Effect of Protein Concentration and Binding on Antibiotic Assays

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Assay curves, using a disk diffusion method for the antibiotics gentamicin and cefazolin, were prepared with: saline, saline plus 10% serum, and ascitic, synovial, cerebrospinal, and pleural fluids. The curves were compared with a standard curve prepared with pooled human serum. The pH, total protein, glucose, blood urea nitrogen, sodium, potassium, calcium, phosphorus, chloride, CO₂ content, uric acid, cholesterol, bilirubin, serum glutamic oxalacetic transaminase, CPK, LDH, and alkaline phosphatase were determined and compared for all fluids. Measurements for cefazolin levels were falsely elevated in those fluids with low protein content when serum was used as a reference standard. There was a linear inverse relationship between the protein content of the fluids and the cefazolin level with serum as the standard for the assay of this highly protein-bound antibiotic. No discrepancies were observed in the assay curves for gentamicin, an antibiotic known not to be bound by serum proteins.

Florey and Tuiton first determined penicillin levels in wound extracts in 1946 (2). Since then advances in antimicrobial therapy have necessitated rapid and accurate serum and tissue antibiotic measurements. Pooled human serum is usually used to ascertain standard curves when determining unknown antibiotic levels in serum. Various solutions have been used to prepare standard curves when other body fluids are investigated. Standard curves generated from saliva, sputum, and buffered saline were very much similar (4, 5). In 1970, Simon and Yin reported differences in standard curves between buffer standards and serum due to protein binding (9). A recent publication reported antibiotic levels in pericardial fluid with buffered saline for the standard reference curve (10). Investigators have often neglected to specify the reference fluid used in the preparation of standard curves (7, 8).

This study was undertaken to evaluate any differences between standard curves generated from body fluids with varying protein concentrations. Standard curves for both cefazolin (Eli Lilly & Co., Indianapolis, Ind.) and gentamicin (Schering Laboratories, Bloomfield, N.J.) were compared.

MATERIALS AND METHODS

Antibiotic fluid concentrations. Gentamicin concentrations tested were 2.5, 5, 10, and 20 µg/ml. Cefazolin concentrations tested were 1.25, 2.5, 5, 10, and 20 µg/ml. Standard concentrations were made

from 1,000 µg of stock solutions per ml of each antibiotic by appropriate dilutions. Fluids in which stock solutions were prepared included isotonic saline, 90% isotonic saline with 10% pooled human serum (Microbiological Associates, Inc., Bethesda, Md.), pooled human serum (Microbiological Associates, Inc.), pleural, ascitic, synovial, and cerebrospinal fluids. All body fluids were also tested for antibiotic activity before adding antibiotics with no activity noted. Twenty microliters of each fluid at the above concentrations were dropped on 0.25-inch paper disks 740-E (Schleicher and Schuell, Inc.) using disposable pipettes (Becton, Dickinson and Co., Rutherford, N.J.) before being placed on test agar.

Experimental model. A disk agar diffusion method was used for measuring antibiotic levels (1). Gentamicin assays were performed with antibiotic medium no. 5 (Difco, Detroit, Mich.) seeded with 0.2% of a 1:5 dilution of *Bacillus subtilis* spore suspension (Difco). Cefazolin assays were performed with antibiotic medium no. 11 (Difco) seeded with a 1:10 dilution of *Staphylococcus* 6538P (Schering) grown for 18 h in Trypticase soy broth (Difco) on a rotary shaker at 37 C.

Each assay was performed by placing one of the 0.25-inch fluid-containing disks and a serum disk with the same antibiotic concentration on the appropriate media. Assays were run in quadruplicate, with a total of 10 assays completed for each sample. Incubation was performed at 37 C for 18 h, and zone sizes were read to the nearest 0.1 mm. Bioassay antibiotic concentrations for the various fluids were read from the standard curve prepared with serum. Antibiotic concentrations determined from the serum standard curve were then compared with the actual amounts of antibiotic added to each fluid.

Fluid analysis. All fluids were tested by a multiple channel automatic analyzer for pH, total protein, glucose, blood urea nitrogen, sodium, potassium, calcium, phosphorus, chloride, CO_2 content, uric acid, cholesterol, bilirubin, serum glutamic oxalacetic transaminase, CPK, LDH, and alkaline phosphatase.

Statistical analysis. Significance was determined by the Student's *t* test.

RESULTS

Antibiotic standard curves in various fluids. (i) Gentamicin. Gentamicin level determinations showed no significant differences between any of the tested fluids when pooled human serum was used for the standard curve. The mean gentamicin levels ± 2 standard deviation were plotted for all fluids and overlap occurred with the values for serum standard curves in all cases (Fig. 1).

(ii) Cefazolin. Significant differences in cefazolin determinations were seen in all fluids except for synovial fluid when compared with serum standards. The use of serum for the generation of the standard curve resulted in falsely elevated antibiotic levels in all cases, except synovial fluid (Fig. 2).

(iii) Fluid analysis. The results of the multi-channel analysis done for each fluid used in preparation of standard curves were plotted against percent false elevation of antibiotic level for cefazolin on graph and semilogarithmic graph paper. Only protein concentration displayed a relationship with percent false elevation of cefazolin level (Fig. 3). This was seen as an inverse, linear correlate with only cerebrospinal fluid falling below the expected level.

FIG. 1. Body fluid, saline, and saline plus serum antibiotic concentrations (bioassay) compared with serum reference standard ($= =$) for gentamicin.

DISCUSSION

In human serum, cefazolin is 86% protein bound (6). Cefazolin levels determined by bioassay by using serum standards were significantly falsely elevated in various body fluids; an inverse relationship between false elevations and protein concentrations was seen.

The fluid, comprised of 10% pooled human serum and 90% isotonic saline, was shown to have the same effect of falsely elevated results of cefazolin tests as did the low protein body fluids when compared to a serum standard.

No erroneous determinations were seen with gentamicin, which is not protein bound (3). The degree of antibiotic binding may thus affect results of antibiotic testing in solutions containing protein.

FIG. 2. Body fluid, saline, and serum plus saline antibiotic concentrations compared with serum reference standard ($= =$) for cefazolin.

FIG. 3. Ratio (in percent) of observed concentration of cefazolin for each fluid, divided by theoretical amount of antibiotic in each fluid, compared to protein (g per 100 ml) in each fluid.

Whenever possible, the same body fluid should be used for developing standard curves as is being tested for antibiotic concentration. If this is not possible, or if large numbers of determinations are required and the protein content of the unknown fluid is known, standard curves can be developed from appropriate mixtures of pooled human serum and isotonic saline. Because commercial pooled human serum often contains hepatitis-associated antigen, care must be exercised in its use for the generation of standard curves.

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