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Fabrication of Chitosan Based Antibacterial Implant for Multibacterial Bone Infection

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Despite advancements in medicines and novel surgical methods, osteomyelitis is still a complex and difficult to treat multi-bacterial bone infection. It generates in tissue necrosis along with rupturing of bone in chronic cases leading towards limited vascularity at infection site, local treatment might not show accountable level of antibiotic at infection site. The present research aims towards development and evaluation of bio-degradable implant of Ciprofloxacin hydrochloride to treat local osteomyelitis. Chitosan is widely investigated biodegradable polymer with hydroxyl moiety which is active and can be altered chemically to develop biomedical and therapeutic dosage forms. Here this active group was modified using epichlorohydrin & crosslinked Chitosan matrices were used as carrier to formulate different Ciprofloxacin implant. *In vivo* parameters and in vitro study was conducted for optimized batch. The formulation having 40% drug loading (EC4) was found to be optimum when all evaluation parameters were tested. The concentration of Ciprofloxacin Hydrochloride (HCl) in bone and surrounding tissues is much higher than minimum inhibitory concentration (MIC) even after a month. After observing the water uptake and extended release of drug from all formulations the drug loading was found to be higher. The present work

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concluded that, the highly branched chitosan remarkably reduce the percent (%) drug release up to a month which could be useful in the treatment of osteomyelitis.

Keywords: Implants; ciprofloxacin; osteomyelitis; chitosan; antibiotics.

1. INTRODUCTION

Osteomyelitis is a long-standing illness that, despite advancements in medicines and surgical methods, remains hard and difficult to treat. It is an pyrogenic disorder of bone inflammation which can be transient and persistent. The main pathogens linked with osteomyelitis are Staphylococcus aureus (80-85 percent) [1,2]. Because of growing usage of prosthetic devices and an increase in the frequency of incidents resulting in severe injuries, osteomyelitis is becoming more prevalent these days [1]. The disease is taking serious mode in both developed and developing nations.

In the early stages of osteomyelitis, systemic antibiotics are required, and in chronic instances, surgical excision of dead tissues from bone with occasional irritation at lesion site required high loading dose of antibiotic [3]. Patient pain, high treatment costs, the development of systemic toxicity, and patient compliance issues are only a few of the drawbacks of extended parenteral therapy [4]. The conventional system may not be therapeutically useful to attain concentration of drug in blood at infection site die to osteomyelitis resulting in bone necrosis as well as degradation. A extended course of parenteral treatment may also cause systemic damage. Localized medication therapy utilizing biologically non degradable PMMA cement for bone implant can be incorporated to correct many treatment issues for osteomyelitis treatment. Local treatment has the benefit of a high, local tissue concentration while limiting possibly harmful effects on other regions of the body.

Previous research on non-biodegradable carriers, on the other hand, has revealed that antibiotic release (in vitro) from PMMA beads is not sufficient and is also regulated poorly. Different polymers like Polycaprolactone (PCL) which are biologically degradable can be used as carrier to develop implant and are expected to show promising results as carriers for antibiotic for local infection treatement. [5]. Additionally, cost of these polymers is a having major limitation on the use of the same.

As the MIC of Ciprofloxacin is less (0.025 g/ml – 2 g/ml) towards large number of causative pathogens of osteomyelitis including *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa,* and *Proteus mirabilis*, and is found to be effective against bacterial bone infections.

Therefore, to avoid drawbacks of conventional therapy, an attempt is made to formulate subcutaneous system of implant incorporating Chitosan releasing drug very slowly from the implant device and help in achieving upper concentration at local tissues at site if infection. N-deacetylation of chitin, a polysaccharide extensively dispersed in nature as a key component of crab and insect shells, produces the biopolymer chitosan. Chitosan is reported to be biocompatible and free of inflammatory responses at the same time acting as release retardant in many reports [6,7].

2. MATERIALS AND METHODS

Ciprofloxacin Hydrochloride HCl and Chitosan (89% deacetylated) was kindly supplied by Glennmark Pharmaceuticals Ltd., (Nashik). All chemicals were of analytical grade.

2.1 Material Characterization

2.1.1 Characterization of simple chitosan

a) Determination of degree of deacetylation by Potentiometry [8,9]

About 2 gm of Chitosan was dissolved in 0.1 N HCl (20 ml) and was titrated using 0.1 N NaOH as titrant. Two inflection points in titration curve were observed showing difference between parallel abscissa quantifying acid level required for protonation of amino group. The general equation can be drawn to calculate deacetylation degree from NaOH utilized between inflection points.

$$DD = 16.1(Y - X)\, f / w$$

Where Y and X are the analogous spots' consumed NaOH volumes, f is the NaOH solution's molarity, and w is the starting chitosan weight (in gms).

b) Fourier Transformation Infra-red (FTIR) analysis

A FTIR of the Chitosan was obtained on a Shimadzu 8400 S FTIR (Tokyo, Japan) in the range of 4000-400 cm^{-1}, using KBr pellet.

2.1.2 Preparation of Cross-Linked Chitosan with Epichlorohydrin [10-12]

a) Around 2.0 gm of Chitosan

About 2 g of chitosan was dissolved in 20.0 mL of acetic acid (5 percent v/v) and 50 mL of distilled water were used to make the chitosan solution. With 1.0M sodium hydroxide solution, the pH was raised from 3.0 to 11.0. Epichlorohydrin solution (0.85 percent V/V) was added, and the mixture was stirred for 24 hours at room temperature (Magnetic Stirrer, Remi). The precipitate was then formed by adding 50mL of 1.0M sodium hydroxide solution to the mixture. To eliminate any unreacted epichlorohydrin, the precipitate was filtered and washed extensively with distilled water. It was then dried for 12 hours in a hot air oven.

2.1.3 Mechanism of Cross-linking

Epichlorohydrin interacts with chitosan's carboxyl groups. The electrostatic interaction between (CH 2O—) on chitosan and (CH 2 +) on Epichlorohydrin results in cross linking. (Fig. 1).

2.1.4 Characterization of Epichlorohydrin Cross-linked chitosan

a) By Potentiometric Titration method

The amounts of protonable (amino) groups in Epichlorohydrin Cross-linked chitosan were measured using potentiometric titration as described for Simple Chitosan.

b) By FTIR

The amounts of protonable (amino) groups in Epichlorohydrin Cross-linked chitosan were measured using FTIR. The drug and polymer were put together in equal proportion and stored in dry vial preferably of glass using normal temperature and humidity conditions up to 7 days. Drug powder as obtained, polymers as obtained and their mixture was then analysed by FTIR.

2.1.5 Compatibility study

a) X-ray Diffraction (XRD) studies [13]

Drug, polymers and mixture triturated must be checked using XRD to check interaction between the excipients. The XRD patterns were taken from diffractometer (PW 3710, Philips) at the following conditions-

time per step: 0.400 s, step size: 2θ = 0.020° (θ is incident angle), current: 30 mA at 40 kV, CuK rays (wavelength =1.542 A°).

b) DSC Study

To verify the compatibility of drug and polymer after cross linking, thermo grams of drug and mixes of drug and cross-linked Chitosan were produced using Shimadzu DSC-60 Differential Scanning Calorimeters and aluminum pans. Different temperature programming was done for different samples. Through the cooling unit, nitrogen was expelled. The DSC temperature was calibrated using an indium standard. The samples were enclosed in aluminium pans and heated at a steady rate of 20 degrees Celsius per minute throughout a temperature range of 0 to 700 degrees Celsius. Purging nitrogen at a rate of 30 mL/min was used to maintain the inert environment.

2.2 Formulation Development

2.2.1 Epichlorohydrin Cross-linked Chitosan as a carrier

Formulations were created in attempt to provide an implanted dosage form with a controlled release. Both the active component (Ciprofloxacin HCl) and the polymer (Epichlorohydrin Cross-linked Chitosan) were precisely weighed and sieved at 60#. Spatulation was used to mix the particles. Drug: The polymer ratio (D: P) and its influence on in-vitro release were the two formulation factors investigated. In all formulations, the weight of the implant pill was kept constant (150 mg). Table 1 shows the formulation code and drug:polymer ratio utilized.

2.2.2 Preparation of implants

The powder mix (EC1 to EC5) was compressed using the direct compression method on a rotary compression machine (General machine, India). 8 mm flat-faced circular punches were used to compress the material.

To obtain a tablet weight of 150 mg and a hardness of 4.5-5.5 kg/cm2 for all formulations, they were crushed at a constant force.

2.2.3 Evaluation of implants

The compressed implants were tested on different parameters as-

2.2.4 Thickness and Diameter variation Test

A Micrometer Screw Gauge (Yamayo classic, Japan) was used to measure the thickness and diameter of the implants. The mean thickness and diameter of five implants from each batch of formulation were determined, together with the respective S.D. for each formulation.

2.2.5 Hardness Test

The Monsanto hardness tester (Cadmach, Ahemedabad, India) was employed to determine the hardness of implants (n=5).

2.2.6 Drug Content [14,15]

The sample milled implant was put in 100 ml (0.1N HCl) and maintained at room temperature for 24 hours with magnetic stirring (50 rpm). The drug concentration was measured spectro-photometrically at 277 nm after the solution was filtered using Whatmann filter paper.

2.2.7 Water Uptake Study [14,15]

The implants were pre-weighed (t=0). These were kept in phosphate buffer pH 7.4 as medium. The aliquot was withdrawn at predetermined time interval resulting blotting of excessive water. The implants were weighed again. Following equation was used to determine water uptake.

$$\text{Water Uptake (\%)} = \frac{W_w - W_i}{W_i} \times 100$$

Where, Ww is the wet weight,

W_i is the initial weight

2.2.8 Mass Loss (% Erosion) [4,14-17]

The previously weighted implants were kept in 20 ml Phosphate buffer at pH 7.4 (t=0) and taken out after the period of 5 weeks. Excess water was dried out and implants were re-weighed. These were again dried at 105^0C at hot air oven and final weight was determined. Amount of CFX released was calculated using spectrophotometric estimation.

% Erosion was determined using following equation,

$$\%\text{Erosion} = \frac{(W_i - W_{CFX\ Released}) - W_d}{W_i} \times 100$$

Where, W_i is the initial weight,
W_d is the dry weight,

$W_{CFX\ Released}$ is the weight of Ciprofloxacin HCl released after 5 weeks.

2.3 *In Vitro* Testing (Percent Drug Release)

2.3.1 Rotary Shaker Method (Vial method) [4,14-17]

The drug release investigation was carried out in 30 mL screw-capped glass vials (diameter = 25 mm) containing 20.0 mL dissolving medium using this procedure. The implants were submerged in USP phosphate buffer (0.1 M, pH 7.4) containing antibacterial agents of 0.1 percent w/v sodium azide. Samples from each formulation were incubated for 5 weeks (or more) at 370°C in an orbital shaking incubator (Remi, India) (shaking bath) (60rpm) with agitation (60 rpm) (Fig. 3). To maintain sink state, full dissolving medium was withdrawn and replaced with fresh buffer at predetermined time intervals. The drug release investigation was carried out in 30 mL screw-capped glass vials (diameter = 25 mm) containing 20.0 mL dissolving medium using this procedure. The implants were submerged in USP phosphate buffer (0.1 M, pH 7.4) containing antibacterial agents of 0.1 percent w/v sodium azide. Samples from each formulation were incubated for 5 weeks (or more) at 370°C in an orbital shaking incubator (Remi, India) (shaking bath) (60rpm) with agitation (60 rpm) (Fig. 3). To maintain sink state, full dissolving medium was withdrawn and replaced with fresh buffer at predetermined time intervals.

2.3.2 Drug Release Kinetics [18]

To determine the relevant release rate and mechanism of drug release from the implants, the release data was fitted to Zero order, First order, Higuchi, and Korsmayer- Peppas equations. The correlation coefficient was used

to evaluate the model that fit the release data (R). The R value was employed as a criterion for selecting the best fit model to characterise drug release from an implantable drug delivery device.

2.3.3 Effect of degree of crosslinking on drug release

By altering the concentration of epichlorohydrin, several cross-linked chitosan matrices were created. In a nutshell, the chitosan solution was made by dissolving 2 gm of chitosan in 20.0 mL of acetic acid (5 percent v/v) and then adding 50 mL of distilled water. With 1.0M sodium hydroxide solution, the pH was raised from 3.0 to 11.0. Epichlorohydrin solution in the varying concentration ranges from 0.85%, 1.70% and 2.55% was added and the mixture was stirred for 24 hours at room temperature (Magnetic Stirrer, Remi). The precipitate was then formed by adding 50mL of 1.0M sodium hydroxide solution to the mixture. To eliminate any unreacted epichlorohydrin, the precipitate was filtered and washed extensively with distilled water. To eliminate any unreacted epichlorohydrin, the precipitate was filtered and washed extensively with distilled water. It was then dried for 12 hours in a hot air oven. To collect cross-linked chitosan particles, the resultant material was crushed and sieved.

2.4 Preparation of Implants

The cross-linked chitosan matrices by varying the concentration of epichlorohydrin were prepared & their formulations were prepared by direct compression as (Table 2). The change in pattern of percent drug release with varying the concentration of crosslinking agent was evaluated by vial method.

2.5 Stability Study [19-22]

The ICH Guidelines were followed for conducting the stability investigation. Accelerated stability testing (402°C/755 % RH) was performed on optimised formulations made from Epichlorohydrin crosslinked chitosan-based implants. After 6 months at 40°C, the test implants were checked for various parameters like hardness, thickness, diameter, drug content, and drug release. The obtained results were compared to an initial sample reading from the same formulation that was tested at ambient temperature (initial readings).

2.6 *In-vivo* Release Study of Optimized Formulation

2.6.1 Selection of formulation for *In vivo* studies

The formulation which shows prolonged release for five weeks based on *In vitro* release pattern will be consider as an optimized formulation & used further for *In Vivo studies.*

2.6.2 Sterilization of implant

i) Terminal Gamma Sterilization

The selected formulation were dispatched to Isomed, Board of Radiation and Isotope Technology (BRIT), Trombay, Mumbai to analyse effect of terminal gamma ray sterilization. The radiation source used is Co 60 & Dose of Radiatio is 2.5 Mrad which is equivalent to 25kGy (Kilogray)

2.7 Animals

The protocol used in animals handling and performance of experiment was approved by animal ethical committee. Wistar rats were used as test a animal to conduct the In-vivo drug release study of implants and the protocol is sanctioned by ethical committee. The Wistar rats of equal age group and each weighing 175-200 g were employed to test antibiotic release (Ciprofloxacin) from chitosan implants. A standard antibiotic free diet was given with water was given to animals which are housed in separate cages. Such animals were allowed to acclimate to the environment (15 days period) before implantation. Total 24 animals were used for the study as mentioned in the protocol. The 24 animals were allotted into six groups. Each group contains four animals. Initially, the EC4 formulation was implanted into each animal to study *in-vivo* drug release and to determine local concentration of Ciprofloxacin HCl near femur bone. At appropriate time intervals (i.e. 4, 7, 14, 21, 28 & 35^{th} day) each group was scarified by method described below and *In vivo* release was studied. From that mean concentration was determined.

2.8 Procedure

2.8.1 Subcutaneous Administration of Implant [14,20]

Rats were anesthetized by the intra-peritoneal (i. p.) Ketamine HCl injection (70 mg/kg) and

Diazepam (5 mg/kg). The hind leg must aseptically cleaned and surgically prepared. All the hairs on the skin above the femur bone must be clan shaved, an incision to skin (1.5 cm) was marked to exposed deep subcutaneous fascia (inner layer of subcutaneous tissue that enclose muscles and muscle groups), The test sample of Implant was securely placed via subcutaneous route and the incision was sutured with surgical suture.

2.8.2 Determination of Residual Ciprofloxacin Concentration [14,20]

On 4, 7, 14, 21, 28 & 35th days after administration period dislocation at cervical site was performed to sacrifice animal and implantation area was open by cutting it. The skin around the implantation site was sliced open immediately after the sacrifice, and the implant pill that remained at the administration site was removed and put in a Citrate buffer (pH-4). The citrate buffer containing residual implant was placed on magnetic stirrer for 24 hrs to extract all residual concentration of Ciprofloxacin. To assess the quantity of medication left at the implantation site, the solution was evaluated by HPLC using a C-18 reversed phase column (Waters, Austria) and an elution with two mobile phases containing 5% acetic acid and acetonitrile–methanol (80:20v/v), respectively. By subtracting the percent drug remaining at the implantation site from the initial percent concentration, the percent drug discharged may be determined. The initial concentration of the implant is considered as 100 percent.

2.8.3 Determination of Muscle Ciprofloxacin Concentration [14,20,21]

After removal of residual implant tablet, the subcutaneous tissues near femur bone was completely removed to exposed the muscles above the femur bone. All the muscles (2×3 cm) above which the implant is placed were cut down and are accurately weighed. The muscles which are removed and were cropped into small pieces and immediately kept in a Citrate buffer (pH-4). The citrate buffer containing muscles was placed on magnetic stirrer for 24 hrs. to extract Ciprofloxacin. The solution was analyzed by HPLC to determine concentration of drug in extracted solution of muscles. The implantation site is also examined for any signs of inflammation. The concentration of Ciprofloxacin HCl in muscle is determined by dividing extracted solution concentration of muscle by weight of that muscle at each time point.

2.8.4 Determination of Bone Ciprofloxacin Concentration [14,20,21,22]

After removal of muscles, femur bone of rat was separated using surgical scissor and is accurately weighed. The separated femur bone is cut into three to four pieces and immediately placed in a Citrate buffer (pH-4). The citrate buffer containing bone was placed on magnetic stirrer for 24 hrs. to extract Ciprofloxacin. The solution was analyzed by HPLC to check the percentage of drug in extracted bone sample. The concentration of Ciprofloxacin HCl in bone is determined by dividing extracted solution concentration of bone by weight of that bone at each time point.

3. RESULTS AND DISCUSSION

3.1 Material Characterization

3.1.1 Characterization of Simple (Non Cross-Linked) Chitosan

a) Degree of deacetylation

The potentiometric plot of chitosan with aid of chemicals like HCl (0.1 N) and NaOH (0.1 N) is as shown in Fig. 2, which shows two equivalent points. The first one is due to reaction of NaOH with excess of HCl present in the reaction medium and the second one is due to reaction of NaOH with NH_3^+ group of chitosan polymer. The value for the test was found to be to be 88.55%.

b) Fourier Transformation Infra-red (FTIR) analysis

FTIR analysis of the sample of chitosan exhibit all the specific IR peaks as can be co related from literature value. A Fourier-transform infrared (FTIR) spectrum of the Chitosan is presented in Fig. 3.

3.1.2 Formulation of epichlorohydrin cross-linked chitosan

A Chitosan (Cross-linked) weighting 1.83 g was obtained from 2g chitosan powder (Percent yield = 91.50 %). The product yield was found to be 91.5%. Deduction of 8.5% can be result of process of collection and drying of the residue.

3.1.3 Evaluation of Epichlorohydrin Chitosan

a) By potentiometric Titration method

The amounts of protonable (amino) groups in polymer were measured using potentiometric

titration. Epichlorohydrin-cross-linked chitosan presents same amount of proton able amino moieties in vicinity to natural (simple) chitosan. This indicates that the amino terminals were not blocked by Epichlorohydrin groups & this crosslinking agent is attacking on different structures for protein binding to initial amino moiety (OH group).

b) By FTIR spectroscopy

The amounts of free hydroxyl groups in FTIR Spectrum of Epichlorohydrin cross-linked chitosan were measured using FTIR. The peak of O-H Stretching & C-O Stretching of Simple (non crosslinked) chitosan were not observed in FTIR Spectrum of Epichlorohydrin cross-linked chitosan as shown in Fig. 3. This clearly indicates that most of reactive hydroxyl terminals were blocked by Epichlorohydrin. Also the peak of amino groups is not affected, i.e. Epichlorohydrin forms crosslink with hydroxyl groups only & not with amino terminals.

3.1.4 Compatibility study

a) X-ray Diffraction analysis

A spectrum of drug, polymer with their compressed mixture is as shown in Fig. 4. The peaks of pure drug were observed in the spectrum of the compressed mixture, this shows absence of any kind of interaction of drug and the polymer incorporated. Hence the drug was found to be compatible with polymer.

b) Thermal analysis (DSC)

Drug Thermograms are graphs that show the temperature of a substance. To assess the compatibility of drug and polymer after crosslinking, a mixture of drug and chitosan was formed (Fig. 5). The endo-thermic peak was clearly preserved in the DSC thermogram of Drug +All excipient combos. As a result of the aforesaid findings, it may be stated that the drug–excipient combination does not exhibit any significant signs of drug–excipient incompatibility.

3.2 Evaluation of Implants

3.2.1 Thickness, Diameter Test

The diameter, thickness, and hardness of the implants were all measured. Table 3 shows the results. The hardness, thickness, and diameter of all of the formulas were all the same.

3.2.2 Drug Content

In all of the formulations, the medication was distributed uniformly in all of the implants. All formulations' drug content was assessed and presented in Table 4.

3.3 Water Uptake Study

Fig. 6 shows the percent water intake of the EC1 through EC5 formulation.

It has been discovered that EC1 has a lower water absorption ratio when compared to other formulations. This variation in water uptake can be related to the percentage of cross-linked chitosan in various formulations. Because chitosan has a lower water uptake capacity and the fraction of cross linked chitosan in EC1 (i.e. Drug: polymer-1:9) is so high, it has a lower water uptake than other formulations.

3.4 Percent Erosion

Table 5 shows the percent erosion of the EC1 through EC5 formulation. The % erosion of EC4 and EC5 formulations is significantly higher than that of EC1.

3.5 *In vitro* Drug Release Study

The total % release from all formulations (triplicate measurements) is calculated, as illustrated in Fig. 7. In five weeks, the EC1 formulation exhibits just 46.07 percent release, while the EC4 formulation shows 99.46 percent release. The amount of Cross-linked Chitosan in different formulations may be responsible for this effect. The amount of Cross-linked Chitosan in the EC1 formulation is very high (drug: polymer ratios (1:9), resulting in higher drug release retardation. Because the quantity of Cross-linked Chitosan in EC4 is low (drug: polymer ratio (1:1.5)), the drug release is slowed less, resulting in a higher cumulative percent release from the EC4 formulation.

The drug:polymer ratio in the EC2 and EC3 formulations (1:4 and 1:2.33, respectively) is also greater than in the EC4 formulation, resulting in only 77 percent and 89 percent release in five weeks, respectively. The smaller quantity of Chitosan (drug: polymer ratio (1:1) utilised in the EC5 formulation allows it to release 99.77 percent of the medication in 28 days. This clearly indicates that the medicine release is delayed as

the amount of epichlorohydrin crosslinked chitosan in the formulation rises.

The main explanation for this finding is that increasing the quantity of cross-linked chitosan in the implants reduces swelling, which prevents medication release. Water absorption diminishes as the amount of cross-linked chitosan increases. The medication is released from the matrix by swelling, which is followed by diffusion of the antibiotic. Higher cross-linked chitosan concentrations resulted in more stiff implants that swelled less in phosphate buffer. These findings show that ionic crosslinking is a promising method for limiting ciprofloxacin release from cross-linked chitosan matrices.

The quick release of all epichlorohydrin crosslinked chitosan-based formulations is followed by a slow controlled release. When administering antibiotics, a large dose is generally necessary during the first period to successfully battle bacterial infections, and then the release is gradually continued for longer time periods to finish the therapy. Such quick initial release was not observed in citrate crosslinked chitosan matrices study.

3.6 Effect of Drug Loading

Using similarity factor (f2) testing, the effect of drug loading on percent cumulative CFX release from EC1 and EC5 formulations was studied. Medication loading affects the release profile of ciprofloxacin HCl. The cumulative % release from implants for the two formulations EC1 vs. EC5. The Similarity factor is calculated and is found to be,

$$f_2 = 36.42$$

i.e. (f_2 < 50), this indicates dissimilarity of dissolution profile & it arises because of drug loading. With increasing drug loading, the cumulative percent release of Ciprofloxacin HCl increased. This effect on release profile could be attributed to difference in water uptake capacity of both formulations. The water uptake results indicate that EC1 has a comparatively less water uptake capacity than EC5 because the amount of Cross-linked Chitosan is increased in EC1 (drug: polymer ratio (1:9)) and as Cross-linked Chitosan has minimal water uptake capacity which results in excess retardation of CFX release and only 46% amount of Ciprofloxacin HCl is released in five weeks. But in case of EC4 as the drug loading increases the water uptake capacity also increases results in comparatively more hydration and porosity; therefore total amount (99%) of Ciprofloxacin HCl is released in five weeks. In EC5 formulation water uptake capacity is higher than others therefore more hydration produces parallel increased in porosity because of this reason, total amount of drug is released in 28 days only.Therefore, it is proved that from study epichlorohydrin crosslinked chitosan based implants, the Ciprofloxacin release is mainly depends on hydration and porosity.

3.7 Drug Release Kinetics

The R values for the Higuchi equation were high for the EC1 to EC5 formulations, indicating that drug release from these formulations followed Higuchi (matrix diffusion) kinetics. For each formulation, the value of Release Exponent ‘n' is also determined. The drug release mechanism is indicated by the value of ‘n' in Korsmeyer-Peppas equation. The value of ‘n' in the range of EC1 to EC4 is less than 0.50, suggesting that drug release from these implants is solely regulated by diffusion (Fickian diffusion). The value of ‘n' in EC5 formulations is in the range of 0.50 to 1.0, indicating that drug release from EC5 formulations is regulated by both drug diffusion and polymer chain erosion (non-Fickian diffusion or anomalous diffusion).

3.8 Effect of Degree of Crosslinking on Drug Release

The cumulative percent release from G1 through G3 formulations was calculated (triplicate measurements) and is displayed in Fig. 8. The G1 formulation releases 99 percent of the drug in five weeks, whereas the G2 and G3 formulations release 99.85% and 99.67 percent of the drug in 28 and 11 days, respectively. This might be due to concentration of cross-linking agent used in various chitosan matrices which were synthesized. The G1 formulation matrices were synthesized by using 0.85%v/v of epichlorohydrin whereas G2 & G3 formulation matrices were synthesized by using 1.7% & 2.55% v/v of epichlorohydrin. i.e. concentration of epichlorohydrin was lesser in G1 & higher in G2& G3. This indicates that, as the amount of crosslinking agent (epichlorohydrin) decreases, the amount of free hydroxyl groups on chitosan decreases, thus hindering the water uptake of matrices. Therefore, Epichlorohydrin when used as a crosslinking agent for polymers, must be used in lesser concentration (i.e.0.85%v/v) so

that synthesized matrices can retard drug release for longer duration of time.

From this study it is proved that concentration of crosslinking agent added in synthesis of polymer matrices have significant effect on drug release. The optimum concentration of epichlorohydrin as a crosslinking agent was found to be 0.85% w/w for crosslinking of Chitosan matrices.

3.9 Stability Study

The stability of the optimised EC4 formulation was investigated. According to the findings, EC4 implants have a decent stability profile throughout a 6-month storage period. During the whole testing period, the implants' physical appearance, hardness, thickness, diameter, and drug content were unchanged at 402°C/755 percent RH. During the stability testing period, all of the implants preserved their initial physical features such as colour, texture, and diameter. Fig. 9 depicts the disintegration profile. After 6 months of storage under accelerated stability conditions, a similar dissolution profile was achieved.

3.10 *In-Vivo* Release Study of Optimized Formulation

3.10.1 Selection of formulation for *In vivo* studies

The EC 4 formulation of epichlorohydrin crosslinked chitosan based matrices was selected for *In Vivo* study.

3.10.2 Host Response

The experimental animals were found to be healthy through entire duration of study and do not shows any signs of adverse effect. The implantation site was free from any signs of macroscopic changes such as reddening, local necrosis, infection, abscess, edema, ulcer formation etc. during the post implantation period

3.10.3 Residual Ciprofloxacin Concentration

Fig. 11 depicts the in vivo releasing profile. R values for Zero order equations were high for EC4 formulation, indicating that drug release from EC4 formulation followed Zero order kinetics. For in-vivo investigations, the value of ‘n' is in the range of 0.50 to 1.0, indicating that drug release from these formulations is regulated by both drug diffusion and polymer chain erosion (non-Fickian diffusion, Case II transport or anomalous diffusion).

3.10.4 Muscle Ciprofloxacin Concentration

The results depict that concentration of the concentration of Ciprofloxacin HCl in muscles is much higher than minimum inhibitory concentration even after four weeks as shown in Fig. 12.

3.10.5 Bone Ciprofloxacin HCl concentration

The results depict that concentration of Ciprofloxacin HCl in bone is much higher than minimum inhibitory concentration even after four weeks as shown in Fig. 13.

Comparatively, the concentration of Ciprofloxacin HCl in muscle is much higher than bone concentration, because the bones are poorly perfused with blood supply than muscles and also bone is more compact and denser than muscles therefore the diffusion of drug in bone is lesser than in muscles. Thus from the *In vivo* release study it is seen that the Ciprofloxacin-Chitosan composite implant (EC4 formulation) prolongs the release of drug up to four weeks in rat model. Also the concentration of Ciprofloxacin HCl in bone and surrounding tissues is much higher than MIC even after a month in rat model therefore the optimized implant formulation could be useful to combat microbial contamination in osteomyelitis & other bone infection.

Table 1. Formulation Development Experiment using Epichlorohydrin Cross-linked Chitosan

Sr. No.	Formulation Code	Ciprofloxacin HCl (%)	Epichlorohydrin Cross-linked Chitosan (%)	Drug: Polymer	Weight of Implant (mg)
1	EC1	10	90	1:9	150
2	EC2	20	80	1:4	150
3	EC3	30	70	1:2.33	150
4	EC4	40	60	1:1.5	150
5	EC5	50	50	1:1	150

Table 2. Formulation development experiment using different concentration of Epichlorohydrin

Sr. No.	Formulation Code	Ciprofloxacin HCl (%)	(0.85% v/v) Epichlorohydrin Cross-linked Chitosan Matrices (%)	(1.70% v/v) Epichlorohydrin Cross-linked Chitosan Matrices (%)	(2.55%v/v) Epichlorohydrin Cross-linked Chitosan Matrices (%)	Weight of Implant (mg)
1	**G1**	40	60	------	------	150
2	**G2**	40	------	60	------	150
3	**G3**	40	------	------	60	150

Table 3. Diameter, Thickness and Hardness of EC1 to EC5 formulations

Parameters	Formulation Code				
	EC1	**EC2**	**EC3**	**EC4**	**EC5**
Diameter (mm)	8.01 (±0.011)	8.08 (±0.009)	8.04 (±0.014)	8.08 (±0.010)	8.06 (±0.011)
Thickness (mm)	2.28 (±0.011)	2.30 (±0.018)	2.33 (±0.022)	2.30 (±0.017)	2.32 (±0.010)
Hardness (Kg/cm2)	5.2 (±0.034)	5.2 (±0.041)	5.1 (±0.048)	5.3 (±0.056)	5.3 (±0.053)

Table 4. Drug content of EC1 to EC5 formulations of Ciprofloxacin HCl

Formulation Code	Drug Content (%)
EC1	98.90 (±0.012)
EC2	99.45 (±0.023)
EC3	98.72 (±0.011)
EC4	99.56 (±0.009)
EC5	99.18 (±0.014)

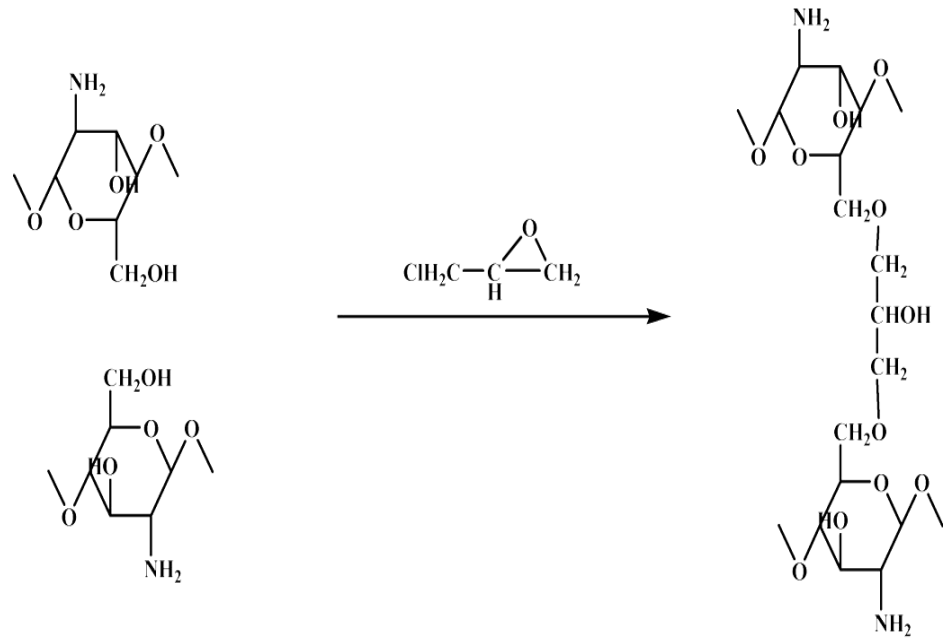

Fig. 1. Crosslinking reaction of chitosan with epichlorohydrin

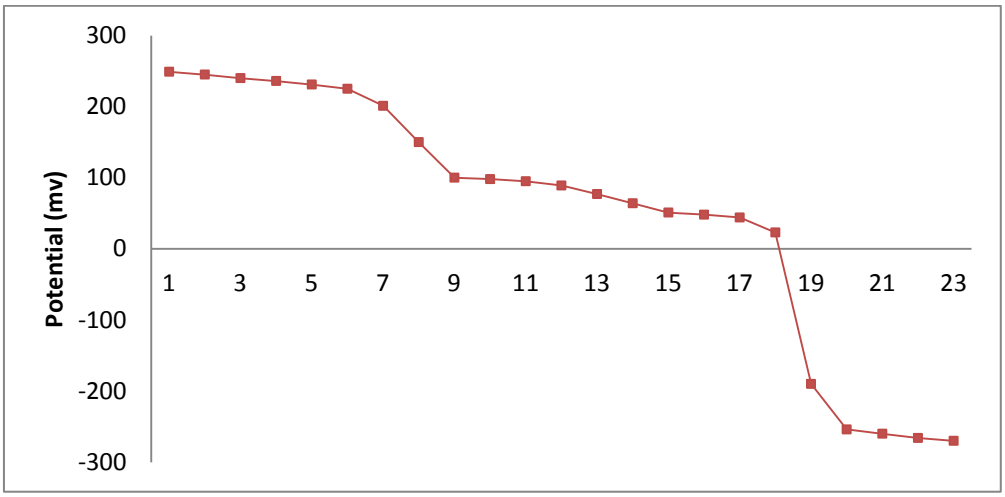

Fig. 2. Potentiometric titration curve of Simple (Non Cross-linked) Chitosan showing two equivalent points (The first one is due to reaction of NaOH with excess of HCl present in the reaction medium and the second one is due to reaction of NaOH with NH_3^+ group of chitosan polymer)

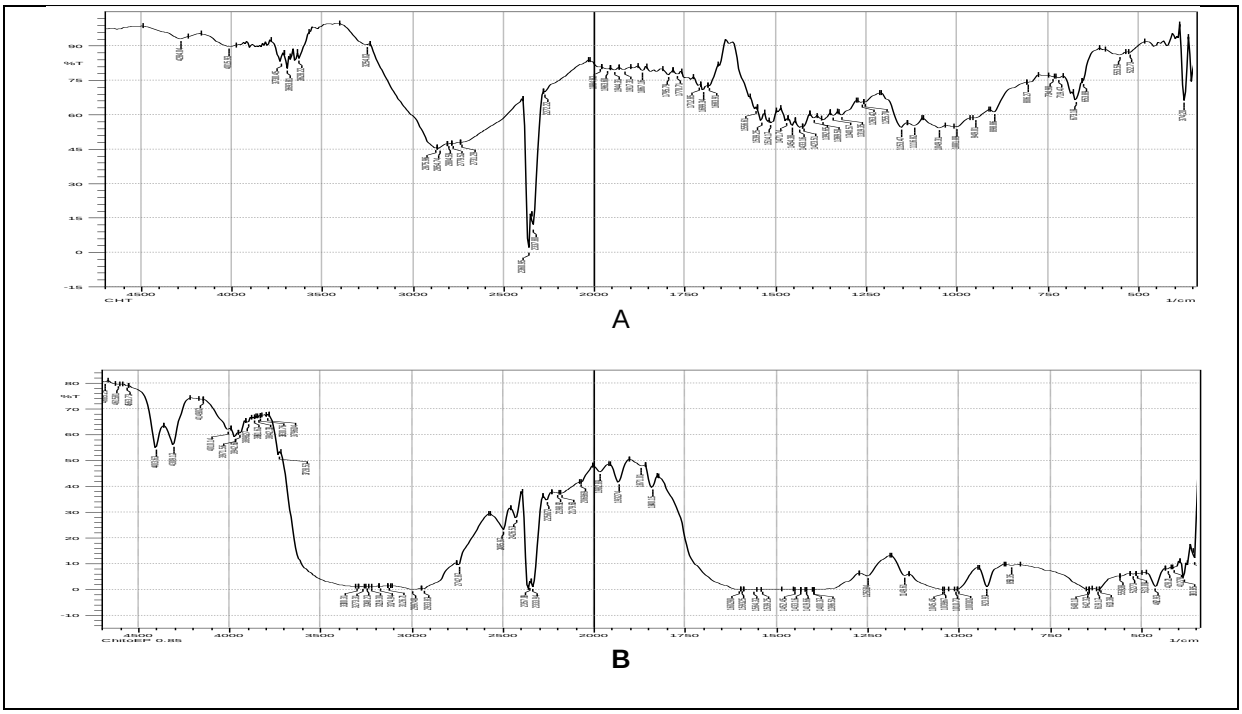

Fig. 3. FTIR Spectrum of Chitosan (A) & Epichlorohydrin crosslinked chitosan (B)

Table 5. Percent erosion of EC1 to EC5 formulations

Formulation Code	% Erosion (w/w)
EC1	5.87 (±0.56)
EC2	6.12 (±0.22)
EC3	8.84 (±0.78)
EC4	11.51 (±0.90)
EC5	14.32 (±0.74)

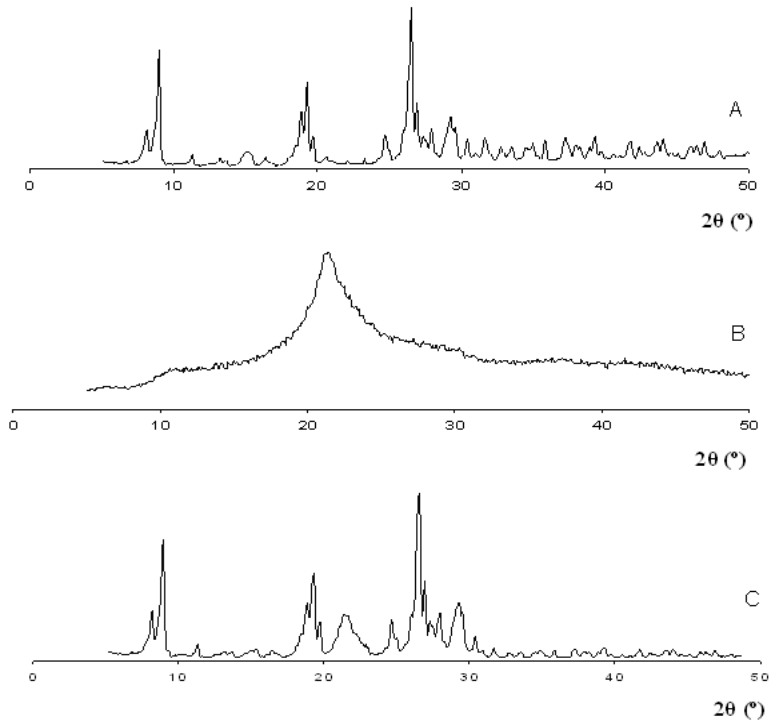

Fig. 4. XRD spectra of Ciprofloxacin HCl (A), Chitosan (B) & there mixture (C)

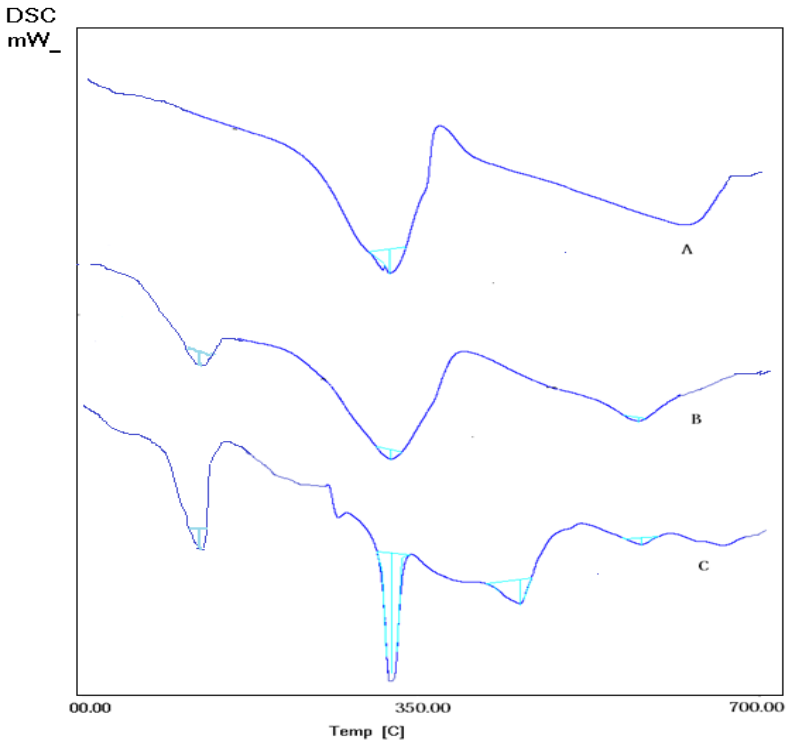

Fig. 5. Thermograms of Drug (A) & Mixture of Drug & Citrate cross-linked chitosan (B) & Mixture of Drug & Epichlorohydrin cross-linked chitosan (C)

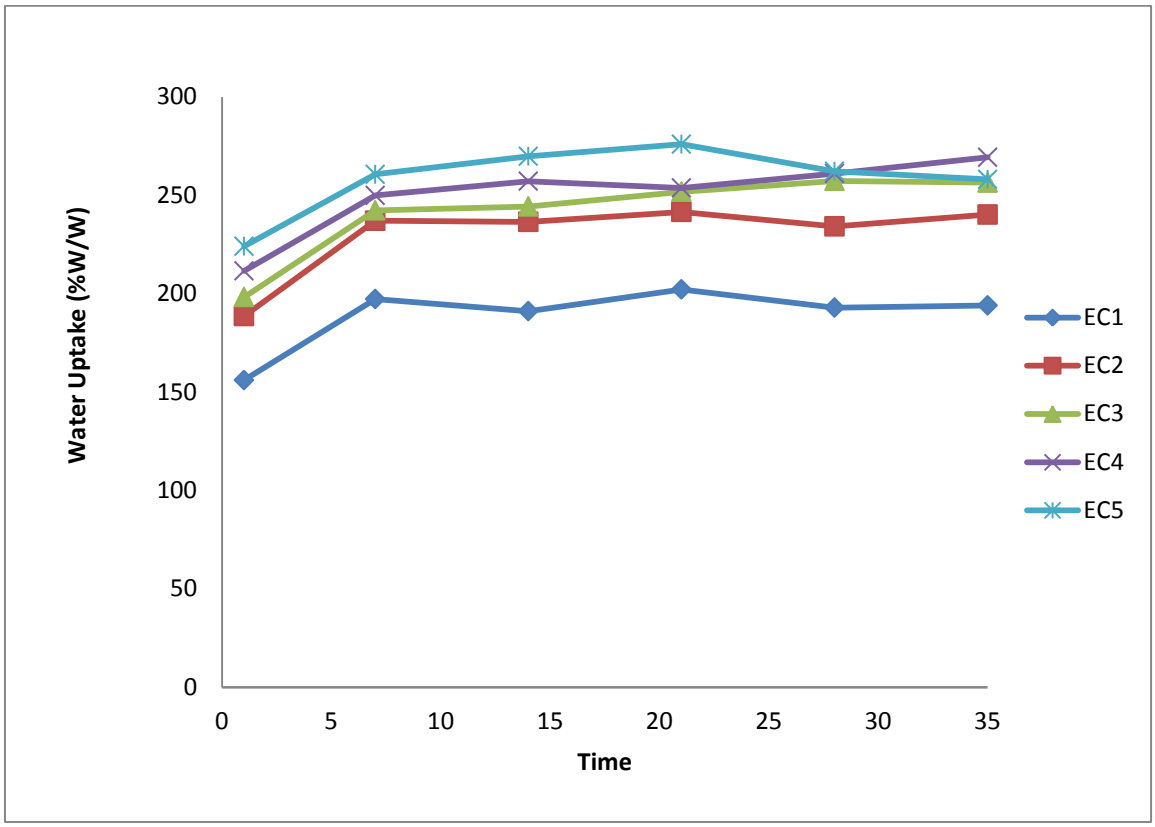

Fig. 6. Water uptake study of EC1 to EC5 formulations

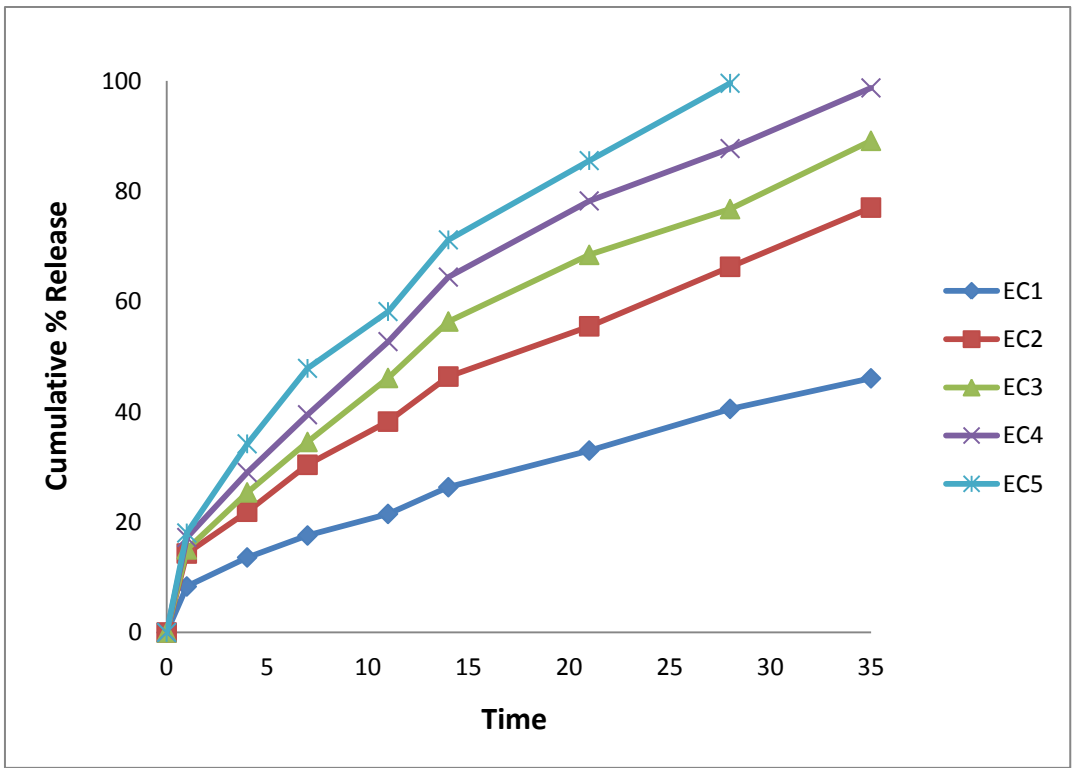

Fig. 7. Cumulative percent drug release from EC1 to EC5 formulations

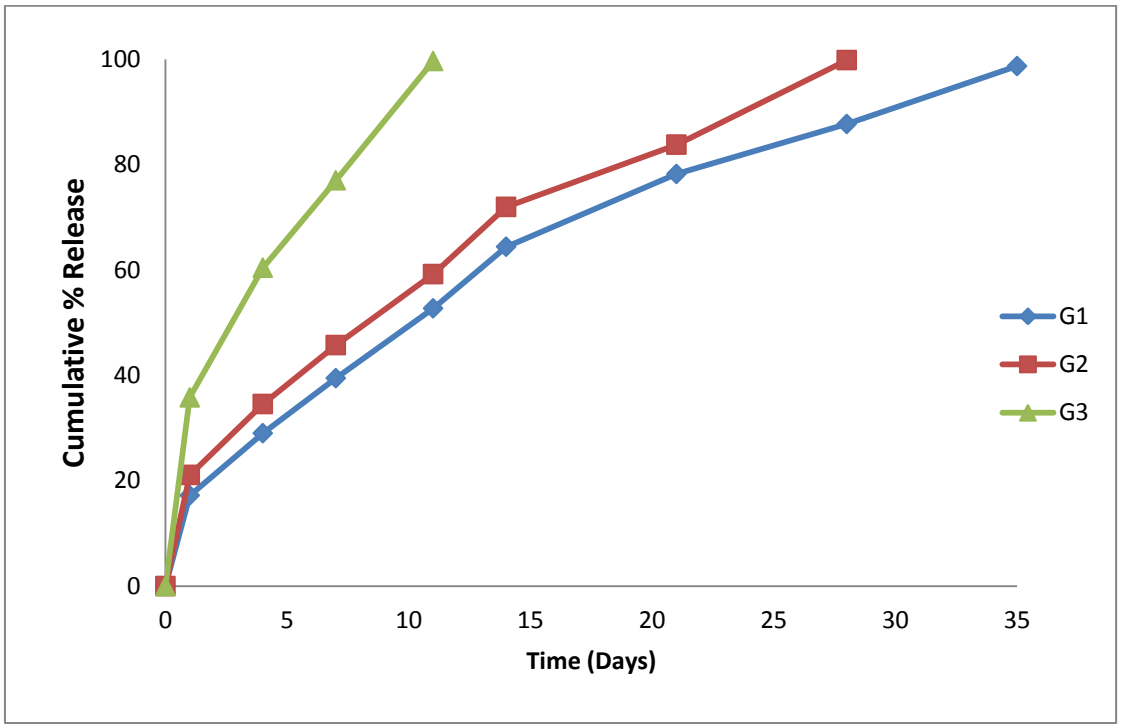

Fig. 8. Cumulative percent drug release from G1to G3 formulations

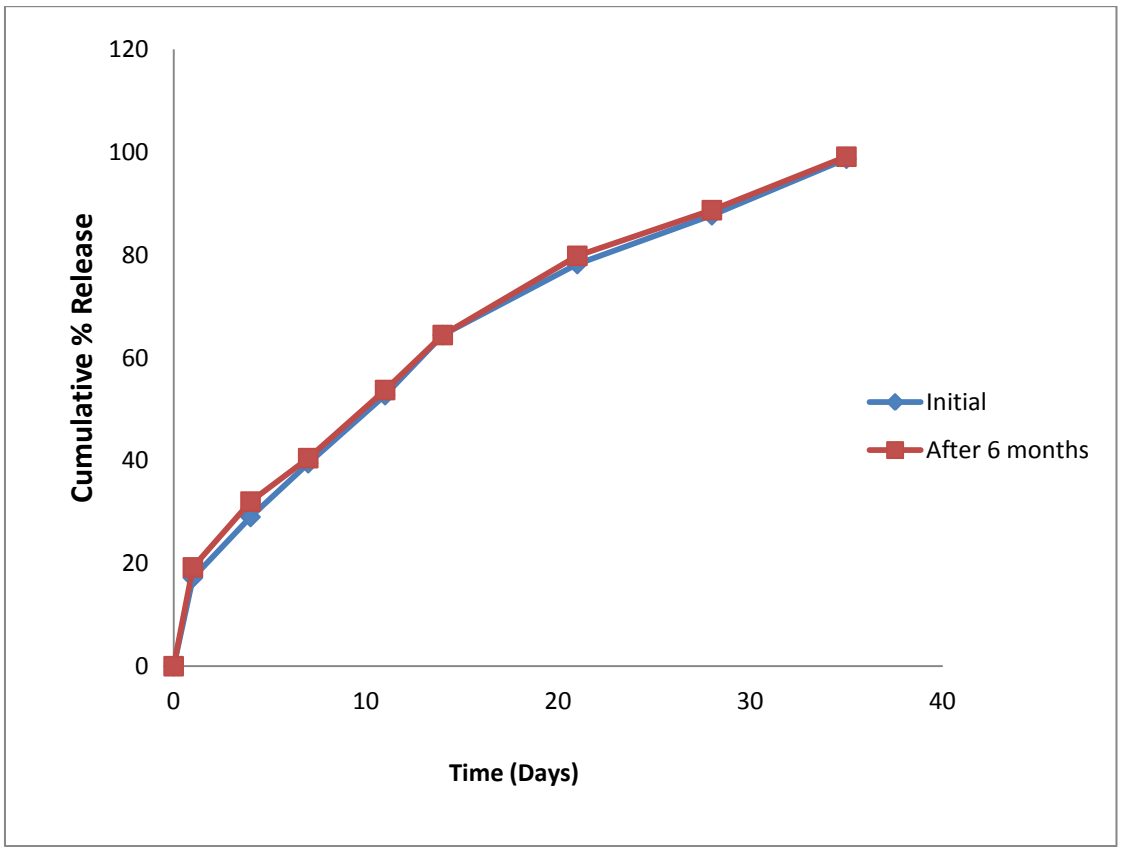

Fig. 9. Effect of temperature on drug release profile of EC4 formulation

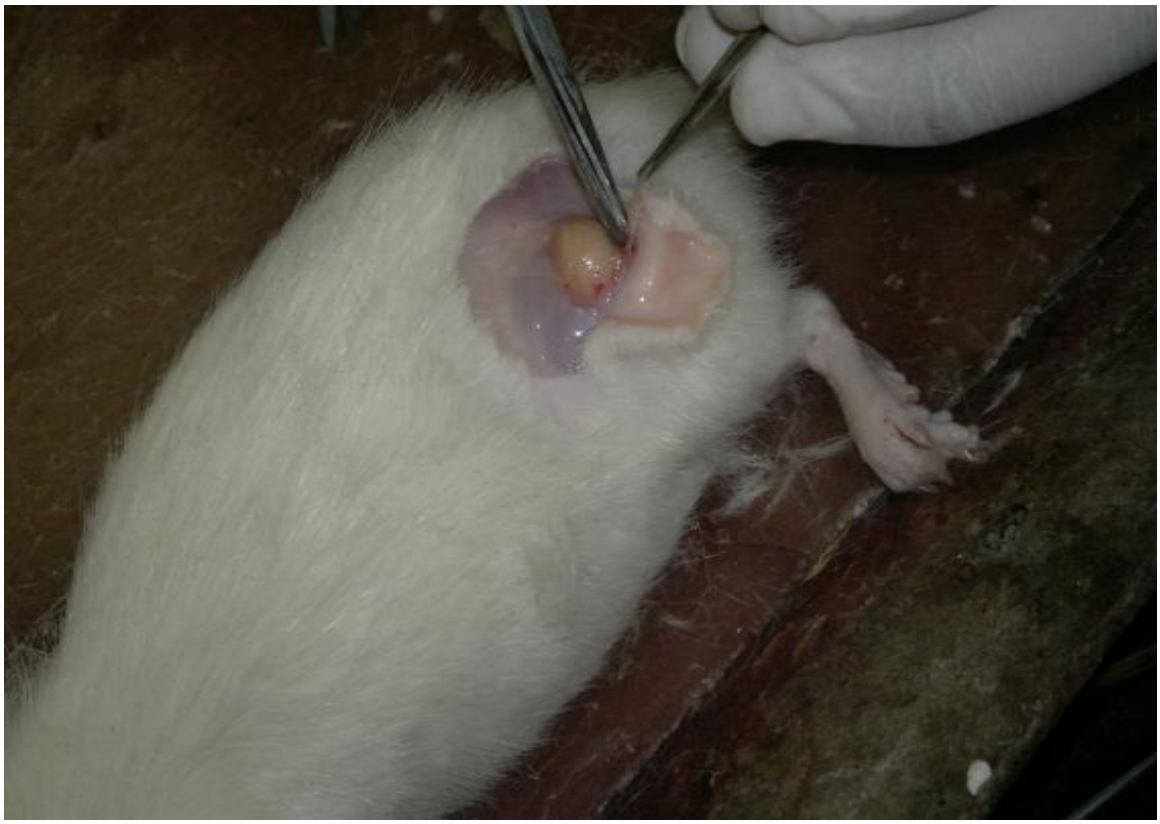

Fig. 10. Residual implant tablet after 28 days

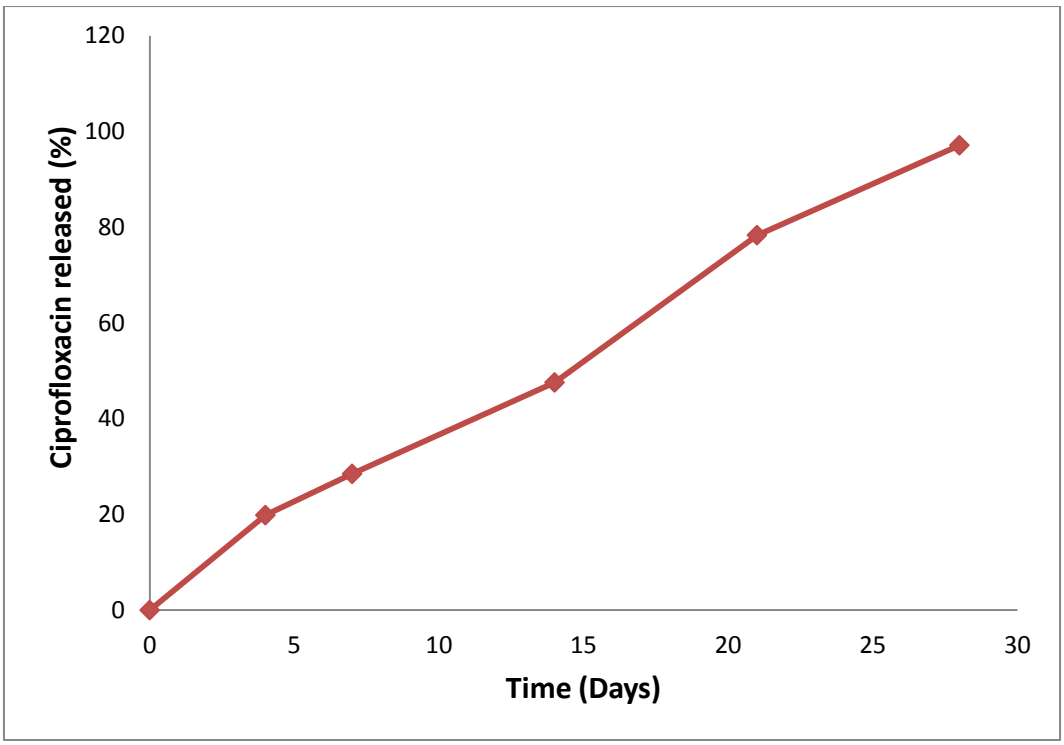

Fig. 11. *In – vivo* released profile of EC4 formulation

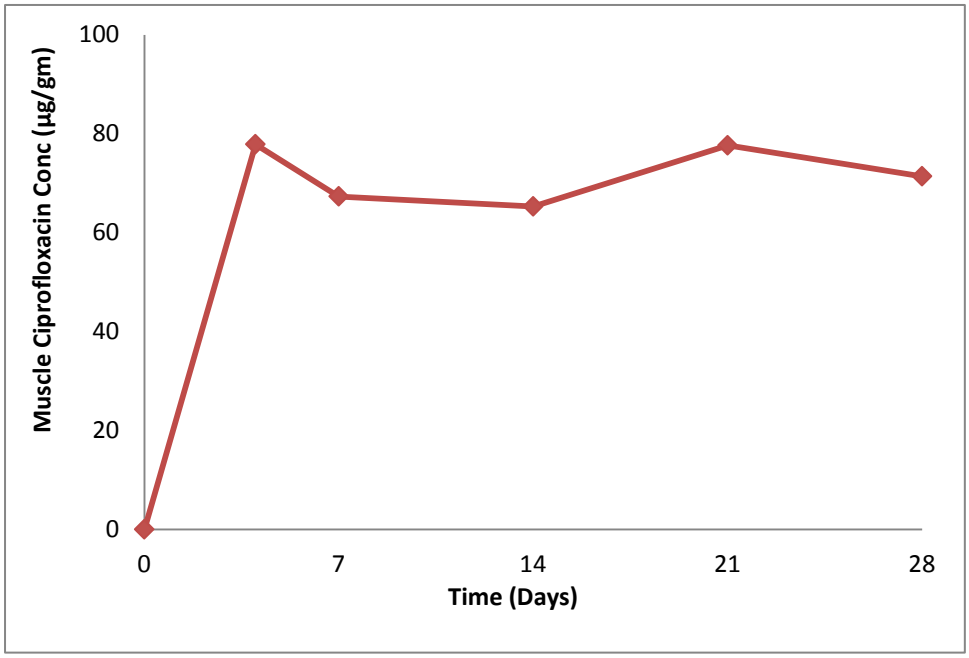

Fig. 12. Concentration Ciprofloxacin HCl achieved in muscle

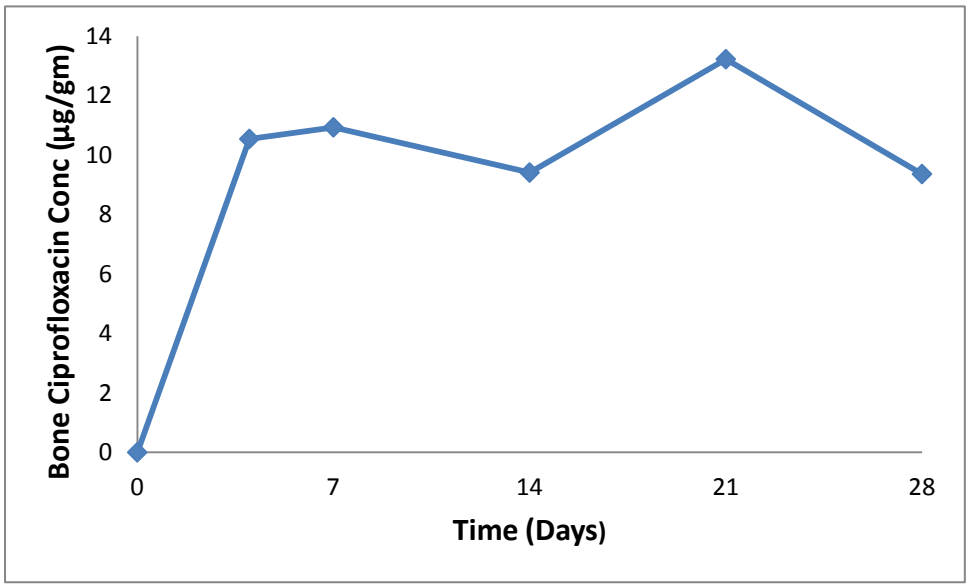

Fig. 13. Concentration Ciprofloxacin HCl achieved in bone

3.10.6 *In vitro–In vivo* correlation (IVIVC)

In the present investigation *In vitro* data of EC4 formulation was correlated with *In vivo* data. The *In vitro-In vivo* drug release data from the optimized implant formulation was correlated using USP established **Level A** correlation. For level-A IVIVC, the correlation between *In vitro* drug release methods (Vial method) is compared to the *In vivo* drug release method for near about four weeks. The *In vitro* method shows good (higher) correlation with *In vivo* release, i.e. **(r^2>0.9).** Therefore from IVIVC, it is proved that EC4 formulation can be used to prolong the % drug release of Ciprofloxacin HCl for more than four weeks.

4. CONCLUSION

The investigation conclusively supported implant being an advantageous technique with sustained delivery of the drug for extended time period.

Cross-linked Chitosan implants were evaluated on basis of swelling index, water absorption, and erosion. With more amount of drug loading in Chitosan can extend the release rate. Water uptake is key factor to mark the release rate. The selected epichlorohydrin Chitosan based EC4 implant formulation shows

Fast release at initial time then follows slow release pattern. This is required in the treatment of osteomyelitis to inhibit the early growth of organisms. Also from *In vivo* study it is proved that EC4 shows prolonged drug release for near about a month & concentration of antibiotic in bone and surrounding tissues is much higher than MIC. Thus present investigation confirms that when used cross linked polymer which can potentially releases drug up to 5 weeks can be used in treating diseases like osteomyelitis and infections of bone.

Therefore from this study it is proved that, the crosslinked chitosan have potential to retard the drug release for near about five weeks which could be useful in the in the treatment of osteomyelitis & other bone infections.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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