Serum Sulfonamide Concentration After Oral Administration: Comparison of Results of Chemical Assay with Two Bioassay Techniques

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Sulfonamide concentrations were studied in 210 serum samples from 10 volunteers after ingestion of sulfactine, sulfoisoxazole, and sulfadiazine. Results obtained by chemical assay were compared with bioassay values determined by two techniques: twofold broth dilution and agar diffusion. Although the correlation for individual samples was rather poor for the twofold broth-dilution method, the agar-diffusion bioassay correlated well with chemical determinations. The agar-diffusion assay tended to "underread" the chemical assay by an amount characteristic of each drug.

The relationship between in vitro antibacterial activity and chemically determined serum or plasma concentrations of sulfonamides has been variously reported to range from "little or no correlation" to "a good correlation" by different workers. Weinstein and Samet (8, 9) studied serial serum samples obtained from subjects after administration of sulfadiazine and triple sulfapyrimidines, and reported that a given titer of antimicrobial activity was associated with a wide range of sulfonamide levels determined by chemical assay. On the other hand, Madsen and co-workers (5, 6) noted a good correlation between chemically determined serum sulfonamide concentrations and antibacterial activity when they examined sulfadiazine and a number of long-acting sulfonamides.

The purpose of the present study was to investigate this relationship by use of methods of bioassay different from those used in previous studies. In addition, it was decided to study sulfactine, an experimental highly soluble sulfonamide. This agent is rapidly excreted in the urine in apparently unmetabolized form, providing high urinary antibacterial levels (2, 3).

MATERIALS AND METHODS

The drugs examined were sulfactine, sulfoisoxazole, and sulfadiazine. The test organism employed for bioassay was a strain of Escherichia coli highly susceptible to each of the drugs.

Ten normal male volunteers, 20 to 27 years of age, were the subjects of this study. Each was given a single dose of 2 g of sulfactine orally after a fast of 8 hr; no food was allowed until 4 hr after the sulfonamide was administered. Seven and 14 days later, 2 g each of sulfoisoxazole and sulfadiazine, respectively, were administered in the same way. Blood was drawn just prior to, and 1, 2, 4, 8, 12, and 24 hr after drug ingestion. The serum was separated, and stored at −20 C until assayed for both sulfonamide content and antibacterial activity.

The chemical concentration of sulfonamide in serum was determined by the method of Bratton and Marshall (1). Pure powder of the specific drug, obtained from the manufacturers, was dissolved in distilled water, and used as a reference standard. (Sulfactine was supplied by Parke Davis & Co.; sulfoisoxazole, by Hoffman-La Roche, Inc.; and sulfadiazine, by Smith Kline & French Laboratories.) Although total sulfonamide is also determined by this method, only the concentrations of the free form (biologically active) are reported in the present paper.

Two different methods were used to determine the antibacterial activity of serum: (i) serial twofold dilution in broth, and (ii) paper-disc agar diffusion. All studies were carried out in a synthetic liquid (SLM) medium made with the following formula: asparagine, 1 g; sodium phosphate, 6.3 g; potassium phosphate, monobasic, 1.0 g; sodium citrate, 1.5 g; magnesium sulfate, 0.2 g; glycerine, 20 ml; and water to a volume of 1 liter; adjusted to pH 6.6 to 6.8. The same medium containing 1.5% agar was used in the diffusion studies.

In performing the broth-dilution assays, serial
twofold dilutions of each serum sample were made in SLM broth. After inoculation with a $10^{-3}$ dilution of an overnight culture of the test organism, the cultures were incubated at 37°C for 24 hr and examined for turbidity. The maximal dilution of serum required to inhibit bacterial growth was considered to be the antibacterial titer. For each series of assays, the minimal inhibitory concentration (MIC) for the test organism was determined, by use of known concentrations of the appropriate sulfonamide diluted in SLM medium. The MIC for sulfacytine and sulfadiazine ranged from 0.015 to 0.030 μg/ml and for sulfisoxazole, from 0.008 to 0.030 μg/ml. The bioassay concentration was calculated by multiplying the reciprocal of the antibacterial titer by the MIC of the test organism. The following method
was used for the diffusion studies. Seeded-agar plates were prepared by adding 1.25 ml of an 18-hr growth of *E. coli* in SLM medium to 1 liter of SLM agar at 50 C. Samples (8 ml) were poured into sterile plastic petri dishes (100 by 17 mm) and allowed to harden on a level surface for 30 min. The plates were then refrigerated (4 C) and used within 3 or 4 days.

Microassay filter-paper discs (E-740, Schleicher & Schuell Co., Keene, N.H.), 6.45 mm in diameter, were autoclaved and dried. Discs were touched to the sample to be assayed, so that absorption occurred by capillary action. Care was taken to avoid over-soaking, and excess liquid was removed by touching the disc to a sterile glass surface. The discs were laid on the surface of the seeded agar. After overnight incubation at 37 C, the diameters of the zones of inhibition were measured and recorded in milli-
meters. Standards for each drug were prepared by diluting known concentrations of the sulfonamide in pooled human serum free from demonstrable antibacterial activity. These were frozen at -20 C when not in use. Values for the reference standards were plotted on semilogarithmic paper. A new reference curve was determined simultaneously with seeded-agar plates from the same batch for each individual experiment.

RESULTS

Correlation of chemically determined concentrations of sulfonamide and antibacterial activity by the broth-dilution methods. The upper graphs in Fig. 1–3 demonstrate, for each of the sulfonamides studied, the relationship between the sulfonamide content and the bioassay concentra-
tion determined by the twofold broth-dilution method. Each of seven serum samples from 10 volunteers is represented by a point on the graph. Because twofold dilutions were used, the values are grouped at intervals along the vertical axis.

When the results were subjected to regression analysis, a positive correlation was demonstrated for each of the drugs, with correlation coefficients of 0.727, 0.823, and 0.704 for sulfacytine, sulfoisoxazole, and sulfadiazine, respectively. More striking, however, was the finding that a narrow range of readings as determined by the one method could be associated with a wide range of values as determined by the other. For example, a bioassay titer of 1:512 was associated with a chemical content of 1.1 to 10.8 mg of sulfacytine per 100 ml. Similarly, a chemical content of 2.1 to 4 mg/100 ml was associated with antibacterial titers ranging from 1:128 to 1:1,024, and bioassay titers of 1.9 to 15.4 mg of sulfacytine per 100 ml. Marked divergences of the same kind were noted with sulfoisoxazole and sulfadiazine. Thus, although in many cases the correspondence was fairly close, the wide discrepancies that sometimes occurred with each drug made correlations for individual samples unreliable. When results for specimens from a single individual were studied, the correspondence between chemical and broth-dilution assay values was moderately better, but still not perfect.

**Correlation of chemically determined levels of sulfonamide and antibacterial activity by the agar-diffusion method.** The lower graphs in Fig. 1–3 demonstrate the correlations between the chemically determined and the bioassay concentration as derived by the agar-diffusion method. Again, a positive correlation is demonstrated, the coefficients being 0.924, 0.949, and 0.762 for sulfacytine, sulfoisoxazole, and sulfadiazine, respectively. However, the agar-diffusion method tended to yield consistently lower values than the chemical method. In the case of sulfacytine, the bioassay underran the chemical level by 10 to 15%; sulfoisoxazole and sulfadiazine underran by about 40%.

When individual values over a narrow range, obtained by the one method, were compared to the corresponding values obtained by the other, the degree of scatter was found to be much less than occurred with the broth-dilution assay. When the underreading phenomenon is taken into account, the chemical determination and agar-diffusion bioassay can be seen to correlate reasonably well for individual specimens, in contrast to the findings with the broth-dilution assay. The underreading phenomenon occurred in similar degree in each subject studied. It was noted in specimens taken shortly after ingestion of drug as well as in later specimens.

Table 1 summarizes the statistical analyses of the relationships for all three drugs for the group as a whole. For both sulfacytine and sulfoisoxazole, the agar-diffusion method resulted in a highly significant improvement in the correlation between the bioassay and chemical concentrations over the correlation obtained with the broth-dilution method. In the case of sulfadiazine, although the correlation coefficient with the agar-diffusion method was better (i.e., closer to unity) than with the broth-dilution method, the difference was not statistically significant.

**DISCUSSION**

The present study confirms results previously reported from this laboratory (8, 9) indicating that, for an individual serum specimen, the chemically assayed sulfonamide content and that determined by a twofold broth-dilution method may be widely divergent. This was found with all three sulfonamides studied and occurred despite a positive correlation for the group as a whole with each drug, with an \( r \) value as high as 0.825 for sulfoisoxazole.

In contrast to this, individual values obtained by the chemical method and the agar-diffusion assay demonstrated much less divergence. When the consistently present tendency to underread by the agar-diffusion method is taken into account (most marked with sulfoisoxazole and sulfadiazine), the values obtained by the two methods are quite close. In addition, when the over-all correlation coefficients are taken into account, the results for two of the drugs (sulfacytine and sulfoisoxazole) are considerably better than the value for the best correlation by the broth-dilution method.

The reason for the improved correlation by the agar-diffusion technique is not clear. It may relate to the fact that the broth-dilution method was done in twofold decrements, so...

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**Table 1. Statistical analyses of the relationships for all three drugs for the group as a whole**

| Drug         | Correlation coefficients (r)^
|--------------|----------------------------------|
|              | Broth-dilution method | Agar-diffusion method |
| Sulfacytine  | 0.727 (0.651–0.823)     | 0.924 (0.880–0.952)     |
| Sulfisoxazole| 0.825 (0.730–0.889)     | 0.949 (0.918–0.968)     |
| Sulfadiazine | 0.704 (0.605–0.832)     | 0.762 (0.644–0.847)     |

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The 95% confidence limits are given in parentheses.
that each tube differed by 100% from the adjacent one, whereas the agar-diffusion method permitted recognition of intermediate readings.

The tendency of the agar-diffusion method to "underread" the value obtained by the chemical assay is not explained by our data. The effect was small with sulfaclorine (10 to 15%), but significant (40%) with sulfadiazine and sulfisoxazole. It was not due to intrinsic differences between individual sera. The explanation may lie in the fact that the chemically measured "free" sulfonamide level, while excluding acetylated conjugates, does not eliminate glucuronide and ethereal sulfates which may lack significant antimicrobial activity (7). The degree to which such conjugates are formed presumably depends on the particular sulfonamide, and this may explain the differences in the discrepancy among the three drugs. That the phenomenon did not appear in the twofold broth-dilution method may relate to the relative crudeness of that assay, as discussed above.

Since the present project was undertaken, a report has been published (4) describing an agar-diffusion bioassay method for determining serum and urine concentrations of several sulfonamides. The technique was very similar to that described above. The authors did not, however, compare the method with the broth-dilution assay, nor were studies done in vivo.

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