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**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

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MASTER OF SCIENCE THESIS

OF

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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 ($\mu\text{g/ml}$) 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 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

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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.”¹ 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 2002.² 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 acids³, 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.⁴ 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 protein⁵

(a) Primary structure

–Ala–Glu–Val–Thr–Asp–Pro–Gly–

(b) Secondary structure

(c) Tertiary structure

(d) Quaternary structure

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.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.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.”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.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.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.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.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).¹³ 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.¹⁴ FVIIIa has no independent enzymatic activity, but acts as a cofactor to increase the proteolytic efficiency of factor IXa.¹³ 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 cascade¹⁵

Factor VIII Products

Concentrates of FVIII derived from human plasma have been in use since the 1960's.¹⁶ Plasma derived FVIII ranges from 170Kd - 280Kd depending on the active form of FVIII employed.¹⁷ 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.¹⁴ 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.¹¹ 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.¹⁸ No FVIII products have transmitted HIV, hepatitis B or hepatitis C since 1987.¹⁹ 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.²⁰

Concerns over inhibition formation, limited access to plasma, and potential viral transmissions led to the development of recombinant FVIII products.¹⁶ The first recombinant antihemophilic factor was approved in 1992, but contained blood-

derived additives from humans or animals, most notably albumin.¹⁹ 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).²¹ Recombinant FVIII products may require albumin or other macromolecular stabilizers.¹⁷ 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.¹⁹ 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.²² The mechanism of the intra molecular reaction occurs when an amide side chain is attacked by a succinimide ring intermediate,²³ 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.²⁴ 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.²⁵ Detection systems monitor charge, molecular weight changes and a direct measurement of succinimide or isoaspartic residue formation.²⁶ Lowering the pH of a protein formulation decreases the occurrence of succinimide deamidation, but also increases the chance for hydrolysis deamidation.²³ In Figure 3, reaction A is the primary degradation step to the intermediate, and reaction B completes the reaction to the two possible products.²²

Figure 3. Deamidation reaction of asparagine

Oxidation is the covalent modification of a protein induced by reactive oxygen intermediates or by-products of oxidative stress.²⁷ Oxidation may occur in a number of amino acids, but is most commonly seen in cysteine and methionine (see Figure 4.²⁸). Oxidation results from exposure to air, intense fluorescent light, and/or residual peroxide content.⁷ 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.¹² Antioxidants are

added while pH, and temperature adjustments are made to decrease oxidative product build up.²⁸ Antioxidants act as free radical scavengers and oxygen acceptors to minimize damage to the protein.²⁷ Oxidation occurs optimally at neutral or basic pH and decreasing the pH can diminish aggregation or disulfide scrambling.⁷

Figure 4. Methionine oxidation reaction.²⁹

Physical

Physical Instabilities, such as aggregation/precipitation and surface adsorption, comprise another major destabilizing factors for protein drug formulations.²⁸

Aggregation is the most prevalent means by which a physical instability in protein structure is manifested.³⁰ Aggregation is a microscopic association of protein molecules, while precipitation forms visible protein particles that decrease potency and alter solution appearance,⁶ Fig. 5. The aggregation of proteins through subtle structural changes is responsible for associative behavior.³¹ Physical factors including temperature, ionic strength, agitation, and surface/interface adsorption, all result in an increased hydrophobic surface area, yielding protein aggregation.²⁸ Protein aggregates may be soluble and create a reversible problem.³⁰ Precipitation may result from protein-salt interactions, yielding a “salting-out” effect when high concentrations of salt interact with hydrophobic residues of the protein.³²

Figure 5. Diagram of two potential reactions: A) Normal protein folding. B) Aggregation of protein.³³

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.³⁴ Surface energy has been identified as a major factor influencing protein adsorption, with hydrophobic surfaces adsorbing more protein than hydrophilic surfaces.³⁵

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.³⁶ Filters vary in their composition materials, yielding varying levels of hydrophobicity, reactivity, and extractability into the protein product.³⁷ Therefore, during formulation it is important to assess the protein and filter to ensure avoiding a significant amount of adsorption.⁶

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 cationic³⁸ 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.³⁹ Surfactants such as polysorbates, and pleuronics may greatly reduce adsorption rates to solid surfaces.⁴⁰ 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.⁴¹ 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).³⁸

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 preformed 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."⁴²

Figure 6. An overview of stage 2 of the aseptic manufacturing process.⁴³

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.⁴⁴ 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.⁴⁵

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.⁴⁶ 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.⁴⁵ Thawing is complete when all water molecules are released from the ice matrix into the amorphous phase.⁴⁶ Additional external mechanical agitation of the entire vessel yields quicker and more uniform thaws by stirring the liquid and solid phases together.⁴⁵ Mixing during the thaw will minimize any freeze-induced active pharmaceutical ingredient concentration effects that may occur during the freezing process.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.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.⁴⁷ Chemical characterization identifies product potency, solubility, stability, reactivity, and purity.⁴⁷ Product characterization identifies potential buffer systems, cryoprotectants, lyoprotectants, and/or other excipients required in the formulation.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.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.⁴⁹ 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.³⁰ “Hold up” volume is defined as the amount of residual water in a filter after a flush of pressurized air.⁵⁰ 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.³⁶ A series of multiple filtration steps must occur to remove debris; particles, bacteria and other molecules prior to the final sterilizing fill operation.³⁷ Final sterilization filters are generally less than or equal to a 0.22 μm pore size and must ensure sterility but minimize protein adsorption.⁴⁹ 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.³⁰

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.⁵² 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.⁵¹

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.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.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 (T_f), 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 (T_e).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.⁵⁴ Most drug products do not have fully crystallized solutes, but form an amorphous glass with no eutectic temperature. Instead there is a collapse temperature (T_c), which defines the maximum product temperature for the freeze-drying cycle, and is customarily a few degrees above ($T'g$).⁵⁶

The ramp rates for product cooling vary and must be understood because the slower the crystal growth, the larger the crystal.⁵⁵ 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.⁵⁶ 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.⁵⁴

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.⁵³ 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).⁵⁸

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.⁵⁸ Final products exhibit reduced protein unfolding in the dried solids, less aggregation and fewer bubbles after reconstitution, and improved pharmaceutical elegance.⁵⁹

Primary Drying

The ice formed during the freezing step is removed by sublimation at sub-ambient temperatures (-30° - $+10^{\circ}\text{C}$) 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.⁵⁷ The partial pressure of water vapor must be reduced below the triple point pressure (P_{TP}) of water to allow sublimation to occur.⁵⁶ 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.⁵⁴ 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.⁶⁰ 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.⁵⁸ 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.³⁰ After the ice sublimes, the water vapor formed passes through the dried portion of the product to the surface.⁵⁷ 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.⁵⁴ Escaped water vapor is transferred from the surface of the product through the chamber and then condensed on the condenser.⁵⁷ 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).⁵⁴ When all crystalline solvents have disappeared, the temperature is raised for the extraction of the remaining unfrozen liquids, known as secondary drying.⁵²

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.⁵⁷ Remaining bound moisture may be water of crystallization, randomly dispersed water in a glassy material, intracellular water, or absorbed water.⁵⁶ 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.⁵³ The product temperature

during secondary drying must be considered for heat labile products which may experience significant degradation at high temperatures.⁵⁷ 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.⁶¹ 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.⁵³ Zero percent moisture in the product is unattainable, therefore the goal is <1% for optimal drug stability.⁵⁷

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.⁶ 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.³⁰ 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 8.⁶² 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.¹⁶ BDDrFVIII SQ, where SQ refers to amino acid linkage (serine 743-glutamine 1738), is a genetically engineered recombinant product.⁶³ The secondary structure of this protein consists of 41% β -sheets, 14% α -helix, 26% random structure, and 19% turns as defined by circular dichroism (CD)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.⁶⁴ This product does not require the addition of human serum albumin as a stabilizer in the final formulation.⁶²

Figure 8 The structure of the B-domain deleted factor VIII¹⁶

The molecule is sensitive to physical and chemical degradation because the molecule contains: glycosylated protein chains, seven disulfide bridges, five sulfhydryl groups, and a metal ion bridge.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.⁶³ 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.⁶³

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.¹⁷

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

	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

	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
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.

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)⁶⁵, 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. 12.⁶³

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

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 13.⁶³

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

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.⁶⁶ 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 ($\mu\text{g/ml}$) 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).⁶⁷ 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 15.⁶⁸

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.⁶⁹

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.

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.⁴ The rate of freezing determines the size of the ice crystals formed, with slow freezing yielding larger ice crystals.⁵⁵ 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.⁷⁰ 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”,⁵³ 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.⁷¹ 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.⁷² Rapid freezing also may decrease the amount of water removed from proteins.⁴

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.⁷³ 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°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°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.⁷³ 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.³⁴ 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 ($\mu\text{g/ml}$), 4 ml of 1.8% NaCl yielded 50.63 ($\mu\text{g/ml}$), and 4 ml of water yielded 48.61($\mu\text{g/ml}$). 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 ($\mu\text{g/mL}$) 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.⁷⁴ Annealing decreases the variation in initial ice crystal size distributions caused by unpredictable nucleation temperatures and allows the uniform drying rates of the product.⁵⁸ 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.⁷⁵ 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.⁴⁶

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 loss⁶³ 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 ($\mu\text{g/mL}$) and potency (IU/mL).

Figure 19: Siliconized vs. untreated vials- Concentration ($\mu\text{g/mL}$) by Mono Q HPLC

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

Type 1 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 ($\mu\text{g/mL}$) 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.⁷⁶ 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.³⁹ 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.⁷⁶ 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.³⁹ 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.³⁸ 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.⁷⁷ Proteins bind to silicone treated glass through hydrophobic bonding between aliphatic (hydrocarbons without benzene rings)⁷⁸ residues on proteins and silicone residues on coated glass.³⁸

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 ($\mu\text{g/mL}$) 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 58mg/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.⁸⁰

The effects of increased concentrations of surfactants (Polysorbate 80) in a BDDrFVIII SQ formulation were previously studied.¹⁶ 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.⁸¹

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.

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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 system consisting of the following:	Waters	2695	
<i>Waters 600S Controller 6DC</i>	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 Biotech	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 μ m filters				
<i>Millipore Stericup</i>	Millipore	N/A	SCGPU02RE	
Milli-Q Water	Wyeth	N/A	N/A	Andover K2100
pH 4.0 standards	Fisher	Research	SB101-500	
PH 7.0 standards	Fisher	Research	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

Oneway Anova Summary of Fit

Rsquare	0.475531
Adj Rsquare	0.460547
Root Mean Square Error	1.900872
Mean of Response	25.6989
Observations (or Sum Wgts)	145

Analysis of Variance

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	Lower 95%	Upper 95%
Coated	30	25.9953	0.34705	25.309	26.681
Pre-Lyo Coated	25	24.5016	0.38017	23.750	25.253
Pre-Lyo PPE	30	28.8600	0.34705	28.174	29.546
Pre-Lyo Tubing	30	23.7660	0.34705	23.080	24.452
Tubing	30	25.1720	0.34705	24.486	25.858

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

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

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

Oneway Anova
Summary of Fit

Rsquare	0.960408
Adj Rsquare	0.959292
Root Mean Square Error	1.59899
Mean of Response	21.06707
Observations (or Sum Wgts)	147

Analysis of Variance

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 Oneway 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	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

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

Oneway 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	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 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

Rsquare	0.166062
Adj Rsquare	0.142098
Root Mean Square Error	1.645729
Mean of Response	28.07817
Observations (or Sum Wgts)	180

Analysis of Variance

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	28.3580	0.30047	27.765	28.951
Pre-Lyo Coated	30	29.1227	0.30047	28.530	29.716
Pre-lyo PPE	30	28.5617	0.30047	27.969	29.155
Pre-lyo Tubing	30	26.9540	0.30047	26.361	27.547
Siliconized	30	28.0737	0.30047	27.481	28.667
Tubing	30	27.3990	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

Oneway Anova- Summary of Fit

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

t Test

Pre-lyo PPE-Tubing

Assuming equal variances

Difference	1.16267	t Ratio	2.007229
Std Err Dif	0.57924	DF	58
Upper CL Dif	2.32214	Prob > t	0.0494
Lower CL Dif	0.00319	Prob > t	0.0247
Confidence	0.95	Prob < t	0.9753

Analysis of Variance

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	N	Mean(Ug/mL)	Std Dev
Pre-lyo PPE	30	28.5616667	2.79719451
Tubing	30	27.399	1.49708533

B. Freeze and Hold with 0.9% NaCl Dilution

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

Oneway Anova- Summary of Fit

Rsquare	0.259821
Adj Rsquare	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 > t	<.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 Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Coated	27	11.7270	0.14470	11.437	12.017
Tubing	30	10.8507	0.13727	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	10.8506667	0.94602228

3. 1mL, 2mL, 4mL Reconstitution studies with different diluents

A. 4mL Reconstitution with three different diluents

Oneway Analysis of Amount (Ug/mL) By ID

Oneway Anova

Summary of Fit

Rsquare	0.24949
Adj Rsquare	0.232237
Root Mean Square Error	1.508886
Mean of Response	49.78367
Observations (or Sum Wgts)	90

Analysis of Variance

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			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
.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	48.6137	0.27548	48.066	49.161

Std Error uses a pooled estimate of error variance

ID	N	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

Oneway Analysis of Amount (Ug/mL) By ID

Oneway Anova Summary of Fit

Rsquare	0.058987
Adj Rsquare	0.037355
Root Mean Square Error	4.881897
Mean of Response	102.9313
Observations (or Sum Wgts)	90

Analysis of Variance

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 Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
0.9 NaCl Recon 2mL	30	104.476	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	104.476333	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 (Ug/mL) By ID

Oneway Anova Summary of Fit

Rsquare	0.084041
Adj Rsquare	0.062489
Root Mean Square Error	7.609981
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

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

Oneway Anova Summary of Fit

Rsquare	0.960408
Adj Rsquare	0.959292
Root Mean Square Error	1.59899
Mean of Response	21.06707
Observations (or Sum Wgts)	147

Analysis of Variance

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 Oneway 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

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

Oneway 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	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 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

B. Full Cycle without annealing

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	1.0000

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

C. Comparison of annealed and unannealed products

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

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 Unsilicized Tubing-Type 1 Unsilicized 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 Unsilicized Tubing	24	10.4008	0.21213	9.975	10.827
Type 1 Unsilicized 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 Unsilicized Tubing	24	10.4008	1.16054304
Type 1 Unsilicized 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	1.0000

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

B. Siliconized vs. Untreated Vials- Potency by COBAS

Oneway Analysis of U/ml By Lot

Oneway Anova

Summary of Fit

Rsquare 0.900712
 Adj Rsquare 0.897402
 Root Mean Square Error 39.28993
 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	Number	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	58.1202	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
Summary of Fit**

Rsquare	0.247365
Adj Rsquare	0.234161
Root Mean Square Error	26.80439
Mean of Response	147.2105
Observations (or Sum Wgts)	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 > t	<.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
Oneway Analysis of Mean (Conc. Ug/mL) By ID

Oneway 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	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 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

Section 3: Formulation Studies

A. 1X vs 2X formulation

Oneway Analysis of mean conc. ug/ml By ID

Oneway Anova Summary of Fit

Rsquare	0.996542
Adj Rsquare	0.996439
Root Mean Square Error	0.914972
Mean of Response	20.90572
Observations (or Sum Wgts)	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	11.3520	0.16705	11.022	11.682
Coated 2X NaCl 6ml	30	11.5450	0.16705	11.215	11.875
PPE 1X	30	26.5730	0.16705	26.243	26.903
PPE 2x	29	52.5079	0.16991	52.172	52.843
Tubing 1X NaCl 6ml	24	10.7829	0.18677	10.414	11.152
Tubing 2X NaCl 6ml	30	11.7023	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

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