Microbiological Assay for Estimating Salivary Concentrations of the Cariostatic Antibiotic Actinobolin

D. E. HUNT, E. L. BRADLEY, JR., AND JOAN W. BACHMANN

Institute of Dental Research and Department of Microbiology, University of Alabama Medical Center, Birmingham, Alabama 35233

Received for publication 8 May 1970

A logarithmic-ratio microbiological assay has been developed for the estimation of concentrations of actinobolin (or inhibitory equivalents) in human saliva. The utility of this assay has been demonstrated by presenting data that show concentrations of this antibiotic (or inhibitory equivalents) detected in saliva or saline after incubation at 37°C for periods as long as 6 hr. The results of some statistical analyses of the assay data have been included.

Actinobolin, an antibiotic isolated from cultures of Streptomyces griseoviridis var. atrofaciens (T. H. Haskell et al., U.S. Patent 3,043,830, 1962), has been reported to be strongly and somewhat specifically inhibitory in vitro for cariogenic streptococci (5) and to have strong cariostatic activity in vivo (D. E. Hunt et al., Abstr. 48th Genl. Mtg. Int. Ass. Dent. Res., New York, 1970). Additionally, actinobolin has various chemical, biochemical, and pharmacological properties that appear to make it uniquely suited as an orally applied cariostatic antibiotic.

A microbiological assay was developed for actinobolin because of the need for an assay for clinical dental trials which are planned and because no microbiological assay which uses a cariogenic streptococcal strain is presently available for this cariostatic antibiotic. This report describes a logarithmic-ratio microbiological assay for the determination of actinobolin concentrations (or inhibitory equivalents) in human saliva.

MATERIALS AND METHODS

Actinobolin sulfate (80% base, lot no. X8061; Parke, Davis & Co., Detroit, Mich.) was used in this study.

A previously reported method (4) was used to select an appropriate bacterial strain for the assay of actinobolin in human saliva. From a variety of cariogenic streptococci, Streptococcus mutans HHT (6) was selected as the assay organism. This culture was maintained on a medium with the following percentage composition (7): sucrose, 1.0; Trypticase, 0.5; yeast extract, 0.5; K₂HPO₄, 0.5; salt solution, 0.05. The salt solution contained: MgSO₄·7H₂O, 11.5 g; MnSO₄·4H₂O, 2.5 g; FeSO₄·7H₂O, 0.68 g; and distilled water, 100 ml. The pH was adjusted to 7.1. For the inoculation of agar assay plates (no. 1029, Falcon Plastics, Los Angeles, Calif.), a Tryptic Soy Broth (Difco, Detroit, Mich.) culture of S. mutans was incubated anaerobically for 14 to 16 hr at 37°C. With additional sterile broth, this culture was adjusted to 53% light transmittance (540 nm) in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.), and 10 ml of this adjusted culture was added to 1 liter of cooled (50 to 55°C) tryptic soy agar containing 1% sucrose and 2 ml of a 0.1% aqueous solution of fast green (FD & C Green no. 3, H. Kohnstamm and Co., New York, N.Y.). The addition of this dye to agar has been found to be a useful adjunct in agar-diffusion assays (3). A 5-ml amount of the inoculated agar was added to the previously described plates containing 8 ml of congealed sterile agar.

Stock solutions of actinobolin were prepared in sterile saline and in pooled, human saliva (heated to 70°C for 20 min) so that when 0.08 ml of the various solutions was added to paper discs (1.27 cm in diameter, no. 740-E, Schleicher and Schuell Co., Keene, N.H.), the following concentrations (micrograms of actinobolin per disc) were obtained: 6, 8, 12, 16, 24, 32, 48, 64, 96, and 128. These discs, used for the preparation of standard assay curves together with discs charged with experimental samples, were prepared in duplicate. The reported experiments were repeated three times on successive days. To insure clearly defined zones of inhibition, no more than five discs were placed on each assay plate. All plates were incubated anaerobically for 14 to 16 hr at 37°C. The resulting zones of growth inhibition were measured and were then plotted on semilogarithmic graph paper with drug concentrations per disc on the logarithmic scale and zone diameters on the arithmetic scale.

Standard curves were constructed through the resulting points by the method of least squares. Concentrations of actinobolin in the experimental samples were
Diameter of Zone of Inhibition

FIG. 1. Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of actinobolin in saline and in pooled human saliva. Assay microorganism, Streptococcus mutans HHT.

TABLE 1. Analysis of data (diameters of zones of inhibition versus concentrations of actinobolin) used for constructing standard curves for the estimation of concentrations of actinobolin in saline or saliva

| Determination               | Expt | Intercept | Slope  | s   | \(\lambda\) | r   | "t" test of slope | F test of deviation from linearity |
|-----------------------------|------|-----------|--------|-----|-------------|-----|------------------|----------------------------------|
| Saline standard curves      | 1\(^b\) | 0.76512 | 0.88369| 0.04799 | 0.05431 | 0.9929 | 35.43***             | 1.71 NS\(^c\)                     |
|                             | 2\(^b\) | 0.64671 | 0.95363| 0.07482 | 0.07914 | 0.9855 | 24.62***             | 0.88 NS                          |
|                             | 3\(^d\) | 0.64205 | 0.92740| 0.04847 | 0.05226 | 0.9934 | 36.82***             | 2.27 NS                          |
| Saliva standard curves      | 1\(^b\) | 0.74389 | 0.85508| 0.05529 | 0.06466 | 0.9900 | 29.76***             | 1.37 NS                          |
|                             | 2\(^d\) | 0.64114 | 0.87778| 0.09954 | 0.11340 | 0.9701 | 16.97***             | 4.15*                            |
|                             | 3\(^d\) | 0.62742 | 0.87663| 0.08027 | 0.09136 | 0.9803 | 21.06***             | 13.25***                         |

* Abbreviations: s, standard deviation of points from fitted line based on 18 degrees of freedom; \(\lambda\), index of precision of standard curves; r, correlation coefficient; "t," test of slope equals \(b(S(S/\sigma)^{1/2})\), where \(SS_x\) is the sum of squares of deviation of the logarithmic concentrations around their mean; \(F\), test equals ratio of mean square deviation from linearity (8 degrees of freedom) to mean square within concentrations (10 degrees of freedom).

\(^b\) For these calculations, data (zones of inhibition versus concentrations of actinobolin) were taken from Fig. 1.

\(^c\) Not significant.

\(^d\) Standard curves not shown for these experiments.

\(^*\) Significant at \(P \leq 0.05\) level.

\(^***\) Significant at \(P \leq 0.001\) level.

determined by reading antibiotic concentrations on the ordinate of the standard curves corresponding to the diameters of the zones of inhibition surrounding discs charged with the experimental samples.

RESULTS AND DISCUSSION

*S. mutans* HHT was selected as the microorganism for the assay of actinobolin because of sensitivity to this antibiotic, linear dose response, and rapid growth rate. Representative standard assay curves, obtained with actinobolin dissolved in saline and in pooled saliva, are presented in Fig. 1. In either diluent, concentrations of actinobolin as low as 6 \(\mu g\) per disc (75 \(\mu g/ml\), which is approximately 0.03 of 1 \(LD_{50}\) oral dose (R. F. Pittillo, personal communication), could be de-
TABLE 2. Estimates and 95% confidence limits of known concentrations of actinobolin dissolved in saline or saliva

| Expt | Known concn | No. of discs | Saline | Saliva |
|------|-------------|--------------|--------|--------|
|      |             |              | Estimated concn | 95% CL | Estimated concn | 95% CL |
| 1    | 10          | 6            | 10.5   | (9.2-12.0) | 8.6 | (7.2-10.1) |
|      | 20          | 6            | 21.4   | (19.0-24.3) | 15.0 | (12.9-17.5) |
|      | 40          | 4            | 44.9   | (38.8-52.0) | 42.6 | (35.8-50.7) |
|      | 80          | 4            | 88.9   | (75.8-104.3) | 75.6 | (62.8-91.0) |
| 2    | 10          | 6            | 9.6    | (7.9-11.7) | 8.0 | (5.9-10.8) |
|      | 20          | 6            | 16.9   | (14.1-20.2) | 15.1 | (11.5-19.7) |
|      | 40          | 4            | 41.3   | (33.5-50.9) | 28.1 | (20.8-37.9) |
|      | 80          | 4            | 69.0   | (55.3-86.0) | 104.2 | (74.0-146.8) |
| 3    | 10          | 6            | 11.7   | (10.3-13.3) | 9.8 | (7.8-12.4) |
|      | 20          | 6            | 18.9   | (16.7-21.3) | 18.5 | (15.0-22.9) |
|      | 40          | 4            | 39.7   | (34.5-45.7) | 35.3 | (27.7-45.0) |
|      | 80          | 4            | 89.0   | (76.3-103.8) | 104.1 | (79.0-137.1) |

- In micrograms per disc.
- Containing known concentrations of actinobolin.
- From saline or saliva standard curves.
- Confidence limits.

![Graph](image1)

FIG. 2. Microbiological assay of actinobolin dissolved in saline or saliva and incubated at 37 C for indicated time. Initial concentrations of actinobolin in both diluents were 225 μg/ml. Each point represents the mean assayed values from two experiments.

tected. Preliminary statistical evaluation of the assay revealed no significant plate-to-plate variation in zone size.

Analysis of data taken from Fig. 1 and from two additional sets of standard curves (not shown) is presented in Table 1. For these calculations, Y (dependent variable) represented a diameter of a zone of inhibition for a corresponding logarithmic concentration, X (independent vari-

![Graph](image2)

FIG. 3. Parallel relationship between the saline standard curve for the microbiological assay of actinobolin and assayed values of actinobolin in saliva. (A) Saline standard curve reconstructed from Fig. 1. (B) Assayed values from undiluted saliva containing actinobolin and a 1:10 dilution thereof.
able), of antibiotic. Thus, $Y$ and $X$ are assumed to satisfy the equation $Y = \alpha + \beta X + \epsilon$, where $\epsilon$ is a random variable with expectation of zero and variance of $\sigma^2$. The method of least squares was used to obtain estimates of $\alpha$, $\beta$, and $\sigma$ for the saline and saliva standard curves. The averages of the indexes of precision ($\lambda$) were 0.06 and 0.09 for the saline and saliva standard curves, respectively, where $\lambda$ equals the standard deviation of points from the fitted line/the estimated slope of the line. The slope of all standard curves was found to be highly significant ($P \leq 0.001$).

A test for nonlinearity between diameters of zones of inhibition and logarithmic concentrations of actinobolin revealed no significant deviation with the saline standard curves. Two of three saliva standard curves, however, showed statistically significant deviation from linearity. Since this was attributable mainly to a possible curvilinear effect at very high and very low antibiotic concentrations and since there was limited scatter of points around the curves, the deviation from linearity was found to be of no practical importance. A further measure of the accuracy of the assay is shown in Table 2, in which known concentrations of actinobolin are compared with their estimates obtained from the standard curves. The confidence limits in Table 2 are calculated from the anti-logarithms of the fiducial limits for the logarithmic concentrations of actinobolin. Fiducial limits are commonly used for establishing limits on estimates of independent variables when values of the dependent variables are observed (2). These limits show the range that should contain the known concentrations 95% of the time. With known concentrations of antibiotic dissolved in saline, only 1 of the 12 calculated confidence intervals failed to show the known concentration of antibiotic. With known concentrations of antibiotic dissolved in saliva, 3 (25%) of the 12 calculated confidence limits did not contain the known concentration of actinobolin. This is higher than the expected 5% and can probably be attributed to the failure of $s$, the estimate of $\sigma$, to reflect all sources of variation, i.e., ions in saliva which have been reported (8) to antagonize the inhibitor activity of actinobolin. Additionally, estimates within each experiment are correlated, thus the true confidence level may be less than 95%. The concentrations of actinobolin detected in saline or pooled saliva, which had been incubated at 37 C for periods as long as 6 hr, are shown in Fig. 2. These data show that actinobolin dissolved in saline or saliva essentially retained its full inhibitory activity for 4 hr. At the end of 6 hr, some degradation of actinobolin occurred in saliva. In microbiological assays, it is assumed that the product assayed in the experimental samples differs only from the standard antibiotic solution by some dilution factor. Consequently, dilutions of experimental samples, when assayed, should yield points that parallel the standard curve in a logarithmic-ratio assay (1). The data presented in Fig. 3 substantiate this assumption.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant DE-02670 from the National Institute of Dental Research. The assistance of Gayle Renfro and Sue Flowers is gratefully noted.

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