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OPTIMIZATION OF THE FORMULATION AND *IN-VITRO* EVALUATION OF CHLOROQUINE LOADED CHITOSAN NANOPARTICLES USING IONIC GELATION METHOD

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ABSTRACT

Biodegradable nanoparticulate carriers have important potential applications for administration of therapeutic molecules. Chitosan based Nanoparticles have attracted a lot attention upon their biological properties such as biodegradability, biocompatibility and bioadhesivity. The aim of the present study is to optimize the formulation and the *In-vitro* evaluation of Chloroquine Phosphate loaded Chitosan Nanoparticles. Chloroquine Phosphate is an anti-malarial drug. In this present study, Chloroquine loaded Chitosan–tripolyphosphate Nanoparticles were prepared by ionic gelation method in five different batches with variable drug to polymer ratios (1:3, 1:4, 1:5, 1:6 and1:7). The drug follows linearity in the concentration range 5-30 µg/ml with regression coefficient value of 0.994. The drug content of Nanoparticles increases on increasing the polymer concentration up to a particular level. Entrapment efficiency of 92.87% was achieved with drug to polymer ratio 1:6. *In-vitro* release of Chloroquine Phosphate from Chitosan Nanoparticles was 85.13% within 24 h. TEM image indicates that the nanoparticles have a discrete spherical structure and particle size was in the range nanometer. FTIR studies show the evidence of cross linking between positively charged amino group of Chitosan and negatively charged Phosphate group of TPP (TriPolyPhosphate) without any significant interaction between Chloroquine Phosphate and Chitosan Nanoparticles after encapsulation. Good stability is observed at refrigeration condition compared to other temperature conditions during eight weeks of storage.

KEYWORDS: Chitosan, Chloroquine Phosphate, Ionic gelation, Nanoparticles

1. INTRODUCTION

Malaria is a major health concern in the developing world including India. The outcome of treatment in malaria depends on the right diagnosis, selection of the right drug and its efficacy, correctness of the advice given to the patient and compliance of the patient (Bajpai and Jyoti Choubey, 2006). Nanoparticles have unique physicochemical properties such as, ultra small size, large surface area to mass ratio and high reactivity which are different from bulk materials of the same composition. These properties can be used to overcome some of the limitations found in traditional therapeutic and diagnostic agents (Zhang, 2008). Thus Nanostructure mediated drug delivery enhances drug bioavailability, improves the timed release of drug molecules and enables precision drug targeting. Chitosan is of major importance in industry because it possesses excellent properties such as biocompatibility, biodegradability, non-toxicity and absorption properties. These properties make chitosan a good candidate for conventional and novel drug delivery systems.

The use of controlled release system has certain advantages over the conventional dosage forms, as they can minimize side effects, prolong the efficacy of the drug release rate and can reduce the frequency of administration of the drug. Thus assuring better patient compliance. The pH is another important factor, since the interaction is higher at slightly acidic pH (Ines panos et al., 2008). Ionic gelation is based on the ability of polyelectrolytes to cross link in the presence of counter ions to form hydro gels (Patel JS et al., 2010). Simply an ionic gel is produced by physical cross-linking between a polycationic and a polyanionic component. The chosen polyanion is the sodium TriPolyPhosphate (TPP). It is largely used in food industry, in detergents and biomedical applications for its non – toxicity and fast gelling ability. Chloroquine phosphate is the first drug of choice among the available anti-malarial drugs and it is an inexpensive drug. However, Chloroquine Phosphate is limited by its cytotoxic effect, which is induced by the higher effective concentration and the low bioavailability in transfection. A systemic administration of Chloroquine Phosphate in aqueous solution would cause retinopathy in patients and even life threatening toxicity (Gandhi AM, 2011). Long term and large amount of consumption of chloroquine phosphate to prevent or treat malaria causes severe side effects. To control these factors chloroquine phosphate loaded Chitosan Nanoparticles are formulated using Ionic gelation method.

2. MATERIALS AND METHODS

Chloroquine phosphate was purchased from IPCA Laboratories Ltd, Chennai. Chitosan is bought from Sigma Aldrich- Bangalore, Sodium tripolyphosphate (TPP) is purchased from Alfa Easer- Chennai. Tween 20, Sodium chloride (NaCl), Potassium Chloride (KCl), Disodium hydrogen phosphate, Potassium dihydrogen phosphate are purchased from R.K Scientifics-Chennai and other reagents were of analytical grade.

Preparation of Chloroquine Phosphate loaded Chitosan Nanoparticles: Chitosan Nanoparticles were prepared according to the Ionic gelation method. This method is based on the conjugation of oppositely charged macromolecules with negatively charged Sodium tripolyphosphate (TPP) and positively charged Chitosan polymers. TPP is nontoxic, multivalent and able to form gelate through ionic interaction between positively charged amino groups of Chitosan and negatively charged TPP. The interaction could be controlled by the charge density of TPP and Chitosan, which is dependent on the pH of solution (Chenguang LIU, 2007). Chitosan were weighed, dissolved in 0.1% v/v Glacial acetic acid with magnetic stirring at room temperature. 0.25%w/v sodium tripolyphosphate (TPP) was also dissolved in distilled water. The surfactant tween 20 is dissolved in deionized water and then added to the above solutions. These two solutions (TPP & Tween20) were then added drop wise simultaneously under constant magnetic stirring to the solutions containing other counter ions (Chitosan) with continuous stirring for about 3-5h. Meanwhile the chloroquine is dispersed in D.I. Water and added dropwise to the chitosan nanoparticles. The amount of Chitosan was varied such that various batches were obtained with drug polymer ratios as shown in the table no 1.

Table no 1. Optimization of Chloroquine Loaded Chitosan Nanoparticles

Formulation Code	Amount of drug (mg)	Amount of Chitosan (mg)	Amount of TPP w/v %	Drug + Polymer ratio
F1	10	30	0.25	1:3
F2	10	40	0.25	1:4
F3	10	50	0.25	1:5
F4	10	60	0.25	1:6
F5	10	70	0.25	1:7

RESULTS

Powdered X-Ray Diffractometry: X-ray Diffraction analysis was performed with a PANalytical Xpert Pro X-ray Diffractometer using Ni filtered Cu k_α radiation. The powdered samples evaluated was taken on the glass slide and placed on the X-Ray Diffractometry. The scanning rate was 10 minutes over a 2θ range of 10 to 90^0 (Jelvehgari, 2010).

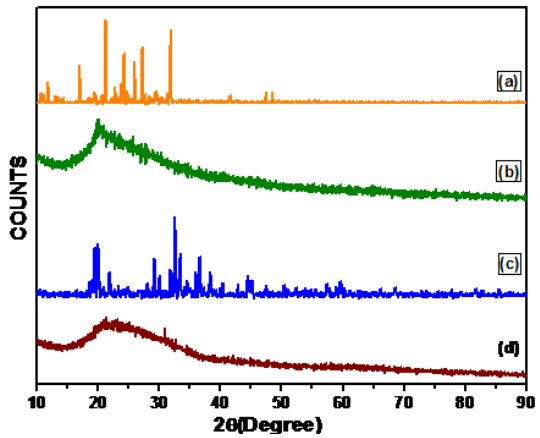


Figure 1: XRD spectrum of (a) Chloroquine, (b) Chitosan, (c) TPP, (d) Chloroquine formulationF_4

Fourier Transforms Infrared Spectroscopy: The FT-IR spectra of Chloroquine phosphate, Chitosan, sodium tripolyphosphate and Chloroquine loaded Chitosan Nanoparticles formulation were recorded to check drug polymer interaction and stability of drug samples of Chloroquine Phosphate, Chitosan, sodium tripolyphosphate, were triturated with an equal quantity of KBr. Each sample was then compressed to obtain pellet for IR analysis (Ashok

Kumar Tiwary and Vikies Rand, 2010) (Tamizharasi S, 2010). The spectra of these samples were recorded on a Perkin Elmer FT-IR Spectrometer, USA in the spectral region of 450 to 4000 cm^{-1}(Colthup, 1950).

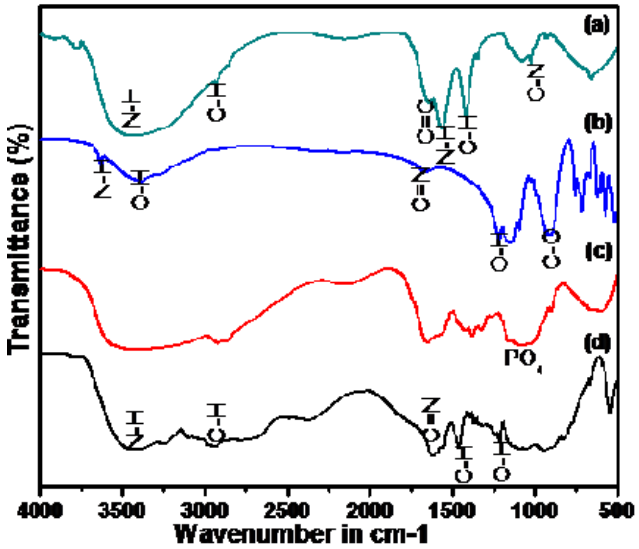


Figure 2: FTIR Spectrums of (a) Chloroquine, (b) Chitosan, (c) TPP and (d) Formulation F_4

Drug Content: An ultracentrifugation technique was used to separate the free drug from formulated Nanoparticles. The Nanoparticles suspension was centrifuged at 15000 rpm for 40 min to separate free drug in the supernatant. Concentration of Chloroquine in the supernatant was determined by UV – Vis spectrometer at 334 nm after suitable dilution using Phosphate buffer saline of pH 7.4 (Simar Preet Kaur et al., 2011). The Entrapment efficiency (EE %) was calculated from the following expression :

$$\text{ENTRAPMENT EFFICIENCY} = \frac{\text{Total amount of drug} - \text{free amount of drug}}{\text{Total amount of drug}} \times 100$$

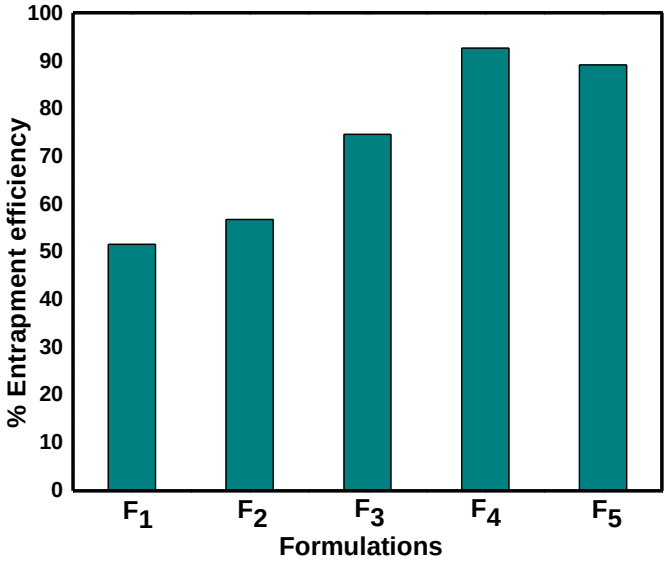
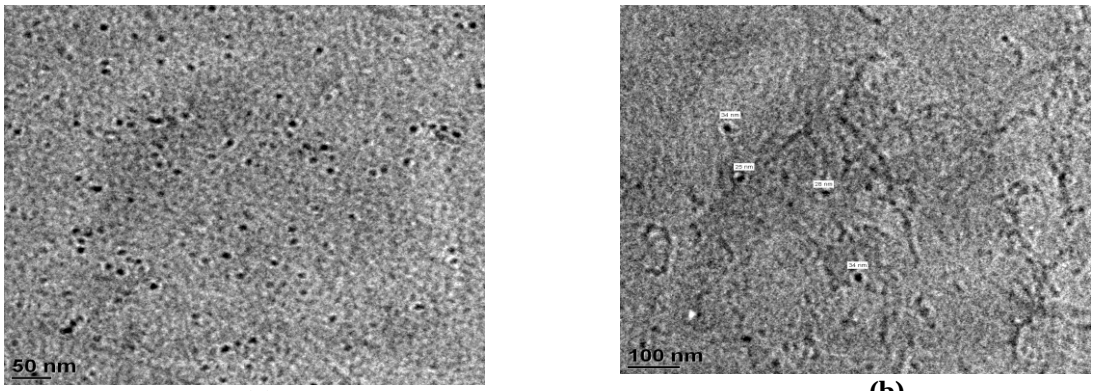


Figure 3: Entrapment Efficiency of all the Formulations F_1-F_5.

Table no: 2 Entrapment Efficiency of all the formulations F_1-F_5.

Formulation Code	Drug + Polymer ratio	Drug Entrapment Efficiency (%)
F1	1:3	51.70
F2	1:4	56.95
F3	1:5	74.80
F4	1:6	92.87
F5	1:7	89.34

Transmission Electron Microscopy (TEM) Analysis: A FEI Technai 20 HR-TEM, Japan (120kv) was used to characterize the size and morphology of the dried Chloroquine loaded Chitosan Nanoparticles. To analyze the sample, a small drop of aqueous sample solution was placed on copper grid, dried completely in vacuum desiccator and then examined using a TEM without any further modification or coating (Magdolna Bodnar, 2005) (Prachi Joshi, 2011). The size and morphology of Chloroquine loaded Chitosan Nanoparticles were obtained by TEM images as shown below figure 4(a) and 4(b).



(a) **(b)**

Figure 4: TEM Images of (a) and (b) were F_4 Formulations

Particle Size Analysis: The Malvern particle size analysis was done for the optimized formulation of Chloroquine loaded chitosan nanoparticles (F_4). The maximum numbers of particles size and size distribution were in the range of 150-500 nm. We obtained the average particle size of the formulated Chloroquine loaded chitosan nanoparticles as shown in the figure 5.

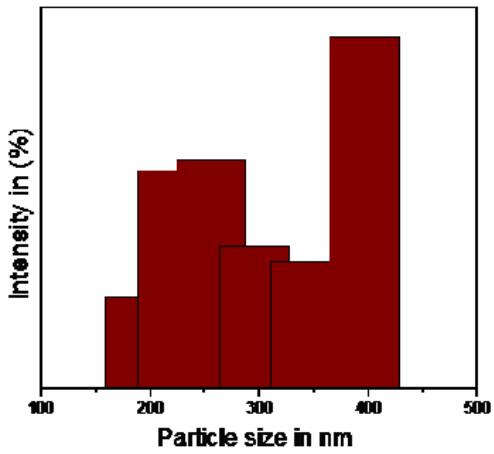


Figure 5: Particle size Analysis of Formulation F_4.

***In-Vitro* Release Studies:** *In-Vitro* release studies of prepared Chloroquine loaded Chitosan Nanoparticles were carried out using Franz diffusion cell (FDC) apparatus. The Chloroquine loaded Chitosan Nanoparticles were placed in donor compartment. The receptor compartment contained phosphate Buffer (pH 7.4). The experiment was carried out at room temperature for a period up to 24 hours. Sample was withdrawn at a time interval of 1h. 3 ml of sample was withdrawn from the receptor compartment using syringe, simultaneously 3 ml of fresh phosphate Buffer was replaced in order to maintain the sink condition. The withdrawn samples were analyzed for the presence of free drug by UV -Vis spectrophotometer. The peak absorbance of the drug was found to be 334 nm. Depend upon the absorbance value we obtained the concentration from the standard calibration curve. Then cumulative percentage drug release at regular time interval was calculated (Badarinath AV, 2010) as shown in the figure 6.

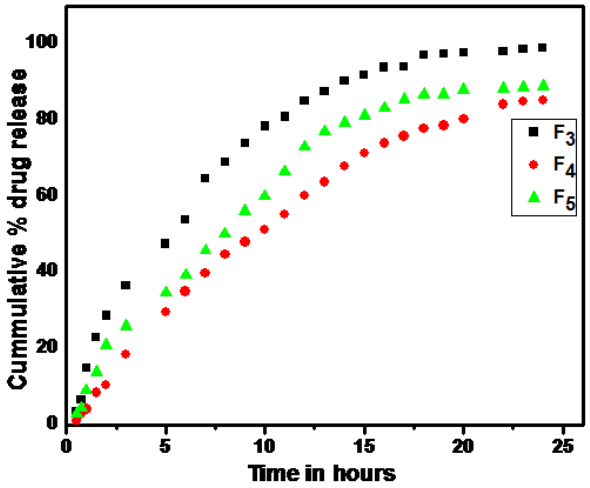


Figure 6: *In-vitro* release of Chloroquine formulations F_3-F_5. F_3 Formulation (1:5), F_4 Formulation (1:6) and F_5 Formulation (1:7) formulations

Stability Studies: The stability studies were carried using the formulation (F_4) (1:6). The stability of Chloroquine loaded Nanoparticles was evaluated in terms of its drugs content and shown in the figure 7. Nanoparticles formulations were stored at refrigerating conditions (4ºC), at room temperature (25ºC) and at 40^0C for the period of 8 weeks. The samples were analyzed for drug content every week by UV-Vis spectrophotometrically at 334 nm (Badarinath AV et al., 2010). It reminded stable during the specified duration of time.

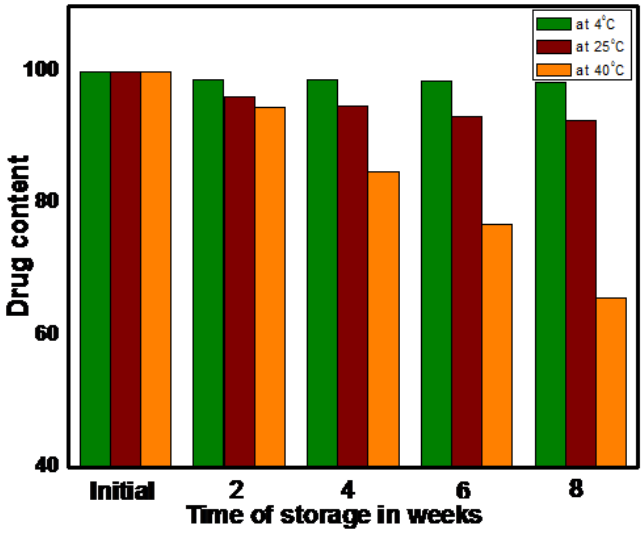


Figure 7: Stability studies of Chloroquine loaded chitosan Nanoparticles F_4

DISCUSSIONS

Chloroquine loaded Chitosan Nanoparticles were prepared and optimized (Formulations F_1-F_5) by Ionic gelation technique. The hydro gel beads are produced by dropping drug and Na-TPP into the aqueous solution containing Chitosan. Chitosan in acidic media interact with negatively charged Na-TPP forming inter and intra molecular cross linkages, yielding ionically cross – linked Chitosan Nanoparticles loaded by Chloroquine drug. This method results in spontaneous formation of Nanoparticles of smaller size (nm). X–ray diffraction spectrum was taken for pure drug Chloroquine, TPP, Chitosan and Chloroquine loaded chitosan nanoparticles (F_4). The Chloroquine shows peak at 2Ɵ positions are 16.96º, 21.21º, 24.32º, 25.98º, 27.19º and 32.02º because of its crystallinity. The polymer chitosan basically amorphous in nature, show peak at 2Ɵ positions 20.16º. The TPP shows peak at 2Ɵ positions 19.79º, 29.30º, 32.62º and 36.70º of its crystallinity. Whereas the Chloroquine loaded chitosan nanoparticles also exits in amorphous state as shown in the figures 1. XRD depends only on crystalline nature of the sample. The Chloroquine in dissolved or in molecular state shows crystallinity whereas the formulation F_4 shows amorphous state, as the individual drug molecules are coated by Chitosan polymer. The XRD graph shows that it is unable to identify the signals, so we obtained noise in the formulation (F_4). In order to confirm the Chloroquine Chitosan interaction between the samples IR spectroscopy were performed. The FTIR spectra of Chloroquine, Chitosan, TPP and Chloroquine loaded Chitosan Nanoparticles formulations are shown in the Figure 2. From the figure 2(a) the Chloroquine shows bend peak at 3462cm^{-1} N-H stretching, at 2933cm^{-1}C-H stretching, 1657cm^{-1}C=C stretching, at 1562cm^{-1} N-H bending, at 1403cm^{-1}bending and at 1012cm^{-1} C-N stretching. The figure 2(b) chitosan shows the broadening peak at 3625cm^{-1} N-H stretching, at 3389cm^{-1} O-H stretching, at 1642cm^{-1} C-N stretching, at 1208cm^{-1} O-H bending and at 918cm^{-1} C-O stretching. The figure 2(c) TPP shows board peaks at 1103cm^{-1} PO_4^+ phosphate ion. From the figure 2(d) the optimized formulation F_4 were taken for FTIR spectrum, the peak at 3440cm^{-1} N-H stretching, at 2925cm^{-1} C-H stretching, at 1635cm^{-1}C=N stretching, at 1461cm^{-1} C-H bending and 1200cm^{-1} bending vibrations. The drug and polymer does not have any inter molecular bond between each other. The drug entrapment efficiency of all formulations F_1 (1:3), F_2 (1:4), F_3 (1:5), F_4 (1:6) and F_5 (1:7) was reported in the table 2. The Nanoparticles exhibited an increase in drug content with increase in polymer ratio, up to a particular concentration (1:7). But the highest entrapment efficiency is around 92.87% for formulation F_4 as shown in the figure 3.

Transmission Electrons Microscope (TEM) images figure 4 (a) and 4(b) confirmed the nanosize of the formulation F_4. Images of Chloroquine loaded Chitosan Nanoparticles indicated that the Nanoparticles have a discrete spherical structure. The particle size was in the range of nanometer. The particle size analyzer (Malvern) is used to analyze the particle size distribution of the formulation F_4. The figure 5. Confirms the average particles size ranges from 175nm to 396nm.

The *In-vitro* drug release studies of different formulations were performed and the cumulative percentage drug release was observed. The formulations 1:5, 1:6 and 1:7 containing 50%, 60% and 70% Chitosan respectively showed a release of 98.81%, 85.13% and 88.85% after 24 hours. This shows that more sustained release was observed with increase in the percentage of Chitosan. The cumulative percentage drug release after 24 hours is reported in the figure 6. An initial fast release suggests that some drug was localized on the surface of the Nanoparticles. Formulation F_4 (1:6), showed good sustain and controlled release as compared to other formulations and it is found to be best.

Stability studies of formulation F_4 (1:6) formulation were showed in the figure 7. In refrigeration condition at 4ºC there was no remarkable change in the drug content compared to room temperature condition 25ºC and 40ºC. The formulations kept at higher temperature get degradation when compared to other conditions. This indicates that formulation F_4 (1:6) was stable in refrigeration condition at 4ºC.

CONCLUSION

Chloroquine Phosphate was successfully encapsulated into Chitosan Nanoparticles by ionic gelation method. Various formulations of Chloroquine Phosphate loaded Chitosan Nanoparticles were developed using various drug to polymer ratio. The prepared formulations were evaluated for drug entrapment efficiency; formulation F_4 (1:6) ratio shows higher drug entrapment efficiency. *In-vitro* release profile shows that Chloroquine Phosphate loaded Chitosan Nanoparticles is capable of releasing the drug in a controlled manner. The *In-vitro* release of Formulation F_4 is found to be 85.13% over 24 h. TEM characterization reveals that the Nanoparticles have a discrete spherical structure. Chloroquine is successfully encapsulated by Chitosan Nanoparticles with a particle size distribution in the range of nanometer. Stability studies indicate that the Optimized formulation was stable in refrigeration condition at 4ºC than room temperature and 40º C. From the present investigation it may be

concluded that Chloroquine Phosphate loaded Chitosan Nanoparticles are effective carriers for the design of controlled drug delivery for anti-malarial disease.

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