The application of MicroCal VP Capillary DSC at a contract development organization

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MICROCALORIMETRY

PROTEIN STABILITY

Introduction

Although biopharmaceuticals represent a rapidly growing segment for ethical products, the ability to progress these products from research to development and ultimately manufactured products can be hindered by the complex structures and multiple degradation pathways that are associated with biopolymers.[1,2,3]

Traditional pharmaceutical development has often relied on real-time stability studies and standard analytics to develop product formulations that confer the two year shelf life typically required for drug products. Although these approaches have proven useful, there is a need to develop and apply techniques that report on the structural stability of biopharmaceuticals and reduce the need to rely on real-time stability studies so extensively. Differential scanning calorimetry, which monitors the apparent excess heat capacity of a protein solution as a function of temperature, is one of several tools that have been proven to be useful in this setting. [4,5] Even when used in a comparative or rank-ordered fashion DSC can provide data on protein stability that correlates well with real-time stability studies.

A key element in the ability to apply DSC or any other technique that may report on protein structure or stability is an organized approach that allows for the rigorous statistical evaluation of preformulation data. Recent regulatory guidance[6] has highlighted the need both for well-characterized biopharmaceutical products as well as a systematic development approach that allows for the understanding of both critical parameters that affect product stability as well as any interactions between those factors that may exist. Here we describe a systematic approach to preformulation development that leverages biophysical characterization and standard analytics in the context of statistical design with the goal of shortening the pharmaceutical development cycle while improving the quality of the development studies.

Tools for pharmaceutical development

A variety of techniques that are capable of monitoring either protein secondary or tertiary structure have been extensively used in support of protein preformulation development; although this is not an exhaustive list, these include differential scanning calorimetry (DSC), circular dichroism (CD), Fourier-transform infrared spectroscopy (FTIR), and fluorescence spectroscopy.[7,8,9] While each of these techniques has its advantages and disadvantages, we have found DSC to be one of the more generally applicable biophysical techniques for preformulation development for the following reasons: most proteins exhibit measureable thermal transitions during unfolding, the technique is relatively insensitive to a variety of potential buffer components and there is no requirement to know the protein structure or relative proportion of alpha-helical or beta-sheet segments in order to generate useful preformulation data.

In addition to DSC or other biophysical techniques, however, standard analytical techniques are also employed to more fully understand product quality in the context of accelerated stability studies. The suite of analytical tools typically includes size exclusion chromatography (SEC) for the detection of aggregates, ion exchange (IEX) for monitoring charge variants and deamidation, peptide mapping with mass spectrometry for chemical degradants and either SDS-PAGE or capillary gel electrophoresis (CGE) for monitoring covalent aggregates and degradants.

The system that our organization employs extensively to provide an organized framework for experimental design and data interpretation is design of experiments (DOE),[10] because it maximizes the quantitative nature of DSC and many of the standard analytical techniques used during preformulation. DOE allows for the identification and statistical assessment of critical factors and their interactions and an example of a DOE interaction plot is shown in Figure 1. The data in Figure 1 illustrate the effect of buffer type and pH on aggregation as assessed by size exclusion chromatography. Had either buffer system alone been used for the pH study, one would have incorrectly concluded that pH either did or did not have an effect on aggregation; however, by conducting a DOE it was possible to demonstrate that an interaction exists between pH and buffer type which was helpful in ruling out one buffer system for subsequent studies.

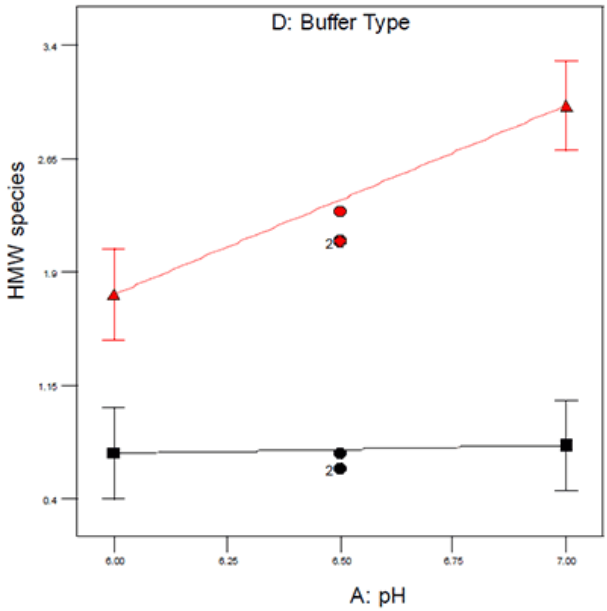


Figure 1: Interaction plot showing the effect of buffer type on aggregation. For each sample, the percent high molecular weigh (HMW) species were summed and then reported in the statistical design as a function of pH and buffer type. For the first buffer system (shown in red), the percent HMW species varies as a function of pH and is generally higher than is observed for the second buffer system. The black data points show the same data for the second buffer system, but in this case the percent HMW species is generally lower and also does not appear to vary as a function of pH, suggesting that buffer two is a more suitable formulation both from a stability standpoint and from a manufacturing standpoint, since in this buffer system the pH would not have to be as tightly controlled to produce a product of acceptable quality.

The type of design selected usually reflects the level of understanding and/or place in the product development lifecycle for a particular therapeutic: for pre-clinical products or products where there is limited information, factorial or fractional factorial designs are used to identify critical factors/interactions from a larger set of potential factors. For later-stage products or those products where some initial studies to identify critical factors have been performed, response surface designs such as central composite or Box-Behnken designs are more suitable because those designs offer greater granularity within the design space and can be used to model complex or quadratic surfaces.

Platform preformulation

Workflow for Preformulation Development

In addition to the desire to more rapidly develop products, biopharmaceutical manufacturers also have a strong desire to leverage core competencies and create economy of scale across multiple products and processes. For preformulation activities in a contract development setting, this has evolved into a defined workflow designed to clarify scope, timelines and deliverables at each point in the development process. A high-level example of a typical preformulation project is shown in Figure 2 and will be discussed in more detail in the following sections:

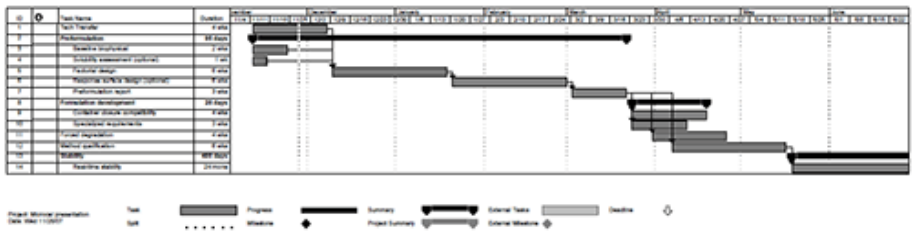


Figure 2: Example Workflow for Preformulation Projects.

Baseline Biophysical Studies

During the initial stages of preformulation development, several candidate biophysical techniques are employed as part of a “baseline biophysical” study (Figures 3 and 4) to assess their utility and those techniques found to be suitable for a particular molecule are then used as part of a screening design. Because of its general applicability to a wide variety of proteins and tolerance for a large number of buffers and excipients, DSC is almost always found to be suitable for both the initial and subsequent preformulation studies. The goals of this initial task are twofold: first it allows identification of the most suitable techniques for a particular molecule while giving the formulation scientist a chance to optimize and standardize acquisition parameters and more importantly, it allows the scientist to eliminate categoric factors that do not appear to be suitable for a particular molecule so subsequent designs are centered around buffer, pH and ionic strength regimes where the molecule appears to be most stable.

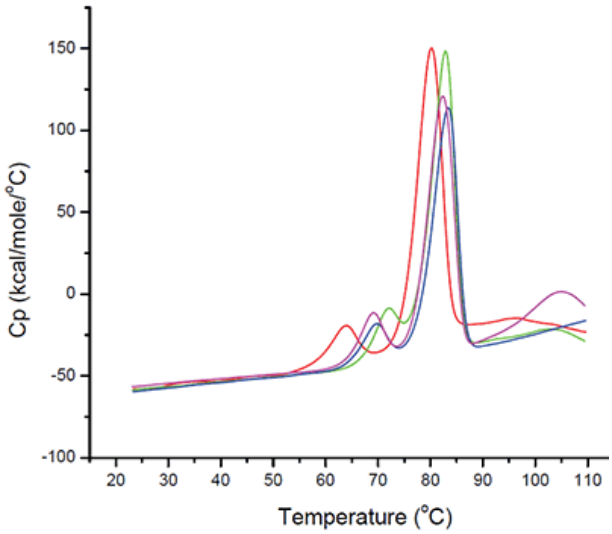


Figure 3: Differential Scanning Calorimetry for several different buffer types. The selected monoclonal antibody was exhaustively dialyzed into four different buffer types prior to analysis. Sample concentrations were approximately 2 mg/mL with a scan rate of 60°C/hr. Data presented here are after buffer subtraction and concentration normalization

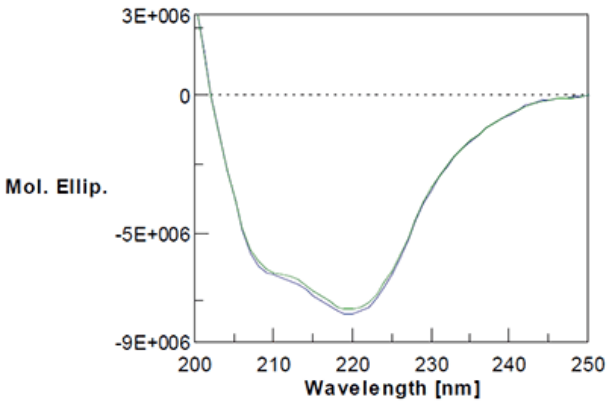


Figure 4: Example circular dichroism scans for baseline biophysical screening. Although CD is not as generally applicable as DSC because of potential buffer incompatibilities, certain proteins lend themselves well to CD analysis. Data above show far-UV CD scans of a protein that contains an appreciable amount of alpha-helix, a secondary structural element for which CD is well-suited.

Solubility

Increasingly, sponsors are requesting solubility studies to generate high concentration (>100 mg/mL) therapeutics because more concentrated drug products allow in-home administration as opposed to restricting administration solely to a clinical setting. While appealing from a marketing point of view, the need for high concentrations where the same dose is delivered in a small volume places extreme demands on the stability of the protein therapeutic. These studies often employ a variety of pH and buffer candidates, typically with a variety of potential excipients.

It is important to note, however, that conditions where the molecule is most soluble do not necessarily reflect conditions where the therapeutic is most stable.

Statistical Design

Statistical design has gained increasing regulatory[6] and industry[11,12] acceptance, and both the FDA and ICH recommend the use of statistical design during pharmaceutical development to better characterize critical factors that may impact product stability as well as interactions between those factors which are not discernable using a one factor at a time (OFAT) approach. There are two general classes of statistical designs that are frequently employed during development: factorial designs and response surface designs. Factorial designs are used when the primary goals of the study are to identify the critical factors and their interactions. Response surface designs, by contrast, are more often used later during development when critical factors have been identified but the development scientists need to optimize the process and establish suitable ranges for manufacturing. Because of its excellent repeatability and intermediate precision, DSC fits well with either of these statistical designs and even factors that have fairly subtle effects on product stability can be identified and optimized.

For each numeric factor in the design, factorial designs are typically set up as low (-) and high-coded (+) values that are decided by the user based on a series of initial studies (in this case the baseline biophysical studies). It follows for a two-level factorial design with all numeric factors that the number of runs is given by:

No. runs = 2x where x = the number of factors

For a two-level three factor design, this would translate into a minimum of eight runs. For any given factor, however, runs that are set to each level are averaged to estimate the effect of that factor using the equation below, meaning that an eight-run DOE has the statistical power of an OFAT experiment with sixteen runs:

$$Effect = \frac{\sum Y_+}{n_+} - \frac{\sum Y_-}{n_-}$$

where Y is the response at the low (-) and high (+) levels and n refers to the number of data points collected at each level In addition to the core design, however, it is advisable to employ three to four center points to estimate curvature within the design space and to allow for a more robust estimation of error.

Central composite designs are response surface designs that are essentially “augmented” factorial designs: they are built around a core factorial design to which has been added a series of “star” or axial points that project outside of the core factorial design space and more easily allow for the identification of effects because they span a wider range than is included within the factorial design.

Additional replicates are also added at the center points that allow for error estimation.

Between the center points, factorial design and axial points, central composite designs have a total of 5 levels that are varied for each individual factor, giving a very high degree of granularity to these designs that allows the use of quadratic functions during the optimization phase of preformulation.

Either the factorial or central composite designs are applied as accelerated stability studies where the stress conditions have been selected based on the thermal transitions for a particular therapeutic. For antibodies, there are typically two to three unfolding transitions corresponding to the C_H2, C_H3 and FAb domains, respectively and the stress temperature employed for the study is targeted to be below the onset of the first observed unfolding transition. The goal of the study is to induce the maximum amount of stress without entering the region where an antibody or other therapeutic would begin to unfold because unfolding may induce degradation or stress that is not indicative of stability under normal storage conditions. Operationally this means that the DSC unit must exhibit excellent baseline stability to ensure that these initial unfolding events can be distinguished from baseline fluctuations.

Once the accelerated stability/preformulation phase of a project is complete, the formulation development activities such as syringeability and container closure compatibility are conducted to ensure that the proposed product formulation can be administered as intended and that there are no major compatibility problems with the container closure system.

Case study

The following study was conducted to develop a high-concentration monoclonal using the general approach outlined above. Initial preformulation development began with a baseline biophysical study that evaluated the effect of buffer type/pH against a standard panel of excipients (Figure 5), each of which represents a particular class or type of excipient (ionic, polyol, disaccharide, etc.).

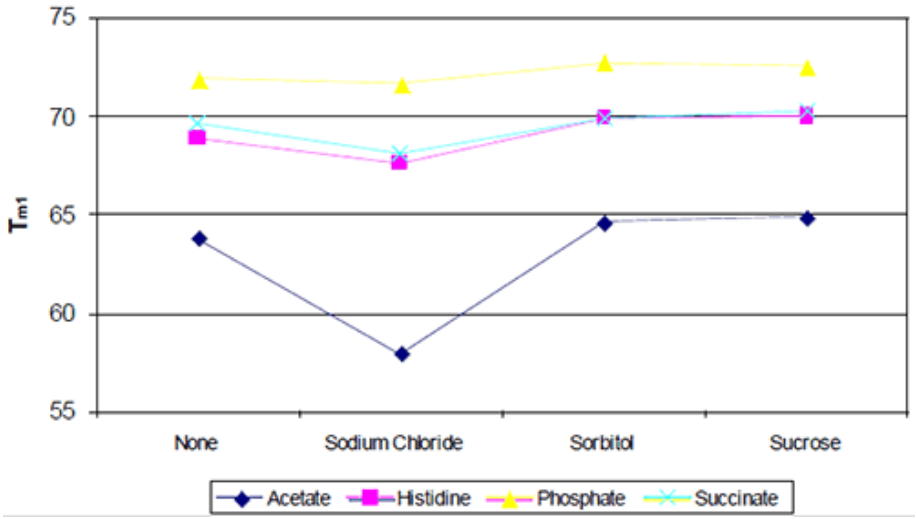


Figure 5: Temperature of the first unfolding transition (T_m1) as a function of buffer type & excipient. The study presented above is typically input as a general factorial design. Although this design type does not lend itself to numerical optimization, it is particularly good at screening a large design space and ruling out buffer types and excipients that do not appear suitable

Even based on a simple rank-ordering approach, it was apparent that acetate did not appear to be a suitable buffer system. Although the effect of sodium chloride on the initial thermal transition was generally moderate, in the acetate buffer system there appeared to be an interaction which made the combination of acetate and sodium chloride poor from a thermal stability point of view. Example thermograms for the acetate and histidine buffer systems are shown in Figure 6.

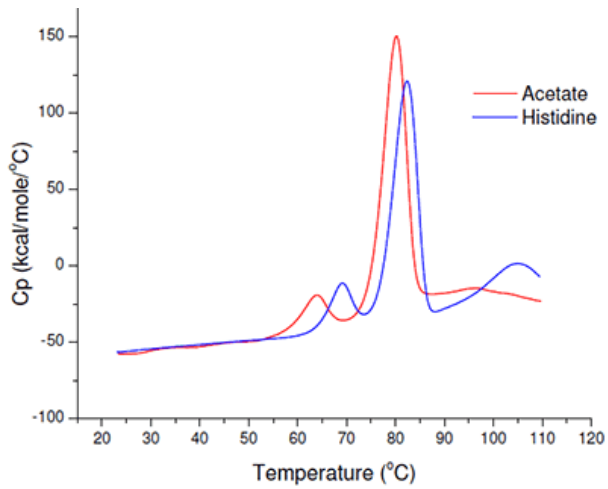


Figure 6: Example; thermograms for a monoclonal antibody. The data shows selected thermograms as a function of buffer type. Additional studies were performed to confirm that the first observed transition was reversible (data not shown).

Although phosphate-containing buffers appeared to show the best thermal stability, standard analytics conducted during accelerated stability indicated that

the phosphate buffer system was not as suitable as formulations containing histidine.

Based on these data and on previous studies, a response surface design was constructed that was used to optimize the pH, buffer concentration and excipient concentration within a more narrow design space (Table 1).

Table 1: Statistical Design for Formulation Optimization. The design above is a central composite design where for the core factorial design, pH is varied from 5.25 – 6.75, buffer concentration is varied from 20 – 50 mM, and excipient concentration is varied from 80 – 150 mM. Axial points outside of this core design space facilitate identification of critical factors

pH	Buffer Conc. (mM)	Excipient Conc. (mM)
6	35	115
6	35	115
6.75	20	150
5	35	115
6.75	50	80
5.25	50	150
6	35	115
5.25	20	80
5.25	20	150
6.75	20	80
6	35	115
6	55	115
6	15	115
7	35	115
5.25	50	80
6	35	50
6	35	115
6.75	50	150
6	35	115
6	35	180

Since some of the lower temperature stress conditions from early studies did not show significant degradation, DSC data was used to target a temperature (50°C) that would offer the maximum chance of stressing the molecule without partially

unfolding the antibody. DSC analysis was performed on all samples at time zero and for the center point formulations after four weeks accelerated stability. For all other analytical techniques, analysis was performed on control (4°C) and stressed (50°C) samples following a four-week incubation (Table 2).

Table 2: Testing Summary for Accelerated Stability Study. The assays listed in this table represent a typical set of assays that might be used in support of an accelerated stability study; however, other assay platforms such as capillary gel electrophoresis (CGE) are excellent substitutes for less quantitative assays such as slab gel electrophoresis

Test	Method	Time - 0	Time - 4 weeks
Physico-Chemical	Differential Scanning Calorimetry	X	
	Viscosity		
	Appearance	X	X
	pH	X	X
Purity	Peptide map (ESI-MS and UV)		X
	SDS - PAGE (reduced and non-reduced)		X
	CEX-HPLC		X
	SEC-HPLC		X
Strength	UV A_{280}		X
Potency	ELISA		X

Although a variety of thermodynamic parameters can be determined using DSC, the analysis of the DSC data for the formulations in Table 1 was focused on the midpoints of the thermal transitions using Design-Expert (Stat-Ease, Minneapolis MN). Based on the model, each of the individual factors was found to be significant along with quadratic terms for each factor. Interestingly, no interaction terms were found to be significant with an alpha of 0.05 (Table 3). Diagnostic plots did not reveal any outliers or deviations from a normal distribution that would require a transformation.

Table 3: ANOVA for DSC Data. While identification of effects is suitably performed by taking the difference between the average response at the high and low-coded conditions, confirmation of the validity of the selected factors should be confirmed using statistical analysis. In this case, the overall model and each of the factors are statistically significant at a 95% confidence level. Equally important, the "lack of fit" is not statistically significant, indicating that the data fit the model well

Source	Sum of squares	df	Mean square	F value	p-value Prob > F
Model	246.0185	6	41.00309	1089.692	< 0.0001
A-pH	236.2554	1	236.2554	6278.691	< 0.0001
B-Buffer conc.	0.287701	1	0.287701	7.645901	0.0161
C-Excipient Conc.	0.695344	1	0.695344	18.47936	0.0009
A2	8.286562	1	8.286562	220.2225	< 0.0001
B2	0.619167	1	0.619167	16.45491	0.0014
C2	0.2832	1	0.2832	7.526291	0.0167
Residual	0.489166	13	0.037628		
Lack of Fit	0.341832	8	0.042729	1.450081	0.3546
Pure Error	0.147333	5	0.029467		
Cor Total	246.5077	19			

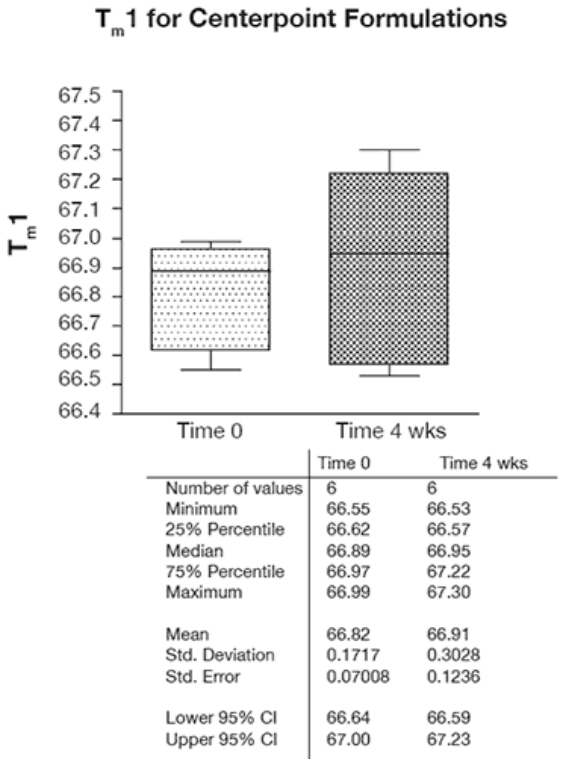
Definitions:

Mean square: $\frac{Sum\ of\ squares}{df}$

F value: $\frac{Mean\ square\ for\ Model,\ pH,\ etc.}{Mean\ square\ of\ residuals}$

p-value: probability that the observed F-value would exceed the critical F-value

In the context of preformulation projects, capillary DSC has shown both the throughput and precision that enables the statistical analysis of fairly subtle trends and effects, as is shown by the excellent precision for six replicate center point formulations in Figure 7.



	Time 0	Time 4 wks
Number of values	6	6
Minimum	66.55	66.53
25% Percentile	66.62	66.57
Median	66.89	66.95
75% Percentile	66.97	67.22
Maximum	66.99	67.30
Mean	66.82	66.91
Std. Deviation	0.1717	0.3028
Std. Error	0.07008	0.1236
Lower 95% CI	66.64	66.59
Upper 95% CI	67.00	67.23

Figure 7: Statistical analysis of the typical precision from MicroCal VP Capillary DSC. Due to the baseline stability of the MicroCal VP Capillary DSC system, data such as these are routinely observed, where a difference of 0.2°C between candidate formulations is statistically significant.

As this Figure shows, the typical precision ranged from 0.2 – 0.4% RSD and even over a temporal span of 4 weeks, the mean values for the first thermal transition were within 0.1°C. Examination of the DOE 3-D and one-factor plots also showed the effects of this precision because although pH, buffer concentration, and excipient concentration are shown to be statistically significant, the only factor that appears to have an appreciable slope or effect on T_m1 is pH as shown in Figure 8. By contrast, for buffer concentration (shown in Figure 9), the slope approaches zero, suggesting that although buffer (and excipient) concentration may be statistically significant, they are not likely to be operationally significant in terms of product stability.

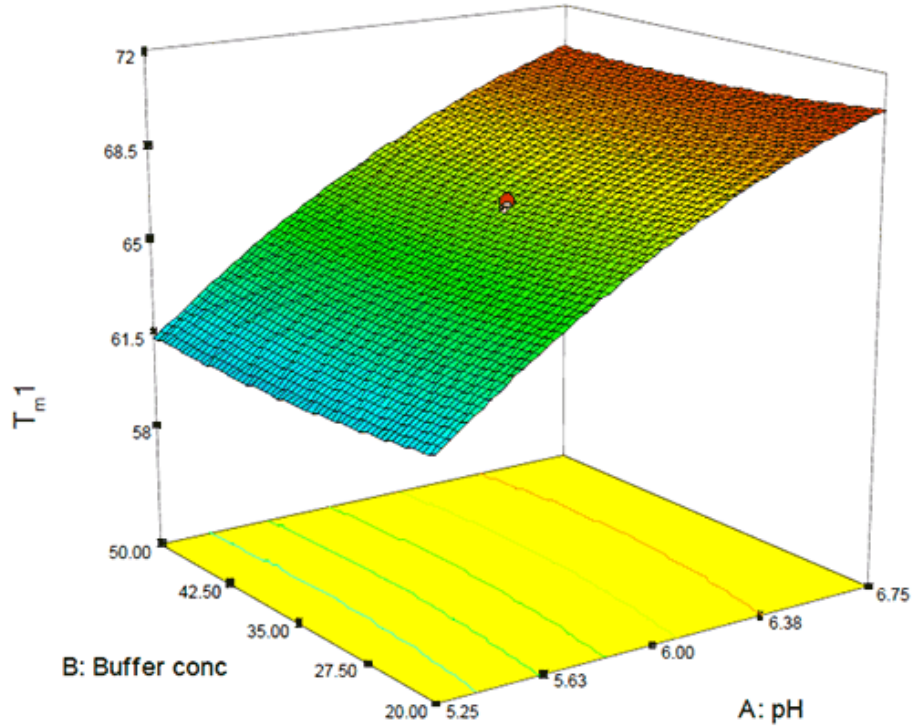


Figure 8: 3-D surface showing the effect of pH and buffer concentration on thermal stability. The data demonstrates the relative effects of pH and buffer strength on the midpoint of the first thermal transition for this antibody. The thermal stability of the antibody changes approximately 9°C as the pH is varied from 5.25 to 6.75

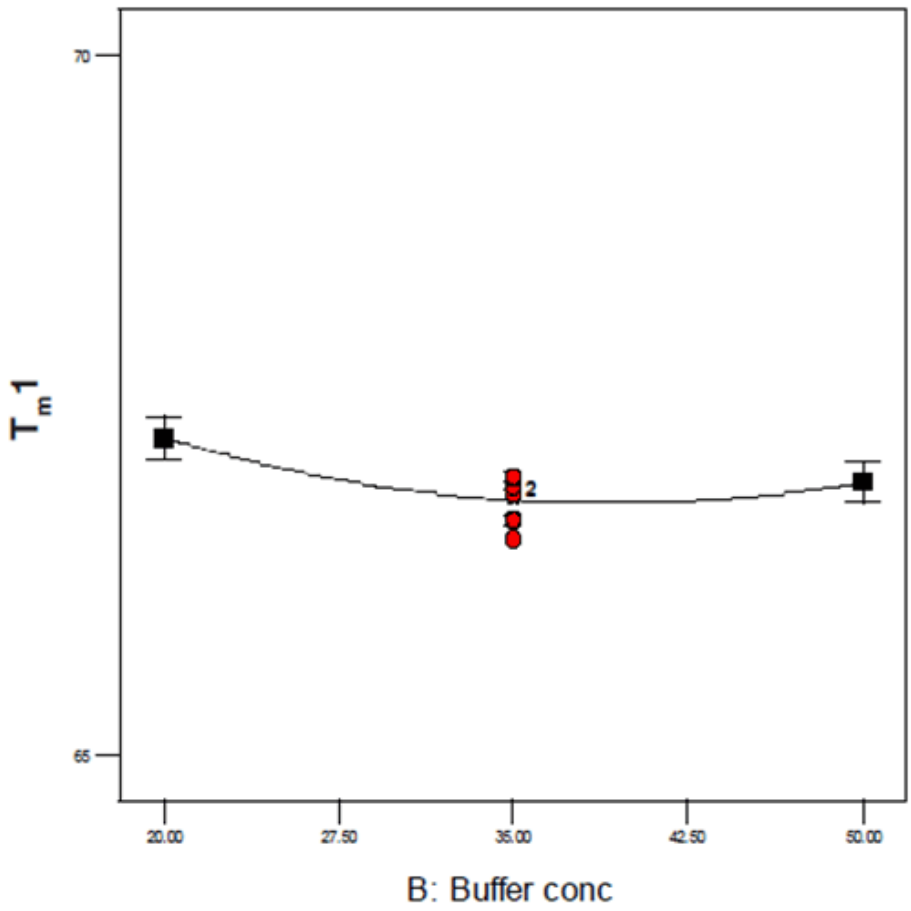


Figure 9: Thermal stability as a function of buffer concentration. In contrast to the data shown in Figure 8, the change in the midpoint of the first thermal transition is negligible (0.3°C) as buffer strength is varied from 20 to 50 mM. These data indicate that while the effect may be statistically significant, it is unlikely that buffer strength will have a dramatic effect on product stability. Other assays used in support of this preformulation study showed similar trends.

While DSC is an important part of most preformulation studies, the ability to demonstrate protein stability relies not on one technique but rather on a series of biophysical and analytical methods to provide a more complete picture of protein stability. Other assays such as size exclusion, dynamic light scattering, ion exchange, and potency or activity assays are frequently employed to assess the effect of a particular factor on protein stability. While first order or screening designs typically show a reasonable correlation between thermal and other forms of stability, second-generation designs such as response surface designs often show differences between the various techniques and scientific staff must balance these assays as well as the proposed indication or means of administration in selecting the final formulation.

In this case, while DSC showed a general trend of increasing thermal stability as a function of pH (Figure 8), analysis of the percent monomer by SEC showed a saddle plot centered around pH 6.3 (Figure 10). The data from these and several

other assays were combined in order to target several candidate formulations that showed suitable conformational and chemical stability. Several of these potential formulations, however, did not meet the criteria for isotonicity that would be required for a low-volume injectable drug product so the data were re-evaluated with respect to both buffer and excipient strength in order to create an isotonic formulation. As was observed for Figure 9, neither buffer nor excipient strength were observed to have a practical effect on product quality following accelerated stability which permitted the addition of a limited amount of excipient in order to create a stable, isotonic formulation for this antibody.

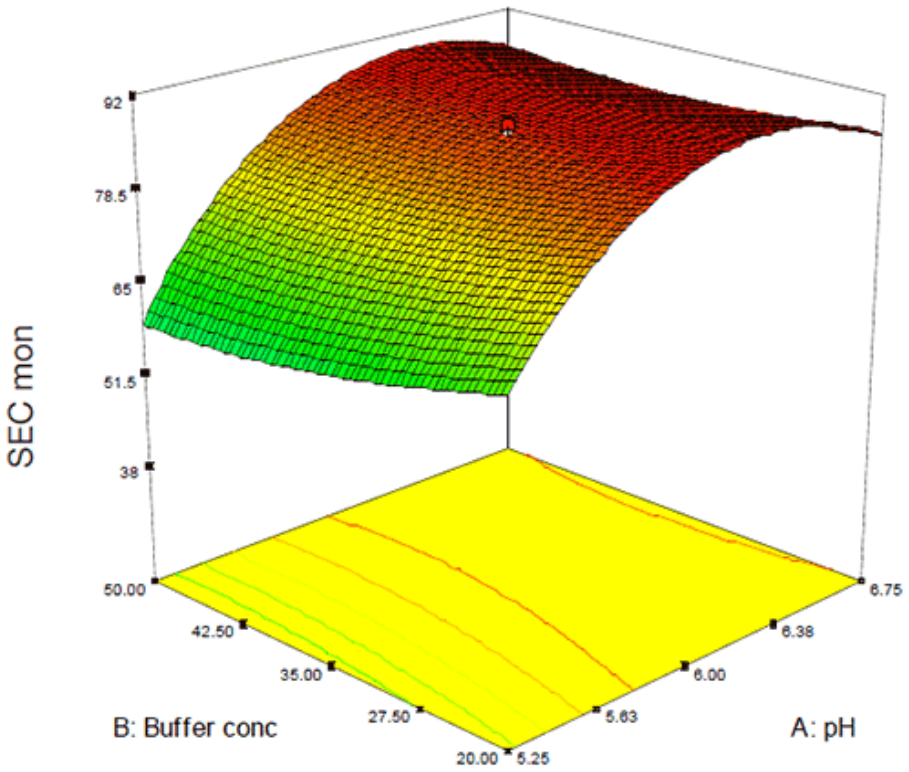


Figure 10: 3-D surface plot showing the effect of pH and buffer concentration on the percent monomer following accelerated stability. To a first order, the SEC data confirm the DSC results but there are some apparent differences: both assays indicate that pH appears to be the most significant factor with a more limited contribution from buffer or excipient concentration. The effect of pH on thermal stability, however, is markedly different than the effect of pH on purity by SEC because while thermal stability tends to increase linearly as a function of increasing pH, the SEC data indicate that above pH 6.4 increasing pH has the effect of decreasing stability as assessed by SEC.

Conclusion

The approach outlined here represents a defined, rational approach to the formulation development of biotherapeutics. It relies heavily on quantitative, automated techniques such as MicroCal VP Capillary DSC where a large region of design space may be examined efficiently and with sufficient precision to allow for the identification and analysis of critical factors and their interactions. Excellent repeatability and intermediate precision of DSC fits well with factorial design and response surface design approaches as even factors that have fairly subtle effects on product stability can be identified and optimized.

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