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Laser micronization of beclomethasone dipropionate: proof-of-concept for pulmonary drug discovery

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Abstract – In this work, we used femto- and nanosecond lasers to produce micron-sized particles of beclomethasone dipropionate (BDP) in water. Laser-micronized BDP particles were compared with BDP particles micronized by traditional milling methods in terms of particle size, degradation and physicochemical properties. The results showed that chemical degradation and partial amorphization might be induced during laser process, but these effects are reasonable and are similar compared to the standard methods. Laser micronization is thus a suitable tool for pharmaceutical R&D, especially for pulmonary drug discovery.

Keywords**:** laser fragmentation, particle-size reduction, pulmonary drug, drug discovery, beclomethasone dipropionate, femtosecond laser, nanosecond laser.

1. Introduction

Since its first introduction (Patil et al., 1987), laser fragmentation in liquid has become a well-established technique to produce nanoparticles (Besner and Meunier, 2010). However, it has been mainly used to produce inorganic nanoparticles, such as metals, alloys and semiconductors. In contrast, organic materials are fragile and have functional structures, so producing organic nanoparticles by laser is more challenging. So far, only a few organic nanoparticles have been produced with lasers (Tamaki et al., 2002; Jeon et al., 2007; Hobley et al., 2007; Asahi et al., 2008; Wagener and Barcikowski, 2010), and fewer works on laser-fabricated drug nanoparticles, including our previous studies on paclitaxel (Kenth et al., 2011) and megestrol acetate (Sylvestre et al., 2011), have been reported.

Laser fragmentation of drugs presents an advantage for drug discovery. In the pharmaceutical industry, reducing the particle size has long been a common practice for poorly water-soluble drugs. Smaller drug particles have a higher surface/volume ratio, which helps to increase their dissolution kinetics and improve their absorption in the body (Noyes and Whiteney, 1897; Mosharraf and Nyström, 1995; Rasenack et al., 2003). Several particle size-reduction methods, such as milling and homogenisation (Rabinow, 2004; Merisko-Liversidge et al., 2003; Keck and Müller, 2006), are mainly used for industrial production (in grams to kilograms). However, these methods are not applicable for drug discovery, as drug candidates at an early stage are available only in minute amount (milligrams). In contrary, laser fragmentation can be operated on small amount of drugs and is well adapted for drug discovery.

As a drug particle-size reduction tool, laser fragmentation presents also a limitation. The studies of Kenth et al. (2011) and Sylvestre et al. (2011) showed that both micron-sized (or "microparticles", 1~10 µm) and sub-micron-sized (or "nanoparticles", 400 nm ~ 900 nm) drug particles were successfully produced with lasers, but sub-micron-sized particles were more degraded (~ 10%) than microparticles (~

2%). From a pharmaceutical viewpoint, drug purity should be as high as possible to avoid drug toxicity and inefficiency. As a result, laser fragmentation seems to be suitable for drug micronization, but is less adequate for nanonization.

While most poorly water-soluble oral and intravenous drugs are best absorbed as nanoparticles, it is different for pulmonary drugs. Because of the lung's branch-like anatomy, particles between 0.5 and 5 μm are well deposed and absorbed in the lung. Particles bigger than 5 μm are often eliminated by the musco-cilary movements, whereas those smaller than 0.5 μm are easily exhaled (Taylor, 2007). Thus, it is of particular interest to evaluate the laser technique on a pulmonary drug. In this paper, we used beclomethasone dipropionate (BDP) as a pulmonary drug model. We investigated several laser conditions in order to produce BDP particles in the 0.5-5 μm range, evaluated the physicochemical properties of the laser-processed drug, and compared laser technique with the standard milling methods.

2. Materials and methods

Materials Beclomethasone Dipropionate (BDP) (> 99%) was purchased from Sigma-Aldrich. The methanol (HPLC grade) used for HPLC analyses was purchased from Fisher Scientific (Ottawa, ON, Canada). All aqueous solutions were prepared using deionised water (18.2 MΩ·cm) generated with a Millipore Milli-Q system (Bedford, MA).

Laser fragmentation Suspensions of the BDP were prepared at concentrations of 0.5 and 2 mg/mL in 2 or 10 mL of deionised water. After 30 min of sonication, laser fragmentation was performed by focusing a laser radiation on the magnetically stirred suspension. Femtosecond laser (120 fs, 800 nm, 1 kHz, Ti:sapphire Hurricane laser, Newport Corporation, power range: 50-400 mW) and nanosecond laser (5 ns, 1064 nm, 10 Hz, Nd:YAG Brilliant B laser, Quantel, Paris, France, power range: 1.5-5 W) were used. In all cases, the laser beam was focused by a lens with a focal length of 10 cm. The irradiation duration varied from 0.5 to 20 hours. BDP particles were fragmented by varying the following parameters: suspension concentration and volume, laser power, and radiation duration. Experiments were produced in triplicates.

Particle size and morphology The BDP particles' average size was first measured with dynamic light scattering (DLS) (Malvern Zetasizer NanoSeries) as a rapid screening method. BDP microsuspensions were then produced at selected conditions, and the more accurate particle size distribution was then measured with Laser Diffraction (LD) using the Horiba LA-950 particle size distribution analyzer (Horiba Instruments, Inc., Kyoto, Japan) equipped with a MiniFlow circulation system. The dispersant was water and the complex refractive index used for the drug particles was 1.55 - i0.1. For observation with scanning electron microscopy (SEM), a drop of the BDP suspension irradiated by laser was dried on a silicon substrate under vacuum, and observed with a FE-MEB S-4700 field-emission scanning electron microscope.

Degradation and content The drug purity and degradation was evaluated with high performance liquid chromatography (HPLC) (1100-LC, Agilent Technologies). Aliquots of the drug suspensions were diluted (10x) in methanol in order to dissolve all BDP. The analyses were conducted with a mobile phase of methanol and water (3:1, v:v) at 25°C. BDP retention time was 5.98 min and the wavelength of the detector was 242 nm. Chromatographic purity was calculated from relative ratio of the area under curve (AUC) of BDP peak and AUC of all peaks. For content assays, BDP was precisely weighted before treatment. Samples after laser fragmentation were entirely dissolved in methanol. The concentration of the samples was then measured by HPLC based on a concentration calibration line ($R^2 > 0.99$). The content was then calculated as the ratio of measured concentration and theoretical concentration, normalized with the average ratio of suspension controls (as 100%). Samples for content assays were performed in duplicata.

Physicochemical properties BDP suspensions were frozen at -80°C and dried under vacuum (< 100 x 10^{-3} bar) for 72 hours. BDP powders were then extracted from lyophilized samples for physicochemical characterization. The chemical composition was analyzed with Fourier transform infrared spectroscopy (FTIR) (FTS3000, Bio-Rad Laboratories) using the potassium bromide (KBr) pellet technique and with elemental analysis (EA) (ECS 4010, Costech). The cristallinity was evaluated with X-ray diffraction

(XRD) (X'Pert X-ray, PANalytical Inc.) and with differential scanning calorimetry (DSC) (DSC7, Perkin Elmer).

Jet milling and media milling For comparison with laser micronization, BDP powder was treated with Fluid Energy jet mill (Telford, PA) at 86 psi (air), 86 psi (grinding) and 77 psi (pusher). For XRD comparison, a nanosuspension of BDP was prepared using a roller mill. A 30-mL glass bottle was charged with 10 mL of 0.8 mm diameter zirconium oxide beads, 150 mg of the drug and 7.5 mL of an aqueous solution of poloxamer 188 (0.8% w/v). The bottle was rolled at 70 rpm for 72 h. The media milled sample was washed by ultracentifugation (3 cycles of centrifugation at 17,000 x g for 20 min followed by redispersion in deionized water) to remove the excess of poloxamer.

3. Results and discussion

We first conducted a screening study in order to gain insight on BDP particle size and impurity after laser fragmentation. We irradiated BDP with femto- and nanosecond lasers and varied the fabrication parameters such as suspension concentration, volume, duration and laser power. The size, polydispersity index and impurity levels are presented in Table 1. We conclude from the screening study that higher irradiation power and duration produce smaller BDP particles, but at a cost of higher impurity. This conclusion is coherent with our previous studies on paclitaxel and megestrol acetate.

Table 1. BDP particle size, polydispersity index (PDI) and chromatographic impurity of BDP fragmented with fs and ns lasers under different conditions

Condition	Power/duration	Average size (nm)	PDI	Impurity (%)
Suspension control	0	7460 ± 4680	0.7 ± 0.3	0.8 ± 0.2
fs, 0.5mg/mL, 2mL, 30min	50 mW	1490 ± 170	0.3 ± 0.1	1.8 ± 0.1
	200 mW	1130 ± 300	0.3 ± 0.1	2.2 ± 0.7
	400 mW	1010 ± 200	0.2 ± 0.1	2.7 ± 0.5
fs, 0.5mg/mL, 10mL, 1h	50 mW	1610 ± 200	0.4 ± 0.2	1.0 ± 0.3
	200 mW	1460 ± 240	0.4 ± 0.2	1.4 ± 0.1
	400 mW	990 ± 120	0.4 ± 0.1	2.1 ± 0.8
fs, 2mg/mL, 2mL, 30min	50 mW	3380 ± 2630	0.4 ± 0.3	0.8 ± 0.1
	200 mW	2150 ± 690	0.5 ± 0.3	0.9 ± 0.1
	400 mW	1290 ± 260	0.4 ± 0.1	1.0 ± 0.1
fs, 2mg/mL, 10mL, 50mW	6 h	1300 ± 300	0.6 ± 0.2	1.0 ± 0.2
	12 h	1010 ± 310	0.6 ± 0.1	1.2 ± 0.3
	16 h	880 ± 260	0.7 ± 0.2	1.6 ± 0.6
ns, 0.5mg/mL, 10mL, 1h	1.5 W	7250 ± 2290	1.0 ± 0.1	1.1 ± 0.5
	3.5 W	3230 ± 2590	0.9 ± 0.1	1.0 ± 0.4
	5 W	2490 ± 1080	0.9 ± 0.1	1.2 ± 0.5
ns, 0.5mg/mL, 10mL, 2h	3.5 W	2500 ± 820	0.7 ± 0.3	1.5 ± 0.2
	5 W	1700 ± 720	0.5 ± 0.3	2.0 ± 0.6

To further compare the size distribution and physicochemical properties with milled BDP particles, we selected four laser micronization conditions: (a) fs laser, 0.5 mg/mL, 10 mL, 400 mW, 1h; (b) fs laser, 2 mg/mL, 2mL, 400 mW, 30 min; (c) fs laser, 2 mg/mL, 10 mL, 50 mW, 16 h; (d) ns laser, 0.5 mg/mL, 10 mL, 5W, 1h. According to the screening study, BDP particles produced at these conditions are inside the 0.5-5 μm size range with acceptable purity.

3. 1. Size distribution and degradation

In order to confirm the BDP particle size distribution after laser fragmentation, we evaluated one sample of each condition by laser diffraction (Table 2). Laser fragments the BDP particle size to a

micrometric level. The majority of laser-micronized particles are inside the 0.5-5 µm range. Laser-micronized particles are more dispersed than jet-milled particles, but longer duration of laser irradiation narrows the particle size dispersion (see Table 2, the condition of ns laser 2h compared to 1h, other parameters are identical). The presence of large particles in laser-micronized samples is mainly due to their sedimentation during laser fragmentation, as magnetic stirring may not generate enough movements at the vial periphery.

Table 2. BDP particle size distribution (mean and cumulative undersize distribution at 10%(d_{10}) and 90%(d_{90})) measured by laser diffraction and relative content calculated from HPLC-measured/theoretical concentration ratio (mean ± standard deviation)

Condition	Size (µm)	d_{10} (µm)	Median (µm)	d_{90} (µm)	Content (%)
Suspension control	10.8 ± 7.9	3.2	9.2	19.4	100 ± 4.7
Jet-milled	1.5 ± 1.0	0.5	1.2	2.7	99.0 ± 0.1
fs, 0.5mg/ml, 10mL, 1h (a)	4.5 ± 5.4	0.6	2.6	10.6	93.6 ± 1.7
fs, 2 mg/mL, 2 mL, 0.5 h (b)	5.1 ± 4.3	0.9	4.0	10.5	89.5 ± 3.1
fs, 2 mg/mL, 10 mL, 16 h (c)	3.5 ± 4.8	0.5	1.8	8.9	95.8 ± 1.2
ns, 0.5 mg/mL, 10 mL, 1h (d)	4.2 ± 2.1	1.9	4.0	6.9	103.9 ± 2.5
ns, 0.5 mg/mL, 10 mL, 2h	2.4 ± 1.5	1.1	2.2	4.0	-

The degradation of BDP evaluated by content assay (calculation explained in materials and method section) is shown in Table 2. The "content" indicates the percentage (by weight) of the pure BDP active in the sample treated by laser fragmentation. Degradation induced by ns laser and jet milling is negligible, when the standard deviation is taken into account. Fs laser induced slightly more degradation than ns laser and jet milling. However, in terms of efficiency, laser fragmentation is clearly advantageous. The output of laser fragmentation is nearly 100%, as the particles were directly irradiated in their glass container. As a comparison with jet milling, only 0.49 g milled particles out of the 2.07 g loaded BDP powder could be retrieved (output 24%).

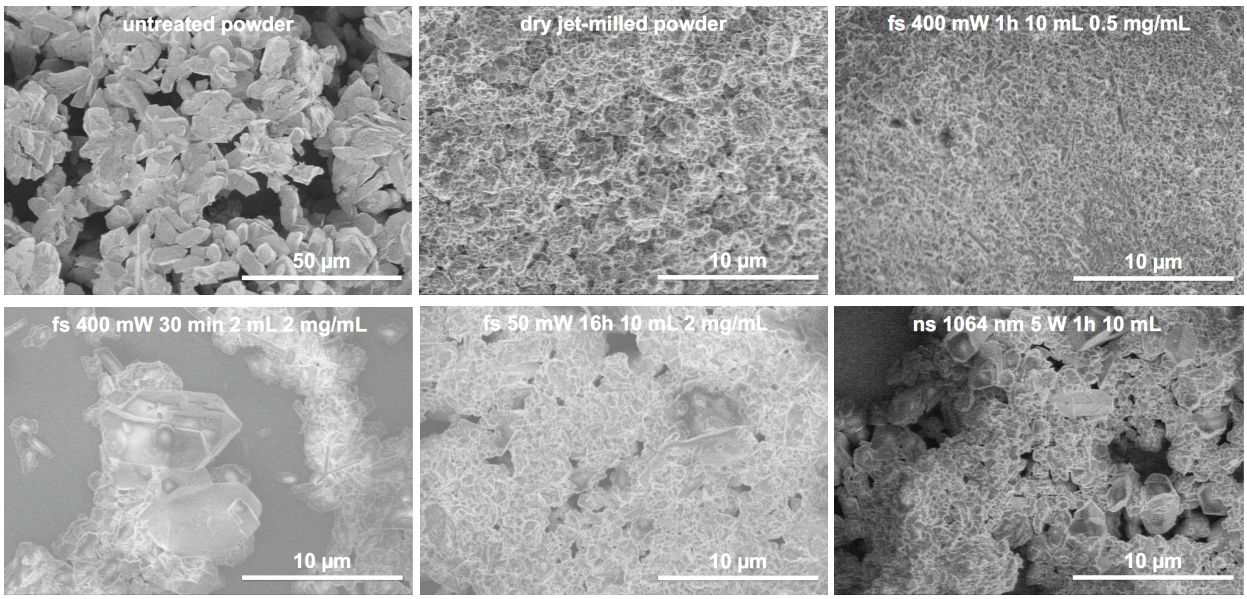

Fig. 1. Electron micrographs of original BDP powder, jet-milled powder and laser fragmented particles at selected conditions (a)-(d). Laser-micronized and jet-milled BDP particles have similar size and morphology. Short laser fragmentation duration may result in a less homogenized suspension with large particle residues (bottom-left).

Untreated, jet-milled and laser-fragmented BDP particles were observed with SEM (Fig.1). Laser-micronized BDP particles have similar size and morphology as jet-milled particles. According to Fig.1, in most cases, laser-micronized BDP particles are inside the 0.5-5 µm range. Larger particles may be present in less homogeneous suspensions, such as in the condition (b) (Fig.1 bottom-left).

3. 2. Physicochemical properties

In order to evaluate the chemical composition of BDP particles after treatment, we analysed untreated BDP powder, lyophilized suspension control, lyophilized laser-fragmented suspensions and dry jet-milled powder with Fourier transform infrared spectroscopy and elemental analysis (Table 3). FTIR spectrogram (Fig.2) shows no changes in the fingerprint region (500-1800 cm^{-1}), but new peaks are observed at 3500-3600 cm^{-1} (OH band) in all BDP samples in aqueous suspension. This modification corresponds to the FTIR spectra of BDP monohydrate (for comparison, BDP•H_2O FTIR spectra can be found in Hunt and Patfield, 1989). BDP is transformed into BDP•H_2O due to its contact with water, and the transformation is not induced by the laser irradiation. The transformation does not affect the therapeutic properties of the drug, as both anhydrous BDP and BDP•H_2O are commercialized anti-asthmatic drugs.

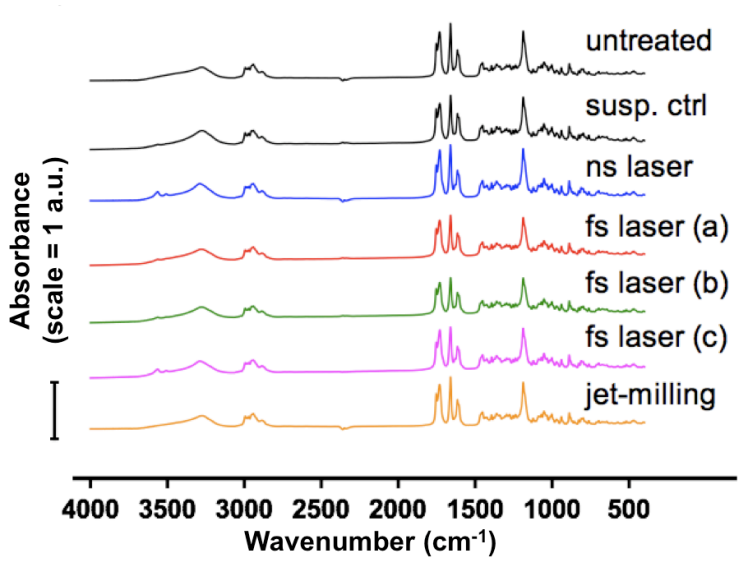

Fig. 2. FTIR spectrogram of untreated BDP powder, lyophilized suspension control, laser fragmented (conditions (a)-(d)) and jet-milled BDP particles. Changes at 3500-3600 cm^{-1} are observable in lyophilized BDP suspensions. This is caused by the formation of BDP monohydrate in water and is not induced by laser process. Laser micronization does not change the chemical composition of the drug.

Elemental analyses (Table 3) were used to measure the content of carbon and hydrogen in BDP particles, and determined by subtraction the content of oxygen and chlorine (the BDP formula is $C_{28}H_{37}ClO_7$). As neither chlorine nor carbon was introduced into the sample, the increase of (O+Cl)/C ratio (Table 3) indicates an increase of oxygen in the samples. Again, this is mainly due to the formation of BDP•H_2O in water. As a dry method, jet-milled BDP shows a similar profile with untreated powder. For BDP samples in contact with water, compared to the suspension control, laser-micronized BDP showed no obvious changes in its elemental composition. Thus, we conclude from FTIR and elemental analyses that the chemical properties of the laser-micronized drug are conserved.

Table 3. Elemental analyses of BDP particles. Oxygen is slightly more present in lyophilized BDP suspensions.

Conditions	C (%)	H (%)	O+Cl (%)	H/C	(O+Cl)/C
Untreated	64.7	7.3	28.0	0.11	0.43
Suspension control	63.0	7.2	29.8	0.11	0.47
Dry jet-milled powder	64.4	7.2	28.5	0.11	0.44
fs, 0.5mg/ml, 10mL, 1h (a)	63.0	7.1	29.9	0.11	0.47
fs, 2 mg/mL, 2mL, 0.5h (b)	62.5	7.0	30.5	0.11	0.49
fs, 2 mg/mL, 10mL, 16h (c)	63.0	7.2	29.9	0.11	0.47
ns, 0.5 mg/mL, 10mL, 1h (d)	62.5	7.1	30.4	0.11	0.49

In order to evaluate the physical structure of the BDP crystals, we conducted X-ray diffraction and differential scanning calorimetry analyses. The XRD spectrogram (Fig. 3) showed two groups with different spectra: 1) Dry powders that were never in contact with water – jet-milled BDP and untreated BDP – have identical spectra; 2) The spectra of laser-micronized BDP show a mixture of original BDP and BDP•H_2O. The peaks at 8° and 12° correspond to BDP monohydrate (see referential XRD spectrogram of raw BDP and BDP•H_2O in Valo et al., 2010). These peaks are more intense when BDP is immerged in water for a long time (fs laser condition (c) and ns laser condition (d)). The spectra of BDP micronized with fs laser under conditions (a) and (b) are similar to that of the suspension control. Finally, the media-milled BDP sample shows a more pronounced presence of BDP•H_2O. In summary, laser micronization does not induce changes in the crystalline phases of drug crystals. But for drugs that are sensitive to hydration, their hydrate forms may appear in the water-suspension during laser fragmentation.

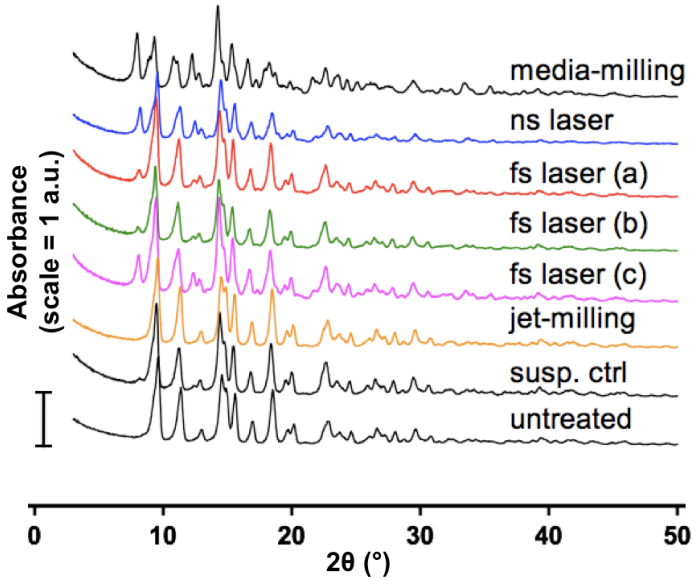

Fig. 3. XRD spectrogram of BDP particles. New peaks appear at 8° and 12° in all lyophilized suspensions, and their intensity is proportional with the duration of BDP immersion in water. These changes in the crystalline structure are caused by the formation of BDP monohydrate and not by the laser process. As a comparison, the untreated BDP powder fragmented by the standard media-milling shows modified spectra, which correspond to the crystalline phases of BDP•H_2O. Laser micronization does not change the crystalline structure of the drug.

By using DSC, we measured the melting point and fusion enthalpy of BDP particles (Table 4). Relative enthalpy is calculated by taking the fusion enthalpy of untreated BDP powder as 100%. Both jet-

milled and laser-micronized BDP particles have a lower melting point. Their enthalpy is also decreased and the decrease in laser-fragmented BDP is more important than jet-milled BDP. These decreases may be caused from three factors: 1) As fragmented particles' surface/volume ratio is increased, less energy is needed for melting; 2) Impurity may be present in fragmented particles; 3) Part of the particles may be amorphous. Considering our previous conclusions that the crystalline phases are conserved, we suggest that the loss in crytallinity is mainly caused by a partial amorphization on the surface of micronized particles and by the presence of chemical impurity in the samples.

Table 4. DSC analyses of BDP particles. (Experiments conducted in duplicata).

Conditions	Melting point (°C)	Fusion enthalpy (J/g)	Relative enthalpy (%)
Untreated powder	209.6 ± 0.1	71.6 ± 17.2	100
Suspension control	208.5 ± 0.1	62.9 ± 3.6	88
Jet-milled	205.6 ± 0.4	61.6 ± 5.0	86
fs, 0.5mg/ml, 10mL, 1h (a)	197.5 ± 0.1	38.6 ± 3.9	54
fs, 2 mg/mL, 2 mL, 0.5 h (b)	201.8 ± 0.1	53.1 ± 0.6	74
fs, 2 mg/mL, 10 mL, 16 h (c)	203.3 ± 0.4	51.7 ± 6.6	72
ns, 0.5 mg/mL, 10 mL, 1h (d)	205.8 ± 0.1	55.8 ± 1.2	78

4. Conclusion

We used femto- and nanosecond lasers to micronize a pulmonary drug model (BDP) as a proof of principle. Laser fragmentation requires only minute amount (several milligrams) and has a higher output than standard milling methods. BDP particles were reduced to the size range of 0.5-5 μm with limited degradation. The laser process induced limited changes in chemical composition and in crystalline structure. However, as drug particles were laser-fragmented in suspension, the hydration-sensitive drug co-existed with its hydrate form. Furthermore, the surface of the micronized drug particles may be amorphous after laser treatment.

In conclusion, drug particles were successfully micronized by laser fragmentation with negligible degradation and physicochemical transformations. Laser fragmentation is thus a simple and efficient micronization tool, and is well suited for pharmaceutical R&D, especially for pulmonary drug discovery.

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