Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product IV

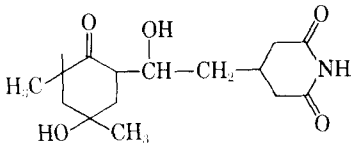
Screening Bulk Drug Stability and Defining the Assay of Choice

By EDWARD R. GARRETT† and ROSS R. HERR

Statistical evaluation of biological assays of bulk streptovitacin A subjected to 70° and room temperature for 60 days permitted the conclusion that the drug was stable in the bulk form. The assay of choice was defined against *Saccharomyces pastorianus* rather than against *Trichomonas vaginalis*. The point is made that operational potency is best defined by assay replication on various days but comparison of lot potencies and production procedures is best made by assay on one given day against the same standards.

WHEN a potential product results from pharmaceutical research, a facile and fast screen of stability in the bulk form of the drug is needed. When biological assays are the major method of potency determination, the stability study must be designed so that confidence in stability estimates must take into account the large inherent error in such assays. Such a design can also contribute supplementary information which can permit a choice of assay and evaluate the effects of variation among days or sampling procedures (1, 2, 3).

A case in point was the antitumor agent streptovitacin A (4–10)



The screening of stability was conducted on samples maintained at both 70° and room temperature. Only if significant differential degradation occurred would a more elegant accelerated stability study at several temperatures for purposes of stability prediction be warranted.

EXPERIMENTAL

Samples of streptovitacin A were weighed and placed in separate vials. One set of vials was subjected to 70° and the other was maintained at room temperature. At intervals, vials were removed and submitted for assay. The two assay procedures used are given in the literature (5), paper chromatographic separation on bioautograph development against *Saccharomyces pastorianus* and *Trichomonas vaginalis*.

RESULTS AND DISCUSSION

The analyses of variance (11) of the assays of streptovitacin A subjected to 70° and room temperature for 60 days are given in Tables I and II, the former for the *S. pastorianus* assay (5) and the latter for the *T. vaginalis* assay (5).

No significant differences between the assays of streptovitacin A maintained at room temperature and 70° for 60 days could be determined. These facts are graphically shown in Fig. 1. No significant trend of assays with days of sampling was apparent for the material maintained at 70°. The differ-

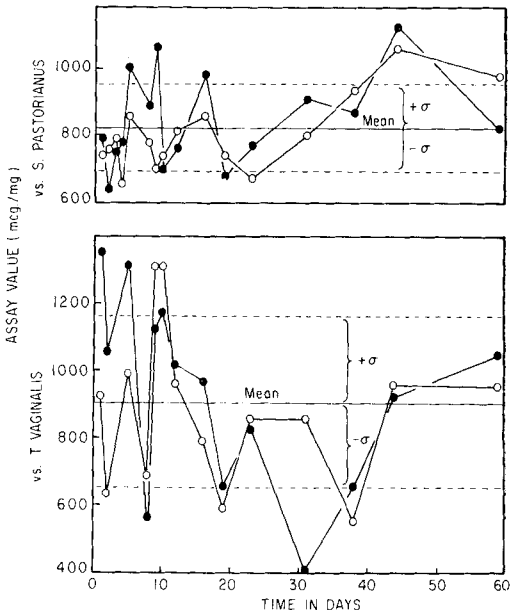


Fig. 1.—The assay of solid streptovitacin A at 70°, ●, and at room temperature, ⊙, as a function of time. The solid line represents the mean of the assays; the dashed lines represent the limits of the mean ± standard deviation.

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TABLE I.—ANALYSIS OF VARIANCE OF STREPTOVITACIN A MAINTAINED AT TWO DIFFERENT TEMPERATURES[a] AND ASSAYED ON VARIOUS DAYS (ASSAY AGAINST *S. Pastorianus*)

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Quantities Estimated by Mean Squares
Days[b]	323,242	13	24,864[c]	$2\sigma_D^2 + \sigma_E^2$
Temperatures	11,401	1	11,401[d]	$14\sigma_T^2 + \sigma_E^2$
Error	117,013	13	9,001	σ_E^2
Total[e]	451,657	27	16,728	

[a] Average of room temperature samples: 813; average of 70° samples: 854; overall average, $\bar{x} = 834$. [b] Estimated variance, $\sigma_D^2 = 7{,}930$. [c] F test: days:error = 2.77; 5% F = 2.6 ∴ sign. @ 5% level. [d] F test: temp.:error = 1.27; 5% F = 4.7 ∴ no significance. [e] Estimate of variance among days and error: $\sigma_E^2 + \sigma_D^2 = 16{,}931$

$$\therefore \sigma = 130 \therefore \frac{\sigma}{\bar{x}} = \frac{130}{834} \therefore 15.6\%$$

ences in the mean values of the assays of the 70° and room temperature materials can be attributed solely to assay variation.

Additional information may be obtained from the analyses of variance. For both assay methods, either against *S. pastorianus* or *T. vaginalis*, the assay variation among days is significant. In fact, when an assay is replicated on several days, the variation is twice that for replication on a given day.

The fact that this variation occurs in the same direction for samples run on the same day is apparent from inspection of the plots in the attached Fig. 1. When the 70° assays give results above the mean on given days, so do the room temperature assays; when they give results below the mean, so do the room temperature assays. That this synchronization of daily deviations from the mean for both samples is not a phenomenon attributable to some inherent variation in the day of sampling may be concluded from the analysis of variance for the total data from both assays as given in Table III.

The interaction days × assay is significant on the 1% level whereas variation among days and assays is not significant with respect to this interaction. Yet, as from Tables I and II, variation among days is significant within each assay. Thus it may be concluded that the variation among days is arbitrary with a particular assay; that there is not a common reason for the highs and lows of assay values *vs.* days to be synchronized for the several assays. Assays yield high and low values independently; results with a particular assay show a daily bias peculiar to that assay.

This is graphically shown in Fig. 1 where the assay values against *T. vaginalis* for a given day may be in excess of the mean, whereas those for *S. pastorianus* are just as likely to be less than their mean on that day. Thus no bias is apparent when the data from both assays are averaged.

These facts permit one point to be stressed. Variation among days of assay is a phenomenon common to microbiological methods (12, 13). Fundamental investigations of the causes of diurnal microorganism or biological variation may eliminate the source of increased error among assays replicated on various days. In this particular study, such elimination would have halved the assay variance.

If an assay is to serve as an operational definition of drug potency and since potency may be evaluated on any arbitrary day, replicate assays on several days may most adequately define the drug. How-

TABLE II.—ANALYSIS OF VARIANCE OF STREPTOVITACIN A MAINTAINED AT TWO DIFFERENT TEMPERATURES[a] AND ASSAYED ON VARIOUS DAYS (ASSAY AGAINST *T. Vaginalis*)

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Quantities Estimated by Mean Squares
Days[b]	1,359,035	13	104,541[c]	$\sigma_E^2 + 2\sigma_D^2$
Temperature	19,189	1	19,189[d]	$\sigma_E^2 + 14\sigma_T^2$
Error	384,431	13	29,572	σ_E^2
Total[e]	1,762,654	22	65,283	

[a] Average of room temperature samples: 880; average of 70° samples: 924; overall average: 906. [b] Estimated variance; $\sigma_D^2 = 34{,}485$. [c] F test; days:error = 3.54; 5% F = 2.6 ∴ sign @ 5% level. [d] F test; temp. error <1 ∴ no significance. [e] Estimate of variance among days and error: $\sigma_E^2 + \sigma_D^2 = 64{,}057$.

$$\therefore \sigma = 253 \therefore \frac{\sigma}{\bar{x}} = \frac{253}{906} \therefore 27.9\%.$$

TABLE III.—ANALYSIS OF VARIANCE OF TWO ASSAYS BY DAYS OF ASSAY[a] WITH 70° AND ROOM TEMPERATURE MATERIAL CONSIDERED AS REPLICATES (ASSAYS AGAINST *S. Pastorianus* AND *T. Vaginalis*)

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Quantities Estimated by Mean Squares
Days	873,545	13	67,196[b]	$\sigma_E^2 + 4\sigma_D^2 + 2\sigma_{D\times A}^2$
Assays	73,732	1	73,732[b]	$\sigma_E^2 + 28\sigma_D^2 + 2\sigma_{D\times A}^2$
Day × Assay	808,732	13	62,210[c]	$\sigma_E^2 + 2\sigma_{D\times A}^2$
Error	532,034	28	19,001	σ_E^2
Total	2,288,000	55	41,601	

[a] Average among days: 883; average among assays: 906; over-all average: 870. [b] Days and assays are not significant with respect to D × A interaction. [c] F test: D × A:error = 3.22; 1% F = 2.9 ∴ sign. on 1% level; estimated variance $\sigma_{D\times A}^2 = 21{,}600$.

ever, drug lots or development procedures to give higher purity or yield would be most accurately compared by assay on one day with the same standard curves. Of course, the possibility of sample × assay × day interaction must not be ignored.

CONCLUSIONS

A screening procedure was used to evaluate the stability of a drug in the bulk form, and assays of streptovitacin A at 70° and room temperature for 60 days were compared. It is concluded that streptovitacin A is stable in the bulk form and is not subject to any thermal degradation which results in loss of biological potency.

The quantitative papergram assay against *S. pastorianus* for streptovitacin A is the method of choice rather than against *T. vaginalis*. The standard deviation, per cent of the mean, for the estimation of error of a single assay is 16% for the former, and 28% for the latter.

The variation of an assay within a single day is only one-half of the variation of that assay on various days. This phenomenon of daily variation has to do with the bias of the assay and not the day of sampling. Elimination of such daily assay variation could halve the variance.

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Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product V

Importance of Assay Validity, a Case in Point

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Microbiological assays are used to measure drug potency but unwanted degradation products or impurities may positively interfere. A case in point is the tetracycline salts with normal moisture content in the cylinder-plate assay against *Bacillus subtilis*. Thermal degradation doubles the potency of tetracycline in this microbiological assay. Ultraviolet and infrared spectrophotometry indicate that aromatization and decarboxamidation are implicated. The resultant products have greater diffusivity and sufficient potency in the agar of the cylinder-plate assay method so that an *apparently* greater drug assay results. Dry tetracycline salts are thermally stable. Normal moisture content does not induce instability at usual storage temperatures. The kinetics of acid degradation of tetracycline phosphate and hydrochloride are the same.

The prediction of stability of drugs in pharmaceutical preparations (1) can be made only within the validity and reliability of the assay for drug. Previous papers in this series (2) have essentially considered the reliability or reproducibility of the assay.

The validity of an assay may be defined as its ability to measure that which it is supposed to measure. When biological assays are necessary, it is frequently assumed that degradation products of the drug do not demonstrate potency under the conditions of assay. This is not necessarily so. Biological assays are a complex sequence of physical, biological, and chemical effects. It is possible that the degradation products of a drug may not have the same pharmacological properties as the drug itself and yet give anomalous estimates of potency.

An interesting case in point is tetracycline and the assay in question is a classical microbiological method of antibiotic evaluation, the cylinder-plate assay (3) with *Bacillus subtilis* as the test organism.

EXPERIMENTAL

Biological Assay of Heat-Treated Tetracycline by Cylinder-Plate Assay.—Bulk tetracycline phosphate[1] with 2% moisture content was subjected to

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[1] Marketed by The Upjohn Co. as Panmycin Phosphate.