rabbits at each point. Peak serum concentrations ranged from 120 to 148 $\mu\text{g/ml}$ at 5 h, 120 to 144 $\mu\text{g/ml}$ at 4.5 h, 113 to 133 $\mu\text{g/ml}$ at 8.5 h, and 100 to 108 $\mu\text{g/ml}$ at 12.5 h. Trough serum concentrations ranged from 3.4 to 8 $\mu\text{g/ml}$ at 4 h, 5 to 5.8 $\mu\text{g/ml}$ at 8 h, 6.5 to 17 $\mu\text{g/ml}$ at 12 h, and 4.9 to 6.6 $\mu\text{g/ml}$ at 16 h. Chamber concentrations are represented by the mean $\pm$ range of three samples (one from each rabbit) at each point. The range of the log mean total serum concentration is represented by the top shaded bar and derived from the range of the peak and trough serum concentrations found after each cefazolin injection. The range of the log mean free serum concentration is represented by the bottom shaded bar and calculated from the range of the log mean total serum concentration assuming 95% cefazolin binding to rabbit serum at 10 to 20 $\mu\text{g/ml}$.

FIG. 3. Semilogarithmic plot of experiment 2 with cefazolin concentrations in serum and subcutaneous Visking chambers containing serum or saline. Serum concentrations represent the mean of five rabbits at each point. Serum standard deviations were ± 13 $\mu\text{g/ml}$ at 0.5 h, ± 1.78 $\mu\text{g/ml}$ at 4 h, ± 27.6 $\mu\text{g/ml}$ at 4.5 h, and ± 1.0 $\mu\text{g/ml}$ at 8 h. Chamber concentrations are represented as the mean $\pm$ standard deviation of 10 samples (2 from each rabbit) at each point. The range of the log mean total serum concentration is represented by the top shaded bar and derived from the range of the peak and trough serum concentrations found after each cefazolin injection. The range of the log mean free serum concentration is represented by the bottom shaded bar and calculated from the range of the log mean total serum concentration assuming 95% cefazolin binding to rabbit serum at 10 to 20 $\mu\text{g/ml}$.

tions based on the two cefazolin injections are shown by the shaded areas in Fig. 2 and 3. The range of the log mean total concentration was calculated by determining the logarithmic mean of the highest peak and highest trough for the upper end of the range, and the logarithmic mean of the lowest peak and lowest trough for the low end of the range. The range of the log mean free concentration was determined by multiplying the range of the log mean total concentration by 0.05. By using equation 1 and the protein binding data from serum, the pre-

dicted total cefazolin peak concentration in the serum-filled chambers for the experiment in Fig. 2 was 29.5 $\mu\text{g/ml}$ (equal to the log mean total serum concentration, because the free drug fractions F_s and F_c are identical). The actual concentration (mean $\pm$ range) found 2 or 3 h after the second to fourth cefazolin dose was 26.4 ± 6.5 $\mu\text{g/ml}$. The predicted peak, or equilibrium, concentration of cefazolin in the saline-containing chambers using the estimated binding data for fluid with 1.1 mg of protein per ml was 2.5 $\mu\text{g/ml}$. The actual concentration of cefazolin in

these chambers at 2 h after the second to fourth antibiotic injection was $5.3 \pm 1.6 \mu\text{g/ml}$. The second experiment, depicted in Fig. 3, which compared 10 saline chambers and 10 serum chambers in five rabbits, gave a predicted total cefazolin peak concentration in the serum-filled chambers equal to $10.5 \mu\text{g/ml}$. The actual concentration (mean $\pm$ standard deviation) found 4 h after the second cefazolin dose was $12.9 \pm 2.6 \mu\text{g/ml}$. The predicted peak, or equilibrium, concentration of cefazolin in the saline-containing chambers using the estimated binding data for fluid with 1.1 mg of protein per ml was $0.87 \mu\text{g/ml}$. The actual concentration of cefazolin in the saline chamber for this experiment 4 h after the second antibiotic injection was $1.8 \pm 0.4 \mu\text{g/ml}$. The low predictions in the saline-containing chambers may have been due to a low estimation of protein binding in the chamber fluid based on serum dilution studies. Small changes in protein concentration at the low level found in the saline chambers markedly affected results of protein binding obtained. Serum diluted to 0.6 mg of protein per ml decreased cefazolin binding to $22.5 \pm 5.4\%$ ($n = 4$) compared with $36.9 \pm 4.9\%$ at 1.1 mg of protein per ml.

Calculation of penetration based on peak chamber to peak serum concentration ratios revealed marked differences between the two types of intrachamber fluid. The serum-containing chambers in Fig. 2 gave a ratio of peak chamber to peak serum cefazolin of 21.8%, whereas the saline-filled chambers only resulted in a ratio of peak chamber to serum of 5.3%. The ratio of cefazolin serum chamber to serum in the second experiment (Fig. 3) was 12.4% at 8 h, whereas the ratio of saline chamber to serum was 1.7%.

DISCUSSION

The results of these experiments demonstrate the importance of drug-protein binding in the extravascular fluid as well as the drug binding of intravascular serum proteins. The findings help to explain why Tan and Salstrom found low ratios of fluid to serum cefazolin concentration when using a saline-filled cutaneous chamber (11). Tan and Salstrom found a penetration ratio of 0.04, which is comparable to our saline chamber results of 4.6 and 1.7% or ratios of 0.05 and 0.02. Experimental models incorporating extravascular fluid containing significant amounts of protein, especially albumin, demonstrate good penetration with highly protein-bound drugs such as cefazolin (1, 3, 4, 8). The cefazolin penetration into these extravascular fluid models was similar to that found in our serum-containing subcutaneous chambers. Also, Cole and Pung have reported good cefazolin penetration

into pleural fluid in humans, which again is a high protein-containing fluid (2). Because of the important effect of protein binding on total drug concentration, it would appear that comparison of free drug extravascular fluid concentration to free drug serum concentration would be more useful than comparison of total drug levels in extravascular fluid and serum. Calculation of penetration ratios based on free cefazolin levels in extravascular fluid and peak free serum levels yield 21.3 and 12.5% penetration for serum chambers in experiments one and two, respectively, and 57.4 and 21.2% for saline chambers. This demonstrates that for each experiment the free or unbound serum cefazolin penetrated into low-protein extravascular fluid at least as well as into high-protein-containing extravascular fluid.

The two experiments provide additional support for the concept of the log mean serum level being important in determining extravascular drug levels when antibiotics are administered intermittently. The first experiment had consistently higher peak and trough antibiotic concentrations which resulted in a higher log mean serum level. Comparison of Fig. 2 with Fig. 3 demonstrates that the higher log mean serum level in the Fig. 2 experiment resulted in higher extravascular antibiotic concentrations in that study.

The variation in subcutaneous chamber antibiotic concentration after equilibrium is achieved was small. The serum chambers only varied $\pm 3.1 \mu\text{g/ml}$ from the mean concentration after the second cefazolin dose, and the saline chambers only varied $\pm 1.6 \mu\text{g/ml}$. The apparent wide variation in saline chamber antibiotic levels in Fig. 2 is due to the representation on a logarithmic scale, because the variation in concentration after equilibrium was achieved is actually less than that for the cefazolin concentration in the serum-filled chambers.

The results of this study suggest that differences in drug penetration, especially involving highly protein-bound antibiotics, can be explained on the basis of the binding properties of the extravascular fluid of the experimental model. We would recommend that to interpret drug penetration studies, antibiotic-protein binding should be evaluated in both the serum and extravascular fluid under evaluation.

The use of serum and extravascular fluid protein binding along with the log mean serum drug level (equation 1) to predict interstitial drug concentration is based on assumptions that (i) free antibiotic readily distributes with body water and that at equilibrium free drug levels are equal in both the intravascular and extravascular space; (ii) that the log mean serum concentration approximates a steady-state infusion

with the same area under the curve and is representative of equilibrium conditions; and (iii) that the total drug concentration in a given fluid is a summation of the free and protein-bound antibiotic levels. The predicted interstitial fluid cefazolin concentrations were similar to levels found after two or more intramuscular doses. Results of this study support all these assumptions.

ACKNOWLEDGMENTS

We thank Carlos Costas-Martinez for technical assistance and Evelyn Glatt for preparation of the manuscript.

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