RESEARCH PAPER

Accepted 16 October 2001
Revised 10 October 2001
Received 29 December 2000
Indian J. Pharm. Sci., 2002, 64(1): 48-52

Preparation and Evaluation of Chloroquine Phosphate Microspheres using Cross-Linked Gelatin for Long-Term Drug Delivery

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Microspheres of chloroquine phosphate (CP) were prepared using cross-linked gelatin and were investigated for the *in vitro* release profile and *in vivo* drug release following subcutaneous injection. A series of batches of microspheres were prepared by cold congealing method to optimize parameters like the media of encapsulation (mixture of heavy and light mineral oil in the ratio of 1:3), stirring rate (250 rpm) and pH of the media (4.5) to obtain discrete microsphere in the size range of 50 to 100 µm. The study indicated the significance of the parameters like gelatin to drug ratio and the percentage of formaldehyde (crosslinking agent) to obtain microspheres with high drug entrapment and optimum drug release behaviour. Multiple regression analysis of the values of the cumulative percent drug released in 8 d for ten batches prepared by varying the above two parameters yielded a polynomial equation. This equation was used to calculate the optimum values of gelatin to drug ratio (2.0) and percentage of the formaldehyde (3.99%) for preparing microspheres with ideal drug release behaviour. Subcutaneous injection of microspheres as concentrated suspension in HPMC base in Wistar rats showed blood level concentration between 5.98 and 18.46 µg/ml for a period of 8 days in comparison to 34.8 µg/ml for 6 h following subcutaneous injection of CP solution. Mean residence time of the drug in the body calculated as the ratio of the area of the first moment curve to the area under the concentration vs time curve was 71.4 h for microspheres as against 2.4 h for the drug solution.

Chloroquine is still a drug of choice in the treatment of acute malarial attack as it is inexpensive, widely available and well tolerated under controlled dose. Due to increasing cases of resistance development for this drug particularly in malaria caused by *Plasmodium falciparum*, management of this disease has become extremely difficult under acute conditions and so the demand for a simple prophylactic measure is always felt[1]. World Health Organisation[2] recommends a minimum of 3 m protective measure against malaria in endemic area by using an injectable prolonged release formulation. Many dosage forms containing anti-malarial drugs have been developed for prolonged release using various approaches like low soluble drug derivatives, pro-drugs, and use of non-biodegradable and biodegradable polymer carrier[3-5]. The present study is an attempt in this direction to develop a long term injectable suspension of microsphere containing CP, prepared using biodegradable polymer like cross-linked gelatin. The purpose of this study is also to investigate the influence of the process variables in the preparation of the microspheres using cross-linked gelatin and the application of multiple regression analysis in optimizing such formulation exercise.

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MATERIALS AND METHODS

CP was a gift sample from M/s. IPCA Laboratories, Mumbai. Gelatin powder was obtained from Burgoyne

Burbidges and Co. India, Mumbai. Formaldehyde (34%w/v aqueous solution)was obtained from BDH, Mumbai; Span 80 (Sigma Chemicals Co., USA), Isopropyl alcohol (Ranbaxy fine Chemicals Limited, S.A.S. Nagar, India) and liquid paraffin was procured from local suppliers. All other chemicals used were of analytical grade.

Preparation of microspheres[6]:

Aqueous gelatin solution (20%) containing the drug was heated to 55° and was slowly added drop wise with stirring into mineral oil containing sorbitan monooleate (4%) and aluminum tristearate (8%) dispersed in it. Temperature was gradually elevated to 70° for 45 m with controlled continued stirring. The vessel was then immediately stepped into ice bath and left there for 20 min with stirring. Microspheres obtained were dehydrated by the addition of isopropyl alcohol with gentle stirring and then were decanted and redispersed in isopropyl alcohol. Formaldehyde solution in isopropyl alcohol (20% v/v) was added with stirring and the micropheres were then recovered by decantation, washed thrice with isopropyl alcohol followed by twice with acetone. They were tested for the absence of free formaldehyde[7] and then dried at room temperature and stored in refrigerator.

Particle size analysis:

Samples of microspheres were analyzed for particle size by optical microscopy. Linear diameters of 500 microspheres were measured per field for every sample. Logarithms of size of microspheres were plotted against the cumulative percent frequency on a probability scale to abtain a linear plot from which mean geometic diameters were calculated[8].

Drug content[9]:

Weighed quantity of microspheres were settled in 3N HCl at 55° till they hydrolyzed and released drug completely. The mixture was filtered through a 0.45 µm filter, diluted and assayed spectophotometrically at 343 nm against a reagent blank. Corresponding drug concentrations in the samples were calculated from the calibration plot generated by regression of the data taken in triplicate.

Dissolution study[10]:

Weighed quantity of micorspheres equivalent to 100 mg of CP was taken for dissolution study. USP XXIII (paddle type) dissolution test apparatus at a rotational speed of 100 rpm and 37° in 900 ml distilled water was used for the study. Samples (10 ml) were withdrawn at convenient intervals and filtered through 0.45 µm filter. Same volume (10 ml) of the dissolution medium was replenished after each sampling. The drug content in the samples was determined in the filtrate by the method described above.

Kinetics of drug release:

In order to understand the mechanism and kinetics of drug release, the results of the *in vitro* dissolution study of the optimized batch of microsphere (batch V) was fitted with various kinetic equations like zero order (% release vs. t), first order (log % release vs. t), Higuchi's model[11] (M_t/M_∞ vs. $\sqrt{t}$), Korsmeyer and Peppas [12] (M_t/M_∞ vs. t^n), Hopfenberg analysis[13] for spherical systems [$(1- M_t/M_\infty)^{1/3}$ vs. t]. R^2 values were calculated for the linear curves obtained by regression analysis of the above plots.

***In vivo* study:**

In vivo study of the CP microspheres was conducted on Wistar albino rats of either sex in two groups of six each (weight range 250-300 g). The first group of rats was injected subcutaneously with CP solution (4.5 mg) and the second group with the suspension of CP microspheres in HPMC base (quantity equivalent to 9 mg of CP) prepared aseptically. Blood samples were withdrawn by puncturing the sino-orbital plexus of the rat eye with a fine capillary at different time intervals for 8 d. Blood sample (0.2 ml) was mixed with tricloroacetic acid to make up the volume up to 1 ml. Sodium hydroxide solution (2 ml, 0.1N) was added and extracted successively with three fractions of 1, 1 and 0.5 ml of dichloromethane respectively. These fractions were pooled and re-extracted with 1, 1 and 0.5 ml of 0.01N HCl. Concentration of CP was determined by the method described above.

RESULTS AND DISCUSSION

The microspheres of CP were prepared by cold congealing method using gelatin cross-linked with formaldehyde. Percentage of gelatin was optimized at 20% and suspending medium selected for the preparation of microspheres was the mixture of heavy and light liquid paraffin in the ration 1:3. Aluminium tristearate (8%) was used as antitacking agent with stirring rate of 250 rpm. The microspheres obtained under these conditions were found to be spherical and without aggregation. Gelatin exhibits two isoelectric points depending on the source and so the influence of pH of the gelatin solution used was found to be a critical factor. Based on the percent drug entrapment efficiency, optimum pH of the gelatin solution was assessed to be 4.5. Mean geometric particle size and percent drug

loading of different batches of microspheres prepared are tabulated in Table 1.

Gelatin being biodegradable could be used for designing long-term delivery systems only after cross-linking to reduce the rate of diffusion of the entrapped drug and also to keep the device intact with out disintegration until the device releases the entrapped drug near to exhaustion. Formaldehyde was selected as crosslinking agent due to its high rate of cross linking and easy removal of the un-reacted free formaldehyde. Batches of microsphere prepared using varied quantities of formldehyde (1% to 7% w/w) and with different gelatin to drug ratio was evaluated for *in vitro* release rate and the results are tabulated in Table 1.

Reduction in the particle size and an increase in percent drug entrapment was observed with decreasing the gelatin to drug ratio from 4 to 0.5. However, considering the *in vitro* release rate, the microspheres prepared with gelatin/drug ratios in the range of 1 to 2 were found optimum. All batches of microspheres prepared with 1 to 7% w/w of formaldehyde were found spherical, rigid and were within the particle size range of 50 µm to 100 µm, but those prepared with 2% to 5% formaldehyde showed suitable drug release behavior in comparison to other batches. In order to optimize these two parameters, gelatin:drug ratio and percentage of cross-linking agent for the controlled release of CP over a period of 15 d, the following drug release profile was hypothesized.

TABLE 1: PHYSICAL CHARACTERISTICS AND *IN VITRO* DISSOLUTION DATA OF CHLOROQUINE PHOSPHATE MICROSPHERES.

Batch No.	L	M	N	O	P	Q	R	S	T	U	V
G/D Ratio	4:1	2:1	1:1	1:2	2:1	1:1	2:1	1:1	2:1	1:1	2:1
CLA(%)	1.0	1.0	1.0	1.0	2.67	2.67	5.0	5.0	6.67	6.67	4.0
Particle size	112	75.5	70.0	65.74	82.4	77.7	88.6	80.4	96.4	88.4	85.9
(µm)	±1.5	±1.3	±1.3	±1.2	±1.6	±1.8	±2.2	±2.0	±2.3	±2.2	±1.8
%drug											
Entrapment	2.4	14.3	19.2	20.1	15.4	19.8	16.2	20.0	16.1	20.1	16.8
Time (d)	***In vitro* cumulative % drug release**										
1	30.6	42.7	40.9	42.9	32.4	38.4	32.5	33.9	26.4	27.6	31.5
2	38.9	46.9	47.2	55.8	40.7	45.6	37.6	40.7	30.9	31.8	37.4
4	52.9	56.1	56.0	70.9	45.8	51.2	40.7	43.8	35.6	36.4	42.7
6	58.4	70.9	72.9	85.0	54.8	59.4	46.8	50.7	40.5	43.2	48.6
8	75.7	80.9	86.0	96.0	60.6	65.9	52.9	56.8	48.7	52.8	61.0
10	86.9	95.5	95.8	97.8	69.9	73.5	60.6	64.9	54.7	59.2	69.6
12	93.8	98.0	98.0	98.1	76.7	79.8	69.8	72.9	60.9	65.2	79.0
14	98.5	98.4	98.7	98.5	94.0	85.0	76.9	80.8	68.8	72.4	87.5
16	98.6	98.7	99.0	98.9	96.5	90.4	82.7	86.9	72.9	79.5	92.1
20	98.7	99.0	99.0	99.1	98.4	93.5	96.9	91.2	84.5	85.9	97.6

Comparison of *in vitro* dissolution data presented as percent drug release as function of time from various batches of cross linked gelatin microspheres containing CP prepared with varied Gelatin to Drug ratio (G/D Ratio) Cross linked with varied percentage of formaldehude. Particle size was measured by optical microscopy.

Cumulative % drug release = 20%<Y1<40%, 50%<Y8<70%<Y12<85%

Where Y1, Y8 and Y12 are the cumulative percentage drug release in 1, 8 and 12 d during *in vitro* dissolution study. As per the above release profile, all batches of microspheres except the batches M, N and O showed optimum release at 1 d. Drug release after 8 d was found to be very high in case of batches L, M, N, and O and very low in case of T and U. Though the drug release in 8 d in case of batches P, R and S were in acceptable range, batch P showed an ideal value (60.6%). Batches L, M, N and O also showed very high release while the batch T and U showed low values at 12 d. Overall, batches P, Q, R and S showed drug release in the expected range throughout the period of the study.

To study the influence of these two factors, the cumulative percentage drug release in 8 d (Y8) from microsperes of batches M to U were subjected to multiple regression analysis[14] and the statistically valid model obtained is Y8=90.99-4.6X1-5.45X2. Where, X1 is the ratio of gelatin to drug and X2 is the concentration of formaldehyde. This polynomial equation indicates the nature and extent of the influence of the variables, X1 and X2 on Y8 values. Both X1 and X2 show almost the same influence with inverse proportionality (due to the negative sign). The equation can be used to calculate Y8 values for batches prepared with varied values of X1 and X2 in an identified range. Setting Y8 for any required value and keeping one of the variables constant, the other variable may be calculated. In the present case keeping Y8 to be 60% (average in the range 50% to 70%) and gelatin to drug ratio at 2, the calculated value of the percentage of formaldehyde is 3.998. Based on this calculation, batch 'V' was prepared and evaluated for *in vitro* dissolution rate. This batch of microcapsules showed optimum cumulative percent release of 31.5, 61 and 79 in 1, 8 and 12 d, respectively. The *in vitro* dissolution profile of this batch of microsphere is best explained by the equation for kinetics of zero order release (fig. 1). The data on regression analysis yielded linear relationship between cumulative drug release and time with the R^2 value of 0.9929 (Y=4.1884 X + 27.005).

Based on the *in vitro* drug release profile, batch "V" was selected for *in vivo* studies in Wistar rats. The microsphere suspension being designed for subcutaneous route of administration, the comparative study was conducted with CP solution administered subcutaneously. Blood concentration vs time data generated during the study is plotted in fig. 2 and salient pharmacokinetic

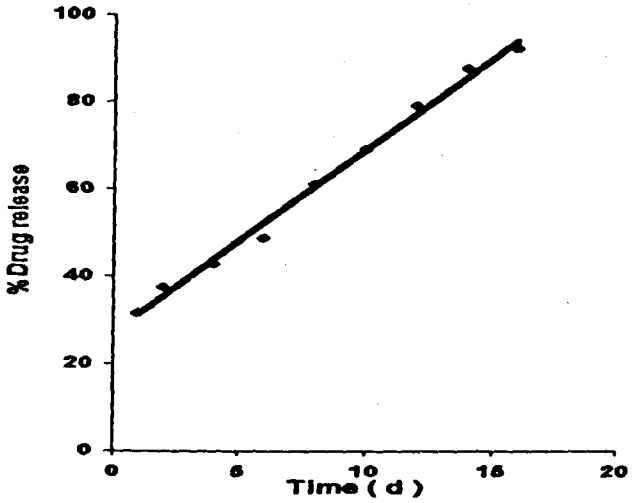

Fig. 1: *In vitro* release profile of CP from microspheres

Drug release expressed as a function of time for chloroquine phosphate microsphere, batch 'V', prepared using 2:1 gelatin to drug ratio cross linked with 4% formaldehyde. Dissolution study conducted as per USP XXIII method using paddle type apparatus in distilled water media.

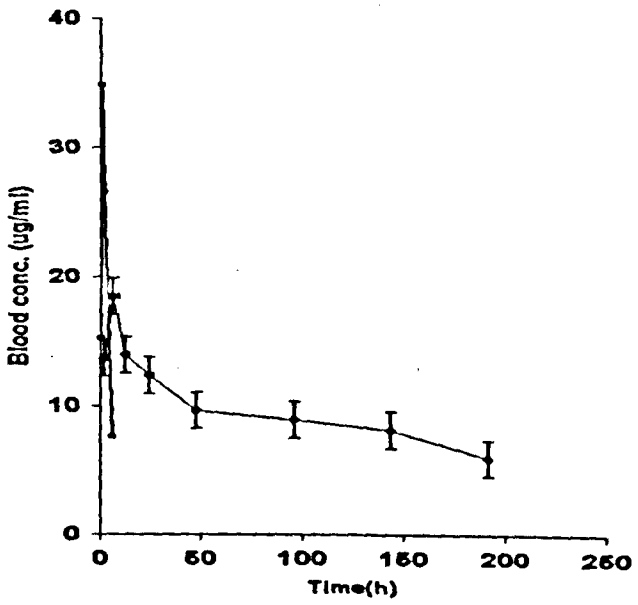

Fig. 2: Blood concentration vs. time curve of CP.

Blood level of CP at different time points after subcutaneous administration of CP solution (■) and suspension of CP microsphere (◆) in Wistar rats.

parameters are calculated. Blood concentration of CP in rats following subcutaneous injection of CP solution reached maximum level (34.8 µg/ml) in 1 h and the drug concentration could be estimated upto 6 h. The microsphere suspension showed the C-max of 18.46 µg/ml after 4 h post subcutaneous injection and the concentration maintained above 4 µg/ml for 8 d. The comparative bioavailability of microsphere suspension with respect to drug solution administered subcutaneously was found to be 8.93. The mean residence time[15] of the drug in the body calculated as the ratio of the area under the first moment curve to the area under the curve was found to be 71.4 h for the microsphere as against 2.4 h for the drug solution.

Based on the above results it is evident that the subcutaneous administration of a slow release formulation like microsphere suspension is beneficial in many respects like optimum blood level maintenance, longer duration and ease of administration in comparison to implants. Zuidema *et al.*[16] reported lesser possibility of intact microspheres of particle size more than about 150 µm to infiltrate into lymphatics, and hence particle size control may be one of the parameter to monitor the distribution of microsphere administered subcutaneously and drug release from it. A significant change is observed between the results obtained in *in vitro* and *in vivo* study for the batch 'V', where, 90% drug release was noted in 15 d of dissolution study while the CP level in blood following subcutaneous injection of the formulation was significant only for 8 d. Slow biodegradation of the injected microspheres may be the probable reason for the comparative faster drug release of the formulation in the bioenvironment. However, the blood level concentration of CP observed (>6 µg/ml) was well above the minimum concentration of 30 ng/ ml, as suggested by Hardman and Limbird[17] for antimalarial activity with any type of *Plasmodium* parasite.

ACKNOWLEDGEMENTS

Financial assistance by All India Council for Technical Education and the University Grants Commission, New Delhi is gratefully acknowledged.

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