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Tetrandrine delivery to the lung: The optimisation of albumin microsphere preparation by central composite design

Xian Ming Zeng, Gary Peter Martin *, Christopher Marriott

Department of Pharmacy, King's College London, Manresa Road, London SW3 7LX, UK

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Abstract

Tetrandrine is an alkaloid extracted from the roots of *Stephania tetrandra S. Moore*. It is employed in China to treat silicosis; its site of activities being within the lungs. Chronic treatment with tetrandrine is limited by unacceptable toxicity to the liver. Entrapment of tetrandrine within albumin microspheres with a view to promoting deposition within the lung may result in an improved therapeutic modality. Accordingly, the preparation of tetrandrine-entrapped albumin microspheres (MS) was optimised using central composite design. The influence of albumin concentration, drug concentration and pH of albumin solution on MS particle size and drug entrapment was investigated. An optimum preparation was established, which produced albumin MS with mean diameter 11.65 ± 0.31 μm ($n = 3$) and 113 ± 12.5 μg entrapped tetrandrine per mg MS ($n = 3$). Such MS could have the potential for targeting tetrandrine to the lung.

Key words: Albumin microspheres; Tetrandrine; Central composite design; Particle size; Drug entrapment; Optimization

1. Introduction

Tetrandrine (6,6',7,12-tetramethoxy-2,2'-dimethyl-(1β)-Berbaman) is a bisbenzylisoquinoline alkaloid extracted from the roots of *Stephania tetrandra S. Moore*. In recent years, it has been used in China to treat silicosis, an occupational disease caused by the inhalation of silica particles. Tetrandrine has been reported to bind to alveolar macrophages, prevent their activation after inhalation of silica and inhibit respiratory burst activity of pulmonary phagocytes. Consequently, it can prevent the excretion of fibrogenic factors and the formation of fibroblasts (Castranova et al., 1991a,b; Ma et al., 1991). It can also interfere with the recruitment of neutrophils and monocytes into silicotic lesions and reduce the collagen concentration of the lungs (Liu and Zou, 1983; Seow et al., 1986). These actions make it effective not only in preventing the formation of fibrocytes but also in inducing the decomposition of collagen fibres already formed. Pharmacokinetic studies have shown that tetrandrine is metabolized slowly and accumulates in the liver. Thus, some hepatic damage is observed in the

* Corresponding author.

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form of scattered small areas of necrotic lesions and elevated liver enzymes are also found in the blood (Suffness and Cordell, 1985; Li et al., 1986). These side effects have largely limited the use of tetrandrine in the treatment of silicosis which requires chronic medication. Hence, the aim of this study is to develop a delivery system for tetrandrine which would optimize its delivery to the lung so as to promote its antisilicotic activity but to minimize its side effects to other organs. The entrapment of tetrandrine within albumin microspheres of tightly defined particle size suitable for lung deposition provides a mode of therapy worthy of investigation.

Albumin microspheres (MS) are biodegradable colloidal particles which have been used for lung scanning and circulatory studies in both animal and human subjects (Rhodes et al., 1969). Since the use of albumin MS in drug delivery was first suggested by Kramer et al. (1974) many drugs have now been entrapped or incorporated. It has been demonstrated that the in vivo disposition of albumin MS depends largely on their particle size (Burger et al., 1985a). Particles with diameter 7–15 μm, when given intravenously, are trapped by the first capillary bed encountered, i.e., that of the lung and exploitation of this characteristic has been used to attempt the delivery of drugs to the lung (Gupta and Hung, 1989). Maximizing drug deposition within the lung is not solely dependent upon particle size. An increase in drug entrapment should reduce the amount of carrier required to deliver a specified dose of the drug, and hence increase its uptake by target tissues. Therefore, optimisation of the preparation is dependent on two parameters, namely, control of particle size and maximizing entrapment.

Both albumin and drug concentration have been reported to influence both the particle size and the efficiency of drug entrapment in albumin MS (Gupta and Hung, 1989). The solubility of drug in water has also been reported to be important in the achievement of a high degree of drug entrapment (Tomlinson et al., 1984). As tetrandrine is a lipophilic alkaloid, it is practically insoluble in water at neutral pH. However, its water solubility is greatly improved at lower pH values. An increase in water solubility may increase entrapment in albumin MS as a result of reducing its partitioning into the non-aqueous phase during the high speed homogenization, heat denaturation and washing process. However, the pH of the aqueous solution has a profound influence on the particle size of albumin MS (Zeng et al., 1993). Hence, the effects of albumin concentration, drug concentration and pH of albumin and drug solution on particle size and drug entrapment were investigated in an attempt to optimise their levels.

Several workers have suggested the use of factorial design when optimizing a formulation (Bolton, 1983; Gupta et al., 1989). Such mathematical models allow all factors to be varied simultaneously, thus enabling evaluation of both the single effect of each variable and showing interactions among them. The most commonly used factorial design involves the use of two levels of each variable, allowing estimation of a linear relationship only (Hinchen, 1969). When the relationship is quadratic, more levels must be investigated and this greatly increases the number of experiments. To solve this problem, an alternative design can be used which includes only central and star points in a two-level factorial design. This design is referred to as a central composite design and can be used to investigate quadratic relationships of multiple variables with a reduced number of experiments (Myers, 1971). Consequently, the purpose of this study was to employ such a design to optimise the preparation of albumin MS with maximum entrapment of tetrandrine and suitable particle size for delivery of the drug to the lung capillary.

2. Materials and methods

2.1. Materials

Bovine serum albumin fraction V (96–99% albumin) was purchased from Sigma Chemical Co. (Poole, U.K.). Tetrandrine (99% purity) was generously donated by Professor C.Q. Zou (Institute of Occupational Medicine, Chinese Academy of Preventive Medicine, Beijing, P.R. China). Butan-1-ol (general grade) and oleic acid (speci-

fied laboratory reagent) were obtained from BDH Laboratory Suppliers (Poole, U.K.) and Fisons Scientific Equipment (Loughborough, U.K.) respectively. Albendazole and Hepes (minimum purity 99.5%) were purchased from Sigma Chemical Co. (Poole, U.K.). Acetonitrile and triethylamine were of HPLC grade while other chemicals were general laboratory reagents.

2.2. Preparation of tetrandrine and albumin solutions

A stock solution of tetrandrine was prepared by dissolving 60 g tetrandrine in 100 ml of 0.1 M citric acid buffer solution (pH 4) and stored in a air-tight container at room temperature.

The drug-albumin solution was prepared by dissolving albumin in tetrandrine solution so that the concentrations of both drug and albumin were slightly higher than required. After the solution had been adjusted to the desired pH with dilute HCl or NaOH solution, its volume was carefully adjusted to obtain the required concentrations. If the drug did not dissolve completely, the suspension was sonicated for 15 min before use (FS100b, Decon Laboratory Ltd, Hove, U.K.). All the solutions were freshly prepared to minimise any hydrolysis of albumin under acidic conditions.

2.3. Preparation of albumin MS

Albumin and drug solution or suspension (3 ml) was added dropwise under high-speed homogenization to 50 ml oleic acid, which was contained in a 100 ml round bottom flask, and then homogenized for 5 min. The speed of the homogenizer (Kika Werk Ultra-Terrax) was fixed at 6000 rpm and this was monitored with a stroboscope (Xenon Stroboflash Model 60). The resultant w/o emulsion was transferred into a dropping funnel and added dropwise at the rate of 40 ± 5 drops min^{-1} into 100 ml preheated oleic acid (80°C) with constant stirring at 1000 rpm. The oleic acid was maintained at 115 ± 5°C, after which it was allowed to cool to room temperature. The suspension was centrifuged at $1900 \times g$ for 10 min, the supernatant was decanted and sedimenting microspheres resuspended in 50 ml of butan-1-ol. After sonication for 5 min, the suspension was centrifuged under similar conditions. The microspheres were washed three times as described. Finally, they were transferred to a petri dish, allowed to dry at room temperature and were then stored as a dry powder in the dark.

2.4. Particle size measurement

Albumin MS (2 mg) were added to 10 ml water containing two drops of Tween 80 and then sonicated for 15 min. The particle size was measured by laser light scattering (Series 2600c, Malvern Instruments Ltd, U.K.).

2.5. Determination of drug entrapment in albumin MS

2.5.1. Sample preparation

A weighed amount of albumin MS (about 20 mg) was added to 10 ml chloroform and dispersed using sonication for 5 min. After centrifugation at $1900 \times g$ for 15 min, the washing was repeated to remove all the surface drug. To the washed microspheres, 25 ml of 5% (w/v) sodium hydroxide solution was added and the mixture was sonicated for 30 min. The suspension was then stored at room temperature until the digestion of microspheres was complete as determined visually by viewing for the absence of MS using a light microscope. The sodium hydroxide solution was transferred into a separating funnel and extracted with chloroform (40 ml) three times. After bulking, the chloroform extract was washed with water twice and then adjusted volumetrically with acetonitrile to 50 ml.

About 2 ml, accurately measured chloroform extract was added to a 10 ml volumetric flask into which 1 ml of 200 $\mu g\ ml^{-1}$ albendazole solution (as internal standard) had been previously placed. The volume was carefully adjusted to 10 ml with acetonitrile.

2.5.2. HPLC assay

Sodium chloride (8.48 g), potassium chloride (0.37 g) and Hepes (2.38 g) were dissolved in 935 ml distilled water. To this solution 5 ml trieth-

ylamine and 60 ml glacial acetic acid were added to produce Hepes buffer solution. Hepes buffer (1 l) and acetonitrile (1 l) were mixed together and sonicated for 30 min to produce the mobile phase.

The HPLC assay employed a spheric ODS column (Fisons Scientific Equipment, Loughborough, U.K.), a multiple solvent delivery system (CM 4000, LDC Analytical Instrument for HPLC, U.S.A.) and an ultraviolet detector (Spectro-Monitor 3200, LDC Analytical Instrument for HPLC, U.S.A.). The flow rate was 1 ml min^{-1}; UV wavelength, 242 nm and sensitivity, 0.02.

Samples (60 μl) were injected using an autosampler (Marathon, Spark Holland Instrument Accesories for HPLC, The Netherlands). The drug concentration was determined from the ratio of the peak area of drug to that of the internal standard.

2.6. Central composite design

The three independent variables and their levels are listed in Table 1. For a three-factor central composite design, 20 experiments are required (Myers, 1971) and the combinations are shown in Table 2. Experiments 1–8 represent the simple factorial design using two levels of each variable. Experiments 9–14 are the central point and experiments 15–20 represent the star points.

3. Results and discussion

Table 3 shows the mean diameter of albumin MS produced using the experimental conditions summarised in Table 2. The particle size was

Table 1
The independent variables and their respective levels in central composite design

Levels	[Albumin] (mg/ml)	[Tetrandrine] (mg/ml)	pH
−1.68	116	23.2	3.32
−1	150	30	4
0	200	40	5
+1	250	50	6
+1.68	284	56.8	6.68

Table 2
Experimental design of albumin MS preparation according to central composite design

Experiment no.	[Albumin] (mg/ml)	[Tetrandrine] (mg/ml)	pH
1	150	50	4
2	150	50	6
3	250	50	4
4	250	50	6
5	150	30	4
6	150	30	6
7	250	30	4
8	250	30	6
9	250	40	5
10	200	40	5
11	200	40	5
12	200	40	5
13	200	40	5
14	200	40	5
15	116	40	5
16	284	40	5
17	200	23.2	5
18	200	56.8	5
19	200	40	3.32
20	200	40	6.68

Table 3
Particle size of tetrandrine-entrapped albumin MS prepared under different conditions and the amount of tetrandrine incorporated (μg drug per mg MS)

Experiment no. [a]	Mean diameter n ± SD (μm)	Drug incorporation
1	14.25 ± 1.13	112.3
2	10.32 ± 0.95	62.4
3	16.75 ± 1.06	89.5
4	12.48 ± 0.74	47.8
5	13.69 ± 1.25	93.1
6	9.98 ± 1.20	45.0
7	15.45 ± 1.18	12.9
8	11.37 ± 1.02 [b]	
9	12.69 ± 1.28	82.7
10	10.75 ± 0.79	65.8
11	13.10 ± 0.98	75.9
12	11.56 ± 1.09	71.2
13	11.96 ± 1.21	53.6
14	12.45 ± 1.19	65.0
15	10.49 ± 1.35	86.7
16	13.47 ± 1.12	44.3
17	11.08 ± 1.15 [b]	
18	12.91 ± 1.19	73.6
19	18.64 ± 2.14	124.6
20	9.66 ± 0.67	23.4

[a] See Table 2.
[b] No entrapped drug detected.

found to vary between 9.66 and 18.64 μm, indicating that the variables had marked effects upon the particle size over the range investigated. The results of experiments 9–14 demonstrate the overall reproducibility in particle size of the MS when the same conditions were used to prepare a number of batches. The coefficient of variation of these data is 7.03% while that of the total data is 18.1%. Based upon these observed results, the following polynomial equation was generated to establish the relationship between the independent variables and particle size:

$$\begin{aligned}\text{Mean diameter } (\mu\text{m}) &= 18.265 + 0.0141 \times [\text{albumin (mg ml}^{-1})] \\ &+ 0.00442 \times [\text{drug (mg ml}^{-1})] \\ &- 1.645 \times (\text{pH value}) + 0.00034 \\ &\times [\text{albumin}] \times [\text{drug}] - 0.002075 \\ &\times [\text{albumin}] \times (\text{pH value}) - 0.006625 \\ &\times [\text{drug}] \times (\text{pH value}) + 0.0000075 \\ &\times [\text{albumin}] \times [\text{drug}] \times (\text{pH value})\end{aligned}$$

In order to determine the influence of each variable on particle size, response surface diagrams of this equation were plotted. Fig. 1–3 are the response surface diagrams for each pair of variables while the third one was maintained at the central point value.

From Fig. 1 and 2 it can be seen that the pH value of albumin solution exerted a strong influence upon MS particle size. Decreasing the pH from 6.68 to 3.32 increased the mean diameter from about 9 to more than 15 μm. This phenomenon is likely to be due to the change in electric charge of albumin molecules caused by the variation in pH. Albumin and drug concentration exhibited only a slight influence upon particle size (Fig. 1–3), although higher albumin concentrations tended to produce larger albumin MS. This can be attributed to the increase in viscosity of albumin and drug solution at higher albumin concentrations, as increasing the viscosity would decrease the subdivision of albumin solution into smaller droplets during emulsification and finally increase the particle size of albumin MS prepared (Ishizaka et al., 1981). Increase in tetrandrine concentration also slightly increased particle size and this is in agreement with a previous

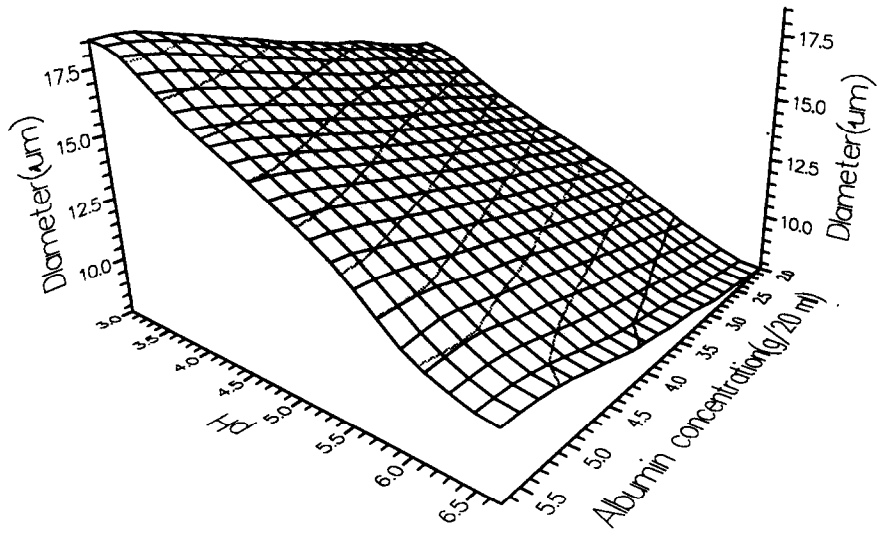

Fig. 1. A three-dimensional response surface diagram showing the effect of pH and albumin concentration on the particle size.

report which showed that an increase in drug concentration resulted in an increase in MS particle size (Burger et al., 1985b).

Table 3 shows the drug entrapment attained under the experimental conditions outlined in Table 2. The value varied over a wide range from 0 to more than 100 units. The data of experiments 9–14 had a coefficient of variation of 14.5%, indicating a larger batch-to-batch variation than in the case of particle size. Employing these data, a polynomial equation was generated in order to reveal the influences of each variable:

$$\begin{aligned}\text{Entrapment } (\mu\text{g drug per mg MS}) &= 642.4 - 3.113 \times [\text{albumin } (\text{mg ml}^{-1})] \\ &\quad - 7.14 \times [\text{drug } (\text{mg ml}^{-1})] - 84.044 \\ &\quad \times (\text{pH value}) + 0.0557 \times [\text{albumin}] \\ &\quad \times [\text{tetrandrine}] + 0.3785 \times [\text{albumin}] \\ &\quad \times (\text{pH value}) + 0.9675 \times (\text{pH value}) \\ &\quad \times [\text{drug}] - 0.00675 \times (\text{pH value}) \\ &\quad \times [\text{albumin}] \times [\text{drug}]\end{aligned}$$

The response surface diagrams of this equation were also plotted (Fig. 4–6). Each diagram represents the effects of two variables on the drug entrapment while the third one was kept at the central point value.

These diagrams demonstrate that all of the three variables have significant effects upon the drug entrapment. The lower pH value employed resulted in the production of albumin MS with more drug entrapped (Fig. 4 and 5). Since tetrandrine is a lipophilic alkaloid, it forms a water-soluble salt under acidic conditions. The increase in water solubility may be the main reason for higher entrapment, since its partitioning into the oil phase during preparation may be reduced. At neutral pH, most of the drug was insoluble in albumin solution. During high-speed homogenization, heat denaturation and the washing process, the lipophilic drug particles can diffuse through the aqueous layer and dissolve in the oil phase, resulting in only a small amount of drug becoming entrapped in the final product. Whether or not the aqueous solubility of the drug is important for its entrapment in albumin MS is still a matter of dispute. Morimoto et al. (1981) were able to prepare albumin MS with more than 15% w/w of adriamycin but with only 3.3% w/w of 5-fluorouracil. The variability in the entrapment was suggested to be due to the difference in their aqueous solubility (6% w/v for adriamycin com-

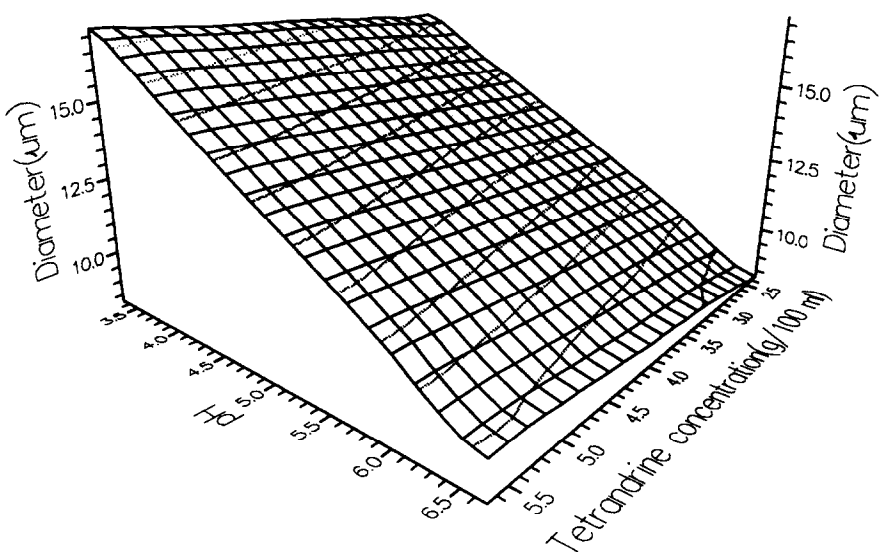

Fig. 2. A three-dimensional response surface diagram showing the effect of tetrandrine concentration and pH on the particle size.

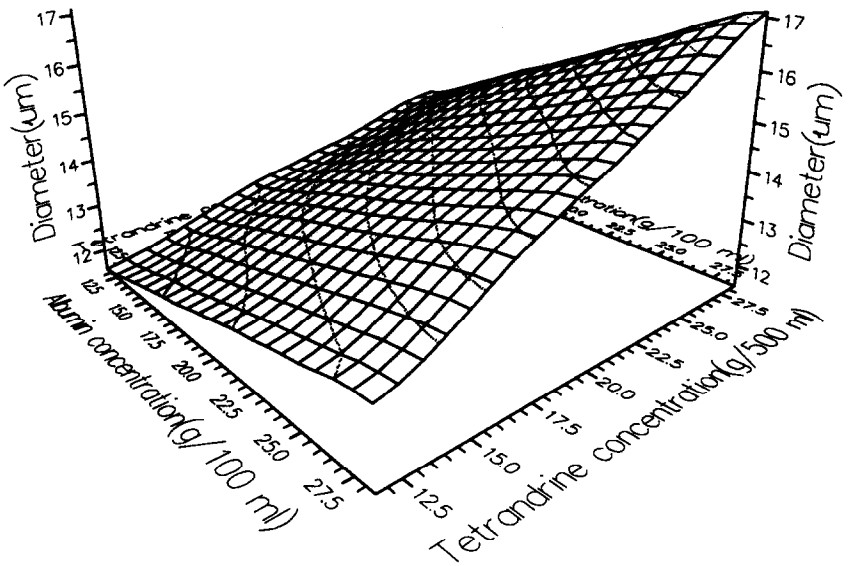

Fig. 3. A three-dimensional response surface diagram showing the effect of albumin and tetrandrine concentration on the particle size.

pared with 1.25% w/v for 5-fluorouracil in water). Other workers have entrapped water insoluble sex hormones or magnetite in albumin MS up to the extent of 20–50% of the carrier and concluded that aqueous solubility was not important (Senyei et al., 1978; Gallo et al., 1989). However, none of the previous studies have investigated the influence of the change in the aqueous solubility

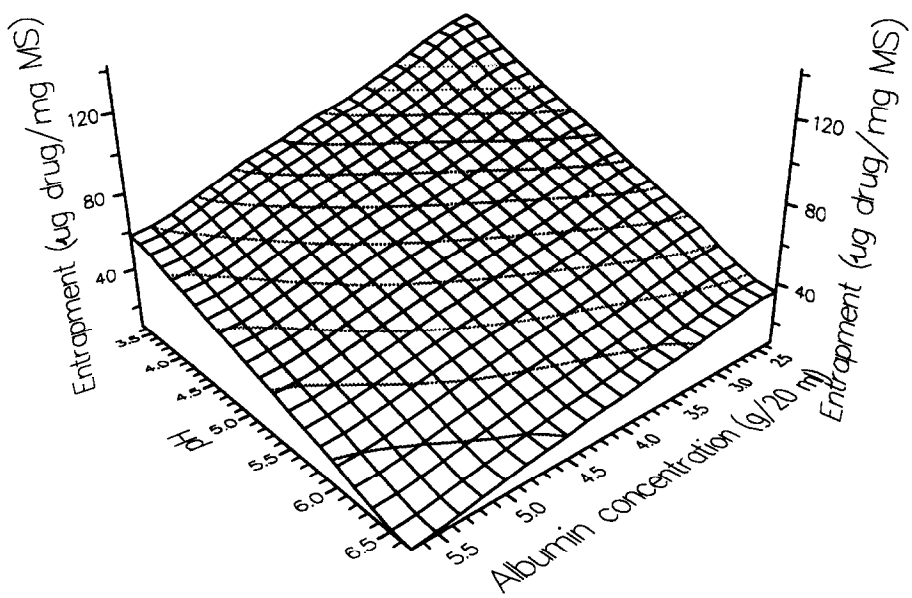

Fig. 4. A three-dimensional response surface diagram showing the effect of pH and albumin concentration on drug entrapment.

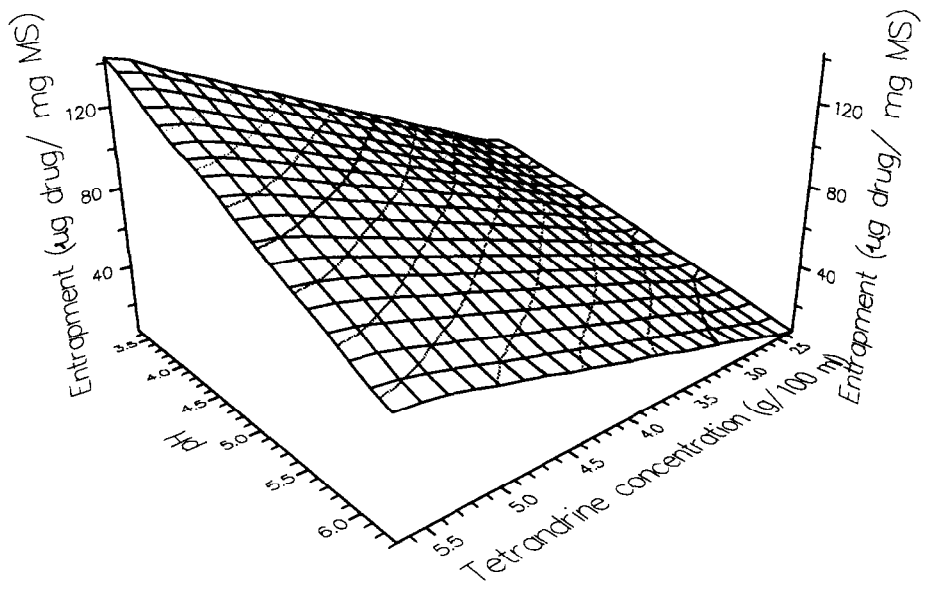

Fig. 5. A three-dimensional response surface diagram showing the effect of pH and tetrandrine concentration on drug entrapment.

of one drug on its entrapment in albumin MS. On the basis of the results from the present study, other factors such as lipophilicity, molecular weight or morphological characteristics of insoluble drugs and drug protein binding should be taken into consideration when comparing the entrapment in albumin MS of drugs with different aqueous solubilities.

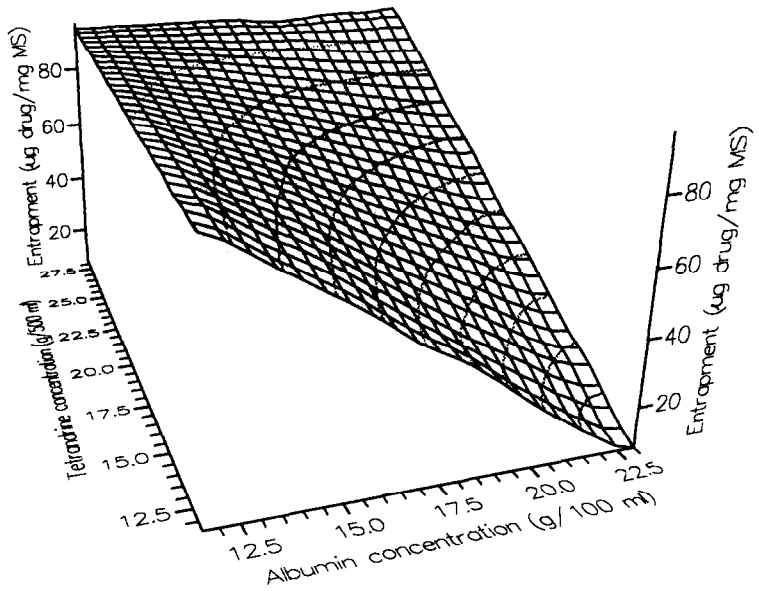

Fig. 6. A three-dimensional response surface diagram showing the effect of albumin and tetrandrine concentration on drug entrapment.

Albumin and drug concentration also had marked effects on drug entrapment (Fig. 4–6). Higher drug concentration led to greater drug entrapment while higher albumin concentration tended to reduce the entrapment. Generally, the higher the ratio of drug to albumin, the greater the amount of drug entrapped in the MS. This observation is also in agreement with a previous report (Tomlinson and Burger, 1985) which demonstrated that at a constant sodium cromoglycate concentration, a decrease in albumin concentration resulted in an increase in drug entrapment. However, excessively low albumin concentration may lead to immediate release of the entrapped drug following in vivo administration (Tomlinson et al., 1984) so that a large fraction of the drug cannot be targeted to the desired sites. Furthermore, low albumin concentration would give a poor yield of microspheres, making the formulation commercially non-viable. Hence, a concentration of albumin lower than 100 mg ml^{-1} was not investigated in this study.

Since particle size plays a decisive role in lung targeting, this parameter was first considered when optimising preparative conditions. The problem was thus to select the level of albumin, tetrandrine and pH value that maximized tetrandrine entrapment, such that mean diameter was suitable for lung targeting after parenteral administration (11–12 μm). As an increase in tetrandrine concentration increases entrapment to a much greater extent than its effect on particle size, a higher initial drug concentration appeared preferrable. Hence, tetrandrine was maintained at 40 mg ml^{-1} which was close to its saturation concentration at pH 4.5. In order to optimize albumin concentration and pH value, contour curves were drawn which illustrate the combinations of independent variables producing the same response (Fonner et al., 1970). Fig. 7 shows the levels of albumin and pH which would produce albumin MS with mean diameters of 11, 12, 14 μm and entrapment of 80, 100, 120 μg drug mg^{-1} MS. The shaded portion of the graph defines the region where particle size was 11–12 μm and entrapment 100–120 μg drug. Any combination of pH and albumin concentration in the region would therefore produce albumin MS with

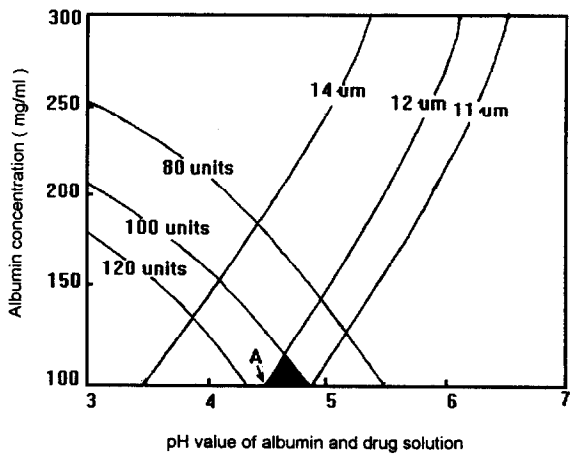

Fig. 7. Contour curves for particle size and drug entrapment, illustrating levels of pH and albumin concentration producing the same response.

mean diameter 11–12 μm and entrapment 100–120 units. Other combinations may lead to the production of albumin MS with either large diameter (> 14 μm) or low entrapment (< 80 units), since greater entrapment could result in larger particles and vice versa. In Fig. 7, region A could be considered to represent the optimum conditions since entrapment was maximized whilst the resultant particle size was 12 μm. Consequently, the optimised MS were prepared under the conditions employing an albumin concentration of 100 mg ml^{-1}, a drug concentration of 40 mg ml^{-1} and a pH of 4.4.

In order to evaluate the predictive power of this mathematical model, three batches of albumin MS were prepared using the optimum conditions. A scanning electron micrograph of one batch is shown in Fig. 8. The particle size was measured by laser light scattering and the drug entrapment was determined by HPLC as described previously. The resultant particle size and entrapment were 11.65 ± 0.31 μm and 113 ± 12.5 units, respectively. The predicted particle size and entrapment were 12 μm and 117 μg tetrandrine mg^{-1} MS, respectively. Since the experimentally described data were close to the predicted values, this indicates that central compos-

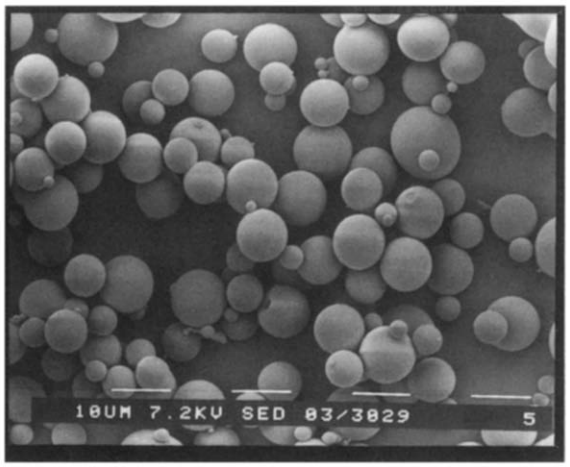

Fig. 8. Scanning electron micrograph of albumin MS prepared under the optimised conditions.

ite design is an adequate model for the optimisation of albumin MS preparation.

In conclusion, this work deals with the optimization of albumin MS preparation using central composite design. Of the three factors investigated, the pH of albumin solution exhibited a strong influence on both the particle size and drug entrapment, whilst albumin and tetrandrine concentration exerted a marked influence only on entrapment with only minor effects on particle size. Optimum preparative conditions required the use of 100 mg ml^{-1} albumin, 40 mg ml^{-1} tetrandrine and pH 4.4, producing albumin MS with diameter suitable for lung targeting and with more than 100 μg entrapped drug per mg MS. After further characteristion, the albumin MS prepared in this study will be used to investigate the delivery of tetrandrine to the lung.

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