Quantitative Spectrophotometric Papergram Assays II

Sparsomycin, A New Antitumor Antibiotic

By T. F. BRODASKY

The paper chromatographic characteristics of a new cytotoxic agent, sparsomycin, on a variety of solvent systems are reported. The chromatographic pattern of this antibiotic on Eagle's KB human epidermoid carcinoma cells in agar was found to correlate with the pattern on *Bacillus subtilis* UC-564. A quantitative papergram assay was developed on agar seeded with *B. subtilis* and compared statistically with a quantitative papergram assay based on the ultraviolet absorption of sparsomycin. The equivalence of the assay methods was determined by testing the equality of the means of the population of assay values using the pairing technique. The effect of the edges of the papergram on the chromatographic zone and its influence on the ultraviolet assay curve are described. There is a favorable agreement between the assays; however, the ultraviolet method reduces the assay time by 50% in comparison to the biological method.

A NEW cytotoxic agent, sparsomycin,[1] has been isolated from *Streptomyces sparsogenes* culture. This paper describes the paper chromatographic characteristics of sparsomycin and the development of a biological and physical quantitative papergram assay. Fermentation, isolation, and characterization studies are described in other papers (1, 2).

Since sparsomycin is coproduced with three other biologically active components, a disk plate assay is not feasible except as a measure of total bioactivity. Paper chromatography in suitable solvent systems yields resolved zones and allows quantitative evaluation of doses of individual components on the papergram. A statistical comparison between the two assays was made to determine the most rapid and reliable means of estimating yields in culture media and purity of preparative samples.

EXPERIMENTAL

Whatman No. 1 paper strips, 15 × 44 cm., containing 10 mcg. of sparsomycin per strip were developed descending for 16 hours (5 hours, in the case of V and VI), using the solvent systems listed in Table I. After a one-hour drying period, the strips were plated on 19 × 50 cm. trays containing 0.2 mg. of Eagle's KB cell protein per ml. of modified Miyamura agar (3). The trays were incubated for 16 hours at 37° with the strips remaining in contact with the agar. Upon removal of the strips, the agar was sprayed with a 0.4% solution of 2,6-dichlorophenol-indophenol in methanol-saline (1:20 by volume). One hour was allowed for dye reduction, during which zones of activity appeared blue against a colorless or very light blue background.

Received July 27, 1962, from the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

Accepted for publication September 28, 1962.

This work was supported by contract No. SA-43-ph-1933, Cancer Chemotherapy National Service Center, National Cancer Institutes, National Institutes of Health, Bethesda, Md.

[1] Sparsomycin was formerly called sparsogenin.

The biological assay was established on agar seeded with *Bacillus subtilis* UC-564. The agar was inoculated with 0.3 ml. per liter of a suspension containing 1.3 × 10^{10} spores/ml. The $^1/_2$-inch Whatman No. 1 paper strips containing 60, 40, 20, and 10 mcg. of sparsomycin per strip (used in both assays) were developed descending for 16 hours in solvent system IV. This system was found to yield the greatest resolution of the various components in the sparsomycin fermentation broths. After one-hour drying, the strips were plated on *B. subtilis* seeded trays and incubated for 16 hours at 30°. Three strips containing assay samples and two strips containing standard (purity > 95%) were plated on each tray. On removal of the strips,

TABLE I.—PAPERGRAM SOLVENT SYSTEMS

System	Composition
I	1-butanol:water::84:16 (v/v)
II	1-butanol:water::84:16 (v/v) plus 0.25 % *p*-toluenesulfonic acid (w/v)
III	1-butanol:acetic acid:water::2:1:1 (v/v/v)
IV	1-butanol:water::84:16 (v/v) plus 0.2% (v) piperidine
V	1-butanol:water::4:96 (v/v)
VI	1-butanol:water::4:96 plus 0.25% *p*-toluenesulfonic acid (w/v)

the diameter of the zones in the R_f 0.1 region were measured. These values were plotted against the log dose per strip to obtain a standard assay curve.

An ultraviolet papergram assay was established on $^1/_2$-inch Whatman No. 1 paper strips using a strip scanner (4) in conjunction with a Cary spectrophotometer. The strips were developed in system IV as before. After drying, the strips were scanned from the origin at 302 mμ with a blank of Whatman No. 1 paper in the reference beam of the spectrophotometer. A set of strips containing standard was run for each group of three unknown samples. Only one peak in the pertinent R_f region appeared and the area under this peak was calculated by the peak height times the half-band width method. The areas were plotted against the log dose per strip to obtain a standard assay curve.

RESULTS AND DISCUSSION

Figure 1 shows the typical six solvent system pattern of sparsomycin against Eagle's KB epidermoid carcinoma cells in agar. A similar pattern was obtained against *B. subtilis* and *Proteus vulgaris*.

Standard curves for the biological and ultraviolet assays appear in Figs. 2 and 3. The points represent an average of 11 determinations and the indices of precision (S/b) of the curves for the biological and ultraviolet methods are 0.047 and 0.072, respectively. The 95% confidence limits for each point are indicated. The 10 mcg. point was not included in the biological curve because of the poor sensitivity of *B. subtilis* to sparsomycin at this level. There was considerably more variation in the higher dose responses in the ultraviolet assay curve than in the biological assay curve and this can be accounted for by irregularities in the chromatographic zone. At high doses on 1/2-inch strips, there is a tendency for some materials to give broad zones exhibiting an effect of the edge of the paper strip. This effect is manifested as a variation in the density of the material across the width of the strip. When the strips are plated on agar, diffusion tends to mask the true density gradient in the zone. When a spectrophotometric determination is made, only the central portion of the strip is scanned because of geometric considerations. Under these conditions, the variation in density of a zone across the width of a strip is manifested as a variation in the area of the response peak. Figure 4 shows an ultraviolet curve indicating the density gradient across a 1/2-inch zone of sparsomycin perpendicular to the direction of development.

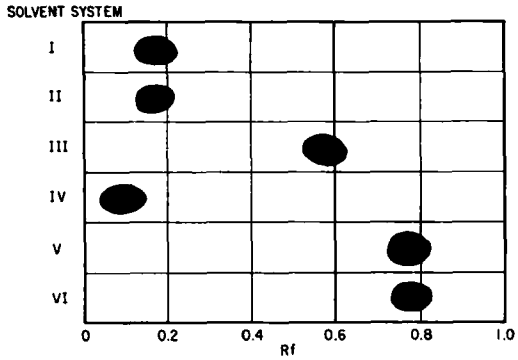

Fig. 1.—Six solvent system patterns of sparsomycin on KB cells.

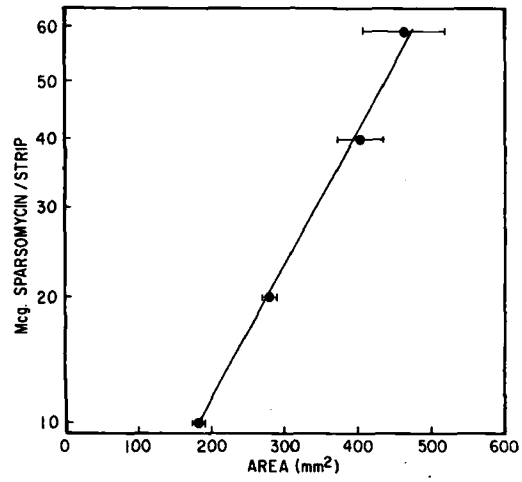

Fig. 2.—Standard *B. subtilis* assay curve for sparsomycin.

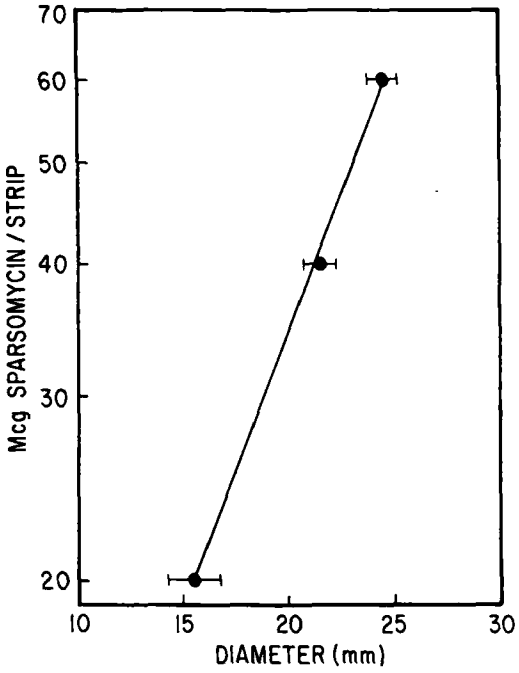

Fig. 3.—Standard ultraviolet assay curve for sparsomycin at 302 mμ.

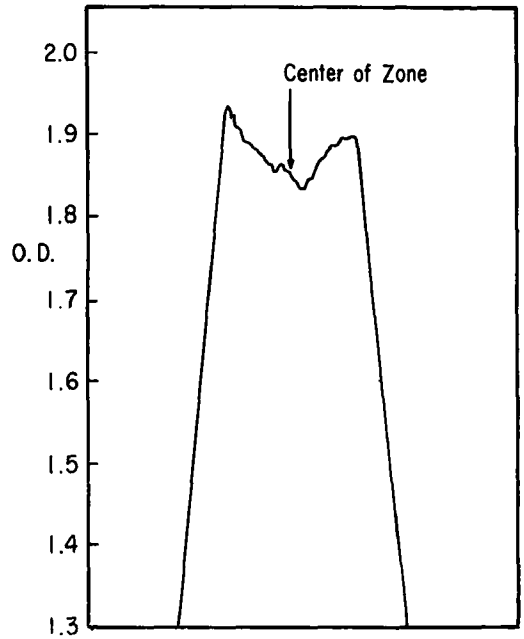

Fig. 4.—Density gradient of sparsomycin zone perpendicular to solvent flow.

Table II summarizes the variation between assay methods. The fact that the mean per cent variation is positive (bioassay with respect to ultraviolet assay) indicates that the ultraviolet assay did not include extraneous material during the recording of the sparsomycin peaks. Large variations in some assay results cannot be explained at this time.

TABLE II.—VARIATION IN UV AND BIOASSAY OF SPARSOMYCIN

Mcg. Sparsomycin/mg. Sample		Variation, %
a UV	*b* Bio	100 (*b* − *a*)/*a*
743	875	+17.8
670	974	+45.3
425	676	+59.7
423	392	− 7.3
676	957	+41.6
1025	1147	+11.6
453	493	+ 8.8
1115	973	−12.7
843	892	+ 5.8
867	910	+ 5.0
785	749	− 4.6
940	934	− 0.6
	Mean	+14.2
	S. D.	22.9

Table III was prepared from data recorded during the evaluation of a crystalline preparation of sparsomycin. The assay results were obtained over a 3-day period using a set of standards for each group of three samples. The 95% confidence limits, determined using a *t* distribution [$\bar{x} + (t_{\alpha/2}\ S)/\sqrt{N}$; $\bar{x} + (t_{1-\alpha/2}\ S)/\sqrt{N}$] (5), indicate considerably greater precision for the ultraviolet assay procedure. The values obtained from ultraviolet assays were consistent with values expected on the basis of ultraviolet and melting point data for sparsomycin. The accuracy of the UV assay was not determined directly. In a previous paper (6), the accuracy of the UV assay for one of the components of a sparsomycin culture in the presence of sparsomycin was found to be −6.3 ± 5.3% at 95% confidence. It is assumed that the sparsomycin assay will be as accurate. Testing for the

TABLE III.—PRECISION OF ASSAY METHODS

Mcg. Sparsomycin/mg. Sample		
UV	*da*	*B. subtilis*
863	− 70	933
950	− 38	988
865	− 60	925
938	+ 38	900
950	+100	850
925	− 42	967
933	− 55	988
900	+ 25	875
890	+ 90	800
900	+ 67	833
Mean 911	5.5	906
S. D. 33	21	66
95% Confidence Limits ± 27 γ/mg.		± 53 γ/mg.

[a] *d* = Difference of paired values of assays.

equality of the mean of the population of values for both assays by the pairing technique indicated that the procedures were equivalent. This is done by determining the value of $t = [\bar{d} - (\mu_1 - \mu_2)]/(S_d/\sqrt{N})$ (7) assuming $\mu_1 - \mu_2$, and comparing it with values obtained from a table giving percentiles for a *t* distribution. The value of *t* was 0.73 and one rejects the hypothesis that μ_1 is equal to μ_2 only if *t* is greater than 2.26 or less than −2.26 at a 5% level of significance.

The data lead to the conclusion that the UV assay compares favorably with the biological method. The UV assay is more rapid than the biological method and eliminates the need for handling biological systems routinely, although it is important to periodically check the ultraviolet method biologically to ascertain that the original bioactivity is being followed.

SUMMARY

Paper chromatographic characteristics of a new cytotoxic agent, sparsomycin, against KB epidermoid carcinoma cells in agar and *B. subtilis* UC-564 are reported.

An ultraviolet assay has been developed and compared statistically with a biological assay. The methods are statistically equivalent with respect to the mean values, but the ultraviolet method is preferred because of greater precision and a reduction in assay time.

GLOSSARY OF TERMS

μ_1–Mean of the population values for the ultraviolet assay.

μ_2–Mean of the population values for the biological assay.

$\bar{d}$–Mean of the differences between the paired values (assays).

S_d–Standard deviation of the differences between the paired values (assays).

α–Probability of rejecting true hypotheses; level of significance.

N–The number of samples (assays).

$t_{\alpha/2}$ − $t_{1-\alpha/2}$–The limits for the critical region, of a *t* distribution, as determined by α.

b–Slope of the standard curve.

REFERENCES

(1) Camiener, G. W., *et al.*, "Sparsomycin, A New Antibiotic I. Discovery and Biological Properties," to be published.

(2) Argoudelis, A. D., and Herr, R. R., "Sparsomycin, A New Antibiotic II. Isolation and Characterization," to be published.

(3) Grady, J. E., Lummis, W. L., and Smith, C. G., *Proc. Soc. Expil. Biol. Med.*, **103**, 727(1960).

(4) Parke, T. V., and Davis, W. W., *Anal. Chem.*, **24**, 2019(1952).

(5) Dixon, W. J., and Massey, F. J., Jr., "Introduction to Statistical Analysis," 2nd ed., McGraw-Hill Book Co., Inc., New York, N. Y., p. 127.

(6) Brodasky, T. F., and Lummis, W. L., THIS JOURNAL, **52**, 230(1963).

(7) Dixon, W. J., and Massey, Jr., F. J., "Introduction to Statistical Analysis," 2nd ed., McGraw-Hill Book Co., Inc., New York, N. Y., p. 124 (1957).