AAPS PharmSciTech, Vol. 10, No. 4, December 2009 (© 2009)
DOI: 10.1208/s12249-009-9327-x

Research Article

Stability Improvement of a Liquid Enzyme Product

Núria Jiménez,[1,4] Maria Luisa Garcia,[2] Javier Galán,[1] Alberto Vallet,[1] Geoffrey Owen,[3] and G. Michael Wall[3]

Received 9 June 2009; accepted 13 October 2009; published online 4 November 2009

Abstract. The shelf-life of a previously developed two-part liquid–liquid enzyme ceruminolytic product was improved maintaining the same final reconstituted composition and re-formulating the liquid enzyme portion as a drug granulate by a double wet granulation process. The critical steps for the preparation of the granulate were studied (mixing/granulating times and drying) determining the proteolytic activity, the residual ethanol, and the moisture content of the granulates. The original liquid–liquid formulation had been proven effective as a ceruminolytic agent, but only had stability of greater than 75% enzyme activity for up to 18 months and up to 1 day at room temperature after combining the two parts. The resulting improved product was proven to be stable for up to 24 months at 30°C, and up to 3 days at room temperature after combining the two parts. Therefore, maintaining the enzyme in a granulated form until reconstitution afforded an improvement in stability compared with the original two-part liquid–liquid formulation.

KEY WORDS: ceruminolytic; drying; liquid enzyme; methyl trypsin; wet granulation.

INTRODUCTION

In a previous study, a proteolytic enzyme-based ceruminolytic product was developed which was proven more efficacious than comparator products *in vitro* (1). The enzyme methyl trypsin (MT) was chosen as a ceruminolytic enzyme based on its efficacy in digesting human cerumen and relative stability compared to other enzymes (1,2). Shelf-life was a challenge with this product since enzymes in aqueous solutions are inherently unstable due to a variety of intramolecular and intermolecular chemical reactions including hydrolysis, aggregation, deamidation, oxidation, β-elimination, and changes in conformation that may result in a loss of biological activity (3,4). Stability was ultimately achieved through the use of a novel two-part bottle that kept a stable, acidic enzyme solution and a basic vehicle separated until just prior to use when the two parts could be easily combined inside the container. Though marginally acceptable stability was obtained, work continued to enhance the shelf-life of the original product.

While keeping the final composition of the reconstituted product constant, it was thought that proteolytic stability could be enhanced by formulating the enzyme as a solid through an inexpensive process. Dry process-roller compaction was not considered due to the small volumes involved in this product (the normal batch size for this product was about 0.5–10 kg) and because it might cause a relatively large amount of waste as dust and fines (5). Lyophilisation was disfavored due to the risks of freezing and drying (6–8), and protein aggregation (9,10). Supercritical fluid antisolvent has been used to obtain microparticulate protein powders, but it exposes proteins to potentially deactivating environments including organic and supercritical non-aqueous solvents, high pressure, and shearing forces (11). Direct compression was not considered because it would have required the use of different excipients to enhance flowability and compressibility (such as microcrystalline cellulose, magnesium stearate, or cross-linked sodium carboxymethylcellulose) (12,13) which would have involved altering the original reconstituted formula. Wet granulation, which is commonly used in the pharmaceutical industry to process fine powder blends (14), was ultimately chosen as an inexpensive and most straightforward method to formulate the solid enzyme.

The aim of this study was to develop a two-part, solid–liquid (S–L) product with improved shelf-life (i.e., proteolytic activity) compared to the previously developed two-part, liquid–liquid (L–L) product, while maintaining the same final composition as the original L–L product. This paper describes the double wet granulation processing employed and the improved stability of the new product.

MATERIALS AND METHODS

Materials

Glycerol, Ph. Eur./USP grade, was obtained from Uniquema, Wilton, UK; Tetronic® 1304 (poloxamine) from

[1] Department of Pharmaceutical Development, Alcon R&D Laboratories, Camil Fabra 58, 08320, El Masnou, Spain.
[2] Department of Physical Chemistry, Faculty of Pharmacy, Barcelona University, Barcelona, Spain.
[3] Research & Development, Alcon Research, Ltd., Fort Worth, Texas, USA.
[4] To whom correspondence should be addressed. (e-mail: nuria.jimenez@alconlabs.com)

ABBREVIATIONS: ANOVA, Analysis of variance; BAC, Benzalkonium chloride; KF, Karl Fischer; L–L, Liquid–liquid; MT, Methyl trypsin; RH, Relative humidity; RT, Room temperature; SE, Standard error; S–L, Solid–liquid; *w/v*, Weight/volume.

BASF Corporation, Washington, DC, USA; sodium bicarbonate, Ph. Eur./USP grade, from MERCK kGaA, Darmstadt, Germany; benzalkonium chloride (BAC) solution 50% (*w*/*v*), Ph. Eur./USP grade, from FeF CHEMICALS A/S, Koge, Denmark; sodium citrate, dihydrate, Ph. Eur./USP grade, from Panreac Quimica S.A., Montcada i Reixac, Spain; and citric acid, anhydrous, Ph. Eur./USP grade, from Citrique Belge, N.V., Tienen, Belgium. All other chemicals were of analytical reagent grade. MT was obtained from Sigma-Aldrich, St. Louis, MO, USA, in powder form.

Composition of S–L and L–L Formulations

The goal of this project was to develop an S–L formulation with improved stability compared to the original L–L formulation, while maintaining the composition of the original reconstituted product. The novel two-part bottle was the same as previously described (1,15). The compositions of the S–L and L–L formulations are listed in Table I.

Preparation of Final S–L Formulation for Stability Studies

In the S–L formulation, one compartment contained the enzyme as granulate (Part I) and the other, the basic vehicle (Part II). The granulate was prepared by a double wet granulation process as indicated in Fig. 1. The granulating fluid used was anhydrous ethanol. To manufacture a 10-kg batch size lot, the granulation was performed in a Prat beater (Prat, Barcelona, Spain) with a high speed Glatt siever (model TR-120-02, Glatt-Labortecnic, Barcelona, Spain). The drying process was carried out in a drying chamber (model CET-0/830, Dycometal, Barcelona, Spain) at 20–25°C, with an air cycle of 10 s per hour for at least 16.5 h for the preliminary granulate and 6 s every 30 min for at least 18 h for the final granulate. To manufacture a 0.5- and a 2-kg batch size lot, the granulation was performed in a Kenwood beater (Kenwood, New Lane, UK), and the drying process was performed in a climatic chamber (model HC-0100/S, Heraeus, Hanau, Germany) at 25°C. After drying, the small size granulates were sieved at 500 μm. The temperature and the relative humidity of the room (RH) were monitored on a recorder (Haenni, Vendôme cedex, France). The in-process controls for preliminary and final granulates were pH of granulate, moisture content (by Karl Fischer analysis), sieve test (16), residual alcohol, and proteolytic activity (only final granulate). In practice, Part I was combined just prior to use with Part II to achieve the final solution (reconstituted sample).

Storage Stability Studies

The long-term stability study of the enzyme was carried out with three primary stability batches of final S–L formulation in accordance with current International Conference on Harmonization stability guidelines for liquids in semipermeable containers: (17) 5±3°C, 25±2°C/40±5% RH, 30±2°C/60±5% RH, and 40±2°C/20±5% RH. Samples were assayed for proteolytic activity and BAC content for 2 years (0, 3, 6, 9, 12, 18, and 24 months) following reconstitution at each time point. After 24 months of storage at 25°C/40% RH, samples of two lots of final formulation were reconstituted and analyzed for their proteolytic activity, initially, at 1 and at 3 days after reconstitution. Proteolytic activity results were expressed as means (±standard error (SE)) of three determinations, and BAC results were expressed as means of two determinations.

Methyl Trypsin Proteolytic Activity Assay

The proteolytic activity of MT was determined spectrophotometrically as previously described (1).

Benzalkonium Chloride (BAC) Assay

The BAC content was determined by high-performance liquid chromatography as previously described (1).

Table I. Composition of Different Parts of S–L Formulation *vs* Different Parts of L–L Ceruminolytic Formulation

Ingredients	mg (*w*/*w*)[a]		% (*w*/*v*)		
	S–L formulation		L–L formulation		Reconstituted solution (Part I + Part II − 10 mL)
	Part I (200 mg granulate)	Part II (10 mL solution)	Part I (1.5 mL solution)	Part II (8.5 mL solution)	
Methyl trypsin	2.7[b]	–	1,333 AU[e]/mL +10% excess[d]	–	200 AU[e]/mL
Glycerol	2.0	6.98	46.7	–	7.0
Sodium bicarbonate	–	5.0	–	5.882	5.0
Sodium citrate, dihydrate	187.1	1.103	–	3.529	3.0
Poloxamine (Tetronic®1304)	4.0	0.21	–	0.294	0.25
BAC (50% *w*/*v*)		0.02	–	0.024	0.02
Citric acid, anhydrous	4.2	qs to pH 8.0	–	qs to pH 8.0	qs to pH 8.0
Purified water	–	qs	qs	qs	qs
Ethanol, anhydrous	28[c]	–	–	–	–

[a] Quantities for 200 mg of granulate
[b] Includes 20% excess to compensate losses during the granulate manufacture. One milligram methyl trypsin=900 AU (activity units)
[c] It is evaporated during the manufacturing process
[d] Overage to compensate losses during manufacture
[e] AU=activity unit (microgram of tyrosine per minute per milliliter)

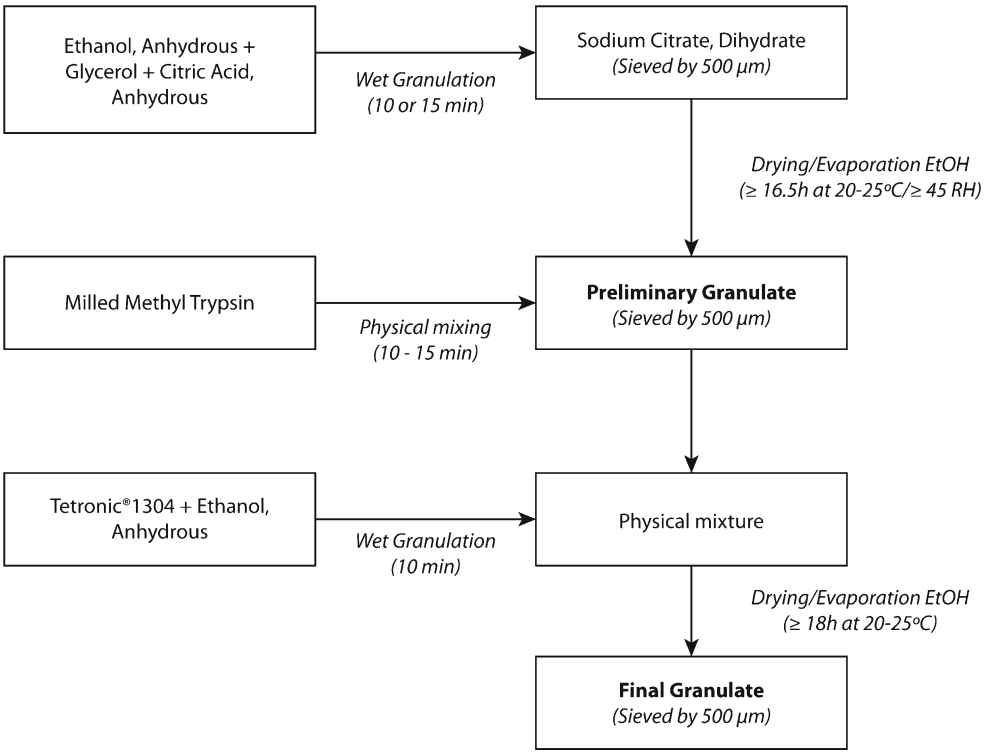

Fig. 1. Schematic representation of the granulate preparation by the double wet granulation process

Residual Ethanol Assay

Residual ethanol content of the preliminary and final granulates after drying was determined by gas chromatography (HP6890 Series II GC equipped with flame ionization detector, HP6890 autoinjector, 9100 Packard Hydrogen Generator, and HP ChemStation, Santa Clara, CA, USA). Diluted solutions were injected (3-mL loop volume) onto a fused silica capillary column (Tracer TR-WAX, polyethylene glycol phase, 30 m×0.53 mm i.d., 1.0 µm film coating) with helium carrier flow rate of 5 mL/min. Results were expressed as means (±SE) of three determinations.

Moisture Content by Karl Fischer (KF)

To determine the water content of the granulate, a Karl Fischer (KF) titration was performed (using a Titrinum equipment model KF701, Metrohm, Herisay, Switzerland). Samples were weighed and immediately put into the chamber. The extraction time was 10 min.

MT Milling Process

Two different lots of raw material, 100 g each, were milled for a period of time (30, 60, 90, and 120 s) using a blender (model 36BL12, Waring, Torrington, CT, USA) at <30°C and ≤40% RH. The temperature and RH of the room were recorded (Haenni recorder, Vendôme cedex, France) at the beginning and at the end of each milling, as well as the temperature of raw material after each milling, to evaluate the effect of the temperature on proteolytic activity. The proteolytic activity of the enzyme was determined by USP method (32nd Edition, Vol. 3, Monograph 3823) after each milling using unmilled samples as control. Results were expressed as means (±SE) of three determinations. The particle size was tested using a microscope (BX40, Olympus, Center Valley, PA, USA). The proteolytic activity data in Table II show that there was not a significant loss in enzyme activity after 120 s of milling ($p>0.05$). The specification criteria for particle size was that not less than 90% of particles were below 10 µm to achieve a homogeneous distribution of the enzyme in the granulate. This size was reached after 120 s of milling.

The temperature of the raw material increased as a function of the milling time (maximum temperature obtained was 43.5°C for a milling time of 90 s), but it did not affect the proteolytic activity. The moisture content of MT was not affected by the RH of the room (40–44% RH). During the milling process, the enzyme moisture content raised maximum up to 1.0% (Table II).

Statistical Analysis

Where appropriate, data were analyzed using analysis of variance. Statistical significance was determined using the regular t test, with p values of <0.05 being significant.

RESULTS

The critical steps for the double wet granulation process were studied, and a stability study of the resulting S–L product was followed. Proteolytic activity of at least 75% was sought since this activity was previously shown to be efficacious (1).

Table II. Testing Performed in Methyl Trypsin Raw Material During the Milling Process

Raw material lot	Milling time (seconds)	Particle size (% particles <10 μm)[a]	Temperature of raw material after each milling time (°C)	Raw material moisture content (%)[a,b]	Proteolytic activity (USP/mg)[c]
A	Unmilled	–	–	1.48±0.01	3,415±12.3
	90	86.5±1.8	43.5	–	–
	120	92.5±1.1	40.1	2.37±0.06	3,356±70.6
B	Unmilled	–	–	1.45±0.01	3,431±47.7
	30	91.3±1.4	28.5	–	–
	60	90.0±0.8	35.5	–	–
	90	93.1±1.7	37.8	–	–
	120	98.0±0.5	36.9	2.49±0.05	3,407±1.3

[a] Three samples collected from the upper, middle, and lower part of the raw material bulk
[b] Determined by Karl Fischer titration method
[c] USP Pharmacopeia, 32nd Edition, Vol. 3, Monograph 3823; USP=trypsin USP units

Influence of Mixing Time/Granulating Time on Homogeneity of Final Granulate

To evaluate the physical mixture between MT and preliminary granulate, three lots of 2 kg (C1–C3) each were prepared with three different lots of MT raw material: two mixing times were tested 10 and 15 min (Fig. 1). These physical mixtures were granulated afterwards with an alcoholic mixture made of anhydrous ethanol and poloxamine for 10 min to obtain the final granulate. An appropriate mixing time for the granulate had to be determined due to the difference between the particle size of the preliminary granulate (>95% particles between 63 and 250 μm) and MT (>90% particles <10 μm) The optimum mixing time was identified as 10 min since there was no statistically significant difference ($p>0.05$) in proteolytic activity between 10 and 15 min of mixing (data not shown). The process provided an adequate homogeneity of MT in the final granulate since SE was <10%. In addition, the homogeneity was maintained during the filling process in Part I of the two-compartment bottle because proteolytic activity results were similar to those obtained for the bulk granulate (Table III). The 10-min mixing and granulating times were confirmed during the manufacture of three lots of 10 kg each (D1–D3; Table III).

Although there is a difference in proteolytic activity results of small- and large-scale batches (Table III), the reproducibility is assured. Variability in the manufacturing process can be attributed to analytical variation and non-homogeneity. The analytical error for proteolytic activity assay, an enzymatic digestion of casein, is approximately 5%. Furthermore, there may be as much as 10% variability due to the lack of homogeneity attributable to the dispersion of the active (MT) in the preliminary granulate, both with different particle size. Taken together, the reproducibility target may be as much as 15%.

Drying Process of the Preliminary and Final Granulates

Exploratory trials gave evidence of a relationship between RH and drying efficiency to obtain residual ethanol less than 100 ppm. Five preliminary granulate lots (E–I) of the same size (0.5 kg) were manufactured in a beater and dried at 25°C and at different RH values for 16.5 h (Fig. 1). Not less than 45% RH was required inside the drying chamber for evaporating the granulating fluid, anhydrous ethanol. The granulate moisture content was not affected by the increment in ambient RH (Table IV). Three 10-kg scale-up granulates (D1–D3) were prepared using a beater and a high speed Glatt. The preliminary granulates were dried in a drying chamber at 20–25°C for 18.5–20 h (Table V). The ambient conditions recorded were 21–25°C/40±10% RH. The moisture content of these lots was between 11.7% and 12.5% (Table V). Considering that the Eur. Ph. Monograph for sodium citrate (18) establishes a moisture content between 11.0% and 13.0% and the water content of the

Table III. Proteolytic Activity Results of Homogeneity of Final Granulates C and D and Release Results During Filling Process of Granulates C

Lot	Batch size (kg)	Proteolytic activity (%)			
		Homogeneity of final granulate ($n=6$)	During filling process		
			Initial ($n=3$)	Middle ($n=3$)	End ($n=4$)
C-1	2	108±2.9	100±2.0	101±2.1	98±4.9
C-2	2	107±4.8	106±1.1	103±2.4	103±3.3
C-3	2	102±2.9	105±1.0	101±5.0	100±2.4
D-1	10	98±4.0			
D-2	10	94±2.9			
D-3	10	91±3.0			

Table IV. Influence of the Relative Humidity on the Drying Process of the Preliminary Granulate

Lot	Chamber conditions	Residual ethanol (ppm)[a]	Granulate moisture content (%)[a]
E	25°C/40% RH	287	13.1
F	25°C/45% RH	<4	12.4
G	25°C/50% RH	<4	12.9
H	25°C/60% RH	<4	13.1
I	25°C/70% RH	<4	13.3

Manufacturing conditions: batch size, 0.5 kg; drying time, 16.5 h
[a] Each point represents the mean of two replicates

preliminary granulate was mainly due to this excipient, the granulate moisture results were considered satisfactory.

Temperature control was critical during drying of the final granulate since the enzyme was easily degraded above 40°C for a long periods of time. The 10-kg final granulates (D1–D3) were dried in a drying chamber at 20–25°C in 20–22 h (Table V). The ambient RH did not affect the process (recorded ambient conditions: 21–22°C/36–40% RH). The moisture content was optimum, between 11.7% and 12.2%. The particle size of both granulates, preliminary and final, was repetitive in all three lots. Not less than 95% of particles were between 63 and 250 µm (Ph. Eur. sieve test (16)). The pH in solution of preliminary and final granulates was maintained at 6.5±0.1.

Long-Term Stability Study of the Final S–L Product

The main reason to develop the S–L formulation was to improve the long-term stability of the enzyme in aqueous solution once reconstituted compared with the original L–L formulation. Therefore, three lots of S–L formulation were prepared, stored, pulled, Parts I and II combined, and tested for proteolytic activity and BAC content at time intervals for 2 years. The BAC content did not significantly decrease over the 2-year period ($p>0.05$) at all storage conditions (data not shown).

Long-term stability results for the S–L formulation demonstrated that proteolytic activity in the finished product tends to degrade with time and increased temperature above and including 25°C (Fig. 2). Proteolytic activity of at least 75% was maintained when stored at 5°C, 25°C, and 30°C for 24 months prior to reconstitution. The rate of loss of proteolytic activity per month was 0.00–0.38% at 5°C, 0.13–0.46% at 25°C, 1.04–1.08% at 30°C, and 0.25–0.42% at 40°C. This represented an improvement over the original L–L product which maintained 75% proteolytic activity for only up to 18 months at 30°C (1).

At various time points during the stability program, the S–L product stored at 25°C/40% RH was pulled and the two parts combined, and the proteolytic activity was assessed for 1, 3, and 7 days. These data were compared to data previously gathered for the L–L product (1). At the beginning of the stability study, the enzyme activity of S–L formulation was significantly higher ($p=0.0024$) than previously observed for the L–L formulation after 7 days of reconstitution at room temperature (RT; Fig. 3). Even after 24 months of storage at 25°C/40% RH, the enzyme activity for two S–L lots tested after 1 day of reconstitution was 88% and 83% and after 3 days of reconstitution was 77% and 76%, respectively (Table VI). These results represented an improvement over the original L–L product which maintained 75% proteolytic activity for only up to 1 day at RT after storage for 2 years at 25°C/40% RH (1).

DISCUSSION

The objective of this study was to develop a two-part S–L formulation with improved stability compared to the original two-part L–L formulation, while maintaining the composition of the original reconstituted product that had previously been shown to be efficacious (1). This was achieved by formulating the enzyme as a granulate, Part I, that could be stored separately in a special two-part bottle until combination with the vehicle, Part II, just prior to use.

The enzyme in granulate form was manufactured by a double wet granulation process (Fig. 1). The first wet granulation at pH 6.5 stabilized MT with regard to degradation. A second wet granulation was performed to achieve a homogeneous mixture with regard to particle size: mixing time and granulation time were found to be critical steps in order to obtain good homogeneity of MT in the final

Table V. Drying of 10 kg Batch Size Preliminary and Final Granulates

Granulate type	Samples	Drying time (hours)			Residual ethanol (ppm)[a]		
		Lot D-1	Lot D-2	Lot D-3	Lot D-1	Lot D-2	Lot D-3
Preliminary	Upper tray[b]	18.5	18.5	20	24.5±11.3	14.4±6.8	60.4±8.4
	Lower tray[c]				9.9±1.3	13.6±4.4	26.8±7.6
Final	Upper tray[b]	20	22	22	<4	7.2±0.5	<4
	Lower tray[c]				<4	6.7±0.0	<4

[a] Each result represents the mean of four individual results ± SE
[b] Two samples collected from the middle and front right corner sides
[c] Two samples collected form the middle and back left corner sides

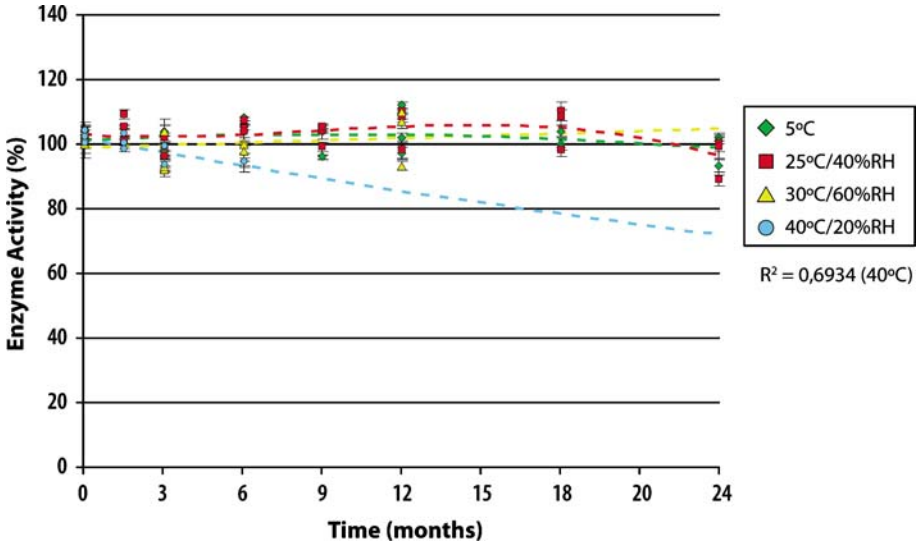

Fig. 2. Long-term stability of methyl trypsin at 5±3°C, 25±2°C/40±5% RH, 30±2°/60±5% RH, and 40±2°C/20±5% RH in S–L formulation. The figure shows cumulative data from three lots of the same formulation. Each point corresponds to the mean of three replicates ± SE. The regression line is shown for each storage condition

granulate. The drying process of both granulates was also found critical.

Since the final composition of the combined product could not be modified, the first approach in the reformulation was to consider which ingredients could help to form a granulate with the appropriate physical characteristics for Part I, based on their solubility, hygroscopicity, and particle size. A preliminary granulate was created using sodium citrate, citric acid, and glycerol. The use of citric acid combined with sodium citrate afforded a pH of 6.5, sufficient to provide good enzymatic stability and an optimum granulate appearance. The addition of a low concentration of glycerol as a binder prevented a sticky appearance of the granulate. This may be due to a polyol-enzyme complex that stabilizes the enzyme since reactivation by dilution of a similar enzyme/stabilizing complex in an aqueous medium has been reported (19).

The effects of milling on the facilitation of physical and chemical degradation of drugs have been extensively investigated (20–22). When materials are subjected to mechanical stress, defects are formed and may result in the formation of nucleation sites for physical transformations or chemical reactions (23). Previous to the manufacturing process, it was found helpful to mill the MT enzyme to homogenize the raw material (>90% of particles with a particle size <10 μm): this treatment reduced the enzyme particle size, facilitating uniform dispersion of the enzyme during the first granulation process. When room conditions for this process were maintained at

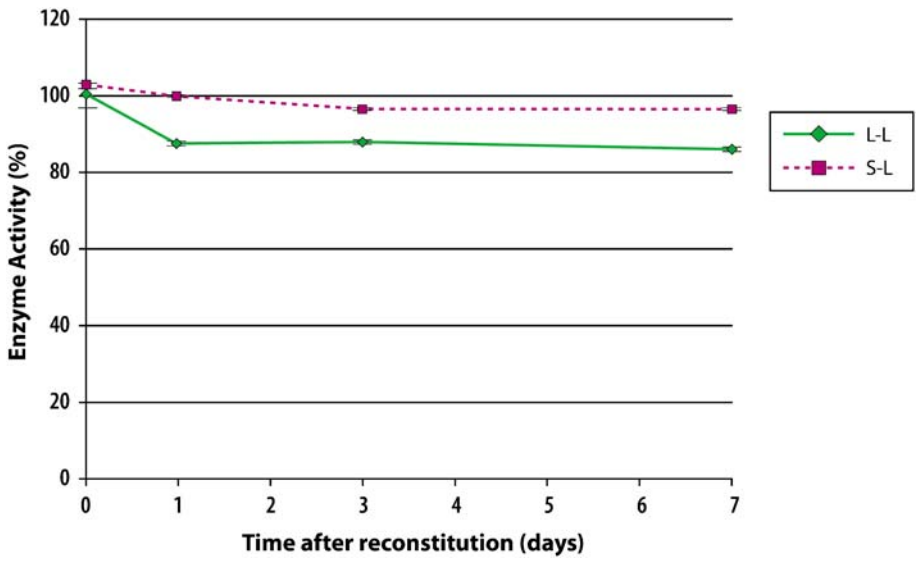

Fig. 3. Enzyme activity after reconstitution of S–L formulation *vs* L–L formulation stored at 25°C/40% RH (Time 0 of 24 months long-term study). Each point represents the mean of three replicates ± SE

Table VI. Comparison of Proteolytic Activity After Reconstitution of S–L and L–L Formulations After 24 months of Storage at 25±2°C/40± 5% RH (means ± SE; n=3; two lots)

Time after reconstitution (days)	Proteolytic activity results (%)	
	L–L	S–L
0	91±1.0	96±0.0
	80±0.2	92±0.3
1	81±0.2	88±0.6
	77±1.0	83±0.9
3	71±1.2	77±0.7
	73±0.6	76±1.8
7	63±0.3	72±0.3
	59±1.0	70±0.6

20–25°C, and below 40% RH, no increment in water content due to the milling process occurred. For a 100-g lot, the particle size of not less than 90% of particles below 10 μm was reached after 120 s of milling without affecting the proteolytic activity although the temperature rose up to 43.5°C.

Homogeneous mixing between MT (initial particle size: >90% particles are <10 μm) and the preliminary granulate (initial particle size: >95% particles between 63 and 250 μm) was achieved (SE <10%) after 10 min (final particle size: >95% particles between 63 and 250 μm). The MT was added as powder to the preliminary granulate because the addition of the enzyme as a solution was found to decrease the proteolytic activity. A second granulation was performed to distribute homogeneously the enzyme in the preliminary granulate by the addition of an alcoholic solution containing ethanol and poloxamine. Anhydrous ethanol was selected as the granulating fluid because it provided a consistent, non-sticky, granulate, probably because the sodium citrate dihydrate was not solublized by the ethanol. The poloxamine was used as binder that enhanced the flowability and the appearance of the final granulate. Three 2-kg lots (C1–C3) were manufactured to evaluate the mixing time (10 min) and the granulating time (10 min) which were confirmed with the manufacturing of three 10-kg lots (D1–D3). The process provided an adequate homogeneity of MT in the final granulate since SE was <10% in both batch sizes.

The drying process involved the evaporation of the granulating fluid from both the preliminary granulate and also the final granulate, with or without the application of heat. Thermal energy could be applied to the granulates by convection, conduction, or vacuum drying (24). However, the evaporation was performed at RT due to the heat sensitivity of MT. Again, anhydrous ethanol was the best granulating fluid because it did not require high temperature for evaporation. The drying of the preliminary alcoholic granulate required a minimum of 45% RH in the room. It was thought that glycerol present in the preliminary granulate was miscible with alcohol, impeding its evaporation (from 45% RH and above, an increment in vapor pressure is generated facilitating a superficial evaporation of ethanol at RT) (25). We evaluated whether an increase in the moisture content of the preliminary granulate could facilitate the drying process. Several 1-kg preliminary granulates prepared with granulating fluids containing increasing concentrations of water up to 30% (EtOH-H_2O (95:05, 90:10, 80:20, and 70:30)) revealed that a minimum of 10% water enhanced evaporation of the ethanol from the preliminary granulate at 23°C and low RH (≤36%). This may be because in presence of water, a positive azeotrope was formed (a liquid mixture of EtOH-H_2O with a boiling point lower than either of its constituents (78.1°C)), displacing the glycerol and aiding in the evaporation of ethanol at lower RH (26).

Long-term stability studies demonstrated that S–L formulation maintained more than 75% enzyme activity for 24 months at 5°C, 25°C, and 30°C for up to 3 days at RT after reconstitution. This represented a significant improvement (p<0.05 for all three temperatures) over the original L–L formulation that was stable for only up to 18 months at 30°C, and up to 1 day at RT after reconstitution (Fig. 4) (1). In addition,

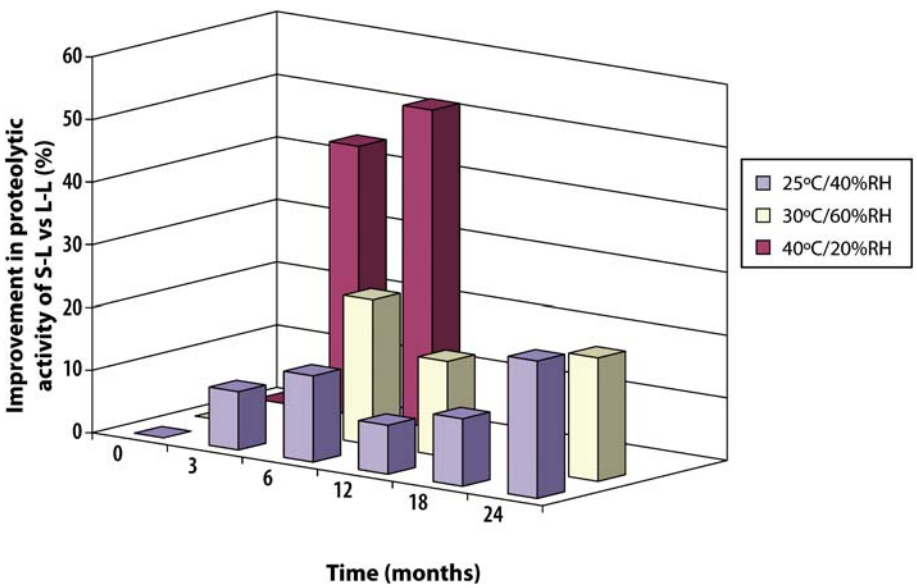

Fig. 4. Improvement in proteolytic activity of S–L *vs* L–L formulation at different storage conditions (25°C/40% RH, 30°C/60% RH, and 40°C/20%RH) with time

if we compare the drop in proteolytic activity after 3 days of reconstitution of samples stored 24 months at 25°C/40% RH, the S–L product lost 21–24% of activity while the L–L product lost 27–28% of activity, which corroborates the significant improvement (p=0.0000). Therefore, reformulation of the Part I into a granulate enhanced the stability of the enzyme.

CONCLUSIONS

An improved two-part S–L enzymatic ceruminolytic product was developed. The liquid enzyme portion was reformulated as a granulate while keeping the final composition of the combined product constant. An improvement in stability was achieved with the S–L formulation compared with the original two-part L–L formulation.

ACKNOWLEDGMENTS

The authors thank colleagues Josep Guasch, Ph.D., Carmen Martínez, Ph.D., Jaume Costa, Lupe Almera, Marta Martínez, Mónica Cuchi, and Paloma Mazoteras for their support in these studies. The authors thank Beverly Loyd for assistance in preparation of the manuscript and graphics. Work was performed at Alcon R&D Laboratories, Camil Fabra 58, 08329, El Masnou, Barcelona, Spain and Alcon Research, Ltd., 6201 South Freeway, Fort Worth, TX 76134, USA. Funding was provided by Alcon.

REFERENCES

1. Jiménez N, García ML, Galan J, Vallet A, Owen GR, Wall GM. Development of a liquid enzyme-based ceruminolytic product. J Pharm Sci. 2008;97(11):4970–82.
2. Asgharian B, Hong BS. Liquid enzyme compositions and methods of use in contact lens cleaning and disinfecting systems. US patent US 6,214,596 B1; 2001.
3. Costantino RH, Langer R, Klibanov M. Solid-phase aggregation of proteins under pharmaceutically relevant conditions. J Pharm Sci. 1994;83(12):1662–9.
4. Winters MA, Debenedetti PG, Carey J, Sparks HG, Sane SU, Przybycien TD. Long-term and high temperature storage of supercritically-processed microparticulate protein powders. Pharm Res. 1997;14(10):1370–8.
5. Ingelbrecht S, Remon JP. Reducing dust and improving granule and tablet quality in the roller compaction process. Int J Pharm. 1998;171:195–206.
6. Constantino HR, Griebenow K, Mishra P, Langer R, Klibanov AM. Fourier-transform infrared spectroscopic investigation of protein stability in the lyophilized form. Biochim Biophys Acta. 1995;1253:69–74.
7. Prestrelski SJ, Tedeschi N, Arakawa T, Carpenter JF. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys J. 1993;65:661–71.
8. Kendrick BS, Dong A, Allison SD, Manning M, Carpenter JF. Quantitation of the area of overlap between second-derivative amide I infrared spectra to determine the structural similarity of a protein in different states. J Pharm Sci. 1996;85:155–8.
9. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Crit Rev Ther Drug Carr Syst. 1993;10:307–77.
10. Pikal MJ. Freeze-drying of proteins, Part 2: formulation selection. Biopharm. 1990;3(9):26–30.
11. Yeo SD, Lim GB, Debenedetti PG, Bernstein H. Formation of microparticulate protein powders using a supercritical fluid antisolvent. Biotechnol Bioeng. 1993;41:341–6.
12. De Souza TP, Bassani VL, González OG, Dalla Costa TCT, Petrovick PR. Influence of adjuvants on the dissolution profile of tablets containing high dose of spray-dried extract of *Maytenus ilicifolia*. Pharmazie. 2001;56:730–3.
13. Plaizer-Vercamen JA, Bruwier C. Evaluation of excipients for direct compression of the spray-dried extract of *Harpogaphytum procumbens*. STP Pharma Sci. 1986;2:525–30.
14. Schenck LR, Plank RV. Impact milling of pharmaceutical agglomerates in the wet and dry states. Int J Pharm. 2008;348:18–26.
15. Gimenez FX, Bergamini MVW. Pharmaceutical container with two separate substances, with means for mixing and for the dosed application of the substances, and assembly process thereof. European patent EP 0 778 221 B1. 2000.
16. European Directorate for the Quality of Medicines & HealthCare of Council of Europe. European Pharmacopoeia. 6th Edition. Strasbourg: European Directorate for the Quality of Medicines & HealthCare of Council of Europe; 2008. p. 20912.
17. European Agency for the Evaluation of Medicinal Products. Note for guidance on stability testing: stability testing of new drug substances and products (Revision 2). CPMP/ICH/2736/99. London: European Agency for the Evaluation of Medicinal Products; 2003.
18. European Directorate for the Quality of Medicines & HealthCare of Council of Europe. European Pharmacopoeia. 6th Edition. Strasbourg: European Directorate for the Quality of Medicines & HealthCare of Council of Europe; 2008. p. 00412.
19. Chowhan MA, Quintana RP, Asgharian B, Hong BS, Bilbault T, Rosental RA. Stable liquid enzyme compositions and methods of use in contact lens cleaning and disinfecting systems. US Patent number 5,604,190; 1997.
20. Buckton G, Choularton A, Beezer AE, Chatham SM. The effect of the comminution technique on the surface energy of a powder. Int J Pharm. 1988;47:121–8.
21. Brittain HG, Fiese EF. Effect of pharmaceutical processing on drug polymorphs and solvates. In: Brittain HG, editor. Polymorphism in pharmaceutical solids. New York: Marcel Dekker, Inc.; 1999. p. 332–61.
22. Chan HK, Doeler E. Polymorphic transformation of some drugs under compression. Drug Dev Ind Pharm. 1985;19:315–32.
23. Byrn SR, Pfeiffer RR, Stowell JG. Role of defects in solid-state reactions. In: Byrn SR, Pfeiffer RR, Stowell JG, editors. Solid-state chemistry of drug. 2nd ed. West Lafayette: SSCI, Inc.; 2000.
24. Fox B. True grit: granulation and drying of delicate products. Chem Eng. 2005;115:35–8.
25. Clark J. Nonideal Mixtures of Liquids. Chemguide.co.uk. http://www.chemguide.co.uk/physical/phaseeqia/nonideal.html. Accessed 27 May 2008.
26. Hilmen EK. Separation of azeotropic mixtures: tools for analysis and studies on batch distillation operation. 2000. Trondheim: Norwegian University of Science and Technology, Dept. of Chemical Engineering. Retrieved on 24 March 2007.