Accelerated Stability Model for Predicting Shelf-Life

Robert T. Magari,* Kevin P. Murphy, and Tracey Fernandez

Beckman Coulter, Inc., Miami, Florida

Second- and higher-order degradation reactions sometimes cannot be approximated with linear or exponential relationships and need to be appropriately modeled. Events above the COULTER[®] HmX Analyzer white blood cell (WBC) counting threshold were recorded for the HmX PAK reagent system stored at five elevated temperatures. An accelerated stability model for a second-degree polynomial degradation pattern was used. The shelf-life of the reagent, along with 95% lower bound confidence intervals, is predicted using the same pattern of degradation as well as the Arrhenius approximation. Experiments indicated that the degradation of the HmX PAK reagent occurred in two phases, the lag phase and the degradation

phase, in all tested temperatures. The phase durations are temperature-dependent, and the Arrhenius approximation is appropriate ($P = 0.639$). The degradation of the reagent during the lag phase was experimentally undetectable. Changes of the reagent were nonsignificant for a predicted period of 164 days at 25°C. The rate of degradation increased significantly later on during the degradation phase. The lower bound of the 95% confidence interval of this prediction indicated that it would take at least 326 days before the HmX PAK reagent would have any performance issue related to aging at storage temperature. *J. Clin. Lab. Anal.* 16:221–226, 2002.

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INTRODUCTION

Degradation is the change of the characteristics of a product as it ages. Nevertheless, the product is considered to be stable as long as its characteristics remain within the approved specifications of the manufacturer. The period of time that the product is stable depends on storage conditions. Products degrade faster when they are subjected to elevated stress conditions such as temperature, humidity, pressure, etc. The period of time that the product is stable at the recommended storage conditions is referred to as the shelf-life.

Real-time and accelerated stability tests are used to assess the shelf-life of products. Accelerated tests are preferred for industrial uses since they shorten the time required to get a product to market (1). The principle of accelerated stability testing is to expose a product to stress conditions in order to increase the rate of degradation (i.e., samples of the materials stored at elevated temperatures will degrade more rapidly than at normal temperatures). By measuring the degradation rate at high temperatures, and by modeling or assuming a degradation rate–temperature relationship, extrapolation at the storage temperature will predict the

shelf-life of the material. Thus, accelerated stability modeling consists of the following steps: 1) estimating the degradation rate at each elevated temperature, 2) relating the degradation rate to the temperature, and 3) predicting stability at the intended storage temperature.

Products show different degradation patterns as they age. King et al. (2) described three main types of degradation reactions: zero-, first-, and second-order. Only one kind of molecule is involved in zero- and first-order reactions, and the degradation rate is constant. The exponential degradation pattern in time of the first-order reaction is probably the most common in the literature, although at optimum storage conditions the differences between the patterns of the first- and the second-order reactions are indistinguishable (2–4). Furthermore, the degradation rate of the zero- and

*Correspondence to: Robert T. Magari, Beckman Coulter Inc., mail code 22-B02, 11800 SW 147 Ave., Miami, FL 33116-9015.

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first-order reactions can be related to temperature by the Arrhenius equation.

Statistical modeling is primarily related to the degradation pattern and the nature of the product. Least-squares or maximum-likelihood approaches are used to estimate the parameters (5). Maximum likelihood is generally preferred over the least-squares method because it can better accommodate nonlinear models, as well as models with random effects (6). Most pharmaceutical and biological models are based on the exponential pattern. However, in some biological products two or more molecules interact and the stress factor has a cumulative effect on degradation. Lower degradation rates observed at the beginning increase gradually with time. These second- and/or higher-order reactions may have specific degradation patterns that need to be appropriately modeled. Thus, the objective of the current work is to provide a statistical model for analyzing a second-degree polynomial degradation pattern with a time-dependent degradation rate, and for predicting shelf-life based on accelerated stability tests.

MATERIAL AND METHODS

Experiments

Experiments were conducted at the Beckman Coulter Inc. (Miami, FL) hematology laboratory. Analyses were performed with a COULTER[®] HmX Analyzer (Beckman Coulter Inc.), an automated hematology analyzer and white blood cell (WBC) five-part differential counter designed for in vitro diagnostic use. The reagent system for enumerating the WBC five-part differential was an HmX PAK reagent system that was intended to be stored at room-temperature conditions. The stability of the HmX PAK needed to be evaluated prior to introduction to the market.

Two lots of the HmX PAK were stored at each of the following elevated temperatures (T_E) ($n=5$): 60°C, 55°C, 50°C, 45°C, and 40°C; and at storage temperature (T_S): 25°C. During scheduled time periods, samples of each lot were removed from each T_E and tested with five fresh in-house donor blood specimens in duplicate on a COULTER[®] HmX Analyzer for events above the instrument WBC counting threshold, according to the manufacturer's protocol. Samples from each T_E tested at a particular date were paired-tested with samples from T_S on the same set of specimens. The statistics, $Y = C_E/C_S$, where C_E was the average WBC count of each individual specimen using the reagent stored at each T_E , and C_S was the average WBC count of each individual specimen using the reagent stored at T_S , were calculated for each time period. Data were electronically collected from the instrument and stored in a Micro-

soft[®] Excel 97 format. SAS[®] 8.1 (SAS Institute Inc., Cary, NC) was used for statistical analysis. The averages across blood specimens and lots for each time point were used for modeling.

Statistical Model

In general, the true degradation pattern (D) at any temperature as a function of time can be expressed as:

$$D = f(t, \beta) \quad (1)$$

Where t is the vector of observed time points (predictor variable) and β is the vector of the parameters. The numbers of parameters (dimensions) of β depend on the degradation pattern. For a second-degree polynomial degradation pattern:

$$\beta = \begin{bmatrix} \beta_0 \\ \beta_1 \\ \beta_2 \end{bmatrix}$$

Where β_0 is the lot average at time zero, and β_1 and β_2 are the linear and quadratic parameters, respectively. For the same pattern, D can be expressed as

$$D = \beta_0 + (\beta_1 + \beta_2 t)t$$

Where t is time. The observed response is

$$Y = D + \varepsilon = \beta_0 + (\beta_1 + \beta_2 t)t + \varepsilon \quad (2)$$

Where ε is the experimental error. All parameters of the model are considered to be fixed except for errors, which are random and assumed to be normally distributed, independent with $E[\varepsilon] = 0$ and constant $\text{Var}[\varepsilon] = \sigma_\varepsilon^2$. Thus, Y will be $N[\beta_0 + (\beta_1 + \beta_2 t)t, \sigma_\varepsilon^2]$.

The predicted stability at any temperature is obtained as

$$t_{\text{Stab}} = \frac{(-\beta_1 - \sqrt{\beta_1^2 - 4\beta_2(\beta_0 - Y_{\text{Crit}})})}{2\beta_2} \quad (3)$$

The value of Y_{Crit} depends on the type of product as well as the level of the response variable (Y) where the prediction is needed. $Y_{\text{Crit}} = 0.63$ was chosen as the failure point for stability for the HmX PAK reagent. This corresponds to the minimum reagent activity required for an accurate determination of the WBC five-part differential, according to the manufacturer's protocol.

The degradation rate at each temperature is a function of time, as $\delta = \beta_1 + \beta_2 t$, and is functionally related to temperature as described by an Arrhenius approximation (7).

$$\delta = A \exp\left(\frac{-E_a}{RT}\right),$$

Where A is an Arrhenius factor, E_a is the activation energy (kcal mol^{-1}), $R = 0.00199$ ($\text{kcal mol}^{-1} \text{K}^{-1}$) is the

gas constant, and T is temperature in Kelvin (K) = $^{\circ}C + 273$.

The ratio of the degradation rate at elevated temperature, δ_e , to the degradation rate at storage temperature, δ_s , is the acceleration factor (λ) calculated as

$$\lambda = \frac{\delta_e}{\delta_s} = \frac{A \exp\left(\frac{-E_a}{RT_E}\right)}{A \exp\left(\frac{-E_a}{RT_S}\right)}$$

$$\lambda = \exp\left[\frac{E_a}{0.00199} \left(\frac{1}{T_S} - \frac{1}{T_E}\right)\right]$$

Thus, the observed response of the temperature-accelerated model can be expressed as

$$Y = \beta_0 + (\beta_1 + \beta_2 t)\lambda + \varepsilon, \quad (4)$$

When λ , as a function of activation energy, is product-specific and is considered as fixed in the model. Since the other assumptions are the same as in Eq. [2], Y will be $N[\beta_0 + (\beta_1 + \beta_2 t)\lambda, \sigma_e^2]$.

The maximum likelihood approach available in PROC MLMIXED of SAS (8) is used for estimating the parameters of Eqs. [2] and [4], and their approximate confidence intervals. We selected the Gaussian quadrature method for approximating the integral of the likelihood, and the Newton-Raphson approach for optimization (6,8). The standard errors of the estimates were computed from the inverse of the Hessian matrix

(8). The predicted duration of the lag phase and shelf-life are obtained from Eq. [3]. Standard error of the time estimates and 95% confidence intervals are calculated from the variance-covariance matrix of the estimates of the parameters, using the delta method (9).

RESULTS AND DISCUSSION

Degradation at Elevated Temperatures

The trends of changes of Y vs. time for each T_E are represented by second-degree polynomial curves in Fig. 1. The estimates of the parameters of the polynomial model Eq. [2] and statistical testing are given in Table 1 for each T_E . Intercepts were not statistically different from 1 ($P > 0.05$), confirming the fact that C_E and C_S were the same (within experimental error) in fresh reagents. The inverse relationship between Y and time was clearly related to temperature (Fig. 1). The steepest decline occurred at higher temperature and the rate of decline decreased as temperature went down. The consistent pattern of degradation for all T_E is the first indication for the appropriateness of the Arrhenius relationship in our modeling (3,7).

While the observed Y trends for each temperature could be modeled by a second-degree polynomial, the kinetics of the reaction indicated that the degradation occurred in two phases (Fig. 1): a first phase in which the degradation was not experimentally detectable, and a second phase in which there was obvious degradation. We will refer to the first phase as the lag phase, and to

Fig. 1. Estimated degradation response for elevated temperatures.

TABLE 1. Estimates of the degradation of WBC count ratios vs. time as elevated temperatures using Eq. [2] and tests for intercept

Temperature °C	$\hat{\beta}_0$	$\hat{\beta}_1$	$\hat{\beta}_2$	H ₀ : Intercept (β_0) = 1	
				<i>t</i> -test	<i>P</i> -value
60	1.0211	0.00284	−0.00810	0.3448	0.7362
55	1.0043	−0.00568	−0.00175	0.0578	0.9544
50	0.9634	0.02032	−0.00161	−0.3817	0.7067
45	0.9925	0.01045	−0.00064	−0.1291	0.8987
40	1.0407	−0.00112	−0.00007	0.5352	0.5962

the second phase as the degradation phase. The expected value of *Y* was 1, if no degradation was present. The changes of *Y* during the lag phase were within the range of experimental error. The 95% confidence interval of *Y* = 1 was calculated based on the WBC count data from the first day of testing. The calculated lower bound of this interval was 0.95. Thus, the reagent was considered to be in the lag phase if $Y \geq 0.95$. Degradation in this phase was not experimentally detectable at most of the temperatures because of the relatively low degradation rates and limited number of days, particularly for higher storage temperatures. This pattern can be explained by examining the optimization of the HmX PAK reagent system (7). During optimization of the HmX PAK reagent system, increases in potency produced an increase in the WBC counts until the reagent was saturated with respect to increased WBC counts. Greater potency did not produce higher WBC counts. The optimum potency was set in this saturation zone.

The degradation phase began when *Y* dropped below 0.95. The number of days of the lag phase at each temperature is obtained from Eq. [3] by substituting $Y_{\text{Crit}} = 0.95$, which corresponds to the extreme level of the reagent performance in the lag phase. The number of days that the product was stable at each temperature is obtained similarly by substituting $Y_{\text{Crit}} = 0.63$, which corresponds to the reagent failure point. The estimated days for each phase clearly showed the effect of temperature on degradation (Table 2). Testing of the reagents at each T_E was stopped only after the reagent recovered below $C_E = 5,000$ a couple of times consec-

tively. Our model indicated that the reagent continued to degrade even after the failure point ($Y_{\text{Crit}} = 0.63$). We suspect that *Y* converged to a constant value and then, depending on storage temperature, leveled off and formed a plateau. However, data were not collected and modeled for this region because there was no interest from the product shelf-life prediction aspect.

Prediction of Shelf-Life

A polynomial degradation is also assumed for T_S , and Eq. [4] is used to obtain the estimates of the degradation parameters of WBC count ratios vs. time and predicting the numbers of days at storage temperature (Table 3). For a period of 164 days the degradation of the reagent at 25°C was considered to be nonsignificant (lag phase). Degradation changed significantly after that, but these changes did not affect the performance for a period of 356 days (Fig. 2). Since all of these predictive values were sampling estimates, they were computed with a statistical error. The value of the lower bound 95% confidence interval (Table 3) indicated that it would take at least 326 days before the reagent would have any performance issue related to aging.

An assumption in the accelerated stability tests is that a single degradation reaction takes place (7). This implies the involvement of only one kind of molecule in simple reactions that depend on temperature, as described by the Arrhenius equation. Since the interaction between two or more molecules was expected in our reactions, the appropriateness of the Arrhenius relationship needed to be statistically tested. Let $\hat{\delta}_e$ and $\hat{\delta}'_e$

TABLE 2. Estimated number of days and 95% confidence lower and upper boundaries for each phase at elevated temperatures

Temperature °C	Lag phase (days)			Stability (days)		
	Estimated	Lower	Upper	Estimated	Lower	Upper
60	3	1	5	7	6	8
55	4	0	8	13	12	14
50	13	9	18	22	20	24
45	20	16	24	33	30	36
40	28	17	39	67	58	76

TABLE 3. Estimates, standard errors, 95% confidence boundaries of the degradation parameters of WBC count ratios vs. time, and number of days at storage temperature (25°C)

Parameter	Estimate	Standard error	Lower	Upper
β_0	1.038000	0.0189900	1.000300	1.075600
β_1	-0.000010	0.0000048	-0.000020	-0.000004
β_2	-0.000003	0.0000003	-0.000004	-0.000003
Activation energy, E_a (kcal mol ⁻¹)	41.3749	0.0000530	41.3748	41.3750
Lag phase (days)	164	15.73	133	195
Shelf-life (days)	356	15.09	326	386

Fig. 2. Predicted degradation response and 95% confidence intervals for 25°C storage temperature.

denote the estimates of the degradation rates at each T_E calculated from Eqs. [2] and [4], respectively. The variances of the degradation rates were calculated as $s_{\hat{\beta}_1}^2 + t^2 s_{\hat{\beta}_2}^2 + 2ts_{\hat{\beta}_1, \hat{\beta}_2}$, where $s_{\hat{\beta}_1}^2$ and $s_{\hat{\beta}_2}^2$ are the variances of $\hat{\beta}_1$ and $\hat{\beta}_2$, $s_{\hat{\beta}_1, \hat{\beta}_2}$ their covariance, and t is time. We used $t = t_{stab}$ in our calculations. Furthermore, let s_{δ}^2 denote a pooled variance calculated from the variances of the degradation rates. For large samples, the ratio $\hat{\delta}_e - \hat{\delta}'_e / \sqrt{s_{\delta}^2}$ is approximately distributed as normal, and the X^2 statistic, as given below, is distributed as chi-square with n degrees of freedom if the Arrhenius relationship was appropriate.

$$X^2 = \sum \frac{(\hat{\delta}_e - \hat{\delta}'_e)^2}{s_{\delta}^2}$$

Degradation rates and variances used for calculating the X^2 statistic are given in Table 4. Our calculated $X^2 = 3.397$ had a P -value of 0.639, indicating that there was enough evidence to support the appropriateness of

TABLE 4. Degradation rates at elevated temperatures and variances for testing the Arrhenius model

Temperature °C	Degradation rates at t_{stab}		
	$\hat{\delta}_e$	$\hat{\delta}'_e$	s_{δ}^2
60	-0.0551506	-0.0548864	0.000116
55	-0.0324499	-0.0285602	0.000017
50	-0.0183954	-0.0151124	0.000007
45	-0.0097195	-0.0107919	0.000002
40	-0.0064019	-0.0058037	0.000001

the Arrhenius equation for the range of the reagent potency that prediction of shelf-life is needed.

Other Modeling Considerations

Our experimental design aimed at having uniform conditions for obtaining constant variances at each time point for each temperature. Most of the blood specimens responded similarly as the reagent aged. However, as the reagent approached the failure point at T_E , some

specimens exhibited a large significant interaction effect for C_E . Since the value of C_S did not show any interaction effect, the ratio $Y = C_E/C_S$ was significantly affected for that particular specimen, which increased or decreased the overall mean response at that time point. This was a characteristic of the reagent response to aging, hence it could not be considered as an outlier and removed from the data. In order to compensate for this effect, more blood samples and/or time points were analyzed. The increased number of samples minimized the influence of the observed interaction effect on the average Y response for that time point, while the increase of time points improved the trending that might have been distorted. Finally, analysis of the residuals did not show any evidence that the necessary statistical modeling assumptions were violated.

Lot-to-lot variability can be another factor in stability testing. This variability is a characteristic of the manufacturing process and is of a random nature. There are two components that contribute to this variability: lot-to-lot variability when no degradation is present, and different lot-to-lot responses due to aging (lot-to-lot variability of the degradation rates). While the first component is established by the manufacturing specifications prior to stability testing, the second one is usually unknown and must be estimated from the experimental data (10). The degradation pattern can be expressed as in Eq. [1], while some or all of the parameters of the β vector are considered as random with the necessary assumptions of their distributional properties (6,10,11). If it is significant, lot-to-lot variability affects confidence intervals and final stability predictions. The differences between two lots in our experiments were negligible, so we decided to pool them together and model the averages.

CONCLUSIONS

The degradation of the HmX PAK reagent system occurred in two phases: the lag phase and the degrada-

tion phase. The dependence of both phase durations on the temperature were approximated with the Arrhenius relationship. The estimated average duration of the lag phase was 164 days, and it was predicted that the reagent would perform within the specification for at least 326 days at the recommended storage temperature (25°C). The degradation of the reagent continued after the failing point, and theoretically it will reach a value where it will level off to form a plateau.

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