SHORT COMMUNICATION

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# A comparative pharmacokinetic study of a novel sustained release danofloxacin formulation and the conventional product in rabbits

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## Abstract

Sustained release drug formulations are frequently developed to reduce dosage frequency and to improve outcomes of drug therapy. This study evaluates the pharmacokinetic (PK) parameters of a novel injectable danofloxacin (DANO) formulation in comparison with a conventional product in an animal model. A recently synthesized DANO formulation, prepared by incorporation of DANO-loaded mesoporous silica nanoparticles in liposomes and integration of liposomes in chitosan and β-glycerophosphate solution (lipogel) along with the conventional DANO product were injected subcutaneously (SC) in rabbits. Blood samples were collected at specific time points and DANO concentrations in plasma samples were measured. The PK parameters including maximum concentration (Cmax), time to reach Cmax (Tmax), area under the concentration versus time curves (AUC), area under the first moment concentration-time curve (AUMC) and mean residence time (MRT) were studied by non-compartmental analyses. The values of MRT (156.00 ± 20.00 hr), AUC (15.30 ± 3.00 μg $mL^{-1}$ per hr) and Tmax (4.70 ± 1.60 hr) for lipogel formulation were higher than those of the conventional product (8.50 ± 3.60 hr, 3.70 ± 2.00 μg $mL^{-1}$ per hr and 0.80 ± 0.26 hr, respectively). However, Cmax values for lipogel formulation (0.41 ± 0.15 μg $mL^{-1}$) were significantly lower than those of the conventional drug product (0.68 ± 0.09 μg $mL^{-1}$). It was concluded that the novel DANO lipogel effectively slowed down the drug absorption and the incorporation of liposomes in hydrogel could be a useful approach to maintain the therapeutic drug level for a longer period; however, more studies are needed in this field.



## Introduction

The use of injectable sustained release delivery systems for antimicrobials may be advantageous in veterinary medicine, since therapeutic levels of these drugs could be maintained for longer times without the need for repeated injections.[1] By application of these delivery systems, the entrapped drugs are released at extended period of time and result in longer duration of pharmacological actions. Consequently, these dosage forms can reduce the stress associated with repeated injections and animal handling through less frequent dosing. In addition, they reduce the cost of anti-microbial therapy and side effects of drugs and improve the compliance of the owner and herd management.[2]

Recent developments in chitosan/β-glycerophosphate (C/GP) *in situ* forming gels drug delivery systems in animals are promising, since they are thermosensitive and could be converted readily to semi-solid gels at injection site following the administration of C/GP solutions [3] As a result, they could act as reservoirs of entrapped drugs and release their drug content over a longer period of time [2]

Among drug-loaded particles, mesoporous silica nanoparticles (MSNs) offer several interesting features including large surface area, easily modifiable pore size and being chemically inert providing better control of drug loading and release profile.[4]

Danofloxacin (DANO) is a fluoroquinolone anti-microbial drug developed for use in veterinary medicine. It has a rapid bactericidal activity against a great number of

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Gram-negative and some Gram-positive bacteria, mycoplasmas and some intracellular pathogens.[5] It is highly active at low concentrations with an elimination half-life of 3 - 6 hr in most animal species being used. It is mainly used for control and treatment of respiratory infections in cattle, swine and poultry.[6]

Due to high water solubility of DANO, initially drug-loaded MSNs were encapsulated in liposomes and then incorporated into C/GP solution.[7] Drug encapsulated by liposomes maintains its therapeutic level for a longer period of time, since the drug must first be released from the liposomes before being exposed to the metabolism and excretion processes.[8] Incorporation of liposomes into the C/GP hydrogel makes more sustained release formulation along with better stability compared to liposomal suspension.[9]

Since current DANO formulations are immediate-release products, this study was aimed to evaluate the pharmacokinetic (PK) parameters of this newly designed *in situ* forming gel of DANO (lipogel) in comparison with a conventional formulation in rabbit as an animal model.

## Materials and Methods

**Drugs and reagents.** Danofloxacin mesylate (the conventional DANO product) and DANO reference standard (DANO content 99.57%) were provided by Kimia Faam Pharmaceutical Company (Tehran, Iran). Medium molecular weight chitosan with degree of deacetylation of 85.00% and GP disodium salt pentahydrate were purchased from Sigma-Aldrich (St. Louis, USA). N-cetyltrimethylammonium bromide (CTAB; 98.00%) and acetic acid were purchased from Merck (Darmstadt, Germany). Tetraethyl orthosilicate (TEOS; 99.00%), egg phosphatidylcholine and cholesterol were purchased from Sigma Aldrich (Seelze, Germany). Other chemicals were reagent grade.

**Preparation of the DANO lipogel.** Preparation and characterization of properties of the lipogel formulation were based on previous report.[6] Briefly, DANO lipogel was prepared by preparation of MSNs using CTAB and TEOS. Liposomes containing DANO-loaded MSNs were prepared by thin film hydration method and then incorporated in C/GP solution to form lipogel.

**Animals.** Eighteen apparently healthy white albino male rabbits, weighing 2.80 - 3.50 kg, purchased from Razi Institute (Tehran, Iran) were used in this study. The rabbits were housed in cages with 12 hr dark/light cycle having a free access to a balanced feed and water. Temperature was maintained at 26.00 ± 2.00 ˚C and humidity at 50.00 - 70.00%. The rabbits were monitored for one week, as an adaptation period, prior to drug administration. This experimental study was approved by the Ethical Committee of Faculty of Veterinary Medicine, University of Tehran (Tehran, Iran) and carried out according to the University of Tehran Animal Welfare Guidelines and Policies (Ethical Code No. 7506006-6-9).

**Experimental design.** The rabbits were randomly divided into three equal groups, with six animals in each group. The first group (conventional DANO) was given a SC dose of conventional DANO product at 1.00 mg $kg^{-1}$.[10,11] The second group (DANO lipogel) was given a single SC dose of lipogel formulation, equivalent to 10.00 mg $kg^{-1}$ of DANO. The third (control) group received equal volumes of blank lipogel formulation which had the same composition and formulating process as a lipogel; but, without addition of DANO in the formulation. The SC administration was performed directly beneath the loose skin at the back of the neck of the rabbit following the zero-time point blood sampling. Rabbits had free access to feed and water during the study period.

**Blood sampling.** Blood samples (~ 1.50 mL) were collected from the jugular vein of rabbits into sterile heparinized micro-tubes at different time points. In each experimental group, the samples were collected prior to drug administration (0 hr) and at 1, 2, 4, 8, 24, 48, 72, 96 and 120 hr post dosing. In addition, one more blood sample was collected at 30 min from each animal in the group of conventional product. Within 1 hr after collection, samples were centrifuged at 3,500 rpm (Model 5810 R; Eppendorf, Hamburg, Germany) for 10 min. The harvested plasma samples were stored at – 20.00 ˚C until further use.

**Analytical method.** For sample preparation, 50.00 µL of 1N NaOH was added to 500 µL of rabbit plasma sample and shaken for 30 sec. Then, 100 µL perchloric acid and 100 µL deionized water were added to each sample, mixed for 1 min using a vortex and consequently centrifuged at 3,500 rpm for 5 min. The supernatant was transferred into special glass tube and 20.00 µL of each sample was injected into the high performance liquid chromatography (HPLC) system for analysis.

Concentrations of DANO in plasma were measured using an HPLC system (Waters, Milford, USA) equipped with a multi-solvent pump, a solvent degasser, an ultraviolet (UV) detector, an auto-sampler, an interface and using ChromGate® Software (version 3.3.2; Knauer, Berlin, Germany). The HPLC column was Chromolith-RP 18e (50.00 × 4.60 mm) from Merck. The method of McKellar *et al*. was used for determination of DANO concentrations in plasma.[10] The HPLC analysis was conducted using a mobile phase consisted of 14.00% acetonitrile and 85.00% water, 0.40% triethylamine and 0.60% phosphoric acid. Mobile phase was filtered under vacuum through a 0.45 µm membrane filter. Chromatographic separation was achieved at a flow rate of 1.00 mL $min^{-1}$ using UV detection at 276.80 nm. Stock solution of 1.00 mg $mL^{-1}$ DANO was prepared by adding 10.00 mg of DANO standard to 10.00 mL of acetonitrile: water (1:1; v/v). Then, it was further diluted in rabbit plasma to yield 0.10, 0.25, 0.50, 0.75, 1.00,1.50, 2.00 and 2.50

µg $mL^{-1}$ concentrations. These concentrations were based on providing a linear calibration curve for HPLC analysis. Validation of the HPLC method for the measurement of DANO in rabbit plasma was done by assessing the linearity, accuracy, precision, recovery rate, selectivity and sensitivity. Standard calibration curve was provided using eight different concentrations of DANO ranging between 0.10 and 2.50 µg $mL^{-1}$. Then, it was used for the calculation of DANO levels in the plasma samples.

**Pharmacokinetic data analysis.** Data of DANO plasma concentrations in each rabbit were used to produce the concentration versus time profile. Maximum concentration (Cmax) and time to reach Cmax (Tmax) values were directly obtained from the DANO concentration versus time curve. Non-compartmental model was used to estimate the PK parameters (area under the concentration versus time curves (AUC), area under the first moment concentration-time curve (AUMC) and mean residence time (MRT).[12] The linear trapezoidal rule was used to calculate areas under concentration-time curves from time zero to 24 and 120 hr post dosing ($AUC_{0-24}$ and $AUC_{0-120}$, respectively) and AUMC using Excel (version 15.0;. Microsoft Corp., Redmond, USA). The $Cmax/AUC_{0-120}$ value as an estimate for DANO absorption rate was also calculated.[13]

**Statistical analysis.** Data were expressed as mean ± SD and analyzed using SPSS Software (version 19.0; IBM Corp., Armonk, USA). The PK parameters were compared using independent samples *t*-test. A $p < 0.05$ was considered statistically significant.

## Results

The standard calibration curve for HPLC analysis of DANO was linear over the range of 0.10 - 2.50 µg $mL^{-1}$ in rabbit plasma samples as indicated by $R^2 = 0.998$. The limit of detection was 0.05 µg $mL^{-1}$ and the limit of quantification was 0.10 µg $mL^{-1}$. Method validation data for determination of DANO in plasma (n = 5) including intra-day variability as relative standard deviation (RSD; %) and accuracy (%) were in the range of 4.20 - 7.60 and 98.20 - 101.20, respectively; intra-day variability as RSD and accuracy were 3.80 - 6.90 and 97.80 - 101.80, respectively and recovery rates (%) were also in the range of 96.70 - 100.20. In addition, there was no detectable interfering peak in chromatograms obtained using the plasma samples of control group.

Overall, DANO was well tolerated by the rabbits and no adverse effects were observed in any of the rabbits. The mean concentrations versus time curves for conventional product and DANO lipogel are shown in Figure 1. The PK parameters of DANO formulations are shown in Table 1.

The Cmax of conventional formulation was significantly higher than that of DANO lipogel formulation ($p < 0.05$). On the other hand, the mean value of MRT for the lipogel was significantly higher than that of conventional formulation ($p < 0.05$). The $Cmax/AUC_{0-120}$ values of two formulations were also significantly different ($p < 0.05$).

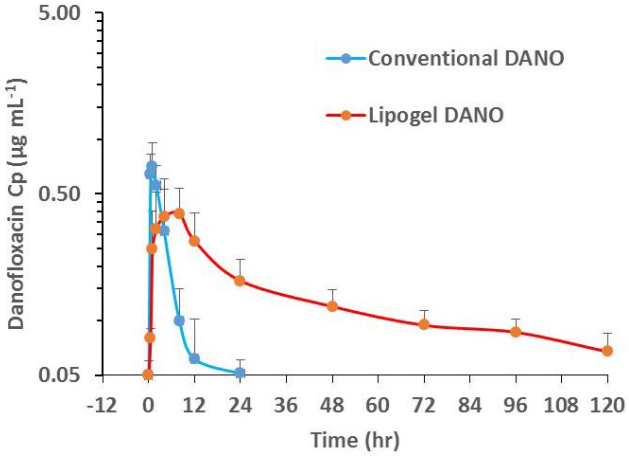


**Fig. 1.** Semi-logarithmic plot of danofloxacin (DANO) plasma concentration (Cp) versus time curve after SC use of a single dose of DANO lipogel (10.00 mg $kg^{-1}$) and the conventional formulation (1.00 mg $kg^{-1}$) in rabbits. Each point represents mean Cp data and standard deviation (n = 6).

**Table 1.** Pharmacokinetic parameters of danofloxacin after SC administration of a single dose of lipogel (10.00 mg $kg^{-1}$) and conventional formulations (1.00 mg $kg^{-1}$) in rabbits.

| Parameters | Lipogel | Conventional |
|---|---|---|
| **$AUC_{0-24}$ (µg $mL^{-1}$ per hr)** | 6.30 ± 2.10 | 3.40 ± 1.80 |
| **$AUC_{0-120}$ (µg $mL^{-1}$ per hr)** | 15.30 ± 3.10 | 3.70 ± 2.00 |
| **$AUMC_{0-120}$ (µg $^2$ $mL^{-1}$ per $hr^2$)** | 2450.00 ± 328.00 | 33.10 ± 27.90 |
| **MRT (hr)** | 156.00 ± 20.00* | 8.50 ± 3.60 |
| **Cmax (µg $mL^{-1}$)** | 0.41 ± 0.15 | 0.68 ± 0.09 |
| **Tmax (hr)** | 4.70 ± 1.60* | 0.83 ± 0.26 |
| **$Cmax/AUC_{0-120}$ (per hr)** | 0.02 ± 0.00* | 0.19 ± 0.08 |

AUC: Area under the plasma concentration-time curve; AUMC: Area under the first moment curve; MRT: Mean residence time; Cmax: Maximal concentration; Tmax: Time to reach maximal concentration; $Cmax/AUC_{0-120}$: An estimate of drug absorption rate.

* Data with significant difference compared to those of conventional formulation ($p < 0.05$). As the doses between lipogel and conventional formulations were different, the significances of analyses of the PK parameters including AUC, AUMC and Cmax, which are positively correlated with the dose, are not logic.

## Discussion

The present study showed that the novel DANO lipogel formulation could maintain drug plasma levels above the detection limit (0.05 μg $mL^{-1}$) up to 120 hr in rabbits indicating the sustained-release nature of the formulation. A single SC injection of DANO lipogel at 10.00 mg $kg^{-1}$ in this animal model was able to provide drug plasma concentration above 0.12 μg $mL^{-1}$ for 72 hr which is above the minimum inhibitory concentration $(MIC)_{90}$ reported for the field isolates of *Pasteurella multocida* and *Mannheimia hemolytica* in bovine respiratory disease.[14] This period is more than five times longer than that of the conventional product. However, it is unlikely that the obtained DANO plasma levels in the present study, both conventional and lipogel formulations, will be effective against less susceptible bacteria such as *Staphylococcus aureus* with $MIC_{50}$ value of 0.50 μg $mL^{-1}$ and as a result, larger doses will be needed.[12]

The benefit of lipogel as a drug delivery system may be largely due to creation of a depot formulation from which drug is slowly released and as a result, the rate of absorption is reduced. In the present study, significant differences were seen between Tmax and Cmax/AUC values, indicators of drug absorption rate, of lipogel formulation in comparison with the conventional DANO formulation.[13] These types of sustained-release formulations could present some pharmacological advantages such as increasing the therapeutic index as well as the efficiency of drug therapy.[2,15] However, from the clinical point of view, some other factors should also be considered including the $MIC_{90}$ of causative agent, effectiveness indices of the anti-bacterial drug such as AUC/MIC or Cmax/MIC, the species of animal to be treated, the risk of drug resistance and the duration of anti-microbial therapy.

In the present study, it was found that the MRT values of lipogel were much longer than those of conventional formulation. The MRT value of the conventional formulation was 8.50 ± 3.60 hr and the plasma concentrations of DANO decreased sharply and fell into unquantifiable levels at 24 hr. But, DANO plasma levels following the use of the lipogel formulation (with MRT value of 156.00 ± 20.00 hr) showed much slower elimination phase, which lasted for more than five days. Regarding MRT values, Shi *et al*. have reported the PK of an injectable hydrogel of diminazene aceturate after SC administration in rabbits.[16] They concluded that hydrogel formulation could significantly increase MRT values (69.00 ± 3.85 hr) in comparison with the aqueous solution (6.53 ± 3.52 hr). Mulik *et al.* have also prepared liposomal hydrogel of cytarabine to reduce dosage frequency and to sustain drug action.[9] They reported that hydrogel could prolong the half-life of liposomes and improve PK parameters (t1/2 and AUC) of cytarabine in rats. The C/GP hydrogel containing cytarabine-loaded liposomes could sustain cytarabine release *in vivo* for more than 60 hr in comparison with cytarabine-loaded liposomal suspension and C/GP containing free cytarabine which showed drug release up to 42 and 10 hr, respectively.[9] Overall, these findings indicated that the *in situ* forming gel system can increase the drug MRT and as a result, reduce the dosing frequency and will be especially useful in long-term treatment protocols.

With regard to Cmax values, the conventional formulation with a lower dose (1.00 mg $kg^{-1}$), produced significantly higher values in comparison with the lipogel formulation (10.00 mg $kg^{-1}$), which is consistent with the sustained release nature of the lipogel. The entrapment of DANO in lipogel networks prolonged the drug release and provided plasma levels above the detection limit until 120 hr. Thus, DANO plasma concentrations were kept more stable; but, at lower levels. Similarly, Geng *et al*. evaluated the PK parameters of an *in situ* forming gel system for delivery of florfenicol in pigs.[17] They also found significantly lower Cmax values for *in situ* forming gel compared to conventional florfenicol injectable formulation (6.87 ± 0.66 versus 12.01 ± 0.66 μg $mL^{-1}$). Moreover, Elmas *et al*. studied the PK parameters of a liposome-encapsulated enrofloxacin (LEE) formulation in rabbits in comparison with free enrofloxacin (FE) using intra-muscular route at the same dose (5.00 mg $kg^{-1}$). The Cmax values for LEE formulation were also significantly lower than those of FE (0.40 ± 0.05 versus 1.44 ± 0.11 μg $mL^{-1}$).[18]

Fernández-Varón *et al*. studied the PK properties of DANO in healthy rabbits following three routes of administration (intravenous, SC and oral) at 6.00 mg $kg^{-1}$. For SC route, they reported Cmax values of 1.79 ± 0.14 μg $mL^{-1}$ which were much higher than those of our findings (0.68 ± 0.09 μg $mL^{-1}$), due to six-times higher dose size (6.00 mg $kg^{-1}$). However, the Tmax values (0.68 ± 0.24 hr versus 0.83 ± 0.26 hr in our study), showed similarity.[12]

Xiao *et al*. also reported PK profile of DANO after a single oral dose of 1.00, 10.00 and 30.00 mg $kg^{-1}$ in *Salmonella typhymurium* infected rabbits with Cmax values of 0.18 ± 0.04, 1.80 ± 0.98 and 3.49 ± 0.91 μg $mL^{-1}$, respectively. The Tmax and $AUC_{0\text{-}24hr}$ values for 1.00 mg $kg^{-1}$ dosing rate were 1.60 ± 0.55 hr and 1.55 ± 0.39 μg hr $mL^{-1}$, respectively.[11] Comparison of these data indicated that DANO absorption rate and extent from oral route were delayed and much less than SC route in rabbits, respectively.

In recent decades, anti-microbial resistance has become a worldwide health challenge in both human and veterinary medicine and the main risk factor for the increased rate of antibiotic resistance seems to be the misuse of anti-microbial drugs.[19] In this regard, the use of long lasting anti-microbial formulations in veterinary medicine, particularly in food animals, is controversial considering their potential risks and benefits. These long-

acting products are able to make drug therapy more convenient and practical by avoiding repeated injections in livestock. But, they can increase the persistence of drug residues in animal tissues which may increase the risks associated with drug residues in food-producing animals. These issues should be handled properly in the development and usage of long-acting formulations.

At present, it seems that these types of sustained release formulations can be more promising in companion animal practice compared to food-producing animals. However, further studies are needed to reach the anti-microbial formulations with the desired properties in target animal species.

In conclusion, the novel *in situ* forming lipogel was able to sustain the release of DANO and to increase its persistence in blood circulation. This DANO lipogel deserves to be considered as a sustained-release preparation for further researches and eventually for the treatment of susceptible microbial infections in veterinary practice.

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## Conflict of interest

The authors declare no conflict of interest.

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