Quantitative Spectrophotometric Papergram Assays I

Tubercidin

By T. F. BRODASKY and W. L. LUMMIS

The paper chromatographic characteristics of the cytotoxic agent tubercidin in a number of solvents is reported. The technique for the production of bioautographs on Eagle's KB carcinoma cells is described. A study of the quantitation of tubercidin on KB cells and on papergrams by ultraviolet analysis was undertaken. Using the same chromatograms, ultraviolet analysis followed by bioautographic assay allowed the compilation of data which were statistically analyzed by the pairing technique. This indicated that the two methods were equivalent; however, the ultraviolet assay is the method of choice inasmuch as it requires only half the time required for the biological method.

BECAUSE of the inherent nature of pharmaceutical compounds, laboratories rely upon biological methods to detect, identify, and assay new drug candidates. This often requires handling pathogenic materials or systems requiring lengthy procedures for quantitative evaluation. It would be advantageous, once biological activity has been established, to use a more direct method, one not based on a biological process but which compared favorably with the biological procedure in regard to quantitative features. In this regard, a series of papers (1, 2) has been written concerning the statistical comparison of biological and spectrophotometric assay methods. The principal aim of the work is to establish the usefulness of existing nonbiological techniques in quantitating biological materials and to point out, in a limited way, the nature of these techniques with regard to accuracy, precision, and time comsumption. Three compounds, each presented in a separate paper, have been chosen because of their dissimilar biological nature and response to light absorption techniques.

Considerable work has been done on quantitating papergrams by ultraviolet spectroscopy (3) and a number of unique devices for scanning papergrams has been developed (4). This paper describes the use of spectrophotometry to follow a biologically active compound. Since paper chromatography is a basic tool in the study of pharmaceuticals, the quantitative absorption of ultraviolet radiation by tubercidin (5) on chromatographic paper was studied. Tubercidin was chosen because it represents a class of compounds principally active on carcinoma tissue culture. The use of this system entails some difficulty, and the ability to detect tubercidin by a physical method would to some extent alleviate this problem. The technique of producing bioautograms of this antibiotic with human carcinoma cells in tissue culture and subsequent development of a quantitative papergram assay are also described. An ultraviolet quantitative papergram assay was developed using the 272 mμ absorption peak of tubercidin.

A statistical comparison of the two assays was made to determine if the absorption and scattering errors inherent in the spectrophotometric papergram assay would limit the usefulness of this method.

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.

EXPERIMENTAL

Bioautograms and Quantitative Papergram Assay.—Whatman No. 1 sheets, 15 × 44 cm., were spotted with 100-μl. samples of fermentation liquor containing tubercidin. The papergrams were developed 16 hours (5 hours, for V and VI) descending in the six solvent systems listed in Table I in a sealed chromatographic chamber. After development, the strips were air-dried (System III strips were neutralized in an ammonia atmosphere for 10 min.), then plated on 19 × 50 cm. trays containing 125 ml. of modified Miyamura agar seeded with 0.2 mg. Eagle's KB epidermoid carcinoma cell protein per ml. (6). The trays were incubated at 37° for 16 hours with the strips in contact with the agar. The zones of activities on the trays were detected by spraying the agar with an 0.4% solution of 2,5-dichlorophenol-

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)

indophenol in methanol-saline (1:20 by volume). Within 1 hour, during which time the unaffected cells reduced the blue dye, the zones of activity appeared dark blue against a colorless or light blue background. None of the solvents resulted in inhibition of KB cells as shown by plating strips washed in each of the mobile phases.

The KB cell assay was developed using essentially the procedure described above. The crystalline standard (purity >95%) was spotted at 10, 20, 40, and 60 mcg. per 0.5 in. strip of Whatman No. 1 paper and developed in solvent system IV. A set of standard strips were developed for each assay tray. The maximum width of the zones of activity were plotted against the log of the dose per strip to construct a standard assay curve.

Ultraviolet Quantitative Papergram Assay.— Using measured volumes of a 10 mcg./ml. dimethylformamide solution, a 0.5 in. strip of Whatman No. 1 paper was spotted with 50 mcg. of tubercidin (purity >95%) and developed descending in system IV for 16 hours. After drying, the strip was placed on an ultraviolet scanning box and the center of the tubercidin zone marked. This zone was then scanned on a Cary spectrophotometer from 365 to 210 mμ with a sheet of Whatman No. 1 paper in the reference beam. Two maxima were noted, 238 and 272 mμ.

Strips spotted with 10, 20, 40, and 60 mcg. per strip of tubercidin were developed descending in system IV for 16 hours. After scanning the strips at 272 mμ using a strip scanner, the areas under the response peaks were calculated by the height times the half band width method and plotted against log dose per strip to construct a standard curve.

RESULTS AND DISCUSSION

The chromatographic pattern for tubercidin in six solvent systems on Eagle's KB cells is shown in Fig. 1. This pattern correlates with the patterns on *Bacillus subtilis* and *Proteus vulgaris*. The activity on the latter organisms was considerably weaker than on KB tissue culture and for this reason the bioassay was developed on KB cells. The 272 mμ peak of tubercidin was chosen for the ultraviolet papergram assay for several reasons. The 272 mμ peak was more intense than the 238 mμ peak, $a_{272} = 33$, $a_{238} = 8$. The 238 mμ peak failed to give a linear relationship between log dose per strip and either optical density or area of response peaks.

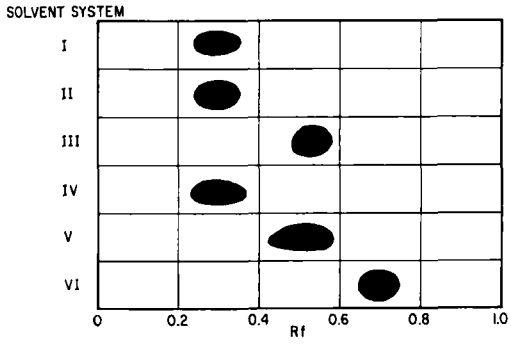

Fig. 1.—KB tissue culture papergram pattern of crystalline tubercidin.

The 238 mμ peak also showed an intensity variation depending upon the pH of the solvent system used in development. The 272 mμ peak did not display any of these variations.

The standard assay curves for the biological and ultraviolet method appear in Figs. 2 and 3, respectively. The points represent the average of 7 determinations and the indices of precision (S/b) of the curves for the biological and ultraviolet method are 0.22 and 0.06. The 95% confidence limits about the points are indicated. Data have been obtained for a limited number of runs indicating the accuracy of the assay methods. Tubercidin was chromatographed in system IV at known doses in the presence of compounds which are coproduced with this antibiotic. Strips were first analyzed by ultraviolet spectroscopy and subsequently assayed by the tissue culture process. The errors were -6.4 ± 5.3 and $25 \pm 34\%$, respectively. The large errors incurred in the tissue culture procedure can be traced to variable sensitivity of the assay organism

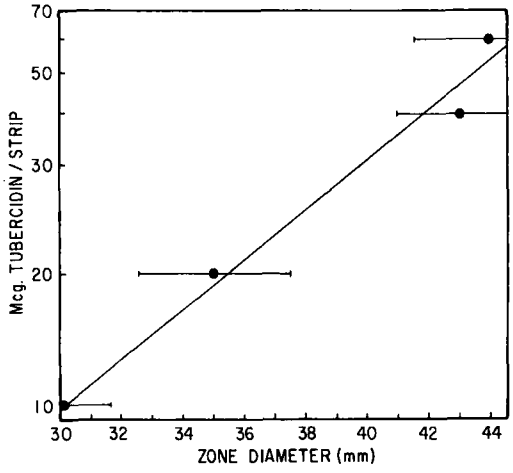

Fig. 2.—Tissue culture assay standard curve for tubercidin.

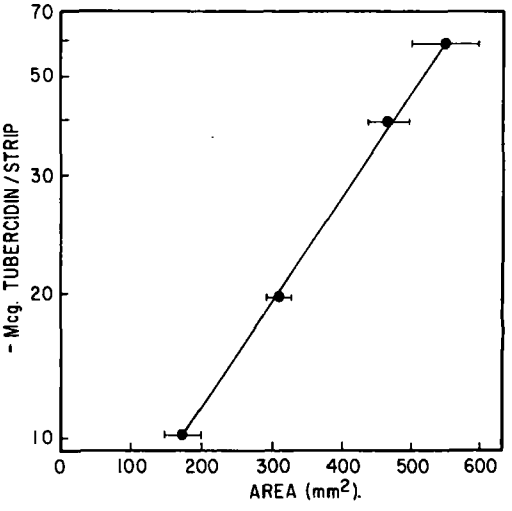

Fig. 3.—Ultraviolet assay standard curve for tubercidin at 272 mμ.

in this early stage of development. A great deal of care must be taken in preparing the trays since such factors as agar thickness can result in a large day-to-day variation in response.

The precision of the assay procedures is given in Table II. This data was accumulated from assays of

TABLE II.—PRECISION OF ASSAY METHODS

	Mcg./mg. of Tubercidin		
	Ultraviolet	d[a]	Biological
	930	−100	1030
	1050	− 50	1100
	960	160	800
	900	120	1020
	1050	30	1020
	1000	− 20	1020
	900	50	850
	1200	180	1020
Mean	999	16	983
S	101	109	102
95% Confidence limits	±85 mcg./mg.		±86 mcg./mg.

[a] d = differences between paired assay values.

crystalline tubercidin (purity >95%) performed first by ultraviolet analysis and then, using the same strips, by the tissue culture method. This was done to allow testing of the equality of the means of the population samples by the pairing technique (7). The difference between paired values of each set of assay results represents a random sample of a population of unknown variance. The population is assumed to have a normal distribution. The pairing procedure allows testing of the mean of the differences for zero and therefore whether the procedures are equivalent. The mean is tested using the t distribution statistic, $t = [\bar{d} - (\mu_1 - \mu_2)]/(Sd/\sqrt{N})$. Assuming that $\mu_1 = \mu_2$, the value of t equals 0.41 and one rejects the hypothesis that $\mu_1 = \mu_2$ only if t is greater than +2.36 or less than −2.36 at a 5% level of significance. The 95% confidence limits shown in Table II were obtained using the statistics for a t distribution $(\bar{x} + (t_{\alpha/2}S)/\sqrt{N}; \bar{x} + (t_{1-\alpha/2}S/\sqrt{N})$ (8). The data indicate a favorable agreement between the two methods. Since the incubation of biological trays is eliminated in the spectrophotometric method, the time necessary for assays is reduced by one-half.

The use of Whatman No. 1 paper in the reference beam of the spectrophotometer during the recording of the response curves does not eliminate the errors due to scattering and absorption by the paper but the inherent advantages of the method offset in part the lack of ultimate quantitation. The spectrophotometric method has been successfully used to estimate the amount of tubercidin in fermentation cultures down to 38 mcg./ml. of culture. In the assay of crystalline preparations the ultraviolet method is capable of detecting biologically inactive materials. This eliminates the inherent error in the biological assay of basing assays on materials which appear pure by biological standards but may actually contain biologically inactive material. For this reason, preparative samples can be selected by the ultraviolet method for standards which are of the highest purity.

SUMMARY

Tubercidin has been chromatographed in a number of solvents to obtain a pattern of Eagle's carcinoma cells. The chromatographic pattern was found to correlate with the *B. subtilis* and *P. vulgaris* patterns although the latter were considerably weaker.

Quantitative papergram assays were developed on carcinoma tissue culture and by using spectrophotometric techniques on papergrams. A statistical analysis of both methods indicates that the assays are essentially equal. However, the spectrophotometric method is more accurate and reduces the assay time by one half.

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).
S—Standard deviation of the samples (assays).
$\bar{x}$—The mean of the samples (assay).
$t_{\alpha/2}$; $t_{1-\alpha/2}$—Limits of the critical region for a t distribution as determined by α.
b—Slope of standard curves.

REFERENCES

(1) Brodasky, T. F., THIS JOURNAL, **52**, 233(1963).
(2) Finland, M., and Savage, G. M., "Antimicrobial Agents and Chemotherapy," Brown-Brumfield, Inc., 1962, p. 198.
(3) Brown, J. A., and Marsh, M. M., *Anal. Chem.*, **25**, 1952(1952).
(4) Parke, T. V., and Davis, W. W., *ibid.*, **24**, 2019 (1952).
(5) Suzuki, S., and Marumo, S., *J. Antibiotics Tokyo, Ser. A*, XIII-5, 360(1960).
(6) Grady, J. E., Lummis, W. L., and Smith, C. G., *Proc. Soc. Expil. Biol. Med.*, **103**, 727(1960).
(7) Dixon, W. J., and Massey, F. J., Jr., "Introduction to Statistical Analysis," 2nd ed., McGraw-Hill Book Co., Inc., New York, N.Y., 1957, p. 124.
(8) *Ibid.*, p. 128.