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Preparation and Evaluation of Chitosan and PLGA Based Implants for the Delivery of Cefotaxime

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Abstract: The poor bioavailability of cefotaxime and limitation of conventional system limits the delivery of antibacterial drug cefotaxime for bone infection treatment. In the current study in situ chitosan implant containing cefotaxime was developed. Injectable implantable drug delivery system containing Cefotaxime was prepared by physically mixing β-glycerophosphate with chitosan in different concentrations. The objective of this study was to standardize the concentrations of the ingredients so as to develop formulation that remains liquid when stored at 4ºC but forms a gel, in minimum time, when injected or when its temperature is raised to 37ºC. Injectable in-situ implant before injection was clear and transparent. Gelation temperature significantly increase from 32.6°C ±0.1 to 48.43°C ±0.1 of all formulation. Percentage drug content of all formulation were found in the range of 82.42±1.93 to 99.43±0.55. The syringeability of the final solutions greatly decreased with the increase of chitosan concentration. The release pattern for all formulations was biphasic, comprising an initial burst effect followed by an almost sustained continuous phase. After an initial burst release, the drug entrapped into the chitosan/Gp gel was released slowly. Local delivery of chemotherapeutic agent by controlled- release polymers is a new strategy with the potential to maximize the antibacterial effect of a drug to treat bone infection. The system formulated with cefotaxime was found to be stable and the release profiles of a formulation with chitosan and beta-GP showed almost Higuchi equation release kinetics. The drug release of chitosan imlant containing cefotaxime was found to be more as compare to PLGA implant.

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1. INTRODUCTION

Treatment of bone infection is a problematic concept and it is very difficult to deliver an antibiotic direct at the site of infection (Christenson *et al*, 2006). Main complication in this treatment is that bacteria adhere to the implant surface and elude host defense mechanism and attain resistance to antibiotic slowly. Due to this aim plant changes into a site of dreadful infection (Harvey *et al*, 2002). A high systemic dose of antibiotics to facilitate sufficient tissue and bio-film penetration is undesirable due to possibility of causing grave toxic side effects. Therefore, control release of antimicrobial from an orthopedic device presents an alternate solution to this problem (Eiff *et al*, 2005). In orthopedics, biomaterials are used in a range of surgical applications including joint replacements, fracture fixation plates, bone defect fillers, artificial tendon and ligaments and bone cements (Hannallah *et al*, 2002). Microbial adherence to foreign bodies depends on the cell surface characteristics of the microorganisms and on the nature of the foreign body material.

Duration of antibiotic therapy is also an important issue while treating osteomyelitis and related bone disorders. There is a little published evidence for determining the most effective duration of these implants and two major trends were found: most treated the patients for about six weeks, while a minority treated patients for about six months (Lazzarini *et al*, 2005).The success of both orthopedic implant and the tissue engineered construct is highly dependent on the selected biomaterial. One of the key factors identified in the failure of both types of implants was insufficient tissue regeneration around the biomaterial immediately after implantation.

Treatment of Osteomyelitis, either associated with orthopedic implants or due to open fractures, needs long term and sustained action of antibiotics on microorganisms at the site of infection. This cannot be achieved by orally and systemically administered antibiotics because lack of blood supply to the inflamed bone and necrotic tissue does not allow antibiotics to reach the infected bone and therefore therapy remains unsuccessful in eradicating microorganisms. (Wu *et al*, 2006) High dose of orally and systemically administered antibiotics causes toxicity like nephrotoxicity and oto-toxicity and emergence of bacterial resistance to antibiotics is the major problem faced in curing this disease. Hence antibiotic with wide broad spectrum activity in the form of injectable implants is proposed to be used to solve these problems(Mirzaee *et al*, 2009).

The above mentioned problems related to treatment of bone infections like toxicity due to high dose of orally and systemically administered antibiotics, increased bacterial resistance to antibiotics and inability of antibiotics to reach infected bone is proposed to be solved by using the implant containing antibiotic in injectable form. It is expected to sustain the release of drug to provide prolonged

action and low toxicity by not administering the drug in systemic circulation. Implant in injectable form is intended to be injected and is expected to form depot by polymer precipitation method. The injectable implant is also proposed to prevent the need for surgical insertion of implant. It is also intended to deliver the dose at the specific site of action in controlled manner for extended period of time. Also repeat administration will be possible. The drug proposed to be used is antibiotic is a bactericidal agent with broad spectrum of antimicrobial activity.

Cefotaxime is a third-generation cephalosporin antibiotic use in joint and bone infection cause by *S. aureus, Streptococcus spp*. In conventional form Cefotaxime has low bioavailability and its half life is 0.8-1.4hr. Apart from short half life, Cefotaxime has high hepatic first pass metabolism. Above limitation of Cefotaxime make this drug a suitable candidate form in situ injectable implant for sustained as well as local action. High local antibiotics levels facilitate diffusion to the poor vascular areas reaching the resistant organisms (World Health Organization. 2013).

The aim of this study was to develop Cefotaxime injectable in-situ chitosan hydrogel or implant system for treatment of orthopedic infections.

2. MATERIAL AND METHODS

2.1 Materials

Cefotaxime (Alkem Laboratories Ltd), Chitosan (UniSpeed Pharmaceuticals Private Limited), Beta glycerophosphate, PLGA (PGI), and other HPLC grade chemicals.

2.2 Methods

2.2.1 FT-IR Spectroscopy

The KBr disk technique was employed for preparation of sample. Pellet of 1mg Cefotaxime and 100 mg dried spectroscopic grade KBr was prepared in a die with the application of pressure and pellet was analyzed using FT-IR Spectrophotometer (NICOLET-380, Thermo, USA).

2.3 Preparations of Implants

2.3.1 Preparation of Chitosan Implants

Injectable implantable drug delivery system containing Cefotaxime was prepared by physically mixing β-glycerophosphate with chitosan in different concentrations. The objective of this study was to standardize the concentrations of the ingredients so as to develop formulation that remains liquid when stored at 4ºC but forms a gel, in minimum time, when injected or when its temperature is raised to 37ºC. The preparation of implants involved following steps.

2.3.2 Sterilization of glass apparatus and chemicals

All the glass apparatus were soaked in chromic acid solution, properly cleaned, dried and packed for autoclaving as per the standard procedure. The packed glass apparatus, chitosan dry powder, water (double distilled) and mechanical stirrer paddle were sterilized by autoclaving at 121^{0}C, 15lb pressure for 15 minutes. All the apparatus and chemicals were transferred to the aseptic area immediately after removing from autoclave. All further processing was done in the laminar air flow bench which was previously prepared as per the standard procedures.

2.3.3 Preparation of Chitosan solution

Weighed 180 gm of Chitosan, by method of difference, on a digital balance kept in the aseptic area and transferred to a sterilized beaker. To this 100 ml of 0.1 N HCl was added and the solution was stirred with mechanical stirrer (Remi) at 1500 rpm for 4 hours. The resultant solution of chitosan (1.8%w/v) was transferred to sterile iodine flask and stored in refrigerator for further use. The other concentrations, 1.5%w/v, 2%w/v, 2.1%w/v and 2.4 %w/v of chitosan were similarly prepared using 150gm, 200gm, 210gm and 240gm of chitosan. All the solutions were labeled and stored in a refrigerator till further use.

2.3.4 Preparation of β-glycerophosphate solution

Accurately weighed 480mg, 560mg and 640mg of β-glycerophosphate separately and transferred each to sterilized 10ml volumetric flasks. Dissolved in sufficient quantity of sterilized water and made up the final volume to 10ml. The resultant solutions of β- glycerophosphate were having final concentrations of 48%w/v, 56%w/v and 64% w/v were made. The solutions were stored in refrigerator till further use.

2.3.5 Preparation of Injectable Implant

Transferred 5ml of the prepared chitosan solution of required concentration in sterilized glass vial under aseptic conditions. To each vial 50 mg of drug cefotaxime was added. The vial was stoppered and sonicate for 15 minutes on bath Sonicator to dissolve the drug. The vial was then transferred to an ice bath for 15 minutes in order to bring down the temperature of chitosan-drug solution to 4^0 C. β-glycerophosphate in required quantity was then added dropwise to the chitosan solution while stirring with a sterile glass rod under aseptic conditions. Formulations were stored below 4^{0}C till further use.

Formulations containing different concentration of chitosan (1.5%, 1.8%, 2%, 2.1%, 2.5%) were prepared while concentration (i.e. 48%w/v, 56%w/v, 64%w/v) of β-glycerophosphate disodium salt of and different ratios of chitosan and β-glycerophosphate i.e. 4:1, 5:1 and 6:1 were same in all formulations. Compositions of all formulations were given in a table1.

Table 1: Formulation Table.

Formulation Code	Cefotaxime (mg)	Chitosan Solution (w/v)	β-Glycerophosphate Solution (w/v)	Ratio (C/GP)
F1	50	1.5%	64%	4:1
F2	50	1.5%	64%	5:1
F3	50	1.5%	64%	6:1
F4	50	1.5%	56%	4:1
F5	50	1.5%	56%	5:1
F6	50	1.5%	56%	6:1
F7	50	1.5%	48%	4:1
F8	50	1.5%	48%	5:1
F9	50	1.5%	48%	6:1
F10	50	1.8%	64%	4:1
F11	50	1.8%	64%	5:1
F12	50	1.8%	64%	6:1
F13	50	1.8%	56%	4:1
F14	50	1.8%	56%	5:1
F15	50	1.8%	56%	6:1
F16	50	1.8%	48%	4:1
F17	50	1.8%	48%	5:1
F18	50	1.8%	48%	6:1
F19	50	2%	64%	4:1
F20	50	2%	64%	5:1
F21	50	2%	64%	6:1
F22	50	2%	56%	4:1
F23	50	2%	56%	5:1
F24	50	2%	56%	6:1
F25	50	2%	48%	4:1
F26	50	2%	48%	5:1
F27	50	2%	48%	6:1
F28	50	2.1%	64%	4:1
F29	50	2.1%	64%	5:1
F30	50	2.1%	64%	6:1
F31	50	2.1%	56%	4:1
F32	50	2.1%	56%	5:1
F33	50	2.1%	56%	6:1
F34	50	2.1%	48%	4:1
F35	50	2.1%	48%	5:1
F36	50	2.1%	48%	6:1
F37	50	2.4%	64%	4:1
F38	50	2.4%	56%	4:1
F39	50	2.4%	48%	4:1

2.3.6 Preparation of Poly (D, L Lactic acid-co-Glycolic Acid) (PLGA) Implant

Preparation of PLGA {Poly (D, L lactic acid-co-glycolic acid)} solution Weighed 1 gm of PLGA {Poly (D, L lactic acid-co-glycolic acid)}, by method of difference, on a digital balance kept in the aseptic area and transferred to a sterilized beaker. To this 5 ml of triethyl citrate was added and the solution was stirred at about 70^0C on magnetic stirrer (Remi) at 900 rpm for 1 hours. The PLGA solution thus obtained was cooled to room temperature. The resultant solution of PLGA was transferred to sterile iodine flask and stored in refrigerator for further use. This resultant solution of PLGA had final concentration of 20 %w/v.

2.3.7 Preparation of PLGA Implant

Measured and transferred 5ml of, the above prepared PLGA solution, into a sterilized glass vial using a sterilized syringe. Weighed 50 mg of the drug (cefotaxime) and transferred to the vial aseptically. The vial was stoppered and sonicated for 30 minutes on bath sonicator to dissolve the drug. The vial was then transferred to an ice bath for 15 minutes in order to bring down the temperature of PLGA drug solution to l4^{0}C. Formulation thus obtained was stored at 4^0C till further use.

2.4 Screening of formulations for gel forming ability

In situ gelling forming ability was determined by visual inspection in a simulated physiological condition. The condition was obtained by exposing the formulation to the physiological conditions of temperature and pH. For this, specified volume of each gel was injected into a vial containing phosphate buffer pH 6.8 maintained at 37^{o}C following the procedure as outlined below.

2.4.1 Determination of gelling temperature

Phosphate buffer (pH 6.8) was prepared and warmed on a water bath to 37^0C. Formulations were introduced into 1ml of phosphate buffer (pH 6.8) with the help of a syringe. Changes in consistency of formulations were visually inspected after every five minutes and results were noted as positive and negative for gel formation. The formation of gel was determined by flow or no-flow criterion over 30 seconds when the vial was tilted at an angle of 90^{o}c.

2.4.2 Determination of Gelation time of formulations

The prepared liquid implant formulations which showed gel formation in the gelling ability test were then subjected to determination of gelation time for comparative evaluation. For this the formulations were placed in to vials

containing1ml phosphate buffer (pH 6.8 maintained at 37°C). The vial was then placed on water bath set at 37°C. The formulations were examined every two minutes by tilting the vials gently, until the liquid didn't flow. Time was noted at which liquid turned into solid form or gel.

2.4.3 Determination of Drug contents

The vials containing the formulation were shaken for 1-2 minutes. 1ml of the formulation was transferred to 15ml centrifuge tube with a micropipette. To this 10ml of Methanol was added to completely precipitate the polymer. The contents of the tube were centrifuged at 1500rpm for 15 minutes. 0.1ml of the clear supernatant was diluted with methanol in 10 ml volumetric flask and the final volume was made up with methanol.

2.4.4 Determination of Syringeability

The formulation was withdrawn into the syringe up to 2 ml mark by inserting the tip of the syringe in the glass vial. The plunger of the syringe was held in the position at 2ml mark. The tip of the syringe was cleaned and 21 gauge needles was fixed. A one kilogram weight was placed on the platform attached to the plunger head, while holding the plunger in position. The plunger was allowed to move down under the influence of 1 kg weight pushing the contents of the syringe out through the 21 gauge needle.

Time taken by the plunger tip to move from 2ml mark to 1ml mark was noted with the help of the stop watch. Syringeability in terms of time taken for 1ml of the contents to pass through 21 gauge needle was recorded.

2.4.5 Determination of Viscosity

Viscosity of the five selected formulations gel was measured by Brookfield viscometer (spindle no. 22) at different rpm at 37^0C.

2.4.6 Determination of In-vitro drug release

For determination of in-vitro drug release from the formulation after gel formation, 1ml of each formulation was placed in the vials containing10ml of phosphate buffer (pH 6.8) maintained at 37°C and were allowed to form gel. After gel formation, the study was carried out by placing the vials in a shaker bath at 37°C and 60 oscillations per minute. 1ml samples were withdrawn at predetermined intervals during 21days. Further dilutions were made wherever necessary. The volume removed from the vial was replaced with equivalent amount of phosphate buffer.

3. RESULT AND DISCUSSION

IR spectra represent the purity of drug. On evaluation of IR spectra of drug cefotaxime, following peaks was found 3441&3346, N-H (Stretching),

2825&2946 C- H (asym& sym) Stretching, 1759 C=O (Lactam) Stretching, 1730 C=O (Carboxylic ester) Stretching, 1608 C=C Stretching, 1541 C=N Stretching, 1047 C-O Stretching

Injectable in-situ implant before injection was clear and transparent. Upon injection of polymer solutions, solution will convert into a gel structure and formed implants. The gelation of a Ch/β-GP solution is thermally induced via several types of interactions including electrostatic attractions/repulsions, hydrogen bonding and hydrophobic effects (Zhi-peng *et al*, 2012, Limin *et al*, 2010). When a weak base, as disodium β-GP solution, is added to an acidic Ch solution, the pH increases and electrostatic attractions between positively charged Chitosan ($NH3^{+}$) and negatively charged phosphate molecules (-HPO4-1 or -PO4-2) of β-GP can occur. Moreover, the charge neutralization caused by β-GP anion causes a reduction in electrostatic repulsion between the Chitosan chains leading to an increase in hydrogen bonding interactions along Chitosan inter-chains. Furthermore, glycerol molecules of β-GP form a shield of water around Chitosan molecules inhibiting their aggregation at low temperature. By raising the temperature, Ch-Ch interactions become dominant resulting in a phase transition from a liquid to a gel while hydrogen-bonding interactions are reduced. Phosphate groups function as a sink for the protons released as a result of breaking of Chitosan hydrogen bonding. Ch chains are subsequently brought close to precipitation resulting in the gelation of the Ch/β-GP formulation.(Heuzey *et al*, 2005, Dass *et al*, 2008)

Ph and Gelation temperature were an important parameter for determination of efficacy of implant. All the formulations showed pH in a range of 6.54-7.65. Gelation temperature depends on the concentrations of beta glycerophosphate and chitosan.

From figure 2 it was found that on increasing Chitosan concentration, gelation temperature significantly increase from 32.6°C ±0.1 to 48.43°C ±0.1

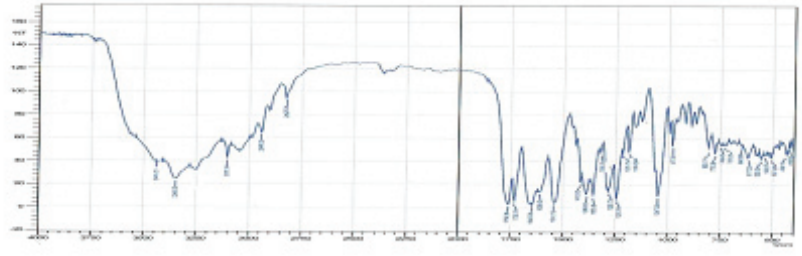

Figure 1: IR Spectra of Pure drug Cefotaxime.

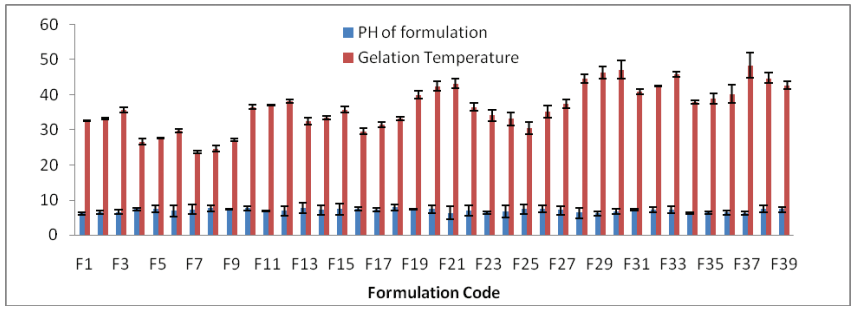

Figure 2: Ph and Gelation temperature of different formulations.

This can be justified by the greater viscosity of Chitosan with higher density of hydrogen bonding requiring higher energy (temperature) to breakdown these bonds and allow for the formation of Chitosan / β-GP thermo reversible hydrogel. A significant decrease in gelation temperature could be noticed by increasing β-GP concentration using the same Chitosan concentration. There was an inverse relation between the gelation temperature and the concentration of β-GP used. The phosphate group of β-GP is the counter-ion for Chitosan, when its concentration increases, enhancement of Chitosan ammonium groups ($–NH3^+$) charge neutralization occurs. Therefore, reducing both Chitosan chain charge density and electrostatic repulsion between the $NH3^+$ groups and increasing the chain flexibility. Chitosan chains are then easier to close, entangle, interact and turn into gel. (Chen *et al*,1996)

3.1 % Drug content and Gelation time and Syringeablity

Table 2 shows Percentage drug content, Gelation time and syringeability of different formulations. it was found that by increasing the concentration of beta glycerophosphate, the required time for gelation decreases. With the increase of beta glycerophosphate concentration, more chitosan amino groups were neutralized. Therefore, electrostatic repulsive force between chitosan chains was damaged, and polymer chains were aggregated more easily. This led to obvious reduction in gelation time. Percentage drug content of all formulation were found in the range of 82.42±1.93 to 99.43±0.55.

The syringeability of the final solutions greatly decreased with the increase of chitosan concentration. The lowest syringeability was observed with chitosan concentration above of 1.8% (w/v). Beta glycerol phosphate concentration had no effect on syringeability of final solutions (Kempe *et al*, 2008).

On the basis of above parameters F11, F12, F14, F15, F17 formulations were selected for Viscosity as well as In-vitro drug release study.

Table 2: Percentage drug content and viscosity of different formulation.

S.No.	Formulation code	Gelation time (min.)	Syringbility	Percent drug content
1	F1	28±1.45	Easily	82.43±1.93
2	F2	29±2.53	Easily	83.54±2.06
3	F3	31±2.64	Easily	85.33±1.34
4	F4	32±1.67	Easily	87.26±1.45
5	F5	33±2.44	Easily	86.93±1.34
6	F6	34±1.97	Easily	88.33±0.56
7	F7	36±1.34	Easily	85.22±1.44
8	F8	38±1.64	Easily	89.01±1.39
9	F9	37±0.95	Easily	90±1.02
10	F10	40±0.34	Drop wise	92.34±0.34
11	F11	42±0.11	Drop wise	99.43±0.55
12	F12	43±0.43	Drop wise	98.35±0.10
13	F13	45±0.67	Drop wise	95.85±0.346
14	F14	47±0.19	Drop wise	98.12±0.61
15	F15	46±0.52	Drop wise	96.63±0.66
16	F16	48±0.56	Drop wise	91.23±0.23
17	F17	47±0.21	Drop wise	95.98±0.53
18	F18	49±0.77	Drop wise	95.34±1.48
19	F19	50±0.43	Hardly	89.45±1.20
20	F20	52±1.33	Hardly	90.23±1.46
21	F21	55±1.43	Hardly	93.35±1.02
22	F22	57±1.53	Hardly	94.88±2.22
23	F23	58±0.55	Hardly	95.67±2.67
24	F24	60±0.67	Hardly	96.22±2.92
25	F25	61±2.55	Hardly	94.22±2.01
26	F26	63±1.26	Hardly	91.08±1.94
27	F27	65±1.33	Hardly	97.98±1.00
28	F28	69±0.88	Hardly	96.90±1.88
29	F29	72±0.56	Hardly	94.89±1.51
30	F30	74±1.33	Hardly	93.71±1.78
31	F31	75±1.77	Hardly	90.02±1.56
32	F32	76±1.11	Hardly	96.02±1.29
33	F33	79±2.54	Hardly	97.75±0.34
34	F34	80±1.53	Hardly	96.34±0.56
35	F35	82±1.33	Hardly	91.34±0.25
36	F36	84±2.35	Hardly	96.05±1.73
37	F37	88±4.76	Hardly	95.34±1.04
38	F38	90±1.96	Hardly	97.04±1.43
39	F39	94±0.35	Hardly	97.44±1.02

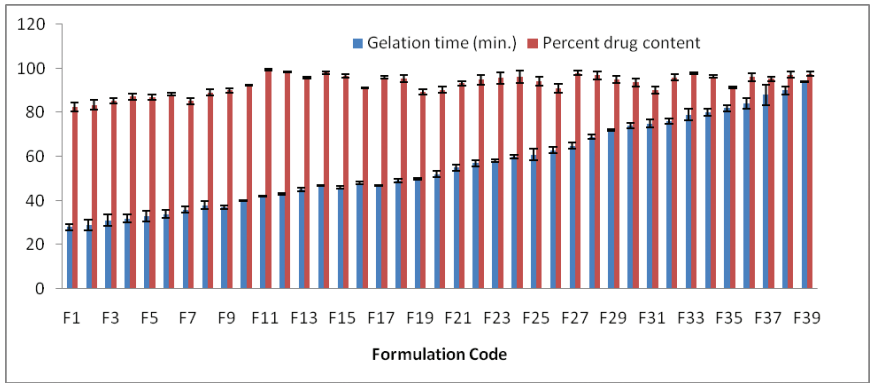

Figure 4: Percentage drug content and Gelation time of different formulation.

Table 3: Viscosity of different formulation at different RPM.

	Viscosity of different formulation (CPS)				
RPM	F11	F12	F14	F15	F17
0	0±0	0±0	0±0	0±0	0±0
10	8342±1.34	7345±1.21	5112±0.92	4782±0.42	4011±1.22
25	6031±1.64	5002±1.77	4893±1.56	3910±0.75	2904±1.55
50	5946±1.09	4289±1.23	3834±1.76	2450±0.31	1707±1.89
100	4211±1.34	3612±1.01	2814±0.42	1190±1.43	990±1.33

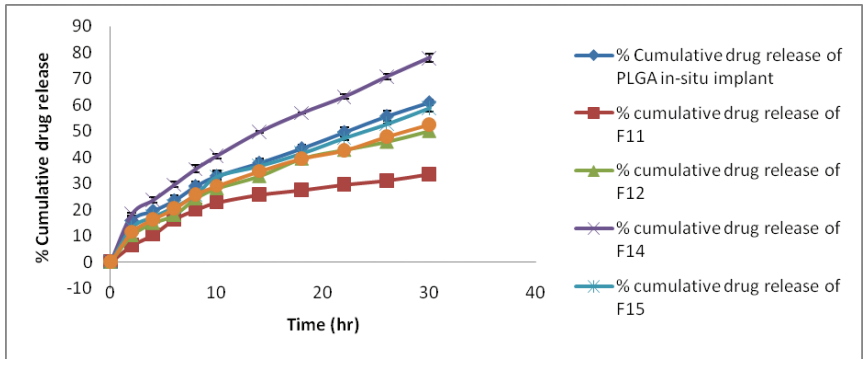

Figure 5: *In-vitro* drug release study of different chitosan in-situ implant.

3.2 Viscosity

Viscosity of F11, F12, F14, F15, F17 gel were evaluated using Brookfield viscometer at different RPM.

From table 3 it was found that on increasing ration of chitosan and beta glycerol phosphate viscosity of formulation will increase. F11, F12 formulation has more viscosity as compare to F14, F15, and F17. At different rpm formulation shows different viscosity that represent its strength.

3.3 In-vitro drug release study

In vitro drug release study of formulations was performed in buffer solution.

From figure 5 it was found that, the release pattern for all formulations was biphasic, comprising an initial burst effect followed by an almost sustained continuous phase. After an initial burst release, the drug entrapped into the chitosan/Gp gel was released slowly. Both rate and total drug release were decreased by increasing the Glycerol phosphate ratio. As discussed before, addition of Glycerol phosphate to aqueous solution of chitosan can directly modulate electrostatic and hydrophobic interactions, and especially hydrogen bonding between chitosan chains, which is the main molecular forces involved in gel formation. Increasing the Glycero phosphate concentration will result in extensive hydrogen bonding via OH– NH and O–HN of chitosan chains, which formed the tightest network and effectively hindered the drug release from the gel. From figure 6 it was also found that release of drug from F14 was grater then PLGA implant gel (Zhou *et al*, 2011). From drug release kinetic study it was found that the in vitro drug release of cefotaxime was best explained by Higuchi equation as the plot showed the highest linearity and R^2 was found 0.993.

4. CONCLUSION

Local delivery of chemotherapeutic agent by controlled- release polymers is a new strategy with the potential to maximize the antibacterial effect of a drug to treat bone infection.

In this study, developed an injectable cefotaxime Ch/β-GP thermoreversible gel bone infection treatment with sustained delivery of cefotaxime was developed. The Ch/β-GP solution underwent thermoreversible gelation around body temperature. Chitosan concentration, β-GP concentration, and ration of chitosan and β-GP effects on thermogelation process were evaluated. Manipulating the chitosan concentration, β-GP concentration, and ration of chitosan and β-GP could lead to a gelation temperature for the Ch/β-GP thermoreversible hydrogel close to body temperature. The prepared system

was able to sustain cefotaxime release for 30hr. The system formulated with cefotaxime was found to be stable and the release profiles of a formulation with chitosan and beta-GP showed almost Higuchi equation release kinetics. The drug release of chitosan implant containing cefotaxime was found to be more as compare to PLGA implant. These findings show chitosan/GP gel to be a safe, effective, homogeneous, injectable and stable formulation for delivery of cefotaxime and this approach represents an attractive technology platform for the delivery of other clinically important hydrophobic drugs

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