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Low-Temperature Micronization of a Peptide Drug in Fluid Propellant : Case Study Cetrorelix

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ABSTRACT Aim of this study was to elaborate an efficient method for the micronization of the decapeptide cetrorelix (a GnRH-antagonist), in order to obtain a microsuspension as basis for other pharmaceutical preparations, such as e.g. inhalation aerosols. A modified pearl-mill coupled with a cryostat was used for the micronization of cetrorelix in fluid propellant and operated under different conditions. The obtained cetrorelix suspensions were analyzed for particle size distribution, purity of cetrorelix, and for metal contamination through abrasion from parts of the mill. The method allowed an effective micronization of cetrorelix . The mean particle size of the initial cetrorelix lyophilizate bulk ware was reduced from 52.5 µm (Volume Mean Diameter, VMD) down to 14.9, 6.1 and 3.1 µm, respectively, respectively. The HPLC analysis of all cetrorelix suspensions after micronization did not show signs of decomposition as compared to the initial product. The elementary analysis of the suspensions performed by inductively coupled plasma mass spectrometry revealed a negligible amount of contaminants in the suspension (Zr = max. 0.6 ppm; Fe, Cr, Ni, Ba, below limit of quantification, i.e. < 0.14 ppm). The only appreciable contaminant, Aluminum (Al = 1.1 ppm), was derived from the mechanical capping of aluminum canisters prior to analysis. The Zr determination in the suspension of 0.6 ppm, is still considered to be negligible as compared to the legally tolerated limit of air contamination. By low-temperature micronization in fluid propellant, fine drug suspensions of cetrorelix for pMDIs can be directly manufactured in one-step procedure without destruction of the peptide structure and without appreciable product contamination.

KeyWords: Peptide delivery; pearl mill; aerosol; inhalators; systemic pulmonary delivery.

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INTRODUCTION

Micronization is one of the most important techniques for ameliorating the formulation characteristics of certain drugs. This is particularly important for substances with low solubility and/or low absorption rate at the application site, but also to reach a particle size range suitable for pulmonary deposition of pharmaceutical aerosols. Unfortunately, the micronization of peptide and protein powders is often limited by the sensitive nature these compounds against the relatively harsh processing conditions. This is particularly true for preparing micronized powders, where a small mean particle size (e.g. < 5 µm) and a narrow size distribution are pivotal [1, 2]. Spray drying and jet milling are probably the most popular methods of micronization but they are not completely satisfactory with respect to the stability of these substances: oxidation, degradation, aggregation, electrostatic charges, cohesion and adhesion problems have to be considered using these techniques [3]. Supercritical fluid antisolvent and spray freeze drying have been recently investigated for producing micronized (or microencapsuled) powder [2], but practical applications have yet not reached the market. An alternative method for overcoming the above mentioned technical difficulties and safety hazards connected to the micronization of biotherapeutics has been described by Adjei et al. [4, 5] and describes the preparation of protein/peptide suspensions for pMDIs. For example, Adjei performed a liquid milling of the nonapeptide leuprolide (a GnRH-agonist) direct in fluid propellant (trichlorofluoromethane and/or dichlorodifluoromethane) at approximately -20°C using a pearl mill (Dyno Mill) containing glass or tungsten pearls. Although microsuspensions of

peptide drugs obtained using this method have already been patented for pMDIs [4, 5], particle size reduction and contamination through abrasion of the tungsten or glass pearl have not adequately been described. In order to investigate this aspect and to further improve this technique we performed a series of micronization experiments using a decapeptide drug (cetrorelix) in a fluid propellant by an abrasion-resistant pearl mill.

The new milling system described here, allowed the micronization in heptafluoropropane at low temperatures (down to -50°C), for direct manufacturing of fine drug suspensions which may be used as basis for other pharmaceutical preparations, such as for pressurized metered dose inhalers (pMDIs), but also for solid dosage forms in which a micronised material is required. The study was conducted to demonstrate the applicability and the effectiveness of this technology in the pharmaceutical field. Particular attention was focused on the reduction in particle size, stability of the peptide (cetrorelix) and on contamination of the final product.

MATERIALS AND METHODS

Materials

Cetrorelix acetate (in ths article simply referred to as cetrorelix) was furnished in the form of a lyophilized powder from Degussa-Hüls AG (Hanau, Germany). Solkane® R227 (Heptafluoropropane, HFA 227) used as milling and suspension medium was purchased from Solvay Fluor und Derivate GmbH (Hannover, Germany). Cyclohexane and Span® 85 (Sorbitan trioleate) were used for the measurement of the particle size distribution, as well as saccharose, polyoxyethylene-25 glyceroltrioleate (Tagat® TO; Goldschmidt AG, Essen, Germany). Anhydrous ethanolas an additional excipient for formulating the cetrorelix suspensions, and denatured ethanol 96%, used as coolant, were purchased from Merck Eurolab GmbH (Darmstadt, Germany). The 17 ml aluminum container (cans) were purchased from 3M Neotechnic Ltd. (Lancashire, UK) Acetonitrile, water and trifluoroacetic acid used as mobile phase for HPLC analyses were purchased from Merck Eurolab GmbH (Darmstadt, Germany).

Apparatus

A pearl-mill "Dispermat® SL-C 12" was specifically modified by VMA-Getzmann GmbH (Reichshof, Germany). Principal modifications concerned the milling chamber, which was provided with abrasion-resistant materials, such as iridium-stabilized ZrO_2 (ZrO_2-Ir), the pearls consisting of ZrO_2-Ir (pearls diameter = 1.1 mm and 0.6 mm), and the positioning of a three way valve for sample collection on the reflux tube connecting the milling chamber with the reservoir (Figure 1).

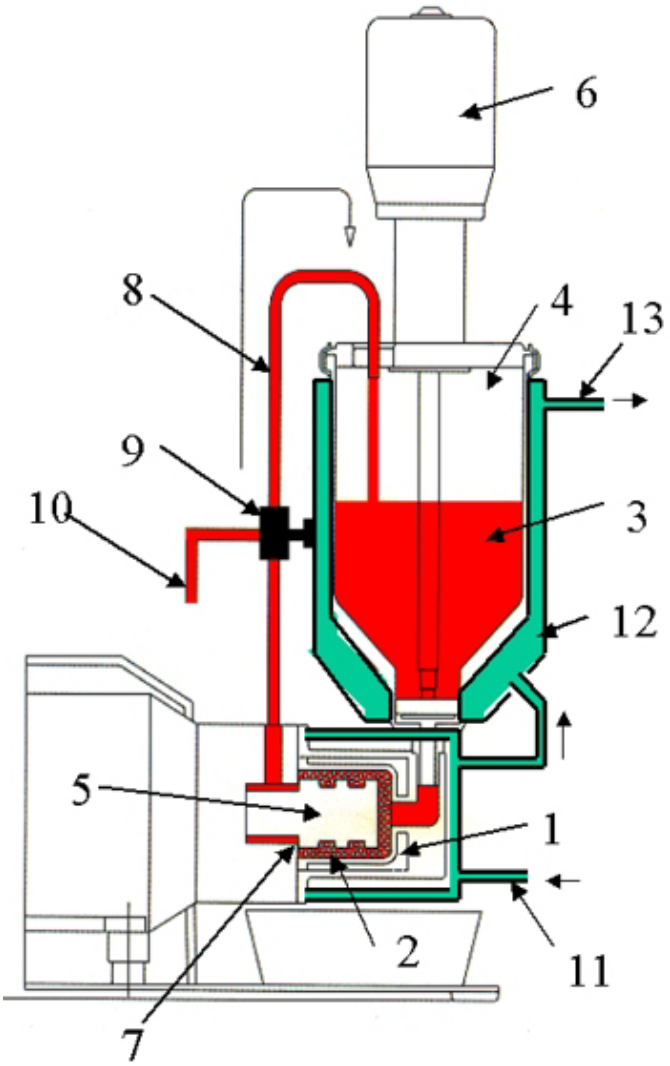

Figure 1. Schematic section of the pearl mill from VMA-Getzmann GmbH showing: 1) the milling chamber; 2) the rotating/milling pearls; 3) the drug suspension in 4) the reservoir; 5) the rotor; 6) the centrifugal pump; 7) the split sieve; 8) the suspension reflux; 9) the three-way-valve; 10) the filling tube for samples; 11) the refrigerant inlet; 12) the cooling jacket and 13) the refrigerant outlet. (used with permission).

A standard cryostat (N8-KT50W) for temperatures down to -50 °C (Gebrüder HAAKE GmbH, Karlsruhe, Germany) was coupled with the pearl-mill for the first two experiments, while a modified cryostat (N8-KT90W) for temperatures down to -90°C, supplemented with a special centrifugal pump, capable to operate up to 2.5 bar, was used in one particular experiment. An UltraTurrax® from IKA Werke, Janke & Kunkel GmbH & Co KG (Staufen, Germany) was used for premixing the cetrorelix suspension-concentrates. A Microscope Laborlux D (Leitz GmbH, Wetzlar, Germany) was used for monitoring the particle size reduction during the milling process. A laser diffractometer Malvern Mastersizer® X from Malvern Instruments GmbH (Herrenberg, Germany) with a small volume sample dispersion unit and a 45 mm lens was used for measuring particle sizes from 0.1 up to 80 µm. An ultrasonic bath (Bandelin electronic, Berlin, Germany) was used for removing the HFA 227 from the cetrorelix/cyclohexane suspensions prior to measuring the particle size distribution by Mastersizer® X. The content and impurities of cetrorelix were determined using a reversed phase HPLC system (Hewlett Packard, Avondale, USA) consisting of a binary pumping system for gradient analy-ses, a vacuum degaser, an autosampler, a diode array detector, an RP-18-column Nucleosil 120-3 (Macherey & Nagel, Düren, Germany) and the chromatography software ChemStation (Hewlet Packard, Avondale, USA). The two mobile phases used for the HPLC gradient analysis consisted of 970 ml purified water/30 ml acetonitrile and 1 ml trifluoroacetic acid (phase A) and 300 ml purified water/700 ml acetonitrile and 1 ml trifluoroacetic acid (phase B).

Methods

Micronization

Three experiments were performed to micronize cetrorelix. Two experiments were performed coupling the "normal" cryostat to the pearl-mill and the third by coupling the "modified"-one. As shown in Figure 1, the pearl mill is equipped with a horizontal milling chamber and a stainless steel reservoir positioned directly on the top of the milling chamber. Both parts are provided with a cooling jacket, each, in which the cooling fluid circulates. The suspension in the reservoir flows by means of an incorporated centrifugal pump directly into the milling chamber, where the particles are micronized by impaction through the rotating pearls. The suspension flows then through a slit sieve (of 0.2 mm) back to the reservoir for a new cycle. The milling chamber and reservoir are provided with a supplementary insulating layer consisting of foam rubber.

Immediately before each micronization experiment, the milling chamber was completely filled with 100 mL ZrO_2-Ir pearls (diameter of 1.1 mm for processes A and B; 0.6 mm for process C), and both the reservoir and the milling chamber were cooled down to -44°C with the normal cryostat (process A and B). The micronization process in the three experiments was performed as follows:

Process A. 1.1 g cetrorelix, 1.1 g ethanol 100% and 1.1 g Tagat® TO were pre-suspended in approx. 30 ml HFA 227 using an UltraTurrax® (5 min, 15000 rpm) in a cooling bath of dry ice/ethanol 96%. This suspension was then filled into the mill, in which 260 ml of HFA 227 have been previously filled. The centrifugal pump was started, the milling rotor speed was turned up to 2150 rpm and a mechanical power input of 56 W was measured on the device display. The operating mode of constant power input was selected and maintained throughout the duration of the experiment. Under these conditions, the temperature of the suspension (measured in the reflux line) reached values between -33 and -30°C. The milling procedure was monitored by sampling the cetrorelix suspension and observing the particle size reduction using the light microscope. After 30 min the cetrorelix suspension (Susp. A) was filled into 17 ml aluminum cans directly from the three way valve of the reflux tube. The cans were then closed using a semi-automatic lock system "Pamasol Crimp Station" from Willi Mäder AG, Pfäffikon, Switzerland.

Process B. For the second experiment 3.3 g cetrorelix, 3.3 g saccharose, 2.1 g TagatÒ TO and 2.1 g ethanol 100% were suspended in approx. 30 ml HFA 227 using an UltraTurrax® (5 min, 15000 rpm) in a dry ice/ethanol 96% bath.

The suspension was then filled into the milling system, in which 258 ml HFA 227 were previously filled. The milling process was performed using the

operative parameters described in the first experiment. In this case the mechanical power input transmitted by the rotor to the product was 62.5 W. After milling the suspension (Susp. B) was filled into 17 ml aluminum cans, which were closed as reported previously.

Process C. The third experiment was performed connecting the more powerful cryostat to the pearl-mill. The isolated pearl-mill was filled with 100 ml ZrO_2-Ir stabilized pearls (diameter = 0.6 mm). The system was cooled down to -60°C (coolant temperature) and a predispersed suspension of 5 g cetrorelix in 243 ml HFA 227 was added into the reservoir of the pearl-mill. The rotor speed was elevated within 5 min, up to 5000 rpm (516 W), while the temperature of the suspension reached -42°C After 15 min the process was stopped. The temperature of the suspension at this time had reached a value of -30°C. The obtained suspension (Susp. C) was directly filled into aluminum cans as reported previously.

Particle-size analysis

In order to determine the particle size distribution of the cetrorelix suspensions, aluminum cans from each process were first cooled to approx. -40°C in a dry ice/ethanol 96% bath, and then opened with a pipe cutter (MS-TC-308, Swagelok, Solon, OH). The suspension was added to 50 ml cyclohexane/Span 85 solution (previously cooled to -20°C) and, under stirring for about 15 minutes, the HFA 227 was evaporated. The newly obtained suspension was taken out of the cooling bath and the temperature was increased to approx. 15°C, then the particle size was measured with the Mastersizer®. The particle size distribution and the volume mean diameter were determined by interpolating the distribution frequency of all particle size classes (measurement data were given as histogram plot) and by constructing an undersize cumulative plot, respectively.

Determination of cetrorelix amount and of degradation products

In order to check for some possible degradation of cetrorelix resulting from the milling process, the content of cetrorelix in the suspensions and of its impurities were determined by reversed phase HPLC. For the analysis, three canisters from each procedure, were cooled, opened and the content of each canister was transferred into a 500 ml volumetric flask containing 100 ml of the cooled mobile phase B solvent mixture. The mixture was first stirred for approximately 60 min and then immersed in an ultrasonic bath for about 5 min until the HFA 227 was eliminated. The flask was then exactly filled up with the mobile phase B at ambient temperature. HPLC analysis was performed at a flow rate of 1 ml/min operating in a progressive gradient of the mobile phase B at 45°C, as follows:

Minute	0	25	27	31	35	40
% of B	30	55	100	100	30	30

Detection was performed at a wave length of 226 nm. The content of cetrorelix per can was determined by first calculating the volume of suspension (per weight difference full vs empty) and then determining the concentration of cetrorelix in the test solution using the external standard solution for carrying out the calculation. The results were expressed as percentage of the theoretical value of concentration. The determination of the cetrorelix purity after micronization was carried out by calculating the peak area of all impurities and expressing them as percentage against the cetrorelix peak area. The obtained results were then compared with the impurity content of the bulk material.

Assay of process contaminants: Contents of metals

During the milling process the cetrorelix suspensions were in contact with various materials like stainless steel and ZrO_2-Ir. To show an eventual contamination deriving from the abrasion of these materials, inorganic elements such as Zr, Fe, Cr, Ni were determined in Susp. B by inductive coupled plasma mass spectrometry (PQ2Plus from TJA-Solutions, Offenbach, Germany), while only the content of Zr was determined in Susp. C. The content of Ir was not determined because it is present in low percentage in the ZrO_2- structure as stabilizer.

The analysis were performed externally at Infracor GmbH (Degussa-Hüls Group, Hanau, Germany) as follows: A sample of Susp. B and C, respectively, was evaporated for removal of HFA 227 until a pasty substance was obtained. The substance was

decomposed using concentrated nitric acid in a microwave oven. The resulting residual substance was further heated with a mixture of sulfuric, nitric, and hydrofluoric acid in a platinum bowl until a dry powder was obtained. This residual powder was then dissolved in diluted nitric acid and used for measuring the metal contents. Additionally, the content of other metals in the Susp. B, such as Al, Ba, Cu, Ti, and Zn was assayed. The results of the analysis were expressed as part per million (ppm) of the residual powder and they have been recalculated as ppm of elements contained in the suspension for a better representation of the results.

RESULTS AND DISCUSSION

Particle-size reduction

The first micronization experiment allowed a particle size reduction from 52.5 µm to 14.9 µm (VMD) as shown by Mastersizer analysis (Figure 2). In this graphical representation, some bimodal distribution is apparent, which is probably due to an incomplete milling. Although the cumulative undersize distribution shows that 50% (d 0.5) of the measured particles were found below 6.7 µm, the value under 90% (d 0.9) was still too big (34.8 µm) and not yet completely satisfactory for use in inhalation aerosols [6]. Only 10% (d 0.1) of particles were found under 3.1 µm. Unfortunately, it was impossible to continue the milling process because of the formation of propellant bubbles in the reflux of the suspension. Obviously, the heat exchange between the milling chamber and the cooling fluid in the jacket was not adequate to control the local temperature spikes, produced during pearl impacting, below the boiling point of HFA 227 (-17°C).

To improve the milling process, a second experiment was performed using saccharose as milling adjuvant and a more concentrated suspension. In this manner, using the same operative parameters, a more effective particle size reduction was achieved. It is important to note that the particle size distribution resulting from this process is relative to a mixture 1:1 of cetrorelix and saccharose. As already demonstrated by other micronization techniques, such as jet milling or spray dry {7, 8], the addiction of crystalline, friable powder as milling adjuvant to an amorphous substance (such as cetrorelix) leads to a higher efficiency of the process and therefore to a smaller mean particle size

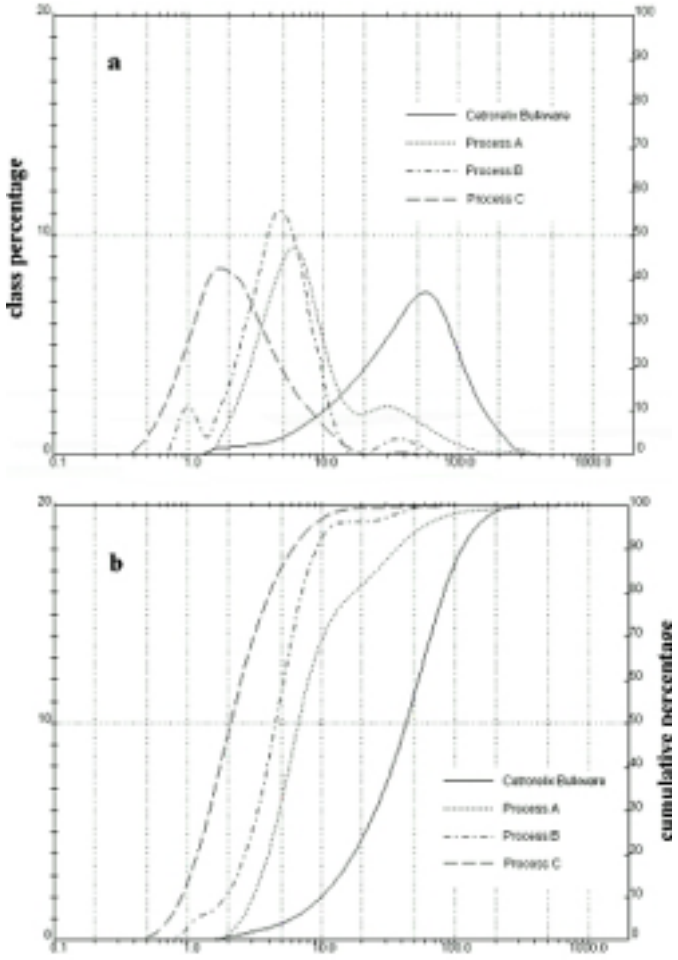

Figure 2. Particle size distribution of the cetrorelix suspensions produced from process A, B, and C, as compared to the initial cetrorelix bulk ware in a class frequency distribution (graphic a) and in an undersize cumulative plot (graphic b).

of the micronized product. In this experiment the smaller mean particle size is evident by a VMD of 6.1 µm. The corresponding values for d 0.9, d 0.5, and d 0.1 were 9.2, 4.6, and 1.9 µm, respectively.

Both formerly described experiments were performed by selecting the operative parameters in the middle range of the pearl mill because the cooling power of the normal cryostat did not allow an adequate heat exchange between milling chamber and coolant, and therefore did not allow the use of a bigger shear stress (i.e. higher speed of rotation) or a longer milling time. For this reason, a third experiment was performed with a more powerful cryostat supplemented with a more powerful pump and capable of generating a turbulent flow of coolant within the refrigerating jackets of the system. In this manner it was possible to increase the shear stress in the milling chamber within a grinding time of 20 minutes and without use of

adjuvants, to obtain a very fine mean particle size. The VMD of cetrorelix particles achieved a value of 3.1 µm, while the values of d 0.9, d 0.5 and d 0.1 were 6.1, 2.1, and 0.9 µm, respectively (Figure 2). The size reduction mechanism is primarily through impaction with the rotating pearls. The milling principle regulating the efficiency of the micronization depends from various factors, such as physico-chemical characteristics of the substance and of the suspending medium; temperature; dimension, nature and rotational speed of the pearls; and finally dimension and geometry of the rotor and of the milling chamber. The influence of all these factors has been well elucidated [9] and a simple mathematical description for practical application has been given [10]. In conclusion, the mechanical power input into the dispersed material is closely connected with the micronization result. It defines the energy which is transmitted by the rotor via the grinding pearls to the product. The power input "P" can be defined by the rotational speed "n" of the rotor and its torque "M" by the following equation:

$$P = 2\pi nM$$

Where:
P = power [Nm/s = J/s = W]
π = 3.1415
n = speed
M = torque [Nm]

In case of a set suspension type, pearl charge and dispersion time, the dispersion result will depend only on the value of the mechanical power input [10].

The pearl mill used in our study allowed to operate in a "constant power input" mode: the integrating motor of the device continuously measures the torque absorbed by the product and the speed of the rotor. The values of these parameters are displayed and can be monitored for all the duration of the experiment. The "constant power input" mode is achieved by automatic adaptation of these two parameters in such a way that the mathematical product of their values leads exactly to the pre-selected mechanical power. In this operating mode, even complicated micronization processes can be performed in a reproducible way, and formulation processes worked out in the laboratory can be easily transferred into production.

Amount of cetrorelix and grade of degradation

The amount of cetrorelix in each canister of the Susp. A, B, and C, determined by HPLC using the external standard was 59.4, 175.5 and 112.1 mg, respectively, which correspond to 115.8%, 107.2% and 101.3% of the theoretical concentration of cetrorelix as determined immediately after micronization for the Susp. A (3.8 mg/ml), B (11.9 mg/ml), and C (8.2 mg/ml), respectively. Probably, the increased concentration of cetrorelix in the final product is due to evaporation of HFA 227 during the filling into canisters. However, these data are indicative for a complete recovery of cetrorelix. The HPLC analysis of the test solutions obtained from the three suspensions did not show any appreciable sign of decomposition as compared to the initial product. The total sum of the impurities detected in the cetrorelix suspensions after micronization were 1.17 % for the Susp. A, 0.89 % for the Susp. B and 0.35 % for the Susp. C. Similarly, the content of impurities present in the bulk material averaged 0.15 % . Although a very small increase in the impurity content is shown in all suspensions, this can be considered negligible as compared with other procedures of micronization and/or processing of peptides and proteins [3]. In previously performed pilot experiments involving Jet milling and spray drying, we noted an elevated cohesion/adhesion of the micronised particles and a high degradation rate of cetrorelix, respectively (data not shown). It is important to remember that, in contrast to the Susp. C, the other two suspensions contained excipients, such as Tagat TO and ethanol (Susp. A and B), required for stabilizing the suspensions; and saccharose (Susp. B), as milling adjuvant. These excipients may be responsible for an increased amount of impurities, as compared to the Susp. C. Moreover, the HPLC analysis of the suspensions obtained from process A and B have been performed after a storage time of more than 4 months at ambient temperature, giving an indication of the stability of the cetrorelix suspension in HFA 227.

Assay of process contaminants: Contents of metals

Because the milling procedure for the Susp. A and B were similar, the determination of eventual metal

contaminants deriving from the system was performed on the Susp. B and C, only. The analysis of contaminants in the Susp. B deriving from the static parts of the pearl mill, such as reservoir and conducting tube (all of stainless steal) were below the limit of quantification. Nevertheless, it was possible to give a qualitative indication. The amount of Fe was found to be below 0.14 ppm (or µg/g) of the suspension and other elements such as Cr, Ni, Ba, Cu, Ti, and Zn were below 0.054 ppm. Zr and Al were the only quantifiable elements in the Susp. B. The amount of Zr was 0.14 ppm and represents a measure of the abrasion in the milling chamber, while the Al contamination (1.1 ppm) was most probably derived from the mechanical capping of the canister for effectuating the analysis. Therefore, only the content of Zr was investigated in the Susp. C as unique indicative element of abrasion. The determination of this metal in the Susp. C revealed an increased contamination of 0.6 ppm (6 µg Zr /g of suspension) whiwas, however, still negligible as compared, to the tolerated limit of air contamination for Zr (5 mg/m^3) reported by the German Federal Department of Work and Social Order [11].

CONCLUSION

The low-temperature micronization of cetrorelix in fluid propellant by the described modified pearl mill yielded an efficient particle size reduction, especially when coupled with an efficient cryostat, down to a mean particle size of 3.1 µm (VMD) which would appear suitable for inhalation aerosols.. The selected conditions and modifications of the process allowed to obtain suitable suspensions of cetrorelix in HFA 227 to be prepared with a pharmaceutical grade of purity. Thus, the absence of degradation products or of contaminants deriving from the process may enable this method to be used as an one step-procedure for the production of micro-suspension for pharmaceutical applications. Moreover, the possibility offered by the system for measuring the milling parameters, such as rotor speed and torque, and the possibility to operate at constant power input guarantees the reproducibility of the experiments and the easy up-scaling of the results obtained in the laboratory.

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