Optimized Formulation of Magnetic Chitosan Microspheres Containing the Anticancer Agent, Oxantrazole

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A combined emulsion/polymer cross-linking/solvent evaporation technique was used to prepare magnetic chitosan microspheres (MCM) containing the anticancer drug, oxantrazole. A central composite experimental design was used to simultaneously evaluate a variety of formulation factors on a number of response variables, such as the percentage of oxantrazole entrapped in the MCM. In association with the study design, statistical optimization procedures indicated the factors that significantly influence MCM preparation and what levels of the factors are needed to produce optimum MCM. Entrapment of anticancer agents into biodegradable microspheres is difficult because of low aqueous drug solubility and porosity of the particles. The latter effect was circumvented by a chitosan cross-linking step that resulted in ~3% (w/w) oxantrazole entrapment in the MCM via the optimization procedures. The combined formulation and statistical optimization strategy provide a basis to develop other microparticulate systems and led to a dosage form that can be used for future *in vivo* investigations.

KEY WORDS: magnetic microspheres; oxantrazole; statistical optimization.

INTRODUCTION

Targeting drugs to specific organs is actively pursued as a means to increase significantly a drug's therapeutic index. Magnetic microspheres containing chemotherapeutic agents have shown the ability to enhance greatly target organ drug concentrations compared to conventional modes of drug administration (1,2). Magnetic microspheres, typically formed from albumin, are retained in target site capillaries by the force of an external magnetic field. It has been suggested that albumin, and specifically magnetic polysaccharide microspheres prepared from chitosan, can also be retained in the vasculature by biochemical forces (3).

Attainment of high percentages (w/w) of drug entrapment into targeted microparticulate carriers will permit flexible dosage regimen design and lower microsphere doses. Lower carrier doses may enhance particle extravasation by preventing saturation of vascular endocytotic process. Further, potential adverse reactions to carrier material would be limited at lower microsphere doses (4,5).

Efforts to improve entrapment of bioactive materials into microparticulate carriers have resulted in the development of various strategies of formulation. The applied techniques include covalent bonding of antibodies to a microsphere matrix (6), drug complexation with macromolecular matrix components (7), and surface or matrix cross-linking (8,9). Covalent bonding and complexation techniques are usually limited by the chemical structure of the drug. On the other hand, cross-linking provides a more general approach for entrapment of a wide variety of biologically active compounds (8–10). A further advantage of carrier cross-linking is that both the extent of drug entrapment and the drug release rates can be controlled by varying the experimental conditions of cross-linking (8–11). Magnetic chitosan microspheres without drug have been formulated and were shown to bind to heparin, a model vascular endothelial glycosaminoglycan receptor (3). The objective of this investigation was to formulate magnetic chitosan microspheres containing oxantrazole (Fig. 1) that would be suitable for *in vivo* use (i.e., sufficient drug entrapment and particle size). Oxantrazole is an anthrapyrazole derivative shown to have antitumor activity similar to that of adriamycin but with fewer cardiotoxic side effects (12,13). A central composite experimental design (14,15) was utilized to develop microspheres with optimal characteristics.

MATERIALS AND METHODS

Chemicals

Chitosan (MW 200,000) was purchased from Proton Laboratories (Redmond, WA). Extra heavy mineral oil was purchased from Ruger Chemical Co. (Irvington, NJ). Ferrofluid (EMG) was obtained from Ferro Fluidics Corp. (Nashua, NH). Glutaraldehyde was purchased from Alfa Products (Danvers, MA). Oxantrazole was kindly provided by Warner-Lambert (Ann Arbor, MI). All other reagents were either HPLC or analytical grade.

Equipment

A Bransonic 200 ultrasonic water bath (Danbury, CT), a Bransonic ultrasonic probe, and a Brinkman Rotovapor-R rotary vacuum drier (Westbury, NY) were used for the preparation of the microspheres. Drug content analyses were performed with a HPLC system that included a Waters Model 510 pump (Milford, MA) and a Waters Model 481 variable-wavelength spectrophotometric detector. The analytical column was a C-8 Zorbax RX (25 × 0.4 cm, 5 μm; Mac Mod, Chadds Ford, PA), preceded by a C-8 Zorbax RX guard column (1.25 × 0.4 cm, 5 μm).

Preparation of Microspheres

Preparation of oxantrazole-loaded magnetic chitosan microspheres was based on a solvent evaporation technique reported earlier (3). The original technique was modified by cross-linking chitosan with gluturaldehyde, in the presence of oxantrazole solution, prior to emulsification. The base procedure for microsphere preparation was as follows. In a 75-ml double-walled beaker, 1.5 g of 5% (w/v) chitosan solution in 10% (v/v) acetic acid was vortexed with specified amounts of oxantrazole until a clear solution was obtained.

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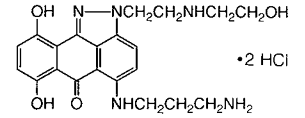

Fig. 1. Chemical structure of oxantrazole, 5-[(3-aminopropylamino)-7,10-dihydroxy-2-[(2-hydroxyethyl)amino]ethyl]anthra[1,9-cd]pyrazol-6(2H)one, dihydrochloride.

About 250 mg of 25% (w/v) ferrofluid colloidal suspension was then added and the mixture was sonicated on a water bath at room temperature to yield a homogeneous dispersion. Two hundred fifty microliters of different concentrations of a glutaraldehyde solution in 1:1 (v/v) ethanol:10% (v/v) acetic acid was added to the contents of the beaker and vortexed for 30 sec. The beaker was further sonicated for determined time periods to allow cross linking. To the mixture, 50 ml of mineral oil containing various amounts of Arlacel-83 was added and an emulsion was formed by sonication for 2.5 min at 200 W with an ultrasonic probe. The temperature of the emulsion was controlled with a circulating water system maintained at 25°C. The emulsion was then transferred to a 250-ml round-bottom flask containing 300 μl of Arlacel-83 and placed in a rotary vacuum drier under nitrogen atmosphere at 70°C for 3 hr to evaporate the aqueous phase. Following evaporation, the microsphere suspension was diluted with 60 ml of hexanes and centrifuged at 5000 rpm for 20 min. The supernatant was decanted and the microsphere pellet was resuspended in about 45 ml of hexanes and transferred to a 50-ml centrifuge tube with two 300-G bar magnets positioned to retain any free magnetite. After centrifugation at 2000 rpm for 5 min and decantation of the supernatant, the pellet was washed two times with 50 ml of hexanes. The final microsphere pellet was dried under nitrogen gas at room temperature and stored at 4°C until further analysis.

The factors and their levels considered in the optimization procedure are shown in Table I. The central composite experimental design required preparation of 27 microsphere batches with 25 combinations of factor levels (15).

Analysis of Microspheres

Yield (%, w/w)

Yield (%, w/w) was calculated as the weight of the dried microspheres recovered from each batch divided by the sum of the initial dry weight of the starting materials multiplied by a hundred, i.e.,

$$\text{yield (\% w/w)} = \frac{\text{weight of dried microsphere recovered} \times 100}{\text{mg oxantrazole} + 79.44 \text{ mg chitosan} + 0.25 \text{ mg ferrofluid} + \text{mg glutaraldehyde}}$$

Table I. Factors and Levels Investigated in the Optimization of Magnetic Chitosan Microspheres Containing Oxantrazole

	Level				
Factor	−2	1	0	+1	+2
Amount of oxantrazole (mg)	5	25	40	55	70
Glutaraldehyde concentration (mg/ml)	10	20	30	40	50
Cross-linking time (min)	10	20	30	40	50
Arlacel 83 (μl/50 ml)	50	100	150	200	250

Drug Loading

About 5 mg of oxantrazole-loaded microspheres was washed twice with 2 ml of cold isotonic phosphate buffer (pH 7.4) containing 0.1% (w/v) Tween 80, to remove surface associated drug. Microspheres were digested in 2 ml of 10% (v/v) acetic acid for 12 hr at 4°C to release entrapped drug. Oxantrazole was analyzed in each surface wash and in the digestion medium by reversed-phase HPLC, with a mobile phase system consisting of 17.5% (v/v) acetonitrile/40 mM sodium acetate buffer, pH 4.0. The mobile phase flow rate was 1 ml/min, and spectrophotometric detection was made at 514 nm. Drug concentrations were calculated from a standard curve of oxantrazole prepared each day of analysis.

Microsphere Morphology and Particle Size

Several electromicrographs were taken for each of the 27 batches with a scanning electron microscope. Average particle size ($n = 300$), standard deviation, and a particle size histogram were generated for each batch that produced spherical particles, using the univariate procedure of the SAS program (16).

Data Analysis

Desirability Function

To compare formulations, a desirability function, *di*, was computed for each of the observed variables [i.e., percentage (% w/w) of drug entrapped, percentage (% w/w) of surface-associated drug, average particle size, and standard deviation]. Table II shows maximum and minimum acceptable values for the observed variables. For variables that are desired to be minimized such as particle size and % w/w of loosely bound drug, *di* can be calculated as follows:

$$di_{\text{min}} = \frac{Y_{\text{max}} - Y_i}{Y_{\text{max}} - Y_{\text{min}}}$$

where Y_{max} and Y_{min} are the maximum and minimum acceptable values for the observed variable, Y. Y_i is the actual

Table II. Maximum, Y_{max}, and Minimum, Y_{min}, Acceptable Values of the Investigated Factors

Factor	Y_{max}	Y_{min}
% (w/w) drug entrapment	5	1
% (w/w) surface-associated drug	10	0
Average particle size, μm	1	0.1
Standard deviation of particle size, μm	0.5	0.01

observed value for each formulation. For % w/w drug entrapment,

$$di_{\max} = \frac{Y_i - Y_{\min}}{Y_{\max} - Y_{\min}}$$

An overall desirability function, *DF*, was calculated to identify optimum conditions for preparation of microsphere. *DF* is the geometric mean of the different desirability factors, *di*, so that

$$DF = \left[\prod_{i=1}^{n} d_i\right]^{1/n}$$

Optimization Procedure

Response surface regression procedures (16) were utilized to correlate responses, e.g., amount of drug entrapped, surface-associated drug, microsphere yield, and *DF*, to the coded values of the studied factors (Table I). Surface response graphs were then generated for the above responses as functions of the significantly contributing factors as determined by regression analysis. Based on the response surface graphs, ranges of optimum experimental conditions for microsphere preparation were identified. Ranges of factor values were chosen such that, when combined, they produced high desirable response values for the percentage of oxantrazole entrapped (see Fig. 2) and the overall desirability function (see Figs. 4–6) and low values for surface-associated drug (see Fig. 3). The selected ranges of the factors do not necessarily correspond to single maximum and minimum responses. If single values of the factors were chosen to produce individual maximum or minimum responses, the values of the factors may not overlap to produce a desired combination of responses. Three batches of microspheres were prepared at a selected point within the optimum range of conditions and subsequently analyzed as stated under Analysis of Microspheres.

Model Simplification and Prediction

Since response surface regression procedure takes into account all of the two factor interactions, in addition to effects of a single factor on response, a polynomial equation relating a response to the studied factors includes 15 parameters. Although it yields a higher correlation coefficient (r^2), this equation may contain some parameters that are not significantly contributing to the response in question. Thus, model simplification, by eliminating insignificant parameters ($P > 0.05$), was undertaken using the multiple regression procedure (16). Simplified models were then used to predict responses at optimal conditions to which experimental values of responses were compared.

RESULTS

Table III summarizes the data obtained from the 27 batches prepared according to the central composite experimental design. Total microsphere yield ranged from 39.1 to 80.6% (w/w). The percentage surface-associated drug was from 0.07 to 28.92% of the final weight of the microspheres, while the percentage of entrapped drug ranged from 0.9 to 4.77%. Spherical microparticulates were obtained in 12 of 27 batches, with a particle size ranging from 0.30 ± 0.12 to 1.4 ± 0.93 μm. The other 15 batches produced large irregularly shaped clumps.

Optimization Procedure

Response surface regression analyses indicated no significant correlation between the yield and any of the studied factors. Figures 2–6 show the response surface curves describing the relationship among the amount of drug en-

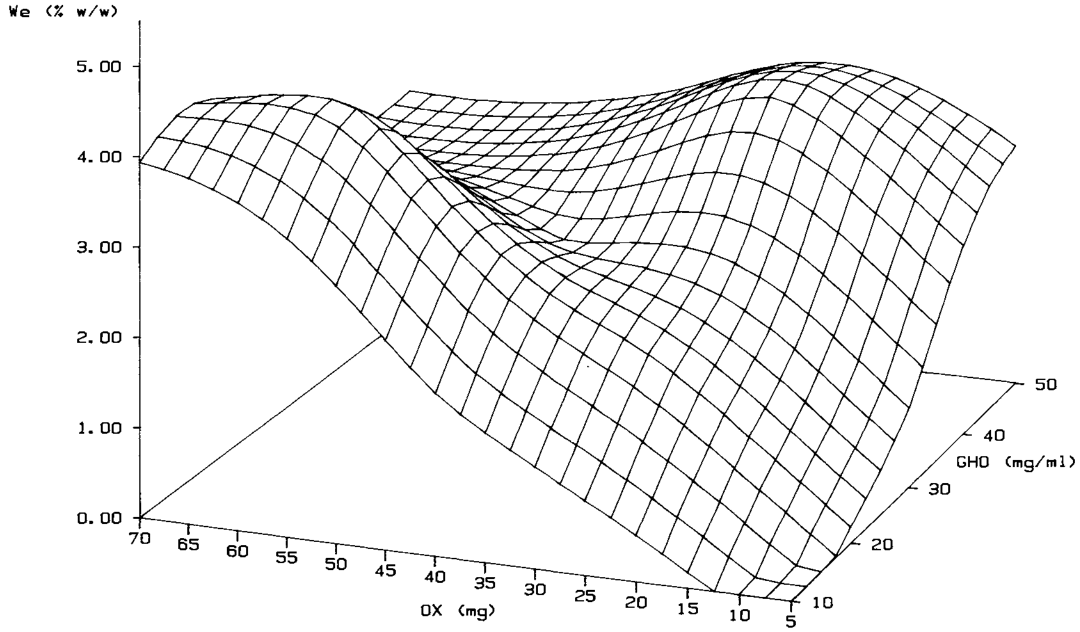

Fig. 2. Response surface graph of percentage (w/w) of entrapped oxantrazole (W_e) as a function of amount of oxantrazole added (OX) and glutaraldehyde concentration (GHO).

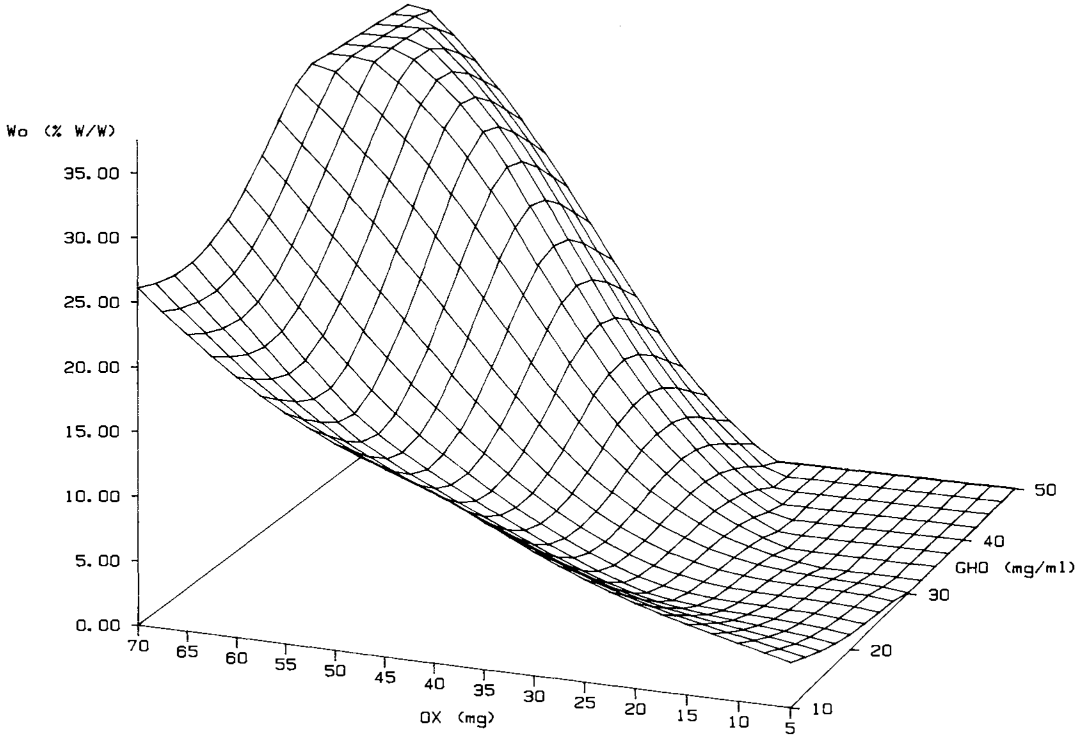

Fig. 3. Response surface graph of percentage (w/w) of surface-associated oxantrazole (W_0) as a function of amount of oxantrazole added (OX) and glutaraldehyde concentration (GHO).

trapped, the surface-associated drug, and the total desirability function as functions of the amount of drug added, glutaraldehyde concentration, amount of surfactant, and cross-linking time. Table IV indicates the range for each factor where an optimum formulation can be obtained, i.e., 20 to 25 mg of oxantrazole, 40–45 mg/ml of glutaraldehyde, and 210–230 μl of Arlacel-83. The optimum cross-linking time was 10 min. Three batches of microspheres were prepared with the following optimal conditions: 20 mg oxantrazole, 45 mg/ml glutaraldehyde, 220 μl Arlacel-83, and a cross-linking time of 10 min. This formulation gave an overall desirability function of 0.61 ± 0.06, a yield of 79.7 ± 6.6%, 0.94 ± 0.02% surface-associated drug, and 3.0 ± 0.1% entrapped drug. The microsphere linear diameter was 0.45 ± 0.2 μm.

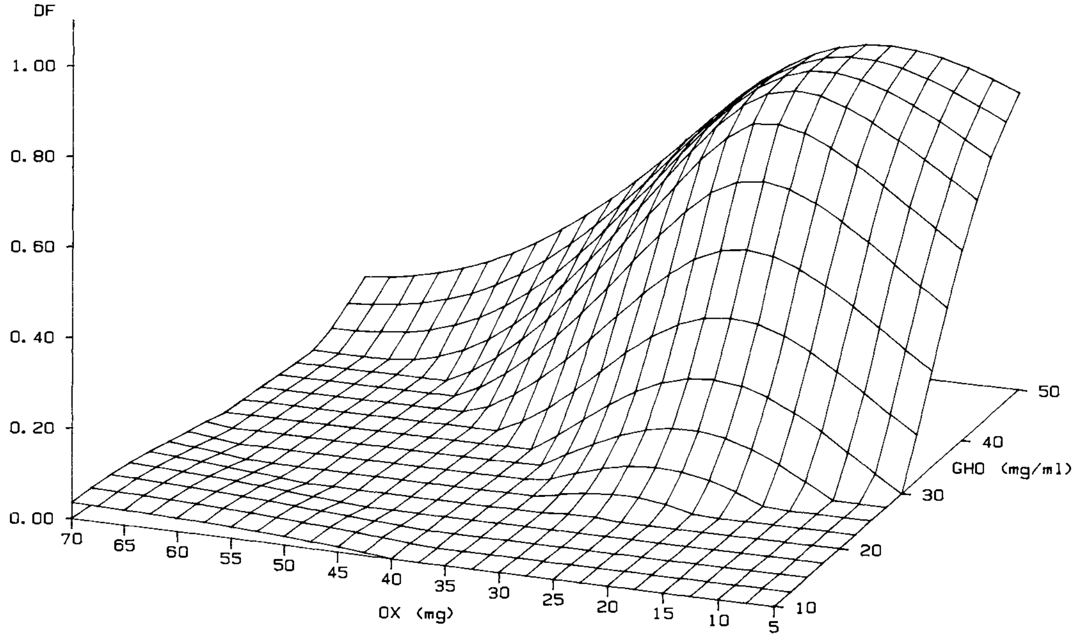

Fig. 4. Response surface graph of the overall desirability function as a function of amount of oxantrazole added (OX) and glutaraldehyde concentration (GHO).

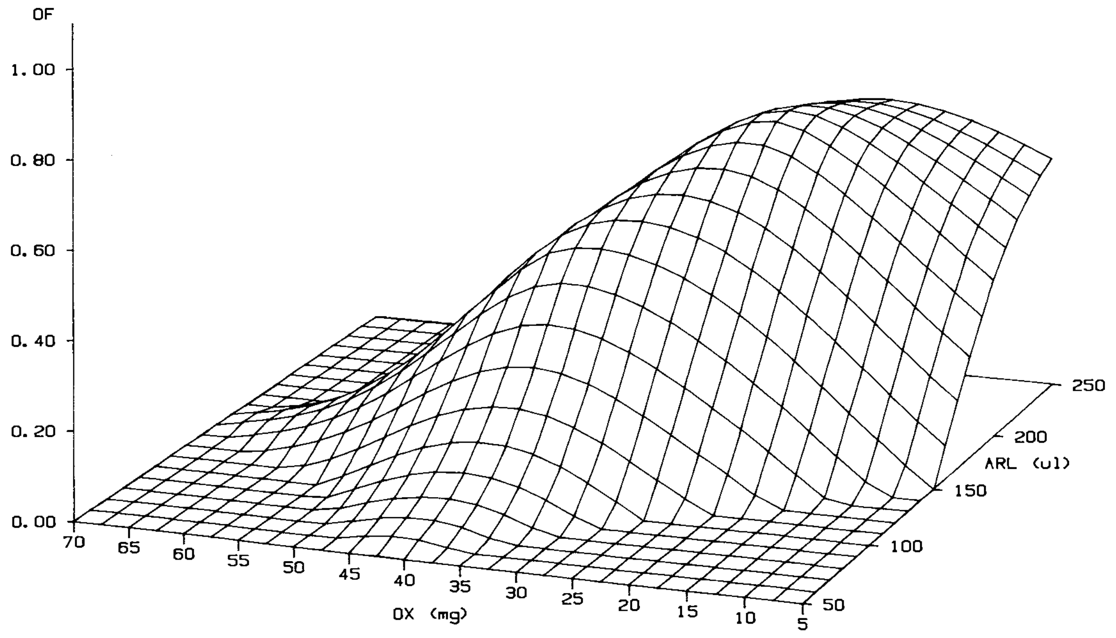

Fig. 5. Response surface graph of the overall desirability function (*DF*) as a function of amount of oxantrazole added (OX) and amount of Arlacel-83 (ARL).

Model Simplification and Prediction

Using the multiple regression procedure (16), models for percentages (w/w) of amount of drug entrapped (W_e) and surface-associated drug (W_o) and total desirability function, *DF*, are given in Eqs. (1), (2), and (3), respectively.

$$W_e = 0.064\,(\mathrm{OX}) + 0.055\,(\mathrm{GHO}) - 0.001\,(\mathrm{OX})\,(\mathrm{GHO}), \quad r^2 = 0.917 \qquad (1)$$

$$W_o = 4.69 \times 10^{-3}\,(\mathrm{OX})^2, \quad r^2 = 0.920 \qquad (2)$$

$$DF = 10^{-3}\,[27.06\,(\mathrm{OX}) - 11.44(T) - 0.27\,(\mathrm{OX})^2 - 0.32\,(\mathrm{GHO})^2 - 0.08\,(\mathrm{OX})\,(\mathrm{ARL}) + 0.17\,(\mathrm{GHO})\,(\mathrm{ARL})], \quad r^2 = 0.74 \qquad (3)$$

where

(OX) = amount of drug added (mg)

(T) = cross-linking time (min)

(GHO) = glutaraldehyde concentration added (mg/ml)

(ARL) = amount of Arlacel-83 used in emulsification step (μl)

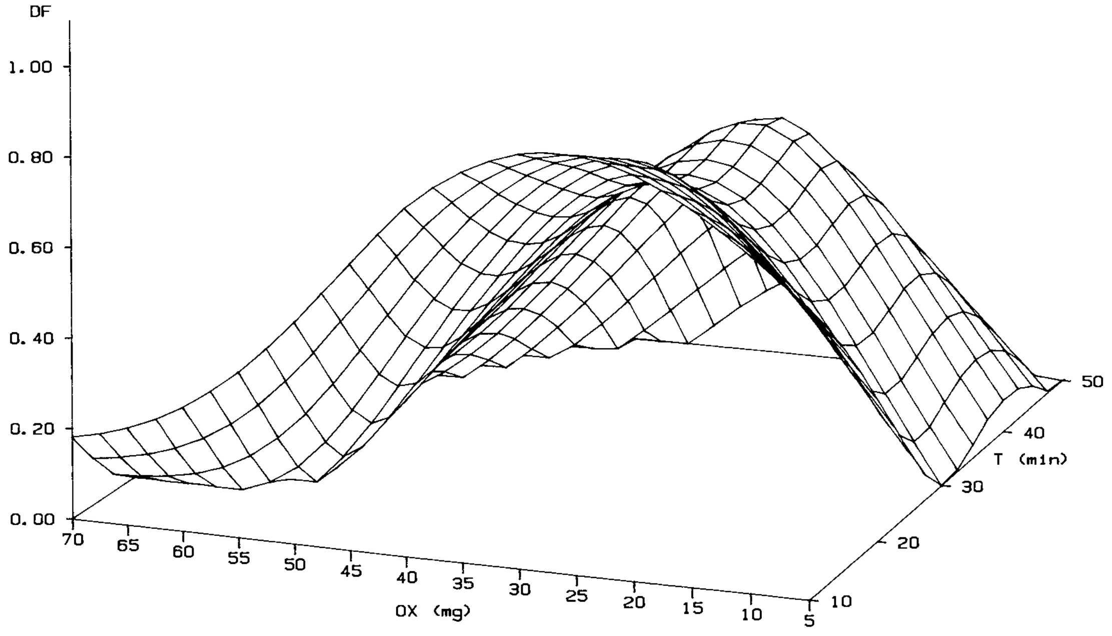

Fig. 6. Response surface graph of the overall desirability function (*DF*) as a function of amount of oxantrazole added (OX) and time of cross-linking (*T*).

Table III. Response Variable Data Following the Preparation of Magnetic Chitosan Carriers Containing Oxantrazole

Expt No.	% yield (w/w)	% (w/w) surface-associated drug	% (w/w) drug entrapped	Formation of carriers[a]	Mean particle size ± SD	Total desirability function
1	79.5	16.94	2.60	0	—	0
2	76.7	15.81	2.60	0	—	0
3	69.7	16.49	1.80	0	—	0
4	66.1	12.48	4.55	0	—	0
5	68.0	13.74	4.19	0	—	0
6	78.0	10.83	2.81	0	—	0
7	60.4	8.24	3.55	0	—	0
8	73.8	13.74	4.77	0	—	0
9	67.0	0.44	3.63	1	0.47 ± 0.25	0.67
10	77.2	2.33	3.13	0	—	0
11	54.2	0.66	3.45	1	0.30 ± 0.12	0.78
12	73.9	7.06	1.87	1	1.4 ± 0.93	0
13	73.7	2.47	1.82	0	—	0
14	75.3	3.68	2.07	0	—	0
15	75.7	2.65	2.37	1	0.73 ± 0.39	0.35
16	39.1	3.71	1.10	1	0.51 ± 0.33	0.24
17	73.6	28.92	3.10	0	—	0
18	70.2	0.07	0.90	1	0.43 ± 0.16	0
19	67.7	7.04	3.04	1	0.50 ± 0.29	0.45
20	80.6	12.99	1.78	0	—	0
21	69.8	8.16	2.35	0	—	0
22	73.1	2.46	4.25	1	0.38 ± 0.15	0.76
23	77.3	3.45	1.84	1	0.63 ± 0.33	0.38
24	71.2	9.52	1.41	0	—	0
25	77.4	8.27	2.23	1	0.70 ± 0.37	0.27
26	75.7	3.12	3.18	1	0.31 ± 0.18	0.67
27	79.3	4.46	3.14	1	0.58 ± 0.34	0.47

[a] 1 indicates spherical carrier formation; 0 indicates failure to produce spherical carriers.

Observed and model-predicted [viz. Eqs. (1)–(3)] values of % W_e, % W_o, and DF are given in Table V.

DISCUSSION

A solvent evaporation technique has been reported earlier for preparation of plain magnetic chitosan microspheres (3). This technique, when applied to the preparation of oxantrazole-loaded MCM, resulted in the majority of the drug on the microsphere surface and little drug entrapped in the microspheres. Failure of drug entrapment was attributed to a low microsphere matrix density. Increasing microsphere density by increasing the polymer concentration in the formulation was not possible due to the limited solubility of chitosan. Matrix cross-linking was chosen in the current investigation since it could increase the microsphere density and limit polymer chain expansion upon hydration. Thus, diffusion of water into the microsphere matrix and drug diffusion to the microsphere surface could be minimized.

Glutaraldehyde has been used in the synthesis of cross-linked microspheres made of albumin (8,11) or gelatin (10,17). In most cases, the concentration of glutaraldehyde and the time of cross-linking affected the extent of drug entrapment and the rate of drug release. The cross-linking interaction involves bridging of polymeric chains at free amino group positions by the aldehydic functions of glutaraldehyde (18). This reaction has been the basis of insolubilizing numerous enzymes and other proteins (19). Since chitosan contains free amino groups, it could undergo a similar cross-linking reaction. Glutaraldehyde cross-linked chitosan was synthesized to have enhanced metal adsorption (20) and to form sustained-release indomethacin granules (21).

Mechanisms of oxantrazole association with magnetic

Table IV. Optimal Range of Formulation Factors Obtained from Response Surface Graphs

Factor	Range
Oxantrazole	20–25 mg
Glutaraldehyde	40–45 mg/ml
Arlacel-83	210–230 μl
Time	10 min

Table V. Observed and Model-Predicted Values of Significantly Correlated Responses at Optimum Conditions

Response	Observed values	Model-predicted value
% (w/w) drug entrapment	3.0 ± 0.1	2.7 ± 0.4
% (w/w) surface-associated drug	0.94 ± 0.02	1.9 ± 1.8
Overall desirability	0.61 ± 0.06	0.97 ± 0.20

chitosan microspheres prepared by glutaraldehyde cross-linking include physical entrapment of oxantrazole in the polymer matrix and covalent bonding of oxantrazole's amino groups to that of the polymer backbone via a bialdehydic bridge (22). Since the cross-linking reaction involves competition, based on the low molar concentration of glutaraldehyde, of the polymer chains and drug molecules for aldehydic functional groups, molar ratios of the competing entities will determine which of the above mechanisms is more probable. In many of the experimental formulations including the optimal formulation chitosan amino groups were more than eight times the oxantrazole molar concentration. Thus based on the molar ratios of the reactants, the favored product is cross-linked chitosan rather than covalently bound chitosan and oxantrazole. Regardless of the nature of oxantrazole's association with the microspheres, the strong acid conditions employed in the assay procedure for the determination of the quantity of oxantrazole entrapped or associated should release physically entrapped and covalently bound drug.

Optimizing a formulation is most frequently carried out by studying each factor separately. Such procedures, also referred to as empirical optimization, usually require a large number of experiments and ignore the effects of possible interactions between factors on the formulation (23). Another approach would be to use a factorial experimental design where all factors and their interactions can be observed simultaneously. Among the wide variety of experimental designs, the central composite design was chosen for this study. Alternatives, such as a two-leveled factorial design, requiring preparation of only 16 batches of microspheres, assumes linear relationships between factors and measured responses. This assumption may not be valid, especially when a wide region of factor values is to be investigated. A three-leveled factorial design for the current study would require examination of 81 microsphere batches, and reproducibility data are not generated to examine the lack of model fit. The central composite design required 27 batches, does not assume linearity, and allows the lack of model fit to be determined. The associated analytical procedure required fitting data to second-order polynomial equations that relate the observed responses and the studied variables.

Although numerous factors have to be set in the formulation of microspheres, the factors studied were considered to have the greatest effect on the measured responses. As stated earlier, cross-linking conditions should impact on drug entrapment due to differences in matrix porosity. Amounts of surfactant and drug added were also selected for this optimization study due to their influence on drug loss by partitioning during formulation and washing of microspheres.

Dependence of drug entrapment on the amount of drug added and the glutaraldehyde concentration is evident from Eq. (1) and Fig. 2. Increases in these parameters increase the quantity of drug entrapped to a certain limit, followed by a decrease in drug entrapment at higher drug and glutaraldehyde levels. The latter phenomenon may be due to a greater probability of the drug (containing free amino group) to interact with glutaraldehyde, prohibiting the polymer cross-linking process. The negative coefficient of the (OX) (GHO) term in Eq. (1) indicates that the negative effect of (OX) (GHO) interaction on oxantrazole entrapment will be more significant at high drug and aldehyde concentrations. Surface-associated drug [see Eq. (2)] was dependent on the squared amount of oxantrazole. As the amount of oxantrazole is increased, the fraction of the microsphere surface area occupied by drug molecules would increase since oxantrazole is water soluble. The presence of oxantrazole at the microsphere surface would enhance water penetration into the microsphere matrix, leading to greater drug loss during the aqueous washing steps.

Desirability functions are useful when several responses are to be evaluated simultaneously (24). Assignment of desirability function values from zero to one simplifies comparisons between experiments. However, being a compound function, interpretation of the desirability function [i.e., Eq. (3)] is more complex. The positive (OX) term [see Eq. (3)] reflects the contribution of increased amounts of oxantrazole to increasing drug entrapment (desirability increasing), while the negative sign for the $(\mathrm{OX})^2$ term corresponds to the effect of starting amounts of oxantrazole on surface associated drug (desirability decreasing). The negative effect of cross-linking time [second term in Eq. (3)] on desirability may be due to an increased viscosity of the aqueous phase as the cross-linking time increases. Higher aqueous-phase viscosities could hinder small globule formation during emulsification and result in either large-sized globules or even incomplete emulsification and, finally, in large irregular masses. Similar effects of the cross-linking time could occur by increasing the cross-linking concentration [negative $(\mathrm{GHO})^2$ parameter in Eq. (3)]. The surfactant, Arlacel-83, has a rather complicated role in microsphere desirability. Arlacel can increase drug loss to the oil phase due to its solubilizing power. This effect will also be dependent on the amount of drug added [negative (OX) (ARLA) parameter]. On the other hand, Arlacel may contribute to increasing overall desirability by producing smaller microspheres. As Arlacel reduces water/oil interfacial tension, smaller aqueous globules can be stabilized during the evaporation step. The positive effect of Arlacel on the desirability will be further amplified after the drug has been entrapped by the glutaraldehyde cross-linking process [positive (GHO) (ARL) parameter].

In conclusion, a statistical central composite optimization procedure was used to obtain equations that identified significantly contributing formulation factors. Subsequent response surface graphic analysis allowed optimal formulation conditions to be located, and finally, model simplification allowed comparison of experimental data to model-predicted results. The optimal formulation, being of a submicron size, high drug entrapment and low surface-associated drug, will be suitable for *in vivo* drug targeting studies.

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