Identification and Minimization of Protein Loss During the Manufacturing of a Beta Domain Deleted Recombinant Human Factor VIII

Cyrus Agarabi
University of Rhode Island

Follow this and additional works at: https://digitalcommons.uri.edu/theses

Recommended Citation
Agarabi, Cyrus, "Identification and Minimization of Protein Loss During the Manufacturing of a Beta Domain Deleted Recombinant Human Factor VIII" (2006). Open Access Master's Theses. Paper 914. https://digitalcommons.uri.edu/theses/914

This Thesis is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Master's Theses by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons@etal.uri.edu.
IDENTIFICATION AND MINIMIZATION OF PROTEIN LOSS DURING THE MANUFACTURING OF A BETA DOMAIN DELETED RECOMBINANT HUMAN FACTOR VIII (BDDRFVIII SQ) ALBUMIN FREE PRODUCT.

BY
Cyrus Agarabi

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN BIOMEDICAL AND PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND
2006
ABSTRACT

**Purpose:** To identify factors affecting the concentration loss during the manufacturing process of Beta Domain Deleted Recombinant Human Factor VIII (BDDrFVIII SQ)

**Methods:** Lyophilization cycles were stopped after each stage to characterize the protein through the cycle. Three different types of glass tubing vials; untreated, silicon dioxide coated, and siliconized were evaluated. Two formulations of BDDrFVIII SQ were evaluated to determine the effects of a concentrated formula. Factor VIII concentration (µg/ml) was determined using an anion exchange high performance liquid chromatography (HPLC) assay, with ultraviolet (UV) and fluorescence detection.

**Results:** The freezing and annealing stages were found to be critical aspects of the lyophilization cycle. Comparison of the three types of glass vials demonstrated that treated vials (siliconized or silicon dioxide) yielded greater protein concentrations. The formulation experiment indicated that a concentrated formula (2X) is superior to the standard formulation for protein recovery.

**Conclusions:** The current freezing cycle produces an ice crystal structure that damages the protein and may increase susceptibility to denaturation and aggregation upon reconstitution. Treated vials showed a significant benefit over untreated glass vials in the recovery of protein, but did not ameliorate the problem entirely. This indicates that the protein loss is only partially due to protein-surface interactions resulting in adsorption. Through fortification of the formula and decreasing the amount of water, there is less protein lost during lyophilization and reconstitution.
ACKNOWLEDGEMENTS

The research for my Master’s degree has provided a valuable learning opportunity in a “real world” setting. The opportunity to assess, plan, perform, and interpret has encouraged the development of my professional skills as a researcher. I would have not been able to accomplish this task without the tremendous support and encouragement from many gifted scientists. I would like to thank my major advisor Dr. Thomas Needham for his guidance and encouragement to pursue this joint Doctor of Pharmacy and Master’s of Pharmacuetics for his flexibility and support of all my academic goals.

I thank the talented scientist at Wyeth BioPharma for providing laboratory and scientific support for this project. I thank Dr. Kasra Kasraian and Edie Neidhardt of Wyeth BioPharma for their hands on approach to data analysis, experimental planning and execution. I thank my colleagues and friends Donovan Quinn and Christian Ruitberg for their expertise in manufacturing and lyophilization.

I would like to thank the University of Rhode Island College of Pharmacy and would not have lasted seven years at this university without the help of a talented and supportive faculty and staff. I thank Dr. John Babson for his encouragement to “rock the boat every now and then” and his jocularity. I sincerely thank Dean Lausier, Dean Letendre, and Dr. Chichester the department chair, for their unwavering support during my time at this university.

This list would not be complete without a special thanks to my family, who has indoctrinated the values of perseverance, and hard work. Their support both fiduciary and emotional has turned my goals into achievements throughout my life.
# TABLE OF CONTENTS

ABSTRACT ................................................................................... .ii

ACKNOWLEDGEMENTS ........................................................................................................ iii

TABLE OF CONTENTS ........................................................................................................ iv

LIST OF TABLES .................................................................................................................... v

LIST OF FIGURES .................................................................................................................. vi

BACKGROUND ...................................................................................................................... 1

STATEMENT OF THE PROBLEM .......................................................................................... 24

MATERIALS ............................................................................................................................ 25

METHODS ................................................................................................................................ 27

RESULTS AND DISCUSSION ............................................................................................... 35

STUDY LIMITATIONS ........................................................................................................... 49

CONCLUSIONS AND FUTURE WORK ................................................................................ 50

REFERENCES ......................................................................................................................... 52

APPENDICES .......................................................................................................................... 56

BIBLIOGRAPHY ...................................................................................................................... 78
LIST OF TABLES

Table 1: Lyophilization cycle with annealing.................................28
Table 2: Lyophilization cycle without annealing.........................29
Table 3: Freeze/Hold Study- Diluted with 0.9% NaCl.......................39
Table 4: Protein Recovery as a percentage between lyophilization cycles that anneal and do not...............................41
Table 5: Comparison of the recovery of protein as a percentage of the initial frozen polypropylene concentration..........................47
LIST OF FIGURES

Figure 1: The four structures of protein ........................................................... 2
Figure 2: Blood clotting cascade ........................................................................ 4
Figure 3: Deamidation reaction of asparagine .................................................. 8
Figure 4: Methionine oxidation reaction ............................................................ 9
Figure 5: Diagram of two potential reactions: A) Normal protein folding.
               B) Aggregation of protein .................................................................... 10
Figure 6: An overview of stage 2 of the aseptic manufacturing process .......... 13
Figure 7: Product temperature versus shelf temperature in the stages of a
               standard lyophilization cycle without an annealing step ................. 16
Figure 8: The structure of the B-domain deleted factor VIII ......................... 22
Figure 9: Schematic diagram of physical and chemical transformations affecting
               r-VIII SQ ......................................................................................... 23
Figure 10: Full lyophilization cycle with annealing ......................................... 28
Figure 11: Lyophilization Startup: Comparison between annealing and
               not annealing .................................................................................... 29
Figure 12: COBAS-FARA (2 Step Kinetic Chromogenic Bioassay)- Step 1 ........ 31
Figure 13: COBAS-FARA (2 Step Kinetic Chromogenic Bioassay)- Step 2 .... 31
Figure 14: Sample of Factor VIII HPLC Chromatogram .................................. 33
Figure 15. An explanation of means one-way ANOVA diamonds and
               x-Axis proportional model .................................................................. 34
Figure 16: Protein concentration recovery as a percentage for each step
               of the lyophilization cycle ................................................................... 36
Figure 17: Concentration (µg/mL) results for the Freeze/Hold study without
dilution.......................................................................................... 38

Figure 18: Concentration (µg/mL) of lyophilized products with or without an
annealing step after reconstitution....................................................... 41

Figure 19: Siliconized vs. untreated vials- Concentration (µg/mL) by
Mono Q HPLC.................................................................................... 43

Figure 20: Siliconized vs. Untreated Vials- Potency(U/mL) by COBAS-FARA.... 43

Figure 21: Mean Concentration (µg/mL) of untreated vials vs.
silicon dioxide (1+) vials................................................................. 44

Figure 22- Recovery of protein (µg/mL) when comparing 1X and 2X
formulations in both coated and untreated vials.................................... 46
Background

The Food and Drug Administration (FDA) defines biologics as, “any virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product or analogous product, or arsphenamine (arsenic compound), or its derivatives, applicable to the prevention, treatment, or cure of disease or injuries of man.” Protein drugs are classified as biologics and are increasingly used for a broad range of indications from arthritis to cancer. Biologics have a high degree of specificity for receptor targets, yielding greater efficacy and lower side effect profiles. Protein drugs are one of the fastest growing sectors within the pharmaceutical industry with nearly $33 billion dollars in worldwide sales in 2002. Development of protein drugs represents new avenues of therapy with hundreds of drugs either in the pipeline or on the market. The development of biological products has been considered a challenge because of the protein’s complex structural nature and the propensity for chemical and physical reactivity.

In order to understand the complexity of proteins, the structural characteristics must first be described. Proteins are composed from the covalent bonding of some or all of the twenty basic amino acids, as seen in Figure 1a. The primary structure of a protein contains at least 50 amino acids, while structures are classified as polypeptides if they contain less than 50 amino acids. The secondary structure consists of local conformations of the polypeptide chains, and folding of the proteins into alpha helices, beta strands and beta turns, Figure 1b. Tertiary structures are three-dimensional polypeptide chains, comprised of domains (specific functional units), which form a functional protein under physiologic conditions, Figure 1c.
Quaternary structures are the assembly of large individual polypeptide chains in large multi subunit proteins,\(^3\) Figure 1d. A simplified overview of protein structures can be seen in Figure 1.

**Figure 1.** The four structures of protein\(^5\)

(a) Primary structure

\[-\text{Ala-Glu-Val-Thr-Asp-Pro-Gly-}\]

(b) Secondary structure

\[\alpha\text{ helix}\]

\[\beta\text{ sheet}\]

(c) Tertiary structure

(d) Quaternary structure

Domain

Complex protein structures result in higher molecular weights than found in conventional drugs, and an increased chance of destabilizing reactions. Protein stability is dependent on multiple factors, including: proper folding, covalent
(disulfide) bonding and non-covalent (Hydrogen, ionic, Van der Waals, and hydrophobic) bonding.\textsuperscript{6} In a drug, instability can cause: loss of active potency, altered pharmacokinetics, loss of content uniformity, loss of pharmaceutical elegance, and the formation of toxic degradation products.\textsuperscript{7} According to the FDA, sec 211.166 “There shall be a written testing program designed to assess the stability characteristics of drug products. The results of such stability testing shall be used in determining appropriate storage conditions and expiration dates.”\textsuperscript{8} Therefore, to maximize a drug's shelf life, a complex product must be effectively stabilized when formulated and manufactured.

**Hemophilia**

Hemophilia is a hereditary blood disease marked by prolonged coagulation time, yielding a failure of the blood to clot and abnormal bleeding.\textsuperscript{9} Hemophilia A, which is called classical hemophilia is caused by a deficiency of factor VIII (FVIII); Hemophilia B, known as The Christmas disease is caused by a deficiency of factor IX.\textsuperscript{10} This thesis will address only Hemophilia A and therapies associated with treatment of this disorder. The gene for FVIII is a 186-kilo base (kb) of the X chromosome and comprised of 26 exons and 25 introns, with mature FVIII protein consisting of 2332 amino acids.\textsuperscript{11} Exons are DNA sequences that code information for protein synthesis, while introns are DNA sequences between exons which are removed and do not function in the synthesis of protein.\textsuperscript{12}
**Factor VIII**

FVIII is a homologous protein, which circulates in the plasma and is bound to the transporter protein, von Willebrand Factor (vWF), until FVIII is cleaved by thrombin to yield activated factor VIII (FVIIIa). As well as acting as a transporter protein, vWF is involved in primary hemostasis by promoting adhesion of platelets to the sub endothelium, resulting in a plug formation at sites of vascular injury. FVIIIa has no independent enzymatic activity, but acts as a cofactor to increase the proteolytic efficiency of factor IXa. These factors are essential for successful completion of the coagulation cascade and the formation of a blood clot, seen below in Figure 2.

Figure 2. Blood clotting cascade
Factor VIII Products

Concentrates of FVIII derived from human plasma have been in use since the 1960's. Plasma derived FVIII ranges from 170Kd - 280Kd depending on the active form of FVIII employed. FVIII is found only in trace amounts (0.11 mg/L) in human plasma, and between 1500 Kg and 5000 Kg of human plasma is processed and enriched, to provide a yield of 18-22% of concentrated FVIII-VWF. The plasma used in the manufacturing of FVIII is extracted from donated human blood and must be viewed as a finite resource.

Prior to 1983, more than half of the hemophiliacs treated with plasma derived FVIII were infected with HIV. New technology and standards have been developed over the past twenty years to decrease the transmission of viruses through therapeutic products. Current FVIII products (plasma-derived and recombinant) are considered safe due to viral inactivation and removal steps in the manufacturing process, which can reduce viral load by several orders of magnitude. No FVIII products have transmitted HIV, hepatitis B or hepatitis C since 1987. Second only to viral infection, FVIII inhibitor development has been the most serious complication associated with plasma derived hemophilia treatment. Inhibitors are antibodies that react with FVIII to hinder the pro-coagulant function, and may present in varying severity in approximately 20% of severe hemophiliacs.

Concerns over inhibition formation, limited access to plasma, and potential viral transmissions led to the development of recombinant FVIII products. The first recombinant antihemophilic factor was approved in 1992, but contained blood-
derived additives from humans or animals, most notably albumin. Recombinant FVIII is synthesized by a genetically engineered Chinese Hamster Ovary (CHO) cell line, then purified with a series of chromatography columns, before viral inactivation with a solvent-detergent treatment step (product dependent). Recombinant FVIII products may require albumin or other macromolecular stabilizers. In 2003, Advate®, a recombinant factor VIII free of additives derived from human or animal sources was approved and provided added reassurance against risk of infection. This thesis will discuss issues associated with another albumin and plasma free product currently in development, namely the beta domain deleted recombinant factor VIII (BDDrFVIII SQ).
Degradation Pathways for proteins

Chemical

Deamidation and oxidation are two important reactions that may cause chemical instability. Deamidation results in spontaneous non-enzymatic degradation and loss of amino acid sequence homogeneity, commonly affecting asparagine, which is changed to aspartate or isoaspartate. The mechanism of the intra molecular reaction occurs when an amide side chain is attacked by a succinimide ring intermediate, Figure 3 reaction A. In a basic environment, the succinimide ring is hydrolyzed into either an aspartyl residue or an isoaspartyl residue, Figure 3 reaction B. This inverts the charge of the amino acid from positive to negative, and increases susceptibility to protease activity and denaturation yielding a decrease in the protein's activity. Detection systems monitor charge, molecular weight changes and a direct measurement of succinimide or isoaspartic residue formation. Lowering the pH of a protein formulation decreases the occurrence of succinimide deamidation, but also increases the chance for hydrolysis deamidation. In Figure 3, reaction A is the primary degradation step to the intermediate, and reaction B completes the reaction to the two possible products.
Oxidation is the covalent modification of a protein induced by reactive oxygen intermediates or by-products of oxidative stress. Oxidation may occur in a number of amino acids, but is most commonly seen in cysteine and methionine (see Figure 4). Oxidation results from exposure to air, intense fluorescent light, and/or residual peroxide content. Oxidation may have varying effects on amino acids, but the formation of free radicals is considered the most dangerous. Free radicals are unstable and highly reactive molecules associated with cellular tissue damage, and are believed to accelerate the progression of serious disease states. Antioxidants are
added while pH, and temperature adjustments are made to decrease oxidative product build up. Antioxidants act as free radical scavengers and oxygen acceptors to minimize damage to the protein. Oxidation occurs optimally at neutral or basic pH and decreasing the pH can diminish aggregation or disulfide scrambling.

![Methionine oxidation reaction](Figure 4)

**Physical**

Physical Instabilities, such as aggregation/precipitation and surface adsorption, comprise another major destabilizing factors for protein drug formulations. Aggregation is the most prevalent means by which a physical instability in protein structure is manifested. Aggregation is a microscopic association of protein molecules, while precipitation forms visible protein particles that decrease potency and alter solution appearance, Fig. 5. The aggregation of proteins through subtle structural changes is responsible for associative behavior. Physical factors including temperature, ionic strength, agitation, and surface/interface adsorption, all result in an increased hydrophobic surface area, yielding protein aggregation. Protein aggregates may be soluble and create a reversible problem. Precipitation may result from protein-salt interactions, yielding a "salting-out" effect when high concentrations of salt interact with hydrophobic residues of the protein.
Adsorption to containers, equipment, and other surfaces for protein drugs present more challenges in production and storage than traditional pharmaceuticals. Two mechanisms: hydrophobic interactions accompanied by dehydration and electrostatic (charge-charge) interactions between the protein and the surface result in adsorption. Surface energy has been identified as a major factor influencing protein adsorption, with hydrophobic surfaces adsorbing more protein than hydrophilic surfaces.

Protein administration is parenteral, which requires a sterile environment for manufacturing and a sterile dosage form. Filters are used to keep products free from contamination but may result in adsorption of proteins through electrostatic
interactions. Filters vary in their composition materials, yielding varying levels of hydrophobicity, reactivity, and extractability into the protein product. Therefore, during formulation it is important to assess the protein and filter to ensure avoiding a significant amount of adsorption.

Once placed in product vials, proteins may adhere to glass surfaces, further reducing available drug concentrations in a product. Untreated glass surfaces are anionic, and adsorb cationic and amphipathic biologics resulting in loss of concentration. Initial concentrations of protein drugs are generally low; with a small loss resulting in a significant relative loss of active ingredient. Surfactants such as polysorbates, and pleuronics may greatly reduce adsorption rates to solid surfaces. Another strategy for decreasing protein adsorption is coating pharmaceutical glass with silicone or polymers to render the glass surface inert. Glass is dipped or sprayed with a solution of silicone or polymer, then cured in an oven under defined time and temperature conditions. This coating repels a water layer and decreases the adsorption of some proteins, but may inadvertently adsorb other proteins (i.e. albumin, IgG, and IgM).

Liquid formulations of protein drugs are the preferred dosage form, from a cost and ease of production viewpoint. The addition of excipients (surfactants, antioxidants, etc.) to some protein drugs will resolve their instability and create a viable liquid dosage form. Drugs that do not respond require a different approach to formulation, and manufacturing; lyophilization is often used for these difficult cases.
Drug Product Manufacturing

Manufacturing a protein product is a long and complex process beginning with amino acids and ending with a final lyophilized product. The process can be divided into two stages: Stage one is the production of a bulk protein substance and the freezing of that bulk for shipment and further processing. Stage two, as presented in figure 6, consists of thawing the bulk drug substance and processing it into a viable drug product for the consumer. This research will address the second stage of protein manufacturing and issues associated with this process.

It is important to understand that the overall manufacturing process must be performed in appropriate environments, in order to minimize the risk of product contamination. According to the FDA “Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing- Current Good Manufacturing Practice.”

“In an aseptic process, the drug product, container, and closure are first subjected to sterilization methods separately, as appropriate, and then brought together. Because there is no process to sterilize the product in its final container, it is critical that containers be filled and sealed in an extremely high-quality environment (Class 100). Before aseptic assembly into a final product, the individual parts of the final product are generally subjected to various sterilization processes. Any manual or mechanical manipulation of the sterilized drug, components, containers, or closures prior to or during aseptic assembly poses the risk of contamination and thus necessitates careful control.”
Thaw

Drug substance or active pharmaceutical ingredient (API), is defined as the unformulated active substance which may be subsequently formulated with excipients to produce the drug product. Pooled lots of bulk drug substance are often frozen in vessels for easier storage and shipping. Thawing these vessels may be thermodynamically different than simply using the opposite requirements for freezing the vessel, and must be carefully evaluated.

When drug substance is needed for processing to a final product, the containers are thawed under temperatures ranging from refrigerated conditions (2–8 °C) to as high as 30 °C. During thawing, the liquid phase appears first at the surface of heat transfer, then quickly separates the frozen product mass from the heating surface. Thawing may be accomplished through externally jacketed vessels, which circulate a thermodynamic transfer medium around the outside of the container. Internally thawed material is pumped to the top of the frozen mass, around the ice mass, and then return to the vessel bottom to increase the melting rate and
homogeneity of the substance.\textsuperscript{45} Thawing is complete when all water molecules are released from the ice matrix into the amorphous phase.\textsuperscript{46} Additional external mechanical agitation of the entire vessel yields quicker and more uniform thaws by stirring the liquid and solid phases together.\textsuperscript{45} Mixing during the thaw will minimize any freeze-induced active pharmaceutical ingredient concentration effects that may occur during the freezing process.\textsuperscript{46}

Formulation

The thawed drug substance is then formulated into the drug product. Formulation is the ordered addition of the recommended excipients at the appropriate concentrations, and the adjustment of protein concentration, pH, and ionic strength.\textsuperscript{30} Proper formulation must be developed and validated rigorously to provide a viable final product. Physical characterization identifies particle size and distribution, shape, surface area, density and porosity of a product.\textsuperscript{47} Chemical characterization identifies product potency, solubility, stability, reactivity, and purity.\textsuperscript{47} Product characterization identifies potential buffer systems, cryoprotectants, lyoprotectants, and/or other excipients required in the formulation.\textsuperscript{48} The order of addition of excipients may also impact product quality, as a result of interim reactions of higher local concentrations of excipients than their end concentrations in the mixed formulated bulk.\textsuperscript{30}

Filter & Filling

The formulated drug product must be sterilized to remove microorganisms and other harmful materials. Filtration is the preferred method for sterilization of
expensive and heat-sensitive proteins. Filters physically remove microorganisms through retention of particulates greater than the pore size of the filter. If the pore size is too small, the filters may become clogged resulting in increased filtration times. Conversely an inappropriately large filter pore size may yield greater product loss due to an increased “hold-up” volume. “Hold up” volume is defined as the amount of residual water in a filter after a flush of pressurized air. Other important factors influencing the removal of microorganisms are: electrical charge interactions, the pH of the solution, the temperature and the pressure or vacuum applied to the filtration system. A series of multiple filtration steps must occur to remove debris; particles, bacteria and other molecules prior to the final sterilizing fill operation. Final sterilization filters are generally less than or equal to a 0.22 µm pore size and must ensure sterility but minimize protein adsorption. The sterilized product is then aseptically filled into vials and partially stoppered, before being loaded into the lyophilizer. Excessive fill speeds may result in product foaming and protein denaturation, while long filling periods may cause protein interactions with tubing and equipment.

Lyophilization

Lyophilization is the process of “freeze-drying” thermally sensitive products in order to preserve them. During World War II, lyophilization was heavily adopted to preserve biological materials, namely human plasma. Since that time, lyophilization technology has improved but the basic method has remained the same. As shown in Figure 7, the product is first frozen in order to solidify the liquid, then a
majority of the ice is removed through sublimation during primary drying, and then the small amount of remaining water is removed by desorption during secondary drying.\textsuperscript{51}

![Graph showing temperature vs. time during freezing, primary drying, and secondary drying.](image)

Figure 7. Schematic diagram showing how the product temperature (---) differs from the shelf temperature (–) in the stages of a standard lyophilization cycle without an annealing step. ‘A’ denotes the water supercooling in the solution.\textsuperscript{57}

**Freezing**

The freezing step is required to bring the material to a solid state before drying by sublimation and evaporation. Freezing is responsible for the internal structure of the final product, its specific area, and orientation.\textsuperscript{52} When freezing is complete, the system consists of a solid (in the ice form), with concentrated amorphous solutes.
Only rarely do the solutes become crystalline, but more commonly, the solute is amorphous below the glass transition state (T’g). Crystalline molecules are arranged in a highly ordered repeating fashion, while amorphous molecules are disordered and irregular.53

Product vials containing the filled formulation are placed on the lyophilizer’s shelves, which may be pre cooled, and use circulating chilled fluid to remove heat. Freezing cycles then ramp the temperature of the shelves well below the customary phase transition freezing temperatures (Tf), known as supercooling.53 The ice nucleation event occurs when the first ice crystal(s) form and then grow in size away from the cold surface.54 Ice nucleation initiation may be accelerated by supercooling, but is also dependent on the condition of the solution and its container.55 The release of heat from crystal formation temporarily raises the product temperature, and then progressively decreases relative to the equilibrium melting point of the concentration solution.56 Ice crystals grow throughout the system, leaving “interstitial fluid” (remaining liquid solution), yielding more concentrated proteins and excipients in the solute (between the ice crystals).57 Solidification is completed slowly as the heat of crystallization is transferred from the solidification interface through the solidified layer and the vial bottom to the shelf.55 Solidification of the maximum concentration of the solutes occurs below a derived temperature, known as the eutectic temperature (Te).54 If the solute does not form a true eutectic, a “glass transition” (T’g) is reached. After reaching the T’g, ice begins crystallizing out as the solute solubility is exceeded and solute precipitation occurs as either an amorphous mass, crystals, or a combination of both.57 Viscosity and rigidity increase as the solute concentration
increases and product temperature decreases, until water is no longer removed from the concentrated solution by freezing and the material solidifies into the amorphous form. Most drug products do not have fully crystallized solutes, but form an amorphous glass with no eutectic temperature. Instead there is a collapse temperature (Tc), which defines the maximum product temperature for the freeze-drying cycle, and is customarily a few degrees above (T'g).

The ramp rates for product cooling vary and must be understood because the slower the crystal growth, the larger the crystal. The crystal size affects sublimation rates and the size of the voids created in the dried material after ice has sublimed away during drying. Larger ice crystals leave larger voids for sublimed water vapor to escape through. These voids will improve the mass transport of water out of the drying cake, but larger product crystals can act as a barrier which slows this process.

**Annealing**

For pharmaceutical products, the crystallization of the water into ice is not often a problem; but the crystallization of the solutes can be difficult. Annealing is a process step that holds the product at a specified temperature in order to enable solute crystallization growth. Generally, the product is held at 10°-20°C above T’g for several hours to allow crystallization. When the product is held above T’g, the smaller ice crystals will melt first and cause a surface-tension-driven process that consolidates high-surface area particles (small crystals) into crystals with lower-surface area particles (large crystals).
The thermal cycle or “annealing” step is not mandatory for the successful lyophilization of all products, but has been shown vital for many products. The benefits of annealing are seen throughout the remainder of the cycle. These benefits include increased drying rates and reduced vial-to-vial heterogeneity in primary drying, and subsequently result in a shortened lyophilization cycle overall. Final products exhibit reduced protein unfolding in the dried solids, less aggregation and fewer bubbles after reconstitution, and improved pharmaceutical elegance.

**Primary Drying**

The ice formed during the freezing step is removed by sublimation at sub-ambient temperatures (-30°-10°C) under vacuum (40-400 Torr). Heat is transferred from the shelf to the frozen solution through the tray and vial, then conducted to the sublimation front. The partial pressure of water vapor must be reduced below the triple point pressure ($P_{TP}$) of water to allow sublimation to occur. During sublimation, the product temperature remains low due to evaporative cooling and vacuum conditions, therefore heat must be provided to the product to ensure the process continues at an acceptable rate. To prevent the melting of ice back into the product, the temperature and pressure must be kept below the triple point. The T’g is the maximum allowable product temperature during the primary drying step. Exceeding T’g during primary drying may result in the collapse of the highly porous amorphous matrix. The point at which the collapse occurs is referred to as the collapse temperature and is generally a few degrees higher than T’g and results in rejection of the final product. A collapsed product has lost its pharmaceutical
elegance, and may lead to increased residual moisture content, uneven distribution of
moisture, longer reconstitution times, and possible changes in protein
conformations. After the ice sublimes, the water vapor formed passes through the
dried portion of the product to the surface. The formation of the crystal structure of
the product is the rate limiting factor for sublimation. Slowly frozen products with
large ice crystals experience less resistance to the sublimed water vapor, due to large
openings and interconnected channels. Quickly frozen products, with smaller ice
crystals, will encounter greater resistance to water vapor because vapor flow must
pass directly through dried material in order to escape. Escaped water vapor is
transferred from the surface of the product through the chamber and then condensed
on the condenser. The condenser operates between -50° and -70°C, with a vapor
pressure gradient resulting in a “vacuum pump,” driving the process from the product
(high pressure) to the condenser plates (low pressure). When all crystalline solvents
have disappeared, the temperature is raised for the extraction of the remaining
unfrozen liquids, known as secondary drying.

**Secondary Drying**

Secondary drying removes absorbed water from the product that had not
previously separated out as ice during freezing and subsequently had not undergone
sublimation. Remaining bound moisture may be water of crystallization, randomly
dispersed water in a glassy material, intracellular water, or absorbed water. The rate
of drying is fast initially but slows as the product dries; drying is increased by higher
shelf temperatures (25°-50°C), but is product specific. The product temperature
during secondary drying must be considered for heat labile products which may experience significant degradation at high temperatures. The glass transition curve \( T_g \) gives the maximum temperature at which the “dry” product remains in the glassy state. If the temperature exceeds \( T_g \) for a sustained period of time or too great a temperature margin, there will be significant rates of deterioration. The rate of drying is also dependent on product consistency with dilute solutions drying quicker than concentrated solutions because of higher surface areas. The rate limiting step is the diffusion in solid and/or evaporation at the solid/vapor boundary, and is not chamber pressure dependent. Zero percent moisture in the product is unattainable, therefore the goal is <1% for optimal drug stability.

Upon sufficiently drying the product, the vials are stoppered in place by compression of the chamber shelves onto the top of the stoppers. The final stoppering phase is essential to ensure that the newly formed solid products are not contaminated later on the manufacturing line. The vials are then capped and crimped to further protect the product during shipping and consumer handling. The vials are then inspected, manually or automated, under appropriate criteria with an established predefined rejection rate for failed products. Finally, vials are labeled and packaged for shipment.

**Specific Problems with FVIII**

(BDDrFVIII)- Background

The structure of human FVIII protein can be categorized into three major regions: the N-terminal 90-kd (Kilo Dalton) heavy chain, a C-terminal 80-kd light
chain, and a large central region designated as the Beta domain, seen in Figure 8.\textsuperscript{62} The B-domain has been deleted to create the smallest active form of factor VIII, an 80 and 90 kDa heterodimer linked by a metal ion.\textsuperscript{16} BDDrFVIII SQ, where SQ refers to amino acid linkage (serine 743-glutamine 1738), is a genetically engineered recombinant product.\textsuperscript{63} The secondary structure of this protein consists of 41\% $\beta$-sheets, 14\% $\alpha$-helix, 26\% random structure, and 19\% turns as defined by circular dichroism (CD).\textsuperscript{40} Due to the complexity of this molecule, a complete high resolution three dimensional understanding has not yet been achieved, and only specific domains have been identified.\textsuperscript{64} This product does not require the addition of human serum albumin as a stabilizer in the final formulation.\textsuperscript{62}

![Diagram of the B-domain deleted factor VIII](image)

Figure 8 The structure of the B-domain deleted factor VIII\textsuperscript{16}

The molecule is sensitive to physical and chemical degradation because the molecule contains: glycosolated protein chains, seven disulfide bridges, five sulfhydryl groups, and a metal ion bridge.\textsuperscript{40} Physical modifications affecting the molecule include: elevated environmental temperatures inducing conformational
changes, as well as higher ionic strength yielding precipitation, which results in irreversible aggregation. Potential chemical reactions including: oxidation, deamidation, dimerization, disulfide exchange, that yield chain separation and ultimately irreversible aggregation. Extensive research on the chemical and physical degradation of BDDrFVIII SQ has been explored previously and is summarized in Figure 9.

Figure 9. Schematic diagram of physical and chemical transformations affecting r-VIII SQ.
Statement of Problem

Recombinant FVIII was chosen as a model protein for this experiment due to observed sensitivities during manufacturing. The primary aim of this study is to explore the root causes of protein loss and approaches to minimize the loss of BDDrFVIII SQ during the manufacturing of the drug product. Three areas of interest were defined in order to create a comprehensive and methodical approach.

1. Analysis of the effects of the lyophilization process through a “step-wise” approach, by dividing the process into three separate steps; freezing/annealing, primary drying, and secondary drying. Each step was individually tested to determine product concentration in an effort to evaluate “critical” process steps and potential sites of protein loss.

2. Comparison of the protein recovery between untreated vials, siliconized vials, and silicone dioxide treated vials. Using a different vial represented a potential change that could be made to the existing product without altering other aspects of the process.

3. A fortified formulation, referred to as the 2X formulation because it contained twice the concentration of excipients and proteins, was assessed. The 2X formulation was studied under processing conditions to determine protein recovery. The protein lost between the traditional formula and the fortified formula was compared to further evaluate the current formula.
**Materials**

Bulk r-VIII SQ (5000 IU/mL) was produced by Wyeth BioPharma (US) and frozen at -80°C, and manufactured in a pilot scale laboratory described below in the methods section. Standard product samples were formulated to 275 IU/mL with a 2mL fill volume. A 550 IU/mL product for the 2X formula study contained double the formulation buffer and only a 1mL fill volume.

The excipients: L-Histidine, sodium chloride, sucrose, calcium chloride dihydrate, and polysorbate 80 (veg) were of pharmaceutical quality. Sodium chloride was used as a bulking agent and to enhance the solubility of the protein. Sucrose (beet) acted as a product stabilizer (cryoprotectant) by substituting for water in the amorphous phase of the lyophilized product. L-Histidine buffered the formula to approximately pH 7.0, while calcium chloride dihydrate preserved the metal ion bridge linkage in the protein. Polysorbate 80 was used as a surfactant to minimize surface denaturation and adsorption.

Durapore Polyvinylidene Fluoride (PVDF) 0.22 µm filters (Millipore, Sweden) and Millipore Stericup® were the sterile filters and collection containers used. Three different type 1 glass product vials were evaluated based on their surfaces to determine their effects on protein yield after reconstitution. Schott® (Germany) Type I tubing vials size 10 ml and 6 ml were used. Untreated glass vials were used as the standard by which all other vials were compared. Siliconized vials, contain a non-
ionic aqueous emulsion of a medium-viscosity Baysilone® Fluid M (Bayer, Germany) surface coating. Schott Type I+® coated vials contain a quartz like inner surface of pure 100% silicon dioxide (SiO$_2$) coating of 0.1-0.2 µm thickness, while the glass matrix is comprised of 75% SiO$_2$. Vials were stoppered with a bromylbutyl rubber stopper FM 157 (Helvoet, Sweden) and crimp sealed with an aluminum flip-off seal.
Methods

The bulk drug substance was thawed at 22°C (+/-2°C) by complete submersion in a water bath. The bulk drug volume needed to formulate 275 IU/ml and the amount of (beet) sucrose needed for a concentration of 6 mg/ml based on bulk substance volume was calculated. Sucrose was dissolved in a portion of the buffer while stirred. Bulk drug volume was diluted with buffer and sucrose in buffer to 275 IU/mL, in a non-sterile bottle, and then agitated/inverted. Formulated drug solution was filtered through a 500 ml Millipore® polyvinylidene fluoride 0.22 µm unit under aseptic conditions in a biological safety cabinet.

Vials were labeled and filled with formulated drug using a repeater pipette to 2 mL. Ten 1 mL aliquots were taken in polypropylene containers for pre-lyophilized samples and frozen at -80°C. Thermocouples were added to each type of vial in order to monitor the thermodynamic environment of the product. Vials were stoppered according to the experimental variation planned. Freezing studies stopped prior to the drying stages were fully stoppered and crimped. Experiments evaluating drying steps only partially stoppered and subsequently crimped at the end of the cycle.

Lyophilization was performed on pilot freeze-driers (Edwards, Germany and Lyostar, Netherlands), with approximately 0.43m² usable shelf area and 25 kg ice condenser capacity. The vials were placed on the lyophilizer shelves at ambient temperature and the lyophilization cycle was run as seen in Table 1 and Figure 10.
Table 1. Lyophilization cycle with annealing

| Step               | Temp (°C) | Time (min) | Pressure (mTorr) | Elapsed time (hrs) |
|--------------------|-----------|------------|------------------|-------------------|
| Loading            | 25        |            | 760000           | 0                 |
| Pre-cool ramp      | 0         | 30         | 760000           | 0.5               |
| Pre-cool           | 0         | 30         | 760000           | 1                 |
| Freeze ramp        | -55       | 270        | 760000           | 5.5               |
| Freeze             | -55       | 120        | 760000           | 7.5               |
| Anneal ramp        | -35       | 60         | 760000           | 8.5               |
| Anneal             | -35       | 240        | 760000           | 12.5              |
| Re-freeze ramp     | -40       | 30         | 760000           | 13                |
| Re-freeze          | -40       | 120        | 50               | 15                |
| Primary drying ramp| -25       | 60         | 50               | 16                |
| Primary drying     | -25       | 1800       | 50               | 46                |
| Secondary drying ramp | 40   | 480        | 35               | 54                |
| Secondary drying   | 40       | 180        | 35               | 57                |

Figure 10- Full lyophilization cycle with annealing

The lyophilization cycle was stopped and tested for concentration at each stage of the cycle. An extended hold at the approximate product temperature during primary drying (after carrying out the cycle through annealing) to isolate temperature as a variable, and exclude water removal as a variable. The removal of the annealing step from the lyophilization cycle was evaluated and the modified cycle can be seen in
Table 2. A comparison between the cycle with and without annealing during lyophilization start-up can be seen in Figure 11.

Table 2. Lyophilization cycle without annealing

| Step                | Temp (°C) | Time (min) | Pressure (mTorr) | Elapsed time (hrs) |
|---------------------|-----------|------------|------------------|-------------------|
| Loading             | 25        | 0          | 760000           | 0                 |
| Pre-cool ramp       | 0         | 30         | 760000           | 0.5               |
| Pre-cool            | 0         | 30         | 760000           | 1                 |
| Freeze ramp         | -55       | 270        | 760000           | 5.5               |
| Freeze              | -55       | 120        | 760000           | 7.5               |
| Primary drying ramp | -25       | 120        | 50               | 9.5               |
| Primary drying      | -25       | 1800       | 50               | 39.5              |
| Secondary drying ramp | 40    | 480        | 35               | 47.5              |
| Secondary drying    | 40        | 180        | 35               | 50.5              |

Figure 11. Lyophilization Startup- Comparison between annealing and not annealing.

Note Figure 11 does not illustrate the full cycle, beyond the points shown above the cycle is identical to Figure 10.

Upon completion of the lyophilization cycle, the vials were fully stoppered under full vacuum. The vials were removed from the lyophilizer and aluminum caps were
crimped to seal the product. The final product was reconstituted with 4 ml of normal saline (0.9%) injection (Abbott, Illinois). For the lyophilization study, samples were reconstituted or diluted dependent on their final form. A 4 ml of normal saline injection was added to all products that had a cake structure; including samples stopped after primary drying and secondary drying. While samples removed prior to drying, (frozen and annealed samples) were diluted with 2 ml of 1.8% sodium chloride solution to ensure equivalent ionic strength of commercial products. Only the extended freeze and hold study received 2 ml of 0.9% sodium chloride solution, (half of the standard salt concentration addition), which resulted in further ionic strength studies to evaluate the effects of varying ionic strengths and volumes for reconstitution. Four milliliters of reconstituted solution contained 137.5 IU/ml of FVIII, 18mg/ml sodium chloride, 3.0 mg/ml sucrose, 1.5 mg/ml L-histidine, 0.25 mg/ml calcium chloride dihydrate, and 0.1 mg/ml polysorbate 80.

The concentration of FVIII was determined after reconstitution or dilution, using HPLC. A chromogenic bioassay was used to determine potency. Due to the inherent variability in bioassays and data showing similar trends as the concentration assay, potency was not determined for the majority of the samples.
Analytical Methods

Chromogenic bioassay of Factor VIII activity

The Cobas Fara II (Roche) employs a two-step kinetic chromogenic substrate method (Coatest® Factor VIII, Chromogenix AB, Sweden) used to measure FVIII activity. Activated factor X (Xa) is generated via the intrinsic pathway where factor VIII:C acts as a co-factor, seen in fig. 12.

Figure 12. COBAS-FARA (2 Step Kinetic Chromogenic Bioassay)- Step 1

\[
\begin{align*}
\text{X} & \xrightarrow{\text{IX a + Ca}^{++} + \text{phospholipid}} \text{VIII:C} & \text{Xa}
\end{align*}
\]

Factor Xa is then determined by synthetic chromogenic substrate, S-2222, in the presence of a thrombin inhibitor, I-2581, to prevent hydrolysis of the substrate by thrombin, as seen in figure 13.

Figure 13. COBAS-FARA (2 Step Kinetic Chromogenic Bioassay)- Step 2

\[
\begin{align*}
\text{Substrate} & \xrightarrow{\text{Xa}} \text{Peptide + pNA}
\end{align*}
\]

The reaction is stopped with acid, and the release of pNA (para-nitroaniline), which is proportional to the VIII:C activity, is measured photometrically at 405 nm against a reagent blank. The activity of factor VIII:C is expressed in international units (IU) as defined by the current International Concentrate Standard (IS) established by the World Health Organization (WHO). The assay variability was found to be 10-12%
and was subsequently used only initially to find a correlating trend between concentration and potency.

**Factor VIII concentration**

Factor VIII concentration (µg/ml) was determined using an anion exchange high performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence detectors. Components from a sample were separated by differences in their charge, and an exchange of their charged analytes with the counter ions electrostatically bound to the oppositely charged functional groups on the column’s stationary phase. The HPLC system (Waters®, US) consisted of: 2695 HPLC, 600S Controller 6DC, 474 Scanning Fluorescence Detector, 2487 Dual Absorbance Detector, 717 Plus Autosampler. A Mono Q® HR5/5 (Pharmacia Biotech, US) anion exchange column was employed. Two mobile phases were used to create the anion gradient; mobile phase A (20mM Tris, pH 7.5) and mobile phase B (20mM Tris, 1M NaCl, pH7.5).

The system was first flushed with purified water and then equilibrated using the mobile phases. The anion gradient is formed by running a weak eluent with low mobility (mobile phase A) and then running a strong eluent with high mobility (mobile phase B). A standard curve was prepared using five samples from a diluted stock solution of FVIII (Wyeth BioPharma, US) from a linear regression model. The lyophilized samples were reconstituted with 4.0 ml of 9 mg/ml NaCl. The liquid is then swirled gently, and the solution should not touch the stopper during mixing. 350 µL of the mixed sample is transferred to the Millipore® sample vials.

Samples were then loaded into the HPLC autosampler (4°C) and drawn in triplicate.
injections. Each sample injection ran for 17 minutes, all vials had their first sample drawn before beginning, the second and third injections. This sequenced method for multiple injections from the same vial ensured that vials tested later in long runs did not experience vastly different environmental exposure times to those vials tested first.

A minimum of 10 vials per sample with triplicate injections yielded a total of 30 data points (n=30) for greater power in the studies. The 550 IU/vial products that were analyzed for concentration demonstrated approximately 4% relative standard deviation. Figure 14 is an example of an FVIII chromatogram.

Figure 14. Sample of Factor VIII HPLC Chromatogram

**Statistical Methods**

JMP 5.1 (SAS Inc., US) was used for all statistical analysis of the data. The data underwent a summary of fit, a student t test, and a one-way analysis of variance
ANOVA. The ANOVA data is presented in this paper in the form of the one-way ANOVA diamonds and x-Axis proportional model, a sample is seen in Figure 15.

Figure 15. An explanation of means one-way ANOVA diamonds and x-Axis proportional model

ANOVA uses a test of the null hypothesis by comparing two sets of differences; the first set compares external (between groups) differences, and the other is an internal (within-group) differences. This allows the measurement of experimental treatment and experimental error (external), compared with experimental error alone (within-group). The test calculates the F-ratio; the model mean square divided by the error mean square and determines whether the variability between the groups is large enough in comparison to the variability of data within each group to justify the difference in groups.
Results and Discussion

Lyophilization Cycle Experiments

Lyophilization Cycle Examination- A step-wise approach

Based on the assumption that protein concentration loss occurs during lyophilization, partial cycles were run to identify the stage(s) during which it occurred. These partial cycles were stopped after annealing and primary drying, and those recoveries were compared to that of a full cycle.

Figure 16 shows each step of the cycle and the mean quantity of protein recovered as a percentage of the pre-lyophilized samples frozen at -80°C. The loss of protein during lyophilization is negligible within the accuracy of the assay compared to the loss upon freezing. This data demonstrates that freezing may act as the greatest contributor to protein loss, while other stages have less impact. Full data and statistical analysis are found in appendix III.
Figure 16- Protein concentration recovery as a percentage for each step of the lyophilization cycle.

| Stage of Lyophilization | Recovery of Protein (%) |
|-------------------------|-------------------------|
| Pre-Lyo                 | 100                     |
| Product Frozen at -55°C, then annealed at -35°C | 95                     |
| Product Stopped after primary drying with annealing | 85                     |
| Complete Cycle (Standard Cycle w/ annealing) | 75                     |

The degradation of protein as a function of the freezing rate has been investigated in model proteins previously. Lactate dehydrogenase (LDH) was found to lose a greater amount of activity (U/mL) after being frozen slowly in a freeze-drier, than when rapidly frozen with liquid nitrogen. The rate of freezing determines the size of the ice crystals formed, with slow freezing yielding larger ice crystals. Due to larger ice crystal formation in the product there may be greater shear force exerted on the protein molecules by the ice crystals, causing degradation. Additionally, the increased concentration of protein and excipients found in the unfrozen portion of the product during freezing increases the ionic strength, a pH shift, and protein
dehydration. These factors increase the probability of "biomolecular collisions", which can yield instability and aggregation.

Finally, the weakening of hydrophilic interactions between protein and water molecules can result in more "free water" removed during ice crystal growth, and result in greater protein unfolding and/or denaturation. Conversely, the rapid freezing of a product may result in less protein lost because the solution yields a large number of small ice crystals that causes less solutes to concentrate in the remaining unfrozen areas. Rapid freezing also may decrease the amount of water removed from proteins.

There is conflicting literature on the topic of freezing rate. Some hypothesize that the larger ice surface areas stress the proteins greatly and believe that surface denaturation may play a role during the freezing of proteins. The varied views may be the result of protein specific activity during the freezing process, and cannot be generalized by the rate of freezing alone.

**Lyophilization freeze and hold study**

An extended "hold" at the approximate product temperature during primary drying (after carrying out the cycle through annealing) was performed and isolated temperature as a variable. By holding the samples above their T'g, the proteins were exposed to a longer period of time, allowing destabilizing reactions to occur and yield an unstable product. This study excluded the rate of water removal as a variable
because primary drying was extended long enough to ensure all sublimated water was eliminated. As a control, a 1mL sample of pre-lyophilized product in polypropylene (PPE) tubes was frozen rapidly in a \(-80^\circ\text{C}\) freezer. These samples were tested without dilution, and pipetted numerous times to prevent stratification, and exposed to a smaller amount of surface area as compared to the standard product.

Figure 17 below, shows that there is no significant loss when the product is frozen, annealed, and held in a lyophilizer at simulated primary drying conditions without a vacuum, than when samples are frozen in a polypropylene (PPE) tube in a \(-80^\circ\text{C}\) freezer.

Figure 17. Concentration (\(\mu\text{g/mL}\)) results for the Freeze/Hold study without dilution.
In an effort to more closely mimic the actual product, additional samples were diluted with 0.9% NaCl, then agitated and inverted, as they would be during reconstitution. Table 3 shows a significant loss is experienced when the product is diluted.

Table 3. Freeze/Hold Study- Diluted with 0.9% NaCl

| Stage of Cycle                  | Protein Recovery (%) (calculated compared to pre-lyophilization sample) |
|---------------------------------|------------------------------------------------------------------------|
| Freeze/hold with no dilution    | 95.9% (5.5% RSD)                                                       |
| Freeze/hold with 0.9% NaCl dilution | 76.0% (8.7% RSD)                                                      |

During freezing loss of product is not seen until after dilution, suggesting that a potential surface denaturation of protein could occur at the ice-water interface. This denaturation coupled with the change in protein and excipient concentration, and/or the additional handling and agitation may be responsible for magnifying the loss. The process of dilution and agitation/inversion exposed the liquid product to a greater surface area of the glass vial, where there may be a potential for adsorption of protein to the glass surface. The two predominant methods of protein adsorption to pharmaceutical containers are charge-charge (electrostatic) interactions and hydrophobic interactions with dehydration of the protein and the surface. The potential protein-glass interaction was evaluated and can be seen later in this paper.
The thawed product contained a volume of 2mL and received 2mL of 0.9% NaCl to bring the product to the proper volume. This adjustment did not meet the ionic requirements of the commercial product which would have required the addition of 2mL of 1.8% NaCl. Further studies were performed to evaluate the ionic strength of the reconstituted product and the effect on recovery. Three diluents: water, 0.9% NaCl, and 1.8% NaCl were added in different volumes: 1mL, 2mL, and 4mL to lyophilized samples. Reconstitution of the lyophilized cake with 4 ml of 0.9% NaCl yielded 50.10 (µg/ml), 4 ml of 1.8% NaCl yielded 50.63 (µg/ml), and 4 ml of water yielded 48.61(µg/ml). Reconstitution with only water yields a lower amount of protein recovery and the reconstitution volume does not significantly affect the amount of protein recovered after reconstitution. This loss may be attributed to a lack of ionic protection due to a lack of proper NaCl concentrations. Complete experimental data is found in appendix III.

**Evaluation of the annealing step**

Many protein drugs are manufactured through a lyophilization cycle that does not require an annealing step. To investigate the effect of annealing on recovery, a full lyophilization cycle was run with the annealing step omitted, the results are seen in Table 4.
Table 4. Protein Recovery as a percentage between lyophilization cycles that anneal and do not.

| Stage of Cycle                  | Protein Recovery (%) (calculated compared to pre-lyophilization sample) |
|---------------------------------|------------------------------------------------------------------------|
| Complete Cycle                  | 80.8% (8.2% RSD)                                                       |
| Cycle without annealing         | 70.4% (20.2% RSD)                                                      |

Figure 18: Concentration (µg/mL) of lyophilized products with or without an annealing step after reconstitution.

The lyophilization cycle with the annealing step showed greater recovery of protein, as seen in figure 18. Annealing has been shown to relieve residual stress, which is correlated with protein unfolding in the dried solids and less aggregation after reconstitution. Annealing decreases the variation in initial ice crystal size distributions caused by unpredictable nucleation temperatures and allows the uniform drying rates of the product. This heterogeneity of drying rates may have caused the...
unusually large relative standard deviation (20.2%) for the unannealed cycle. Research has also shown that removing the annealing step can have a detrimental effect on the final lyophilized cake structure. Poor pharmaceutical elegance, weak cake structure, the formation of bubbles upon reconstitution, and increased drying rate heterogeneity are examples of negative effects that may occur in unannealed products.

Glass vial experiments

This study compared three different types of Schott® Type I glass tubing vials; untreated, silicon dioxide coated (Type 1 Plus®), and siliconized. Research had shown FVIII adsorption to the vial surface to be a mode of protein loss and was evaluated by comparing the differently treated vials. Because of delayed shipments of siliconized vials, the two types of treated vials available were not the same size. Each of the treated vial types were compared to untreated vials of the same size. Unfortunately comparing the two types of coated vials to each other was not possible from this data because of the difference in surface area.

Siliconized vs. Untreated Glass Vials

Comparison of siliconized vials and untreated vials, when lyophilized but not annealed in a simulated "worst case scenario."
Figures 19 and 20 show that a siliconized vial yields a significantly higher recovery of protein when tested for concentration (µg/mL) and potency (IU/mL).

**Figure 19:** Siliconized vs. untreated vials- Concentration (µg/mL) by Mono Q HPLC

![Figure 19: Siliconized vs. untreated vials- Concentration (µg/mL) by Mono Q HPLC](image)

**Figure 20:** Siliconized vs. Untreated Vials- Potency (U/mL) by COBAS-FARA

![Figure 20: Siliconized vs. Untreated Vials- Potency (U/mL) by COBAS-FARA](image)
Type I Plus® vs. Untreated Glass Vials

This experiment compared silicone dioxide coated vials and untreated vials, when lyophilized under the current commercial cycle. Figure 21 below shows that the Schott Type 1+ coated vial yields a significantly greater recovery of protein than an untreated vial.

Figure 21. Mean Concentration (µg/mL) of untreated vials vs. silicon dioxide (1+) vials

Adsorption of proteins to surfaces due to their amphipathic nature has been widely recognized due to multiple binding sites on the protein and mixed-site nature of substrates. This is especially apparent when products contain low overall
concentrations of active products, and a small concentration of protein adsorbed to
the vial wall may result in a significant loss in product concentration. Previous
studies have shown that structurally altered forms of the protein may appear on
surfaces because of: 1) Pre-existing distribution of conformations in the solution
phase 2) Rapid conformational changes accompanying adsorption 3) Slow
conformational changes after adsorption. Previous studies addressing adsorption of
protein to glass vials, found that in general proteins appear to saturate at
approximately 5µg/mL of protein, with a maximum of 10-15% protein adsorption and
the degree of binding based on individual proteins. This may represent a significant
loss for the product, which contains 10.5-11.5 (µg/mL). Proteins have been shown to
experience different mechanisms of bonding to untreated glass vials and silicone
treated vials. Proteins bind to untreated glass through ionic amine bonding, and an
aggregative force between silica and proteins. Proteins exposed to untreated glass
surfaces two rates of reactions occur: 1) a rapid rate of adsorption based on the
number of amines in the protein, 2) a slower rate of adsorption due to the protein’s
molecular weight, which determines the rate and extent of protein diffusion into the
porous structure of the glass. Proteins bind to silicone treated glass through
hydrophobic bonding between aliphatic (hydrocarbons without benzene rings)
residues on proteins and silicone residues on coated glass.

Both vial coatings (silicone and silicon dioxide) improve recovery and decrease
sample variability compared to untreated vials. It can be speculated that vials that
have been treated have less surface charge and attract less protein to adsorb to the vial
walls. However, substituting a treated vial would yield an approximately 5% greater recovery of protein than an untreated vial. While there is a significant cost associated with the drug, expenses associated with the new vials and their implementation must be considered for a more accurate cost to benefit rationale.

**Formulation Study**

*1X versus 2X formulation*

This study compared the 1X and 2X formulations of BDDrFVIII SQ to determine whether the concentrated formula yields greater protein recovery. Schott Type I Plus® coated vials were tested to determine the effects on recovery for the concentrated formulation.

Figure 22- Recovery of protein (µg/mL) when comparing 1X and 2X formulations in both coated and untreated vials.
Table 5. Comparison of the recovery of protein as a percentage of the initial frozen polypropylene concentration.

| Stage of Cycle                      | Type I Untreated          | Type I Plus® Coated       |
|-------------------------------------|---------------------------|---------------------------|
| 1X formulation w/ 4mL 0.9% NaCl Recon | 80.0% (8.2% RSD)          | 86% (2.2% RSD)            |
| 2X formulation w/ 4mL 0.9% NaCl Recon | 89.2% (5.8%)              | 87.8% (2.3%)              |

Figure 22 and Table 5 show that there is a significant improvement in the untreated vial when the 2X formulation is used. The recovery is approximately the same for both formulation strengths in the Type I Plus® coated vials, which suggests that an increase in formulation strength and the vial coating may alleviate similar problems. This improvement may be attributed to the excipient concentration, which has doubled and provided greater cryoprotection, lyoprotection, and/or overall stabilizing effects. Additionally, the decrease in water per unit volume for the product fill may also result in less potential ice surface area to damage the protein, as discussed in the lyophilization experiment section.

The increased concentration of excipients may protect the protein from denaturation during freezing. This protection during freezing is explained by the Theory of Preferential Exclusion states that excipients are preferentially excluded from protein. This exclusion results in a decreased ratio of the native conformation’s surface area to the solution, which is thermodynamically preferred over the denatured state.79 This
mechanism describes how solutes such as salts and sugars can stabilize proteins during freezing, thawing, and in liquid solution.

Increased ionic strength through the addition of sodium chloride to the formulation may decrease aggregation of BDDrFVIII SQ. Fatorous, et al. compared 5.8 mg/ml and 58 mg/ml of sodium chloride with regard to total aggregate formation, the higher salt concentration formula was found to be advantageous. The stabilization of the drug was attributed to interactions affecting specific ion binding, and the exclusion of salt from contact with the protein to cause preferential hydration. The effects of increased concentrations of surfactants (Polysorbate 80) in a BDDrFVIII SQ formulation were previously studied. The Osterberg, et al study was used to determine the optimal amount of polysorbate 80 for the albumin free formulation of the product. While 0.1 mg/ml of polysorbate 80 was chosen, data presented in the study indicated that at higher concentrations (0.25 mg/ml and 0.4 mg/ml) yielded factor VIII activity that was equivalent (in one circumstance) and usually greater. Surfactants have been shown to reduce protein adsorption to surfaces and aggregation-induced denaturation, while protecting against thermal degradation and freeze-thaw induced degradation.
Study Limitations

This study was designed in a methodical and scientific manner, but caution must be taken not to generalize the results of this study. This study was performed in a research laboratory and not in a commercial or clinical scale plant. All of the processing was performed manually and at a significantly smaller scale than the product would normally undergo. Commercial manufacturing enjoys a greater degree of automation and an advantageous volume to processing surface area than was seen in this study. Additionally the laboratory equipment operates and affects the product differently than would be experienced in the commercial scale. While effort was made to minimize equipment, operator, or scale variability, it is a factor that must be considered when interpreting these results.
**Conclusions and Future Work**

The primary aim of this study was to determine the primary causes of protein loss from the BDDrFVIII SQ during the manufacturing of the drug product. Due to the complexity of proteins and the intricacies of the drug product manufacturing operations, this objective was ambitious. When surveying all of the data, one can conclude that multiple factors contribute to the problem.

Through observation and data analysis it has been determined that the freezing step of the lyophilization cycle is the most important processing component, contributing to the loss of protein concentration. The current freezing cycle produces an ice crystal structure that damages the protein and may create an increased susceptibility to denaturation and aggregation upon reconstitution. Future studies must further analyze the process of freezing and subsequent reconstitutions. A comparison of different lyophilization cooling temperatures and rates, the use of pre-cooled shelves, and the use of vacuum at different points of the cycle would further characterize the effects of freezing on BDDrFVIII SQ.

The use of treated vials showed a significant benefit over untreated glass vials in the recovery of BDDrFVIII SQ, but did not ameliorate the problem entirely. This indicates that the loss of protein is only partially due to protein-surface interactions resulting in adsorption. Further evaluations should examine siliconized, silicone dioxide, and untreated vials in a “head to head” study of the same vial surface areas. Additional studies to determine the optimal siliconization level for the siliconized vials for maximal protein recovery should be performed.
In general, samples tested from either treated vial showed less inter-sample variability. While this does not affect the primary goal of the study, it is of interest because finished products are required to be tested prior to commercial release. Less variability may result in the difference between the product being commercially released or destroyed. Studies investigating the variability of protein concentrations with respect to product release parameters should be investigated to fully appreciate the trend seen in this study.

The use of the 2X formulation demonstrates that the problems associated with the product lacking protection during processing can be alleviated. Through fortification of the formula and the decreased amount of available ice surface area, there is less protein lost during lyophilization and reconstitution. Future studies should examine the effects of decreasing the volume of ice independently of formulation change, to gain insight into interactions of ice surface and the protein. Further investigation of new fortified formulas, such as 3x, 5x, 10x, etc, should be studied to find the optimum protein yield.
References

1. Winbourne, Pam. Overview of the Drug and Biologics Review Process. Food and Drug Administration, 11/2/00 http://www.fda.gov/oia/embslides/drugreview/index.htm
2. Rajan Malika. Worldwide market for protein drugs to reach nearly $71 Billion by 2008. Business Communications Company, Inc. Press Release, www.bccresearch.com/press September 22, 2003
3. Creighton, Thomas. Encyclopedia of Human Biology, 2nd Ed., Academic Press 1997, pp 189-203
4. Neema, S, Avis, K. Freeze-thaw studies of a model protein, lactate dehydrogenase, in the presence of cryoprotectants. “J Parenter Sci Technol.” 1993 Mar-Apr;47(2):76-83.
5. Horton, Moran, Ochs, Rawn, Scrimgeour. Principles of Biochemistry 3rd ed. Prentice Hall, 2002. http://cwx.prenhall.com/horton/medialib/media_portfolio/text_images/FG04_01.JPG
6. Dal Monte, Paul. Edmond Rouan, Katy, Bam, Narendra. Biotechnology-Based Pharmaceuticals. Chapter 22. Modern Pharmaceutics 4th ed. Eds. Banker and Rhodes. Marcel Dekker, NY 2002. Pg. 700-721
7. Cartensen, Jens, Rhodes, C.T.Drug Stability Principles and Practices Ed. Marcel, Dekker. NY 2000 pp3-8
8. Food and Drug Administration. CGMP for Finished Pharmaceuticals. Sec. 211.166 Stability Testing. http://www.fda.gov/cvm/guidance/guide5part1.html#211166
9. Bickert, Betsy. Kwakowski, Janet. Coagulation Disorders. “Pharmacotherapy: A pathophysiologic approach 5th ed.” Ed. By Dipiro. McGraw Hill Companies, Inc. 2002. pp. 1751-1753
10. Miller, Kim. Factor Products in the treatment of hemophilia. “Journal of pediatric health care, (2004). Vol. 18, pp. 156-157
11. Agliotis, Dimitrios. Hemophilia, Overview. “E-medicine” June 23, 2004 http://www.emedicine.com/med/topic3528.htm
12. Dictionary.com. Lexico Publishing group, 2005. http://dictionary.reference.com/
13. Majerus, P, Tollefsen, D. Anticoagulant, thrombolytic, and antiplatelet drugs. “Goodman & Gilman’s The pharmacological basis of therapeutics 10th ed.” Ed. Hardman, Limbird. McGraw-Hill, 2001. pp. 1520-1521
14. Josic, Dj. Schwinn, H. Stadler, M. Strancar, A. Purification of factor VIII and von Willebrand factor from human plasma by anion-exchange chromatography. “Journal of Chromatography B: Biomedical Applications” No. 662, 1994. pp-181-190.
15. Kwa, Andrew. Blood Clotting Cascade. University of California at San Francisco. Gladstone Institutes, 2000. http://www.genmapp.org/MAPPSetHuman/GenMAPP.org_MAPPs/Other_MAPPs/ Hs_Blood_Clotting_Cascade.htm
16. Osterberg, Thomas. Fatouros, Angelica. Mikaelsson, Marianne. Development of a Freeze-Dried albumin free formulation of recombinant factor VIII SQ. “Pharmaceutical Research” Vol 14, No. 7, 1997 pp. 892-898.
17. Osterberg, T. Fatourous, A. Neidhardt, E. Warren, N. Mikaelsson, M. “B-Domain deleted recombinant Factor VIII formulation and stability.” Seminars in Hematology. Vol. 38, No. 2, Suppl 4 (April), 2001: pp 40-43.
18. Tuddenham, EGD. Laffan, M. Purified Factor VIII: Theoretical advantages, but at a cost. “BMJ” 1995;311:465-466 (19 August)
19. Food and Drug Administration. FDA Talk Paper, “New Recombinant Antihemophilic Factor Licensed” Document #T03-55. July 25, 2003. http://www.fda.gov/bbs/topics/ANSWERS/2003/ANS01241.html
20. Pascual, Virginia. Capra, Donald. Tolerance to Factor VIII inhibitors in hemophilia A patients: A French twist. “Journal of Clinical Investigation.” Vol 97, No. 6, March 1996, 1357-1358.
21. Advate Package Insert. Baxter Healthcare Co. 2003. http://www.advate.com/images/pdf/prescribing_info_english.pdf
22. Teshima, Glen. Deamidation in Proteins and Peptides: Monograph 0001. “Ion Source.com” November 21, 2000. http://www.ionsource.com/Card/Deamidation/mono0001.htm
23. Wang, Yu-Chag John. Parental Products of Proteins and Peptides. Pharmaceutical Dosage Forms: Parental Medications, Vol. 1, 2nd Ed. Edited by: Avis, Lieberman, Lachman. Marcel Dekker Publishing, NY, 1992. pp. 291-296.
Biopharmaceuticals Using Common Techniques—and the Magnitude of Freeze-Concentration.

“Biopharm” Volume 15 Number 5 May 2002, 22-34.

47 Gomez, Adolfo. Delivering the delivery system: A brief guide to outsourcing formulation development. “Contract Pharma” Vol. 6, No. 8, October, 2004. pp.42-46

48 Jennings, Thomas. Lyophilization made easy. “Contract Pharma.” Vol. 6, No. 2 March 2004, pp. 56-60.

49 Gabriels, Joseph. A fast-flow, low-protein-binding membrane for sterile filtration. “American Biotechnology Laboratory.” April 1999 pp. 34-35.

50 Millipore Industries. Technical Publication-Validation Guide: Non-sterile 33 mm Millex® Filter Units with Durapore® PVDF Membrane. http://www.millipore.com/publications.nsf/dda0cb48e91c0fb6852567430063b5d6/2e3c458f53db40085256eb0068847d/$FILE/VG002EN00.pdf

51 Bedu-Addo, Frank Kofi. Lyophilization: A technology primer, Understanding lyophilization formulation development “Supplement to Pharmaceutical technology, 2004.”pp10-19.

52 Rey, L.R. Basic aspects and future trends in the freeze-drying of pharmaceuticals. “International symposium on biological product freeze-drying and formulation.” Bethesda, USA 1990 Develop. Biol. Standard., Vol. 74, pp3-8

53 Pikal, M. Overview of Freeze Drying Science and Technology. Seminar July 14, 2004. pp. 4-6

54 Trappler, E. “Fundamental Aspects of Lyophilization.” HULL Corportaion, Hatboro, Pennsylvania. www.hullcompany.com/html/sem_desc.html

55 Searles, Carpenter, Randolph. The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf. “Journal of Pharmaceutical Sciences, Vol. 90, No.7, July 2001.” pp.860-871

56 Snowman, J.W. Formulation and cycle development for lyophilization: First steps. “Pharmaceutical Engineering” Nov/Dec 1993, pp 26-34.

57 Williams, Polli. The lyophilization of Pharmaceuticals: A literature review. “Journal of parenteral science and technology.” Vol. 38, No. 2/ March-April 1984, 48-59.

58 Searles, Carpenter, Randolph. Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine Tg’ in pharmaceutical lyophilization. “Journal of pharmaceutical sciences, Vol. 90, No. 7, July 2001 pp 872-887.

59 Serena, Cleland, Carpenter, Randolph. Effects of annealing lyophilized and spray-lyophilized formulations of recombinant human interferon-γ. “Journal of pharmaceutical sciences, Vol. 92, NO. 4, April 2003, pp.715-729.

60 Franks, Felix. Freeze-Drying of Labile Biologics: An overview. “The science of freeze-drying” seminar organized by Pafra Biopreservation, Cambridge, Ma 14-15 January 1993

61 Hatley, Ross. Storage Stability predictions and quality control. “The science of freeze-drying” seminar organized by Pafra Biopreservation, Cambridge, Ma 14-15 January 1993.

62 Lusher, J.M. Lee, C.A. Kessler, C.M. Bedrosians, C.L. The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. “Haemophilia (2003), 9, pp. 38-49

63 Fatourous, Angelica. Pharmaceutical formulation and impact on protein structure and stability: Recombinant Factor VIII SQ. “The Royal Danish School of Pharmacy, Department of Pharmaceuticals.” Copenhagen, 1998.

64 Wang, Wei. Wang, John. Kelner, Drew. Coagulation Factor VIII: Structure and Stability. “International Journal of Pharmaceutics” No 259 (2003), pp. 1-15.

65 Seghatchian MJ, Miler-Andersson M. A simple and sensitive new technique for screening FVIII activity in blood donors and detection of carriers of haemophiliaics. Proc Xith Congr WFH 1976; 309-311.

66 Rosen, S. Assay of Factor VIII:C with a chromogenic substrate. Scand Journal of Haemotol 1984: 33 (Suppl 40): 139-145.

67 Stevenson, Robert. The World of Separation Science: HPLC '01: NEW TECHNOLOGY FOCUSES ON IMPROVING PEAK CAPACITY—HYPHENATION, MULTIDIMENSIONAL SEPARATIONS, AND MONOLITHS. American Laboratory August 2001: 50-56.
68 SAS Inc. JMP 5.1. Help section. One-way Analysis of Variance and t-Test: Tables and Plots.
October 2003,
69 Gaddis, Monica. Gaddis, Gary. Introduction to Biostatistics. Annals of Emergency Medicine 19:7
July 1990 PP. 137-142.
70 O.P. Chilson, L.A. Costello, and N.O. Kaplan, “Effects of freezing on enzyme,” Fed. Proced., 24,
Supp. 15, s55-s65 (1965).
71 F. Franks, T. H. M. Hatley, and H.L. Friedman, “The thermodynamics of protein stability: Cold
destabilization as a general phenomenon,” Biophysical Chemistry, 31, 307-315 (1988).
72 Hsu, Chung. Nguyen, Hoc. Young, Douglas. Brooks, Dennis. Koe, Gary. Bewley, Thomas.
Pearlman, Rodney. Surface denaturation at solid-void interface- A possible pathway by which
opalaceous particulates form during the storage of lyophilized tissue-type plasminogen activator at high
temperatures. Pharmaceutical Research, Vol. 12, No. 1 1995. 69-77.
73 Chang, Beyong. Kendrick, Brent. Carpenter, John. Surface-Induced Denaturation of Proteins
during Freezing and Its Inhibition by Surfactants. Journal of Pharmaceutical Sciences. Vol. 85, No.
12, December 1996. PP. 1325-1330.
74 Webb, Serena. Cleland, Jeffrey. Carpenter, John. Randolph, Theodore. Effects of Annealing
Lyophilized and Spray-Lyophilized Formulations of Recombinant Human Interferon-γ. Journal of
Pharmaceutical Sciences, Vol. 92, No. 4, April 2003. PP 715-729.
75 Xiang, Jun. Hey, Jeffery. Lietdke, Volker. Wang, D.Q. Investigation of freeze-drying sublimation
rates using a freeze-drying microbalance technique. International Journal of Pharmaceutics 279 (2004) 95–105
76 Horbett, T.A. and Brash, J.L. (1986) in Proteins at Interfaces- Physicochemical and Biochemical
Studies ACS Symposium Series 343, American Chemical Society.
77 Messing, Ralph. Adsorption of proteins on the glass surfaces and pertinent parameters for the
immobilization of enzymes in the pores of inorganic carriers. Journal of Non-Crystalline Solids. Vol.
19, December 1975. Pages 275-283.
78 Pasto, Daniel. McGraw-Hill Encyclopedia of Science & Technology, 6th ed. McGraw Hill
Publishing, NY. 1987. Pg. 344-345.
79 Arakawa, Tsutomo. Timasheff, Serge. “Stabilization of protein structure by sugars” Biochemistry
Vol. 21, No. 25, December 7, 1982. Pg. 6536-6544
80 Fatorous, Angelica. Osterberg, Thomas. Mikaelsson, Marianne. Recombinant factor VIII SQ-
Influence of oxygen, metal ions, pH and ionic strength on its stability in aqueous solution.
International Journal of Pharmaceutics 155 (1997) 121-131.
81 Parkins, Dave. Lashmar, Ulla. The formulation of biopharmaceutical products. Pharmaceutical
Science & Technology Today. Vol. 3, No. 4 April 2000. 129-137.
## Appendix I (Equipment)

| Equipment Name (Abbr.) | Manufacturer | Model No | Serial No |
|------------------------|--------------|----------|-----------|
| 2-8°C Cold Room        | N/A          | N/A      | N/A       |
| Lyophilizer            | Edwards      |          |           |
| Lyophilizer            | Lyostar      |          |           |
| Waters 2695- HPLC      | Waters       | 2695     |           |
| system consisting of the following: | | | |
| Waters 600S Controller 6DC | Waters | 600S | D006DC |
| Waters 474 Scanning Fluorescence Detector | Waters | 474 | L98474091M |
| Waters Dual Absorbance Detector 2487 | Waters | 2487 | D00487304M |
| Waters 717 Plus Autosampler | Waters | 717 | C9971P049M |
| Cobas-FARA Centrifugal Spectrophotometer | (Roche Analytical Instruments) | | |
| Cobas-FARA Reagent Cups | | | |
| Cobas-FARA Cuvettes | | | |
| Cobas Sample Cups | | | |
| Balance | Mettler | | |
| Calibrated Pippetes (May 04): P20, P100, P200 and P1000 | | | |
| pH meter | Orion | | |
# Appendix II: Reagents and Materials

| Chemical Name                      | Manufacturer | Grade   | Catalog No. | Lot Number   |
|-----------------------------------|--------------|---------|-------------|--------------|
| ReFactoAF Active Substance        | Wyeth/GI     | GMP     | N/A         | #PO34109m01  |
| ReFactoAF Concentration Standard  | Wyeth/GI     |         |             | 02417M01     |
| ReFactoAF Potency Standard        | Wyeth/GI     |         |             | 86759-51     |
| 0.9% NaCl                         | Abbott       | 10 mL vial | NDC0074-4888-10 |             |
| Sodium Chloride                   | Sigma        | USP     | S-1679      |              |
| Trizma Base                       | Sigma        | USP     | #T-1503     |              |
| BSA 1%                            | Sigma        | USP     | #A-7030     |              |
| Mobile phase A for AEX-HPLC       | Wyeth/GI     | Research | N/A         |              |
| Mobile phase B for AEX-HPLC       | Wyeth/GI     | Research | N/A         |              |
| AEX Column                        | Pharmacia    | Research | 17-0546-011 |              |
| Factor IXa+X                      | Chromogenix  | Reagent  | N/A         |              |
| S2222 20 mg-I2581                 | Chromogenix  | Reagent  | N/A         |              |
| Calcium Chloride                  | Chromogenix  | Reagent  | N/A         |              |
| Phospholipid Emulsion             | Chromogenix  | Reagent  | N/A         |              |
| MFR218 buffer                     | Wyeth        | N/A     | N/A         |              |
| Sucrose                           | Sigma        | USP     | S7903       |              |
| MFR 275                           | Wyeth        | N/A     | N/A         |              |
| MFR 323                           | Wyeth        | N/A     | N/A         |              |
| Millipore                         | Millipore    | N/A     |             |              |
| Durapore 0.22 µm filters | Millipore Stericup | Milli-Q Water | pH 4.0 standards | pH 7.0 standards |
|--------------------------|--------------------|---------------|------------------|------------------|
| Durapore 0.22 µm filters | Millipore          | N/A           | SCG9402RE        | N/A              |
| Millipore Stericup       | Wyeth              | N/A           | N/A              | Andover K2100    |
| Milli-Q Water            |                    |               | SB101-500        | SB107-500        |
| pH 4.0 standards         | Fisher             | Research      | SB101-500        | SB107-500        |
| PH 7.0 standards         | Fisher             | Research      | SB101-500        | SB107-500        |
Appendix III

Section 1: Lyophilization Cycle experiments

1. Lyophilization cycle- “Stepwise” approach

A. Lyophilization cycle stopped after annealing

Oneway Analysis of Mean (Conc. Ug/mL) By ID

![Diagram showing mean concentrations by ID]

Oneway Anova

Summary of Fit

| Source          | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|-----------------|----|----------------|-------------|---------|----------|
| ID              | 4  | 458.66274      | 114.666     | 31.7342 | <.0001   |
| Error           | 140| 505.86388      | 3.613       |         |          |
| C. Total        | 144| 964.52662      |             |         |          |

Means for Oneway Anova

| Level           | Number | Mean   | Std Error | Std Dev |
|-----------------|--------|--------|-----------|---------|
| Coated          | 30     | 25.9953| 0.34705   | 1.8414738|
| Pre-Lyo Coated  | 25     | 24.5016| 0.38017   | 0.7771244|
| Pre-Lyo PPE     | 30     | 28.86  | 0.34705   | 3.2294699|
| Pre-Lyo Tubing  | 30     | 23.766 | 0.34705   | 0.47189712|
| Tubing          | 30     | 25.172 | 0.34705   | 1.70311358|

Std Error uses a pooled estimate of error variance

ID         N   Mean     Std Dev
Coated     30  25.9953333  1.8414738
Pre-Lyo Coated 30  24.5016  0.7771244
Pre-Lyo PPE 30  28.86  3.2294699
Pre-Lyo Tubing 30  23.766  0.47189712
Tubing 30  25.172  1.70311358
B. Lyophilization cycle stopped after primary drying

Oneway Analysis of Mean (Conc. Ug/mL) By ID

|   | Coated | Tubing |
|---|--------|--------|
| Mean (Conc. Ug/mL) | 11.5   | 11.5   |

Oneway Anova - Summary of Fit

Rsquare | 0.158533
Adj Rsquare | 0.142351
Root Mean Square Error | 0.542159
Mean of Response | 10.765
Observations (or Sum Wgts) | 54

$t$ Test

Coated-Tubing
Assuming equal variances

| Difference | 0.461852 | $t$ Ratio | 3.129988 |
| Std Err Dif | 0.147557 | DF | 52 |
| Upper CL Dif | 0.757947 | Prob > $|t|$ | 0.0029 |
| Lower CL Dif | 0.165757 | Prob > $t$ | 0.0014 |
| Confidence | 0.95 | Prob < $t$ | 0.9986 |

Analysis of Variance

| Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|---|---|---|---|---|---|
| ID | 1 | 2.879646 | 2.87965 | 9.7968 | 0.0029 |
| Error | 52 | 15.284704 | 0.29394 |
| C. Total | 53 | 18.164350 |

Means for Oneway Anova

| Level | Number | Mean | Std Error | Lower 95% | Upper 95% |
|---|---|---|---|---|---|
| Coated | 27 | 10.9959 | 0.10434 | 10.787 | 11.205 |
| Tubing | 27 | 10.5341 | 0.10434 | 10.325 | 10.743 |

Std Error uses a pooled estimate of error variance

| ID | N | Mean | Std Dev |
|---|---|---|---|
| Coated | 30 | 10.9959259 | 0.25662393 |
| Pre-Lyo Coated | 30 | 26.362 | 1.07727434 |
| Pre-Lyo PPE | 30 | 23.5108333 | 2.03095376 |
| Pre-Lyo Tubing | 30 | 25.7566667 | 2.22171157 |
| Tubing | 30 | 10.5340741 | 0.7225077 |
C. Full Lyophilization cycle

One way Analysis of Mean (Conc. Ug/mL) By ID

\[ \text{Coated} \quad \text{Pre-Lyo Coated} \quad \text{Pre-Lyo PPE} \quad \text{Pre-Lyo Tubing} \quad \text{Tubing} \]

### One way ANOVA
#### Summary of Fit

| Source           | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|------------------|----|----------------|-------------|---------|----------|
| ID               | 4  | 8806.9416      | 2201.74     | 861.1397 | <.0001   |
| Error            | 142| 363.0612       | 2.56        |         |          |
| C. Total         | 146| 9170.0028      |             |         |          |

#### Means for One way ANOVA

| Level            | Number | Mean   | Std Error | Lower 95% | Upper 95% |
|------------------|--------|--------|-----------|-----------|-----------|
| Coated           | 30     | 12.1363| 0.29193   | 11.559    | 12.713    |
| Pre-Lyo Coated   | 28     | 27.4707| 0.30218   | 26.373    | 28.068    |
| Pre-Lyo PPE      | 29     | 28.2148| 0.29692   | 27.628    | 28.802    |
| Pre-Lyo Tubing   | 30     | 26.781 | 0.29193   | 26.204    | 27.358    |
| Tubing           | 30     | 11.3977| 0.29193   | 10.821    | 11.975    |

Std Error uses a pooled estimate of error variance

### Std Error

| ID               | N   | Mean   | Std Dev |
|------------------|-----|--------|---------|
| Coated           | 30  | 12.1363| 0.2688992|
| Pre-Lyo Coated   | 30  | 27.4707| 3.05278613|
| Pre-Lyo PPE      | 30  | 28.2148| 1.46510999|
| Pre-Lyo Tubing   | 30  | 26.781 | 0.91070397|
| Tubing           | 30  | 11.3977| 0.93185645|
Oneway Analysis of Mean (Conc. Ug/mL) By ID

Oneway Anova
Summary of Fit

Rsquare AdjRsquare Root Mean Square Error
Mean of Response Observations (or Sum Wgts)

0.230783 0.217521 0.685807 11.767 60

| Test | Type I Tubing- Untreated-Type I+ Coated Assuming equal variances |
|------|------------------------------------------------------------------|
|      | Difference | Std Err Dif | Upper CL Dif | Lower CL Dif | Confidence |
|      | -0.7387 | 0.1771 | -0.3842 | 0.0001 | 0.95 |
|      | -4.1715 | 58 | 0.0001 | 0.9999 | <0.001 |

Analysis of Variance

| Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|--------|----|----------------|-------------|---------|----------|
| ID     | 1  | 8.184427       | 8.18443     | 17.4014 | 0.0001   |
| Error  | 58 | 27.279233      | 0.47033     |         |          |
| C. Total | 59 | 35.463660      |             |         |          |

Means for Oneway Anova

| Level                | Number | Mean | Std Error | Lower 95% | Upper 95% |
|----------------------|--------|------|-----------|-----------|-----------|
| Type I Tubing- Untreated | 30     | 11.3977 | 0.12521 | 11.147 | 11.648 |
| Type I+ Coated        | 30     | 12.1363 | 0.12521 | 11.886 | 12.387 |

Std Error uses a pooled estimate of error variance
2. Freeze and hold studies

A. Freeze and Hold without dilution

Oneway Analysis of Mean (Conc. Ug/mL) By ID

Oneway Anova
Summary of Fit

| Source        | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|---------------|----|----------------|-------------|---------|----------|
| ID            | 5  | 93.84293       | 18.7686     | 6.9297  | <.0001   |
| Error         | 174 | 471.26577     | 2.7084      |         |          |
| C. Total      | 179 | 565.10869     |             |         |          |

Means for Oneway Anova

| Level          | Number | Mean   | Std Error | Lower 95% | Upper 95% |
|----------------|--------|--------|-----------|-----------|-----------|
| Coated         | 30     | 28.358 | 0.30047   | 27.765    | 28.951    |
| Pre-Lyo Coated | 30     | 29.123 | 0.30047   | 28.530    | 29.716    |
| Pre-lyo PPE    | 30     | 28.562 | 0.30047   | 27.969    | 29.155    |
| Pre-lyo Tubing | 30     | 26.954 | 0.30047   | 26.361    | 27.547    |
| Siliconized    | 30     | 28.074 | 0.30047   | 27.481    | 28.667    |
| Tubing         | 30     | 27.399 | 0.30047   | 26.806    | 27.992    |

Std Error uses a pooled estimate of error variance

| ID          | N  | Mean | Std Dev |
|-------------|----|------|---------|
| Coated      | 30 | 28.358 | 0.750   |
| Pre-Lyo Coated | 30 | 29.123 | 1.564   |
| Pre-lyo PPE | 30 | 28.562 | 2.797   |
| Pre-lyo Tubing | 30 | 26.954 | 1.568   |
| Siliconized | 30 | 28.074 | 0.848   |
| Tubing      | 30 | 27.399 | 1.497   |
A. Continued freeze and hold without dilution

Oneway Analysis of Mean (Conc. Ug/mL) By ID

| Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|--------|----|----------------|-------------|---------|----------|
| ID     | 1  | 20.27691       | 20.2769     | 4.0290  | 0.0494   |
| Error  | 58 | 291.90129      | 5.0328      |         |          |
| C. Total | 59 | 312.17819      |             |         |          |

Means for Oneway Anova

| Level       | Number | Mean | Std Error | Lower 95% | Upper 95% |
|-------------|--------|------|-----------|-----------|-----------|
| Pre-lyo PPE | 30     | 28.5617 | 0.40958 | 27.742 | 29.382 |
| Tubing      | 30     | 27.3990 | 0.40958 | 26.579 | 28.219 |

Std Error uses a pooled estimate of error variance

| ID | Mean(Ug/mL) | Std Dev |
|----|-------------|---------|
| Pre-lyo PPE | 28.5616667 | 2.79719451 |
| Tubing       | 27.3990 | 1.49708533 |

Oneway Anova - Summary of Fit

| R Square | Adj R Square | Root Mean Square Error | Mean of Response | Observations (or Sum Wgts) |
|----------|--------------|------------------------|------------------|-----------------------------|
| 0.064953 | 0.048831     | 2.243386               | 27.98033         | 60                          |

t Test

Pre-lyo PPE-Tubing
Assuming equal variances

| Difference | t Ratio | Prob > t |
|------------|---------|----------|
| 1.16270    | 2.007229| 0.00319  |

ID N Mean(Ug/mL) Std Dev

Pre-lyo PPE 30 28.5616667 2.79719451
Tubing 30 27.3990 1.49708533

Analysis of Variance

-2.0 -1.5 -1.0 -0.5 0 0.5 1.0 1.5 2.0

ID

Pre-lyo PPE 30
Tubing 30
B. Freeze and Hold with 0.9% NaCl Dilution

One way Analysis of Mean (Conc. Ug/mL) By ID

![Graph](image)

One way Anova - Summary of Fit

|          |        |        |
|----------|--------|--------|
| R-square | 0.25921|        |
| Adj R-square | 0.246363|        |
| Root Mean Square Error | 0.75187|        |
| Mean of Response | 11.26579|        |
| Observations (or Sum Wgts) | 57|        |

**t Test**

Coated-Tubing

Assuming equal variances

| Difference | 0.87637 | t Ratio | 4.3939 |
| Std Err Dif | 0.19945 | DF | 55 |
| Upper CL Dif | 1.27608 | Prob > | <.0001 |
| Lower CL Dif | 0.47666 | Prob > t | <.0001 |
| Confidence | 0.95 | Prob < t | 1.0000 |

Analysis of Variance

| Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|--------|----|----------------|-------------|---------|----------|
| ID | 1 | 10.914040 | 10.9140 | 19.3064 | <.0001 |
| Error | 55 | 31.091950 | 0.5653 | | |
| C. Total | 56 | 42.005989 | | | |

Means for One way Anova

| Level | Number | Mean | Std Error | Lower 95% | Upper 95% |
|-------|--------|------|-----------|-----------|-----------|
| Coated | 27 | 11.7270 | 0.1447 | 11.437 | 12.017 |
| Tubing | 30 | 10.8507 | 0.1372 | 10.576 | 11.126 |

Std Error uses a pooled estimate of error variance

| ID | N | Mean(Ug/mL) | Std. Deviation |
|----|---|-------------|----------------|
| Coated | 30 | 11.727037 | 0.44454657 |
| Tubing | 30 | 10.850667 | 0.94602228 |
3. 1mL, 2mL, 4mL Reconstitution studies with different diluents

A. 4mL Reconstitution with three different diluents

One way Analysis of Amount (Ug/mL) By ID

![Box plot showing amount of reconstitution studies with different diluents.]

One way Anova
Summary of Fit

|               | 0.24949 | 0.232237 | 1.508886 | 49.78367 |
|---------------|---------|----------|----------|----------|
| Rsquare       | Adj Rsquare | Root Mean Square Error | Mean of Response | Observations (or Sum Wgts) |
| Source        | DF      | Sum of Squares | Mean Square | F Ratio | Prob > F |
| ID            | 2       | 65.84586    | 32.9229   | 14.4606 | <.0001   |
| Error         | 87      | 198.07603   | 2.2767    |          |          |
| C. Total      | 89      | 263.92189   |           |          |          |

Analysis of Variance

| Source        | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|---------------|----|----------------|-------------|---------|----------|
| .9 Nacl Recon 4mL | 30 | 50.1027        | 0.27548     | 49.555  | 50.650   |
| 1.8 Nacl Recon 4mL | 30 | 50.6347        | 0.27548     | 50.087  | 51.182   |
| H2O Recon 4mL   | 30 | 48.613667     | 0.27548     | 48.066  | 49.161   |

Means for One way Anova

| Level             | Number | Mean (Ug/mL) | Std. Dev. |
|-------------------|--------|--------------|-----------|
| .9 Nacl Recon 4mL | 30     | 50.1026667   | 1.8262963 |
| 1.8 Nacl Recon 4mL| 30     | 50.6346667   | 1.24084713|
| H2O Recon 4mL     | 30     | 48.6136667   | 1.39826613|
B. 2mL Reconstitution with three different diluents

One way Analysis of Amount (Ug/mL) By ID

![Graph showing the analysis of amount (Ug/mL) by ID with different diluents.]

One way Anova  
Summary of Fit

| Source    | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|-----------|----|----------------|-------------|---------|----------|
| ID        | 2  | 129.9745       | 64.9873     | 2.7268  | 0.0710   |
| Error     | 87 | 2073.4641      | 23.8329     |         |          |
| C. Total  | 89 | 2203.4386      |             |         |          |

Means for One way Anova

| Level                  | Number | Mean   | Std Error | Lower 95% | Upper 95% |
|------------------------|--------|--------|-----------|-----------|-----------|
| 0.9 NaCl Recon 2mL     | 30     | 104.47633 | 0.89131 | 102.70    | 106.25    |
| 1.8 NaCl Recon 2mL     | 30     | 102.772 | 0.89131 | 101.00    | 104.54    |
| H2O Recon 2mL          | 30     | 101.546 | 0.89131 | 99.77     | 103.32    |

Std Error uses a pooled estimate of error variance

| ID                  | N  | Mean (Ug/mL) | Std. Dev |
|---------------------|----|--------------|----------|
| 0.9 NaCl Recon 2mL  | 30 | 104.47633    | 6.86423489 |
| 1.8 NaCl Recon 2mL  | 30 | 102.772      | 1.4194059  |
| H2O Recon 2mL       | 30 | 101.545667   | 4.72930532 |
C. 1mL Reconstitution with three different diluents

Oneway Analysis of Amount (µg/mL) By ID

![Graph showing reconstitution with three different diluents]

Oneway Anova
Summary of Fit

- R2: 0.084041
- Adj R2: 0.062489
- Root Mean Square Error: 7.605981
- Mean of Response: 202.6765
- Observations (or Sum Wgts): 88

Analysis of Variance

| Source    | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|-----------|----|----------------|-------------|---------|----------|
| ID        | 2  | 451.6508       | 225.825     | 3.8995  | 0.0240   |
| Error     | 85 | 4922.5038      | 57.912      |         |          |
| C. Total  | 87 | 5374.1546      |             |         |          |

Means for Oneway Anova

| Level                  | Number | Mean     | Std Error | Lower 95% | Upper 95% |
|------------------------|--------|----------|-----------|-----------|-----------|
| 0.9 NaCl Recon 1mL     | 28     | 205.959  | 1.4382    | 203.10    | 208.82    |
| 1.8 NaCl Recon 1mL     | 30     | 201.538  | 1.3894    | 198.78    | 204.30    |
| H2O Recon 1mL          | 30     | 200.752  | 1.3894    | 197.99    | 203.51    |

Std Error uses a pooled estimate of error variance

| ID                  | N  | Mean       | Std. Dev. |
|---------------------|----|------------|-----------|
| 0.9 NaCl Recon 1mL  | 30 | 205.958571 | 6.98180212|
| 1.8 NaCl Recon 1mL  | 30 | 201.538    | 8.36187341|
| H2O Recon 1mL       | 30 | 200.751667 | 7.37812895|
4. Annealing vs. No Annealing full lyophilization cycle

A) Full lyophilization cycle with annealing

One-way Analysis of Mean (Conc. Ug/mL) By ID

| ID       | Sum of Squares | Mean Square | F Ratio | Prob > F |
|----------|----------------|-------------|---------|----------|
| ID       | 8806.9416      | 2201.74     | 861.1397| <.0001   |
| Error    | 363.0612       | 2.56        |         |          |
| C. Total | 9170.0028      |             |         |          |

Means for One-way Anova

| Level              | Number | Mean  | Std Error | Lower 95% | Upper 95% |
|--------------------|--------|-------|-----------|-----------|-----------|
| Coated             | 30     | 12.1363 | 0.29193   | 11.559    | 12.713    |
| Pre-Lyo Coated     | 28     | 27.4707 | 0.30218   | 26.873    | 28.068    |
| Pre-Lyo PPE        | 29     | 28.2148 | 0.29692   | 27.628    | 28.802    |
| Pre-Lyo Tubing     | 30     | 26.7810 | 0.29193   | 26.204    | 27.358    |
| Tubing             | 30     | 11.3977 | 0.29193   | 10.821    | 11.975    |

Std Error uses a pooled estimate of error variance

ID | N | Mean(Ug/mL) | Std Dev  
---|---|-------------|----------
Coated | 30 | 12.1363333 | 0.2688992 |
Pre-Lyo Coated | 30 | 27.4707143 | 3.05278613 |
Pre-Lyo PPE | 30 | 28.2148276 | 1.46510999 |
Pre-Lyo Tubing | 30 | 26.781 | 0.91070397 |
Tubing | 30 | 11.3976667 | 0.93185645 |
A. Continued Full Lyophilization cycle with annealing

One way Analysis of Mean (Conc. Ug/mL) By ID

| ID | Type I Tubing- Untreated | Type I+ Coated |
|----|-------------------------|----------------|
|    | 11.3977                 | 12.1363        |

One way Anova

Summary of Fit

- Rsquare: 0.230783
- Adj Rsquare: 0.217521
- Root Mean Square Error: 0.685807
- Mean of Response: 11.767
- Observations (or Sum Wgts): 60

**t Test**

Type I Tubing- Untreated-Type I+ Coated

Assuming equal variances

- Difference: -0.7387
- Std Err Dif: 0.1771
- Upper CL Dif: -0.3842, Prob > |t|: 0.0001
- Lower CL Dif: -1.0931, Prob > t: 0.9999
- Confidence: 0.95, Prob < t: <.0001

**Analysis of Variance**

- Source: ID, Error, C. Total
- DF: 1, 58, 59
- Sum of Squares: 8.184427, 27.279233, 35.463660
- Mean Square: 8.18443, 0.47033
- F Ratio: 17.4014
- Prob > F: 0.0001

**Means for One way Anova**

- Level: Type I Tubing- Untreated, Type I+ Coated
- Number: 30, 30
- Mean: 11.3977, 12.1363
- Std Error: 0.12521, 0.12521
- Lower 95%: 11.147, 11.886
- Upper 95%: 11.648, 12.387

Std Error uses a pooled estimate of error variance
B. Full Cycle without annealing

Oneway Analysis of Mean (Conc. Ug/mL) By ID

| ID | Mean (Conc. Ug/mL) | 95% CI | Std. Dev. |
|----|--------------------|--------|-----------|
| 10 | 12.7433333         | 12.347 - 13.139 | 0.19631 |
| 11 | 10.4008333         | 10.031 - 10.771 | 0.18363 |

Analysis of Variance

| Source    | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|-----------|----|----------------|-------------|---------|-----------|
| ID        | 1  | 61.457830      | 61.4578     | 75.9431 | <.0001    |
| Error     | 43 | 34.79250       | 0.8093      |         |           |
| C. Total  | 44 | 96.256080      |             |         |           |

Means for Oneway Anova

| Level                  | Number | Mean   | Std Error | Lower 95% | Upper 95% |
|------------------------|--------|--------|-----------|-----------|-----------|
| Type 1 Siliconized Tubing | 21     | 12.7433 | 0.19631   | 12.347    | 13.139    |
| Type 1 Unsiliconized Tubing | 24     | 10.4008 | 0.18363   | 10.031    | 10.771    |

Std Error uses a pooled estimate of error variance

**ID**

| Type 1 Siliconized Tubing | 30 | 12.7433333 | 0.43706216 |
| Type 1 Unsiliconized Tubing | 30 | 10.4008333 | 1.16054304 |
C. Comparison of annealed and unannealed products

Oneway Analysis of Mean (Conc. Ug/mL) By ID

![Graph showing comparison between annealed and unannealed products]

### Oneway Anova - Summary of Fit

- Rsquare: 0.190883
- Adj Rsquare: 0.175323
- Root Mean Square Error: 1.039232
- Mean of Response: 10.95463
- Observations (or Sum Wgts): 54

### t Test

Type 1 Unsiliconized Tubing-Type 1 Unsiliconized Tubing- Annealed
Assuming equal variances

- Difference: -0.9968
- t Ratio: 3.50251
- Std Err Dif: 0.2846
- DF: 52
- Upper CL Dif: 0.4257
  - Prob > |t|: 0.0010
- Lower CL Dif: -1.5679
  - Prob > t: 0.9995
- Confidence: 0.95
  - Prob < t: 0.0005

### Analysis of Variance

| Source     | DF  | Sum of Squares | Mean Square | F Ratio | Prob > F |
|------------|-----|----------------|-------------|---------|----------|
| ID         | 1   | 13.249023      | 13.2490     | 12.2676 | 0.0010   |
| Error      | 52  | 56.160120      | 1.0800      |         |          |
| C. Total   | 53  | 69.409143      |             |         |          |

### Means for Oneway Anova

| Level | Number | Mean  | Std Error | Lower 95% | Upper 95% |
|-------|--------|-------|-----------|-----------|-----------|
| Type 1 Unsiliconized Tubing | 24     | 10.4008 | 0.21213   | 9.975     | 10.827    |
| Type 1 Unsiliconized Tubing- Annealed | 30     | 11.3977 | 0.18974   | 11.017    | 11.778    |

Std Error uses a pooled estimate of error variance

| ID | N  | Mean  | Std. Dev |
|----|----|-------|----------|
| Type 1 Unsiliconized Tubing | 24 | 10.4008 | 1.16054304 |
| Type 1 Unsiliconized Tubing- Annealed | 30 | 11.3977 | 0.93185645 |
Section 2: Vial Experiments

A. Siliconized vs. Untreated vials - Concentration

Oneway Analysis of Mean (Conc. Ug/mL) By ID

Oneway Anova - Summary of Fit

|                  |            |            |            |
|------------------|------------|------------|------------|
| Rsquare          | 0.638483   |            |            |
| Adj Rsquare      | 0.630075   |            |            |
| Root Mean Square Error | 0.89959   |            |            |
| Mean of Response | 11.494     |            |            |
| Observations (or Sum Wgts) | 45        |            |            |

T Test

Type 1 Siliconized Tubing-Type 1 Unsiliconized Tubing
Assuming equal variances

|                  |            |            |            |
| Difference        | 2.34250    | t Ratio    | 8.714533   |
| Std Err Dif       | 0.26880    | DF         | 43         |
| Upper CL Dif      | 2.88459    | Prob > |t|     | <.0001     |
| Lower CL Dif      | 1.80041    | Prob > |t|     | <.0001     |
| Confidence        | 0.95       | Prob < |t|     | 0.0000     |

Analysis of Variance

| Source     | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|------------|----|----------------|-------------|---------|----------|
| ID         | 1  | 61.457830      | 61.4578     | 75.9431 | <.0001   |
| Error      | 43 | 34.798250      | 0.8093      |         |          |
| C Total    | 44 | 96.256080      |             |         |          |

Means for Oneway Anova

| Level                   | Number | Mean     | Std Error | Lower 95% | Upper 95% |
|-------------------------|--------|----------|-----------|-----------|-----------|
| Type 1 Siliconized Tubing | 21     | 12.7433  | 0.19631   | 12.347    | 13.139    |
| Type 1 Unsiliconized Tubing | 24     | 10.4008  | 0.18363   | 10.031    | 10.771    |

Std Error uses a pooled estimate of error variance

| ID | N     | Mean (Ug) | Std. Dev. |
|----|-------|-----------|-----------|
|    | Type 1 Siliconized Tubing | 30 | 12.7433333 | 0.43706216 |
|    | Type 1 Unsiliconized Tubing | 30 | 10.4008333 | 1.16054304 |

73
B. Siliconized vs. Untreated Vials- Potency by COBAS

Oneway Analysis of U/ml By Lot

Oneway Anova
Summary of Fit

| R-square          | 0.900712          |
|-------------------|-------------------|
| Adj R-square      | 0.897402          |
| Root Mean Square Error | 39.28963        |
| Mean of Response  | 213.2786         |
| Observations (or Sum Wgts) | 94              |

Analysis of Variance

| Source            | DF  | Sum of Squares | Mean Square | F Ratio  | Prob > F |
|-------------------|-----|----------------|-------------|----------|----------|
| Lot               | 3   | 1260358.0      | 420119      | 272.1512 | <.0001   |
| Error             | 90  | 138932.9       | 1544        |          |          |
| C. Total          | 93  | 1399290.8      |             |          |          |

Means for Oneway Anova

| Level                          | Number | Mean  | Std Error | Lower 95% | Upper 95% |
|-------------------------------|--------|-------|-----------|-----------|-----------|
| Control                       | 5      | 16.133| 17.571    | -18.8     | 51.04     |
| Pre-Lyo PPE                   | 30     | 376.070| 7.173     | 361.8     | 390.32    |
| Type I Siliconized Tubing     | 30     | 162.061| 7.173     | 147.8     | 176.31    |
| Type I Unsiliconized Tubing   | 29     | 131.848| 7.296     | 117.4     | 146.34    |

Std Error uses a pooled estimate of error variance

Means and Std Deviations

| Level                          | Numbe | Mean  | Std Dev | Std Err Mean | Lower 95% | Upper 95% |
|-------------------------------|-------|-------|---------|--------------|-----------|-----------|
| Control                       | 5     | 16.133| 2.1792  | 0.975        | 13.43     | 18.84     |
| Pre-Lyo PPE                   | 30    | 376.070| 56.1202 | 10.611       | 354.37    | 397.77    |
| Type I Siliconized Tubing     | 30    | 162.061| 30.3346 | 5.538        | 150.73    | 173.39    |
| Type I Unsiliconized Tubing   | 29    | 131.848| 22.5734 | 4.192        | 123.26    | 140.43    |
B. Continued Siliconized vs. Untreated Vials - Potency by COBAS

Oneway Analysis of U/ml By Lot

![Oneway Anova Graph]

Summary of Fit

| Rsquare     | 0.247365 |
| Adj Rsquare | 0.234161 |
| Root Mean Square Error | 26.80439 |
| Mean of Response | 147.2105 |
| Observations (or Sum Wghts) | 59 |

**t Test**

Type I Siliconized Tubing-Type I Unsiliconized Tubing

Assuming equal variances

| Difference | 30.2125 | t Ratio | 4.328271 |
| Std Err Dif | 6.9803 | DF | 57 |
| Upper CL Dif | 44.1903 | Prob > | .0001 |
| Lower CL Dif | 16.2348 | Prob > t | <.0001 |
| Confidence | 0.95 | Prob < t | 1.0000 |

Analysis of Variance

| Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|--------|----|----------------|-------------|---------|----------|
| Lot    | 1  | 13459.862      | 13459.9     | 18.7339 | <.0001   |
| Error  | 57 | 40953.086      | 718.5       |         |          |
| C. Total | 58 | 54412.948     |             |         |          |

Means for Oneway Anova

| Level                  | Number | Mean   | Std Error | Lower 95% | Upper 95% |
|------------------------|--------|--------|-----------|-----------|-----------|
| Type I Siliconized Tubing | 30     | 162.061| 4.8938    | 152.26    | 171.86    |
| Type I Unsiliconized Tubing | 29     | 131.848| 4.9774    | 121.88    | 141.82    |

Std Error uses a pooled estimate of error variance
C. Type 1+ coated vials vs. untreated tubing vials

One way Analysis of Mean (Conc. Ug/mL) By ID

One way Anova
Summary of Fit

| Parameter                          | Value     |
|-----------------------------------|-----------|
| Rsquare                           | 0.230783  |
| Adj Rsquare                       | 0.217521  |
| Root Mean Square Error            | 0.685807  |
| Mean of Response                  | 11.767    |
| Observations (or Sum Wgts)        | 60        |

**t Test**
Type I Tubing- Untreated-Type I+ Coated
Assuming equal variances

| Difference       | -0.7387 | t Ratio | -4.1715 |
|------------------|---------|---------|---------|
| Std Err Dif      | 0.1771  | DF      | 58      |
| Upper CL Dif     | -0.3842 | Prob > [t]| 0.0001 |
| Lower CL Dif     | -1.0931 | Prob > t | 0.9999  |
| Confidence       | 0.95    | Prob < t | <.0001  |

**Analysis of Variance**

| Source     | DF  | Sum of Squares | Mean Square | F Ratio | Prob > F |
|------------|-----|----------------|-------------|---------|----------|
| ID         | 1   | 8.184427       | 8.18443     | 17.4014 | 0.0001   |
| Error      | 58  | 27.279233      | 0.47033     |         |          |
| C. Total   | 59  | 35.463660      |             |         |          |

**Means for One way Anova**

| Level           | Number | Mean   | Std Error | Lower 95% | Upper 95% |
|-----------------|--------|--------|-----------|-----------|-----------|
| Type I Tubing- Untreated | 30     | 11.3977| 0.12521   | 11.147    | 11.648    |
| Type I+ Coated  | 30     | 12.1363| 0.12521   | 11.886    | 12.387    |

Std Error uses a pooled estimate of error variance
Section 3: Formulation Studies

A. 1X vs 2X formulation

Oneway Analysis of mean conc. ug/ml By ID

![Graph showing mean concentrations for different formulations]

Oneway Anova

Summary of Fit

Rsquare Adj R-square Root Mean Square Error Mean of Response Observations (or Sum Wgts)
0.996542 0.996439 0.914972 20.90572 173

Analysis of Variance

| Source    | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
|-----------|----|----------------|-------------|---------|----------|
| ID        | 5  | 40293.111      | 8058.62     | 9625.975 | <.0001   |
| Error     | 167| 139.808        | 0.84        |         |          |
| C. Total  | 172| 40432.919      |             |         |          |

Means for Oneway Anova

| Level               | Number | Mean  | Std Error | Lower 95% | Upper 95% |
|---------------------|--------|-------|-----------|-----------|-----------|
| Coated 1X NaCl 6ml  | 30     | 11.352| 0.16705   | 11.022    | 11.682    |
| Coated 2X NaCl 6ml  | 30     | 11.545| 0.16705   | 11.215    | 11.875    |
| PPE 1X              | 30     | 26.573| 0.16705   | 26.243    | 26.903    |
| PPE 2x              | 29     | 52.507| 0.16991   | 52.172    | 52.843    |
| Tubing 1X NaCl 6ml  | 24     | 10.782| 0.18677   | 10.414    | 11.152    |
| Tubing 2X NaCl 6ml  | 30     | 11.702| 0.16705   | 11.373    | 12.032    |

Std Error uses a pooled estimate of error variance

ID N Mean (ug/mL) Std. Dev.
Coated 1X NaCl 6ml 30 11.352 0.27873359
Coated 2X NaCl 6ml 30 11.545 0.26125361
PPE 1X 30 26.573 1.03509503
PPE 2x 30 52.507931 1.74928226
Tubing 1X Nacl 6ml 30 10.7829167 0.49525118
Tubing 2X NaCl 6ml 30 11.7023333 0.67424381
Bibliography

Agarabi, Cyrus. 11/26/03. An overview of the aseptic manufacturing process. University of Rhode Island, Seminar: Aseptic manufacturing: Guidance documentation, slide 20.

Agliotis, Dimitrios. June 23, 2004. Hemophilia, Overview. E-medicine http://www.emedicine.com/med/topic3528.htm (Retrieved on 01/15/05)

Ansel, Howard, Allen, Loyd, Popovich, Nicholas. 1999. Pharmaceutical dosage forms and drug delivery systems. Lippincott Williams & Wilkins. 408-9.

Arakawa, Tsutomo. Timasheff, Serge. December 7, 1982. Stabilization of protein structure by sugars. Biochemistry Volume 21, Number 25 6536-6544.

Aswad, Dana. 1995. Deamidation and Isoaspartate Formation in Proteins. Promega Notes Magazine 52, 27.

Baxter Healthcare Co. 2003. Advate Package Insert. http://www.advate.com/images/pdf/prescribing_info_english.pdf (Retrieved on 01/12/05)

Bedu-Addo, Frank Kofi. 2004. Lyophilization: A technology primer, Understanding lyophilization formulation development. Supplement to Pharmaceutical technology 10-19.

Bickert, Betsy. Kwaitkowski, Janet. 2002. Coagulation Disorders. Pharmacotherapy: A pathophysiologic approach 5th ed. Ed. By Dipiro. McGraw Hill Companies, Inc. 1751-1753.

Burke, C., Steadman, B., Volkin, D., Tsai, P-K., Bruner, M. Middaugh, R. 1992 The adsorption of proteins to pharmaceutical container surfaces. International Journal of Pharmaceutics 86, 89-93.

Cartensen, Jens, Rhodes, C.T. 2000. Drug Stability Principles and Practices. Introductory overview. Chapter 1. Ed. Marcel Dekker. NY 3-8

Chang, Beyong, Kendrick, Brent. Carpenter, John. 1996. Surface-Induced Denaturation of Proteins during Freezing and Its Inhibition by Surfactants. Journal of Pharmaceutical Sciences. Volume 85, Number 12 1325-1330.

Chilson, O.P. Costello, L.A. and Kaplan, N.O. 1965. Effects of freezing on enzyme, Federal Proceedings 24, Supplement 15, s55-s65.
Creighton, Thomas. Encyclopedia of Human Biology, 2nd Ed., Academic Press 1997. 189-203

Curtis, R.A., Prausnitz, J.M., Blanch, H.W. 1998. Protein-Protein and Protein-salt interactions in aqueous protein solutions containing concentrated electrolytes. Biotechnology and Bioengineering. Volume 57, Number 1, 11-21.

Dal Monte, Paul. Edmond Rouan, Katy, Bam, Narendra. 2002. Biotechnolody-Based Pharmaceuticals. Chapter 22. Modern Pharmaceutics 4th ed. Eds. Banker and Rhodes. Marcel Dekker, NY 2002. Pg. 700-721

Dictionary.com. 2005. Exons. Lexico Publishing group. http://dictionary.reference.com/ (Retrieved on 01/13/05)

Duncan, M. Lee, J. Warchol, M. 1995. Influence of surfactants upon protein/peptide adsorption to glass and polypropylene. International Journal of Pharmaceutics 120, 179-188.

Ellis, John. Pinheiro, Teresa. 2002. Medicine: Danger- Misfolding Proteins. Nature 416, 483-484.

Emilien, Maloteaux, Penasse, Goodeve, Casimir. 2000. Haemophilias: advances towards genetic engineering replacement therapy. Clinical Laboratory Haemophilia 22, 313-323

Fatourous, Angelica. 1998. Pharmaceutical formulation and impact on protein structure and stability: Recombinant Factor VIII SQ. The Royal Danish School of Pharmacy, Department of Pharmaceutics, Copenhagen. Doctor of Philosophy dissertation in pharmaceutics.

Fatouros, Angelica. Brita, Sjostrom. 2000 Recombinant factor VIII SQ- the influence of formulation parameters on structure and surface adsorption. International journal of Pharmaceutics. 194, 69-79.

Fatourous, Angelica. Osterberg, Thomas. Mikaelsson, Marianne. 1997. Recombinant factor VIII SQ-Influence of oxygen, metal ions, pH and ionic strength on its stability in aqueous solution. International Journal of Pharmaceutics. 155, 121-131.

Food and Drug Administration. 1996. Guidance for Industry: For the submission of chemistry, manufacturing, and controls information for a therapeutic recombinant DNA-Derived product or a monoclonal antibody product for in vivo use. Center for Biologics Evaluation and Research.
Food and Drug Administration. CGMP for Finished Pharmaceuticals. Sec. 211.166 Stability Testing. Center for Drug Evaluation and Research. http://www.fda.gov/cvm/guidance/guide5part1.html#211166 (Retrieved on 06/12/05)

Food and Drug Administration. 2004. Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice. Center for Drug Evaluation and Research. http://www.fda.gov/cder/guidance/5882fnl.htm

Food and Drug Administration. 2003. New Recombinant Antihemophilic Factor Licensed Document #T03-55. FDA Talk Paper http://www.fda.gov/bbs/topics/ANSWERS/2003/ANS01241.html (Retrieved on 01/14/05)

Franks, Felix. January 14-15, 1993. Freeze-Drying of Labile Biologics: An overview. The science of freeze-drying seminar. Organized by Pafra Biopreservation, Cambridge, Ma

Gabriels, Joseph. 1999. A fast-flow, low-protein-binding membrane for sterile filtration. American Biotechnology Laboratory. April, 34-35.

Gaddis, Monica. Gaddis, Gary. 1990. Introduction to Biostatistics. Annals of Emergency Medicine 19, 7, 137-142.

Gomez, Adolfo. 2004. Delivering the delivery system: A brief guide to outsourcing formulation development. Contract Pharma Volume 6, Number 8, October, pp.42-46

Grillo, A. Edwards, K. Kashi, R. Shipley, K. Hu, L., Besman, M. Middaugh, C. 2001. Conformational origin of the aggregation of recombinant human factor VIII. Biochemistry. 40, 586-595.

Guzzetta, Andrew. 2000. Mass Spectrometry and Biotechnology Resource: Oxidation of Methionine. Ion Source.com http://www.ionsource.com/Card/MetOx/metox.1.gif (Retrieved on 04/12/04)

Franks, F. Hatley, T. and H.L. Friedman. 1988. The thermodynamics of protein stability: Cold destabilization as a general phenomenon. Biophysical Chemistry. 31, 307-315

Hampton, KK, Preston, FE. 1997. ABC of clinical haematology: Bleeding disorders, thrombosis, and anticoagulation. British Medical Journal. 314, 1026

Hatley, Ross. January 14-15, 1993. Storage Stability predictions and quality control. The science of freeze-drying seminar Organized by Pafra Biopreservation, Cambridge, Ma
Horbett, T.A. and Brash, J.L. (1986) in Proteins at Interfaces- Physicochemical and Biochemical Studies ACS Symposium Series 343, *American Chemical Society.*

Horton, Moran, Ochs, Rawn, Scrimgeour. 2002. *Principles of Biochemistry* 3rd ed. *Prentice Hall publishing*  
http://cwx.prenhall.com/horton/mediaLib/media_portfolio/text_images/FG04_01.JPG  
(Retrieved on 04/13/05)

Hsu, Chung. Nguyen, Hoc. Young, Douglas. Brooks, Dennis. Koe, Gary. Bewley, Thomas. Pearlman, Rodney. 1995. Surface denaturation at solid-void interface- A possible pathway by which opalescent particulates form during the storage of lyophilized tissue-type plasminogen activator at high temperatures. *Pharmaceutical Research.* Volume 12, Number 1, 69-77.

Janetschek, Robert. 1999. Simplifying scaleup for the biopharmaceutical and biologics industries. *American Biotechnology Laboratory.* May, 20

Jennings, Thomas. 2004. Lyophilization made easy. *Contract Pharma.* Volume 6, Number 2, 56-60.

Josic, Dj. Schwinn, H. Stadler, M. Strancar, A. 1994. Purification of factor vIII and von Willebrand factor from human plasma by anion-exchange chromatography. *Journal of Chromatography B: Biomedical Applications.* 662, 181-190.

Kwa, Andrew. 2000. Blood Clotting Cascade. University of California at San Francisco. *Gladstone Institutes.*  
http://www.genmapp.org/MAPPSetHuman/GenMAPP.org_MAPPs/Other_MAPPs/Hs_Blood_Clotting_Cascade.htm  
(Retrieved on 06/20/05)

Lusher, J.M. Lee, C.A. Kessler, C.M. Bedrosians, C.L. 2003. The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. *Haemophilia.* 9, pp. 38-49

Majerus, P. Tollefsen, D. 2001. Anticoagulant, thrombolytic, and antiplatelet drugs. Goodman & Gilman’s The pharmacological basis of therapeutics 10th ed. Ed. Hardman, Limbird. *McGraw-Hill publishing,* 1520-1521

Miller, Kim. 2004. Factor Products in the treatment of hemophilia. *Journal of pediatric healthcare.* 18, 156-157

Messing, Ralph. 1975. Adsorption of proteins on the glass surfaces and pertinent parameters for the immobilization of enzymes in the pores of inorganic carriers. *Journal of Non-Crystalline Solids.* 19, 275-283.

81
Millipore Industries. Technical Publication-Validation Guide: Non-sterile 33 mm Millex® Filter Units with Durapore® PVDF Membrane. http://www.millipore.com/publications.nsf/dda0cb48e91c0fb685256743063b5d6/2e3c458f53dbb40085256ecb0068847d/$FILE/VG002EN00.pdf (Retrieved on 06/11/05)

Mizutani, Takaharu. 1981. Estimation of protein and drug adsorption onto silicone-coated glass surfaces. *Journal of Pharmaceutical Sciences.* Volume 70, Number 5, 493-496.

Neema, S, Avis, K. 1993. Freeze-thaw studies of a model protein, lactate dehydrogenase, in the presences of cryoprotectants. *Journal of Parenteral Science Technology.* 47(2), 76-83.

Osterberg, Thomas. Fatouros, Angelica. Mikaelsson, Marianne. 1997. Development of a Freeze-Dried albumin free formulation of recombinant factor VIII SQ. *Pharmaceutical Research.* Volume 14, Number 7, 892-898.

Osterberg, T. Fatorous, A. Neidhardt, E. Warren, N. Mikaelsson, M. 2001 “B-Domain deleted recombinant Factor VIII formulation and stability.” *Seminars in Hematology.* Volume 38, Number 2, Supplement 4, 40-43.

Parkins, Dave. Lashmar, Ulla. 2000. The formulation of biopharmaceutical products. *Pharmaceutical Science & Technology Today.* Volume 3, Number 4, 129-137.

Pascual, Virgina. Capra, Donald. 1996. Tolerance to Factor VIII inhibitors in hemophilia A patients: A French twist. *Journal of Clinical Investigation.* Volume 97, Number 6, 1357-1358.

Pasto, Daniel. 1987. *McGraw-Hill Encyclopedia of Science & Technology, 6th ed.* McGraw Hill Publishing, NY. 344-345.

Patro, Sugunakar. Freund, Erwin. Chang, Byeong. 2002. Protein formulation and fill-finish operations. *Biotechnology Annual Review.* 8, 55-84.

Pikal, M. July 14, 2004. Overview of Freeze Drying Science and Technology Seminar. *Organized by Wyeth BioPharma.* 4-6.

Rajanm Malika. Worldwide market for protein drugs to reach nearly $71 Billion by 2008. *Business Communications Company, Inc. Press Release,* www.bccresearch.com/press September 22, 2003 (Retrieved on 04/20/04)

Rey, L.R. 1990. Basic aspects and future trends in the freeze-drying of pharmaceuticals. “International symposium on biological product freeze-drying and formulation.” Bethesda, USA. *Developments in Biological Standard.* 74, 3-8.
Robinson, Noah. 2002. Biochemistry: Protein Deamidation. *Proceeding of the National Academy of Sciences in The United States of America.* Volume 99, Number 8, 5283-5288

Rosen, S. 1984. Assay of Factor VIII:C with a chromogenic substrate. *Scandinavian Journal of Haemotology.* 33, Supplement 40, 139-145.

SAS Inc. 2003. JMP 5.1. Help section. One-way Analysis of Variance and t-Test: Tables and Plots. (Retrieved on 07/12/05).

Schacter, Emily. Protein Oxidation: A primer on characterization, detection, and consequences. *Oxygen Society Education Program.* http://www.medicine.uiowa.edu/FRRB/VirtualSchool/Shacter-ProtOx.pdf (Retrieved on 06/19/05)

Searles, Carpenter, Randolph. 2001. Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine Tg’ in pharmaceutical lyophilization. *Journal of pharmaceutical sciences* Volume 90, Number 7, 872-887.

Searles, Carpenter, Randolph. 2001. The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf. *Journal of Pharmaceutical Sciences.* Volume 90, Number 7, 860-871.

Seghatchian MJ, Miler-Andersson M. 1976. A simple and sensitive new technique for screening FVIII activity in blood donors and detection of carriers of haemophiliacs. *Proceedings of the Xith Congress.* 309-311.

Serena, Cleland, Carpenter, Randolph. 2003. Effects of annealing lyophilized and spray-lyophilized formulations of recombinant human interferon-γ. *Journal of pharmaceutical sciences.* Volume 92, Number 4, 715-729.

Snowman, J.W. 1993. Formulation and cycle development for lyophilization: First steps. *Pharmaceutical Engineering.* November/December, 26-34.

Song, Yuan, Schowen, Richard. Borchadt, Ronald, Topp, Elizabeth. 2001 Effect of ‘pH’ on the rate of Asparagine Deamidation in Polymeric Formulations: ‘pH’- Rate Profile. *Journal of Pharmaceutical Sciences* 90, 141-156.

Stevenson, Robert. August, 2001. The World of Separation Science: HPLC ’01: New technology focuses on improving peak capacity—Hyphenation, multidimensional separations, and monoliths. *American Laboratory.* 50-56.
Teshima, Glen. 2000. Deamidation in Proteins and Peptides: Monograph 0001. Ion Source.com http://www.ionsource.com/Card/Deamidation/mono0001.htm (Retrieved on 07/20/05)

Theodorakis, M., Kutris, A., Renitakis, N. 1980. A study on particulate formation of silicone-coated glass surfaces. International Journal of Pharmaceutics 6, 333-337.

Trappler, E. “Fundamental Aspects of Lyophilization.” Hull Corporation, Hatboro, Pennsylvania. www.hullcompany.com/html/sem_desc.html (Retrieved on 08/20/05)

Tuddenham, EGD. Laffan, M. 1995. Purified Factor VIII: Theoretical advantages, but at a cost. British Medical Journal. 311, 465-466

Wahlgren, Marie, Arnebrant, T. 1991. Protein adsorption to solid surfaces. TIBTECH 9, 201-208.

Wang, Yu-Chag John. Parental Products of Proteins and Peptides. 1992. Pharmaceutical Dosage Forms: Parental Medications, Volume 1, 2nd Ed. Edited by: Avis, Lieberman, Lachman. Marcel Dekker Publishing, NY, 291-296.

Wang, Wei. 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. International Journal of Pharmaceutics 185, 129-188

Wang, Wei. Wang, John. Kelner, Drew. 2003. Coagulation Factor VIII: Structure and Stability. International Journal of Pharmaceutics. 259, 1-15.

Webb, Serena. Cleland, Jeffrey. Carpenter, John. Randolph, Theodore. 2003. Effects of Annealing Lyophilized and Spray-Lyophilized Formulations of Recombinant Human Interferon-γ. Journal of Pharmaceutical Sciences. Volume 92, Number 4, 715-729.

Webb, S, Webb, J, Hughes, T, Sesin, D, Kincaid, A. 2002. Freezing Bulk-Scale Biopharmaceuticals Using Common Techniques—and the Magnitude of Freeze-Concentration. Biopharm Volume 15, Number 5, 22-34.

Williams, Polli. 1984. The lyophilization of Pharmaceuticals: A literature review. Journal of parenteral science and technology. Volume 38, Number 2, 48-59.

Winbourne, Pam. 2000. Overview of the Drug and Biologics Review Process. Food and Drug Administration. http://www.fda.gov/oia/embslides/drugreview/index.htm (Retrieved on 05/22/05)

Wisniewski, Wu. 1996. Large-scale freezing and thawing of biopharmaceutical drug product. Biotechnology and Biopharmaceutical Processing and Preservation. K Avis and V Wu, eds, Buffalo Grove, IL, Interpharm Press, Inc. 7-59.
Xiang, Jun. Hey, Jeffery. Lietdke, Volker. Wang, D.Q. 2004. Investigation of freeze-drying sublimation rates using a freeze-drying microbalance technique. *International Journal of Pharmaceutics*, 279, 95–105.