Statistical Techniques in Predicting Thermal Stability

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Abstract □ In developing new pharmaceutical products it is often necessary to predict degradation rates at marketing temperatures from data collected on accelerated degradation taken at elevated temperatures. A technique for predicting degradation rate based on the Arrhenius equation was presented by Garrett in 1956. While his method is characterized by ease of computation involved (necessary due to scarcity of computer facilities at that time), it violates a number of assumptions upon which least-squares analysis is based, and hence inferences made from the results can be misleading. This report presents a method based on weighted least-squares analysis which can easily be adapted for computer analysis. Comparisons are made with the method suggested by Garrett to illustrate differences in technique and the effect the basic assumptions have upon the results obtained by the two methods. A statistical test is presented for determining the applicability of the Arrhenius relation to the data at hand. Finally, the technique is illustrated by application to chloramphenicol.

Keyphrases □ Thermal stability—statistical techniques for prediction, equations derived □ Arrhenius least-squares equations, thermal stability—comparison, evaluation □ Chloramphenicol, thermal stability—statistical determination □ Degradation rates—chloramphenicol

In 1956 Garrett (1) published a paper recognizing the economic benefits of predicting thermal stability of a drug from data collected at elevated temperatures. The method outlined by Garrett was a simple approximation to a method of fitting the Arrhenius relation by weighted least-squares analyses suggested by McBride and Villars (2) in 1954. The approximation suggested by Garrett gained a great deal in ease of computation, yet sacrificed in the extent to which valid inferences could be derived from the results. Until 1960 ease of computation was of primary concern due to scarcity and cost of computer time, but today even the smallest company can easily rent computer time at a reasonable cost. However, the approximate solution suggested by Garrett is the standard method used today (3, 4).

The purposes of this paper are to outline a method for predicting stability as based on the weighted least-squares technique, to illustrate why weighted least squares should be used in lieu of the unweighted approximation, and to present a statistical test for the validity of the Arrhenius assumption which can easily be computed from the results of the weighted method.

Appendix 1 contains the mathematical formulas involved in the weighted least-squares analysis.

THEORETICAL

Arrhenius Relationship—The functional relationship between time and concentration of a drug stored under constant conditions is dependent upon order of reaction and a rate constant which determines speed of reaction. A thorough discussion of methods for picking proper order is outside the scope of this paper and the assumption will be made that correct order can be determined. An example of a typical situation is the first-order reaction given by Eq. 1 where the logarithm of concentration at time t, denoted C_t, is linearly related to time by

$$\ln C_t = \ln C_0 - k_\tau t \qquad \text{(Eq. 1)}$$

where τ is the temperature at which storage took place and k_τ is the rate constant.

The Arrhenius relationship:

$$\ln k_\tau = \gamma + \delta/\tau \qquad \text{(Eq. 2)}$$

states that speed of reaction is dependent upon temperature; that is the logarithm of the reaction rate is a linear function of the reciprocal of absolute temperature. It is this relationship which allows data taken at elevated temperatures to be used to predict the degradation rate at room temperature and hence estimate shelf life of the drug. The rate constants obtained at the elevated temperatures can be used to estimate the parameters in the Arrhenius equation, which in turn can be used to estimate reaction rate at room temperature (or any other desired temperature).

The methods of estimation used in the previous procedure can vary from simple (and quick) eyeball techniques to a thorough statistical analysis based on weighted least squares. The remaining discussion points out the advantages of the latter in comparison to less rigorous techniques.

Simple Linear Regression—Assume a situation in which a variable Y is linearly dependent upon a second variable X. Examples of such a situation include both Eqs. 1 and 2. A general expression for such a relationship is $Y = \alpha + \beta X$. If Y could be measured without error for any value of X, α and β could be determined from two sample points and any further observations would fall on the determined line.

In practice, experimental error enters due to measurement error, biological variation, etc. Hence, an observed Y_i is related to its corresponding X_i by Eq. 3,

$$Y_i = \alpha + \beta X_i + \epsilon_i \qquad \text{(Eq. 3)}$$

where $\alpha + \beta X_i$ is the underlying relationship and ϵ_i is the error term. This situation is illustrated in Fig. 1 in which the dotted line represents the true but unknown relationship and the error is the vertical distance from the point to the dotted line. The statistician's problem is to estimate α and β. A number of methods are available such as: arbitrarily saying $\alpha = 47$ and $\beta = -10$, fitting the data by eye, and least-squares analysis. The first method is obviously of no value, the second might prove useful if rough guesses are desired; but if a thorough analysis including extrapolation, confidence statements, or tests of hypotheses is desired, a proper least-squares analysis must be performed to make better use of the data.

The conditions of a simple least-squares analysis are as follows: assume a sequence of n observation pairs $(Y_1, X_1), (Y_2, X_2), \ldots (Y_n, X_n)$ represented by the points in Fig. 1. If Eq. 3 holds for all $i = 1, \ldots, n$, and if further (*a*) the additive errors ϵ are independent of one another; (*b*) the distribution of the error term has mean zero; and (*c*) the variance of each ϵ_i is σ^2, a constant not depending upon i or X_i; then the "best" estimates (5) for α and β are those which will minimize the right-hand side of Eq. 4.

$$\sum_{i=1}^{n} \epsilon_i^2 = \sum_{i=1}^{n} (Y_i - \alpha - \beta X_i)^2 \qquad \text{(Eq. 4)}$$

In Fig. 1 the least-squares line is represented by the solid line and is the one which minimizes the sum of squared distances in the vertical direction from sample points to fitted line.

Note again assumptions (*a*) that the error ϵ_i is additive, and (*c*) that the ϵ_i's have a common variance; that is, dispersion of error is not proportional to X and hence to the expected value of Y.

The results of a least-squares analysis are not restricted to α and $\hat{\beta}$, the estimates of α and β, respectively. Equation 5 gives the variance

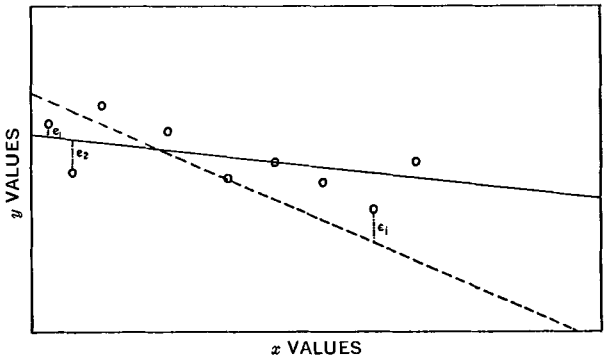

Figure 1—*Example of regression situation. Broken line represents true unknown relationship; solid line represents least-squares fit to data.*

of the estimate of β in which σ^2 is the variance of the ϵ_i's.

$$\sigma_{\hat{\beta}}^2 = \sigma^2 / \sum_{i=1}^{n} (x_i - \sum_{i=1}^{n} x_i/n)^2 \qquad \text{(Eq. 5)}$$

The smaller the variance of the estimate the greater is the confidence which can be placed in it being close to the true unknown parameter.

One further estimate which can be obtained is given by Eq. 6.

$$S^2 = \sum_{i=1}^{n} (Y_i - \hat{\alpha} - \hat{\beta} X_i)^2/(n - 2) \qquad \text{(Eq. 6)}$$

This is an estimate of σ^2, the variance of the error term. This estimate is just an average of the squared deviations of the sample points from the fitted line.

Application of Weighted Least-Squares to Stability Studies—Assume an experiment in which a drug has been stored at various temperatures. Assays are made throughout the period of the experiment to estimate concentration as a function of time. According to the Arrhenius relation a faster degradation is expected to occur at higher temperatures; hence, assays for the higher temperature data might be made more frequently but for a shorter period of time.

A simple least-squares analysis is made by fitting Eq. 1 to the data collected at each temperature τ to determine $\hat{k}_\tau$, the estimate of degradation rate for that temperature. The assumptions mentioned previously as required for a valid least-squares analysis will be sufficiently satisfied except for those nonzero-order reactions in which the range over which concentration varies is extremely large.

Along with each estimate of a k_τ is an estimate of the experimental error variance σ^2 as given by Eq. 6 and the coefficient by which σ^2 must be multiplied in order to obtain the variance of $\hat{k}_\tau$, the estimate of k_τ. This coefficient is given by

$$1/\sum_{i=1}^{n_\tau} (t_i - \bar{t})^2 \qquad \text{(Eq. 7)}$$

where t_i represents the times at which the assays are made, n_τ represents the number of assays made at temperature τ, and $\bar{t} = \sum_{i=1}^{n\tau} (t_i/n_\tau)$.

Figure 2 shows the least-square curves for data collected on chloramphenicol at temperatures 32, 34, 42, 58, and 71°. As each analysis yields an estimate of the same experimental error variance σ^2, these estimates can be combined to form one single estimate of error variance. This estimate will be used later in the analysis and will be referred to as the combined variance estimate denoted S_c^2.

Figures 3 and 4 illustrate the reason for using weighted least squares for fitting the Arrhenius relationship. Figure 3 is a plot of the various k_τ estimates against reciprocal absolute temperature using a linear scale on both axes. Each vertical line represents a 95% confidence interval for the corresponding k_τ. The horizontal mark indicates the point estimate. The width of each confidence interval depends upon the coefficient given by Eq. 7, that is the times and number of observations taken at each temperature.

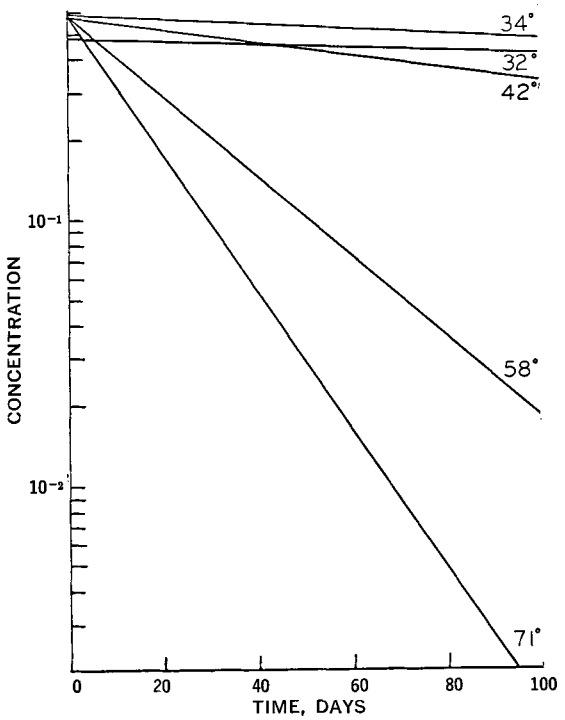

Figure 2—*Least-squares degradation of chloramphenicol data.*

Figure 4 is a plot of the same estimates and same confidence intervals, but drawn on semilogarithm paper. Hence the scale on the vertical axis is $\log_{10} k_\tau$. Note that the length of the confidence intervals for the k_τ at lower temperatures have been lengthened relative to the higher temperatures.

A simple least-squares fit, represented by the broken lines in Fig. 4, was applied to the model:

$$\hat{k}_\tau = e^{\gamma + \delta/\tau + \epsilon} \qquad \text{(Eq. 8)}$$

that is, $\ln \hat{k}_\tau = \gamma + \delta/\tau + \epsilon$ where $\hat{k}_\tau$ is the estimate of k_τ obtained in the previous analyses, and ϵ is an additive error to $\ln k_\tau$ with com-

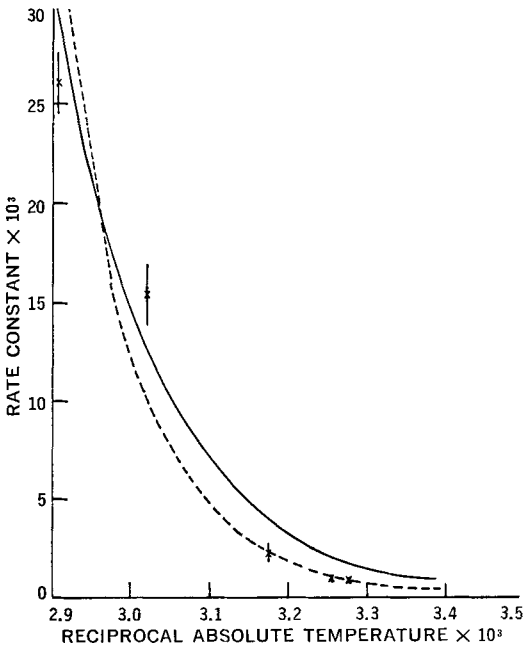

Figure 3—*Arrhenius fit to degradation constants with 71° data included. Vertical lines represent 95% confidence intervals. Solid line is the weighted least-squares line; broken line is the unweighted least-squares line.*

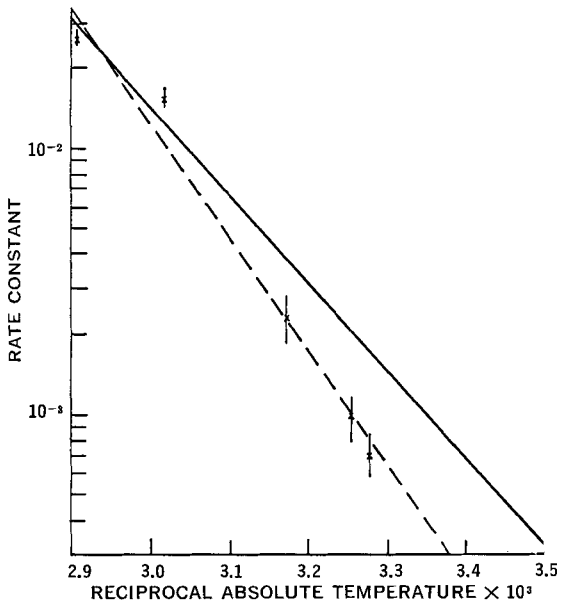

Figure 4—*Arrhenius fit to degradation constants with 71° data included. Vertical lines represent 95% confidence intervals. Solid line is the weighted least-squares line; broken line is the unweighted least-squares line.*

mon variance for all τ. But the true error in $\hat{k}_\tau$ is additive to k_τ, not ln k_τ, because of the method of derivation of $\hat{k}_\tau$; further, the variances of the errors in the $\hat{k}_\tau$ differ according to the coefficient given in Eq. 7. This is illustrated by the extreme difference in the lengths of the lines representing the confidence intervals in Fig. 4. The simple least-squares analysis assumes these lengths to be the same. The effect of the simple least-squares analysis is to force the Arrhenius equation through the low temperature data and essentially ignore the high temperature data. Hence, much more faith is placed in the point estimates of the low temperature $\hat{k}_\tau$ than is warranted. Finally, the usual confidence statements on extrapolated

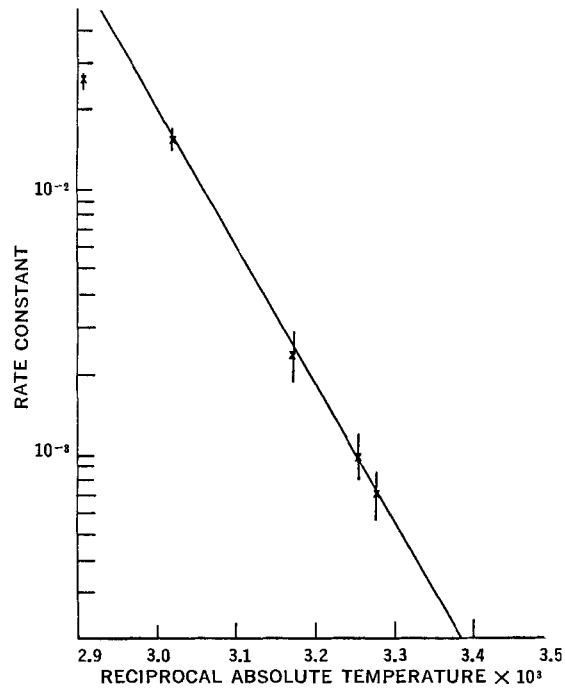

Figure 5—*Arrhenius fit to degradation constants with 71° data excluded. Vertical lines represent 95% confidence intervals; solid line is the weighted least-squares line.*

degradation rates (such as at room temperature) cannot validly be made.

Appendix 2 outlines justification for applying the method of weighted least squares to the model:

$$\hat{k}_\tau = e^{\gamma + \delta/\tau} + \epsilon_\tau \qquad \text{(Eq. 9)}$$

where ϵ_τ is now an additive error to k_τ in the estimate $\hat{k}_\tau$ and has variance σ_τ^2. The method of weighted least squares weights each ln $\hat{k}_\tau$ in inverse proportion to the square of the width of its confidence interval. The weighted least squares fit is represented by the solid lines in Figs. 3 and 4.

Statistical Test of Arrhenius Assumption—The benefits of the weighted least-squares analysis are many. First, the estimates of the parameters of the Arrhenius equation meet the statistician's requirements as the "best" which can be obtained. Second, a confidence interval can easily be constructed around the rate constant for any desired temperature, such as room temperature. Finally, a second estimate of σ^2, the variance of the original experimental error, can be obtained from the fit of the $\hat{k}_\tau$ to the Arrhenius equation. This estimate of variance will be independent of the combined variance estimate S_c^2 based on the original individual temperature analyses, and it will be referred to as the Arrhenius variance estimate and will be denoted S_a^2. Its formula is included in Appendix 1.

The advantage of the two estimates of error variance is as follows. S_a^2 is dependent upon the validity of the Arrhenius assumption. If the Arrhenius relationship does not hold, S_a^2 will tend to be large relative to the true experimental error variance, and therefore large relative to S_c^2 which does not depend upon the Arrhenius assumption. By dividing the Arrhenius variance estimate S_a^2 by the combined variance estimate S_c^2, one obtains an F statistic. By comparing the computed F with a tabled F, which can be found in any elementary statistics book (6), the experimenter can determine the validity of the Arrhenius assumption.

A significantly large F ratio would indicate the Arrhenius relationship does not hold. If the Arrhenius assumption is shown to be significantly invalid (that is the dispersion around the Arrhenius line is greater than could be attributed to chance variation in the $\hat{k}_\tau$), then the least-squares line is not valid and should not be used for predictive purposes. Further, no method of fitting a straight line would be valid for predictive purposes.

On the other hand, if the Arrhenius fit does not yield a significant F value, the high as well as low temperature rate constants fit the Arrhenius line within the measurement error involved in the $\hat{k}_\tau$, and hence all rate constants should be weighted according to their error variances.

Example—The above analysis was performed on a chloramphenicol solution. The results of the analysis are given in Table I. The initial analysis was performed at five temperatures: 32, 34, 42, 58, and 71°. Both the weighted and unweighted least-squares analyses were performed in order to make a comparison between the two methods. The two Arrhenius curves are presented in Figs. 3 and 4. Note that the estimate of degradation from the unweighted method for 23° would have been 2.91×10^{-4} and the analysis terminated at this point. This compares with an estimate of 8.01×10^{-4} computed by the weighted method on the same data.

At this point in the weighted analysis the F test was applied. The computed value was 36.95 with 3 degrees of freedom in the numerator and 167 in the denominator. This value is extremely significant ($F = 5.70$ is significant for $\alpha = 0.001$), indicating the Arrhenius assumption invalid. Upon investigation a precipitate was found to have formed in the 71° data soon after termination of collecting data at that temperature. Hence, these data were excluded from the analysis and another weighted least-squares analysis was performed. The F ratio testing the fit of the four remaining temperatures to the Arrhenius relationship yielded a value $F = 0.1110$ with 2 degrees of freedom for the numerator and 136 for the denominator. Such a small value indicates an extremely good fit to the Arrhenius relationship. The weighted least-squares line is plotted in Fig. 5. No analysis was performed by the unweighted method omitting the 71° temperature to emphasize the fact that the unweighted method would not have led to the detection of lack of fit to the Arrhenius relationship.

The new estimate of degradation rate at 23° was 2.12×10^{-4}. Of primary concern is how well this estimate, based on accelerated data collected over an 89-day period, compares with the value observed on production batches. The actual values observed on two separate

Table I—Results of Analysis on Chloramphenicol Study

Temperature, °C	Reciprocal Absolute Temperature	Computed from Raw Data: $\hat{\alpha}$	Computed from Raw Data: $\hat{K}_\tau \pm$ 95% Confidence Limits	K_τ Predicted from Arrhenius Fit: Unweighted with 71° Data	K_τ Predicted from Arrhenius Fit: Weighted with 71° Data	K_τ Predicted from Arrhenius Fit: Weighted without 71° Data
32	3.28×10^{-3}	−0.313	$7.03 \times 10^{-4} \pm 1.32 \times 10^{-4}$	7.96×10^{-4}	1.72×10^{-3}	7.02×10^{-4}
34	3.26×10^{-3}	−0.227	$9.72 \times 10^{-4} \pm 1.94 \times 10^{-4}$	9.87×10^{-4}	2.03×10^{-3}	9.07×10^{-4}
42	3.18×10^{-3}	−0.232	$2.36 \times 10^{-3} \pm 4.80 \times 10^{-4}$	2.28×10^{-3}	3.82×10^{-3}	2.45×10^{-3}
58	3.02×10^{-3}	−0.215	$1.55 \times 10^{-2} \pm 1.49 \times 10^{-3}$	1.07×10^{-2}	1.24×10^{-2}	1.54×10^{-2}
71	2.91×10^{-3}	−0.234	$2.61 \times 10^{-2} \pm 1.67 \times 10^{-3}$	3.39×10^{-2}	2.98×10^{-2}	6.12×10^{-2}
23	3.38×10^{-3}			2.91×10^{-4}	8.01×10^{-4}	2.12×10^{-4}

production batches, with more than 2 years of data on each, are 2.07×10^{-4} and 2.10×10^{-4}.

APPENDIX 1

Suppose $Y_i = X_{i1}\beta_1 + X_{i2}\beta_2 + \cdots + X_{im}\beta_m + \epsilon_i$ for $i = 1, 2, \ldots n$. Define Y to be the $n \times 1$ matrix $[Y_i]$, X the $n \times m$ matrix $[X_{ij}]$, β the $m \times 1$ matrix $[\beta_i]$, and ϵ the $n \times 1$ matrix $[\epsilon_i]$. Then the n observations satisfy $Y = X\beta + \epsilon$. Further, suppose the covariance matrix for ϵ is $\sigma^2 V$ for a known $n \times n$ matrix V and unknown scalar σ^2. That is the expectation of $[\epsilon\epsilon'] = \sigma^2 V$ where ϵ' is the transpose of ϵ.

The estimate of β is

$$\hat{\beta} = (X'V^{-1}X)^{-1}X'V^{-1}Y \qquad \text{(Eq. 10)}$$

The estimate of σ^2 is

$$S^2 = (Y'V^{-1}Y - Y'V^{-1}X\hat{\beta})/(n - m) \qquad \text{(Eq. 11)}$$

The covariance matrix for the $\hat{\beta}$ vector is

$$(X'V^{-1}X)^{-1}\sigma^2 \qquad \text{(Eq. 12)}$$

which can be estimated by replacing σ^2 by S^2. To estimate a value on the true line $Y_0 = X_{01}\beta_1 + X_{02}\beta_2 + \cdots + X_{0m}\beta_m = X_0\beta$, use the estimate:

$$\hat{Y}_0 = X_0\hat{\beta} \qquad \text{(Eq. 13)}$$

The variance of the estimate $X_0\hat{\beta}$ is

$$\sigma_{Y_0}^2 = X_0(X'V^{-1}X)^{-1}X_0'\sigma^2 \qquad \text{(Eq. 14)}$$

which can be estimated by replacing σ^2 by S^2. A $(1 - \alpha)$ 100% confidence interval on Y_0 can be constructed as

$$X_0\hat{\beta} \pm t_{n-m}^{(1-\alpha/2)}[X_0(X'V^{-1}X)^{-1}X_0'S^2]^{1/2} \qquad \text{(Eq. 15)}$$

where $t_{n-m}^{(1-\alpha/2)}$ is the point on Student's t distribution with $n - m$ degrees of freedom which is exceeded with probability $\alpha/2$.

To apply the weighted analysis to the degradation problem, first perform an individual analysis on the data at each temperature τ setting $V = I$, the $n \times n$ identity matrix. The matrix X is $n \times 2$ with each element of the first column one (1) and the second column the respective times t_i at which assays were made. Y is the vector of the appropriate function of concentration (for a first-order reaction, Y is the vector of the logarithms of concentration). The second element of the $\hat{\beta}$ vector in Eq. 10 is the estimate $\hat{k}_\tau$ of the rate constant.

Call ω_τ the second row, second column element of $(X'V^{-1}X)^{-1}$, $v_\tau = (\hat{k}_\tau)^2/\omega_\tau$ and S_τ^2 the computed value for Eq. 11 at temperature τ based on $n = n_\tau$ and $m = 2$.

The combined variance estimate for the weighted least-squares analysis is

$$S_c^2 = \sum_{\text{all } \tau}(n_\tau - 2)S_\tau^2 / \sum_{\text{all } \tau}(n_\tau - 2) \qquad \text{(Eq. 16)}$$

The method of fitting the Arrhenius relationship to the $\hat{k}_\tau$ is as follows. Set the Y vector to be the logarithms of the $\hat{k}_\tau$'s. The X matrix has each element of the first column one (1) and the second column the reciprocal absolute temperatures, *i.e.*, $1/\tau$. The matrix V, which is $n \times n$ where n is now the number of temperatures at which data were collected, has the corresponding v_τ on the main diagonal and zeros everywhere else.

The Arrhenius variance estimate S_a^2 is computed at this point by Eq. 11 where n is the number of temperatures and $m = 2$. The F statistic is

$$F = S_a^2/S_c^2 \qquad \text{(Eq. 17)}$$

having $n - 2$ degrees of freedom in the numerator and $\sum_{\text{all } \tau}(n_\tau - 2)$ degrees of freedom in the denominator. Reject the applicability of the Arrhenius relationship for large F.

Estimates of logarithm of rate constant at absolute temperature τ_0 can be made from the results of the Arrhenius fit by setting $X_0 = (1/\tau_0)$ in Eq. 13. A confidence interval for logarithm rate constant at τ_0 can be established by Eq. 15. Once these have been done the estimate of time for a given drug to degrade from an initial concentration C_0 to a minimal concentration C_m can be estimated and a confidence interval for length of time for such a degradation can be constructed.

APPENDIX 2

Consider a set of n observations $(y_1, x_1), (y_2, x_2), \ldots, (y_R, x_R)$ where x_i and y_i are related by

$$y_i = e^{\alpha+\beta x_i} + \epsilon_i \qquad \text{(Eq. 18)}$$

Denote by $\tilde{y}_i$ the true value of y associated with x_i; that is $\tilde{y}_i = e^{\alpha+\beta x_i}$. Then $\tilde{y}_i = y_i - \epsilon_i$. Expanding $\ln\tilde{y}_i = \ln(y_i - \epsilon_i)$ in a Taylors series about y_i gives

$$\ln\tilde{y}_i = \alpha + \beta x_i = \ln y_i - \epsilon_i\frac{1}{\tilde{y}_i} + \text{remainder} \qquad \text{(Eq. 19)}$$

where the remainder is a term of order $[\epsilon_i/\tilde{y}_i]^2$. Provided $\epsilon_i/\tilde{y}_i$ is much less than 1, the remainder can be ignored. This gives

$$\ln y_i \simeq \alpha + \beta x_i + \epsilon_i/\tilde{y}_i \qquad \text{(Eq. 20)}$$

where the new error term, call it ϵ_i^*, is now $\epsilon_i/\tilde{y}_i$. It follows immediately that the mean of ϵ_i^* is zero and the variance of ϵ_i^* is $\sigma_{\epsilon_i}^2/\tilde{y}_i^2$. Approximating $\tilde{y}_i$ by y_i gives the weighting used in the Arrhenius analysis described in Appendix 1.

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Whole Body Measurements of ^{131}I-Tetracycline as an Index of Skeletal Growth

R. G. WOLFANGEL*, S. M. SHAW, and J. E. CHRISTIAN

Abstract □ A derivative of tetracycline was tagged with ^{131}I and administered to rats. Whole body retention of the tetracycline was determined by sequential measurements of whole body radioactivity. Statistically significant differences of whole body burdens were found for two age groups of rats (100 g. *versus* 200 g.); the younger animals retaining a greater portion of the administered tetracycline. Subsequent distribution analysis indicated that whole body radioactivity measurements did not precisely assess skeletal burdens of ^{131}I-labeled tetracycline because variable amounts of tetracycline persisted in soft tissue for prolonged intervals after injection, but did provide estimates of skeletal burdens which could be used to recognize differences in skeletal growth rate between groups of young and mature rats. The whole body counting technique may be applicable for the study of metabolic skeletal disorders.

Keyphrases □ ^{131}I-Tetracycline—skeletal growth index, whole body measurements □ Growth, skeletal—index, radioiodinated tetracycline □ Paper chromatography—analysis, identity □ UV spectrophotometry—analysis, identity □ Scintillation counting, whole body—analysis

Since the initial observations of tetracycline-induced fluorescence of bones by Rall *et al.*, investigators have been examining tetracycline fixation in mineralized tissue (1). The following has been reported concerning the deposition of tetracycline in bones and teeth; deposition occurs after introduction by any route, but is greatest following parenteral administration (2); tetracyclines are actively deposited at all sites of newly mineralizing bone and are relatively permanently fixed in the bone until resorption occurs (3, 4). The quantity of tetracycline deposited in bone is proportional to animal age and the dose administered (5, 6), and the presence of tetracycline in bone or teeth can be readily detected by the appearance of a bright yellow fluorescence under UV irradiation (7, 8).

Tetracycline bone labeling, followed by microscopic measurements of the width, area, or volume of yellow fluorescent zones found in bone sections, is used as an index of skeletal metabolic activity (9–11) such as appositional growth rate, radial rate of osteon closure, and osteon maturation rate. Direct determinations of the total quantity of tetracycline bound to the skeleton might also provide an index of skeletal metabolic activity; if so, the necessity of skeletal biopsy, sectioning, and tedious fluorescence microscopy currently employed for tetracycline skeletal observations would be alleviated. Thus, ^{131}I-labeled tetracycline was prepared and used to conduct animal studies. Whole body measurements of tetracycline retention, following administration of labeled tetracycline, were investigated for possible value to assess skeletal metabolic activity in young growing rats as compared to older mature rats. The accuracy of whole body counting for the determination of the total quantity of tetracycline bound to the skeleton was established by the direct measurement of labeled tetracycline bound to the entire skeleton of the two age groups of rats, as well as the residual amount of tetracycline remaining in the soft tissue of the animals.

METHODS

Synthesis and Purity—Hlavka *et al.* (12) reported the preparation of 7-iodo-6-demethyl-6-deoxytetracycline (766 tet) by dissolving 6-demethyl-6-deoxytetracycline (66 tet) and *N*-iodosuccinimide (NIS) in concentrated sulfuric acid at 0°. By substitution of ^{131}I for stable iodine, 7-radioiodo-6-demethyl-6-deoxytetracycline (*766 tet) was prepared in this laboratory according to Hlavka's directions. The ^{131}I-label was introduced by the preparation of *N*-^{131}iodosuccinimide (N*IS) by modification of the method of Benson *et al.* (13). Aqueous solutions of Na^{131}I[1] (25–75 mc.) were transferred to a test tube containing 2 ml. of carbon tetrachloride, and 1 ml. of NaI carrier (5 mg./ml.) was added. The test tube was fitted with a rubber stopper through which a dropping pipet, filled with concentrated nitric acid, had been inserted. Nitric acid was then added to the water–carbon tetrachloride mixture. The closed tube was left for 18–24 hr., during which time free iodine was formed and dissolved in the organic liquid. The aqueous overlayer was removed with a micropipet allowing the $^{131}I_2$ in the carbon tetrachloride to remain in the test tube. Stable elemental iodine (1 g.) was placed in an amber 5-dr. vial, the cap lined with Teflon, and 5 ml. of sodium-dried, distilled dioxane added. The solution of carbon tetrachloride, containing $^{131}I_2$, was transferred to the vial. The test tube was rinsed with 1 ml. additional carbon tetrachloride, and the 1 ml.

[1] Purchased from Nuclear Science and Engineering Corp., Pittsburgh, Pa.