Stability of Antibacterial Preservatives in Parenteral Solutions II

Microbiological Turbidimetric Assay Method for Preservative Content

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An accurate and rapid microbiological method for assaying chemical preservatives of nonantibiotic nature has been developed which employs a time-tested, proven procedure. Preservative concentration is measured by a turbidimetric procedure using *Escherichia coli* as the test organism. The method has been applied to chlorobutanol, phenol, and -chloro-beta-phenylethyl alcohol and compares favorably with chemical analytical methods.

IN CARRYING out stability studies on chemical preservatives in pharmaceutical solutions, it is essential that the methods employed accurately establish the preservative content. Analytical methods invariably have involved chemical procedures of varying degrees of complexity. Although regarded as possessing reliability, such assays may require chemical manipulation and equipment which are not always readily available. From a theoretical standpoint it is conceivable that while a chemical analysis may indicate appreciable loss of preservative content because of degradation, the breakdown products *per se* may possess intrinsic antibacterial activity.

In view of the generally accepted concept that preservative as well as antibiotic content is best expressed on the basis of antimicrobial activity, an attempt was made to develop an accurate and rapid turbidimetric assay similar to those generally employed for antibiotics. An extensive search of the literature has revealed no reports on the existence of a bioassay for chemical preservatives of nonantibiotic nature.

Initial attempts on our part (1) to arrive at a bioassay method involved the addition to the sample being assayed of a standardized number of *E. coli* organisms. At intervals, 1-ml. portions were removed, which were immediately plated for survivor count. Curves were then plotted of per cent survivors *vs.* time for each concentration of preservative. The time required to produce 50% killing was obtained from the plots. A standard curve was then drawn of the time required to destroy 50% of the organisms *vs.* the corresponding concentrations of preservative. Using the 50% killing time value of a sample of preservative solution under test and placing it on the standard curve, an estimate of the concentration of preservative was obtained. However, this procedure proved too cumbersome and time-consuming and, moreover, yielded poorly reproducible results.

As presently applied, the assay has proved to be rapid and reliable for the determination of chlorobutanol, phenol, and *p*-chlorophenylethyl alcohol, the only preservatives investigated thus far. With minor modifications, the procedure is similar to that of the turbidimetric assay employed in our laboratory for viomycin as described by Kersey (2).

In essence, the method consists of the addition graded concentrations of the preservative to a series of test tubes containing nutrient broth seeded with *E. coli*. After an incubation of approximately 3.5 hours the turbidity of each tube is measured for graded response by means of a photoelectric colorimeter fitted with a color filter. The potency of the sample is established by comparing the response of the test organism to the preservative under test with that observed in the standard reference series. Because of the rapidity of the test and the relatively heavy *E. coli* inoculum, strictly sterile techniques need not be necessarily observed.

EXPERIMENTAL

Culture and Medium.—Stock cultures of *E. coli*, strain Su 2, are maintained on BBL trypticase soy agar slants. Prior to the assay, the culture is transferred to two fresh agar slants which are incubated overnight at 37°. The growth from these slants are suspended in sufficient sterile 0.9 per cent sodium chloride solution to yield a light transmission reading of 10–12% (equivalent to an optical density of approximately 1.0 at a wavelength of 580 mμ in a Bausch and Lomb Spectronic 20 colorimeter). One milliliter of this suspension is added to 100-ml.

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TABLE I.—REPRODUCIBILITY OF ASSAYS PERFORMED ON DIFFERENT DAYS

Assay Method	Assay No.	*p*-Chloro-beta-phenylethyl Alc., % Found	Chlorobutanol, % Found	Phenol, % Found
Microbiological	1	0.121	0.255	0.590
	2	0.126	0.270	0.565
	3	0.116	0.249	0.565
	4	0.108	0.262	0.535
	5	0.111	0.256	0.543
	6	0.129	0.238	0.525
	7	0.120	0.262	0.543
	8	0.124	0.250	0.543
	9	0.117	0.262	0.535
	10	0.119	0.250	0.550
	11	0.130	0.245	...
	12	0.131	0.252	...
	13	0.120	0.253	...
	14	0.124	0.270	...
	15	...	0.262	...
Means ± standard errors		0.121 ± 0.0017	0.255 ± 0.0022	0.549 ± 0.0060
95% Confidence limits		0.1173 – 0.1247	0.2503 – 0.2590	0.5354 – 0.5626
Chemical		0.117	0.250	0.560

TABLE II.—ACCURACY OF ASSAYS CALCULATED FROM DIFFERENT POINTS OF STANDARD SLOPE[a]

Preservative	Assay Method	Preservative Content, % in Sample No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Chlorobutanol	Chemical	0.47	0.44	0.34	0.25	0.14	0.04
	Microbiol.	0.46	0.43	0.35	0.22	0.14	0.04
Phenol	Chemical	1.02	0.62	0.27	...	...	...
	Microbiol.	1.05	0.61	0.24	...	...	...
p-Chloro-beta-phenylethyl alcohol	Chemical	0.22	0.19	0.16	0.13	...	...
	Microbiol.	0.23	0.20	0.17	0.13	...	...

[a] All values based upon microbiological assay values for each different preservative were obtained from a single standard slope.

amounts of BBL trypticase soy broth. This inoculated broth is now ready for use in the assay.

Standard Solutions.—The following standard reference solutions are prepared in pH 4.0 (0.275 *M*) citric acid–sodium phosphate buffer: chlorobutanol, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05%; phenol, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, and 0.2%; and *p*-chloro-beta-phenylethyl alcohol, 0.262, 0.225, 0.187, 0.15, 0.112, and 0.075%.

Samples.—All samples for assay are diluted to contain not more than the maximum concentration of preservative employed in the standard reference series.

Assay Procedure: One-milliliter amounts of each standard reference concentration and of each unknown solution are added to test tubes in quadruplicate. As controls, 1.0 ml. of buffer solution containing no preservative is included in each assay. Nine milliliters of inoculated broth is then added to each tube. A syringe pipetting assembly (Cornwall type) fitted with a two-inch cannula is used for the rapid dispensing of the broth. Each tube is thoroughly shaken and placed in a water bath uniformly maintained at 37° until the control tubes have an optical density reading of approximately 0.35 (usually 3.5 hours of incubation). Further growth of the test organism is immediately stopped by the addition of 0.5-ml. amounts of formalin, diluted 1:3, employing the syringe pipet for this purpose.[1] The optical density of each tube of broth is obtained with the colorimeter at a wavelength of 530 mμ.

[1] The use of buffers with pH values greater than 4.0 will result in short periods of incubation.

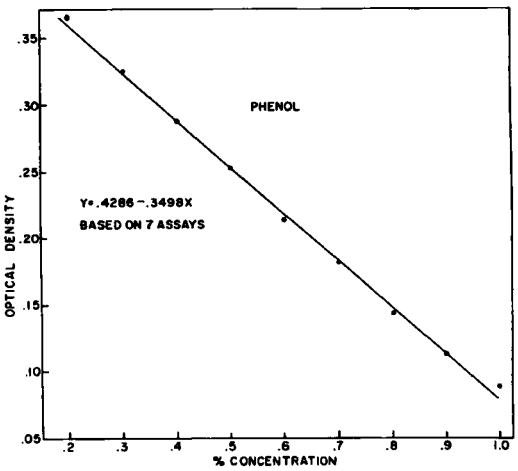

Fig. 1.—Computed line based on the values obtained from 7 assays.

The colorimeter is adjusted with clear, sterile broth to a reading of *O* optical density (*i.e.*, 100% light transmission). For each turbidity reading, the contents of the tube are transferred to a 3/4 in. × 6 in. test tube made expressly for the Spectronic 20 colorimeter by Bausch and Lomb.[2] After each reading, the broth is removed by suction. The values obtained for each set of quadrupli-

[2] Catalog No. 33-29-37.

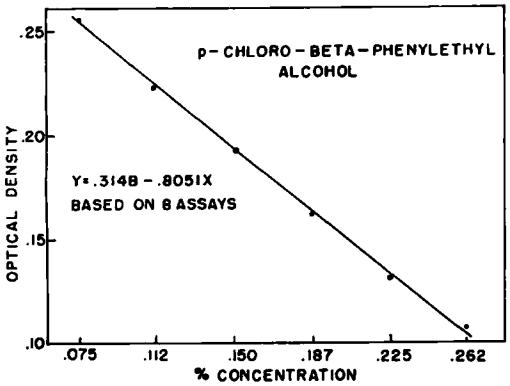

Fig. 2.—Computed line based on the values obtained from 8 assays.

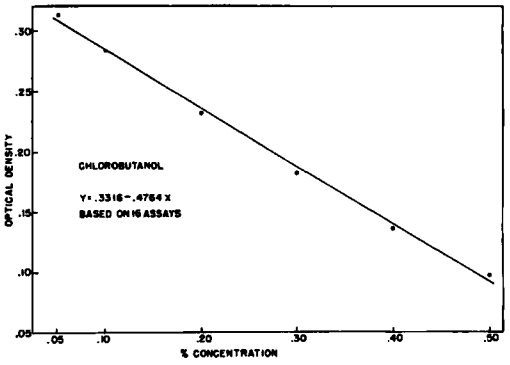

Fig. 3.—Computed line based on the values obtained from 16 assays.

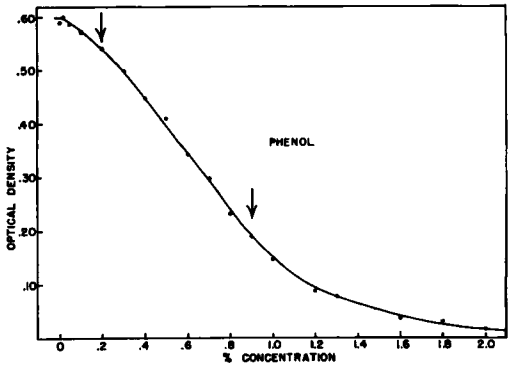

Fig. 4.—A typical sigmoid curve obtained when % concentration is plotted against optical density. The zone of straight line relationship is indicated by the arrows.

cate tubes are averaged. The average optical density values obtained for the standard reference concentrations are plotted on arithmetic paper against the corresponding preservative concentrations, and a straight line is drawn. Potencies of samples being assayed are then determined from the standard slope. Figures 1, 2, and 3 represent computed lines for phenol, *p*-chloro-beta-phenylethyl alcohol, and chlorobutanol based on 7, 8, and 16 assays, respectively, each assay being performed on different days. A straight line relationship was proved by the analysis of variance. Figure 4 shows the typical sigmoid curve for phenol. For our potency estimations only the linear zone, as indicated by the arrows, was used.

RESULTS

Since the relationship between the variables (*i.e.*, the per cent concentration of preservative and the corresponding observed optical density) is linear, we have compared the standard dose-response line as drawn by inspection with one drawn by the method of least squares. In almost all instances these two drawn lines, with respect to each of the three preservatives studied, were virtually superimposable.

As indicated in Table I, there is excellent agreement between the values derived for the three preservatives by chemical analysis and those obtained by the turbidimetric bioassay with *E. coli*.

That assay values of unknown samples calculated from different portions of the standard reference slope agree with corresponding values obtained by chemical analysis is shown in Table II.

SUMMARY

1. An accurate method is described for the turbidimetric assay of chlorobutanol, phenol, and *p*-chloro-beta-phenylethyl alcohol, employing *E. coli* as the test organism and requiring a $3^1/_2$-hour incubation period. The method is essentially the same as that currently used for viomycin and other antibiotics.

2. The procedure for establishing standard reference slopes for these preservatives is described.

REFERENCES

(1) Lachman, L., Weinstein, S., Hopkins, G., Slack, S., Eisman, P. C., and Cooper, J., THIS JOURNAL, 51, 224(1962).
(2) Kersey, R. C., *Antibiot. Chemotherapy*, 1, 173(1951).