Determining Shelf Life by Comparing Degradations at Elevated Temperatures

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The degradation of a biological product follows a specific pattern that depends on the kinetics of the chemical reaction. For most biological and pharmaceutical products, accelerated stability tests are preferred to establish shelf life. We describe a new approach for accelerated stability tests, for situations in which the Arrhenius equation is not appropriate. This approach consists of estimating stability at elevated temperatures and comparing these results with the stability estimates for a similar product with a known shelf life. In this article, "Test" refers to Immuno-Trol™ Low Cells, and "Control" (the product with a known shelf life) refers to Immuno-Trol™ Cells. The degradation rates and stabilities at elevated temperatures of three antigens of the Test are estimated and compared to

their respective Control values. Most of the degradation occurs at the beginning of the experimental period and then slows down until it levels off to form a plateau at the minimum level. Both Control and Test showed similar degradation patterns at three elevated temperatures, indicating that they both have the same mechanism of degradation. Thus, it is expected that they will degrade similarly at storage temperature and have nonsignificantly different shelf lives. The approach for accelerated stability testing discussed here is applicable to situations in which the Arrhenius equation is not appropriate, and the chemical properties of both the Test and Control products are similar. *J. Clin. Lab. Anal.* 18:159–164, 2004. © 2004 Wiley-Liss, Inc.

Key words: accelerated stability; statistics; modeling; shelf life

INTRODUCTION

Product degradation is defined in terms of loss of activity or/and decreased performance with age. It follows a specific pattern depending on the kinetics of the chemical reaction. Chemical reactions that involve only one kind of molecule are referred to as simple reactions. Most of these reactions are described with linear or exponential relationships, and are referred to as zero- and first-order reactions (1). The degradation process does not depend on the number of intact molecules in the case of zero-order reactions, while it is proportional to the number of remaining intact molecules in first-order reactions (2). Second- and higher-order reactions, which are referred to as multiple interactions of two or more types of molecules, are characteristic for most biological materials that consist of large and complex molecular structures. It is common to approximate these reactions with exponential relationships; however, at times the degradation pattern must be specifically modeled.

The loss of activity in a unit of time is defined as the degradation rate. This rate depends on the required activation energy for the chemical reaction, and is

product-specific. The observed responses of different types of reaction orders are indistinguishable for products that degrade slowly. The degradation rate also depends on the environmental conditions under which the chemical reaction takes place. It is known that products degrade faster when they are subjected to stress conditions (acceleration factors) such as temperature, humidity, pH, etc. Temperature is probably the most common acceleration factor used in practice, since its relationship with the degradation rate is well characterized by the Arrhenius equation (2,3). More than one factor can be used to accelerate degradation, but it is necessary to characterize beforehand the interactions of the acceleration factors with the degradation rate (4).

Stability is commonly expressed in time units (e.g., hours or days). Even though product performance

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degrades during this time period, it is still considered to be stable as long as it remains within specifications. The length of time the product is stable at the recommended storage conditions is referred to as the shelf life. Experimental data from stability tests are used to estimate degradation parameters and assess shelf life (3,5). Two types of stability tests are used in practice: real-time tests and accelerated stability tests (5). Accelerated stability tests are preferred in industry because they reduce the time required to market a product. However, under certain conditions, the Arrhenius equation is not applicable for accelerated stability tests, particularly when higher-order reactions are involved. Sometimes the exponential relationship may not be an appropriate basis for approximating the degradation pattern of these reactions. In addition, the prediction of shelf life may be incorrect when the storage temperature is very different from the range of the accelerated temperatures used in the experiment, and/or the relationship between the temperature and the log of degradation rate is not linear.

The objective of this study was to provide an alternative approach to accelerated stability testing for situations in which the Arrhenius equation is not appropriate. Our approach consists of two steps: 1) degradation is modeled and stability is estimated at elevated temperatures, and 2) these estimates are compared with the stability estimates for another product with similar chemical properties and a known shelf life. We present several examples of applications, using real data.

MATERIALS AND METHODS

Experiments

Data from experiments conducted at the Beckman Coulter Inc. immunology laboratory in Miami, Florida, were used in this study. Stability tests were conducted to show that the shelf life of a new product is not different from that of the current product. In this study, Immuno-TrolTM Cells (Beckman Coulter) is the current product, and is referred to as the "Control." Immuno-TrolTM Low Cells reagent is the new product, and is referred to as the "Test." The Control is a single-level, assayed, whole-blood, quality-control product that provides positive cell control and is processed in the same manner as a normal whole-blood specimen. When this product is stored at 2–8°C, it has a known shelf life of 9 months. The Test is a quality-control product that can be used in a similar manner as the Control but provides a lower level of CD4 cells.

The two products were subjected to side-by-side stability testing. Three lots of Test and two lots of Control were each stored at elevated temperatures

(50°C, 45°C, and 37°C). During scheduled time periods, vials from each lot were tested on a COULTER[®] flow cytometer system (Beckman Coulter) with two reagents (R1 and R2). R1 is composed of CYTO-STAT[®] tetraCHROME[™] (Beckman Coulter) CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5, and R2 consists of CYTO-STAT[®] tetraCHROME[™] CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5. The mean fluorescence intensity (MFI) of the antigens CD3+CD8+, CD3+R1, and CD3+R2 were recorded. Vials from the Test sample were tested in parallel with vials from the Control sample at each time point. Data were electronically collected from the instrument and stored in a Microsoft[®] Excel 2000 format. SAS[®] 8.1 (SAS Institute Inc., Cary, NC) was used for statistical analysis.

Statistical Analysis

The true degradation pattern as a function of time at each elevated temperature is expressed as:

$$D = \alpha[1 + \beta_0 \exp(-\beta_1 t)]$$

Where, t is time, β_1 is the degradation rate, $\alpha(1 + \beta_0)$ is the expected MFI at time zero, and α is the minimum MFI after all the degradation has occurred. Products were considered to have the same degradation pattern at each elevated temperature. The observed degradation at each temperature can be expressed as

$$Y = D + \varepsilon = \alpha[1 + \beta_0 \exp(-\beta_1 t)] + \varepsilon, \quad (\text{Eq. 1})$$

where ε is the experimental error. All parameters of the model are considered to be fixed except for errors, which are considered to be random, normally distributed, and independent, with $E[\varepsilon] = 0$ and constant $\text{Var}[\varepsilon] = \sigma_\varepsilon^2$. Thus, Y will be $\text{NOR}[D, \sigma_\varepsilon^2]$.

The predicted stability at any temperature is obtained as

$$t_{\text{sub}} = \frac{\log(Y_{\text{Crit}} - \alpha) - \log(\alpha) - \log(\beta_0)}{-\beta_1}. \quad (\text{Eq. 2})$$

The value of $Y_{\text{Crit}} = \alpha(1 + \beta_0)/2$ is chosen as the failure point for stability, and $\log$ is the natural logarithm. SAS 8.01 (SAS Institute Inc., Cary, NC) is used for data analysis. The parameters of the nonlinear Eq. [1] are estimated based on a maximum likelihood approach (SAS) (6). The Gaussian quadrature method is used for approximation of the integral of the likelihood, and the Newton-Raphson approach is used for optimization (SAS). The standard errors of the estimates are computed from the inverse of the Hessian matrix. The predicted stabilities are obtained from Eq. [2], and their standard errors and 95% confidence intervals are calculated from the variance-covariance matrix of the estimates, using the delta method (8).

RESULTS

Degradation rates and stabilities in hours are given in Tables 1–3 for each individual antigen. The effect of temperature on degradation rates is highly significant and is particularly evident when the reagents are stored at 50°C, where they degrade within 4–23 hr, depending on the antigen. We decided to pool the lot data together since all of them recovered within the expected specification of $\pm 10\%$ at time zero. This variability, along with the imprecision of the

measurements, is accounted for in the confidence intervals of each estimate. The CD3+CD8+ antigen degrades faster than the CD3+R1 and CD3+R2 antigens at all three temperatures, while the last two antigens have nonsignificantly different degradations (Fig. 1). All *P*-values in Tables 1–3 are >0.05 and 0.01 , indicating that, for the respective 95% and 99% confidence levels, there are nonstatistical differences between the stabilities of the Control and Test reagents for each elevated temperature and antigen (Fig. 2).

TABLE 1. Degradation rates and hours of stability for CD3+CD8+ antigen

Temperature (C°)	Parameters ^a	Control lots				Test lots				Comparison (<i>P</i> -value)
		Estimate	Standard error	Lower	Upper	Estimate	Standard error	Lower	Upper	
37	β_1	0.001	0.0002	0.001	0.002	0.001	0.0001	0.002	0.002	0.973
	t_{Stab}	749	115	512	985	752	72	606	897	0.982
45	β_1	0.019	0.0025	0.014	0.024	0.016	0.0013	0.019	0.016	0.361
	t_{Stab}	54	5	43	65	62	4	54	69	0.268
50	β_1	0.206	0.0748	0.033	0.078	0.241	0.0455	0.342	0.140	0.692
	t_{Stab}	5	2	2	9	4	1	3	5	0.387

^a β_1 is the degradation rate and t_{Stab} are hours of stability.

TABLE 2. Degradation rates and hours of stability for CD3+R1 antigen*

Temperature (C°)	Parameters ^a	Control lots				Test lots				Comparison (<i>P</i> -value)
		Estimate	Standard Error	Lower	Upper	Estimate	Standard Error	Lower	Upper	
37	β_1	0.001	0.0002	0.000	0.001	0.001	0.0002	0.000	0.001	0.815
	t_{Stab}	1,614	559	463	2,764	1,474	378	709	2,239	0.837
45	β_1	0.004	0.0011	0.001	0.006	0.003	0.0009	0.002	0.005	0.955
	t_{Stab}	269	50	165	373	281	38	204	359	0.845
50	β_1	0.052	0.0198	0.007	0.096	0.058	0.0233	0.006	0.011	0.832
	t_{Stab}	19	4	9	29	16	3	10	23	0.664

*R1 is composed of CYTO-STAT[®] tetraCHROME[™] CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5.

^a β_1 is the degradation rate and t_{Stab} are hours of stability.

TABLE 3. Degradation rates and hours of stability for CD3+R2 antigen*

Temperature (C°)	Parameters ^a	Control lots				Test lots				Comparison (<i>P</i> -value)
		Estimate	Standard Error	Lower	Upper	Estimate	Standard Error	Lower	Upper	
37	β_1	0.001	0.0001	0.001	0.001	0.001	0.0002	0.000	0.001	0.731
	t_{Stab}	1,288	138	1,003	1,572	1,167	211	739	1,595	0.634
45	β_1	0.004	0.0015	0.001	0.007	0.003	0.0004	0.002	0.004	0.864
	t_{Stab}	247	21	202	291	275	14	247	303	0.266
50	β_1	0.037	0.0296	0.029	0.104	0.085	0.0107	0.060	0.109	0.153
	t_{Stab}	23	7	8	38	11	1	10	13	0.092

*R2 is composed of CYTO-STAT[®] tetraCHROME[™] CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5.

^a β_1 is the degradation rate and t_{Stab} are hours of stability.

Fig. 1. Degradation patterns of CD3+CD8+, CD3+R1, and CD3+R2 Test reagents stored at 45°C.

Fig. 2. Degradation patterns of CD3+R1 antigen, Test, and Control reagents stored at 45°C and 50°C.

The estimate of the initial MFI value at time zero varies because of experimental error. Antigen-to-antigen differences are more evident (Table 4). CD3+CD8+ has an average MFI of 15.3 at time zero, as compared to 23.7 for CD3+R1 and 21.8 for CD3+R2. These values may be affected by lot-to-lot variation, but should be within the expected $\pm 10\%$ for a process under control (5). The estimated minimum MFI value after all degradation has occurred also depends on the antigen, and is positively correlated to the initial value. CD3+CD8+ has an average minimum MFI of 3.4 as

compared to 4.5 for CD3+R1. CD3+CD8+ was the fastest-degrading antigen in this study, and was also the first to reach the minimum MFI. The number of hours to reach minimum (t_{Min}) are obtained by iteratively solving for different values of t in $\hat{Y} = \hat{\alpha}[1 + \hat{\beta}_0 \exp(-\hat{\beta}_1 t)]$, where $\hat{\alpha}$, $\hat{\beta}_0$, and $\hat{\beta}_1$ are the estimates of the respective parameters. The t_{Min} is equal to that t for which the difference of $\hat{Y}$ values of two consecutive iterations is $< 10^{-2}$. The t_{Min} is highly dependent on the temperature. CD3+CD8+ will perform within specifications for about 4–5 hr at 50°C, but

TABLE 4. Initial and minimum MFI values, and number of hours to reach the minimum MFI value for each antigen

Antibody ^a	Parameters ^b	Control lots			Test lots			Comparison (<i>P</i> -value)		
		37C°	45C°	50C°	37C°	45C°	50C°	37C°	45C°	50C°
CD3+CD8+	$\alpha (1 + \beta_0)$	15.1	15.1	14.9	15.7	15.4	15.7	0.064	0.486	0.552
	α	3.3	3.3	3.9	3.6	3.1	3.1	0.855	0.854	0.610
	t_{Min}	7,436	536	48	6036	636	48			
CD3+ R1	$\alpha (1 + \beta_0)$	23.6	24.1	24.9	23.4	23.2	23.2	0.813	0.356	0.323
	α	4.4	4.4	4.7	4.6	4.5	4.3	0.967	0.989	0.925
	t_{Min}	14,436	2,936	168	16,336	3,036	168			
CD3+ R2	$\alpha (1 + \beta_0)$	22.9	22.7	22.3	21.4	20.9	20.9	0.000	0.000	0.433
	α	3.6	3.4	3.0	3.6	3.5	3.8	0.998	0.985	0.926
	t_{Min}	9,936	3,036	240	9,636	2,336	120			

^aR1 is composed of CYTO-STAT[®] tetraCHROME[™] CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5, and R2 is composed of CYTO-STAT[®] tetraCHROME[™] CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5.

^b $\alpha (1 + \beta_0)$ is the initial MFI at time zero, α is the minimum MFI after all the degradation has occurred, and t_{Min} is number of hours to reach the minimum.

Fig. 3. Degradation patterns of CD3+CD8+ Test reagent stored at elevated temperatures.

it will continue to degrade for another 43 hr until it reaches a minimum MFI that plateaus around 3.4 (Fig. 3).

DISCUSSION

Because of the complexity of the chemical systems involved, most of the assumptions required for the use of the Arrhenius model are not satisfied, and therefore comparisons with a product of known stability are used. This approach requires the use of a similar product with a known shelf life as a control. The control and the new product are then tested side by side at different elevated temperatures. The new product and the control are expected to behave similarly at elevated

temperatures because they pertain to the same family and thus exhibit comparable degradation kinetics. The model that best fits the data is then selected to represent the degradation pattern. However, it is necessary to assume that the same model can represent degradation at each elevated and storage temperature. This implies that the form of *D* should be the same regardless of the temperature level, while the value of the parameters of *D* should change according to the temperature. The same assumption is used in the case of the Arrhenius model. The hypothesis that the Test and Control degrade the same at elevated temperatures can be statistically tested with the data collected at the elevated temperatures. Once the null hypothesis is not rejected, it is possible to assume that

the Test and Control will degrade in a similar manner at the storage temperature. The closer the elevated temperatures are to the storage temperature, the more this holds true.

In our accelerated stability tests, Control and Test pertain to the same family of reagents and the same function (different parameter values) is used to fit their degradation trends at each of the three elevated temperatures. They also show nonsignificantly different degradation rates at each elevated temperature, which indicates that they both have similar mechanisms of degradation. Additionally, there is no evidence that the CD4 antigen behaves differently at the different concentration levels. Thus, it is expected that Control and Test reagents will degrade similarly at storage temperature and have nonsignificantly different shelf lives. Since shelf life is based on a 95% estimated upper bound confidence interval of the degradation rate (5), it can be safely concluded that the Test reagent has at least 9 months of stability at storage temperature. Lot-to-lot variability at time zero may affect the expiration date of the product. A lot with lower MFI values at time zero may reach the failure point sooner than a lot with a higher MFI at zero time, even though they may have the same degradation rates. This type of variability is controlled by the manufacturing specifications and is considered in shelf-life specifications. Lots that perform outside these specifications at time zero are discarded. Lot-to-lot variability of degradation rates is another potential factor that may affect the expiration date (5), but it is more difficult to control by specifications. There is no evidence that such variability was significant in our experiments.

Most of the degradation of the reagents occurs at the beginning of the process and then slows down until it levels off and plateaus at a minimum MFI value. The time to reach the minimum is a function of temperature, but it also depends on the activation energy (Table 4). In contrast, the minimum MFI value does not depend on temperature. Table 4 and Fig. 2 show that the estimates of minimum MFI vary slightly due to the sampling error, but are consistent at the three elevated temperatures. In most stability tests, data for this region are not

collected because it is not relevant to the estimation of product shelf life.

Equation [1] considers a degradation situation in which the degradation rate at a particular time is proportional to the amount of degradation that will subsequently occur. This function decreases steadily from the initial point $\alpha(1 + \beta_0)$ to a limiting value α , and has no points of inflections. Equation [1] can be paralleled with the simple exponential model commonly used to fit first-order reaction kinetics, because they both have a constant degradation rate: β_1 (1,5). However, as time increases, Eq. [1] better fits the degradation of the Immuno-Trol family of reagents, particularly in the region where the degradation plateaus.

In conclusion, the present approach for accelerated stability tests is applicable to situations in which the Arrhenius equation is not appropriate and the products have similar chemical properties. The degradation function and the similarity of products must be determined accordingly. In addition, real-time stability studies must be carried out to confirm the results from accelerated testing. Further work is needed to develop models and estimate parameters for cases in which a significant lot-to-lot variability is present in the data.

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