Design of the lyophilization process of a L-leucyl-L-alanine dipeptide formulation based on its thermal properties

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RESEARCH

ABSTRACT

The system for phenylketonuria diagnostic in newborns (UMTEST® PKU) is produced at the Center for Immunoassay (Havana, Cuba). L-leucyl-L-alanine is one of the kit components and requires lyophilization due to its limited aqueous solution stability. Results from Diffferential Thermal Analysis/Impedance (DTA/ Zsinϕ) and lyomicroscopy determinations, identify that during the freezing step product temperature must be below –30 °C, while throughout the primary drying process product temperature at sublimation front cannot exceed –18.9 °C. The use of a procedure based on the critical variables determinations, allow the lyophilization of three industrial batches that finally fulfilled the quality specifications and achieve a reduction of 32 % in the cycle total time.

Keywords: lyophilization, L-leucyl-L-alanine, sublimation, electrothermal analysis, lyomicroscopy

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RESUMEN

Diseño del ciclo de liofilización de una formulación del dipéptido L-leucil-L-alanina basado en sus propiedades térmicas. El sistema para la detección de la fenilcetonuria (UMTEST® PKU) en recién nacidos, se produce en el Centro de Inmunoensayo (CIE, La Habana, Cuba). La L-leucil-L-alanina, uno de los componentes del diagnosticador, requiere ser liofilizado debido a su limitada estabilidad en solución acuosa. La utilización de las técnicas de Análisis Térmico Diferencial/Impedancia (ATD/Zsinϕ) y la liomicroscopía permitieron definir que en la liofilización de este reactivo, la congelación requiere ser realizada a temperaturas inferiores a –30 °C, mientras que en el secado primario la temperatura del frente de sublimación no puede sobrepasar el valor de temperatura de colapso (Tc) de –18.9 °C. La liofilización de tres lotes a escala industrial, ejecutada con el procedimiento diseñado según los resultados de las determinaciones previamente descritas, garantizó la obtención de un producto acorde a las especificaciones de calidad, con una reducción del 32 % en el tiempo total del ciclo.

Palabras clave: liofilización, L-leucil-L-alanina, análisis térmico diferencia, impedancia, liomicroscopía

*I*ntroduction

Phenylketonuria (PKU) is an inborn metabolic error manifested as an autosomic-inherited recessive monogenic disorder [1]. From the biochemical point of view, it leads to the accumulation of phenylalanine (Phe) in blood and other organic fluids and tissues, also slightly decreasing tyrosine levels [2]. PKU patients without control treatment could suffer from post-natal brain damage, further developing irreversible cognitive impairment, skin modifications and absence or difficulty of speech [3].

For these reasons, neonatal screening programs for the massive detection and treatment of hyperphenyalaninemias started as early as in 1961, with the Guthrie's Bacterial Inhibition test [4]. That assay was used in Cuba from 1983 to 2000, with an incidence of 1:50 000 inhabitants been detected [5]. A pilot program started that year for PKU screening using the UMTEST® PKU kit, which consists on a fluorescent ultramicroassay for the quantitative detection of Phe in dry blood on filter paper, by using the ultramicroanalytical system (SUMA®) developed at the Center of Immunoassays (CIE), Cuba [6]. The screening compound, L-Leucyl-L-alanine, requires to be lyophilized to guarantee its two-year validity.

Lyophilization is a freeze-drying operation commonly used for the long-term stabilization of substances unstable in water and sensitive to high temperatures. It occurs in three main steps: freezing, sublimation or primary drying, and desorption or secondary drying [7]. During freezing, most of the water in the product is turned into ice, practically achieving the complete immobilization of the system. The microstructure formed during this step determines mostly the final quality of the material and lyophilizate structure also. Ice is further eliminated by sublimation during primary drying [8]. This step requires the decrease of the chamber pressure below the ice vapor pressure (which is temperature-dependent) in the sublimation front [9]. The water remaining in the structure at the end of the sublimation step (5-20 %) is eliminated during the secondary drying step. This last is applied at temperatures higher enough to guarantee water desorption from the structure, but without compromising the integrity of the product [10, 11].

One of the major challenges during lyophilization resides on shortening the process' duration without compromising the quality of the final product [12-16]. The appropriate characterization of the formulation of interest is a key step prior to freeze-drying cycle design [17, 18]. The knowledge on the temperature of glass transition (Tg') or the eutectic temperature (Teu) determines the freezing temperature value of the product

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required to achieve the complete solidification of the matrix. At the same time, the collapse temperature (T_C) for each formulation determines the operational parameters during primary drying [19], and the proper characterization of the formulation facilitates the execution of a safe and efficient process [20].

There are several analytical tools available to accomplish such characterization. However, all of them have advantages and limitations, their combination being essential to accomplish reliable lyophilization rates [21]. Differential scanning calorimetry (DSC) and Differential thermal analysis (DTA) take advantage of any physical change occurring in the structure of the lyophilized compound as related to either an exo or endothermic phenomenon [22]. Impedance determinations (Zsinφ) provide evidences on matrix mobility according to the temperature [23]. Lyomicroscopy allows the real time observation of the matrix at microscopic level during the entire process, being regarded as the genuine technique to establish the Tc of the structure [18, 23].

The proper combination of several analytical tools offhand for determining the critical temperature values guarantee the quality of the lyophilizate obtained [21, 24]. Hence, this work was aimed to design the lyophilization process of the L-Leucyl-L-alanine dipeptide. It started from results obtained for the characterization of critical variables of the formulation by using DTA/Zsinφ and lyomicroscopy techniques.

Materials and methods

Reagents, bottling materials and lyphilization equipment

The following reagents were used in the formulation of the L-Leucyl-L-alanine dipeptide: 10.12 g/L L-Leucyl-L-alanine (ApplyChem, Germany), 2 g/L Sodium azide (ApplyChem, Germany) and 30 g/L dextranase (9000-11 000 g/mol; Sigma Aldrich, Denmark). Bottling materials were clear tubular glass vials GL 14 × 2.5-3 mL (MG, Germany) and GL 14 rubber caps (West, Germany).

Materials were lyophilized in a Usifroid SMH-200 equipment (Usifroid, France) using the following technical specifications: 1 m^3 chamber volume, 6 shelfs, 3 m^2 total shelf area, condenser with 40 kg of ice capacity, Pt100 product temperature sensors and a Pirani Edwards APG-M-NW16 vacuum-meter chamber pressure gauge.

Electrothermal analysis (DTA/Zsinϕ)

The following reagents were used in the formulation of the L-Leucyl-L-alanine dipeptide: 10.12 g/L L-Leucyl-L-alanine (ApplyChem, Germany), 2 g/L Sodium azide (ApplyChem, Germany) and 30 g/L dextranase (9000-11 000 g/mol; Sigma Aldrich, Denmark). Bottling materials were clear tubular glass vials GL 14 × 2.5-5 mL (MG, Germany) and GL 14 rubber caps (West, Germany).

DTA and Zsinφ analysis were carried out simultaneously in a Lyotherm2 equipment (Biopharma Technology Ltda, Surrey, UK), bearing the Lyotherm2 as main component of the analytical system and the cooling/heating block in which the cuvettes containing the samples (L-Leucyl-L-alanine dipeptide) and the reference substance (distilled water) are placed with temperature (DTA) and impedance (Zsinφ) sensors. Measurements were made in 3 mL each, either for the samples or reference substance. Freezing was carried out at temperatures below 100 ºC by immersion of the block into liquid nitrogen. Once equilibrated the block, samples and reference substance temperatures, they were heated at 2 °C/min speed.

Determinations were made while heating. Data were acquired with the aid of the Lyotherm2 program (Biopharma Technology Ltda, Surrey, UK) and processed using Microsoft Excel 2010 (Microsoft Corporation, USA) for subsequent analysis.

Lyomicroscopy

A Lyostat3 lyomicroscope (BiopharmaTechnologyLtda, Surrey, UK) was used, with 2 µL of the L-leucil-L-alanine formulation. In the first step, the product was frozen at 3 °C/min speed until reaching –40 °C with 20 min of stabilization time. In the second step, the pressure dropped down to 0.01 mBar and the product was heated at 1 °C/min speed until reaching –10 °C. The reported Tc value coincided with the temperature at which significant changes in the structure of the substance were detected as starting to occur.

Determination of residual moisture level

The residual moisture level was determined by using the Karl Fisher technique with the aid of a Mettler Toledo V30 (Greifensee, Switzerland) equipment. For that purpose, six vials were randomly selected (one per shelf). The procedure was carried out by titration using methanol (Riedel de Haen, Germany) and Hydranal-Composite 5 (Riedel de Haen, Germany) reagents.

Equipment and accessories of SUMA® technology for the UMTEST® PKU

L-leucyl-L-alanine lyophilizate batches were certified using a batch previously certified by the UMTEST® PKU test (Center of Immunoassay, Cuba) as standard. Equipment and devices were: manual punch device P-51 and fluorimeter-photometer reader PR-521 (Center of Immunoassay, Cuba), an oven (Memmert, Germany) and the Strip Reader software, SRS, version 9.0 (Center of Immunoassay, Cuba).

Methodology for designing the lyophilization process

The freezing and sublimation safety (T_{SP-S}) temperatures were determined according to the results obtained in characterization studies (DTA/Zsinφ and lyomicroscopy). The T_{SP-S} was set at approximately 3 ºC below the previously determined T_C value [8, 23].
The shelf temperature (T_S) and the chamber pressure (P_C; mBar) remain steady during primary drying. Since the equipment has no temperature manometric measurement (MTM) device, the temperature of the shelf was set to a value similar to Tc. This was intended to guarantee that the Tc value could not be surpassed during primary drying. P_C was calculated according to equation 1 and the set value was controlled by applying a calibrated injection of dry air into the chamber. Product temperature (T_P) was substituted by T_{SP-S} to obtain an optimal pressure value able to guarantee the safety and efficiency of the process [8, 25, 26].

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$$Pc = 1.333 \times (0.29 \times 10^{0.019\,Tp}) \quad [1]$$

The secondary drying step was established by heating the shelf at a 0.4 °C/min rate until reaching 30 ºC, that value maintained until the temperature of all products surpassed 10 °C. Subsequently, shelf temperature was increased to 37 ºC, based on results obtained in previous studies of accelerated stability of the lyophilizate (7 days, 37 ± 2 °C). No significant alterations were detected in the performance of the screening system (data not shown). The chamber pressure was not controlled during this step.

Industrial-scale lyophilization of the L-Leucyl-L-alanine dipeptide component

The batch subjected to lyophilization comprised 7600 vials distributed in 22 trays, accounting for about 85 % of the Usifroid SMH-200 lyophilizer capacity. Vials were filled with 0.5 mL each. The three Pt100 temperature sensors were placed in vials located in different shelfs. The acceptance criteria followed to demonstrate the validity and effectiveness of the designed lyophilization procedures consisted on obtaining three consecutive batches fulfilling quality specifications.

Results and discussion

Industrial-scale lyophilization of the L-Leucyl-L-alanine dipeptide component

The results of impedance (Zsinφ) determinations of the L-Leucyl-L-alanine formulation are shown in figure 1. The negative inflexion in T_1 temperature values (–40.32 °C) demonstrated the destabilization of the matrix, a phenomenon that seems to have no influence on lyophilization parameters. This view was reinforced by the tendency to increase in temperature values immediately after reaching T_2 (–36.29 °C) due to the recrystallization of any of the formulation components. Since T_3 (–22.86 °C), the temperature values steadily decreased, indicating that the interstitial mobility increases until the structure collapse is reached at temperatures above T_3. The minimal impedance value was –3.37 ºC, as associated to the fusion of ice crystals.

Results of DTA analysis (Figure 2) show that at T_1 (–24.5 ºC) starts an endothermic pattern indicative of phenomena related to the destabilization of the frozen matrix. The perfectly defined endothermic peak initiated at T_2 (–8.2 ºC) indicates the start of the fusion of ice crystals.

Tc was determined with the aid of the lyomicroscopy technique of an approximate value of –18.9 °C for the formulation under study. Some of the areas where the start of structural morphology modifications was observed are shown in Figure 3. Such a phenomenon resulted from the increase in water mobility at the matrix interstitial region [18, 19].

Structure collapse was detected at temperatures higher than T_3 (Figure 1) and T_1 (Figure 2), which is plausible since such values correspond to incipient destabilizations of the frozen matrix. The vitreous transition matrix (Tg') is the value at which the frozen

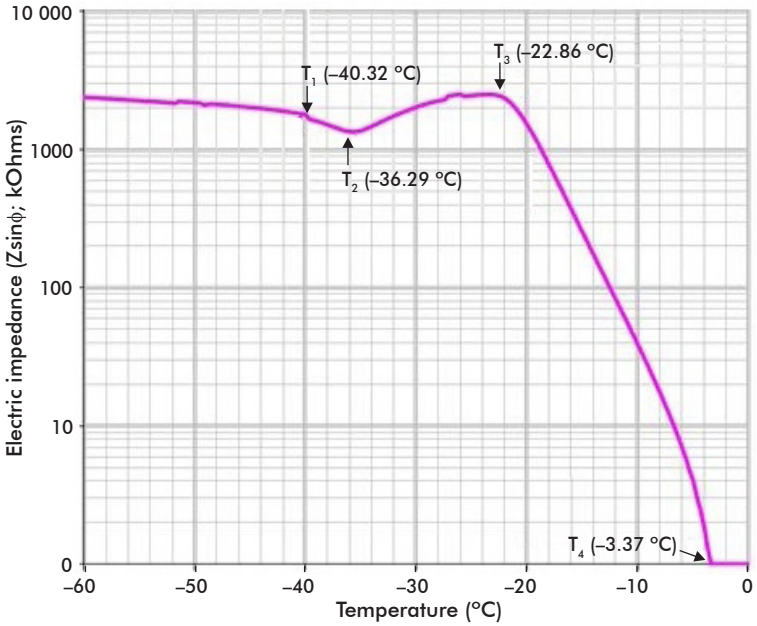

Figure 1. Impedance (Zsinϕ) of the L-leucyl-L-alanine dipeptide formulation. Samples were cooled down to temperatures below –100 °C and immediately heated until room temperature at a rate of 2 °C/min. T_1 and T_3: temperatures of matrix destabilization; T_2: recrystalization temperature; T_4: temperature of ice crystals fusion.

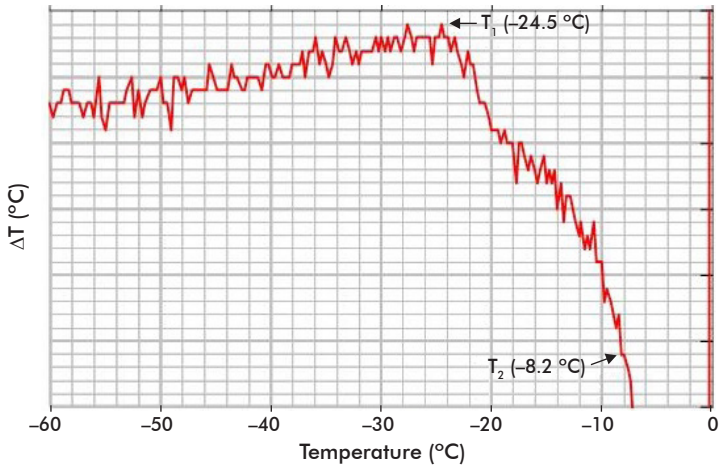

Figure 2. Differential termal analysis (DTA) curve of the L-leucyl-L-alanine curve. Samples were cooled down below –100 °C, and subsequently heated until room temperature at a rate of 2 °C/min. T_1: matrix destabilization tempersature. T_2: temperature of ice crystals fusion.

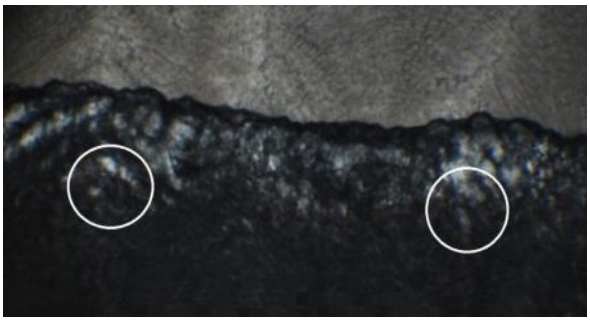

Figure 3. Determination of collapse temperature (T_C) of the L-leucyl-L-alanine formulate, by lyomicroscopy technique. Sample frozen at –40 °C (3 °C/min), followed by heating to –10 °C (1 °C/min) at 0.01 mBar pressure. White circles indicate the area where the structure started to collapse. T_C onset: –18.9 °C.

matrix progressively softens due to the increased mobility in the interstitial zone [8, 27]. The Tg' value is of the same magnitude, being within the range as reported in the literature in respect to T_C [18, 23], considering that DTA graph shows the start of an endothermic profile at T_1 (–24.5 ºC) differing in 5 ºC from Tc. This results need to be confirmed by using the DSC technique.

Design of the lyophilization procedure for the L-Leucyl-L-alanine component

Taking into account the previously discussed results, the freezing temperature of the product was set to –30 ºC, with a margin of 11.1 ºC below Tc. The shelf starting temperature (TS) was set to –45 ºC, with a cryogenic decrease speed of 0.6 ºC/min (maximum capacity of the refrigeration equipment). When the three temperature sensors of the product reached values below –30 ºC, the shelf temperature was increased up to –36 ºC to stabilize the products in the desired range of temperatures. This was followed by a 1-h stabilization period (the liquid column reference height in the vial lower than 10 mm) prior sublimation start [7, 12].

The shelf temperature was set to –19 ºC during primary drying, in order to avoid surpassing the previously established T_C value. In fact, as shown in figure 4, along this step the temperature of the products remained below the $T_{SP\text{-}S}$ (–22 ºC). The chamber pressure was calculated from Eq. 1, being 0.148 mBar, a value kept within the range 0.140-0.160 mBar by injecting dry air in the chamber. The end of primary drying was determined by detecting the change in the slope of the curve of temperature values, recording it by product sensors, which were close to the shelf temperature values. Additionally, the injection of dry air was stopped and Pc was checked together with the condenser temperature (T_{COND}) to decrease to the usual minimal values during this step (Pc < 0.05 mBar and T_{COND} < –70 ºC).

Secondary drying was carried out at a shelf final temperature of 37 ºC. PC and T_{COND} monitoring values were set as primary criteria to determine the end of this lyophilization step. Once P_C values were below 0.03 mBar and T_{COND} below –70 °C, the chamber pressure increase test was done at a 1-h interval, and the process was regarded as complete once the pressure difference was lower than 0.01 mBar/min.

The knowledge acquired on the critical variables associated to the design of the lyophilization cycle supported the increase of the freezing temperature in 5 ºC, and therefore, the shortening of this step duration (Table 1). Shelf values increased during primary drying reaching –19 ºC, as well as chamber pressure to 0.148 mBar, all these increasing the drying kinetics.

The duration of the lyophilization cycle (Table 2), as designed attending to determinations of formulation critical variables, was of 7.7 h, 32 % shorter than the duration of the previous process established by the "trial-and-error" method. This supports an increase in the production capacity, making able to run four processes (30 400 vials) in the same time of four traditional ones (22 800 vials).

Product evaluation

Batches lyophilized using the newly established parameters complied with quality specifications (Table 3). The organoleptic appearance of the lyophilizate showed white pills of regular edges and structures devoid of cavities or deformations, these last associated to alterations during the lyophilization process when present. The pills dissolved immediately, leading to a clear solution lacking mechanical particles. The residual moisture was lower than 3 % in all the cases. The marker performance parameters of the component in the screening device (fluorescence calibrators A and F) were within the established limits.

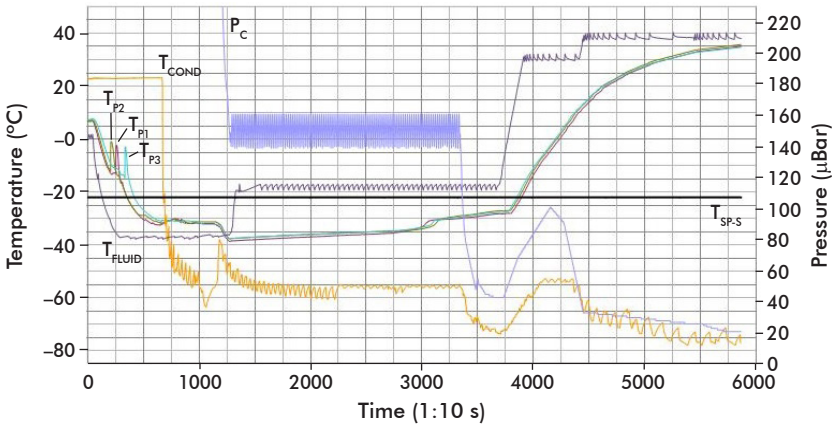

Figure 4. Lyophilization process of the L-leucyl-L-alanine dipeptide. T_{P1}, T_{P2}, T_{P3}: Product temperatures 1 to 3. T_{COND}: Temperature of the condenser. T_{FLUID}: Temperature of the fluid. $T_{SP\text{-}S}$: Safety temperature. P_C: Chamber temperature.

Table 1. Parameters of the lyophilization procedure of the L-Leucyl-L-alanine dipeptide

Lyophilization process	Steps and parameters						
	Freezing		Sublimation			Desorption	
	Freezing temperature (ºC)	Stabilization time (h)	Shelf temperature (ºC)	Pressure (mBar)	Safety temperature (ºC)	Shelf temperature (ºC)	Pressure (mBar)
Previous process	–35	2	–30	0.050	–30	30	< 0.05
New process	–30	1	–19	0.148	–22	37	< 0.05
Difference	5	1	11	0.098	8	7	–

Table 2. Cycle times of the lyophilization process of the L-Leucyl-L-alanine dipeptide at industrial scale

Lyophilization process	Cycle times (h)			Whole cycle
	Freezing	Sublimation	Desorption	
Previous process	2.8	7.7	6.0	16.5
New process	4.1	11.1	9.0	24.2
Difference	1.3	3.4	3.0	7.7

Table 3. Quality specifications for industrial-scale lyophilizate batches of the L-Leucyl-L-alanine dipeptide

Batch	Organoleptic properties of the reconstituted solution	Color change (%)	Residual moisture (%)	Fluorescence (FU)	
				Calibrator A	Calibrator F
1	Comply	0	2.9	7.2	115.7
2	Comply	0	2.2	12.2	136.5
3	Comply	0	2.6	6.1	124.1
Acceptance limits	Comply	0	≤ 4	≤ 20	100-150

Conclusions

The results of the combination of DTA/Zsinφ and lyomicroscopy techniques indicate that freezing has to be

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carried out at temperatures below –30 ºC and sublimation up to –18.9 ºC, for the correct liophilization of the L-Leucyl-L-alanine dipeptide. The batches obtained with the new lyophilization procedure complied with quality specifications, achieving a 32 % reduction in the overall cycle duration.

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