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PREPARATION OF POLY(D,L-LACTIDE/GLYCOLIDE) NANOPARTICLES OF CONTROLLED PARTICLE SIZE DISTRIBUTION: APPLICATION OF EXPERIMENTAL DESIGNS

M.C. Julienne*, M.J. Alonso, J.L. Gómez Amoza, J.P. Benoit*

Laboratorio de Farmacia Galénica. Facultad de Farmacia. Universidad de Santiago. Spain.
*Laboratoire de Pharmacie Galénique. Faculté de Pharmacie. Université d'Angers. France.

SUMMARY

This study investigates the use of the solvent evaporation method for preparing acid and glycolic acid-based copolymer nanoparticles. Initially, appropriate technological and formulation factors for elaboration of polymeric particles were selected by screening. Most favourable results were obtained using polyvinyl alcohol as a dispersing agent and a high pressure homogenizer to reduce the droplet size of the emulsion initially formed. On the basis of the conclusion thus drawn, a composite rotational experimental design was employed to evaluate the joint influence of three formulation variables (phase volume ratio of the emulsion formed, polymer concentration and homogenization pressure) on the micromeritic properties of the suspension finally obtained (mean particle size, coefficient of variation and polydispersity of the particle size distribution). Analysis of variance corresponding to the experimental design, showed a significant influence of the volume phase ratio and the polymer concentration on the mean particle size and the coefficient of variation, whereas the polydispersity is also affected by the homogenization pressure. Considering this information, a 3^2 experimental factorial design was then selected to investigate the possible interaction between the phase volume ratio and the concentration of polyvinyl alcohol in the aqueous phase. Analysis of variance and subsequent sequencial regression analysis evidenced last hypothesis providing the way to determine the experimental conditions required to achieve a specific particle size distribution.

INTRODUCTION

Drug delivery systems consisting of biodegradable polymers have become very popular over the last few years due to their ability to control drug release effectively and to the possibility of their administration by parenteral routes (1). Among these polymers, much attention has been paid to the D,L-lactic and glycolic acids copolymers (PLA/GA) because of the total absence of toxicity of the degradation products and their modulatable degradation rates (2). Consequently, these kinds of controlled release systems have been developed in a wide range of sizes and shapes, as pellets and microparticles, for delivery of many drugs and , in particular, as new dosage forms for peptide delivery (3).

The drawback of these polymeric particles is that they cannot be administered intravenously owing to their size in the micrometer range. In spite of the importance of the size of particles, not only for drug targeting by intravenous administration (4), but also for controlled release purposes (5), no conclusive results have been published yet concerning preparation of nanoparticles of controlled particle size distribution.

The most common techniques for preparing PLA/GA microspheres are the solvent evaporation method (6) and the phase separation processes (7). The first step in both methods requires the formation of an emulsion. The phase separation techniques are more complicated and necessitate the further elimination of the continuous phase. In our study, we have chosen the solvent evaporation technique in order to obtain a final colloidal suspension of nanoparticles. The work presented here further examines the suitability of this technique for preparing nanoparticles with the desired particle size distribution. Although the solvent evaporation process is conceptually simple, many variables can influence the final product (8,9). The process variables include principally the emulsification procedure, the ratio and nature of both phases and the polymer concentration. In this study, a rotatable composite design was initially applied to establish a correlation between some preparation variables and particle size distribution parameters. The interest of this particular design in the formulation of polymeric nanoparticles was demonstrated in a previous paper (10). Once selected the most relevant factor, a factorial experimental design was secondly carried out to study in depth the influence of formulation variables on particle size distribution of PLA/GA nanoparticles and to define the optimal manufacturing conditions.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide-co-glycolide) (PLA85/GA15) was purchased from Boehringer (Resomer RG 858^{R}, Boehringer, Ingelheim, Germany) ($\bar{M}_{GPC}$ 87,000). The emulsifying

agents used were : 88 % hydrolysed poly(vinyl alcohol) (PVA, 4cp) (Rhodoviol 4/125^{R}, Rhône-Poulenc, Vitry s/Seine, France), methylcellulose (MC, 15 cp), (Methocel^{R} A15 LV Prem EP, Dow Chemical, England), gelatin PS 160 (Rousselot, Paris, France) and lecithin (Sigma Chimie, Paris, France) were used as received. Dichloromethane was supplied by Prolabo (Paris, France) and used without further purification.

Methods

The preparation of nanoparticles was based on the solvent evaporation process (6). The polymer was dissolved in methylene chloride. The organic phase was then emulsified in an aqueous solution of surfactant agent. To set the emulsion, three different ways were investigated.

Dispersion by mechanical stirring

The O/W emulsion was stirred vigorously (10,000 rpm) with a high speed agitator (Virtis 45^{R} , Virtis Co., Osi, Paris, France) at room temperature and under ambient pressure for 5 minutes, then the solvent evaporation was completed under reduced pressure in a rotary evaporator. For this study two emulsifying agents were tested, methylcellulose and polyvinyl alcohol at 0.1%, 0.2%, 0.5% (w/v). The polymer concentration in the organic phase was 2% (w/v) and the phase volume ratio described as methylene chloride volume/overall volume (v/v), was 20%.

Dispersion by mechanical stirring and high pressure homogenization

In this case, the two phases were stirred with the high speed agitator for 3 min and the preformed emulsion was passed through the homogenizer (Guerin ALM2^{R}, Mauzé, France), the solvent was then evaporated under reduced pressure. PVA, methylcellulose, gelatin and lecithin at 0.5 % (w/v) were used as emulsifying agents. The polymer concentration and the phase volume ratio were 2 % (w/v) and 20 % (v/v) respectively.

Dispersion by high pressure homogenization

The aqueous phase, a PVA solution, was passed through the homogenizer and the organic phase was added slowly. The emulsification was set 5 min and then, the solvent was evaporated under reduced pressure in a rotary evaporator. Two series of formulations were prepared according to this procedure. In one of the series, the concentration of polymer in the organic phase varied from 0.79 to 9.20 % (w/v), the phase volume ratio was ranged from 8.18 to 41.82 % (overall volume 150 ml) and pressures between 65 to 234 bars were used, the concentration of PVA in the aqueous phase was fixed at 0.5 %. In the other serie, the phase volume ratio was ranged from 10 to 40 % and the PVA concentration in the aqueous phase was varied between 1 and 15 %. The homogenization pressure was maintained at 150 bars.

Particle size analysis

Particle size distributions were determined by photon correlation spectroscopy (P.C.S.). The instrument used was a Coulter N4MD submicron particle analyser (Coultronics, U.S.A.) with a 14 mW helium-cadmium laser irradiation multiple scattering angle detection and a size distribution analysis processor. Each suspension sample was diluted to the appropriate concentration with distilled water.

The principle of PCS or Dynamic Light Scattering (DLS) is the use of autocorrelation spectroscopy of scattered light to determine the time dependence fluctuations of scattered light, which result from brownian motion of the particles in suspension (11). The light intensity scattered at any angle (in our experiments at 90 º) is detected by a photomultiplier whose output current is passed to an autocorrelator, which by correlating sections of the signal with themselves at different times (autocorrelation), evaluates this time dependency, so that, the resulting autocorrelation function can be derived. The time dependency of the scattered light determines the rate of diffusion or Brownian motion of the particles and hence their size.

The Size Distribution Processor (SDP) program analyses the correlation function data and gives the particle size distribution, which is shown as concentration (by photon intensity) against particle diameter. This program makes no assumptions about the size distribution in order to compute results and it also provides an estimation of the distribution width from a term denoted MU2/GAMMA SQ and regularly called the polydispersity (for a monodisperse system, this parameter is close to 0.1 and for a bimodal sample close to 0.2).

Statistical analysis

Most formulation studies involve variation of one factor at a time, keeping other factors constant. Factorial designs enable all factors to be varied simultaneously, thus allowing quantification of the effects caused by independent variables and interactions between them. In an ordinary factorial design, only a limited number of factors at a low number of levels can be investigated because an increase in the number of factors markedly increases the number of experiments to be carried out. In this case, an alternative approach is the use of a rotatable composite design which enables a wide range of variable values to be investigated using only a small number of formulations (12). In this study, two different experimental designs have been chosen: a central composite design and a factorial design. The central composite design allows us to evaluate 3 factors at 5 levels by preparing only 20 formulations. Selection of the extreme real values of the selected variables was carefully made taking into account the feasability of the preparation

while studying the largest range of variable conditions and, the intervals between levels are decided keeping with the orthogonalized values of the design. According to the information provided by the reduced design, a factorial design 3^2 (2 factors, 3 levels), which corresponds to 9 formulations, was established.

On the basis of the data obtained by using both experimental designs, quadratic polynomial equations were generated to establish the correlation between the independent variables (i.e., polymer concentration, phase volume ratio and homogenization pressure) and each of the dependent variables (mean particle diameter, coefficient of variation and polydispersity) estimated by S.D.P. analysis. Analysis of variance specific to the central composite design (12) was performed to determine the significance of each term of the equations fitted and to estimate the goodness of the fit in each case. In these equations, the values of independent variables are orthogonalized. Data obtained from the factorial design were interpretated statistically by an analysis of variance combined with a sequencial regression analysis (13), which allows to deduce the significance of each factor and to calculate the equations which relate dependent and independent variables and their corresponding correlation coefficients.

RESULTS AND DISCUSSION

The main objective of this work was to obtain PLA/GA nanoparticles and also to investigate the possibility of modulating their particle size distribution. Hence screening was carried out to find the most relevant technological factors, which were then extensively studied with the chosen experimental designs. The solvent evaporation technique (6) for preparing nanoparticles involves forming an oil/water emulsion, the oily phase of which is an organic solvent that diffuses adequately in the aqueous phase. In this respect, studies investigating the solubility of poly(D,L-lactide) in a series of organic solvents (methanol, acetone, methylene chloride, chloroform and benzene) and their solubility in water, indicate that it is necessary, not only a high solubility of the polymer in the organic solvent, but also an adequate diffusion of the later in the aqueous phase for perfect spheres to be obtained (9). The diffusion rate depends on the solubility of the solvent in the aqueous phase and its rate of evaporation at the water/air interface. On the basis of these criteria, dichloromethane is usually chosen since it is considered to give the most highly spherical particles(6-9).

Table 1 compares the values of parameters related to the particle size distribution for a series of formulations differing in the nature and concentration of the dispersing agent incorporated in the aqueous phase of the emulsion. The dispersing agents selected were

TABLE 1

Effect of nature of the dispersing agent on particle size distribution parameters. Polymer concentration in organic phase: 2% (w/v). Phase ratio: 20% (v/v). Emulsification procedure: high speed agitator (10,000 r.p.m., 10 min.)

Aqueous phase (% w/v)	Mean particle size (nm)	C.V. (%)	MU2/GAMMA SQ
P.V.A. 0.5%	291	27	0.15
P.V.A. 0.2%	340	30	0.21
P.V.A. 0.1%	342	38	0.31
M.C. 0.5%	1880	82	0.61
M.C. 0.2%	1810	54	0.65
M.C. 0.1%	1950	93	0.78

the most commonly used for preparation of PLA/GA microspheres by the solvent evaporation technique (6,7). The organic phase (dichloromethane), the polymer concentration in the organic phase (2%, w/v), the phase volume ratio (20%, v/v) and the emulsification procedure (high-velocity agitator) were kept constant. This table shows that methylcellulose always gives particles greater than 1 μm in size, whereas with PVA, a notably smaller particle size can be achieved; moreover even though the mean particle size is not appreciably affected by the concentration of PVA, we must be aware that multimodal particle size distributions have been detected at low concentration of PVA. The difference in behaviour of PVA and methylcellulose as regards the nanoparticles obtained using each, becomes clearer looking at the stages of the process and the different nature of the suspending agents. One consideration is that the system evolves from the emulsion state to the suspension state by way of solvent evaporation and the consequent precipitation of the polymer, hence the final particle size is presumably conditioned by the size of the initial emulsion droplet. It is interesting to note that a solution of PVA exhibits a surface tension of 37 dyn/cm at 20ºC whereas, in the same conditions, a solution of methylcellulose presents a surface tension ranging from 47 to 53 dyn/cm (14,15). Considering the sole reduction of interfacial free energy in the studied emulsions, polyvinyl alcohol appears more efficient than methylcellulose. This point might explain why the smallest particles are obtained with PVA. In this case, less coalescence of methylene chloride droplets occurs during stirring.

To evaluate the possible reduction of droplet size in the already prepared emulsion, that could be achieved using a high pressure homogenizer, a series of

TABLE 2

Effect of homogenization step on particle size distribution parameters. Polymer concentration in organic phase: 2% (w/v). Phase ratio: 20% (v/v). A: Emulsification with a high speed agitator (3 min., 10,000 r.p.m.). B: Emulsification with a high speed agitator and homogenization at high pressure (300 bars).

	A			B		
Aqueous phase	Mean size (nm)	C.V. (%)	MU2/GAMMA SQ	Mean size (nm)	C.V. (%)	MU2/GAMMA SQ
P.V.A. 0.5%*	288	37	0.18	231	21	0.11
M.C. 0.5%*	2013	120	0.68	576	31	0.31
Gelatin 0.5%*	1400	69	0.38	493	46	0.28
Lecithin 0.5%*	298	43	0.20	294	44	0.21

*w/v

formulations were prepared, some of which after emulsification with the high-speed agitator went directly through a process of fast solvent evaporation (rotary evaporator under reduced pressure) and some of which after emulsification with the high speed agitator were subjected to high pressure homogenization prior to the evaporation step. Table 2 shows the conditions employed and the corresponding values of the parameters for the particle size distributions. It can be appreciated that the homogenization step reduces in a higher level the particle size for those formulations giving the largest particles in the absence of this step; actually, when the particle size was relatively low, the complementary homogenization procedure merely gave a less spread out particle size distribution. These results, while showing the nature of the tensioactive agent used primarily determines the drop size of the emulsion firstly formed, also suggest that the effectiveness of the homogenization process is dependent on the original size of the droplets.

In line with the preliminary results, a statistical design was chosen in order to evaluate simultaneously the most relevant factors for controlling particle size: phase volume ratio, polymer concentration and homogenization pressure, on the particle size distribution. It may be presumed that these variables influence the droplet size in the emulsion initially formed and also probably the precipitation rate of the polymer as a consequence of solvent evaporation. The extreme values assigned to the independent

TABLE 3

Real values corresponding to the three variables central composite design employed and particle size distribution parameters (mean particle size, coefficient of variation and polydispersity) of the formulations designed. Results are the average of 5 determinations carried out for two preparations. X_1: Phase volume ratio (per cent v/v); X_2: PLA/GA concentration in organic phase (per cent w/v); X_3: Homogenization pressure (bars)

	Independent variables						Response values		
	Orthogonal values			Real values					
Formulation	X_1	X_2	X_3	X_1	X_2	X_3	Size (nm)	C.V. (%)	MU2/GAMMA SQ
1	-1	-1	-1	15.00	2.50	100.00	178	22	0.080
2	1	-1	-1	35.00	2.50	100.00	325	22	0.081
3	-1	1	-1	15.00	7.50	100.00	268	27	0.062
4	1	1	-1	35.00	7.50	100.00	406	33	0.142
5	-1	-1	1	15.00	2.50	200.00	188	38	0.090
6	1	-1	1	35.00	2.50	200.00	332	18	0.080
7	-1	1	1	15.00	7.50	200.00	236	25	0.097
8	1	1	1	35.00	7.50	200.00	466	34	0.178
9	-1.682	0	0	8.18	5.00	150.00	177	32	0.088
10	1.682	0	0	41.82	5.00	150.00	616	40	0.143
11	0	-1.682	0	25.00	0.79	150.00	220	31	0.126
12	0	1.682	0	25.00	9.20	150.00	343	25	0.105
13	0	0	-1.682	25.00	5.00	65.90	312	22	0.107
14	0	0	1.682	25.00	5.00	234.10	273	24	0.107
15	0	0	0	25.00	5.00	150.00	279	21	0.046
16	0	0	0	25.00	5.00	150.00	289	21	0.068
17	0	0	0	25.00	5.00	150.00	308	31	0.055
18	0	0	0	25.00	5.00	150.00	294	22	0.063
19	0	0	0	25.00	5.00	150.00	291	23	0.059
20	0	0	0	25.00	5.00	150.00	278	22	0.065

variables were at first checked to see if they indeed give nanoparticle suspensions. Table 3 displays the structure of the reduced statistical design (real and orthogonalized values of the independent variables) and the average data of the parameters describing the particle size distribution (dependent variables) of the selected formulations.

The equations of the response surfaces corresponding to each of the parameters feature are:

Mean size (nm) = $290.73 + 102.28\ X_1 + 41.03\ X_2$

Variation coefficient = $23.47 + 4.07 X_1^2 + 4.31\ X_1 X_2$

MU2/GAMMA SQ = $5.98\ 10^{-2} + 1.79\ 10^{-2} + 1.68\ 10^{-2} X_1^2 + 1.68\ 10^{-2} X_2^2 + 1.38\ 10^{-2} X_2^2 + 1.38\ 10^{-2} X_3^2 + 2.13\ 10^{-2} X_1 X_2$

Where: X_1, X_2, X_3 represent the orthogonalized values of the independent variables.

Statistical analysis of these results (Table 4), likewise is shown by the response surfaces (Figure 1), indicates homogenization pressure was not significant as regards the mean particle size and coefficient of variation of the particle size distribution; it can be said then, that these principally depend on the formulation variables. This notwithstanding, the polydispersity did show such dependency, its minimum value (indicating an unimodal distribution) corresponding to intermediate pressures. Two relevant response surfaces for polydispersity are shown (Fig. 1c), the uppermost of which corresponds to the extreme conditions of the design (orthogonalized values: $X_3 = +1.682$ and $X_3 = -1.682$), while the lowermost corresponds to the intermediate conditions of the design (orthogonalized value: $X_3 = 0$). The explanation of these results lies in the functioning of the homogenization system, in which, the solution is forced through a series of 10 cells, where the compression and decompression of the arriving wave and the associated phenomenon of cavitation take place. Logically, the intensity of these phenomena is dependent on the homogenization pressure. Unimodal distributions at intermediate pressures point towards a real effect of homogenization of the sample as it goes through successive cells. It is possible that simultaneous fracture and/or fusion of droplets occurs at the highest values of pressure to give bimodal distribution profiles without the coefficient of variation being substantially modified.

The response surface corresponding to the mean particle size (Figure 1a) demonstrates a notable influence of the phase volume ratio at all the polymer concentrations studied, such that the greater the volume of organic phase, the greater the particle size; also increasing the polymer concentration at each of the phase ratios

TABLE 4
Results of the ANOVA for the parameters indicated.

	Source of variation	S.S.	D.F.	F
Size	1st order	165892.5	3	43.7*
	2nd order	19346.4	6	2.5
	Error	12651.3	10	
	Total	197890.2	19	
C.V.	1st order	24.5	3	0.3
	2nd order	474.1	6	3.3**
	Error	239.2	10	
	Total	737.8	19	
MU2/GAMMA SQ	1st order	5.8 10-3	3	5.7**
	2nd order	13.2 10-3	6	6.4*
	Error	3.4 10-3	10	
	Total	22.2 10-3	19	

* Significant at $\alpha < 0.01$
** Significant at $\alpha < 0.05$

studied, drew an increase in particle size, but smaller than did increasing the phase ratio.These effects could be in accordance with a bigger droplet formed in the emulsion and possibly with a greater quantity of polymer to be dispersed. However, the fact that the formulation corresponding to the greatest percentage of organic phase and the lowest polymer concentration presented larger particles than the formulation corresponding to the lowest percentage of organic phase and the greatest polymer concentration, even though the amount of dispersed polymer is superior in the later case, allow us to deduce that the increase in size is not correlated with the increase in amount of polymer. The conclusion from this is that the amount of polymer does not determine particle size, but its concentration in the organic phase and the phase ratio do.

The response surface corresponding to the coefficient of variation of the particle size distribution (Figure 1b) reflects an increase in this parameter at the extreme working conditions. These conditions refer either to a low phase ratio and a low polymer concentration or a high phase ratio and a high polymer concentration, which means that there is a significant interaction between the two factors. What also stands out is the fact that, the decrease in particle size found as the phase ratio decreased, correlated with an

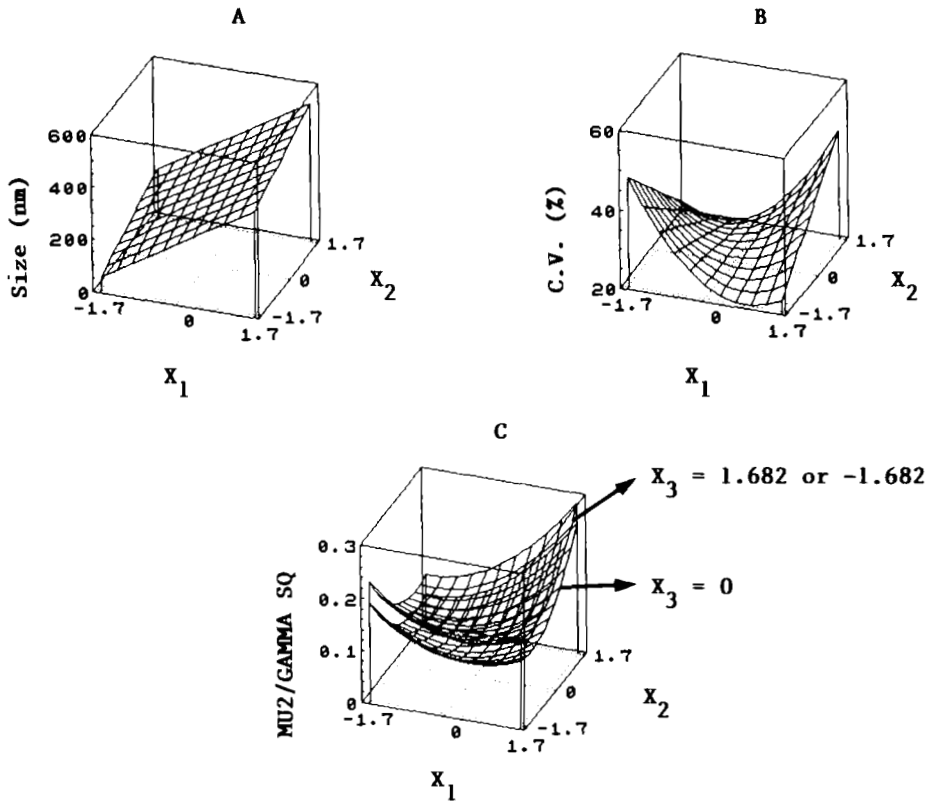

FIGURE 1

Three-dimensional response surface showing the effect of phase volume ratio (O/W) (X_1) and polymer concentration (X_2) on mean particle size distribution (a), coefficient variation (b) and polydispersity (c).

increase in the coefficient of variation, although for intermediate values of both parameters the coefficient of variation was a minimum.

In the last part of this work the previous information was taking into account to select the variables to be investigated according to an experimental factorial design. Table 5 shows the levels of the factors under study(volume phase ratio and concentration of PVA used as an emulsifier) and the parameters characterizing the particle size distribution of the formulations designed. From these data, the surface response equations were calculated by analysis of variance and subsequent sequencial regression analysis (15), showing the feature:

TABLE 5

Particle size distribution parameters (mean particle size, coefficient of variation (CV) and polydispersity (MU2/GAMMA SQ) of the formulations designed according to an 3^2 experimental factorial design. Results are expressed as the average of 5 determinations for each of the two preparations.

	Independent variables		Response variables		
Formulation	Y_1	Y_2	Size (nm)	C.V. (%)	MU2/GAMMA SQ
1	10	1	157.7	31.1	0.080
2	25	1	251.9	36.6	0.075
3	40	1	337.6	37.3	0.094
4	10	8	106.8	43.1	0.229
5	25	8	111.2	29.4	0.096
6	40	8	130.5	16.5	0.075
7	10	15	98.5	30.2	0.333
8	25	15	105.1	35.4	0.141
9	40	15	115.1	28.7	0.097

Y_1: Phase volume ratio (% v/v)

Y_2: PVA concentration in the aqueous phase (% w/v)

$$\text{Mean size (nm)} = 95.99 + 7.26\, Y_1 - 1.21\, Y_1Y_2 + 5.03\, 10^{-2} Y_1Y_2^2$$

$$R^2 = 0.9945$$

$$\text{MU2/GAMMA SQ} = 0.075 + 0.033\, Y_2 - 0.02\, Y_1Y_2 + 2.4\, 10^{-5}\, Y_1Y_2$$

$$R^2 = 0.9655$$

Where Y_1, Y_2 represent the real values of the independent variables (phase volume ratio and PVA concentration respectively)

The coefficient of variation was not significantly affected by the factors under evaluation as it is shown in table 6.

The corresponding surface response diagrams (Figure 2) evidence the influence of the PVA concentration, until the limit of 8-10 %, on the particle size; although this influence is only appreciable for the highest phase volume ratios. Moreover, the observed influence of phase volume ratio on particle size is insignificant when the concentration of the PVA in the dispersion medium is very high. From the observation of the surface response related to the polydispersity, it is clear the interaction between the two factors,

TABLE 6

Results of the ANOVA for the parameters indicated

Parameter	Source of variation	S.S.	d.f.	F
Size	Y_1	16166.1	2	196.3*
	Y_2	76288.1	2	926.4*
	$Y_1 Y_2$	17118.8	4	103.9*
	Error	370.5	9	
	Total	109943.6	17	
C.V.	Y_1	188.2	2	1.2
	Y_2	90.1	2	0.6
	$Y_1 Y_2$	615.6	4	1.9
	Error	712.3	9	
	Total	1606.2	17	
MU2/GAMMA SQ	Y_1	0.056	2	145.6*
	Y_2	0.034	2	88.4*
	$Y_1 Y_2$	0.035	4	45.5*
	Error	0.002	9	
	Total	0.127	17	

* Significant at $\alpha < 0.01$

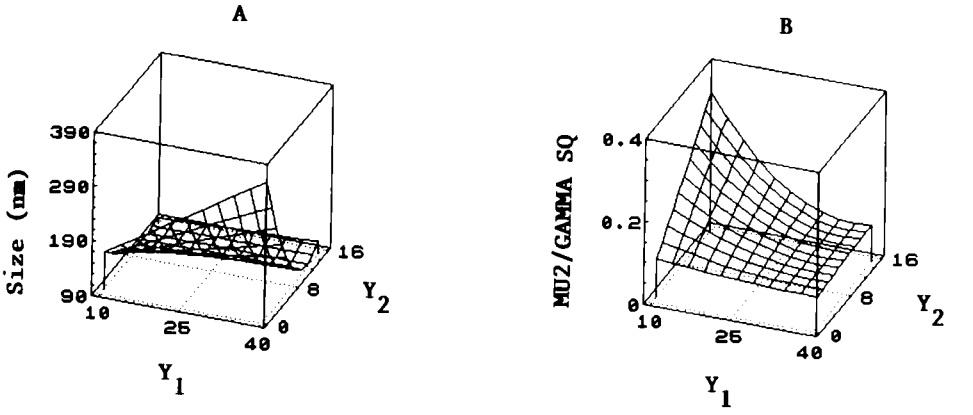

FIGURE 2

Three-dimensional response surface showing the effect of the phase volume ratio (O/W) (Y_1) and dispersing agent (PVA) concentration (Y_2) on the mean particle size distribution (a) and the polydispersity (b).

displaying the broadest particle size distribution pattern for the smaller volume phase ratio and the highest concentration of dispersing agent. From this study we can elicit that, if a small mean particle size and a narrow unimodal distribution is desired, it is necessary to prepare the particles using a low phase volume ratio and a relatively low concentration of PVA or, in other case, the opposite conditions, which means a high phase volume ratio and a great concentration of emulsifier. In addition, taking into account the values of the correlation coefficients calculated by sequencial regression analysis, the described equations could be applied to deduce the necessary conditions to achieve a specific particle size distribution pattern.

CONCLUSIONS

The work presented here describes a rigorous analysis of the formulation and technological variables that might have influence individually and/or jointly on the particle size distribution, revealing, by way of the response surfaces, the importance of the characteristics of the emulsion formed prior to the particles. Furthermore, it is seen that regardeless of the possibility of modulating the particle size by designing the formulations adequately, on occasions, modifying the size implies a fall in the homogeneity of the sample. The study also emphasizes the need to choose experimental designs for the information they can provide about the process to develop new formulations.

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