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Development of 5-iodo-2′-deoxyuridine milling process to reduce initial burst release from PLGA microparticles

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Abstract

The aim of this study was to prepare 5-iodo-2′-deoxyuridine (IdUrd) loaded poly(d,l-lactide-co-glycolide) (PLGA) microspheres with a reduced initial burst in the in vitro release profile, by modifying the drug grinding conditions. IdUrd particle size reduction has been performed using spray-drying or ball milling. Spray-drying significantly reduced drug particle size with a change of the initial crystalline form to an amorphous one and led to a high initial burst. Conversely, ball milling did not affect the initial IdUrd crystallinity. Therefore, the grinding process was optimized to emphasize the initial burst reduction. A first step allowed us to set qualitative parameters such as ball number (7) and cooling with liquid nitrogen to obtain a mean size reduction and a narrow distribution. In a second step, three parameters including milling speed, drug amount and time were studied by a response surface analysis. The interrelationship between drug amount and milling speed was the most significant factor. To reduce particle size it should be necessary to use a moderate speed associated with a sufficient drug amount (400–500 mg). IdUrd release from microparticles prepared by the o/w emulsion/extraction solvent evaporation process with the lowest crystalline particle size (15.3 μm) was studied. Burst effect could be reduced significantly. Concerning the first phase of drug release, the burst was 8.7% for 15.3 μm compared to 19% for 19.5 μm milled drug particles.

Keywords: Drug milling; IdUrd release; Burst effect; PLGA microspheres; Experimental designs

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1. Introduction

The treatment of infiltrating brain tumors, particularly oligodendrogliomas, requires radiotherapy which provides a median survival of 3.5–11 years (Daumas-Duport et al., 1997). Since 5-iodo-2′-deoxyuridine (IdUrd) is a powerful radiosensitizer (Djordjevic and Szybalski, 1960), the intracranial implantation of IdUrd-loaded microparticles within the tumor might increase the lethal effects of γ radiations on malignant cells having incorporated IdUrd. The particles can be administered by stereotactic injection, a precise surgical injection technique (Menei et al., 1994). This approach requires microparticles of 40–50 μm in size releasing in vivo their content over 6 weeks, the standard period during which a radiotherapy course must be applied.

The solvent evaporation process is commonly used to encapsulate drugs into poly(lactide-co-glycolide) microparticles (PLGA) (Benoit et al., 1996). It is well known that the candidate drugs must be soluble in the organic phase. In the case where the active ingredient is not oil soluble other alternatives can be considered. The w/o/w-multiple emulsion method is particularly suitable for the encapsulation of highly hydrophilic drugs. For drugs which are slightly water soluble, like IdUrd (2 mg/ml), other approaches must be investigated to achieve significant encapsulation: dissolution of the drug in the organic phase through the use of a cosolvent or dispersion of drug crystals in the dispersed phase. In the latter case, it is often admitted that the suspension of crystals in the organic phase can lead to an initial drug release which is difficult to control (Bodmeier et al., 1997; Shenderova et al., 1997).

The objective of the present study was to reduce the initial burst in the release of IdUrd from PLGA microspheres by modifying the grinding conditions of the drug crystals, before suspending them in the organic phase. No other process parameter was modified to attain the fixed objective. To reduce IdUrd particle size, two grinding processes were used: spray-drying and planetary ball milling (Gubskaya et al., 1995; Annapragada and Adjei, 1996; Villiers and Tiedt, 1996). The optimal conditions of grinding were studied through experimental design and the impact on in vitro drug release from PLGA microspheres was then examined.

2. Materials and methods

2.1. Materials

5-Iodo-2′-deoxyuridine (IdUrd) was supplied by Sigma (Sigma-Aldrich, St. Quentin Fallavier, France). The coating polymer, PLGA 50/50, was supplied by Boehringer Ingelheim (Resomer® RG 506, BI Chimie, Paris, France). The chains contained 25% d-lactic units, 25% l-lactic units and 50% glycolic units. The average molecular weight ($\overline{Mw}$) (given by size-exclusion chromatography) was 75 000 ($I = 1.56$). Methylene chloride, acetone and poly(vinyl alcohol) (PVA, Rhodoviol®, 4/125, 88% hydrolyzed) were purchased from Prolabo (Paris, France).

2.2. IdUrd particle size reduction

2.2.1. Spray drying process

A solution of drug in methanol (0.3% (w/v)) was sprayed by using a Mini Spray Dryer Büchi B-191 (Büchi, Flawil, Switzerland). The process conditions were: inlet temperature 63°C, outlet temperature 45°C, aspirator setting 100%, feed spray rate 4.5 ml/min, spray flow 600 NL/h. Drug particles were collected and kept in a dessicator under vacuum at + 6°C.

2.2.2. Ball milling

IdUrd crystal grinding was performed with a planetary micro mill (Pulverisette 7®, Fritsch, Idar-Oberstein, Germany). 12-mm balls in a 45-ml jar were used. The milling speed was expressed as a function of the planetary rotation intensity and varied from 6 to 10 corresponding to 2100 to 3300 rpm respectively. At the same time, the acceleration increased from 6 to 18*g*. No more than 67% of the bowl volume was filled with grinding material and balls.

2.2.3. Experimental designs

2.2.3.1. Fractional factorial design. Five experimental factors were studied simultaneously in one experiment in order to determine quickly the relative influences of milling factors on IdUrd particle size (Y_1, μm) in a defined experimental domain and to find the qualitative factors at their best level. The coefficient of variation was taken into account (Y_2, %). Factors were: milling speed (U_1) (6; 10), drug amount (U_2) (200; 500 mg), milling time (U_3) (10; 40 min), ball number (U_4) (3; 7); cooling with liquid nitrogen (U_5) (no; yes). To estimate the five main effects (b_i) and two first-

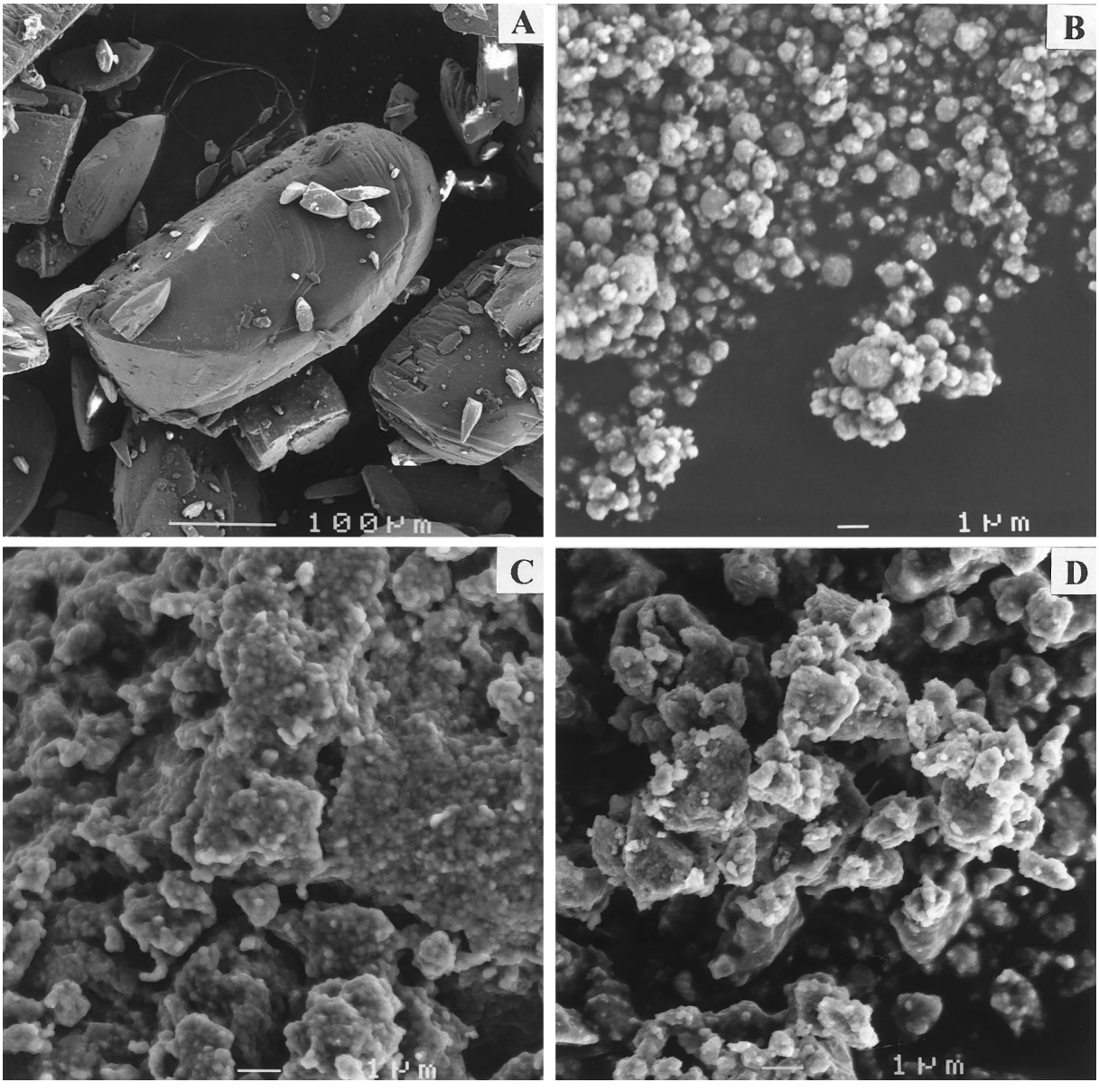

Fig. 1. SEM photomicrographs of IdUrd particles: A, unmilled drug particle; B, 11.5 μm spray-dried drug particle; C, 19.5 μm ball milled drug particle; D, 15.3 μm ball milled drug particle.

Table 1
Characteristics of reduced size IdUrd particles (analysed from light scattering) and IdUrd-loaded microspheres

IdUrd			Microspheres	
Reduction size process	Experimental conditions	Particle size (mean ± S.D.) (μm)	Mean ± S.D. (μm)	Drug content (w/w,%)
Spray-drying	–	11.5 ± 8.2	43.9 ± 20.3; $n = 4$	18.0 ± 1.3
Ball milling	730 mg IdUrd; speed 7.5; 9 min cooling	19.5 ± 18.2	46.1 ± 20.4; $n = 3$	16.3 ± 0.4

order interaction effects b_{12} and b_{23} postulated as most probable, a fractional factorial design (2^{5-2}) was selected with two defining contrasts: 134 and 1235 (Box et al., 1978). The experiment consisted of eight of the 32 possible treatment combinations.

2.2.3.2. Response surface analysis. In a second step, to optimize the grinding conditions, a second-order polynomial model was postulated to express the response Y_1 (mean crystal size) as a function of the three selected factors: milling speed (U_1), milled drug amount (U_2) and milling time (U_3). A Doehlert matrix was used for a response surface analysis (Doehlert, 1970). In order to estimate the coefficients of the model, 13 distinct experiments (+two more center-replicated points to calculate the variability of the experimental results) were carried out. The analysis of variance (ANOVA) was performed in order to determine significance of the fitted equation.

2.3. IdUrd particle characterization

Drug particle morphology and size were characterized by scanning electron microscopy (SEM) (JSM 6301F, Jeol, Paris, France). Crystallinity of the milled sample was assessed using an X-ray powder diffractometer (Innel X RG300, Orléans, France) and the absence of polymorphic transformation was controlled using a Fourier-Transform (FT) Raman spectrometer (RFS 100 /D418-S FT, Bruker, Wissembourg, France). Crystal size distribution was determined with a Mastersizer S (Malvern Instruments, Malvern, Orsay, France). The technique is based on the principle of laser light scattering. The crystals (30 mg) were suspended in 3 ml cyclohexane (Prolabo, Paris, France) under sonication and immediately analysed. At least two samples were analysed and for each sample, the mean size value (volume size distribution) was obtained from the average of four calculation cycles.

2.4. Microparticle preparation and characterization

Microspheres were prepared by an o/w emulsion/extraction method (Boisdron-Celle et al., 1995). The coating polymer (250 mg) was dissolved in 3 ml of methylene chloride and 100 mg of IdUrd were dispersed in this solution. The resulting suspension was emulsified in 100 ml aqueous PVA (7% w/v) for 2 min at 800 rpm (Heidolph RGH 500, Prolabo, Paris, France). The emulsion was subsequently poured into a large volume of extracting water (500 ml). Stirring was continued for 2 min at 500 rpm. The microparticles were collected by filtration (0.8 μm), washed with 100 ml water and freeze-dried (RP2V Serail, SGD, Argenteuil, France).

Particle size was determined using a Coulter® Multisizer (Coultronics, Margency, France). Microparticles were dispersed in a polysorbate 80 aqueous phase by sonication for 5 min and suspended in Isoton® II solution (Coultronics, Margency, France).

The IdUrd content of the microspheres was determined by dissolving 6–8 mg microspheres in dimethylsulfoxide (50 ml) and assaying the solution spectrophotometrically at 287 nm ($n = 3$).

2.5. In vitro release study

The release medium, phosphate buffer 0.13 M, pH 7.4, was agitated at 100 rpm in a USP rotating paddle apparatus at 37°C (Sotax AT7, St Germain en Laye, France). A dialysis bag containing 80 mg microparticles and 7 ml phosphate buffer was placed into 300 ml release medium (molecular cut-off 6–8 kDa, Bioblock, Illkirch, France). IdUrd released from microspheres was assayed spectrophotometrically at 287 nm.

3. Results

3.1. IdUrd particle size reduction

Unmilled IdUrd particles consisted of large crystals with a size ranging from 120 to 750 μm (Fig. 1A). In order to define the most appropriate protocol for crystal size reduction, spray-drying and ball milling were examined (Table 1). Fig. 1B shows an electron micrograph of the spray-dried drug. Spray-drying led to spherical particles of 1–1.5 μm with a narrow size distribution. The distribution size analysis gave mean size values higher than those corresponding to individuals (Fig. 1B, Table 1) due to the difficulty of dispersing drug particles in the organic solvent. Nevertheless, the analysis conditions are close to those met during IdUrd dispersion in the organic phase at the beginning of the encapsulation process. An amorphous structure was witnessed by X-ray diffraction patterns (results not shown). Fig. 1C shows an electron micrograph of the 19.5 μm IdUrd samples produced with ball milling. Drug crystals resulted from the aggregation of small particles leading to a slightly granular surface. The crystalline character of the milled IdUrd crystals was not affected: no polymorphic transformation occurred as supported by FT-Raman spectroscopy. The spectrum of milled IdUrd did not reveal any detectable change (bands and intensity) compared to unmilled crystal spectrum (results not shown).

3.1.1. Fractional factorial design

Experimental results of the fractional factorial design are reported in Table 2. The effect of the experimental factors evaluated was determined using NEMROD® software (Mathieu and Phan-Tan-Luu, 1996). Fig. 2 shows graphically the relative importance of these factors on size reduction (Fig. 2A) and size distribution (Fig. 2B). The reduction of the experiment to eight trials (instead of 32) showed that each main effect is inextricably mixed with one of the two-factor interactions (aliases), but it was assumed in a first step that aliases were negligible. The higher the effect of a factor in absolute value, the more significant the effect it had on particle size. The + and − signs of the effect indicated an increase or a decrease of the response respectively (mean size or coefficient of variation). Concerning the ball number, it ap-

Table 2
Factorial design (real values) and experimental results of the 2^{5-2} fractional factorial design

Trial	Milling speed	Drug amount (mg)	Milling time (min)	Ball number	Cooling	Mean diameter (μm)	Coefficient of variation (%)
1	6	200	10	7	No	29.8	105.4
2	10	200	10	3	Yes	48.30	71.6
3	10	200	10	3	Yes	51.10	76.0
4	10	200	10	3	Yes	53.30	86.6
5	6	500	10	7	Yes	26.40	49.8
6	10	500	10	3	No	47.90	121.7
7	6	200	40	3	Yes	24.4/24.2	81.4/82.0
8	10	200	40	7	No	39.80	96.4
9	6	500	40	3	No	37.10	137.9
10	10	500	40	7	Yes	21.60	67.2

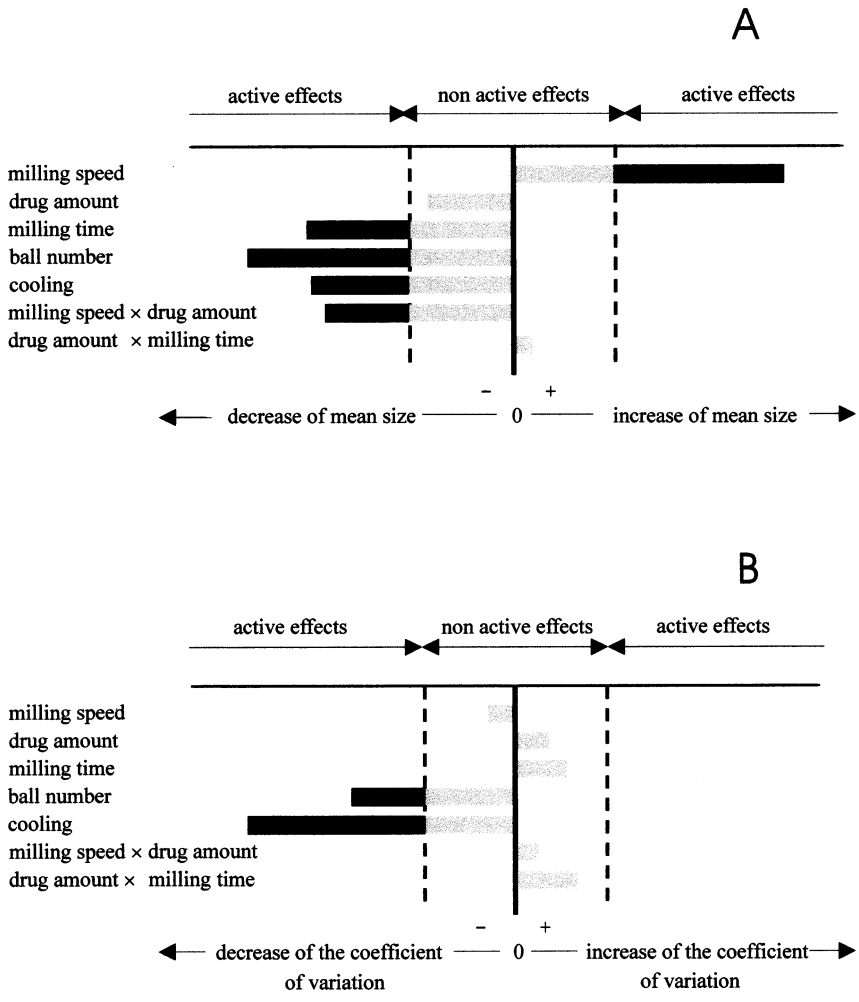

Fig. 2. Fractional factorial design: graphical analysis of the parameter effects on the mean particle size (A) and coefficient of variation (B).

peared that to reduce the mean particle size and size distribution, a maximum ball number was necessary. Cooling was done by the addition of liquid nitrogen in a jar mill before powder deposition, so that a cold environment existed for a short period. This cooling step had an effect on particle size reduction and narrowed the particle size distribution. The influence of milling speed on mean size depended on the amount of drug and revealed the interaction between milling speed and drug amount. Time is also a critical factor on mean particle diameter.

3.1.2. Response surface analysis

To go further, both qualitative factors were set in the sense of a mean size reduction and narrow distribution, i.e. cooling and using seven balls. To optimize the grinding conditions, three parameters including milling speed (U_1), drug amount (U_2) and time (U_3) were studied by response surface analysis (Table 3). Coefficients of the second-order polynomial model were estimated by the least square method. The equation model for Y_1 (mean particle size) was as follows:

$$Y_1 = 16.1 + 2.199X_1 - 0.881X_2 + 0.019X_3 + 7.500X_1^2 + 5.667X_2^2 + 1.635X_3^2 + 9.988X_1X_2 - 5.670X_1X_3 - 3.439X_2X_3$$

The statistical analysis (Table 4) for Y_1 showed that the model represented the phenomenon quite well and the variation of the response was correctly related to the variation of the factors. Fig. 3 shows a graphical representation of the isoresponse surface. However, it must be noted that the mathematical model did not fit the experimental points of the coefficient of variation (Y_2); the phenomenon is probably more complex than a second-order polynomial model.

Milled drug amounts of 400–500 mg and a moderate speed (7) were preferred for good particle size reduction. Drug amounts below 350 mg associated with a milling speed of 6–8 or drug amounts above 350 mg associated with a milling speed between 8.5 and 10 dramatically increased the particle size. The lowest particle size which could be produced in the studied domain is 16 μm predicted by the model. Trial 6 of the experiment was a point included into this optimal zone: 500 mg of drug, speed 7 and 27.5 min, it showed a 15.3 μm experimental value (16.1 μm predicted) with a coefficient of variation of 90.5%. Fig. 1D shows the morphology of the resulting particles. Their crystallinity was not altered as shown in the X-ray diffraction patterns. The Raman spectrum did not reveal any polymorphic transformation.

3.2. In vitro IdUrd release from microspheres

Firstly, microspheres were prepared with spray-dried and milled 19.5 μm IdUrd particles (Table 1). The drug contents were in the same order of magnitude (Table 1) which corresponded to encapsulation yields of 61 and 59% respectively. Fig. 4 contains IdUrd release profiles from microparticles. The spray-dried and milled IdUrd particle structures led to different drug release profiles. The release of spray-dried IdUrd particles from microspheres revealed a large initial burst (47% within the first 24 h). The 19.5 μm ball milled IdUrd crystals were released more slowly from the PLGA matrix, but a burst effect concerning 19% of the content was still present.

Secondly, IdUrd-loaded microspheres were prepared using the 15.3 μm IdUrd crystal (Doehlert matrix, trial 6). Particles exhibited a 17.7% drug content ($n = 2$). Fig. 4 contains the drug release profile exhibited by the microparticles. The burst effect accounting for 8.7% within 24 h, was followed by a significantly lower release rate. Then the IdUrd release rate increased again. This in-

Table 3
Doehlert matrix in coded variables, experimental design and experimental results of the surface response analysis step

Trial	X_1	X_2	X_3	Milling speed	Drug amount (mg)	Milling time (min)	Mean diameter (μm)	Coefficient of variation (%)
1	1.000	0.000	0.000	10	350	27.5	25.1	81.0
2	−1.000	0.000	0.000	6	350	27.5	22.1	76.7
3	0.500	0.866	0.000	9	500	27.5	28.0	135.0
4	−0.500	−0.866	0.000	7	200	27.5	25.1	84.8
5	0.500	−0.866	0.000	9	200	27.5	20.5	88.6
6	−0.500	0.866	0.000	7	500	27.5	15.3	90.5
7	0.500	0.289	0.816	9	400	45.0	18.3	131.9
8	−0.500	−0.289	−0.816	7	300	10.0	17.2	80.1
9	0.500	−0.289	−0.816	9	300	10.0	20.9	80.9
10	0.000	0.577	−0.816	8	450	10.0	19.8	84.1
11	−0.500	0.289	0.816	7	400	45.0	18.3	88.3
12	0.000	−0.577	0.816	8	250	45.0	21.6	96.2
13	0.000	0.000	0.000	8	350	27.5	12.8	89.2
14	0.000	0.000	0.000	8	350	27.5	16.4	84.8
15	0.000	0.000	0.000	8	350	27.5	19.1	82.9

Table 4
Results of the analysis of variance (ANOVA) for the response Y_1 (IdUrd mean particle size)

Source of variation	S.S.	df	Mean square	*F*-value	Significance
Regression	207.85	9	23.09	5.32	*
Residue	26.05	6	4.34		
Validity	5.09	3	1.69	0.24	86.2%
Error	20.96	3	6.98		
Total	233.90	15			

* Significant at $1\% < \alpha < 5\%$.

crease was due to PLGA degradation associated with pore formation inside the matrix.

4. Discussion

The ultimate objective was to increase the radiosensitivity of brain tumor cells over a conventional radiotherapy period (6 weeks) by intracerebral implantation of IdUrd-loaded microspheres. Microencapsulation by the solvent evaporation process of a drug insoluble in methylene chloride such as IdUrd needs some adaptations: dissolution of the drug in the organic phase through the use of a cosolvent or dispersion of drug crystals in the dispersed phase. The latter approach limits the use of organic solvents that generally cause environmental and toxicity problems. The aim of this study was to prepare IdUrd-loaded microspheres from a drug crystal dispersion in the organic phase and to observe the possibility to reduce the initial burst by modifying drug crystal size.

Firstly, IdUrd particle size reduction has been performed using either spray-drying or ball milling to observe which process is better adapted to reduce drug particle size and lower the burst effect. Spray-drying strongly reduced drug particle size associated with a change of the initial crystalline form. Takada et al. (1997) reported that an amorphous form of a water-soluble GPIIb/IIIa antagonist (4–7 μm) dispersed in the organic phase gave a more homogeneous dispersion and higher viscosity than the crystalline form, resulting in a well-controlled release of drug. This was explained by the development of molecular interactions between the drug and PLGA. In the present study, small spherical spray-dried particles were difficult to disperse in the organic solvent because of electrostatic agglomeration and a lack of affinity for the solvent. Moreover, the dissolution rate of spray-dried poorly water soluble compounds was already described as much more rapid when compared to non-spray-dried crystals (Broadhead et al., 1992). The presence of amorphous agglomerates, non-homogeneously dispersed within the polymeric matrix and irregularly coated, led to a high initial burst.

IdUrd crystalline particles were obtained by using a planetary micro mill. The lower initial burst observed was mainly related to the IdUrd particle crystallinity associated with a low dissolution rate as compared to spray-dried particles. Ball milling conditions revealed a milled particle aggregation (Fig. 1C). This milled powder did not reduce sufficiently the burst effect. But the easier dispersion in the organic phase improved the IdUrd release profile in terms of burst effect. Consequently, grinding was selected for particle size reduction; but the challenge was to obtain sufficient particle size reduction whilst limiting secondary aggregates, to keep the burst under control.

Grinding is a well known process to reduce particle size. Milling parameters such as drug amount, milling speed and milling time are known to influence particle size but the interrelation of these parameters is still not clear, their adjustment often being empirical. The complexity of the process requires several factors to be studied simultaneously. Consequently, the experimental design is a tool to explore the possible combination be-

tween milling parameters. The first step allowed us to set up qualitative parameters (number of balls, cooling with liquid nitrogen) to obtain a mean size reduction and a narrow distribution. It appeared that the milling speed, time and quantity of drug were also critical with a possible complex relationship between them (interaction b_{12} was notable). Grinding time dependence on particle size results from the action of two opposite effects: the dispersion and formation of secondary aggregates. The relative ratio of these two effects has a strongly pronounced dependence upon temperature. Low grinding temperatures slowed down the process of aggregation and provided high efficiency (Gubskaya et al., 1995; Annapragada and Adjei, 1996). In this study, cooling was carried out by the addition of liquid nitrogen in a jar mill before powder deposition. It was observed that this short cooling step had an important effect on particle size reduction and narrowed the particle size distribution.

In the second step, a Doehlert network which homogeneously distributed the experimental points in the studied domain was used and the relationship between the parameters was quantified. The model, calculated in the experimental design, clearly showed that the parameters were interrelated. Milling time did not affect size reduction as significantly as the two other variables. Nevertheless, milling time should be relatively short; between 10 and 27.5 min. It was previously found that a prolonged grinding time resulted in a limit in particle size reduction within a relatively short time (20–30 min) using a vibrating (Villiers and Tiedt, 1996) or ball cryomill (Gubskaya et al., 1995). The interrelationship be-

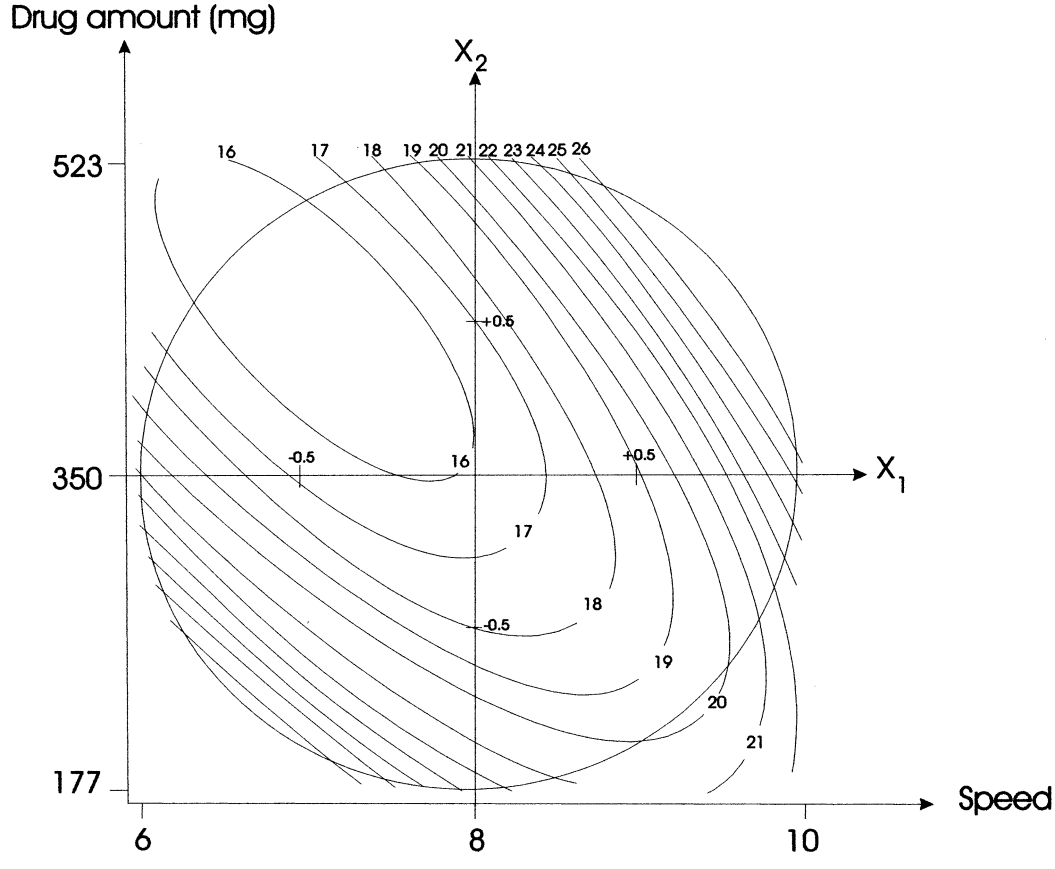

Fig. 3. Graphical representation (2D) of the experimental response Y_1 (IdUrd mean particle size) for 27.5 min milling time.

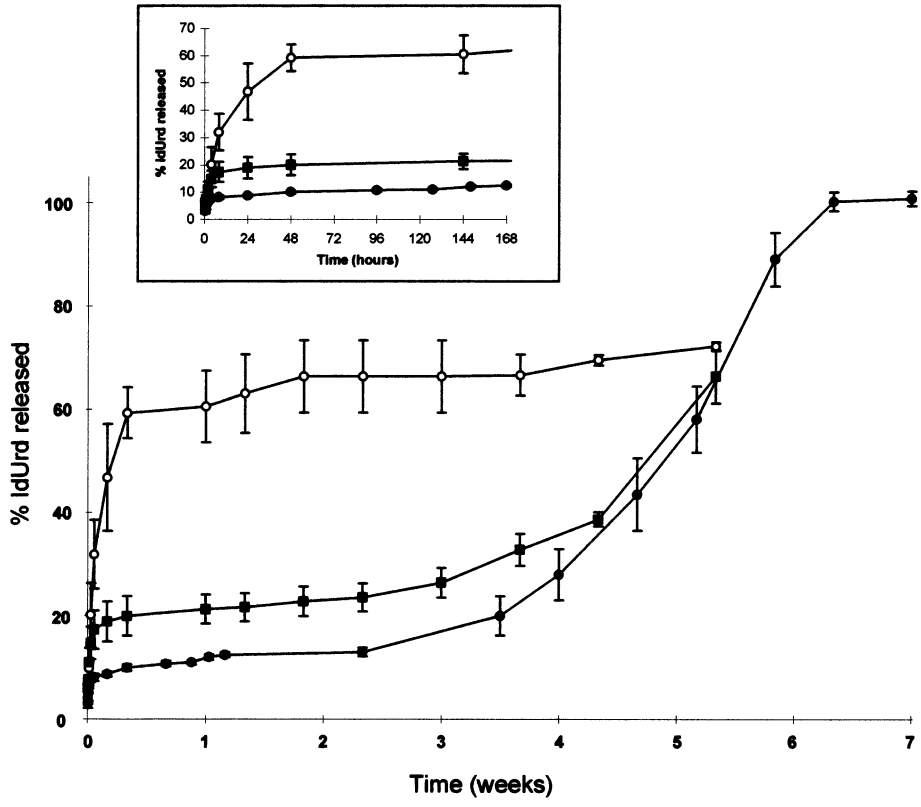

Fig. 4. IdUrd release profiles from microspheres prepared after IdUrd size reduction according to: ○, 11.5 μm (spray-dried); ■, 19.5 μm (ball milled); ●, 15.3 μm (ball milled).

tween drug amount and milling speed is the most significant. To reduce particle size it should be necessary to use a moderate speed associated with a sufficient drug amount. It should be remembered that particle size decrease occurs through fragmentation to smaller particles and particle growth occurs through collisions between particles (Annapragada and Adjei, 1996). If time or speed were too high in comparison to the amount of drug, particle agglomeration was favoured. A compromise should be found to have a sufficient particle size reduction without any agglomeration.

The lowest particle size obtained was 15.3 μm. IdUrd release from microparticles prepared with this drug milled batch was studied. The obtained release patterns have already been observed, particularly in the case of peptides and proteins entrapped in PLGA microparticles (Ruiz and Benoit, 1991; O'Hagan et al., 1994). The profile showed three phases indicating that after the early stage, IdUrd release closely followed the degradation process of the biodegradable matrix. Concerning the first phase of drug release, the burst reached a plateau at 8.7 and 19% from the release profiles obtained with the 15.3 and 19.5 μm IdUrd particles respectively. Usually, particle size reduction increased powder surface areas and dissolution rate of sparingly soluble drugs. Conversely, in the present study, when particle size decreased, the IdUrd release from microspheres was slowed down. If drug particles were assimilated to spheres, the ratio between milled IdUrd particle volume and microsphere volume changed markedly; the ratio was 1/39 for 15.3 μm and 1/13 for 19.5 μm IdUrd particles. During microsphere

preparation, the smallest ratio allowed us to obtain a better coating of IdUrd particles in the matrix allowing a better control of drug release by the matrix. For these reasons, the 15.3 μm IdUrd milled particles were preferred.

The model presented fitted the grinding phenomenon and showed the complex relationship between the parameters. If a link was established between the factorial design and the Doehlert matrix, it appeared that the interaction b_{35} (milling time-cooling, alias of b_{12} in the fractional factorial design) may be not so negligible. In another study it would be interesting to determine this independent of the other factors, although this point does not modify our decision to cool the jar with liquid nitrogen to reduce particle size. The crystal size of the starting drug also had an important effect and depended on the drug batch, therefore the model should be adapted each time a new batch of a drug is used, but the approach is identical: process parameters should be considered simultaneously.

In conclusion, the entrapment of milled drug particles of 15–16 μm mean size in 40–50 μm microspheres allowed us to limit considerably the burst effect exhibited by the IdUrd release profile from biodegradable microspheres. This study outlined the importance of the milling process and the complex dependence of the milling parameters. Although the initial stage could be controlled by drug size, the rest of the release depends strongly on polymer degradation. The drug profile remained to be modulated in order to obtain an ideal zero-order drug release. The addition of additives in the polymer matrix may be an issue to regulate release and is currently being looked at by our group.

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