DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY®
Vol. 30, No. 8, pp. 877–889, 2004

Stabilization of Rasburicase and Physico-Chemical Characterization of the Resulting Injectable Formulation

Alain Bayol,[1,5,*] Thierry Breul,[2] Patrice Dupin,[1] Jérome Menegotto,[3] C. Aleman,[2] Hélène Duplaa,[3] Philippe Faure,[2] Marie Claude Bonnet,[1] and Michel Bauer[4]

[1]Analytical Science Department, Sanofi-Synthelabo Recherche, Labège Cedex, France
[2]Pharmaceutical Science Department, Sanofi-Synthelabo Recherche, Montpellier, France
[3]Analytical Chemistry and [4]International Analytical Sciences Department, Sanofi-Synthelabo Recherche, Toulouse Cedex, France
[5]Analysis and Quality Control Unit, Sanofi-Synthelabo Recherche, Labège Cedex, France

ABSTRACT

Rasburicase (Fasturtec®/Elitek®) is a new generation of recombinant urate oxidase administred therapeutically by intravenous infusion for the prevention or treatment of hyperuricemia during chemotherapy. To ensure a long storage period, a freeze-dried formulation was developed to guarantee the molecular integrity and enzyme activity. Screening of potential excipients was the first stage of the preformulation study. The selection was based on stability results (rasburicase solution with excipient) obtained with the isoelectric focusing profiles and residual enzyme activity. The different excipients were classified as stabilising, neutral or destabilising. A stability study was then carried out on different freeze-dried formulations containing the usual bulking agents for freeze-drying, excipients with a high glass transition temperature or competitive enzyme inhibitors having a stabilising effect. A mannitol/alanine mixture in phosphate buffer was selected from these preliminary results. Finally, the optimal content of mannitol and alanine in the freeze-dried powder was determined by an experimental design study. The water content and the appearance of the ''cake'', the osmolality, pH, clarity, and enzyme activity of the reconstituted solution were assessed. The formula with a mannitol/alanine ratio of 0.7 was found to be the best composition. Differential scanning calorimetry and ThermoStimulated Current technique experiments were carried out to study the amorphous phase. A glass

*Correspondence: Alain Bayol, Analytical Science Department, Sanofi-Synthelabo Recherche, La Pyrénéenne, BP 37202, 31672 Labège Cedex, France; Fax: (33) 5-61-00-40-01; E-mail: alain.bayol@sanofi-synthelabo.com.

DOI: 10.1081/DDC-200034582
0363-9045 (Print); 1520-5762 (Online)

transition temperature of about 45–50°C was found. Glassy state is known to preserve stability, which was verified by the real stability data. X-ray diffraction studies have shown that alanine is in a crystallised state and that mannitol remains amorphous. Crystallised excipients participate in forming the structure of the powder and therefore help to prevent any collapse. Amorphous mannitol creates a surrounding medium favourable to the stability of the protein.

Key Words: Uricase; Lyophilisate; Rasburicase; Differential scanning calorimetry; Thermostimulated current spectroscopy; Chromatography.

INTRODUCTION

Proteins have always raised a particular problem with respect to handling and behaviour in pharmaceutical formulations due to their physico-chemical instability. They can actually undergo two kinds of degradation:

- A chemical process leading to the formation of related impurities, which may involve hydrolysis and oxidation reactions as well as deamidation.
- A physical process giving rise to aggregation thus generating structural alterations which may impact on the biological activity and enhance immunogenicity.

This is the reason why, in order to minimize the degradation, the proteins are rarely formulated as a solution but rather as solid dosage forms. It would be ideal to use the protein in a crystalline state as long as it represents the most stable state from a thermodynamical perspective. Unfortunately at present, it is almost impossible to design industrial processes using this strategy. As well documented in the literature, an alternative strategy employs the use of incorporating the molecule in a highly viscous glassy phase obtained by a freeze-drying process with the aid of compatible excipients such as sugars (saccharides and polyols, amino-acids).[1,2] The interest of this approach originates from the significantly reduced molecular mobility of the active moiety in the glassy phase compared to that in solution. Because each protein has its own characteristics, the way the pre-formulation and the formulation of the rasburicase, a new generation of recombinant urate oxidase recently marketed in Europe and in the US as a freeze-dried product, is considered to be worthy of discussion. This product was shown to be clinically potent, when administered by infusion for the prevention or treatment of hyperuricemia occurring during chemotherapy. Also a more rapid decrease in uric acid plasma level was induced compared with Uricozyme®, a natural urate oxidase that is extracted from *Aspergillus flavus* and which has been marketed for a long time.[3,4]

Several publications on rasburicase, a 34 kDaltons protein, can be consulted for information concerning its tetrameric structure,[5] its isolations and the manufacturing process.[6,7] Also a DSC study concerning urate oxidase in solution at different pHs[8] has been conducted.

In this publication, in addition to physico-chemical techniques classically used in this kind of study (electrophoresis, HPLC, DSC, circular dichroism, XRPD etc...), two others were developed for assessing the stability of the active substance:

- Enzyme activity determination,
- Thermally stimulated currents (TSC).

Enzyme activity determination is a global evaluation which is a measure of the therapeutic effect and does not reflect the subtle changes in conformation accessible by techniques such as raman spectroscopy, circular dichroism etc... In this perspective, the technique has proven to be efficient for screening several sets of excipients during the pre-formulation work.

TSC is a dielectric technique based on the analysis of the dielectrical currents created by the relaxation of dipoles born by the molecules. It is particularly suitable for the analysis and the characterization of semi-crystalline and amorphous phases. Details can be found in literature concerning the theory and the equipment[9–12] and to the application to chemical entities[9] and polymers.[10–12] This technique has allowed a better understanding of the stabilization mechanism of the drug substance by specific excipients and the validation of a second clinical strength without having to reperform full pharmaceutical developments.

MATERIALS AND METHODS

Urate Oxidase Preparations

Rasburicase: Recombinant urate oxidase was obtained from Sanofi-Synthelabo Recherche (Labège-

France). Fastutec®/Elitek® injectable preparations from Sanofi-Synthelabo, (Notre Dame de Bondeville, France).

Freeze-Drying Conditions

- ***1.5 mg/vial strength***: The drug substance (1.5 mg/ml) is freezed-dried in vials stoppered with chlorobutyl rubber stoppers. The buffer solution concentration is indicated in the Results section. After compounding and sterile filtration, the solution is distributed into 3 ml type I glass vials. A temperature of −48°C was chosen for freezing. Primary drying (sublimation) is performed over at least 40 h, with a final shelf temperature of −20°C and a pressure of 12 Pa in an Usifroid freeze drier type SMH15. Secondary drying is performed at a temperature of +25°C and a pressure of 3 Pa over a period of 25 h.
- ***7.5 mg vials***: The drug substance solution concentration and the excipients to drug substance ratios are the same as for the 1.5 mg/ml solution. Only the fill volume is adjusted to 7.5 mg per vial. The components are the same but the primary pack is a 10 ml glass vial.

The primary drying shelf temperature was increased to +25°C to shorten the primary drying time.

Water Content

Water content was determined by micro-coulometry according to the European Pharmacopoeia (section 2.5.32).

Isoelectric Focusing

Ready to use Pharmacia Ampholine PAG plates (ref. 80.1124.82) pH gradient at 5.5 to 8.5 were used. Protein samples (20 μL of a 2 mg/mL solution) and pI marker solution (Pharmacia ref. 17-0473-01) were applied onto paper rectangles. Focusing was performed for 60 minutes at a maximum voltage setting of 1600 volts, maximum intensity of 50 mA, constant power of 25 watts. The paper rectangles were removed, and then the voltage was reapplied under the same conditions as before for a further 90 minutes. Protein bands were visualized by Coomassie blue staining. The procedure is essentially that recommended by Pharmacia.

Enzyme Activity

The enzyme activity was determined as described in reference[6] using a test that simulates in vivo biological activity. One unit corresponds to the enzyme quantity, which converts 1 μmol of uric acid into allantoin per minute in trolamine (TEA) pH 8.9 buffer at +30°C.

Differential Scanning Calorimetry (DSC)

DSC measurements were carried out with a Perkin-Elmer DSC-7 instrument. Thermograms were recorded with a heating rate of 10°C/min from room temperature to 250°C under a dry nitrogen gas purge. High purity indium was used for temperature and enthalpy calibrations (manufacturer's instructions). The glass transition temperature, T_g, was determined as the temperature of the midpoint of the heat capacity increment. The temperature of melting and cold-crystallization was taken as the onset temperature of the endothermic and exothermic peaks, respectively.

X-Ray Powder Diffraction (XRPD)

The XRPD patterns of solid samples were determined at ambient temperature using a Siemens D500TT x-ray powder diffractometer at 40 kV and 25 mA with CuKα radiation. Counts were measured with a scintillation counter from 4° to 40° in the 2θ diffraction angle range with an angle rate of 1°/min.

Thermally Stimulated Current (TSC) Spectroscopy

TSC measurements were carried out with a Thermold® TSC/RMA 9000 instrument. To avoid sample flow at high temperature and to keep an accurate electrical contact between analyzed product and electrodes, samples were placed into a sample cell specially designed to study fine powders. The sample cell was then inserted between the two plane stainless steel electrodes of the TSC apparatus. To eliminate the conductivity phenomenon, related to the movement of free charge carriers from one electrode to the other, which masks, at high temperature, some important events like the dielectric manifestation of the glass transition of amorphous phases, a thin film (25 μm) of Teflon® was inserted as a blocking electrode between the top electrode of the sample cell and the TSC electrode. Before experiments, the sample cell, located in the TSC cryostat, was submitted to a primary vacuum and then flushed with dry helium. To record TSC spectra, a static electrical field, Ep=600V/mm, was applied at the polarization temperature, Tp=70°C, during a time, tp=2 min. This polarization stage allows the orientation, towards the direction of the field, of dipolar species that

are mobile at this temperature. The sample was then quenched to a temperature T0 = −120°C which enables the orientation polarization to be frozen. At T_0, the field was removed and the sample was short-circuited for a time, tcc = 2 min, to remove fast relaxing space charges and to stabilize the sample temperature. The depolarization current I(T) was then recorded, through a very sensitive electrometer (resolution of 10^{-16}A), as the temperature increased from T_0 up to the final temperature, Tf = 80°C, with a constant heating rate of 7°C/min. The variation of the depolarization current versus temperature corresponds to the TSC spectrum.

RESULTS

Compatibility Study of Rasburicase with Various Molecules in Solution

The aim of the study in solution is to detect any major incompatibility between an excipient and the enzyme. It allows also a classification of the excipients, according to their stabilizing effect, neutral or destabilizing effect in solution, knowing that the stabilization mechanisms in the dry state are different.

Binary mixtures as solution in 50 mM phosphate buffer pH 8.0 of rasburicase and each of the potential excipients or reactants were incubated at +35°C and analyzed by isoelectric focusing and the enzyme activity assay, as rough rapid screening tools. The percentage of stabilization based on enzyme activity measurements, for each binary mixture and for a reference solution containing only rasburicase after 10 days of incubation as shown in Fig. 1. The percentage of stabilization is calculated as follows:

$$\text{Percentage of stabilization} = 100 \times \frac{[S - T]}{[T]}$$

Where :

S : is the residual enzyme activity of the enzyme solution with the excipient or reagent to be tested after 10 days at 35°C

T : is the reference without any excipient stored in the same conditions

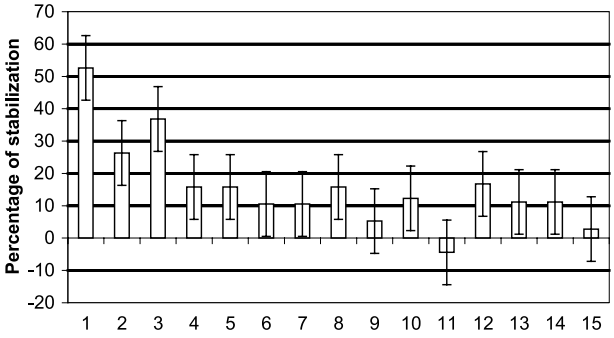

Figure 1. Compatibility study: Percentage of stabilization based on the measurement of the enzyme activity of different binary mixtures, after 10 days at 35°C in solution and related to a reference without any excipient. Rasburicase 1 mg/ml is incubated in a 50 mM phosphate buffer pH8.0 with 8-azaxanthine 50 μM (1), 8-azahypoxanthine 50 μM (2), xanthine 50 μM (3), orotic acid 50 μM (4), histidine 1 mM (5), aspartic acid 1 mM (6), glutamic acid 1 mM (7), sodium edetate 1 mM (8), mannitol 1 mM (9), alanine 1 mM (10), cysteine 1 mM (11), lactose 1 mM (12), alpha-cyclodextrine 1 mM (13), HP beta-cyclodextrine 1 mM (14) and gamma cyclodextrine 1 mM (15).

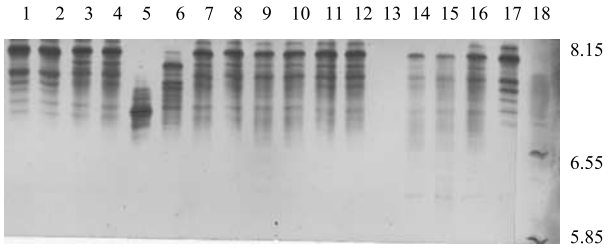

Figure 2. Compatibility study: isoelectric analysis of different mixtures, after 10 days at 35°C. rasburicase 1 mg/ml is incubated in a 50 mM phosphate buffer pH8.0 with 8-azaxanthine 1 mM (1) and 50 μM (2), sodium edetate 1 mM (3) and 50 μM (4), cysteine 1 mM (5) and 50 μM (6) Lanes 17 corresponds to the non-incubated rasburicase reference and line 18 to the isoelectric point reference proteins. Other lines are non relevant.

The results showed that 8-azaxanthine, 8-azahypoxanthine and xanthine had a clear positive effect on the stability of rasburicase in solution. Guanine, adenine, orotic acid, histidine, aspartic acid, glutamic acid, alanine, serine, gluconic acid, malic acid, malonic acid, mannitol, lactose and cyclodextrins had a slightly positive effect or no effect. Cysteine had no significant effect by enzyme activity determination compared to the reference with rasburicase alone, but a negative effect was demonstrated by isoelectric focusing as shown by the shift toward more acidic pH's (Fig. 2).

Freeze-Dried Pre-Formulation

Based on the results of the solution compatibility study several excipients were chosen: the first category which had a positive effect on stability and a second

category which were more common excipients already used in parenteral drugs and with no negative effect. They were used alone or combined within thirteen formulations containing 1.5 mg of rasburicase and a 50 mM sodium phosphate pH 8.0 buffer, and freeze-dried under the same conditions.

The water content determined on freeze-dried powders was between 1.4 and 3.7%. After reconstitution with 1 mL of water for injections, the turbidity, osmolarity, pH, clarity, and enzyme activity were determined at time zero and after 1 month at +35°C.

However, the most relevant results were obtained with the clarity test (performed according to the Ph. Eur. section 2.2.1) and enzyme activity measurements. After reconstitution with water, the best clarity was obtained with the formula containing 8-azaxanthine; the worst results were obtained with alanine alone, glycine alone, mannitol alone, and the mixture glycine/mannitol. The other formulations had intermediate but acceptable results.

The residual enzyme activity after 1 month at +35°C is shown in Fig. 3. Based on these results, the mannitol/alanine combination in phosphate buffer was the best formula. The residual activity was better for this formula than the residual activity observed with the formula with each of the excipients alone and better than the other formulations including those with the competitive inhibitors (8-azaxanthine and xanthine) that were more efficient in solution.

From these results, the solid formulation based on rasburicase mixed with a mannitol/alanine blend, as excipient, was chosen for the next stage of development.

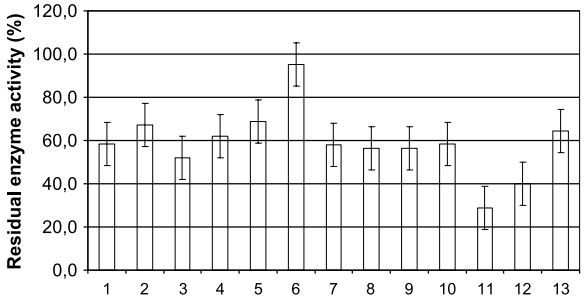

Figure 3. Residual enzyme activity after 1 month at 35°C (related to time zero) in per cent for freeze-dried formulations containing (1) 1.5 mg of rasburicase without any excipients and 1.5 mg of rasburicase with (2) alanine (16 mg); (3) glycine (14 mg); (4) mannitol (33 mg); (5) glycine/mannitol (12 mg/9.5 mg); (6) mannitol/alanine (9.5 mg/21 mg); (7) lactose (80 mg); (8) lactose/alpha-cyclodextrine (54 mg/50 mg); (9) lactose/8-azaxanthine (2 mg/0.12 mg); (10) lactose/8-azaxanthine (80 mg/0.015mg); (11) lactose/xanthine (2 mg/0.12 mg); (12) lactose/xanthine (80 mg/0.015 mg); (13) maltose (100 mg).

Optimization of the Formulation

Biochemical and Pharmaceutical Analysis

To determine the optimal mannitol/alanine ratio (M/A) in the freeze-dried powder that would provide the best chemical and physical stability of the final drug product, an optimization design study was carried out. 50 mM phosphate solutions containing about 1.5 mg of rasburicase in different mannitol/alanine ratios varying from 0.125 to 4 (w/w) were lyophilized and evaluated. The total mass of excipients (alanine, mannitol) was the same in all formulas.

The controls performed on solutions immediately after reconstitution by addition of 1 mL of water for injections were: osmolality, pH, turbidity, clarity, and enzyme activity. These parameters, except turbidity and osmolality, were also measured after 1 month at +35°C.

The results (Table 1) show that the water content of the different powders for injection was lower than 3%, the lower percentages were observed for high alanine contents (ratio 0.25 and 0.125). Water can potentially play a critical role on the stability of proteins. Osmolality and pH, determined by the composition, have acceptable expected values. Clarity less than or equal to II (European Pharmacopoeia test with reference suspensions) is considered acceptable for an injectable solution: the worst clarity is observed for the two extreme ratios (i.e, 4 and 0.125).

It was also noticed that the physical aspect of the powders was not acceptable when the mannitol/alanine ratio was greater than or equal to 2; in fact for these ratios, collapsed or broken ''cakes'' were produced. On the contrary an entire cake was observed for the others ratios (0.125 to 1).

After 1 month storage of the freeze-dried product at +35°C, the M/A ratio of 0.67 exhibited the highest enzyme activity and therefore, constituted the optimal mannitol/alanine association (Fig. 4).

Physicochemical Analysis of the Lyophilized Powder

The physicochemical properties of different formulations and more especially the thermodynamic behavior and physical structure of powders have been investigated by DSC and XRPD to achieve a better understanding of the protein stabilization mechanisms.

Diffractograms recorded for pure excipients (mannitol and alanine) and for the all formulations based on different M/A ratios are shown in Fig. 5. Even if the

Table 1. Results of the optimization design: composition, clarity, osmolarity, water content, pH.

Trial number	Mannitol (mg)	Alanine (mg)	Mannitol/alanine ratio (w/w)	Clarity	Osmolarity (mosm/kg)	Water content (% w/w)	pH
1	27.7	6.9	4	II–III	280	2.1	8.04
2	20.8	10.4	2	<I	300	2.8	8.03
3	14.15	14.15	1	I–II	328	1.35	8.01
4	10.6	15.9	0.67	I–II	322	2.6	7.99
6	7.3	16.0	0.45	I–II	310	2.0	8.06
9	4.7	18.9	0.25	I–II	329	1.04	7.96
10	2.5	20.0	0.125	II–III	324	0.44	7.96

intensity of certain peaks (such as the peak located at 9°) are somewhat altered by preferential orientation artifacts, useful information can be extracted from these results. Indeed, Fig. 5 shows that diffractograms of freeze-dried formulations for which the M/A ratio is lower than 1 contain x-ray patterns close to that of pure alanine.

This result means that when the M/A ratio is lower than 1, whereas the alanine component is in its crystalline form, mannitol remains amorphous. Moreover, Fig. 5 reveals that when the M/A ratio increases from 1 to 4 w/w, recorded diffractograms become more representative of the weighted sum of the x-ray patterns corresponding to the mannitol and alanine crystalline structures, respectively. In this range of concentration, we can then conclude that alanine and at least a part of mannitol contents are in their crystalline states.

DSC thermograms have been recorded for all formulations under the same experimental conditions. Since all mixtures considered exhibited very similar thermodynamic behaviors, we have just reported in Fig. 6, as an example, the formulation containing a 0.67 w/w Mannitol/Alanine blend. The physical interpretation of thermodynamic transitions observed in the DSC spectra has been based on the study of the influence of the M/A ratio on the intensity of the different events. At low temperature, about 40°C, the endothermic heat capacity step and the superimposed enthalpy relaxation peak was attributed to the glass transition of amorphous phases which are composed of a blend of protein, alanine and a large percentage of mannitol molecules. Around 100°C, two exothermic first order transitions have been observed. These transitions have been assigned to the cold-crystallization of mannitol. The endothermic peak noted at high temperature (T_{onset}=156.2°C) corresponds to the melting transition of the mannitol crystalline phase formed during the DSC experiment (during the cold-crystallization located around 100°C). Finally, around 210–230°C depending on the mixture, the melting transition of crystalline alanine has been observed in DSC spectra (not shown here).

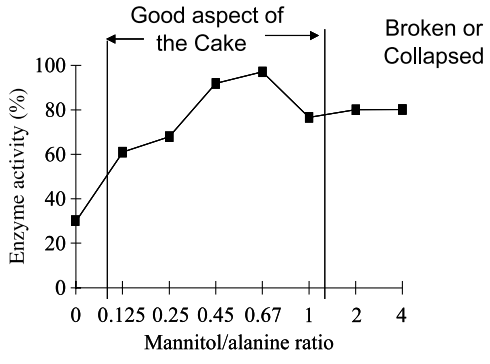

Figure 4. Residual enzyme activity after 1 month at 35°C of the different formula containing rasburicase, 50 mM phosphate buffer with a (w:w) alanine/mannitol ratio between 0.125 and 4.

The DSC results are in very good agreement with the x-ray diffraction measurements. Indeed, whereas areas under the endothermic and the exothermic first order transitions located around 100°C and 156°C, respectively were found to be very similar for formulations with M/A ratio lower than 1, significant differences between these areas were noted when the M/A ratio was higher than 1. So, as concluded from the x-ray diffraction study, the DSC results indicated that although no crystalline mannitol domain was present initially when M/A is lower than 1, some mannitol crystallites were formed during DSC measurements when the alanine was the minor component.

The glass transition temperature dependence on the M/A ratio of all formulations analyzed is reported in Fig. 7. It can be seen that the glass transition of the amorphous phase is constant near 50°C in the [0.125 ; 1 w/w] M/A ratio range and decreases dramatically when the M/A ratio increases from 1 to 4. The decrease of Tg is not due to water as the water content

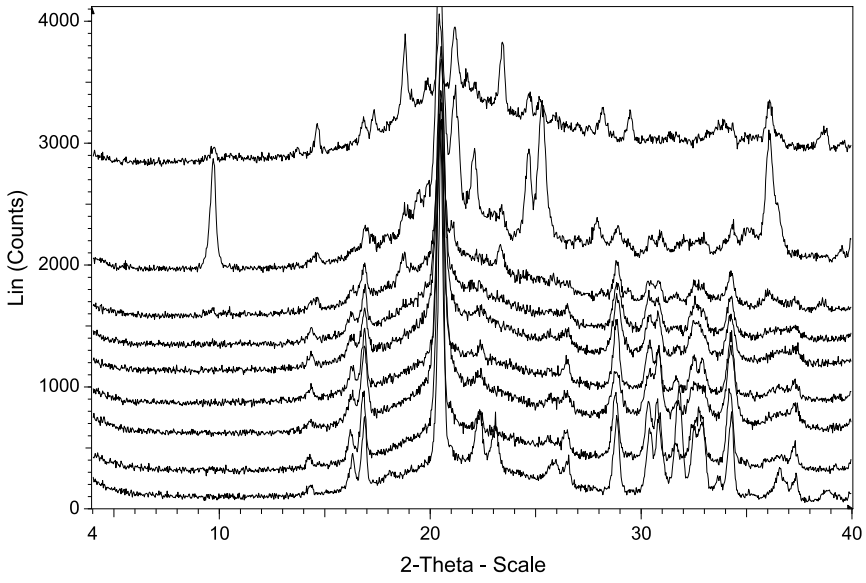

Figure 5. X-ray diffraction patterns recorded for formulation containing mannitol/alanine blend at 0.125, 0.25, 0.5, 0.67, 1, 2 and 4 w/w ratio (from bottom to top) surrounded by diffractograms recorded for pure alanine (bottom of the figure) and for pure mannitol (top of the figure).

is lower for these two M/A ratios. This phenomenon may be related to the crystallization of mannitol compound in this range as supported by X-ray results. The break observed for Tg also correlates with the collapsed nature of the cake.

From the enzyme activity, turbidity and clarity results, the formulation containing a 0.67 w/w M/A ratio was considered optimal. From analyses of the physical structure of all formulations, the 0.67 M/A formulation was characterized by a well-defined crystalline structure (a single crystallized compound: Alanine) and a high glass transition temperature allowing the system to remain partly in a glassy state.

From these results, the solid formulation based on rasburicase mixed with a 0.67 mannitol/alanine blend, as excipient, was selected for the next stage of development.

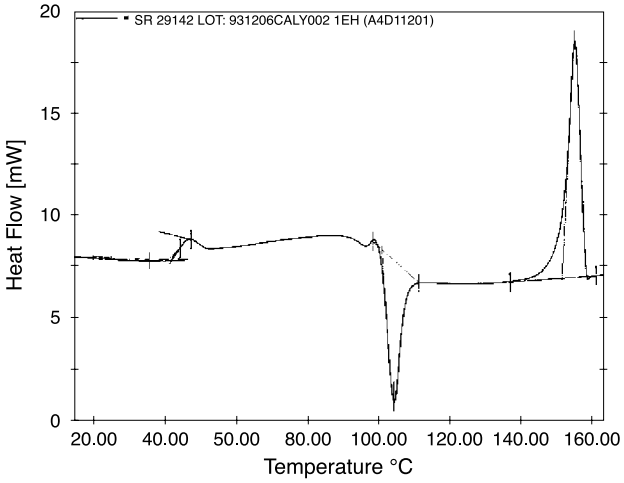

Figure 6. DSC of the freeze-dried powder containing rasburicase, 50 mM phosphate buffer pH8.0 and a 0.67 w/w mannitol/alanine blend, with a constant heating of 10°C/min.

Real Time Stability Results

A comparison of the stability results obtained on the drug substance solution and on the freeze-dried drug product (rasburicase solid formulation based on a 0.67 w/w M/A blend) is reported in Table 2.

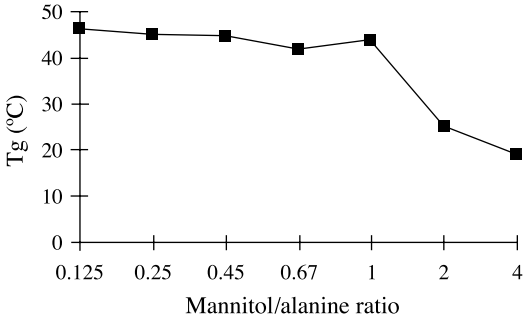

Figure 7. Glass transition temperature determined by DSC as a function of the mannitol/alanine w/w ratio.

Table 2. Slopes of the degradation rates for quantitative methods for the drug substance and the drug product stored at 5°C±3 C and at +25±2°C.

	Drug substance		Drug product	
Parameter	5±3°C	25±2°C	5±3°C	25±2°C
Enzyme activity	NS after 12 months	−0.03% per month	NS after 36 months	−0.001% per month
Rasburicase by SEC	NS after 12 months	−0.09% per month	NS after 36 months	−0.003% per month
Octamer by SEC	+0.01% per month	+0.14% per month	+0.007% per month	+0.03% per month
Sum of related impurities by RPC	+0.09% per month	+2.1% per month	+0.02% per month	+0.43% per month

The difference in stability between the drug substance and the drug product is more pronounced at 25°C than at 5°C. This result was confirmed by IEF as illustrated in Fig. 8. Indeed, after 12 months at 25°C the IEF pattern of the drug substance was dramatically modified. The main band corresponding to the non-modified rasburicase greatly decreased, whereas several new bands at more acidic isoelectric points were observed. On the contrary, even after 36 months at 25°C the IEF profile of the drug product stored at 25°C was almost the same, confirming the stabilizing role of the formulation chosen.

Only a limited degradation was observed as measured by quantitative techniques. The enzyme activity showed only small changes over time: −0.03% per month for the drug substance and only −0.001% for the drug product at 25±2°C. Other techniques like size exclusion chromatography and reverse phase chromatography confirmed this trend, proving the efficacy of the formulation to prevent aggregation or oxidation.

These results showed that stabilization of rasburicase in the drug product by means of the selected 0.67 w/w M/A excipient is effective.

Based on these results obtained at 5°C±3 C, a shelf life of 36 months was claimed for the drug product.

Moreover, it is important to emphasis that the results show that the product stored at +25±2 C/60% RH is stable over a period of 5 months. Thus short periods of storage at room temperature would not be considered to be detrimental.

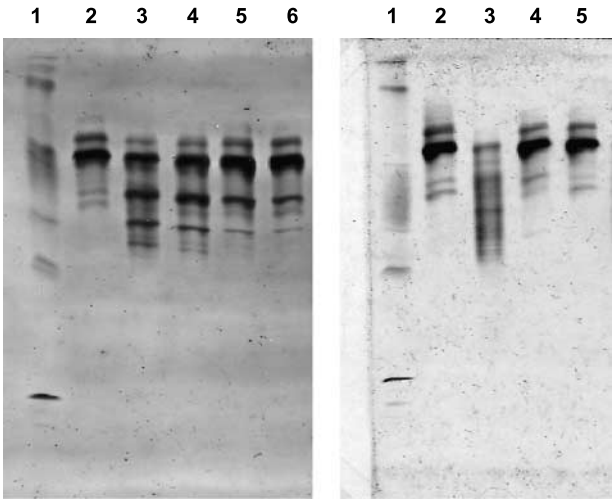

Figure 8. Comparison of the stability of the drug substance solution (b) line 3 and of the freeze- dried powder (a) line 3 by isoelectric focusing after storage 12 months at 25°C. Line 6 (a) and line 5 (b) correspond to the reference standard.

Development of a New Strength

For practical purposes and due to the large range of treated patient weights, a second strength containing 7.5 mg of rasburicase mixed with the same ratio of excipient/drug substance of the optimized 0.67 w/w mannitol/alanine blend was developed. As the lyophilization conditions were adjusted for this new vial size, a comparison of the 1.5 mg and the 7.5 mg powders was judged critical. Moreover, it was also an opportunity to test the robustness of the formulation.

The DSC thermograms and x-ray diffractograms were shown to be comparable suggesting the physical state, crystalline as well as amorphous, is the same for both 1.5 and 7.5 mg strengths of the freeze-dried powder. Glass transition temperatures and x-ray patterns are reported in Table 3 and Fig. 9 respectively.

However, as shown previously, for the preferred formulation where the M/A ratio is equal to 0.67 w/w, alanine was in the crystalline form, whereas rasburicase and mannitol were amorphous. As a consequence, for the 0.67 w/w M/A ratio, only the amorphous phase might have been affected by the increase from 1.5 to 7.5 mg of the rasburicase strengths in the formulation.

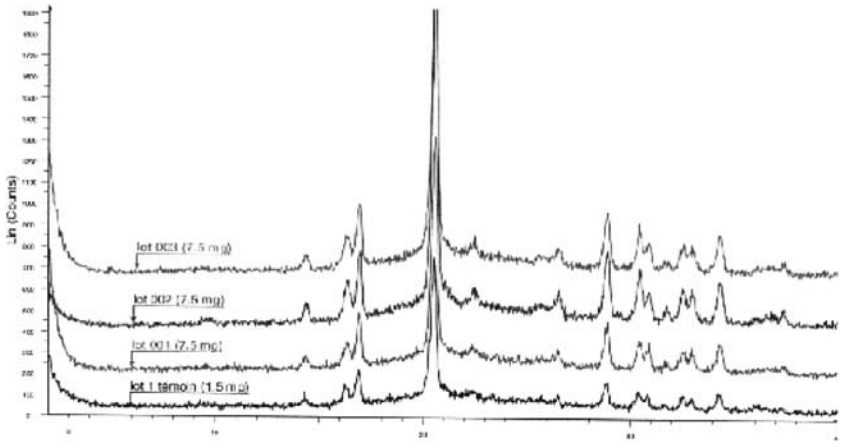

Figure 9. Comparison of the x-ray diffratograms of Fasturtec®/Elitek® 1.5 mg and 7.5 mg batches.

To ensure that the physical state of both 1.5 and 7.5 mg rasburicase formulations were identical, the TSC technique was used as a complementary form of analysis.

The TSC spectra recorded for the two batches from $-120°C$ to $+80°C$ after a polarization of the sample at $+70°C$ are reported in Fig. 10. Only one relaxation mode, labeled α_a, at around $+45°C$, was observed for the two rasburicase strengths. The good agreement between the temperature of the glass transition revealed by DSC for the two batches (Table 3) and the temperature of the α_a TSC peak maximum ($45-50°C$) allow us to associate unambiguiously the α_a relaxation process with the dielectric manifestation of the glass transition of the drug product. The main point to note in Fig. 10 is the very similar shape of TSC spectra recorded for the two rasburicase strengths over the whole temperature range investigated. This fact means that the amorphous phases of the two formulations exhibit identical molecular dynamics, indicating no difference between the physical structure of amorphous phases of both rasburicase formulations (7.5 and 1.5 mg).

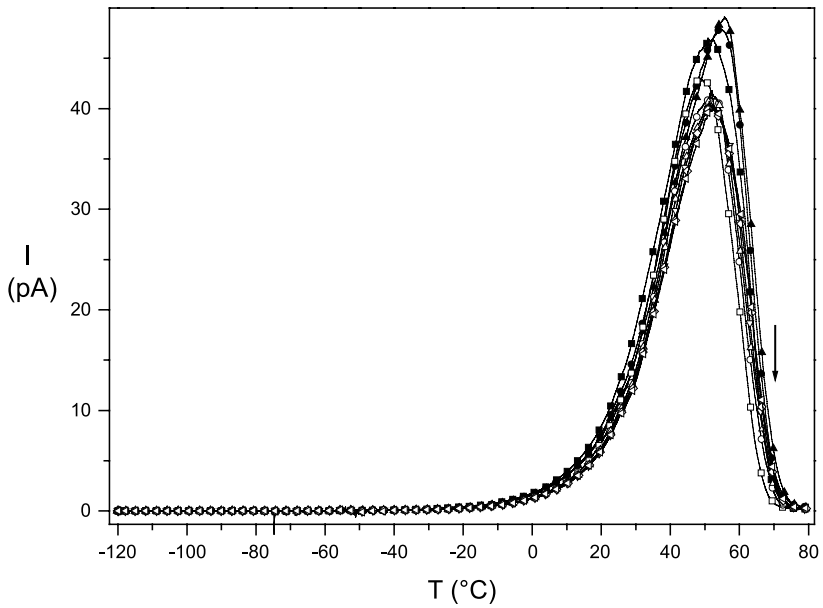

Figure 10. TSC spectra of Fasturtec®/Elitek® 1.5 mg (solid symbols) and 7.5 mg (open symbols) batches, with a constant heating rate of 7°C/min.

Table 3. Glass transition temperature of Fasturtec®/Elitek® 1.5 mg and 7.5 mg batches.

Fasturtec®/Elitek®	Batch	Tg (°C)
1.5 mg	1	Not determined
	2	41.5
	3	43.6
7.5 mg	1	40.6
	2	39.1
	3	39.7
	4	36.9
	5	38.9
	6	40.7

DISCUSSION

Due to the high molecular mass, the purity determination of a protein is a real challenge and a wide set of analytical methods is considered necessary to completely characterize a protein.[a] However, during the early stages of the development of the rasburicase, the combination of isoelectric focusing, having a high-resolution power, and enzyme activity, which are easy to perform and reflect the tertiary structure of the molecule, were shown to be sufficiently discriminating. Obviously, for a different protein, a different combination of analytical methods may be necessary. As an example, for somatropin a protein, which has a high tendency to form aggregates very easily during shaking or freeze-drying, size exclusion chromatography was an important method to optimize formulation.[13]

Although the formulation of proteins presents special concerns because of their potential conformational instability, or other specific well known degradation behaviors (tendency for aggregation, oxidation or deamidation)[14] the same general approach as for more conventional chemical drugs can be adopted.

In this paper the different stages of the development and confirmation of a stable formulation have been discussed. Having the knowledge of the specific behaviour of urate oxidase and the composition of existing parenteral products, a compatibility study was conducted with different molecules or potential excipients in solution. The interaction with the active site may explain the stabilization effect observed with competitive inhibitors of the enzyme, like 8-azaxanthine and xanthine. The presence of three free cysteines per monomer of urate oxidase and particularly the cysteine in position 103 explains the reaction when cysteine[15] is added.

It is interesting to note that the competitive inhibitors of the enzyme, 8-azaxanthine or xanthine, were the most efficient stabilizers in solution but have shown a poor stabilizing effect in the freeze-dried powder. Alanine and mannitol, on the contrary, have a slight effect (if any) in solution but a greater stabilizing effect on the powder. However, it should be emphasize that the mechanisms of stabilization of proteins in solution (i.e. preferential exclusion theory) are different from those in the dry state (i.e; vitrification theory, water replacement theory, particle isolation theory). It is known that crystallized excipients participate in creating the structure of a freeze-dried powder and therefore avoid any collapse during primary drying (sublimation) and secondary drying.[2] They may also participate in the stabilization of the proteins, preventing aggregate formation or stabilizing against heat denaturation. Alanine is in a crystallized state and may have this function in the lyophilizate.

Amorphous mannitol is also known to protect proteins during the freezing stage of the lyophilization process ensuring a good stability of the protein.[16] The loss of activity of a protein is directly linked to the degree of crystallinity of the cryoprotective molecule.[17] Izutsu et al.[18] have studied the stabilizing effect of mannitol on β-galactosidase from *E.coli*, rabbit-muscle L-Lactate dehydrogenase (LDH) and L-asparaginase from *E. chrysenthemi* during freeze-drying. It is well known that mannitol tends to crystallize during lyophilization and therefore loss its stabilizing effect whereas excipients like sucrose or threalose remain amorphous. When other components (salts, sugar, amino-acids) are added to aqueous protein solutions, the physicochemical properties of the frozen concentrate are far more complicated than those of the individual components due to unpredictable interactions between different molecules. The stabilization effect of mannitol was reduced with the appearance of mannitol crystals but surprisingly the presence of sodium phosphate buffer maintains mannitol in an amorphous state.

In an other more recent study Pyne et al.[19] investigate a ternary system containing glycine, mannitol and a phosphate buffer with the same model enzyme LDH. Glycine has a stronger tendency to crystallize than mannitol. An increase of buffer concentration decreases the crystallinity of mannitol and glycine and increases the retention of protein activity.

In our present study the phosphate buffer probably also inhibits the mannitol crystallization. Il our formulation study alanine is crystallized and mannitol has its maximum protective effect when it is totally in

[a]ICH, Specifications, tests and procedures for biotechnological/biological products.

an amorphous state (M/A ratio lower than 1). In the glycine formulation obtained by Pyne the crystallinity for both excipients glycine and mannitol was only reduced and mannitol was partly crystallized.

As the amount of mannitol versus alanine increases, the crystallinity of mannitol increases. This could explain the optimum found for the quantity of mannitol versus the enzyme activity.

The possible crystallization of disodium phosphate during freezing is also an important point to consider because it will induce a pH shift detrimental for the retention of the enzyme activity. However it should also be recognized that the crystallization of the disodium phosphate will depend on the phosphate concentration and other environmental conditions. Chang and Randall[20] described the possible role of different excipients (lactose, sucrose, sorbitol, thehalose), in the prevention of crystallization of disodium phosphate.

Some general comments can be made also on the glass transition temperature. It is interesting to see that the optimum M/A ratio is in the range where the glass transition temperature of the formulation is particularly high, thus predicting good long-term stability. This point is fundamental, since a decrease of glass temperature is synonymous with reduced stability for a given protein.[21] This phenomenon is directly connected to the molecular mobility of amorphous phases. Indeed, at Tg, the relaxation times of molecular motions involved in the glass transition of all amorphous materials are well known to be equal to 100 s[22] ($\tau(Tg)=100s$). However, when the temperature of the system is decreased, the molecular motions slow down due to the establishment of specific physical interactions between molecules which leads to an increase in the local density of the product: the relaxation time of the relaxing species is then increased by many orders of magnitude at temperatures below Tg. From several studies performed on different pharmaceutical drugs, it has been proven that the higher the time scale of molecular motions involved in amorphous phases, the lower the chemical reactivity and the greater the stability of molecules in these domains.[23]

In this context, the two choices usually considered to reduce the chemical degradation by physical means, ie. through a reduction of the molecular motion, are to increase the glass transition temperature of formulation and/or to decrease the storage temperature of drug product. In the case of urate oxidase, both these strategies were considered. Firstly the mannitol/alanine blend giving a high glass transition temperature of the drug product was selected and secondly, the storage temperature selected was 5°C, i.e. some 40°C below the glass transition of the formulation. This large difference between the storage temperature and Tg implies the inhibition of the molecular motions responsible for the degradation in the amorphous phase. Indeed, this storage temperature is very close to the Kauzmann temperature, $T_0=Tg-50°C$, where molecular motions are almost totally frozen. As the molecular mobility is obviously the base of physico-chemical reactivity for any molecular entity, frozen the molecular mobility means better stability.[22,24] However, at 20°C below the glass transition, i.e. at room temperature in this case, the molecular mobility of amorphous phases is non-zero but significantly reduced, assuring the absence of chemical degradation and physical instability during at least 5 months.

At a M/A >1, the Tg drops to lower values when mannitol starts to crystallize. At a ratio lower than 1, the glass transition was observed at about 40–50°C higher than the reported glass transition of the pure amorphous mannitol transition (13–15°C).[25] Sharma V.K. et al[26] have prepared lyophilized mixture of mannitol and β-galactosidase in a 1:1 ratio; a transition temperature of about 31.4°C was found. This higher transition temperature is explained according to these authors, to the coexistence of the mannitol with the protein in a single amorphous phase. At a M/A>1 is the transition temperature tends to reach the transition temperature of pure amorphous mannitol and a concomitant decrease of the stabilizing effect is observed.

As discussed above, it is important that formulations utilizing amorphous phases be in glassy state (T<Tg) when the molecular mobility is very reduced, in order to improve stability. Furthermore, the fact that the TSC patterns as well as their DSC profiles of both the 1.5mg and the 7.5 mg lyophilizates of rasburicase are comparable allows us to anticipate that the stability of the 7.5 mg formulation will be enterely comparable to that of the 1.5 mg product under the same storage conditions.

ABBREVIATIONS

DSC	differential scanning calorimetry
TSC	thermostimulated current spectroscopy
XRPD	X-ray powder diffraction
SEC	size exclusion chromatography
RPC	reverse phase chromatography

ACKNOWLEDGMENTS

We thank Hélène Cayron, Jacqueline Perez, and Pascal Malazzi for technical assistance.

REFERENCES

1. Geigert, J. Overview of the stability and handling of recombinant protein drugs. J. Parent. Sci. Technol. **1989**, *43* (5), 220–224.
2. Pikal, M.J. Freeze—Drying of Proteins, Part II: Formulation Selection. *Biopharm*; October 1990; 26–30.
3. Pui, C.H.; Sima, J.; Camita, B. In *Blood, Meeting Info*, 42nd Annual meeting of the America Society of Hematology, San Francisco, CA, USA, December 01–05, 2000; American Society of Hematology, 2000; 11–96, Part I, pp719a. print.
4. Pui, C.H.; Mahmoud, H.H.; Wiley, J.M.; Woods, G.M.; Leverger, G.; Camitta, B.; Hastings, C.; Blaney, S.; Relling, M.V.; Reaman, G.H. Recombinant urate oxidase for the prophylaxis or treatment of hyperuricemia in patients with leukemia or lymphoma. J. Clin. Oncol. **2000**, *19* (3), 697–704.
5. Colloc'h, N.; El Hajji, M.; Bachet, B.; Lhermite, G.; Schiltz, M.; Prange, T.; Castro, B.; Mornon, J.P. Crystal structure of the protein drug urate oxidase—inhibitor complex at 2.05 Å resolution. Nat. Struct. Biol. **1997**, *4*, 947–952.
6. Legoux, R.; Delpech, B.; Dumont, X.; Guillemot, J.C.; Ramond, P.; Shire, D.; Caput, D.; Ferrara, P.; Loison, G. Cloning and expression in *Escherichia coli* of the gene encoding *Aspergillus flavus* urate oxidase. J. Biol. Chem. **1992**, *267* (12), 8565–8570.
7. Leplatois, P.; Le Douarin, B.; Loison, G. High-level of a peroxisomal enzyme: *Aspergillus flavus* uricase accumulates intracellularly and is active in Saccharomyces cerevisiae. Gene **1992**, *122*, 139–145.
8. Bayol, A.; Dupin, P.; Boe, J.F.; Claudy, P.; Letoffe, J.M. Study of pH and temperature—induced transitions in urate oxidase (Uox-EC 1.7.3.3.) by microcalorimetry (DSC), size exclusion chromatography (SEC) and enzymatic activity experiments. Biophys. Chemist. **1995**, *54*, 229–235.
9. Boutonnet-Fagegaltier, N.; Ménégotto, J.; Lamure, A.; Duplaa, H.; Caron, A.; Lacabanne, C.; Bauer, M. Molecular mobility study of amorphous and crystalline phases of a pharmaceutical product by thermally stimulated current spectroscopy. J. Pharm. Sci. **2002**, *91* (6), 1548–1560.
10. Menegotto, J.; Demont, P.; Bernes, A.; Lacabanne, C. Combined dielectric spectroscopy and thermally stimulated currents studies of secondary relaxation process in amorphous Poly(ethylene terephthalate). J. Polym. Sci., Part B, Polym. Phys. **1999**, *37*, 3494–3503.
11. Menegotto, J.; Demont, P.; Lacabanne, C. Secondary dielectric dielectric-relaxation in amorphous poly(ethylene terephthalate): combined thermally stimulated and isothermal depolarization current investigations. Polymer **2001**, *42*, 4375–4383.
12. Lacabanne, C.; Lamure, A.; Teyssedre, G.; Bernes, A.; Mourgues, M. Study of cooperative relaxation modes in complex systems by thermally stimulated current spectroscopy. J. Non-Cryst. Solids **1994**, *172–174*, 884–890.
13. Bayol, A.; Breul, T.; Dupin, P.; Faure, P. Stable Freeze-Dried Formulation Comprising a Protein Assay kit. Patent, EP0682944B1 1999/US serial no. 5,763,409, 1998.
14. Yu-Chang, J.W.; Hanson, M.A. Parenteral formulations of proteins and peptides: stability and stabilizers (1988). J. Parent. Sci. Technol. **1988**, *10* (Supp.), 42-S4-S26 Technical Report N°.
15. Bayol, A.; Capdevielle, J.; Malazzi, P.; Buzy, A.; Bonnet, M.C.; Colloch's, N.; Mornon, J.P.; Loyaux, D.; Ferrara, P. Modification of a reactive cysteine explains differences between rasburicase and Uricozyme®, a natural *Aspergillus flavus* uricase. Biotechnol. Appl. Biochem. **2002**, *36*, 21–31.
16. Back, J.F.; Oakenful, D.; Smith, M.B. Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry **1979**, *18*, 5191–5196.
17. Izutsu, K.L.; Yoshioka, S.; Terao, T. Decrease protein-stabilizing effects of cryoprotectants due to crystallization. Pharm. Res. **1993**, *10–8*, 1232–1237.
18. Izutsu, K.L.; Yoshioka, S.; Terao, T. Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying. Chem. Pharm. Bull. **1994**, *42* (1), 5–8.
19. Pyne, A.; Chatterjee, K.; Suryanarayanan, R. Solute crystallization in mannitol-glycine systems—imlications on protein stabilization in freeze-dried formulations. J. Pharm. Sci. **Nov 2003**, *11*, 2272–2283.
20. Chang, B.S.; Randall, C.S. Use of subambient thermal analysis to optimize protein lyophilization. Cryobiology **1992**, *29*, 632.
21. Franks, F. Freeze-drying from empiricism to predictability. Cryo-lett. **1990**, *11*, 93–110.
22. Ediger, M.D.; Angell, C.A.; Nagel, S.R. Supercooled liquids and glasses. J. Phys. Chem. **1996**, *100*, 13200–13212.

23. Guo, Y.; Byrn, S.; Zographi, G. Physical characteristics and chemical degradation of amorphous Quinapril hydrochloride. J. Pharm. Sci. **2000**, *89* (1), 128–143.
24. Scherer, G.W. Theory of relaxation. J. Non-Cryst. Solids **1990**, *123*, 75–89.
25. Kim, A.L.; Akers, M.J.; Nail, S.L. The physical state of mannitol after freeze-drying: effects of mannitol concentration, freezing rate, and a non-crystallizing cosolute. J. Pharm. Sci. **1998**, *87*, 931–935.
26. Sharma, V.K.; Kalonia, D.S. Effect of vacuum drying on protein-mannitol interactions: the physical state of mannitol and protein structur in the dried state. AAPS PharmSciTech **2004**, *5* (1), 1–11, Art 10.