Preparation and in vitro evaluation of a novel chitosan-based hydrogel for injectable delivery of enrofloxacin

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Abstract:

BACKGROUND: The development of injectable sustained-release products are of great interest to veterinary pharmaceuticals and animal health business. Recently, great attention has been paid to in situ gel-forming chitosan/beta-glycerophosphate (chitosan/β-GP) solutions due to their good biodegradability and thermosensitivity. **OBJECTIVES:** The general aim of this study was to prepare a novel in situ gel-forming drug delivery system with a sustained release profile for enrofloxacin. **METHODS:** Chitosan, β-GP and enrofloxacin were used in different concentrations and six formulations of chitosan/β-GP were prepared. The properties of the hydrogels including the pattern of drug release, gelation time, syringeability, morphology, FTIR spectra, and in vitro antimicrobial activity were evaluated. **RESULTS:** The release rate of enrofloxacin from the hydrogels and syringeability of the final solutions were decreased by increasing in β-GP and chitosan concentrations. All formulations could release the drug up to 120 hours but formulation 1 (chitosan-2%, β-GP-5% and enrofloxacin-1%) gave the best results based on its optimal drug release profile and viscosity. The FTIR studies showed that there were no interactions between enrofloxacin and hydrogel excipients. Scanning electron microscopy showed that the formed gel had a continuous texture, while the swelled gel in phosphate buffer had a porous structure. Microbiological tests revealed high bactericidal activities for this enrofloxacin- loaded hydrogel which were comparable to those of positive control (enrofloxacin suspension) in terms of inhibition zone, MIC and MBC values. **CONCLUSIONS:** Because of simple preparation and sustained release profile of the drug, this hydrogel could be a promising delivery system for enrofloxacin in animals.

Introduction

Hydrogels are cross-linked, three-dimensional hydrophilic networks that swell but do not dissolve when brought into contact with water (Khodaverdi, et al. 2012). Hydrogels can be formulated in a variety of physical forms, including slabs, micro-

particles, nanoparticles, coatings and films (Kuhwasha, et al., 2013). Their highly porous structure can easily be tuned by controlling the density of cross-links in the gel matrix and the affinity of the hydrogels for the aqueous environment in which they are swollen (Khan, 2014). If a drug is incorporated into the polymer solution, it becomes entrapped within polymer matrix as it solidifies. Drug release occurs over time as polymer biodegrades (Pandya, et al., 2014). The development of injectable sustained-release formulations for intramuscular/subcutaneous (IM/ SC) use has become an increasingly important issue in the animal health business during the last two decades. Such products are of interest in both the farm and companion animal business areas (Matschke, et al. 2002). Injectable hydrogels have attracted much attention during the past decade, due to their rapid gelation from flowable aqueous solution when injected into the desired tissue or organ (Qiu, et al. 2011).

Chitosan, a natural copolymer produced by the deacetylation of chitin, is especially interesting for pharmaceutical application because of its high solubilized capacity, biodegradability, and desired safety profile (Li, et al. 2014). Chitosan is widely used in food and pharmaceutical industries as well as in biotechnology (Parida, et al. 2011). The in situ gelation mechanism involves neutralization of the ammonium groups in chitosan, allowing strengthened hydrophobic and hydrogen bonding between the chitosan chains at elevated temperatures (Chen, et al. 2011). Chitosan solutions that are physically mixed with glycerol-2-phosphate (β-GP) can be injected into the body in liquid form, forming a gel in situ at the body temperature. The rate of gelation depends on the degree of chitosan deacetylation, the concentration of β-GP, and the temperature and pH of the final solution (Chenite, et al. 2000). Chitosan hydrogels can be produced by cross-linking of chitosan macromolecules. These hydrogels can easily swell in water or biological fluids, therefore, they have become a potential candidate for carriers of bioactive macromolecules, wound dressing, and controlled release of drugs in their swollen state (Mirzaei, et al. 2013).

Enrofloxacin is a fluoroquinolone antimicrobial agent developed exclusively for use in animals (Kumar, et al. 2015). It inhibits prokaryotic topoisomerase II (DNA gyrase), which is an important enzyme for bacterial replication (Vancutsem, et al. 1990). It has broad spectrum antibacterial activity, especially against gram negative bacteria, such as Pseudomonas spp (Udomkusonsri, et al. 2010). Enrofloxacin has the maximal lipid solubility among fluoroquinolones. This lipophilicity promotes its diffusion into biological tissues, including bacterial cells (Martinez, et al. 2006).

The pharmacokinetics of enrofloxacin are characterized in general terms by high bioavailability in most species and rapid absorption after IM, SC or oral administration. However, several studies reported that enrofloxacin showed low bioavailability after oral administration in ruminants. This drug has a wide volume of distribution in the organism, excellent tissue penetration and a serum half-life in the range of 3 to 6 hours (López-Cadenas, et al. 2013, Anadon, et al. 1999) Other important characteristics of enrofloxacin include few adverse effects, good therapeutic index and good tolerance in animals (Anadon, et al. 1999).

Major motive forces for the development of innovative veterinary sustained release products include the reductions in the fre-

quency of drug administration, duration of medical treatment and the imposed stress to the animals. As a consequence, an increased ease of drug use by the veterinarians and the pet's owner as well as a decrease in treatment costs seems to be typical for these products. These factors have stimulated the expansion of modified releasing drug delivery systems for use in both companion and farm animals. While oral drug delivery continues to be the main route of administration, the parenteral route suggests an absorbing alternative when oral administration is difficult or cumbersome. The development of new injectable drug delivery systems has received considerable attention over the past few decades. The minimization of dosing frequency enhances patient compliance and comfort. Therefore, injectable drug delivery systems capable of releasing an active ingredient in a controlled manner for a desired period have a high priority. Additionally, biodegradable systems allowing the administration without the need for a subsequent medical procedure to remove the device, contribute to higher patient compliance. However, these innovative therapies are developed at the expense of increased complexity, often leading to issues such as high development and production costs (Sautter 2006).

The focus of the present investigation is to prepare and evaluate beta glycerophosphate-chitosan system as in situ gelling vehicle for controlled delivery of enrofloxacin in a slow release manner. Then, in vitro release profiles of enrofloxacin from the prepared formulations as well as antibacterial activities were investigated.

Materials and Methods

Medium molecular weight chitosan with degree of deacetylation (DDA) of 75-85% and β-glycerophosphate disodium salt pentahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Enrofloxacin standard (99.57%) was purchased from TEMAD Pharmaceutical Co. (Iran). Acetic acid was purchased from Merck (Darmstadt, Germany). Other chemicals were reagent grade.

Preparation of in situ gel: Six formulations were prepared by dissolving different amounts of chitosan powder in 0.1 mol/l diluted acetic acid to achieve concentrations of 2.0 and 3.0% (w/v) along with 5.0, 7.5 and 10.0% (w/v) β-GP and using 5 or 10 g/l enrofloxacin to form hydrogels (Table 1).

Briefly, chitosan powders (200mg/300mg) were dissolved in 8mL of 0.1M acetic acid and gently stirred for 3h to make a homogeneous solution. 50 mg/100 mg enrofloxacin was added to the chitosan solution and stirred for another 1h. Different concentrations of β-GP were dissolved in deionized water. The chitosan and β-GP solutions were placed in an ice-water bath at 4°C. The β-GP solution was added to chitosan and drug solution for 10 min drop-wise. The final 10.0 ml solution was maintained at 4°C for further studies.

Determination of gelation time and syringeability: In this study, gelation was assessed using the inverted tube test, as described by Zhou and coworkers (Zhou, et al. 2011). When a test tube containing a solution is titled, it is defined as a sol phase if the solution deforms by flow, or a gel phase if there is no flow (Fig 1). Firstly, 2 ml of chitosan/ β-GP solutions were maintained for 12 h at 4°C in 5 ml vials with inner diameter of 10 mm to remove air bubbles. The vials were then incubated in a temperature-controlled bath. The sol-gel transition time was determined by inverting the vi-

als horizontally every minute. The time at which the gel did not flow was recorded as the gelation time. The syringeability of the chitosan/ β-GP solutions is how easy it is to expel sample from a syringe and an important parameter for practical administration of gels was also tested.

In vitro drug release studies: In vitro release was performed under sink conditions using the molded 1.0 g gel immersed at 37°C in 500 ml of phosphate buffer pH =7.4 containing 0.5% Tween 80. The dissolution system was shaken at 100 rpm. Samples were removed periodically and the medium was replenished. Because of drug instability, all of the release medium was substituted every 24 hours. The absorbance of the samples was measured at 273 nm by using UV-Vis spectrophotometer. All measurements were performed in triplicate. Data are reported as means ± SD.

Drug release kinetics: To analyze the in vitro release data, various kinetic models were used to describe the release kinetics. The zero order rate in Eq. (1) describes the system where the drug release rate is independent of its concentration (Pandian, et al. 2012). The first order Eq. (2) describes the release from system where release rate is concentration dependent (Shoaib, et al. 2006). Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion Eq. (3). The Hixson-Crowell cube root law Eq. (4) describes the release from systems where there is a change in surface area and diameter of particles or tablets (Hixson and Crowell 1931).

$C=K^0t$ (1)

Where, K0 is zero-order rate constant expressed in units of concentration/time and t is the time.

$\text{Log } C = \text{Log } C^0 - Kt / 2.303$ (2)

Where, C^0 is the initial concentration of drug and K is first order constant and t is the time [9]

$Q = Kt^{1/2}$ (3)

Where, K is the constant reflecting the design variables of the system. Hence drug release rate is proportional to the reciprocal of the square root of time.

$Q^0 1/3 - Qt^{1/3} = KHC\ t$ (4)

Where, Qt is the amount of drug released in time t, Q0 is the initial amount of the drug in tablet and KHC is the rate constant for Hixson-Crowell rate equation as the cube root of the percentage of drug remaining in the matrix vs time.

The kinetic analysis of the release profile was calculated according to the Peppas equation:

$Mt / M\infty = kt^n$ (5)

where 'Mt' is the cumulative amount of drug released at time 't'; 'M∞' is the total amount of drug incorporated; 'k' is the proportionality constant, the value of which depends on the structural and geometrical properties of the matrix; and 'n' is the release exponent, its value depends on the mechanism of drug release. 'R' regression coefficient was also calculated in a set of data; the model showing highest R value was taken as the best model. If 'n' value is < 0.5, the polymer relaxation does not affect the molecular transport, hence diffusion is Fickian. If n > 0.5, the solid transport will be non-Fickian and will be relaxation controlled. If n=1, release follows case II transport (zero order release) and if n > 1, indicates super case II transport (Venkatesh, 2012).

The method that best fits the release data was evaluated by the regression coefficient (r^2). Criteria for selecting the most appropri-

ate model was based on the ideal fit indicated by the values of r^2 near to 1 (Ranjha and Qureshi, 2014).

Morphological studies: For SEM studies, gels containing 5 different chitosan/β-GP ratios were fabricated. For sample preparation, all formulations were initially placed in a freezer at -20°C as a short-term storage and then freeze-dried overnight. Dried gels were cut with a sharp blade to expose internal microstructure and sputter coated with platinum-gold for SEM imaging at 30 kV using scanning electron microscope (FESEM, Hitachi. S4160, Japan).

FTIR spectra: FTIR spectra of enrofloxacin, chitosan, β-GP, dried chitosan/β-GP gel and formulation F1 were recorded in KBr pellets (the samples were triturated with KBr in the ratio of 1:100 and pressed to form pellets) in the range of 400-4000cm-1 using a FTIR spectrophotometer (Nicolet, Model Impact 410; Madison, WI) at room temperature.

Microbiological studies: To determine the antibacterial activity of enrofloxacin hydrogel, the "well diffusion test" was carried out by using *Escherichia coli*, *E.coli* ATCC35218, Pseudomonas aeruginosa, *P. aeruginosa* ATCC10145 and Klebsiella pneumonia, *K. pneumoniae* ATCC13883 as gram-negative pathogenic strains and Staphylococcus aureus ATCC29213 as gram-positive pathogenic strain. The bacterial suspensions with a cell density comparable to 0.5 McFarland (1.5×10^8 CFU/ml) were transferred onto the surface of Muller-Hinton agar plates by using sterile cotton swab. Wells with 8mm diameter were prepared by punching a sterile cork borer onto the solid agar medium. Aliquots of 20 µl of each solution were delivered into the wells (containing 20 µg/ml of enrofloxacin) for enrofloxacin hydrogel, enrofloxacin suspension as positive control, and the blank preparation (formulated exactly the same as hydrogel but without adding enrofloxacin) were used as control to investigate the antimicrobial properties of vehicle. The plates were kept in non-upside down position for 30-60 min to facilitate the diffusion of formulations into the media. After incubation time of about 24h at 37°C, the zones of inhibition around the wells were measured in mm using a caliper (Jahangirian, et al. 2013). The development of a clear zone around the cylinders after 24 h of incubation indicated antibacterial activity against the test organisms. Zone of inhibition data was analyzed using independent t-tests. All experiments were carried out in triplicate.

Determination of MIC and MBC: The broth macrodilution tube method was used to determine MIC and MBC of the formulations against *E. coli* and *S. aureus* bacteria (Yilmaz 2012). A stock solution of enrofloxacin was prepared in sterile water (32µg/ml) that was further diluted in Muller-Hinton broth to reach a concentration range of 0.5 to 32 µg in 4 ml of Muller-Hinton broth. Enrofloxacin hydrogel and enrofloxacin were dispersed in Muller-Hinton broth to reach an equal concentration of enrofloxacin from 0.5 to 32 µg/ml (depending on the percentage of drug loading ratio). Final concentration of bacteria in individual tubes was adjusted to about 5×10^6 CFU/ml by adding 50 µl of *S. aureus* and *E. coli* inoculums. The blank preparations (formulated exactly the same as each formulation without adding enrofloxacin) were used as control and also prepared as above. Control tubes containing just Muller-Hinton broth without any antimicrobial agent and Muller-Hinton broth with enrofloxacin hydrogel formula-

tions and enrofloxacin, both without bacteria, were used as negative control tubes for checking any probable contamination.

To determine MIC values, after 24h incubation at 37°C, the test tubes were examined for possible bacterial turbidity, MIC of each test compound was determined as the lowest drug concentration that could inhibit visible bacterial growth for 24 h The MBC was measured by sub-culturing the broths used for MIC determination onto fresh agar plates. The MBC is the lowest concentration of the drug that kills 99.9% of cells of a given bacterial strain. All experiments were conducted in triplicate (Lalitha 2004, Yilmaz 2012).

Results

Gelation time and syringeability: Chitosan/glycerophosphate gel exhibited thermosensitive property, which was liquid (solution) at refrigerated temperature and solidified into a white semi-transparent hydrogel at body temperature (Fig 1). The influence of Gp salt concentration on the gelation time of chitosan solution was also shown in Table 2. Apparently, by increasing the concentration of Gp salt, the required time for gelation decreases. F3 solution with 10% Gp salt took 17 min to form gel whereas for F1 with 5.0% Gp salt, the gelation time was 25 min. The same situation was observed for other pairs.

The syringeability of formulations has been presented in Table 2. The syringeability of the final solutions greatly decreased with the increase of chitosan concentration. The best syringeability was observed with chitosan concentration of 2% (w/v).

In vitro drug release studies: The cumulative amounts of enrofloxacin released from the hydrogel as a function of time are shown in Fig 2. It was found that cumulative percentage drug release for formulations prepared F1, F2, F3, F4, F5, F6 were 82.8%, 84.2%, 74.0%, 81.7%, 76.8%, 76.2%, respectively. All formulations sustained the drug release for 120h. Formulation F5 did not release enrofloxacin until about 48h, F2 and F3 did not release enrofloxacin until about 4h, whereas F1, F4 and F6 started the release of enrofloxacin within the first hour of the experiment.

Table 1. The compositions of thermosensitive chitosan/ β-GP enrofloxacin formulations.

	Formulation (10 ml)					
Ingredients	F1	F2	F3	F4	F5	F6
Chitosan (mg)	200	200	200	300	300	200
β-GP (mg)	500	750	1000	500	1000	500
Enrofloxacin (mg)	100	100	100	100	100	50

Effect of chitosan concentration on enrofloxacin release behavior: The viscosity of the gel was low when the chitosan concentration was 2.0% (w/v) and grew higher at 3.0% (w/v). As can be seen in Fig 2, the release rates of enrofloxacin were 44.0% and 53.8% for CS/ β-GP thermo-sensitive hydrogels containing 2.0% (w/v) and 3.0% (w/v) CS, respectively along with 5.0% (w/v) β-GP during the first 24 h. It was found that, 41.7% % and 0 % of the trapped drugs were released during the first 24 h for hydrogels containing 2.0% (w/v) and 3.0% (w/v) CS, respectively along with 10.0% (w/v) β- GP. The final cumulative release rates were 82.8%, 74.0%, 81.7 and 76.8% for formulation F1, F3, F4, F5, respectively. CS/β-GP thermo-sensitive hydrogels containing 3.0% (w/v) (F4 and F5) CS showed the abrupt release. On the contrary, hydrogels containing 2.0% (w/v) CS presented the gradual release, which was desirable for controlled release.

Table 2. In vitro drug release kinetics parameters of different enrofloxacin hydrogels.

Hydrogel code	Zero Order	First Order	Higuchi	Hixson-Crowell	Korsmeyer-Peppas	
	r^2	r^2	r^2	r^2	n	r^2
F1	0.8788	0.9600	0.9754	0.9372	1.8923	0.5910
F2	0.8212	0.8923	0.9344	0.8693	1.8961	0.9190
F3	0.8203	0.8556	0.9316	0.8451	1.8327	0.9209
F4	0.8553	0.9264	0.9624	0.9048	0.3688	0.9836
F5	0.9276	0.9157	0.8210	0.9230	0.7834	0.7885
F6	0.9600	0.9935	0.9927	0.9886	0.6850	0.9545

Table 3. Gelation time and syringeability of formulated enrofloxacin hydrogels.

Formulation	F1	F2	F3	F4	F5
Gelation Time (min)	25	20	17	8	5
Syringeability	Easily	Drop-wise	Drop-wise	Drop-wise	Hardly

Table 4. Zone of inhibition of microbial growth around the cylinder containing enrofloxacin hydrogels and enrofloxacin suspension (positive control).

Bacteria	Zone of inhibition (mm ±SD)	
	Enrofloxacin hydrogel	Positive control
S. aureus	32.0±1.01	34.2±1.08
E. coli	35.5±1.26	38.1±1.31
P. aeruginosa	28.3±1.21	33.6±1.04
K. pneumonia	36.6±1.51	37.2±1.12

Table 5. The MIC and MBC of enrofloxacin hydrogels and enrofloxacin suspension (positive control) against *S. aureus* and *E. coli* bacteria. Data expressed as mean ±SD (n=3).

Bacteria		Enrofloxacin hydrogel	Positive control
S. aureus	MIC (µg/ml)	1.0±0.0	1.0±0.0
	MBC (µg/ml)	1.0±0.0	1.0±0.0
E. coli	MIC (µg/ml)	0.5±0.0	0.5±0.0
	MBC (µg/ml)	1.0±0.0	1.0±0.0

Effect of β-GP concentration on enrofloxacin release behavior: In vitro release behaviors of enrofloxacin from chitosan/β-GP hydrogels containing different amounts of β-GP (5.0% (w/v) and 10.0% (w/v)) were shown in Fig 1. The initial release rates of enrofloxacin were 3.14% and 17.5% for hydrogels F1 and F4 (containing 5.0% (w/v) β-GP), respectively. While for both hydrogels containing 10.0% (w/v) β-GP (F3 and F5) the initial release was 0.0%. The pH value of hydrogels increased and got close to the physiological pH by increasing the β-GP concentration. Among all the formulations, hydrogels F4 and F5 with 10.0% (w/v) β-GP showed the most prominent viscosity change during the phase transition.

Effect of drug concentration on enrofloxacin release behavior: The release behavior of chitosan /β-GP hydrogels containing different amounts (5 and 10 g/l) of enrofloxacin (F6 and F1) are shown in Fig 1. Results showed that in the first 24 h, the hydrogel containing 10 g/l enrofloxacin exhibited higher release rate than hydrogel containing 5 g/l enrofloxacin.

Kinetic modelling of drug release profiles: The model fitting for the release profile of formulations by using various models was shown in Table 3. By analyzing regression coefficient values of all formulations, it was found that formulation F6 hydrogel matrix exhibits almost first order kinetics. Formulation F5 followed zero order kinetics; F4 followed Korsmeyer-Peppas kinetics whereas the remaining formulations showed the release kinetic model of Higuchi. So, the predominant drug release mechanism was controlled release. Based on the results of syringeability, in vitro release tests and release profile of formulations, the

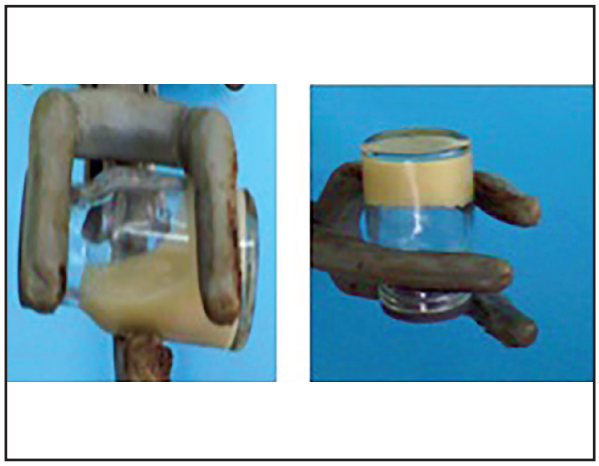

Figure 1. The chitosan/GP formulation at room temperature (left) and at 37°C (right).

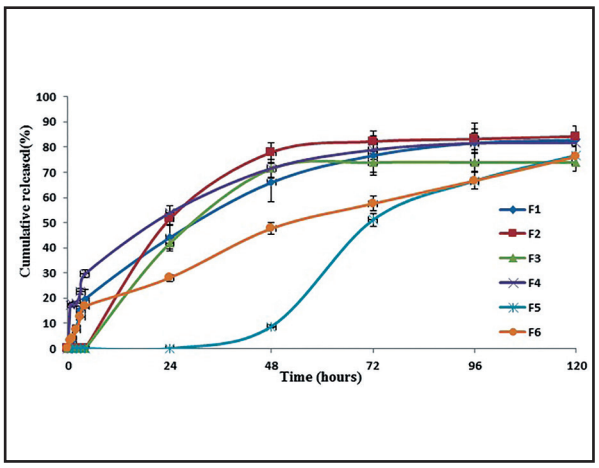

Figure 2. In vitro cumulative release of enrofloxacin (%) from different formulations of in situ gels in PBS + 0.5% Tween 80 at 37°C. Compositions of formulations (F1-F6) have been presented in Table 1. Each point represents the mean value ± SD (n = 3).

formulation F1 was chosen as the appropriate formulation for other tests.

For the drug release, the best fit model was "Korsmeyer-Peppas" model. The values of "n" were calculated from the drug release data (<70%). The obtained values of formulation F4 were between 0 and 0.5, indicating that the release of enrofloxacin was by Fickian diffusion. These values for Formulation F5 and F6 were > 0.5, indicating that the release of enrofloxacin was by non-Fickian diffusion and for other formulations was > 1, indicating that the release of enrofloxacin was by super case II transport.

Fourier Transform Infrared spectroscopy (FTIR) studies: The FTIR spectra of enrofloxacin, chitosan, β-GP, chitosan/β-GP and formulation F1 are shown in Fig 3. The FTIR studies showed that there were no interactions between enrofloxacin and excipients.

Enrofloxacin has two characteristic absorption peaks, 1736 cm^{-1} and 1628 cm^{-1}; the first is the C=O vibration absorption peak from carboxylic acid oxygen, and the second is assigned to keto C=O peak from the ring of enrofloxacin. For the enrofloxacin hydrogel system, the bands at 1736 and 1628 cm^{-1} were shifted to 1742 and 1629 cm^{-1}, respectively.

In the wavenumber range 800 - 1200 cm^{-1}, the FTIR spectrum of chitosan shows three bands at 1155, 1030 and 894 cm^{-1}. The wide band at 1030-1155cm^{-1} represents the bridge -O- stretch of the glucosamine residues. The spectrum of chitosan shows a band at 1595 cm^{-1} that is assigned to the NH_2 group of chitosan. These bands indicate that chitosan is a partially deacetylated product of chitin. The chitosan molecule shows four peaks at 1423, 1380, 1315 and 1255 cm^{-1}. The bands at 1423 and 1315 cm^{-1} are associated with oscillations characteristic for OH and C-H bending of CH2 groups. The band at 1380 cm^{-1} represents the C-O stretching of the primary alcoholic group -CH2-OH. Chitosan exhibited a broad peak at 3434 cm^{-1}, which was assigned to the stretching vibration of N-H and O-H bond. Peaks at 2924 cm^{-1} were due to the C-H stretch vibrations. A peak at 1653 cm^{-1} was due to the C=O stretch of amide bond.

Bands for wave numbers 1076 cm^{-1} and 782 cm^{-1} are characteristic for β-GP and correspond to stretching vibrations P-O-C. A band for wave number 971 cm^{-1} is char-

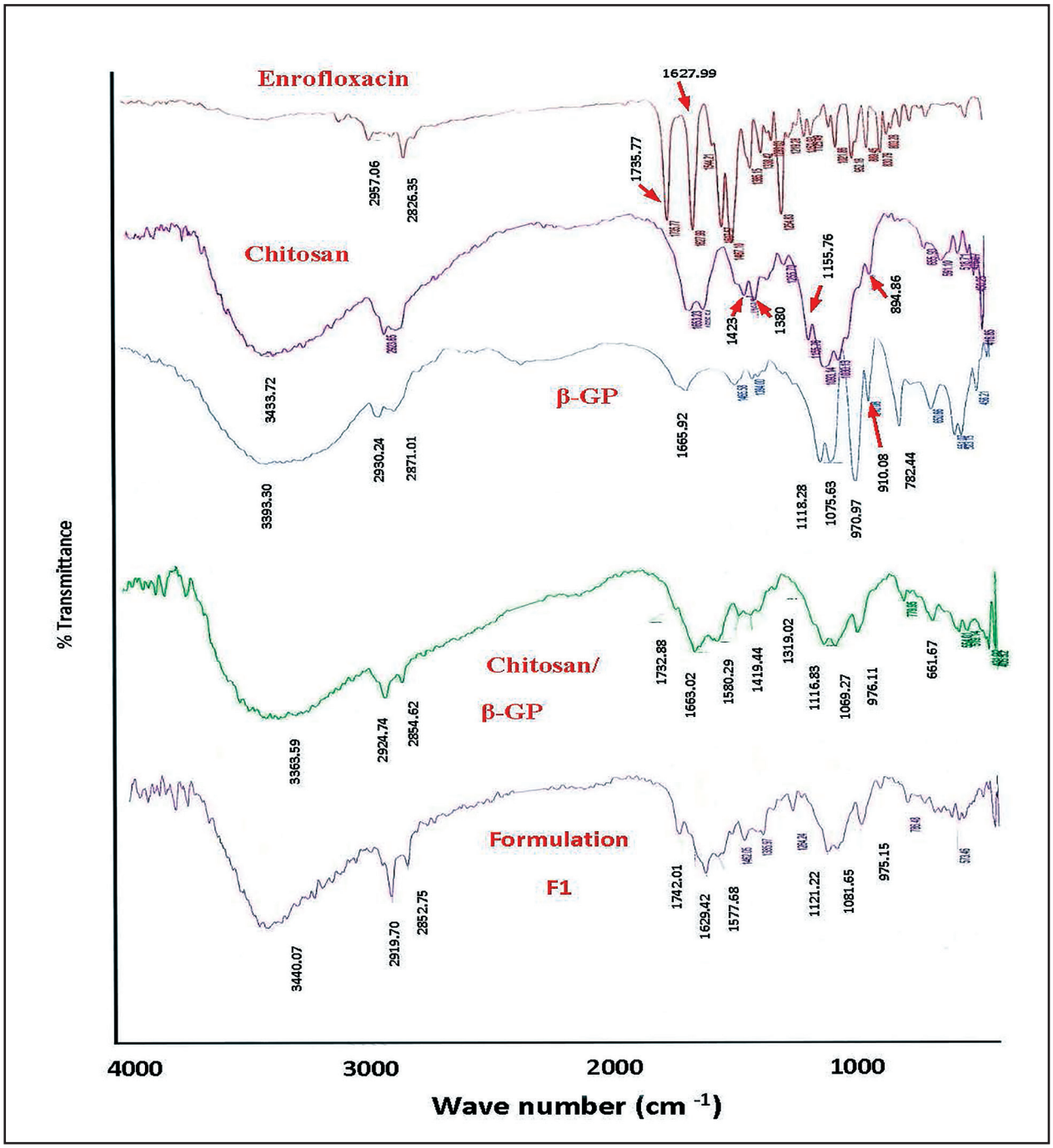

Figure 3. The FTIR spectra of enrofloxacin, chitosan, β-GP, chitosan/β-GP, formulation F1.

acteristic for (-$PO4^{3-}$). A band for wave number 910 cm^{-1} indicating the presence of groups (-$HPO4^{2-}$).

The FTIR spectrum of the chitosan/β-GP system after gelation indicates characteristic bands for chitosan and glycerol phosphate disodium salt. There are no additional bands observed. For the chitosan/β-GP system, the bands of chitosan at 1380 and 1315 cm^{-1} are shifted to 1385 and 1319 cm^{-1}, respectively and the bands of GP at 1076 and 971cm^{-1} are shifted to 1069 and 976 cm^{-1}. We can see from the FTIR spectra between mixture of enrofloxacin, chitosan and β-GP that no significant differences were shown.

Morphological studies: Scanning elec-

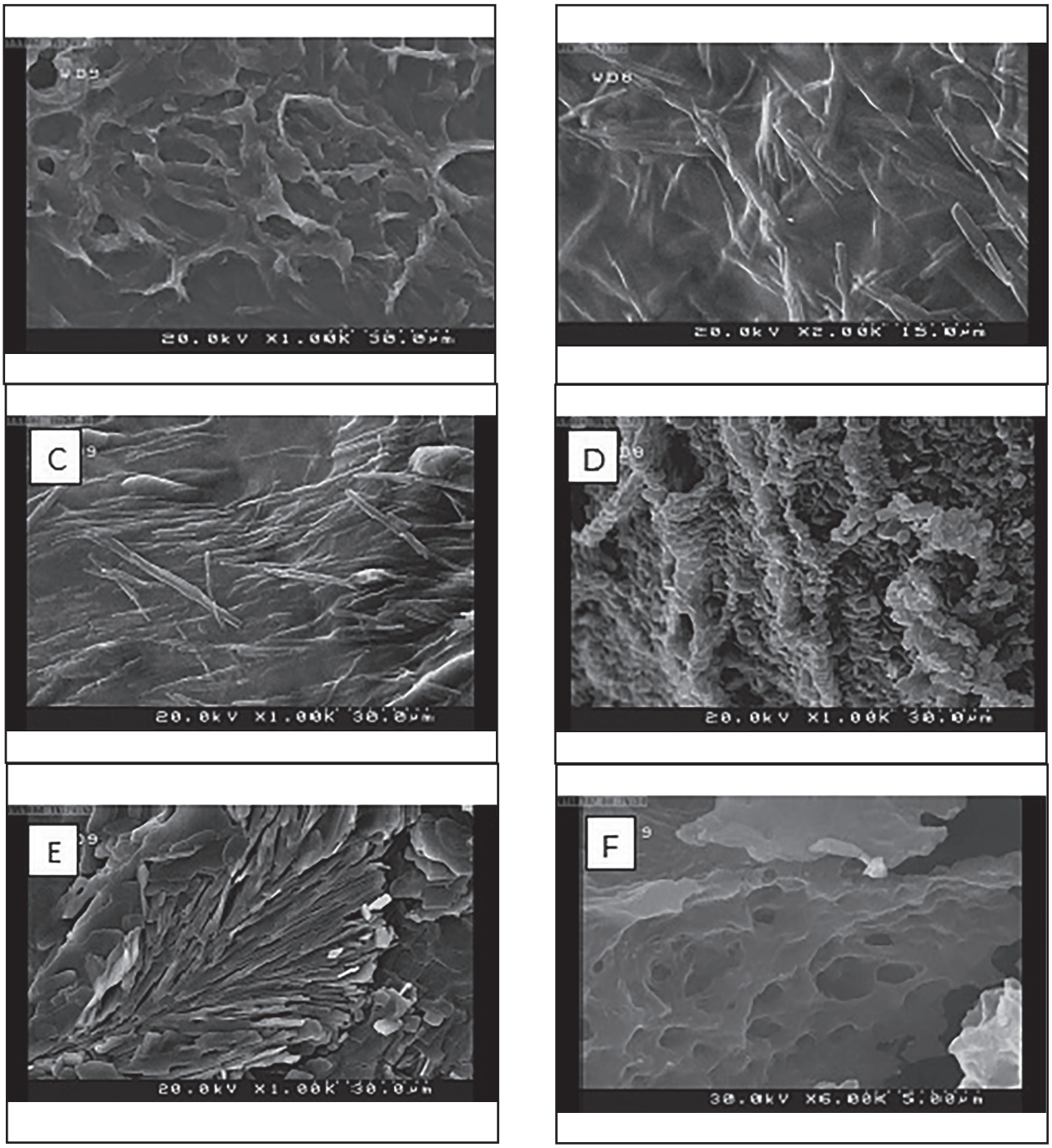

Figure 4. Scanning electron microscopy of freeze-dried chitosan based hydrogels. A:F1, B:F2, C:F3, D:F4, E:F5, F: Swelled hydrogel (F1) in phosphate buffer for 3 days.

tron microscopy was used to investigate morphology and porosity of produced chitosan hydrogels samples. The SEM photograph of the F1-F5 samples is shown in Fig 4 (A-E). It shows that the surface of Chitosan/β-GP hydrogel is rough, obviously dense, non-porous and integrated. The formed gel had a continuous texture for the Chitosan/β-GP hydrogel section (A-E), while the swelled gel in phosphate buffer (pH=7.4) had a porous structure as shown in Fig 4 (F). The SEM graphs of dried hydrogels roughly reflect the pores and water permeation in the hydrogels, because of the small difference of free water between the sol and gel state of poorly-swollen or non-swollen

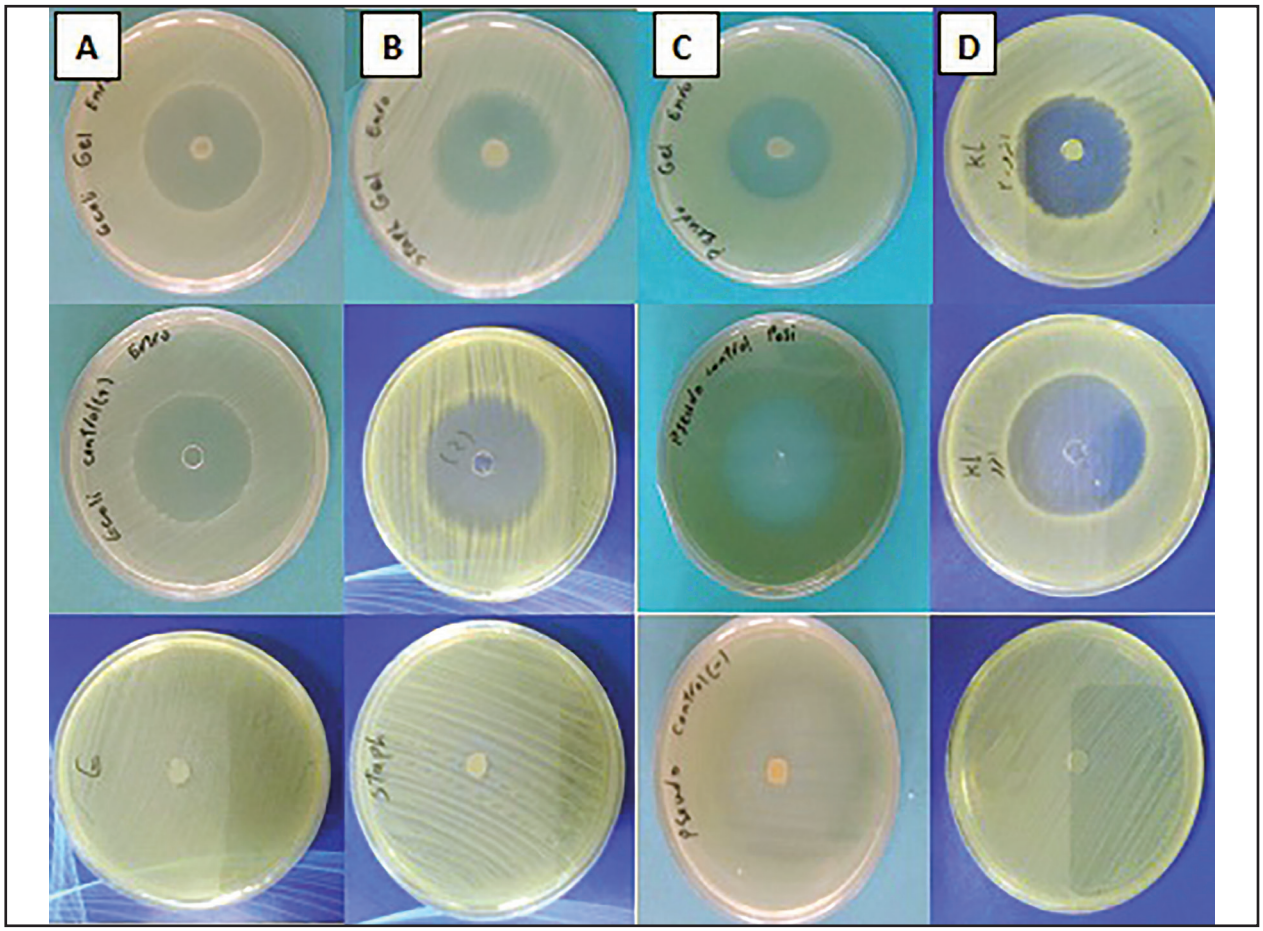

Figure 5. Antibacterial activity of enrofloxacin hydrogel (first row), free enrofloxacin as positive control (second row) and the blank hydrogel (third row) against different bacteria. Column A: *E. Coli*, Column B: *S. Aureus*, Column C: *P. aeruginosa*, Column D: *K. pneumonia*e.

chitosan hydrogels. The observed pores indicate that water once existed over there. The porous structure created a substantial water environment and resulted in burst release of drugs. The structure of the matrix clearly changed with composition. The higher the amount of β-GP salt added to the chitosan solution, the greater the increase in the amount of crystal precipitation, density and integrity of the formulation. The higher the concentration of chitosan added in the solution, the higher the density of polymer chains yielded.

Microbiological activities: The mean diameter of the inhibitory zones (mm) provided by enrofloxacin suspension (positive control) and enrofloxacin hydrogels are presented in Table 4. Both tested materials showed high antimicrobial activity against all microbial strains tested. The result of well diffusion assay showed that the inhibition zone of positive control was slightly greater than that of enrofloxacin hydrogel. However, no statistical differences in inhibitory zones were observed between the positive control and enrofloxacin hydrogels against *E. coli*, *K. pneumonia*, *P. aeruginosa* and *S. aureus* ($p>0.05$). No inhibition zone was observed for the drug-free hydrogel (the blank preparation), which demonstrates that our polymer-drug systems manifest an antibacterial activity and the blank preparation does not present any antimicrobial activity against tested bacteria.

MIC and MBC data of enrofloxacin sus-

pension (positive control), and enrofloxacin hydrogels against *E. coli* and *S. aureus* are presented in Table 5.

Discussion

Infection control in animals is a primary clinical objective and is usually achieved by treatment once or twice a day for at least 3-5 days. In the present work, we evaluated a new thermosensitive formulation as the basis for antibacterial chemotherapy. The objective of the study was to prepare and evaluate enrofloxacin in situ gels as a sustained release formulation. We used enrofloxacin as a model drug for this study, because it is a widely used antimicrobial in veterinary medicine. The chitosan-based solution in the present study remained liquid at room temperature and turned into a gel as temperature increased to body temperature.

The polymeric matrix used in this study consisted of chitosan polymer and β-Glycerophosphate (β-GP). Addition of glycerol-2-phosphate (β-GP) to chitosan solution produces a hydrogel which undergoes sol-gel transition at a temperature close to 37°C, making the formulation a suitable vehicle for drug administration. Our results showed that formulation F4 had the highest burst release (17.5%) but in remaining formulations it was <10%. Drug release in all gel formulations was followed by a sustained release pattern over 120 h. The chitosan/β-GP system could sustain the delivery of hydrophobic drugs such as enrofloxacin for at least 4 days, which is a reasonable time span for antimicrobial therapy. Berrada et al. (2005) used the same chitosan/GP formulation to deliver intratumorally a hydrophobic anticancer agent campothecin by using a mouse tumor model. The implanted hydrogel containing 4.5% campothecin was found to be more effective than systemically delivered campothecin in terms of delaying the tumor growth. Venkatesh et al. (2012) prepared and evaluated a C/GP formulation loaded with pilocarpine for sustained release. The prepared in situ gels released drug for 12 h, and they were non-irritant and well tolerated during the in vivo eye irritancy studies in rabbits. Their study revealed that in situ gels formulation was simple, easy to administer, comfortable, with reduced frequency of instillations and also enhanced the drug activity by releasing the drug in sustained manner. Ruel-Gariepy et al. (2004) used a chitosan/GP formulation loaded with paclitaxel and found it to be as efficacious as four intravenous injections of Taxol by inhibiting the growth of EMT-6 tumors in balb/c Mice.

Gong et al. (2009) prepared enrofloxacin chitosan nanoparticles (EFX-CS-NPs). They reported that the total drug release in 24 h was 79.9% with a sustained release pattern. In addition, Kumar et al. (2015) loaded enrofloxacin in solid lipid nanoparticles (SLNs) with sustained release profile which was characterized by an initial burst release of 18% within 2 h followed by a sustained release pattern up to 96 h.

Our results showed that concentration of chitosan affected the cumulative drug release. Formulations with higher percentage of chitosan retarded the onset of drug release. Similar trend was observed with glycerophosphate. It should be noted that chitosan/β-GP hydrogels containing 3.0% chitosan, retain more enrofloxacin during the sol-to-gel transformation process owing to low viscosity at the sol state but high viscosity at the gel state. The initial burst release decreased with increased polymer

concentration. The initial burst effects also demonstrated that the higher the concentration of β-GP in the hydrogels, the lower the initial burst effect. Venkatesh et al. (2012) reported that concentration of chitosan affected the cumulative drug release. Formulations with higher percentage of chitosan retarded the drug release. Similar trend was observed with glycerophosphate, but not to the extent of chitosan.

The mechanisms of sol-gel transition in the chitosan/GP system include hydrophobic interactions, hydrogen bonding, electrostatic interactions, and molecular chain movement. At low temperature, the solubility of solute is probably due to hydration of the chitosan promoted by glycerophosphate. Upon heating, the chitosan chains lose their water of hydration, which promotes the bonding between chains and proceeds gelation. Three types of interactions may be involved in the process of gelation: (1) electrostatic attraction between the ammonium groups of chitosan and the phosphate group of glycerophosphate; (2) hydrogen bonding between polymer chains due to reduced electrostatic repulsion after neutralization of the chitosan solution with glycerophosphate; and (3) enhancement of chitosan-chitosan hydrophobic interactions by structuring action of glycerol. (John, et al. 1994; Song, et al. 2010).

Degree of deacetylation (DDA) of chitosan, pH of the chitosan/β-GP solution, pressure, ions and temperature are expected to control gel-forming process. DDA is the dominant parameter in controlling gelation time, because the deacetylation of chitosan can increase the hydrophilicity of the system. (Chenite, et al., 2000; Jia, et al. 2006, Purohit Kamlesh, et al. 2013). The primary action of β-GP is to rapidly take the proton from the protonated amino groups of CS; thus, higher GP concentration induces higher pH (Ganji, et al. 2007). The addition of a glycerol-phosphate salt to aqueous chitosan solutions directly modulates the electrostatic and hydrophobic interactions, and hydrogen bonding between chitosan chains, which are the main molecular forces involved in gel formation (Chenite, et al. 2000). With the increase of β-GP salt concentration, more chitosan amino groups are neutralized. Therefore, electrostatic repulsive forces between chitosan chains are damaged and polymer chains are aggregated more easily. This leads to obvious reduction in gelation time (Khodaverdi, et al. 2012). In this study, we found that chitosan solution could be neutralized up to physiological pH by using β-GP due to the neutralizing effect of the phosphate groups (base). Our results apparently showed that by increasing the concentration of β-GP salt, the required time for gelation decreased. The short gelation time observed for formulation F4 and F5 may be attributed to DDA and concentration of chitosan and glycerophosphate. Our results are in agreement with the findings of Khodaverdi et al. 2012 who also reported that, by increasing the concentration of β-GP salt, the required time for gelation decreased. They reported that the Ch4 formulation solution with 0.55 M β-GP salt took 37 min to form gel whereas for Ch3 formulation with 0.45 M β-GP salt, the gelation time was 50 min. (Khodaverdi, et al. 2012). This result is in agreement with the findings of Kempe's group as well (Kempe, et al. 2008). They showed that higher β-Gp concentrations lead to faster gelation at constant temperature. Comparing the gelation behavior of chitosan/Gp solutions of various polymer concentrations, a reverse relationship be-

tween time of gelation and chitosan concentration could be found (F5 vs F3 or F4 vs F1). In other words, solutions with higher polymer concentrations which correspond to more -OH and -NH groups for intermolecular hydrogen binding; thereby, form gel faster than those with lower concentrations.

It was also demonstrated that the initial drug loading affected the release rate. The initial burst value was higher (3.79 vs 3.14%) for the 5 mg/ml-loaded gel vs 10 mg/ml-loaded gel. However, the following release rate was lower for the 5 mg/ml-loaded gel. Li et al. (2014) also reported that the initial burst effects for 1 mg/ml loaded gel and 4 mg/ml loaded gel were 22.06% and 17.56%, demonstrating that the higher the concentration of docetaxel in the hydrogels, the lower the initial burst effect (Li, et al. 2014).

The syringeability increased as the concentrations of β-GP and chitosan of solutions increased. Our results were in agreement with findings of Senyigit et al., 2014. They showed that significant decreases in syringeability observed as the molecular weight and viscosity of chitosan increased ($p<0.05$) (Şenyiğit, et al. 2014).

The higher value for regression coefficient (r^2) in Higuchi model indicates that the drug release mechanism is diffusion controlled (Ranjha and Qureshi 2014). High values of r^2 were obtained for formulations F1-F3 in Higuchi model, which illustrates that the rates of drug release were directly proportional to the square root of time (Murtaza, et al. 2012).

SEM pictures show a change in the gel structure after conditioning in water. Pores in the structure are seen in the micrometer range after conditioning in water. Modrazejewska et al. (2014) also showed that after SEM evaluation of thermosensitive chitosan chloride gels following conditioning in water, the crystals resulting from chloride salt were washed and the pore sizes were about 10 μm (Modrzejewska, et al. 2014).

Regarding microbiological studies, our results indicated that the inhibition zones were rather small for the polymer drug system as compared to the free drug (positive control) but not significantly different ($P>0.05$). It seems that it is mainly due to the fact that the drug was not completely released after 24 hours. The hydrogels loaded with enrofloxacin presented high bactericidal activities. The enrofloxacin retained its antimicrobial activity after formulation into gelling solution. In a similar study, Bhushan et al. (2011) studied antimicrobial efficiency of a controlled release ciprofloxacin gel, CPXF6 hydrogel. The inhibitory effects of ciprofloxacin formulation on some microorganisms were evaluated using agar diffusion test. They showed that the values of zone of inhibition (ZOI) for *S. aureus* for conventional ciprofloxacin eye drop and CPXF6 hydrogel were 31.5 ± 1.1 and 34.4 ± 0.1, respectively. They reported that the higher ZOI values obtained for the formulations in comparison to the conventional eye drop could be attributed to the slow and prolonged diffusion of the ciprofloxacin from the polymeric solution due to its higher viscosity (Bhushan, et al. 2011). On the other hand, the ZOI values for in situ gel formulation were lower than those of a marketed eye drop (Wagh, et al. 2012).

Chitosan/β-GP gels of enrofloxacin showed appreciable in situ gelling properties in the present study. The in situ gels were found to be uniform, isotonic, thermosensitive and released the drug for 5 days. This study revealed that the novel in situ

gel formulation was simple to prepare, easy to administer, comfortable due to reduced frequency of injections, and with enhanced drug activity due to sustained release profile. It was found that the release of enrofloxacin from chitosan/β-GP thermosensitive gel decreases by increasing in β-GP salt. The mechanism of gelation, which does not involve covalent cross-linkers, organic solvent or detergents, combined with a controllable residence time, renders this injectable biomaterial uniquely compatible with sensitive antimicrobial agents.

Conclusion: Chitosan/β-GP in situ gels revealed effective, homogeneous, injectable drug delivery for enrofloxacin and formulation F1 (chitosan-2%, β-GP 5% and enrofloxacin-1%) gave the best results based on its optimal drug release profile and syringeability. Microbiological findings suggest a good efficiency of the hydrogel in terms of antimicrobial activity. Because of simple preparation, easy administration and prolonged drug release, this approach represents an attractive platform for the delivery of enrofloxacin in animals.

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تهیه و ارزیابی آزمایشگاهی یک هیدروژل جدید قابل تزریق انروفلوکساسین بر پایه کیتوزان

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چکیده

زمینه مطالعه: توسعه فرآورده های تزریقی آهسته رهش مورد علاقه شدید صنایع دارویی دامپزشکی و دست اندرکاران بهداشت دام است. در سال‌های اخیر، توجه زیادی به محلول های کیتوزان/بتا-گلیسروفسفات تشکیل دهنده ژل در محل، بخاطر زیست تجزیه پذیری خوب و حساسیت آنها به دما، جلب شده است. **هدف:** هدف کلی این مطالعه تهیه یک سامانه دارورسانی جدید تشکیل ژل در محل با پروفایل آهسته رهش برای انروفلوکساسین است. **روش کار:** با استفاده از غلظت های مختلف کیتوزان، بتاگلیسروفسفات و انروفلوکساسین، ۶ فرمولاسیون کیتوزان/ بتا-گلیسروفسفات تهیه شد. خصوصیات هیدروژل‌ها از جمله الگوی رهایش دارو، زمان ژل شدن، قابلیت کشیده شدن هیدروژل در سرنگ، ریخت شناسی، طیف FTIR و فعالیت ضدمیکروبی آنها در شرایط آزمایشگاهی ارزیابی گردید. **نتایج:** سرعت رهایش دارو و قابلیت کشیده شدن هیدروژل در سرنگ با افزایش میزان کیتوزان و بتاگلیسروفسفات کاهش یافت. تمامی هیدروژل ها قابلیت رهایش دارو را تا ۱۲۰ ساعت داشتند، اما بهترین نتایج براساس رهایش بهینه دارو و ویسکوزیته با فرمولاسیون ۱ (کیتوزان ۲٪، بتاگلیسروفسفات ۵٪ و انروفلوکساسین ۱٪) بدست آمد. مطالعات FTIR هیچ برهمکنشی را بین دارو و اجزاء هیدروژل نشان نداد. میکروسکوپ الکترونی نگاره، ساختار یکنواختی را برای ژل تشکیل شده نشان داد اما هیدروژل متورم شده در بافرفسفات، ساختار متخلخلی داشت. آزمایشات میکروبی، فعالیت باکتری ساید بالایی برای انروفلوکساسین بارگذاری شده در این هیدروژل نشان داد که از نظر میزان منطقه مهار رشد، حداقل غلظت مهاری و حداقل غلظت کشندگی باکتری‌ها مشابه نمونه های شاهد مثبت (سوسپانسیون انروفلوکساسین) بود. **نتیجه گیری نهایی:** این هیدروژل بخاطر داشتن روش تهیه ساده و پروفایل آهسته رهش دارو، دارای چشم انداز روشنی برای دارورسانی انروفلوکساسین در حیوانات می‌باشد.

واژه های کلیدی: بتا-گلیسروفسفات، کیتوزان، انروفلوکساسین، هیدروژل، آهسته رهش

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