

Stability of biopharmaceuticals within the existing guidelines

From a science perspective to a
regulators perspective

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**Some of the biopharmaceuticals
belong to the group of
well-characterised biologicals.**

**The major characteristics of this group are:
proteins whose identity, purity,
impurities, potency, and quantity
can be determined.**

***my presentation deals with
this group of biologicals***



Stability of a biopharmaceuticals is defined to be:

“.... The capacity... to remain within specifications established to ensure its identity, strength, quality, and purity. ”

Stability is interpreted as the length of time under specific conditions of storage that the product will remain within predefined limits for all important characteristics.



Outline presentation

- ◆ Production process
- ◆ ICH guidelines (Q5C)
- ◆ Typical characteristics of the products
- ◆ Protein folding
- ◆ Physical and chemical stability in solution
- ◆ Stability of the products during and after lyophilization
- ◆ Conclusions



Production process of a biopharmaceutical

Upstream part (cultivation steps)

Downstream part (purification steps)

Bulk material

Formulation

Final Product



All biopharmaceuticals are injected

Consequences:

- ◆ Volume and tonicity (i.m. and s.c.)
- ◆ (Volume) and (hyper)tonicity (i.v.)
- ◆ Lyophilization (all routes)



Major characteristics biopharmaceuticals

- ◆ Recombinant DNA technology has developed biopharmaceuticals (proteins) in the past three decades
- ◆ In comparison with drugs, biopharmaceuticals have high specificity and activity at relatively lower concentrations
- ◆ Due to separation technologies, the recombinant proteins are purified to a high level
- ◆ These purified proteins significantly reduce the known and unknown potential side or even toxic effects



Most challenging tasks for biopharmaceuticals

- ◆ Physical and chemical instabilities of proteins
- ◆ Stability of proteins may be significantly different depending on its production process and source of proteins (eu- versus pro-karyotic cells).
- ◆ Any presence of trace amount of enzymes, metal ions, or other contaminants can potentially effect stability.
- ◆ Protein morphism is another factor that influences protein stability.



Structural differences between proteins

- ◆ The structural differences among different proteins are so significant that generalization of universal stabilization strategies will not be successful
- ◆ A rational approach, however, is possible (this presentation)
- ◆ Case-by-case approach (knowledge of your product; knowledge of the intermediates; knowledge of your process are crucial elements)



Specifications are the crucial characteristic of biopharmaceuticals

- ◆ Production process
- ◆ Stability profile
- ◆ Qualities of materials used in preclinical and clinical trials
- ◆ Statistical validity of data analysis

From: Murano (1997)



Product characterisation

- ◆ Physical properties (MW, OD, IEP)
- ◆ Composition/structure (amino acid composition and sequence; glycosylation, disulphide bridges)
- ◆ Subunit structure (SDS-PAGE, under reducing and non-reducing conditions)
- ◆ 3-D structure (UV, fluorescence, CD, infrared, NMR and crystal)
- ◆ Identify indicating patterns (peptide map, sugar map, chromatography profiles, IEF, SDS-PAGE, CE, MS, NMR and SPR)



ICH guidelines (selection of batches)

- ◆ Intermediates
- ◆ Drug substance (bulk material; API)
- ◆ Drug product (final lot; final container material)



ICH guidelines (stability indicating profile)

- ◆ Potency
- ◆ Purity and molecular characterization
- ◆ Other product characteristics (visual appearance; sterility; additives (stabilizers; preservatives); container and closure



ICH guidelines (testing frequency)

- ◆ Shelf-life less than 1 year: monthly during the first three months and at 3 month intervals thereafter
- ◆ Shelf life greater than one year: every 3 months during the first year, every 6 months during the second year and annually thereafter



ICH guideline (specifications)

Recommendations for maximum acceptable of activity, limits for physicochemical changes, or degradation during the proposed shelf-life have to be considered on a case-by-case basis



ICH guideline (storage conditions)

- ◆ Temperature
- ◆ Humidity
- ◆ Accelerated and stress conditions
- ◆ Light
- ◆ Container/Closure

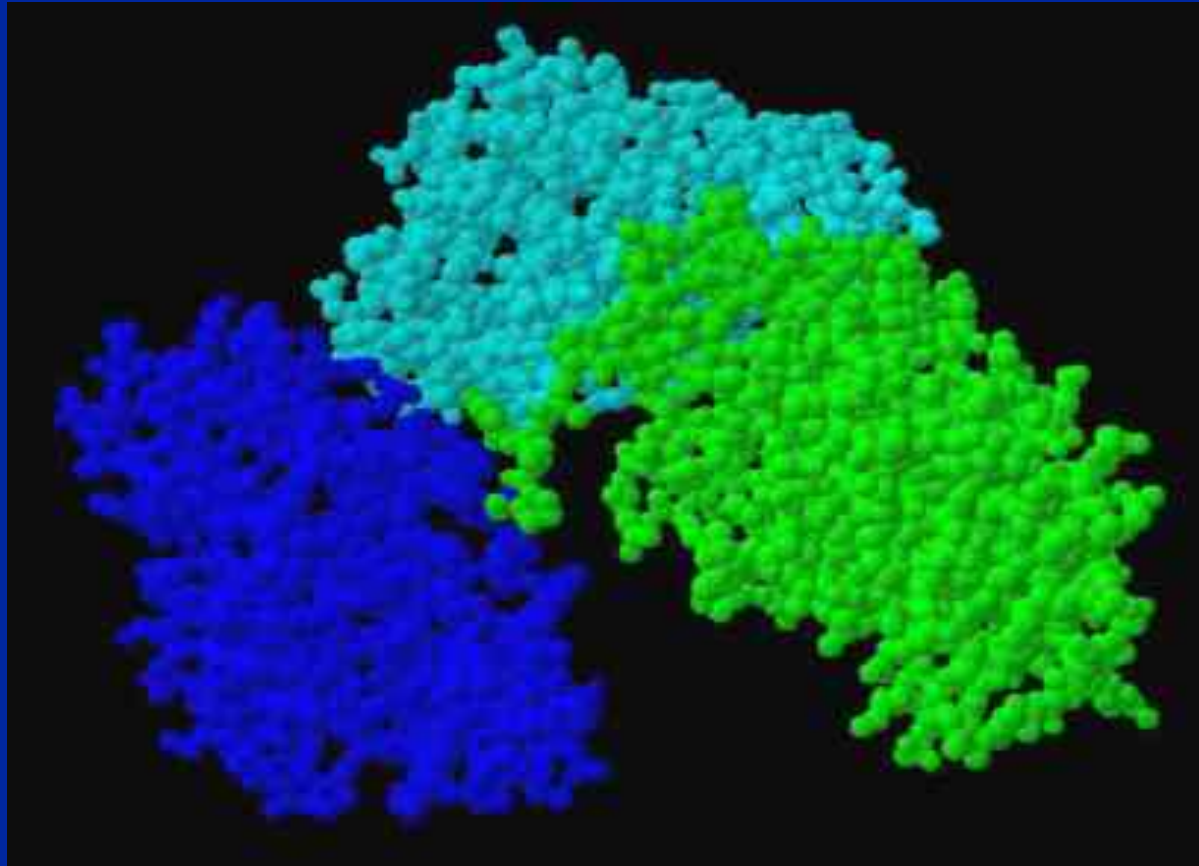


Protein folding

- ◆ Biologically active proteins are folded properly
- ◆ Number of possible conformations with an average domain size is about 10^{80}
- ◆ Most stable conformation of a protein is usually the native state (free energy of stabilization is only about 50 kJ/mol)



3-D structure of diphtheria toxin



Protein folding process

- ◆ Folding of newly-synthesized polypeptide require assistance of chaperone proteins
- ◆ The rate of protein folding is high
- ◆ After folding 80% of the non-polar side chains (Ala, Val, Ile, Leu, Met, Phe, Trp, Cys) are buried in the interior of a protein
- ◆ Amino acids as Glu, Lys and Arg are almost invariable located on the protein surface. These residues are exposed to the solvent



Major forces involved in keeping native state

- ◆ Hydrophobic interactions
- ◆ Electrostatic interactions
- ◆ Hydrogen bonding
- ◆ van der Waals forces



Physical stability (melting temperature)

Proteins unfold above certain temperatures (melting temperature); unfolding results in aggregation



Some melting temperatures (T_m of proteins)

Protein	Matrix	T_m (°C)
DNase	in water, pH 6.8	67
Elastase	in acetate, pH 5.0	66
aFGF	in PBS, pH 7.2	45
bFGF	in phosphate-citrate-borate, pH 4	50
bFGF	in phosphate-citrate-borate, pH 9	64



Protein aggregation (1)

- ◆ Protein aggregation in many cases results from intermolecular association of partially denatured protein chains
- ◆ Aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by nonspecific coaggregation
- ◆ The aggregation process can be roughly divided into three steps: initiation, propagation and termination
- ◆ Proteins aggregate to minimize thermodynamically unfavorable interactions between solvent and exposed hydrophobic residues of proteins



Protein aggregation (2)

- ◆ The balance is so delicate that a change of one amino acid in a protein may substantially change its aggregation behavior
- ◆ Factors as temperature, ionic strength, vortexing, surface/interface adsorption etc. increase the hydrophobic surface area of proteins, causing aggregation
- ◆ Protein aggregation may result from chemical degradations or modifications and subsequent exposure of the hydrophobic surface(s).



Protein aggregation (3)

- ◆ Proteins can directly form covalent aggregates
- ◆ Many agents can be used to probe possible mechanisms of protein aggregation.
- ◆ Increased hydrophobic interactions
- ◆ Formation of extra hydrogen bonds and/or salt bridges
- ◆ More compact protein structures
- ◆ Presence of few Cys, high content of Arg and low content of Lys



Favored pH conditions for some degradation reactions in proteins/peptides (1)

Degradation	Protein	Reaction sites	Favored pH conditions
Hydrolysis	bFGF	Asp-Pro	Very acidic
Deamidation	hEGF	Asn	pH > 7
Deamidation	bFGF	Asn-X	pH > 7
Deamidation	Insulin	Asn	pH < 5



Favored pH conditions for some degradation reactions in proteins/peptides (2)

Degradation	Protein	Reaction sites	Favored pH conditions
Deamidation	Rnase A	Asn-X, Gln-X	High pH
Oxidation	rhPTH	Met	pH = 10
Oxidation	Relaxin	His, Met, etc.	pH 5>5>7>8
Oxidation	His-Met	Met	pH = 7-8
Succinimidation	bFGF	Asx-Gly	pH = 4-5



Chemical instabilities of proteins

- ◆ *Hydrolysis*
- ◆ *Deamidation*
- ◆ *Oxidation*
- ◆ *Succinimidation*
- ◆ Disulfide bond breakage and formation
- ◆ Isomerization
- ◆ Non-disulfide cross linking
- ◆ Deglycosylation
- ◆ Maillard reaction



Factors affecting protein stability (1)

- ◆ *Formulation pH*
- ◆ Temperature
- ◆ Adsorption
- ◆ Salts
- ◆ Metal ions
- ◆ Chelating agents
- ◆ Shaking and shearing
- ◆ Non-aqueous solvents



Factors affecting protein stability (2)

- ◆ Protein concentration
- ◆ Source and purity of proteins
- ◆ Protein morphism



Excipients to keep the integrity of the active component

- ◆ Amino acids (e.g. glycine)
- ◆ Sugars
- ◆ Surfactants
- ◆ Salts
- ◆ Polyols (e.g. cyclodextrin)
- ◆ Antioxidants (e.g. ascorbic acid)
- ◆ Polymers (e.g. PEG)
- ◆ Chelating agents (e.g. EDTA)



Stabilization proteins prior and after lyophilization

- ◆ The most important parameter prior to drying: T_g' . This is the glass temperature associated with the maximum freeze-concentrated, amorphous solute/unfrozen water matrix surrounding ice crystals in frozen condition
- ◆ The most important parameter after lyophilization: glass-rubber transition temperature (T_g). This is the glass temperature of the dried product.



Constraints for formulations for lyophilization of proteins

- ◆ Protein stability
- ◆ Final product configuration
- ◆ Formulation tonicity
- ◆ Product collapse temperature
- ◆ Cake structure
- ◆ Product glass transition temperature



Rational choice of stabilizing excipients

- ◆ Dissaccharide (sucrose or trehalose) and gelatins; formation of glass
- ◆ Crystalline bulking agents as mannitol and glycine do not provide protection during lyophilization; effective sublimation and cake structure
- ◆ (specific) Polymers (dextran) and proteins: increase T_g; cake structure
- ◆ Detergents; prevention of aggregation
- ◆ Buffer; control of pH during lyophilization



Tg' of a pure sucrose solution and sucrose formulations with glycine and mannitol at various concentration ratios

Sucrose	Glycine	Mannitol	Concentration ratio	Tg' (°C)
x				-32
x	x		2:1	-39
x	x		1:1	-44
x	x		1:2	-49
x		x	2:1	-34
x		x	1:1	-37
x		x	1:2	-38



Glass transition (Tg) and residual moisture content

Condition	Tg	Moisture
1% Dextran	78	2.6
1% Sucrose	54	1.8
1% Sucrose + dextran	67	1.2
5% Sucrose	50	1.3
5% Sucrose + dextran	60	1.2

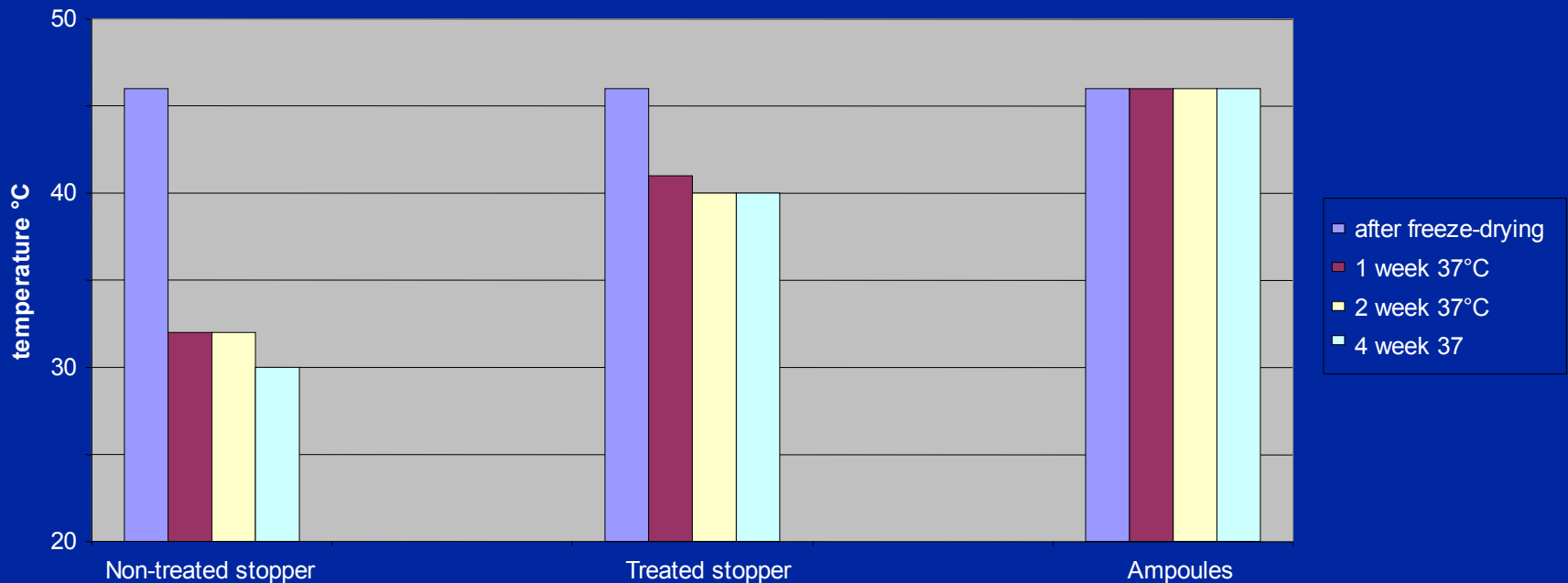


Determination of apparent frozen pH for both potassium phosphate and PBS buffer formulations

Formulation	10 mM potassium phosphate, pH 7.5	
	phosphate, pH 7.5	PBS, pH 7.0
No excipients	8.17	5.21
5% Sucrose	7.23	5.70
0.03% Polysorbate 20	8.24	4.33
5% Sucrose/0.03% Polysorbate 20	7.18	5.77



Consequences of moisture in the treated and non-treated stopper on Tg

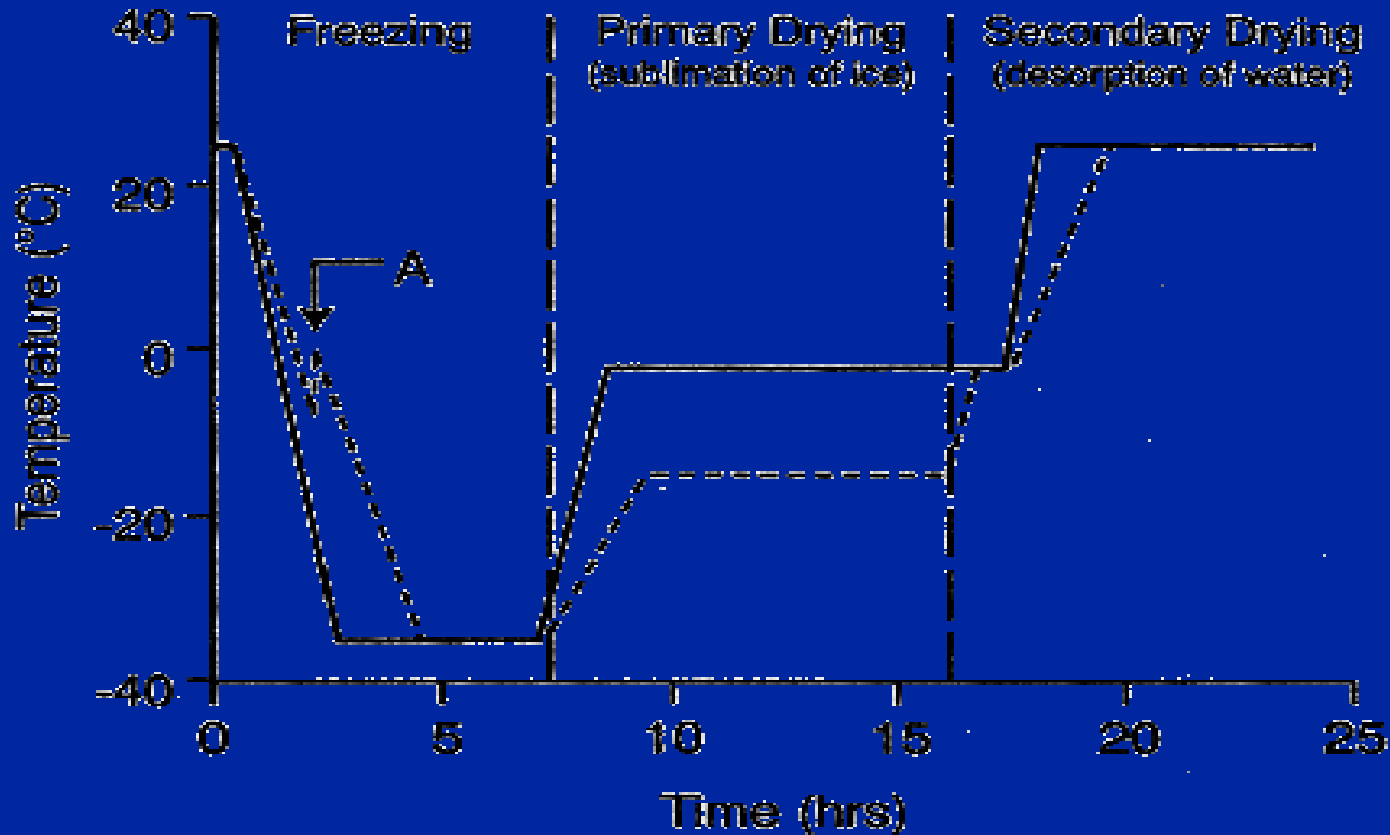


Impurities in the excipients

- ◆ Sugars and mannitol can contain transition metals
- ◆ Surfactants can be contaminated with peroxides, all of which can foster oxidation
- ◆ Evidence of is presented about a reducing sugar in mannitol
- ◆ Moisture transfer from the stopper to the cake



A typical lyophilization cycle

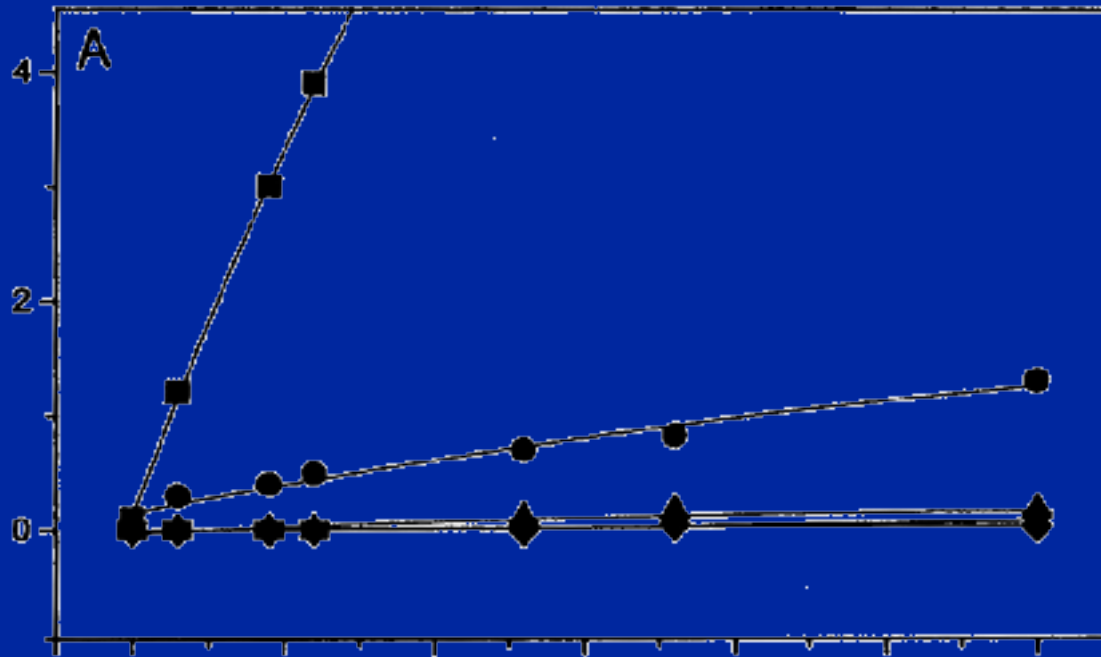


Effect of annealing during freezing in a lyophilization cycle

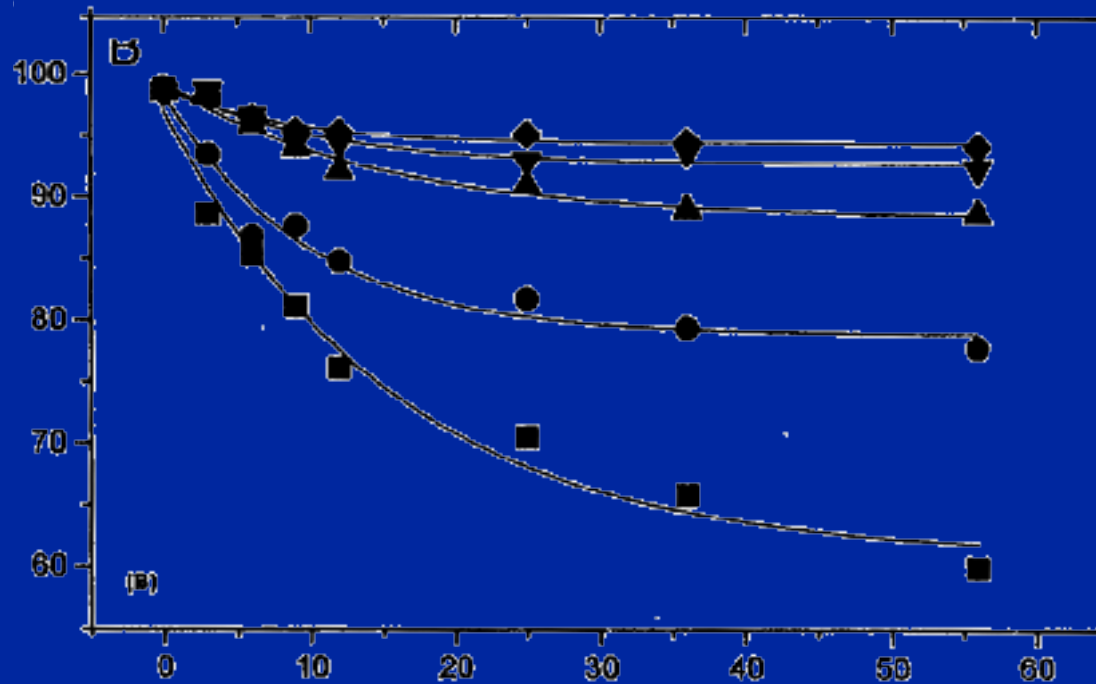
Formulation (mg/ml)	Freeze-drying process, visual appearance	Tg (°C)	% Water
Sucrose 30	<Tg' (shelf: -35°C), stable	66.5	0.7
Sucrose/dextran 30:10	<Tg' (shelf: -35°C), stable	75.0	0.5
Sucrose/glycine 34:17	<Tg' (shelf: -42°C), stable	53.3	0.9
Sucrose/glycine 20:20	<Tg' (shelf: -47°C), stable	37.7	1.9
Sucrose/glycine 20:20	annealed 2h at -30°C, stable	59.2	0.6



Effect of sucrose content on precipitation of rhIL-1



Effect of sucrose content on deamidation of rhIL-1



Leachables from and to stoppers



Conclusions

- Biopharmaceuticals are proteins, but their 3D-structures are totally different. The right 3D-structure is crucial for their biologic activity.
- ICH guidelines are transparent and logic.
- Stability of the proteins both in solution and in lyophilized condition is a complicated issue.
- A rational approach to tackle the issue is very well possible
- Finally, know your product and know your process. Validation studies are extremely informative.



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