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A Spectrophotometric Assay for Measuring the Uptake of Actinobolin by Components of Human Enamel[1] (39195)

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The antibiotic actinobolin appears to have potential clinical utility in the control of dental caries and periodontal disease because of the following: (i) It has most of the properties reported to be desirable or essential in an ideal cariostatic agent (1). (ii) It is effective against microorganisms that cause or are associated with both dental caries and periodontal disease (2, 3). (iii) Bacterial resistance or cross-resistance to this antibiotic appears to represent no significant problem (5, 6). (iv) Actinobolin binds to human enamel (7) and may be synergistic with fluoride in controlling dental caries (8). Exploratory studies concerning the binding of actinobolin to components of human enamel revealed the need for an assay that could detect actinobolin at concentrations <5.0 μg/ml. Although a microbiological assay is available for estimating concentrations of actinobolin in physiological solutions or saliva, the lower limit of the sensitivity of the assay is approximately 75.0 μg actinobolin/ml (9). This report concerns the development and demonstrated utility of a spectrophotometric assay that can detect actinobolin in various solvent systems at <2.0 μg/ml.

Materials and methods. Hydroxylapatite (HAP) lot number 11524 was obtained from Bio-Rad Laboratories, Richmond, California. Actinobolin sulfate lot number X8061 was a gift from Parke, Davis and Company, Detroit. Powered human enamel was prepared by grinding the crowns and necks of extracted, unpolished, and sound teeth with a number 700 tapered burr. For experiments, 10-mg amounts of HAP or powdered enamel were placed in test tubes to which were added 5 ml of: 0.01 *M* sodium phosphate at pH 5.5, 7.0, or 8.0; deionized water; deionized water containing 1% salivary supernatant; or each of the above indicated solvent systems containing 1-5 ppm sodium fluoride. The 10-mg quantities of HAP or powdered enamel were selected because they represented convenient working levels; however, later experiments showed that larger or smaller amounts could be used without any apparent effect on the assay procedure. When the sodium phosphate buffers were used, pH values of 5.5, 7.0, and 8.0 were chosen because these represent, respectively, the "critical pH" at which human enamel begins to undergo acid demineralization, the approximate pH of human saliva, and the pH at which actinobolin exhibits incipient chelator properties. Separate stock solutions of actinobolin were prepared in which this antibiotic was dissolved in the respective solvent systems. Serial twofold dilutions of the stock solutions were prepared so that the following concentrations of actinobolin (micrograms per milliliter) were obtained in each of the solvent systems: 25, 12.5, 6.25, 3.125, and 1.56. One milliliter volumes of each of these dilutions were transferred to matched cuvettes (Far-UV silica, A.H. Thomas Co., Philadelphia) and the optical densities determined in a spectrophotometer (Beckman Model 24, Fullerton, California) at a wavelength of 270 nm. The resulting optical density values were plotted on the Y axis and the actinobolin concentrations plotted on the X axis of arithmetic graph paper. Standard curves were obtained by constructing a line through the respective points by the method of least squares.

For experiments, a sufficient volume of the above indicated stock solutions was added to tubes, containing the respective solvent systems and HAP or powdered enamel and appropriate control tubes without HAP or enamel, to yield a final concentration of 25 μg actinobolin/ml in all tubes. All

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tubes were mixed for 1 min in a Vortex mixer (Scientific Products, Evanston, Illinois), centrifuged at approximately 2000*g* for 10 min to sediment most of the HAP and enamel, and then filtered through a Swinney filter (pore size 0.5 μm, Propper Mfg. Co., Long Island City, N.Y.). Previous studies established that no detectable amount of actinobolin was removed by this filtration. One milliliter volumes of these filtrates were then spectrophotometrically assayed, and the resulting optical density values were used to determine the amount of actinobolin remaining in the filtrates, and by difference the amount of actinobolin taken up by HAP or powdered enamel, by reference to the previously described standard curves.

Results and discussion. A standard assay curve for the spectrophotometric assay of actinobolin dissolved in 0.01 *M* sodium phosphate buffer at pH 5.5 is presented in Fig. 1. Nearly identical curves were obtained when actinobolin was dissolved in: 0.01 *M* sodium phosphate buffer at pH 7.0 or 8.0; deionized water; deionized water containing 1% salivary supernatant; or in each of the indicated solvent systems containing 1–5 parts per million sodium fluoride. In each system, concentrations as low as 1.56 μg actinobolin/ml could be detected. The utility of the assay is demonstrated by data in Fig. 2, which show the uptake of actinobolin by 0.01 *M* sodium phosphate suspensions of HAP or powdered human enamel at pH 5.5, 7.0, and 8.0. The concentrations of actinobolin (~5–7 μg) found to be taken up by 10 mg of HAP or enamel represent levels of actinobolin approximately 10 times below the minimum concentrations of this antibiotic detectable by microbiological assay (9). That actinobolin was adsorbed, and not in some way degraded or inactivated by HAP or powdered enamel, was indicated as follows: HAP or powdered enamel, suspended in phosphate buffer containing actinobolin, was removed and washed twice by centrifuging in deionized water. A portion of the HAP and enamel was used to fill holes (4-mm deep by 8-mm diameter) cut into a tryptic soy agar plate uniformly inoculated with *Streptococcus mutans* AHT. After overnight incubation at 37°, distinct zones of growth inhibition were observed around the washed HAP and enamel. In the same plate, washed HAP or enamel that had been suspended in phosphate buffer without actinobolin, produced no zones of inhibition. The amounts of HAP or powdered enamel used in the assay did not appear to be critical since in exploratory experiments, 5–500 mg of HAP or enamel were used without any apparent effect on the utility of the assay. However, when salivary supernatant was added to the various solvent systems at concentrations >1%, inconsistent results were obtained which were attributed to the turbidity caused by the addition of the salivary supernatant. This type of problem is frequently encountered in spectrophotometric assays for biologically active compounds (10) and must of course be considered in the interpretation of results. Analysis of data taken from Fig. 1 and from five additional standard curves (not shown) is presented in Table 1. For these

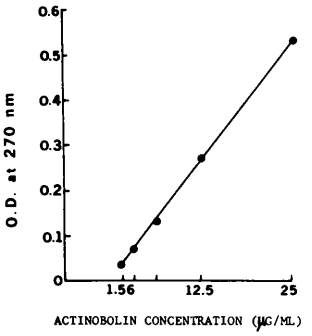

FIG. 1. Standard assay curve for the spectrophotometric assay of actinobolin. Optical densities were determined for serial twofold dilutions of actinobolin dissolved in 0.01 *M* sodium phosphate buffer at pH 5.5.

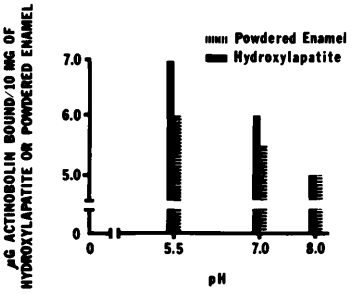

FIG. 2. Uptake of actinobolin by 0.01 *M* sodium phosphate buffer suspensions of hydroxylapatite or powdered human enamel determined by spectrophotometric assay.

TABLE I. ANALYSIS OF DATA (CONCENTRATIONS OF ACTINOBOLIN VERSUS OPTICAL DENSITY) USED FOR CONSTRUCTING STANDARD CURVES FOR THE SPECTROPHOTOMETRIC ASSAY OF ACTINOBOLIN.

Solvent system	Conditions or additions	Slope	λ[a]	"F" test of deviation from linearity[b]
0.01 *M* Sodium phosphate[c]	pH 5.5	0.0211	0.0324	6.412[d]
0.01 *M* Sodium phosphate[e]	pH 7.0	0.0216	0.0416	6.663[d]
0.01 *M* Sodium phosphate[e]	pH 8.0	0.0212	0.042	6.219[d]
Deionized water[e]	–	0.0217	0.037	6.302[d]
Deionized water[e]	1 ppm NaF	0.0208	0.0401	6.401[d]
Deionized water[e]	1% SS[f]	0.0208	0.0408	6.393[d]

[a] λ = Index of precision.

[b] "*F*" test equals ratio of mean square deviation from linearity to mean square within concentrations (4 degrees of freedom).

[c] For the calculations on this line, data were taken from Fig. 1.

[d] Not significant at $P \leq 0.05$ level.

[e] For the calculations on these lines, data were taken from five additional standard curves (not shown) for the indicated solvent systems.

[f] Salivary supernatant.

calculations, *Y* (dependent variable) represented optical density values for corresponding concentrations, *X* (independent variable), of actinobolin. Thus *X* and *Y* are assumed to satisfy the equation $Y = mX + b$. The indices of precision (λ) were calculated, where λ equals the standard deviation of points around the standard curve/the calculated slope of the curve. The λ values for the six standard curves were all <0.05. This indicates a highly precise assay since the smaller the λ value, the greater is the inherent precision of the assay technique (11). An important condition of assays for biologically active compounds is a linear relationship between drug concentration and response (11). This condition is fully satisfied in this assay since visual observation of all standard curves revealed a highly linear relationship between optical density and serial twofold dilutions of actinobolin dissolved in the respective solvent systems. Additionally, an "*F*" test was used to test for nonlinearity between optical density values and concentrations of actinobolin, where *F* equals the ratio of mean square deviation from linearity to mean square within concentrations. None of the standard curves showed any significant deviation ($P \leq 0.05$) from linearity.

Additional data are needed before any meaningful discussion can be undertaken regarding the mechanism or factors involved in the binding of actinobolin to components of human enamel: however, the significance of this report is that a new and sensitive assay has been developed which will allow studies directed toward elucidating the qualitative and quantitative aspects of the binding of actinobolin to components of human enamel.

Summary. A spectrophotometric assay was developed for measuring the uptake of the antibiotic actinobolin by hydroxylapatite (HAP) or powdered human enamel. The assay is sufficiently sensitive to detect <2.0 μg actinobolin/ml of: 0.01 *M* sodium phosphate buffer at pH 5.5, 7.0, or 8.0; deionized water; deionized water containing 1% salivary supernatant; or each of the above indicated solvent systems containing 1–5 parts per million sodium fluoride. The utility of the assay system has been demonstrated by data which show that approximately 5–7 μg of actinobolin are bound per 10 mg of HAP or powdered enamel.

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