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Statistical experimental design based studies on placebo and mitoxantrone-loaded albumin microspheres

Christian P. Luftensteiner, Helmut Viernstein *

Institute of Pharmaceutical Technology, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria

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Abstract

A new technique for the preparation of hydrophilic, nonaggregating placebo albumin microspheres was developed. The influence of albumin concentration, poloxamer concentration, pH of albumin solution, and dispersion energy on mean microsphere size was investigated using a central composite design. The microspheres exhibited mean particle sizes in the range from 15 to 69 μm. Mitoxantrone-loaded microspheres were prepared by dissolving the drug in an albumin solution prior to dispersion in the coherent phase. Effects and interactions of four relevant process factors (type of dehydrating agent, amount of glutaraldehyde, amount of mitoxantrone, and stabilisation time) on drug incorporation and particle diameter were determined according to a factorial design. Microspheres with mitoxantrone content from 6.1 to 11.5% (w/w), drug entrapment efficiency from 58 to 91%, and mean particle sizes from 25 to 36 μm were obtained. Variation of stabilisation time showed no influence on the response variables. Nonaggregating particles with a smooth surface and high drug entrapment efficiency were obtained using a mitoxantrone/albumin ratio of 1/10 (w/w). The drug content was increased by the application of butanol instead of acetone as the dehydrating solvent while simultaneously increasing the amount of glutaraldehyde. The drug release following first order kinetics was delayed by increasing the crosslinking degree.

Keywords: Albumin microspheres; Mitoxantrone; Particle size; Drug content; Drug release; Experimental design

1. Introduction

The aim of targeted chemotherapy is to alter the distribution, uptake, or effects of anticancer drugs in such a way that the tumour cells are damaged substantially more than normal cells. For the vectoring of cytotoxic compounds to target areas liposomes, nanoparticles, and microspheres have been suggested. Since organ distribution of the latter is dependent upon their size and shape, it is reasonable to attempt passive targeting of microspheres on this basis (Ranade and Hollinger, 1995). A simple alteration in particle

* Corresponding author. Tel.: +43 1 313368470; fax +43 1 31336779.

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Table 1
Factors and levels investigated in the preparation of placebo albumin microspheres

Factor	Level				
	−1.547	−1	0	+1	+1.547
Poloxamer concentration (% w/v)	12.3	15	20	25	27.7
Albumin concentration (% w/v)	12.3	15	20	25	27.7
pH of the albumin solution	5.49	5.9	6.65	7.4	7.81
Dispersion energy (speed setting)	3.5	4	5	6	6.5

size and/or the route of administration leads to a manipulation of targeting (Burger et al., 1985).

Recently, Codde et al. (1993) reported that targeting of doxorubicin using microspheres (particle size 32.5 μm) increased the antitumour efficacy in the treatment of liver cancer compared to the delivery of free or liposomal drug; simultaneously systemic toxicity was reduced. Nishioka et al. (1994) examined microspheres of different particle sizes (smaller than 20 μm or 20–37 μm) containing cisplatin for chemoembolisation therapy via the hepatic artery and concluded that microsphere size was strongly correlated with the augmentation of the antitumour effect. Further, Burt et al. (1995) developed 'large' (30–100 μm) and 'small' (10–30 μm) taxol-loaded microspheres for arterial chemoembolisation.

In addition, during the last decade microspheres were examined for intraperitoneal delivery of anticancer drugs, based on the finding that particles showing 15 μm in diameter are retained in the abdominal cavity for a long period (Ugelstad et al., 1984). Hagiwara et al. (1993) reported a marked therapeutic benefit with little systemic side effects following intraperitoneal administration of cisplatin incorporated in microspheres (50–150 μm) to patients with malignant ascites. Recently, Demetrick et al. (1997) demonstrated that taxol-loaded microspheres (30–100 μm) were more effective than drug solution in preventing intraperitoneal tumour seeding.

Mitoxantrone (MXN), a synthetic anthracenedione derivative, has proved successful in the intraarterial treatment of hepatic carcinoma and breast carcinoma, the regional treatment of malignant effusions, and the intraperitoneal therapy of ovarian carcinoma. However, leucopenia may be dose limiting for systemic administration, whereas local toxicity may limit the dosage in intracavitary treatment (Faulds et al., 1991). Little work has been done to overcome these restrictions. Therefore, in this study we investigated the entrapment of MXN within microspheres. This formulation might have potential for targeted delivery of the drug via arterial chemoembolisation or for sustained intraperitoneal delivery of MXN in terms of higher antitumour efficacy and reduced systemic or local tissue toxicity.

For such drug targeting purposes albumin microspheres are well suited due to their biodegradability, biocompatibility, high stability, shelf life, controllable drug-release properties, and high loading capacity for hydrophilic molecules as a result of the drug-binding properties of native albumin (Müller et al., 1996). Unfortunately, albumin microspheres prepared by common techniques, using vegetable oil as the coherent phase, are hydrophobic; also aggregation problems have limited the utility of these methods (Roser and Kissel, 1993; personal communication). Consequently, we present experiments on the preparation of hydrophilic, nonaggregating albumin microspheres.

Table 2
Factors and levels investigated in the preparation of MXN-loaded albumin microspheres

Factor	Level	
	−1	+1
Type of dehydrating agent	Acetone	1-Butanol
Amount of glutaraldehyde (μl)	100	500
Amount of MXN (mg)	17.5	30
Stabilisation time (h)	1	5

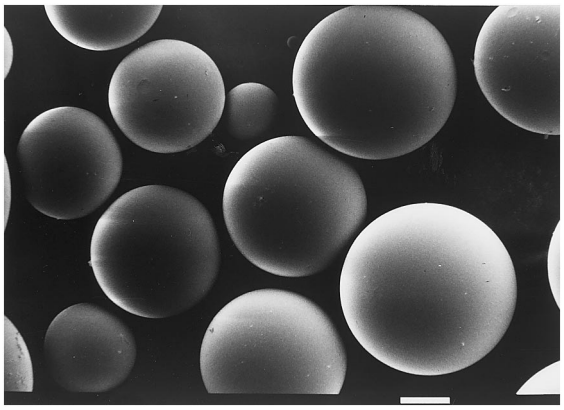

Fig. 1. Scanning electron micrograph (1000 ×) of the placebo albumin microspheres showing a mean diameter of 37.6 μm. Scale bar = 10 μm.

Although it has been demonstrated that many process factors can influence microsphere properties (Gupta and Hung, 1989), rarely statistical experimental design has been employed in the development of a manufacturing technique for albumin microspheres. Mostly single-factor experiments were reported with a lack of information about the potential interactions of two or more process factors. In the present study factorial and central composite design methodology (Leuenberger, 1991; Montgomery, 1997) were employed to find the significant effects and interactions of relevant process factors on the response variables.

2. Materials and methods

2.1. Materials

MXN dihydrochloride (MXN·2HCl; 1,4-dihydroxy-5,8-bis-((2-((2-hydroxyethyl)amino)ethyl) × amino)-9,10-anthracenedione dihydrochloride) was supplied by Wyeth-Lederle (Vienna, Austria). Human serum albumin fraction V, aqueous glutaraldehyde solution (25% w/w; grade II), and polyoxyethylenesorbitan monooleate (Tween® 80) were purchased from Sigma (St. Louis, MO). Poloxamer 188 (Synperonic® F68) was obtained from Serva (Heidelberg, Germany) and pepsin (from porcine stomach mucosa; activity: 3090 U mg^{-1} solid) from Calbiochem-Novabiochem (La Jolla, CA). All other reagents and solvents were of analytical grade.

Glutaraldehyde was purified by distillation (Gillett and Gull, 1972), diluted to a 10% (w/v) solution in dichloromethane and stored at −30°C until use.

2.2. Determination of water- and solvent-content

The described weights of albumin, MXN·2HCl, and microspheres refer to dry solids. Water- and solvent-content of the materials used was determined by thermogravimetric analysis (TGA 7, Perkin-Elmer, Norwalk, CT). Loss of sample weight was measured from 30 to 130°C at a scanning rate of 10°C min^{-1} using nitrogen as the sample purge gas.

2.3. Placebo albumin microspheres

2.3.1. Experimental design

An orthogonal central composite design (Leuenberger, 1991) was created to study the main effects and interactions of four factors on mean particle size. The factors investigated were poloxamer concentration of the organic phase, albumin concentration of the aqueous phase, pH value of the albumin solution, and speed setting of the vortex mixer (dispersion energy); their levels are listed in Table 1. Twenty-seven experiments were required and the combinations were performed in random order. Sixteen runs represent the simple factorial design, 8 the star points, and the center points were replicated three times. The axial distance (α) of the star points was 1.547.

2.3.2. Preparation technique

A technique for manufacturing albumin microspheres has been developed by modification of the emulsion crosslinking method described by Longo et al. (1982).

An aqueous solution of human serum albumin (12.3–27.7% w/v) was adjusted to the desired pH value (5.49–7.81) using hydrochloric acid or sodium hydroxide. The albumin solution (1 ml) was dispersed in 10 ml dichloromethane contain-

Table 3
Results of the design for placebo albumin microspheres

Experiment No.	Poloxamer concentration (% w/v)	Albumin concentration (% w/v)	pH of albumin solution	Dispersion energy (speed setting)	Mean particle size (μm)
1	20	20	5.49	5	37.3
2	25	15	5.9	6	18.7
3	15	25	5.9	4	68.5
4	20	27.7	6.65	5	57.2
5	20	12.3	6.65	5	29.6
6	20	20	6.65	5	39.6
7	27.7	20	6.65	5	22.8
8	15	25	7.4	4	58.1
9	15	25	5.9	6	51.2
10	15	15	5.9	4	58.3
11	20	20	6.65	5	37.6
12	25	25	5.9	4	54.4
13	15	25	7.4	6	41.2
14	12.3	20	6.65	5	55.8
15	25	25	7.4	4	38.9
16	15	15	7.4	4	55.0
17	15	15	5.9	6	34.1
18	25	15	7.4	4	32.8
19	20	20	6.65	6.5	28.1
20	20	20	6.65	5	40.5
21	20	20	7.81	5	31.6
22	20	20	6.65	3.5	55.7
23	15	15	7.4	6	29.7
24	25	15	5.9	4	37.5
25	25	15	7.4	6	15.3
26	25	25	7.4	6	27.9
27	25	25	5.9	6	35.1

Table 4
Placebo microspheres: summary of the ANOVA for mean particle size

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio	p-Value
A: poloxamer concentration	1676.8	1	1676.8	163.1	0.0000
B: albumin concentration	899.47	1	899.47	87.49	0.0000
C: pH of albumin solution	220.60	1	220.60	21.46	0.0002
D: dispersion energy	1793.5	1	1793.5	174.4	0.0000
BB	47.870	1	47.870	4.656	0.0433
BC	46.683	1	46.683	4.558	0.0457
Error	205.62	20	10.281		
Total	4890.5	26			

ing poloxamer 188 (12.3–27.7% w/v) by vortexing (REAX 2000, Heidolph, Kelheim, Germany) for 5 min (speed setting 3.5–6.5). Subsequently 500 μl of a 10% (w/v) solution of glutaraldehyde in dichloromethane were added in order to harden the formed droplets. After initial crosslinking (30 s) 20 ml acetone were added. During stabilisation (5 h) agitation at 200 rpm was performed with a shaking apparatus (SM 25 B digi Swip, Edmund Bühler, Bodelshausen, Germany). Finally the microspheres were washed with acetone (seven times) and water (three times) and recovered by

Table 5
Results of the design for MXN-loaded albumin microspheres

Experiment No.	Type of dehydrating agent	Amount of glutaraldehyde (μl)	Amount of MXN (mg)	Stabilisation time (h)	Drug content (% w/w)	Drug entrapment efficiency (%)	Mean particle size (μm)
1	Butanol	500	17.5	1	7.94	88.6	25.7
2	Butanol	100	30	1	8.35	58.4	32.6
3	Acetone	100	17.5	1	7.22	80.6	25.6
4	Butanol	500	17.5	5	8.13	90.8	26.1
5	Butanol	100	30	5	8.68	60.7	28.6
6	Butanol	500	30	1	11.00	77.0	35.4
7	Acetone	100	30	1	9.20	64.4	32.0
8	Acetone	100	17.5	5	7.53	84.1	27.5
9	Acetone	100	30	5	9.14	64.0	34.4
10	Butanol	500	30	5	11.46	80.2	36.2
11	Acetone	500	17.5	5	6.06	67.7	25.0
12	Acetone	500	30	1	9.80	68.6	35.4
13	Acetone	500	30	5	9.83	68.8	34.5
14	Butanol	100	17.5	5	8.12	90.7	26.5
15	Butanol	100	17.5	1	8.19	91.4	26.3
16	Acetone	500	17.5	1	6.53	72.9	28.3

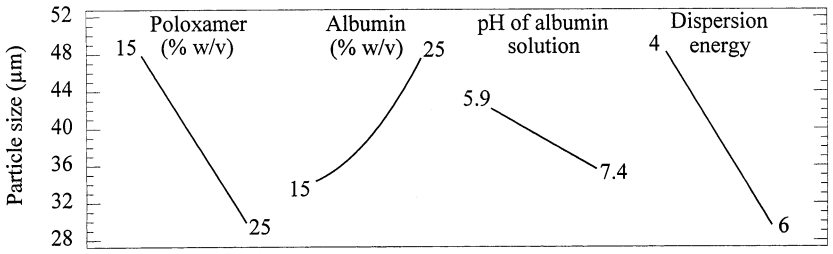

Fig. 2. Central composite design for the placebo albumin microspheres: plot of the main effects of the process factors on mean particle size.

centrifugation at 15600 × *g* and 4°C for 10 min (Z 323 K, Hermle, Wehingen, Germany). After the second washing step with acetone the product was sonicated for 30 s with approximately 20 W at 20 kHz (microtip ultrasonic horn MS 73 connected to a Sonopuls HD 70, Bandelin, Berlin, Germany). An aqueous suspension of microspheres was frozen in liquid nitrogen and lyophilised at 0.1 mbar and −40°C for 24 h (Beta 1-8K, Martin Christ, Osterode am Harz, Germany). The resulting free-flowing powder was stored in a desiccator at 4°C.

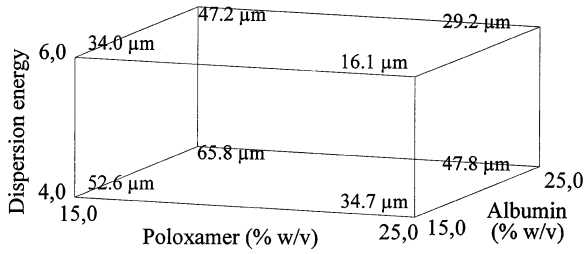

Fig. 3. Central composite design for the placebo albumin microspheres: cube plot of the values for mean particle size for all combinations of low and high levels of albumin concentration, poloxamer concentration, and dispersion energy.

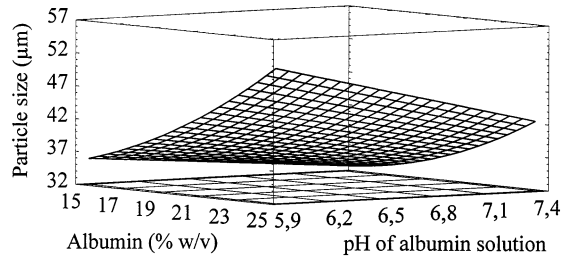

Fig. 4. Central composite design for the placebo albumin microspheres: response surface plot of the interaction between albumin concentration and pH of the albumin solution on mean particle size.

2.4. MXN-loaded albumin microspheres

2.4.1. Experimental design

Four factors were investigated for their main effects and interactions on drug content, drug entrapment efficiency, and mean particle size using a factorial design of 2^4 experiments. The factors studied were type of dehydrating agent, amount of glutaraldehyde, amount of MXN (calculated as free base), and stabilisation time; and their levels are shown in Table 2. The experiments were performed in random order.

2.4.2. Preparation technique

The desired amount of MXN · 2HCl (calculated as free base: 17.5–30 mg) was dissolved in 1 ml of an aqueous solution of albumin (17.5% w/v). The mixture was gently stirred for 1 h. Subsequently

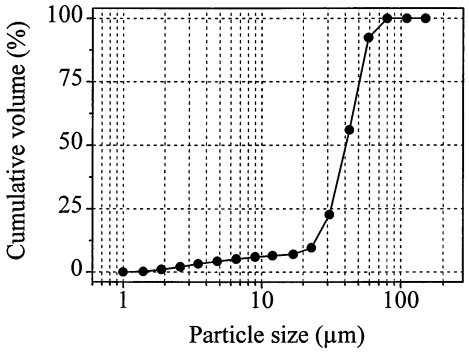

Fig. 5. Particle size distribution of the placebo albumin microspheres which were prepared at the centerpoint levels of the process factors (mean diameter: 40.5 μm).

Table 6
MXN-loaded microspheres: summary of the ANOVA for drug content

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-ratio	*p*-Value
A: type of dehydrating agent	2.6896	1	2.6896	63.01	0.0000
B: amount of glutaraldehyde (μl)	1.1664	1	1.1664	27.32	0.0008
C: amount of MXN (mg)	19.669	1	19.669	460.8	0.0000
AB	2.2952	1	2.2952	53.77	0.0001
AC	0.7744	1	0.7744	18.14	0.0028
BC	5.1984	1	5.1984	121.8	0.0000
ABC	0.3080	1	0.3080	7.215	0.0277
Error	0.3415	8	0.042688		
Total	32.443	15			

the aqueous phase was dispersed in 10 ml of a solution of poloxamer (30% w/v) in dichloromethane by vortexing at speed setting 5. Further manufacturing steps were performed in the same manner as described for the preparation of the placebo microspheres.

2.5. Characterisation of placebo and MXN-loaded albumin microspheres

2.5.1. Particle size measurement

The size distribution of the microspheres was examined using a laser diffraction type particle size analyser (SALD-1100, Shimadzu, Kyoto, Japan). The particles were dispersed in an aqueous solution of 0.2% (w/v) Tween® 80 and 0.9% (w/v) sodium chloride by sonication in an ultrasonic bath with 35 W at 47 kHz for 1 min. The dispersions were diluted until the turbidity was within the optimal analysis range, the particle size distribution was measured and the mean diameter (50% volume average) was calculated.

2.5.2. Morphological analysis

The particles were sprinkled onto a double-sided tape and sputter-coated (Hummer JR, Technics, Dublin, CA) with gold. The morphology was characterised by scanning electron microscopy (JSM-35CF, Jeol, Tokyo, Japan) at 5 kV.

2.5.3. Determination of MXN content

MXN-loaded (5 mg) microspheres were dispersed in 15 ml of a buffer solution (100 mM KCl–HCl, pH 2.0) by sonication in an ultrasonic bath with 80 W at 30 kHz for 30 s. Subsequently 10 ml of a pH 2.0 buffer solution containing pepsin (5 mg ml^{-1}) were added. After shaking in a water bath at 37°C for 48 h the enzymatic degradation of particles was achieved; complete dissolution of the matrix components was verified by light microscopy. The MXN concentration of the solution was determined photometrically at 608 nm (U-2000, Hitachi, Tokyo, Japan). Controls were performed with drug or drug plus placebo microspheres incubated with pepsin. In both cases no loss of MXN was observed.

The drug content was calculated as weight of incorporated MXN (free base) per total weight of microspheres in percent. The drug entrapment efficiency was described according to the following equation:

$$E = D \times (\mathrm{HSA} + \mathrm{MXN} \cdot 2\mathrm{HCl})/\mathrm{MXN}$$

where E (%) is the drug entrapment efficiency, D (%) is the drug content, HSA (mg) is the amount of albumin, MXN·2HCl (mg) is the amount of MXN dihydrochloride, and MXN (mg) is the amount of MXN (free base).

2.5.4. Examination of MXN release

The MXN release from the microspheres was examined in vitro using a continuous flow (open) system. Microspheres containing 2 mg of MXN were placed in a release cell (capacity 20 ml; Desaga, Heidelberg, Germany) and eluted with physiological saline at 37°C under magnetically

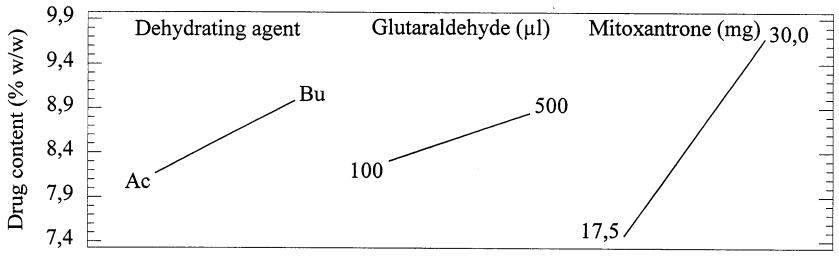

Fig. 6. Factorial design for the MXN-loaded albumin microspheres: plot of the main effects of three process factors on the drug content (Ac, acetone; Bu, butanol).

stirring at 600 rpm and at a flow rate of 10 ml h^{-1}. Samples of the effluent were taken at appropriate time intervals and analysed for the drug concentration photometrically at 608 nm. The release profiles were fitted using χ^2 minimisation (ORIGIN 4.10, Microcal, Northampton, MA).

2.6. Data analysis of the experimental designs

The main effects and interactions of the factors investigated on the response variables were estimated by applying the statistical program STATGRAPHICS (Statgraphics Plus for Windows 2.0, Manugistics, Rockville, MD). Analysis of variance (ANOVA) was used to test the statistical significance of each source of variation in the microsphere characteristics (Montgomery, 1997). The sums of squares of insignificant ($p > 0.05$) effects and interactions were added to the experimental error. Summarising equations for the response variables were obtained by regression analysis (Leuenberger, 1991).

3. Results and discussion

3.1. Placebo microspheres

Preliminary studies indicated that common preparation techniques for albumin microspheres, using vegetable oil as the lipophilic phase, lead to undesirable products, which usually require surfactants to make the aqueous dispersions and show a high aggregation grade. Longo et al. (1982) developed a new method, applying a concentrated solution of polymethylmethacrylate or of a polyoxyethylene–polyoxypropylene block copolymer as the coherent phase, to overcome these problems. Recently, also hydroxypropylcellulose (Roser and Kissel, 1993) and an aliphatic polyurethane (Latha and Jayakrishnan, 1995) were used as dispersion stabilisers and hydrophilic, nonaggregating particles were obtained.

In our opinion the polymer used should exhibit amphiphilic properties to allow superior dispersion stabilisation and easy removal by common washing solvents. The polymer poloxamer 188 fulfils these features. Because of its toxicological safety this polymer is also used in parenteral formulations. In contrast to Longo et al. (1982) and Roser and Kissel (1993) we used dichloromethane instead of chloroform as the solvent for the polymer with the intention of reducing the toxicological risk of residual solvent in the microspheres (Dalby, 1993). The residual dichloromethane content in the microspheres prepared by our technique was less than the limit (500 ppm) given by the US Pharmacopeia XXIII (1995) (unpublished data). Furthermore, in the present study the crosslinking agent was distilled prior to use in order to obtain pure monomeric glutaraldehyde and therefore to increase the reproducibility of the denaturation extent. We found that the addition of a dehydrating agent (acetone or 1-butanol) after initial crosslinking is necessary and the key to obtaining completely nonaggregating particles. Fig. 1 (scanning electron micrograph) shows the smooth spherical geometry of the albumin microspheres typically prepared by the procedure described. The microspheres were easily dispersable in aqueous media without the need for any surfactant.

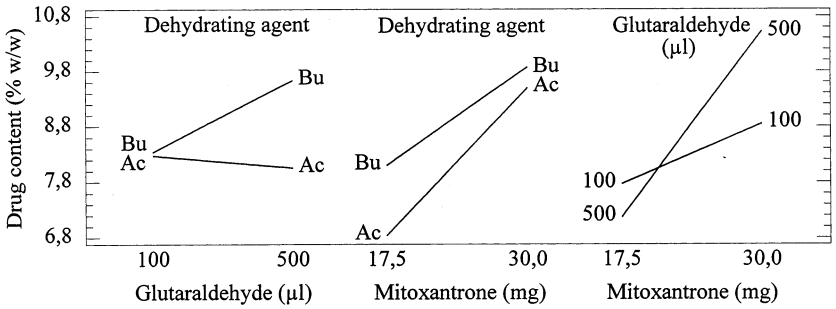

Fig. 7. Factorial design for the MXN-loaded albumin microspheres: plot of the interactions of three process factors on the drug content (Ac, acetone; Bu, butanol).

Using a central composite design we investigated the main effects and interactions of the relevant process factors on mean particle size. Microspheres with mean diameters from 15.3 to 68.5 μm were prepared (Table 3). All the experimental factors exhibited remarkably significant ($p < 0.001$) main effects on the response variable (Table 4). Applying a higher poloxamer concentration, a lower albumin concentration, or a higher dispersion energy input resulted in smaller particles (Figs. 2 and 3). These effects were undoubtedly caused by changes in viscosity or turbulence and are in agreement with studies reported previously for other preparation techniques (Gupta and Hung, 1989). Increasing the pH value of the albumin solution decreased the mean particle diameter (Fig. 2). This effect ($p < 0.001$) and the interaction ($p < 0.05$) with albumin concentration (Fig. 4) can be attributed to a pH dependent conformational change in the albumin, which offers different crosslinking sites and therefore a modification of the matrix density. The albumin concentration also contributed as a quadratic term ($p < 0.05$) to the following summarising equation for mean particle size:

$$\begin{aligned}&\text{Mean particle size } (\mu\text{m})\\ &= 95.97 - 1.796\\ &\quad \times (\text{poloxamer concentration } (\%\text{w/v})) + 1.073\\ &\quad \times (\text{albumin concentration } (\%\text{w/v})) + 4.766\\ &\quad \times (\text{pH of albumin solution}) - 9.289\\ &\quad \times (\text{dispersion energy (speed setting)}) + 8.18\\ &\quad \times 10^{-2} \times (\text{albumin concentration})^2 - 0.4555\\ &\quad \times (\text{albumin concentration})\\ &\quad \times (\text{pH of albumin solution})\end{aligned}$$

The particle size distribution of the microspheres was very narrow and is shown for a batch, which was prepared at the centerpoint levels of the process factors, in Fig. 5.

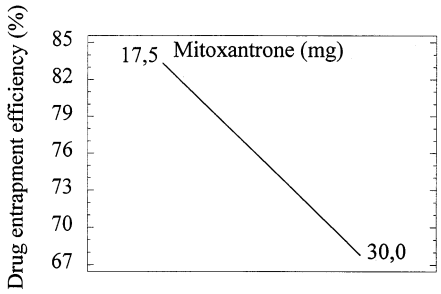

Fig. 8. Factorial design for the MXN-loaded albumin microspheres: plot of the main effect of the applied amount of MXN on the drug entrapment efficiency.

3.2. MXN-loaded albumin microspheres

Albumin microspheres are well suited for the incorporation of MXN due to its high binding affinity to albumin. However, postloading of placebo microspheres by equilibration in a solution of MXN·2HCl resulted in low drug content and low entrapment efficiency (unpublished data). Consequently, the drug was added to the aqueous solution of albumin before dispersion and crosslinking. In order to ensure that a high

Table 7
MXN-loaded microspheres: summary of the ANOVA for mean particle size

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio	p-Value
A: amount of glutaraldehyde (μl)	10.726	1	10.726	4.763	0.0497
B: amount of MXN (mg)	210.98	1	210.98	93.69	0.0000
AB	13.506	1	13.506	5.998	0.0307
Error	27.023	12	2.2519		
Total	262.24	15			

amount of drug was bound to the binding sites on the albumin molecules (Yapel, 1979), the MXN–albumin solution was equilibrated for 1 h.

For good stabilisation of the W/O dispersion poloxamer concentration was increased (30% w/v) in comparison to the preparation of the placebo microspheres (12.3–27.7% w/v). With this modification nonaggregating particles were obtained.

A 2^4 factorial design was chosen for the evaluation of the main effects and interactions of the process factors on the response variables. Microspheres exhibiting MXN content from 6.06 to 11.46% (w/w), drug entrapment efficiency from 58.4 to 91.4%, and mean particle sizes from 25.0 to 36.2 μm were prepared (Table 5). The stabilisation time had no significant ($p < 0.05$) influence on any of the response variables.

The main effect of changing the dehydrating agent from acetone to butanol, increasing the amount of glutaraldehyde, or increasing the amount of MXN was an increase in drug content ($p < 0.001$, Table 6, Fig. 6). In Fig. 7 the two-factor interactions are shown. Changing the dehydrating agent had only an evident effect when a high amount of glutaraldehyde was used ($p < 0.001$) and showed a major effect at a low level of MXN amount in comparison to a high level ($p < 0.005$). It seems that the release of MXN from the microspheres to the coherent phase was slower when butanol was used as the dehydrating agent instead of acetone, especially at the high crosslinking extent or low MXN content in the microspheres. Using a large amount of MXN a decrease in the degree of crosslinking accelerated the release of MXN to the coherent phase or the washing solvents, whereas at a low level of MXN amount this effect seemed to be prevented or competed with another action ($p < 0.001$).

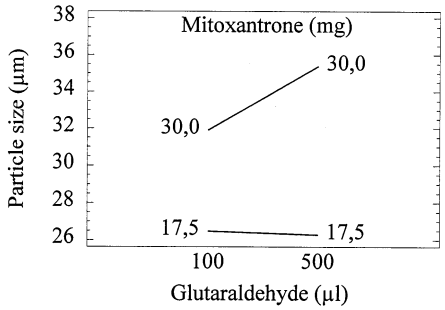

Fig. 9. Factorial design for the MXN-loaded albumin microspheres: plot of the interaction between the applied amounts of MXN and glutaraldehyde on the mean particle size.

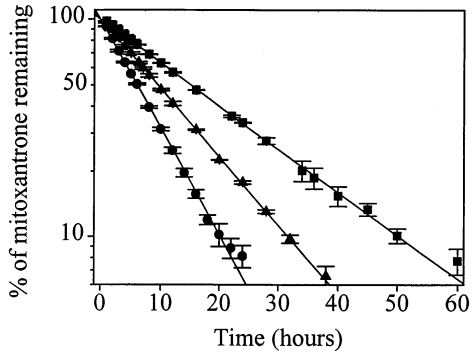

Fig. 10. In vitro release of MXN from the microspheres stabilised with increasing quantities of glutaraldehyde solution: 100 μl (●), 300 μl (▲), and 500 μl (■) ml^{-1} of albumin–MXN solution. Error bars mark the S.D. ($n = 5$). Regression lines were fitted using χ^2 minimisation and indicate first order release kinetics.

In addition, a significant ($p < 0.05$) interaction between all three factors contributed to the summarising equation for MXN content:

$$\begin{aligned}&\text{Drug content (\% w/w)}\\&= 7.205 + 1.469 \times (\text{dehydrating agent}) - 9.48\\&\quad \times 10^{-3} \times (\text{amount of glutaraldehyde } (\mu\text{l}))\\&\quad + 4.06 \times 10^{-2} \times (\text{amount of MXN (mg)})\\&\quad - 7.43 \times 10^{-4} \times (\text{dehydrating agent})\\&\quad \times (\text{amount of glutaraldehyde}) - 6.85 \times 10^{-2}\\&\quad \times (\text{dehydrating agent}) \times (\text{amount of MXN})\\&\quad + 4.56 \times 10^{-4} \times (\text{amount of glutaraldehyde})\\&\quad \times (\text{amount of MXN}) + 1.11 \times 10^{-4}\\&\quad \times (\text{dehydrating agent})\\&\quad \times (\text{amount of glutaraldehyde})\\&\quad \times (\text{amount of MXN})\end{aligned}$$

where the factor 'dehydrating agent' takes the value -1 for acetone and $+1$ for butanol.

Analysis for the drug entrapment efficiency is not described in detail, because it revealed similar effects and interactions of the process factors compared to the analysis for the drug content. This is not surprising considering that these response variables are dependent on each other. One quite interesting main effect should be mentioned: increasing the amount of MXN resulted in a decrease in entrapment efficiency ($p < 0.001$, Fig. 8). The microspheres produced with 30 mg MXN exhibited a very rough surface, whereas the particles prepared with 17.5 mg MXN showed no irregularities. An increase in surface area caused the greater loss of MXN, which was observed in the aqueous washing steps.

The mean particle size was influenced by varying the amounts of MXN ($p < 0.001$, Table 7) and glutaraldehyde ($p < 0.05$). In this case the main effect of the amount of crosslinker seems to be meaningless. This factor had no effect, when the amount of MXN was at a low level. Whereas, the influence of the amount of glutaraldehyde was observable when the amount of MXN used was at a high level (Fig. 9). This interaction ($p < 0.05$) was easy to interpret, because aggregates were found in the products prepared with both factors at their high levels. Enlargement of the particles as a result of using a higher drug amount or, as MXN-loaded particles are larger than expected from the summarising equation for the placebo microspheres, even adding drug is in agreement with other studies (Gupta and Hung, 1989). The summarising equation for the mean particle size is:

$$\begin{aligned}&\text{Mean particle size } (\mu\text{m})\\&= 20.22 - 1.34 \times 10^{-2}\\&\quad \times (\text{amount of glutaraldehyde } (\mu\text{l})) + 0.3605\\&\quad \times (\text{amount of MXN (mg)}) + 7.35 \times 10^{-4}\\&\quad \times (\text{amount of glutaraldehyde})\\&\quad \times (\text{amount of MXN})\end{aligned}$$

MXN release from microspheres was investigated for batches prepared with butanol, 17.5 mg MXN and 1 h stabilisation time. 100, 300, or 500 μl glutaraldehyde solutions were used for stabilising the particles. It was demonstrated that release was delayed with an increase in crosslinking degree (Fig. 10). MXN liberation followed first order kinetics; the relationship is shown in the following equation:

$$A = A_0 \times [\exp(-k \times t)]$$

where A (mg) is the amount of the drug remaining to be released, A_0 (mg) is the original amount of the drug present in the carrier matrix, k (h^{-1}) is the first order release constant, and t (h) is the time in which the release of the drug occurs.

Release constants were 0.1140 ± 0.0013 h^{-1} for 100 μl, 0.0728 ± 0.0009 h^{-1} for 300 μl, and 0.0459 ± 0.0004 h^{-1} for 500 μl glutaraldehyde solution (5 measurements per batch). At least 95% of incorporated MXN were available in vitro. In this study, in our opinion, burst release was prevented due to the aqueous washing steps in the microsphere preparation.

4. Conclusions

By changing the levels of several process factors of the preparation technique described hydrophilic, nonaggregating placebo albumin microspheres exhibiting mean diameters from 15.3 to 68.5 μm were manufacturable. These microspheres might be appropriate for postloading with drugs, though not for MXN.

MXN-loaded microspheres with a high drug content as well as a high drug entrapment efficiency were obtained by applying a MXN/albumin ratio of 1/10 (w/w) and using butanol instead of acetone as the dehydrating solvent. Variation of the stabilisation time showed no influence on the drug content. By increasing the crosslinking degree of the MXN-loaded microspheres the drug release was successfully delayed and followed exactly first order kinetics, which is favourable since quality control is simplified.

To our knowledge, this study is the first developing MXN-loaded albumin microspheres for intraarterial and intraperitoneal drug delivery. The results of our present investigations allow preclinical evaluation of the formulation. In vivo studies concerning the intraperitoneal application of MXN-loaded microspheres are in progress and evaluation for chemoembolisation is suggested.

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