Disc-Agar Diffusion Microbiological Assay Procedure for Determining Serum and Urine Levels of Sulfacytine and Other Sulfonamides

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A reasonably precise, reproducible, and sensitive microbiological procedure for directly assaying sulfacytine and other sulfonamides as antibacterially active drugs has been developed by appropriately modifying the standard disc-agar diffusion technique. Blood and urine levels as low as 3 μg/ml may be determined through the use of a strain of Escherichia coli and a chemically defined agar medium devoid of sulfonamide antagonists. Results indicate that this assay method should be a useful adjunct to the Bratton-Marshall colorimetric procedure, by permitting the direct measurement of antibacterially active drug in clinical specimens.

Sulfacytine (1-ethyl-N-sulfanilylcytosine) is a highly soluble, "short acting" sulfonamide (7, 9). To study its distribution in normal volunteers, it was important to determine the proportion of active (free) sulfacytine present in blood and urine. Classically, this has been accomplished by the Bratton-Marshall (6) colorimetric procedure in which only the nonacetylated, presumably antibacterially active sulfonamide is measured as directly reacting diazotizable amine. However, this method is incapable of eliminating conjugates such as glucuronides and ethereal sulfates, which react as free sulfonamides, although they may be significantly less active antimicrobially than the nonmetabolized compound and should be determined separately (13, 16, 18). Consideration was given to the development of a microbiological assay which would only respond to antibacterially active forms of sulfonamide. The frequently used broth dilution method was not used since it provides only a rough estimate of the concentration of antibacterially active sulfonamide in the sample (3, 11, 12, 14, 16, 22). Although a general parallel relationship has been demonstrated between colorimetric and broth dilution data, individual values are frequently widely divergent. The turbidimetric growth-inhibition method (1) possesses quantitative potential, but its application would be impractical because specimens need to be sterile and the assay range is narrow. Therefore, the well-known disc diffusion antibiotic assay procedure (10) was investigated as a method for determining concentration of various sulfonamides in clinical specimens.

MATERIALS AND METHODS

The assay organism was E. coli Vogel, a human clinical isolate (8) which has been maintained in lyophilized form and by transfer on Trypticase soy agar slants. Cultures were grown in a chemically defined "synthetic" liquid medium (SLM) with the following composition: asparagine, 1.0 g; Na2HPO4·12H2O, 6.3 g; KH2PO4, 1.0 g; sodium citrate, 1.5 g; MgSO4·7H2O, 0.2 g; glycerine, 20 ml; and distilled water, 1 liter. The solution was adjusted to pH 6.6 to 6.8 and sterilized by autoclaving for 20 min at 15 psi at 121 C. All incubations were at 37 C for 16 to 20 hr.

Assay plates were prepared on a level table in disposable petri dishes (25 by 150 mm Falcon Plastics Div. of B-D Laboratories, Inc., Los Angeles, Calif.). Each plate contained a 42-ml base layer and an 8-ml seed layer. A "synthetic" agar medium (SAM), containing 1.5% agar in SLM at 47 to 50 C, was used for both layers. After adding each layer, the medium was allowed to harden with the petri-dish lids removed to avoid excess condensation. Base layers could be stored at 4 C up to 1 week and were warmed to 50 C at 1 to 2 hr before receiving the seed layers.

To prepare standardized inocula containing approximately 10^6 colony-forming units (CFU) per ml, cultures were adjusted with SLM to an optical density (OD) of 0.15 in a Coleman Jr. Spectrophotometer (model 6A) at a wave length of 600 nm. The agar was seeded by mixing one volume of a 100-fold dilution of SLM culture inoculum with nine volumes of SAM. Plates were refrigerated within 30 min after preparation and used the same day.
Aqueous stock solutions of 1,000 μg/ml were made with 1 ml of dimethylacetamide as the initial auto-
sterilizing solvent for each 50 ml. Subsequent stock
solutions (500 to 31.25 μg/ml) were made by serial
twofold dilution. Reference standard solutions (100 to
3.125 μg/ml) were prepared as tenfold dilutions of the
stock solutions into the appropriate vehicle. Pooled,
normal human serum was the diluent for standard
solutions and clinical samples in assays of human sera
or human plasma. For urine assays, 0.01 M (pH 7.2),
phosphate-buffered saline (PBS) was used.

Standard solutions or appropriately diluted clinical
specimens were pipetted as 0.08 ml to paper assay discs
(12.7 mm; no. 740-E; Schleicher and Schuell Co.,
Keene, N.H.) concurrent with their application to the
seeded agar. The reference standard solutions were
tested together on each of the four plates, the
maximum number removed from the refrigerator
at one time, as were each five or six appropriately
diluted clinical samples and the internal plate
control (12.5 μg/ml). Immediately after completion,
the plates were inverted and incubated.

Inhibition zone diameters were measured by caliper
to the nearest 0.1 mm when light, confluent back-
ground growth first appeared, usually after 16 to 20 hr
of incubation. Visualization was facilitated by using a
black background and oblique transmitted light in a
darkened room. The light background growth pre-
cluded the use of commercial zone readers. Zone
diameters on each plate containing clinical samples
were corrected by adding a factor representing the
difference between the internal plate control and the
mean zone diameter, produced by the same solution
on the standard curve plates. The concentration in
the standards were plotted on the logarithmic scale of
semilog graph paper against inhibition zone diameters
mathematically derived from observed data, by using
the least means square equation (2). The mean cor-
corrected inhibition zone diameters obtained with the
appropriately diluted clinical specimens were con-
verted to sulfonamide concentrations (μg/ml) by
reference to this semilog plot.

Influence of inoculum size and incubation time on
inhibition zones. Solutions of 10, 30, and 100 μg of
sulfacytine per ml in PBS were tested on seed layers
containing 300-fold (3 × 10^8 CFU/ml), 1,000-fold
(10^9 CFU/ml), and 3,000-fold (3 × 10^9 CFU/ml)
dilutions of the standardized inoculum. Inhibition
zones were observed and measured after 16, 18, 21, and
24 hr of incubation.

Comparison of within-plate and between-plate vari-
tion of inhibition zone diameters. A PBS solution of
sulfacytine (12.5 μg/ml) was tested at seven locations
on each of seven plates. The zone diameters were sub-
ject to analysis of variance (21) for between-plate
and within-plate variations.

Application to various sulfonamides. Sulfacytine,
sulfadiazine, sulfamethizole, sulfisoxazole, and sulfa-
methoxypyridazine were compared with respect to
regression coefficients (2) and the lowest useful con-
centration providing a linear response. To determine
whether the sulfacytine slope was significantly differ-
ent from the slopes produced by the other sulfon-
amides, the t test (2) was used.

Sulfacytine regression lines in various diluents.
Several concentrations of sulfacytine were compared
in PBS, undiluted human urine, 1 and 10% human
urine in PBS, undiluted human serum, undiluted
human plasma, and an equal mixture of undiluted
human serum and plasma. Slopes obtained at the
standard reference concentrations were compared by
the t test (2).

Degree of accuracy and precision. Unknown PBS or
urine solutions of sulfacytine were assayed in replicate
before their concentrations were revealed. The data
from each run were evaluated for deviations from the
mean assay value and actual content. Replicate assays
were also performed on urine samples from three sub-
jects who had received oral doses of sulfacytine. These
data were evaluated for 95% confidence limits of the
mean and per cent deviation of each assay value from
the mean (2).

RESULTS AND DISCUSSION

The synthetic media are suitable for agar
diffusion sulfonamide assays since they are free of
antagonists, which are contained in ordinary
culture media (15, 24), and yet support good
growth of the assay organism. The Vogel strain

![Graph showing Influence of incubation time and inoculum dilution on inhibition zone diameters produced by a 10-μg solution of sulfacytine per ml in phosphate-buffered saline. Seed layers contained 3 × 10^8 colony-forming units (CFU)/ml (●●●), 10^9 CFU/ml (○○○), and 3 × 10^9 CFU/ml (▲▲▲) derived from inoculum dilutions of 1:3,000, 1:1,000, and 1:100, respectively.](image)

**Fig. 1.** Influence of incubation time and inoculum dilution on inhibition zones produced by a 10-μg solution of sulfacytine per ml in phosphate-buffered saline. Seed layers contained 3 × 10^8 colony-forming units (CFU)/ml (●●●), 10^9 CFU/ml (○○○), and 3 × 10^9 CFU/ml (▲▲▲) derived from inoculum dilutions of 1:3,000, 1:1,000, and 1:100, respectively.
of *E. coli* was chosen because it was highly susceptible to sulfacytine (7).

Sharply defined, easily measured inhibition zones were obtained by carefully following the described assay procedure. Prolonged incubation times and excessively heavy inocula yielded inhibition zones with diffuse, ragged, and poorly defined perimeters, precluding accurate diameter measurements, and (Fig. 1) also markedly decreased their diameters, effects similar to those obtained by Bauer and Sherris (4). A 3,000-fold diluted inoculum (3 × 10⁶ CFU/ml) produced growth that was too light, rendering the inhibition zones extremely difficult to visualize.

The data summarized in Table 1 indicate that the between-plate variations (degrees of freedom, 6; sum of squares, 0.50237; mean square, 0.08372; and variance ratio, 0.24107) in inhibition zone diameters, produced by a solution of sulfacytine (12.5 μg/ml), were not statistically significant with respect to within-plate variations (degrees of freedom, 42; sum of squares, 14.58580; mean square, 0.34728; and variance ratio, not sig-

**TABLE 1. Variation of sulfacytine-produced zone diameters within and between plates**

| Operator | Plate no. | Inhibition zone diameters (mm) | Mean ± se |
|----------|-----------|--------------------------------|-----------|
|          |           | Highest value | Lowest value |           |
| A        | 1         | 29.5          | 28.2          | 28.8 ± 0.2 |
| A        | 2         | 29.2          | 28.5          | 28.8 ± 0.1 |
| A        | 3         | 28.2          | 29.2          | 28.8 ± 0.1 |
| B        | 4         | 28.0          | 30.0          | 28.9 ± 0.2 |
| B        | 5         | 27.5          | 30.0          | 29.0 ± 0.4 |
| C        | 6         | 27.8          | 29.5          | 28.8 ± 0.3 |
| C        | 7         | 28.2          | 29.8          | 29.1 ± 0.2 |

* a Produced by 12.5 μg of sulfacytine per ml in phosphate-buffered saline.
  b Standard error.

![Fig. 2. Sulfacytine least mean square dose-response line; points are the arithmetic mean of four inhibition zone diameters observed at each concentration.](image)

**TABLE 2. Typical standard dose-response regression equations obtained with various sulfonamides in phosphate-buffered saline**

| Sulfonamide       | Lowest useful concn (μg/ml) | Regression equation: Inhibition zone diameter (mm) |
|-------------------|-----------------------------|--------------------------------------------------|
| Sulfacytine       | 3.125                       | 15.7 + 10.8 Xa                                  |
| Sulfadiazine      | 1.5625                      | 15.4 + 12.7 X                                   |
| Sulfamethizole    | 6.25                        | 5.2 + 14.6 X                                    |
| Sulfamethoxpyridazine | 3.125                     | 13.1 + 13.0 X                                  |
| Sulfisoxazole     | 1.5625                      | 14.3 + 12.6 X                                  |

* a X = Log base 10 of sulfonamide concentration (μg/ml).

**TABLE 3. Summary of typical sulfacytine dose-response regression lines in various diluents**

| Test group | Sulfacytine diluent                          | Regression equations: Inhibition zone diameter (mm) |
|------------|---------------------------------------------|--------------------------------------------------|
| A          | PBSa                                        | 15.7 + 10.8 Xb                                  |
|            | 1% human urine in PBS                       | 15.9 + 10.8 X                                   |
|            | 10% human urine in PBS                      | 15.3 + 11.1 X                                  |
|            | Undiluted human urine                       | 16.1 + 10.6 X                                  |
| B          | PBS                                         | 16.5 + 13.9 X                                  |
|            | Undiluted human serum                       | 16.2 + 12.7 X                                  |
|            | Undiluted human plasma                      | 16.3 + 12.8 X                                  |
|            | Undiluted human serum + human plasma (equal parts) | 16.2 + 12.9 X                                  |

* a Phosphate-buffered saline.
  b X = log base of 10 for any concentration of sulfacytine between 3.125 and 100 μg/ml.
significant). (The total no. of degrees of freedom was 48, and the sum of the squares was 15.08817.)

A typical sulfacytine regression line is illustrated in Fig. 2. A linear response was obtained at concentrations as low as 3.0 μg/ml in PBS or undiluted human serum, although some inhibition was detected at slightly lower concentrations. The correlation coefficient of the line was almost

| TABLE 4. Microbiological assay of unknown samples containing sulfacytine, accuracy and reproducibility |
|-----------------------------------------|-----------|----------------|----------------|
| Unknowna sulfacytine sample assayed      | No. of assays | Sulfacytine        | Per cent deviation from |
|                                         |             | assay value (μg/ml) | Mean            | Actual value |
| 1,325 μg/ml in PBSb                      | 1          | 1,350            | 6.3             | 1.9          |
|                                         | 1          | 1,450            | 1.3             | 9.4          |
|                                         | 1          | 1,500            | 5.1             | 13.2         |
| Mean                                     | 1,433      |                 |                 | 8.2          |
| 165 μg/ml in PBS                         | 2          | 170              | 1.6             | 3.1          |
|                                         | 1          | 175              | 1.7             | 5.7          |
| Mean                                     | 172        |                 |                 | 4.2          |
| 2,550 μg/ml in undiluted urine           | 1          | 2,200            | 14.7            | 13.7         |
|                                         | 2          | 2,400            | 7.0             | 5.9          |
|                                         | 3          | 2,500            | 3.1             | 2.0          |
|                                         | 4          | 2,600            | 0.8             | 5.9          |
| Mean                                     | 2,580      |                 | 8.5             | 9.8          |
| 487.6 μg/ml in undiluted urine           | 1          | 400              | 17.5            | 18.0         |
|                                         | 2          | 460              | 3.1             | 5.7          |
|                                         | 7          | 480              | 1.0             | 1.6          |
|                                         | 1          | 500              | 3.1             | 2.5          |
|                                         | 3          | 520              | 7.2             | 6.6          |
| Mean                                     | 485        |                 | 11.3            | 10.7         |

a Actual sulfacytine concentrations were disclosed after the assays were completed.
b Phosphate-buffered saline.

| TABLE 5. Reproducibility studies of urine assay levels from patients receiving oral doses of sulfacytine |
|------------------------------------------|----------|----------------|----------------|
| Patient’s urine sample                   | Assay no. | Sulfacytine | Per cent deviation |
|                                         | (µg/ml)  | in urine     | from mean        |
| A                                       | 1        | 2,100        | 5.6              |
|                                         | 2        | 2,700        | 21.3             |
|                                         | 3        | 2,050        | 7.9              |
|                                         | 4        | 1,800        | 19.1             |
|                                         | 5        | 2,600        | 16.9             |
| Mean ± 95% confidence limits            |          | 2,225 ± 360  |                  |
| B                                       | 1        | 75           | 12.3             |
|                                         | 2        | 100          | 17.0             |
|                                         | 3        | 86           | 0.6              |
|                                         | 4        | 100          | 17.0             |
|                                         | 5        | 75           | 12.3             |
| Mean ± 95% confidence limits            |          | 85.5 ± 12.5  |                  |
| C                                       | 1        | 6,000        | 10.4             |
|                                         | 2        | 5,000        | 8.0              |
|                                         | 3        | 5,300        | 2.4              |
| Mean ± 95% confidence limits            |          | 5,433 ± 1,560|                |
always greater than 0.99, and analysis of variance revealed no significant curvilinear components (2).

That other sulfonamides may be assayed by this same procedure is exemplified by the data summarized in Table 2, although a separate standard curve must be constructed for each sulfonamide. Variations in lowest useful concentrations and regression coefficients may reflect differences in solubility, intrinsic antibacterial activities (7, 9), degrees of diffusion through the agar medium (4), or susceptibility to sulfonamide antagonists in the clinical specimens (20, 23). These considerations preclude the application of this technique to the assay of body fluids from individuals receiving multisulfonamide dosing.

Typical regression equations obtained with sulfacetin in various diluents are summarized in Table 3. At the sulfacetin concentrations employed in the assay standards, no significant differences between regression equations occurred in PBS, undiluted human urine, and their combinations. Also superimposable were the standard curves in undiluted human plasma, undiluted human serum, and a mixture of both.

With low concentrations of sulfacetin, inhibition zone diameters were markedly influenced by the diluent as illustrated in Fig. 3. In undiluted human serum, no inhibition zone was produced by 2 \( \mu g \) or less of sulfacetin per ml. This possibly resulted from protein binding (19) or the presence of sulfonamide antagonists (5, 15).

Low concentrations of sulfacetin produced larger inhibition zones when dissolved in undiluted urine than in PBS. Urine may have enhanced the diffusion or the activity of the sulfonamide, or both (17). This diluent-associated variation, however, would not be expected to influence the assay since much higher sulfacetin levels would be encountered at the recommended clinical dosages.

The results of replicate microbiological assays of "unknown" samples (Table 4) indicate that this procedure provides a reproducible and accurate measure of sulfacetin concentrations. In 36 replicate assays, deviations from the mean exceeded 10\% in only three instances and exceeded 5\% in 12 assays. Deviations from the actual value were greater than 10\% on only four occasions.

Results of repeated assays of clinically derived urine samples are summarized in Table 5. Deviations from the mean varied from 0.6 to 21.3\%, with the majority between 10 to 20% on either side of the mean. In only one instance was the deviation from the mean greater than 20\%.

These data indicate that the microbiological assay procedure described herein should be a useful adjunct to the Bratton-Marshall (6) colorimetric method to determine antibacterially active concentrations of sulfacetin and other sulfonamides in clinical specimens.

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