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Diglyceride prodrug strategy for enhancing the bioavailability of norfloxacin

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ABSTRACT

Prodrug approach using diglyceride as a promoiety is a promising strategy to improve bioavailability of poorly absorbed drugs and the same was explored in the present work to improve oral bioavailability of norfloxacin; a second generation fluoroquinolone antibacterial. The prodrug was synthesized by standard procedures using dipalmitine as a carrier and the structure was confirmed by spectral analysis. Higher Log *P* indicated improved lipophilicity. The ester linkage between norfloxacin and dipalmitine would be susceptible to hydrolysis by lipases to release the parent drug and carrier in the body. *In vivo* kinetic studies in rats indicated 53% release of norfloxacin in plasma at the end of 8 h. The prodrug exhibited improved pharmacological profile than the parent compound at equimolar dose that indirectly indicated improved bioavailability.

1. Introduction

Norfloxacin (NFX) is a second generation fluoroquinolone. The mechanism of entry into cells is by diffusion (Chu and Fernandes, 1991). Because of low lipophilicity, it very slowly diffuses through cell membrane and is unable to attain therapeutic concentration at the site of infection. NFX faces a major shortcoming of low bioavailability (30–40%), single oral dose of 400 mg giving peak serum level of about 1.5 μg/ml. Due to *limited* plasma protein binding (5–15%), NFX is efficiently excreted via renal route that results in its serum half life of approximately 3 h (Coessens et al., 1997). NFX has limited clinical use in the treatment of urinary tract infections, although it is active *in vitro* against many Gram-positive and Gram-negative bacteria because its urine concentration is 100–300 times higher than that in serum which largely exceeds its minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) (Siporin, 1989). Enhancing NFX penetration into the infection site and slowing down renal filtration would improve the antibacterial efficiency of this antibiotic. One of the approaches in improving the bioavailability and hence the antibacterial efficiency is the prodrug design/drug latentiation. Prodrugs are considered as drugs containing specialized non-toxic protective groups utilized in a transient manner to alter or eliminate the undesirable properties of the parent drug molecule. This approach is widely applicable in enhancing bioavailability and bio-membrane passage (Notari, 1987). Drugs such as LK-A, L-dopa, bupranolol, phenytoin and closantel have been conjugated with diglyceride for improved bioavailability (Sugihara and Furuuchi, 1988; Garzon-Aburbeh et al., 1986; Mantelli et al., 1985; Scriba, 1993a,b; Scriba et al., 1995; Lambert et al., 1995; Poupaert et al., 1995). Review of literature reveals that efforts are mainly focused on derivatization of piperazine nitrogen of NFX for designing its prodrugs with improved pharmaceutical, pharmacokinetic and pharmacodynamic properties. Mannich bases (Abuo-Rahma et al., 2009) and N-masked prodrugs (Hirosato et al., 1989) of NFX have been reported for enhancing its lipophilicity, bioavalability and *in vivo* activity. (Acyloxy) alkyl carbamate esters of NFX were designed to mask its bitter taste (Alexander et al., 1991). Macromolecular dextran prodrug of NFX with improved water solubility has been developed for its intravenous administration (Veerle and Etienne, 1997). However there are no reported attempts where carboxylic group of NFX has been derivatized for designing its prodrug. The present work explores dipalmitin as a promoiety to improve oral bioavailability of NFX.

Temporary masking of carboxylic group can also protect NFX from forming chelates with polyvalent metal ions which would indirectly help to enhance its bioavailability. The ester linkage between NFX and diglyceride would be hydrolyzed by lipases to release the parent drug and carrier in the body.

2. Chemistry (Scheme 1)

Dipalmitate of 1,3-dihydroxyacetone (**3**) was synthesized by stirring dihydroxyacetone (**1**) and palmitoyl chloride (**2**) in chlo-

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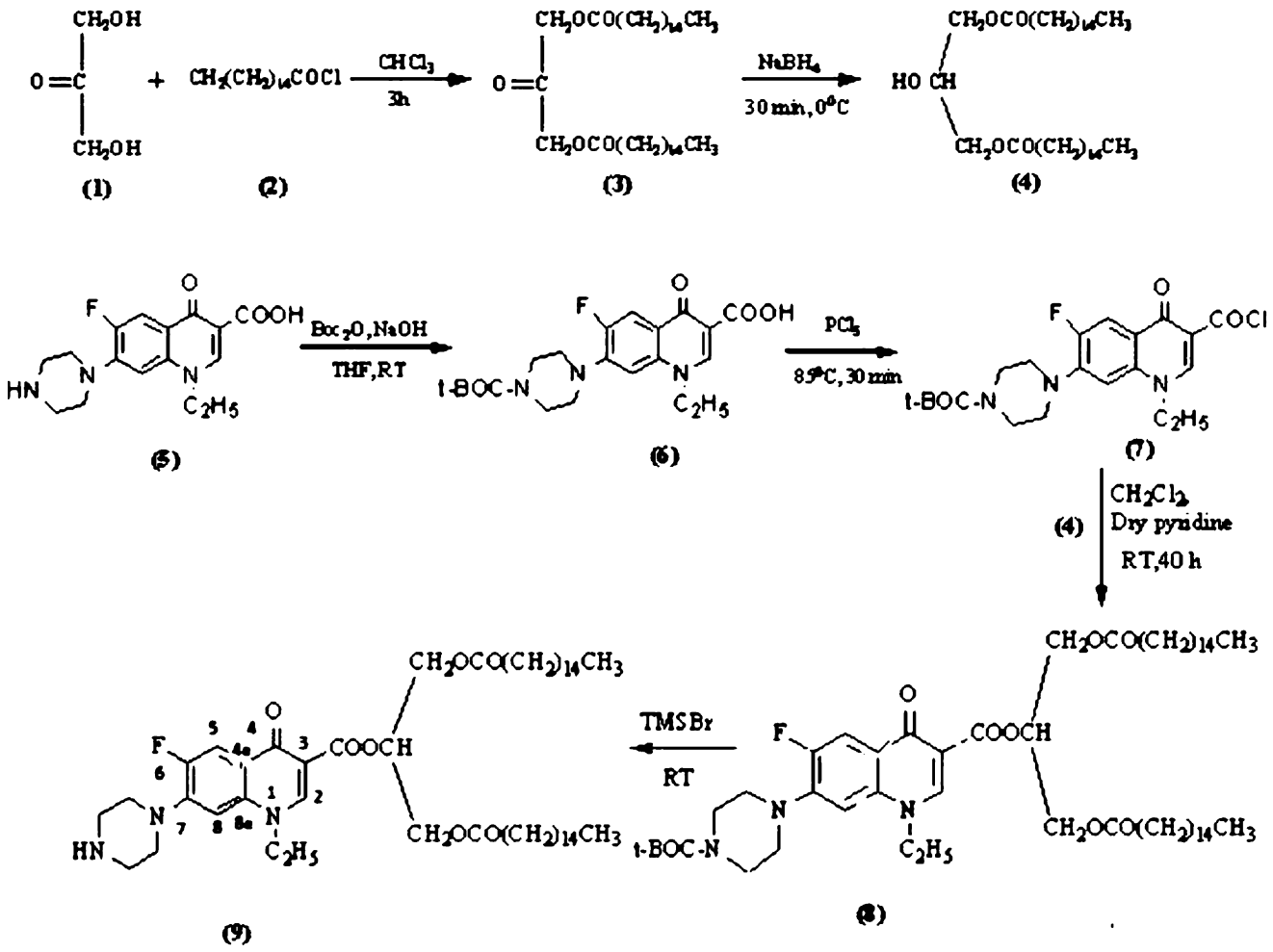

Scheme 1.

roform in presence of anhydrous pyridine for 3 h and was then reduced to 1,3-dipalmitin (**4**) with the help of sodium borohydride at 5 °C by external cooling (Bentley and McCrae, 1970). The piperazine –NH of NFX (**5**) was BOC-protected by stirring it with Boc_2O, 1 M NaOH and THF overnight at room temperature (Tanaka et al., 2008). The BOC-protected NFX (**6**) was converted into its acid chloride (**7**) by heating it with PCl_5 for 30 min in a water bath (Furniss et al., 1989). Compounds (**4**) and (**7**) were coupled by stirring the solution for 40 h at room temperature to give BOC-protected prodrug (**8**) which was recrystallized from petroleum ether (Khan and Akhter, 2005). Deprotection was done by adding TMSBr in one portion to stirring solution of (**8**) in CH_2Cl_2 to get prodrug NFXDG (norfloxacin-1,3-dipalmitin ester or 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1,4-dihydro-quinoline-3-carboxylate of 1,3-dipalmitoyl-glycerol). It was further purified by preparative TLC using solvent system benzene:ethyl acetate:methanol:glacial acetic acid (60:60:15:30 drop, v/v).

3. Experimental

3.1. In vitro hydrolysis kinetics

A new HPLC method was developed for estimation of NFX released from NFXDG in HCl buffer (pH 1.2), phosphate buffer (pH 7.4), stomach and intestinal homogenates (Rainsford, 1975; Morris et al., 1989; Nielson and Bundgaard, 1988; Jung et al., 2001; Scheline, 1973). The HPLC system used for this purpose consisted of a pump (Jasco LC Net II/ADC, Serial No. B224461095), with sampler programmed at 20 μl capacity per injection and a UV/VIS detector (Jasco UV 2075). Data were integrated using Jasco Borwin version 1.5. The column used was HiQ Sil C18HS (4.6 mm I.D. × 250 mm *L*; Batch: #080253; Column Number: 0HS00422) in the reversed phase partition chromatographic condition. Particle size of the column was 5 μm. The system was used in an air-conditioned HPLC laboratory atmosphere (20 ± 1 °C). Before analysis, the mobile phase was degassed using sonicator and filtered through a 0.45 μm membrane filter. Sample solutions were also filtered through the same. The system was equilibrated before making an injection. The column was monitored for UV absorbance at a detection wavelength selected after considering the overlay spectra of all the components. All the kinetic studies were carried out in triplicate. The *K* values from the plots were calculated separately and average *K* and S.D. value was determined. The half lives were calculated using the software 'PCP Disso' developed by Department of Pharmaceutics, Poona College of Pharmacy, Pune. The process was validated as per U.S.P. XXIV edition using different parameters like accuracy, selectivity, sensitivity and reproducibility.

HCl buffer (100 ml) was taken in a 250 ml beaker kept on a magnetic stirrer at a temperature of 37 °C with continuous stirring. NFXDG (13.6 mg) was added to this buffer solution. Immediately 0.1 ml of sample was withdrawn (0 min reading) from beaker with help of 1 ml pipette whose end was tied with muslin cloth and simultaneously 0.1 ml of buffer was replaced in the beaker. 20 μl of this sample was injected in the C_{18} column and eluted with the mobile phase phosphate buffer 0.05 M (pH 3.0 adjusted

with o-phosphoric acid):acetonitrile (25:75, v/v) at a flow rate of 1.2 ml/min and elute was monitored at wavelength of 279 nm and chromatograms of all the components were taken by measuring the absorption with a sensitivity of AUFS 0.01. Similarly samples were withdrawn after every 15 min till 1 hand then on an hourly basis till 6 h and injected in the column.

Same procedure as described earlier was followed for phosphate buffer. The kinetics was monitored by the increase in NFX concentration with time.

To study the release of NFX from NFXDG in stomach homogenate, a male Wistar rat was anesthetized by ether and midline incision was made. Sections of stomach were collected and washed to remove their contents, homogenized and diluted to half concentration with isotonic HCl buffer (pH 1.2). NFXDG (13.6 mg) was dissolved in minimum amount of DMF (1–2 ml) and volume was made up to 10 ml with HCl buffer (pH 1.2) (1360 μg/ml). 5 ml of this solution was added to 10 ml volumetric flask and volume was made up to 10 ml with HCl buffer (pH 1.2) (680 μg/ml). This was considered as the stock solution. To each eppendorf tube, 0.8 ml of the stock solution of prodrug and 0.2 ml of stomach homogenate was added and kept in incubator at 37 °C. The eppendorf tubes were taken out at different time intervals and centrifuged at 5000 rpm at 4 °C for 10 min. The supernatant (0.1 ml) was taken in fresh eppendorf tube and 0.9 ml of methanol was added to it and centrifuged again at 5000 rpm and 4 °C for 10 min. The sample (20 μl) was injected in the C_{18} column and eluted with the mobile phase phosphate buffer 0.05 M (pH 3.0 adjusted with o-phosphoric acid):acetonitrile (25:75, v/v) at a flow rate of 1.2 ml/min and elute was monitored at wavelength of 279 nm. Similar procedure was followed for small intestinal homogenate and release was studied till 10 h.

3.2. *In vivo hydrolysis kinetics (Scheline, 1973; Nakamura et al., 1992)*

For *in vivo* kinetic studies, a new HPLC method was developed for simultaneous estimation of NFXDG and NFX in rat plasma, feces and urine of Wistar rats. Same HPLC instrument with its accessories was used for this study as described in Section 3.1. Human plasma used for method development was purchased from Serological Institute of India, Navi Peth, Pune, India.

A male Wistar rat was starved for 24 h prior to use. The reading obtained prior to drug treatment was considered as zero min reading. An oral dose of NFXDG (36.84 mg/kg) was administered and blood samples were collected by retro-orbital puncture from both the eyes of the rat in EDTA-coated tubes at an interval of 15 min for the next 1 h. Then subsequently blood was collected on an hourly basis till 12 h and finally at 24th hour. The tubes were centrifuged at 5000 rpm at 0–5 °C for 10 min. Supernatant solution (0.1 ml) of centrifuged blood was added to eppendorf tube and 0.9 ml methanol was added to it for plasma protein precipitation. All the solutions were then vortexed for 2 min and again centrifuged at 5000 rpm for 10 min at 0–5 °C. Then these samples were analyzed by HPLC, using same procedure as mentioned in Section 3.1. The concentration of NFX released from NFXDG in plasma was calculated using the equation $Y = 189902x - 104132$ which was generated from calibration curve in plasma.

In order to study the release of NFX in urine and feces, male Wistar rats were starved for 24 h prior to use but had free access to water. The animals were then placed in metabolic cages and NFXDG (36.84 mg/kg) was orally administered. The pooled samples of urine and feces were collected at 24 h and were diluted with isotonic phosphate buffer solution (pH 7.4) by 10-fold and then centrifuged at 5000 rpm at 0–5 °C for 10 min. The supernatant solutions (0.1 ml) were added to another set of eppendorf tubes (1 ml capacity) and 0.9 ml methanol was added to each one of them. The solutions were then vortexed for 2 min and again centrifuged at 5000 rpm for 10 min at 0–5 °C. The samples were analyzed by HPLC, using same procedure as mentioned above for *in vivo* release in blood. The concentration of NFX in rat urine and feces was calculated using the equations $Y = 32072x + 14879$ and $Y = 26713x + 40315$ respectively which were generated from calibration curves in rat urine and feces.

3.3. *Antimicrobial screening (Cheng et al., 2009; Kim et al., 2010; Desboisa et al., 2010)*

NFXDG was screened for *in vivo* antimicrobial activity in Swiss albino mice infected with *S. aureus* in a mouse renal abscess model described by Kim et al. (2010) and the results were compared with the standard i.e. NFX. The bacterial strain *S. aureus* (NCIM 2079) was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India which was grown in nutrient broth (100 ml) culture medium at 37 °C for 18–20 h. Growth of colonies of bacteria was monitored on mannitol salt agar base. Nutrient broth and mannitol salt agar were procured from HIMEDIA, India. Average of six reading was calculated and all data were expressed as mean ± S.E.M. Statistical differences between the groups were calculated by two-way ANOVA followed by Bonferroni's test.

Suspension of microorganism (*S. aureus*) was prepared by adding one loopful of bacterial culture into 100 ml of sterilized nutrient broth. It was incubated for 24 h at 37 °C in incubator shaker. Optical density (OD) was measured at 600 nm and the solution was diluted until absorbance was equal to 0.5 which corresponded to 1×107 CFU/ml. Mice were weighed, marked and segregated into various treatment groups. Tail vein of mouse was dilated with absolute alcohol and then inoculated by injecting 1 ml of *S. aureus* suspension using a 30 gauge needle and syringe. After 12 h of infection, they were orally administered with 3 doses of NFX (5.5 mg/kg) and NFXDG (14.98 mg/kg) at the intervals of 6 h. On 4th day of infection, animals were sacrificed by cervical dislocation. A midline incision was made and kidneys were identified and dissected out. They were washed with sterilized phosphate buffer saline (PBS) or sterile water for injection (SWFI) to remove blood. Adrenal glands were carefully removed without damaging the kidney. One kidney was fixed in 10% formalin and sent for histopathological studies. 5 μm sections of kidney were taken and stained with haematoxylin and eosin. The other kidney was quickly transferred to a sterilized eppendorf tube containing 500 μl of sterilized PBS or SWFI. It was homogenized in aseptic conditions using sterilized pestles. 1 ml of homogenate was added to 9 ml of sterile distilled water in a test tube (dilution 1:10) and subsequently serially diluted till dilution of 1:108 was achieved. One ml of each dilution was mixed with 15 ml of sterile nutrient agar medium (cooled to 45 °C) and poured in a sterile Petri dish. All the Petri-dishes were incubated at 37 °C for 24 h. Incubator was pre-sterilized by fumigation. *S. aureus* colonies showing golden yellow pigmentation were counted and documented for each plate. Colonies were counted by a blinded observer who was unaware of experimental protocols. The results were recorded as CFU g^{-1} of tissue and are expressed as Log_{10} (CFU) g^{-1} of kidney tissue.

Histopathological studies of the kidneys of mice were carried out at Dhande Laboratory, Pune, India. Photomicrographs of the same were taken on Leica Microscope attached with a camera (10×/20×), provided by Department of Histopathology, Ruby Hall Clinic, Pune.

3.4. *Evaluation of survival rate in mice (Ikeda et al., 2000)*

Swiss albino mice were divided in 3 groups ($n = 10$). All the animals were infected by *S. aureus*. Group I (negative control) did not receive any treatment, whereas groups II and III received 3 doses of NFX (5.5 mg/kg) and NFXDG (14.98 mg/kg) respectively at a time interval of 6 h. No further treatment was given to the animals after-

wards. All the three groups were monitored over the next seven days for mortality and % survival was calculated for each day.

3.5. General procedure

All chemicals used in the synthesis were of AR grade. Norfloxacin was obtained as gift sample from Emcure Pharmaceutical Pvt. Ltd., Pune, India and dihydroxyacetone was purchased from Merck (Germany). Palmitoyl chloride was purchased from SAS Chemicals and Co., Mumbai, India. The reactions were monitored by TLC on pre-coated silica gel plates-60 F264 (Merck) using solvent system of benzene:ethyl acetate:methanol:glacial acetic acid (2:2:0.5:1 drop, v/v) and iodine vapours/UV light as detecting agents. The IR spectrum of the synthesized compound was recorded on JASCO, V-530 FTIR in potassium bromide (anhydrous, IR grade). NMR spectra of the intermediate and synthesized compound were recorded in $CDCl_3$ using ^1H NMR Varian Mercury 300 MHz with superconducting magnet using TMS as internal standard while ^{13}C NMR spectra were recorded on Bruker Instruments Model WM 250 NMR spectrometer at Department of Chemistry, University of Pune, Pune, India. Chemical shifts are reported in ppm downfield on δ scale. Elemental analysis was carried out on Hereaus Vario EL apparatus at CDRI, Lucknow, India. The λ_{max} of synthesized compound was determined on JASCO V530, UV–visible double beam spectrophotometer in hydrochloric acid buffer (pH 1.2), phosphate buffer (pH 7.4) and chloroform. Partition coefficient was determined in chloroform/phosphate buffer (pH 7.4). Pharmacological screening of the synthesized compound was carried out in the Department of Pharmacology, Poona College of Pharmacy and its animal facility is approved by Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA). The experimental protocols for the same were approved by the Institutional Animal Ethical Committee.

3.5.1. Synthesis of dipalmitate of 1,3-dihydroxyacetone (**3**)

Dihydroxyacetone (22 mmol, 2.0 g) was stirred in chloroform (85 ml) under nitrogen at room temperature. To this was added palmitoyl chloride (45.5 mmol, 13.9 ml) followed by anhydrous pyridine (4.3 ml). The heterogeneous mixture was stirred for 3 h and diluted with water and the chloroform layer was separated. The aqueous layer was extracted with two 20 ml portions of chloroform and the chloroform solutions were combined and washed once with water. Concentration of the chloroform to small volume resulted in the precipitation of a crystalline solid which was recrystallized from methylene chloride–ether (20:20) to give (**3**) as small plates.

Mp: 70–75 °C, R_f = 0.81 in hexane:ethyl acetate (6:1, v/v), % yield: 58.

3.5.2. Synthesis of 1,3-dipalmitin (**4**)

A mixture of tetrahydrofuran (75 ml) and benzene (15 ml) was used to dissolve (**3**) (5.3 mmol, 3.0 g). Water (4.5 ml) was slowly added to this solution with stirring and the temperature of the mixture was reduced to approximately 5 °C by external cooling in an ice bath; a milky-white suspension resulted. Sodium borohydride (7.9 mmol, 0.3 g) was added to this heterogeneous mixture; after 30 min, excess borohydride was destroyed by drop-wise addition of glacial acetic acid (0.5 ml) and the solution was diluted with chloroform (30–40 ml) and transferred to a separating funnel, washed with water, aqueous sodium bicarbonate followed by water again and the chloroform layer dried over magnesium sulfate to give (**4**) as a waxy white solid, which was recrystallized from chloroform. Thin layer chromatography did not show any traces of the 1, 2 isomer.

Mp: 78–80 °C, R_f = 0.75 in hexane:ethyl acetate (6:1, v/v), % yield: 65.

3.5.3. Synthesis of BOC-NFX (**6**)

A mixture of the norfloxacin (**5**) (1.56 mmol, 0.5 g), Boc_2O (1.64 mmol, 0.35 g) and 3.3 ml of 1 M NaOH solution in 15.5 ml of THF was stirred at room temperature overnight. After the removal of the organic solvent, the residue was neutralized with saturated aqueous ammonium chloride solution. The mixture was extracted with ethyl acetate (3× 15 ml) and dried over anhydrous sodium sulfate. The combined organics were concentrated in vacuum to provide (**6**).

Mp: 240–245 °C, R_f = 0.87 in glacial acetic acid:acetone:n-butanol:toluene:water (1:1:0.5:1:1, v/v), % yield: 94.2.

3.5.4. Synthesis of BOC-NFX acid chloride (**7**)

In a round bottom flask (**6**) (3.3 mmol, 1 g) and pure PCl_5 (3.3 mmol, 0.7 g) were mixed. Flask was fitted with $CaCl_2$ guard-tube and connected latter to gas absorption device. Flask was heated on water bath, with occasional shaking, until reaction commenced and then for further 30 min or until vigorous evolution of HCl had almost ceased.

Mp: 265–270 °C, R_f = 0.65 in benzene:ethyl acetate: methanol:glacial acetic acid (2:2:0.5:1 drop, v/v), % yield: 95.

3.5.5. Synthesis of BOC-NFXDG (**8**)

Freshly distilled dichloromethane (35 ml) was used to dissolve (**4**) (2.6 mmol, 1.5 g). Dry pyridine (3.2 mmol, 0.3 ml) and (**7**) (2.96 mmol, 1.0 g) were added at once, the contents were stirred for 40 h at room temperature and then diluted with 50 ml of water followed by extraction with 2× 15 ml of CH_2Cl_2. The CH_2Cl_2 extracts were combined, washed with 1% HCl, dried over anhydrous sodium sulfate and evaporated to dryness. Solid mass so obtained was crystallized from petroleum ether.

Mp: 325–330 °C, R_f = 0.80 in benzene:ethyl acetate: methanol:glacial acetic acid (2:2:0.5:1 drop, v/v), % yield: 75, IR (KBr; cm^{-1}) 3080 olefenic and aromatic C–H stretching, 1733 C=O stretching (ester), 1626 C=O stretching (pyridone), 1474 C–C and C–N stretching (quinoline), 1249 C–F stretching. ^1H NMR ($CDCl_3$; δ ppm): 8.76 [s; 1H] – C_2 proton, 7.69 [d; J_{H-F} = 12.6 Hz; 1H] – C_5 proton, 7.15 [d; J_{H-F} = 7.12 Hz; 1H] – C_8 proton, 4.49 [q; 2H] –N–C*H*$_2$CH$_3$, 2.2–2.3 [q; 4H] – C*H*$_2$–NH–C*H*$_2$, 1.58 [t; 3H] –N–CH$_2$C*H*$_3$, 1.40 [s; 9H] [–C(CH$_3$)$_3$]. 1.25 [s; 56H] – (C*H*$_2$)$_{14}$ × 2, 1.03 [m; 6H] – C*H*$_3$ × 2. ^{13}C NMR ($CDCl_3$; δ ppm): fluoroquinolone backbone: 177.5 (C_4), 169.9 (–*C*OO), 149.5 (C_2), 108.2 (C_3), 121.5 (C_{4a}), 113.1 (C_5), 134.6 (C_6), 146.1 (C_7), 106.9 (C_8), 138.6 (C_{8a}), 51.1 CH$_3$–*C*H$_2$–N–, 14.9 *C*H$_3$–CH$_2$–N–, 47.67 C_α, 44.7 C_β, 27.7 [–C(*C*H$_3$)$_3$], 66.8 [–*C*(CH$_3$)$_3$], 150.7 (N–*C*OO) dipalmitin backbone: 64.3 (–*C*H$_2$–CH–*C*H$_2$–), 69.9 (–CH$_2$–*C*H–CH$_2$–), 168.2 (2× –OCO–), 33.6–23.1 (2 × 14 –*C*H$_2$), 14.2 (2× –*C*H$_3$).

3.5.6. Synthesis of 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1,4-dihydro-quinoline-3-carboxylic acid 2-hexadecanoyloxy-1-hexadecanoyloxymethyl-ethyl ester (NFXDG)

TMSBr (15.2 mmol, 2.0 ml) was added in one portion to a stirring solution of (**8**) (1.0 mmol, 1.0 g) in CH_2Cl_2. After 18 h, the solvent was removed at reduced pressure. The solid thus obtained was re-suspended in water and the pH adjusted to 7.4 by addition of NaOH. Product obtained was filtered, dried and purified by preparative TLC using solvent system benzene:ethyl acetate:methanol:glacial acetic acid (60:60:15:30 drops, v/v).

Mp: 295–300 °C, R_f = 0.76 in benzene:ethyl acetate: methanol:glacial acetic acid (2:2:0.5:1 drop, v/v), % yield 95. IR (KBr; cm^{-1}) 3501 –NH stretching (piperazine), 3082 olefenic and aromatic C–H stretching, 1731 C=O stretching (ester). ^1H NMR ($CDCl_3$; δ ppm): 8.88 [s; 1H] – C_2 proton, 7.87 [d; J_{H-F} = 13.0 Hz; 1H] – C_5 proton, 7.2 [d; J_{H-F} = 7.0 Hz; 1H] – C_8 proton, 4.55 [q; 2H] –N–C*H*$_2$CH$_3$, 2.298–2.370 [q; 4H] – C*H*$_2$–NH–C*H*$_2$, 2.0 [s; 1H] – N*H*

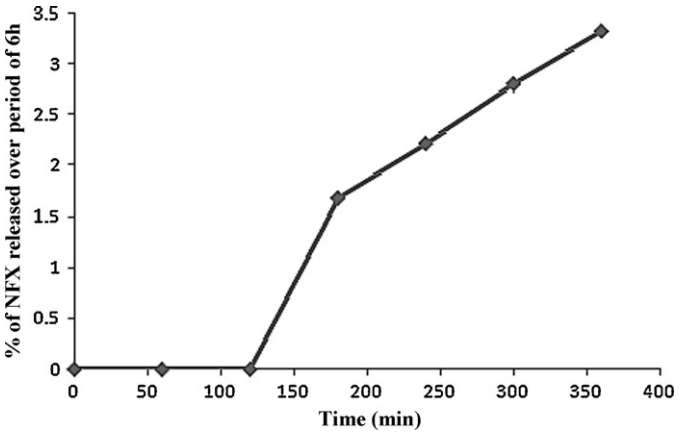

Fig. 1. *In vitro* kinetic studies: % release of NFX in HCl buffer (pH 1.2).

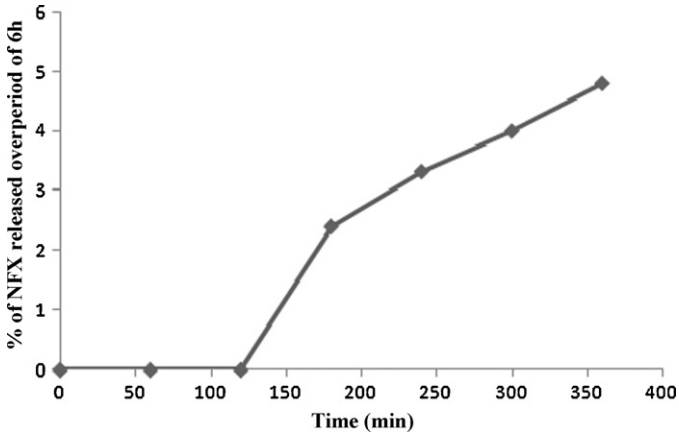

Fig. 3. *In vitro* kinetic studies: % release of NFX in stomach homogenate.

piperazine, 1.58 [t; 3H] –N–CH_2CH_3, 1.254 [s; 56H] – $(CH_2)_{14} \times 2$, 1.05 [m; 6H] – $CH_3 \times 2$. ^{13}C NMR ($CDCl_3$; δ ppm): fluoroquinolone backbone: 177.5 (C_4), 169.9 (–COO), 149.5 (C_2), 108.2 (C_3), 121.5 (C_{4a}), 113.1 (C_5), 134.6 (C_6), 146.1 (C_7), 106.9 (C_8), 138.6 (C_{8a}), 51.1 CH_3–CH_2–N–, 14.9 CH_3–CH_2–N–, 47.67 C_α, 44.7 C_β, dipalmitin backbone: 64.3 (–CH_2–CH–CH_2–), 69.9 (–CH_2–CH–CH_2–), 168.2 (2× –OCO–), 33.6–23.1 (2×14 –CH_2), 14.2 (2× –CH_3) Anal. calculated for $C_{51}H_{84}FN_3O_7$: C, 70.39; H, 9.73; N, 4.83; found: C, 70.35; H, 9.75; N, 4.82.

4. Results and discussion

The structure of NFXDG was confirmed by IR, ^{1}H NMR, ^{13}C NMR and C, H, N analysis and was found to be in accordance with the anticipated structure. Protection of NFXDG with Boc was confirmed by disappearance of NH piperazine peak at 3501 cm^{-1} in the IR spectrum and its reappearance after final deprotection of Boc-NFXDG. ^{1}H NMR and ^{13}C NMR also confirmed that protection–deprotection was achieved successfully. Melting point of NFXDG was found to be 295–300 °C (uncorrected). Partition coefficient of NFXDG was determined in chloroform: phosphate buffer (pH 7.4) and was found to be 5.25 which was 2.7 times higher as compared to NFX (1.94). This enhanced lipophilicity would improve the ability of NFXDG to cross the bacterial membrane in a positive manner because it has been reported that partition coefficient is an important parameter for biological activity of fluoroquinolones. *In vitro* kinetic studies were performed in HCl buffer (pH 1.2) and phosphate buffer (pH 7.4). Release studies were also carried out in tissue homogenates of stomach and small intestine of rat. The kinetics was monitored by increase in concentration of NFX released from NFXDG at 385 nm in HCl buffer (pH 1.2) and at 415 nm in phosphate buffer (pH 7.4). Percent release of NFX from NFXDG in HCl buffer (pH 1.2) and stomach homogenates is shown in Figs. 1 and 3 respectively. The studies revealed that hydrolysis of the prodrug started after 2 h and released negligible amounts (3.4% and 5%) of NFX in HCl buffer (pH 1.2) and stomach homogenates respectively over a period of 6 h. However in phosphate buffer (pH 7.4; Fig. 2) and intestinal homogenates (Fig. 4), the activation of the prodrug started early (after 1 h) furnishing 21% and 32% release of NFX respectively over a period of 10 h. The release of NFX from

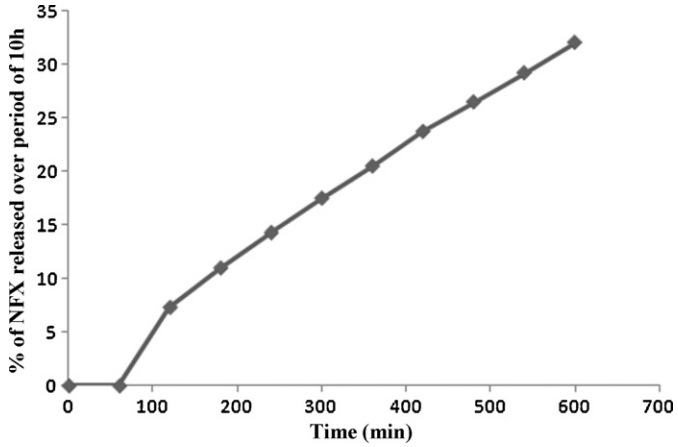

Fig. 4. *In vitro* kinetic studies: % release of NFX in intestinal homogenate.

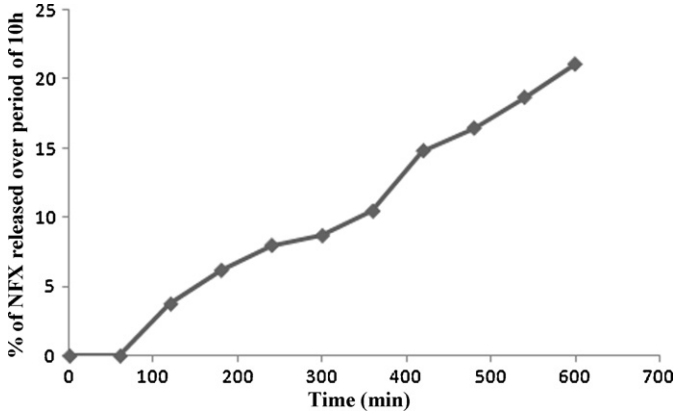

Fig. 2. *In vitro* kinetic studies: % release of NFX in phosphate buffer (pH 7.4).

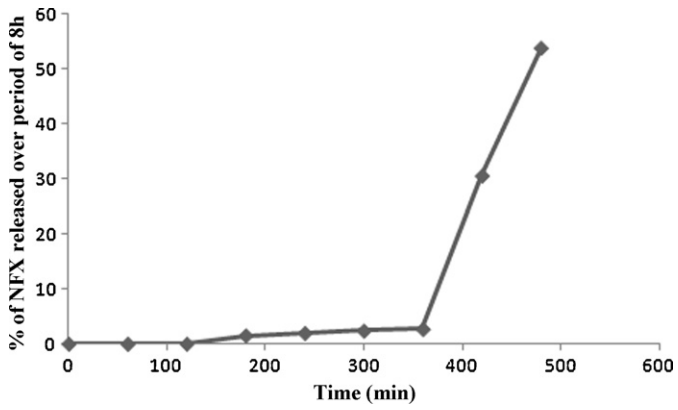

Fig. 5. *In vivo* kinetic studies: % release of NFX after oral administration in rat.

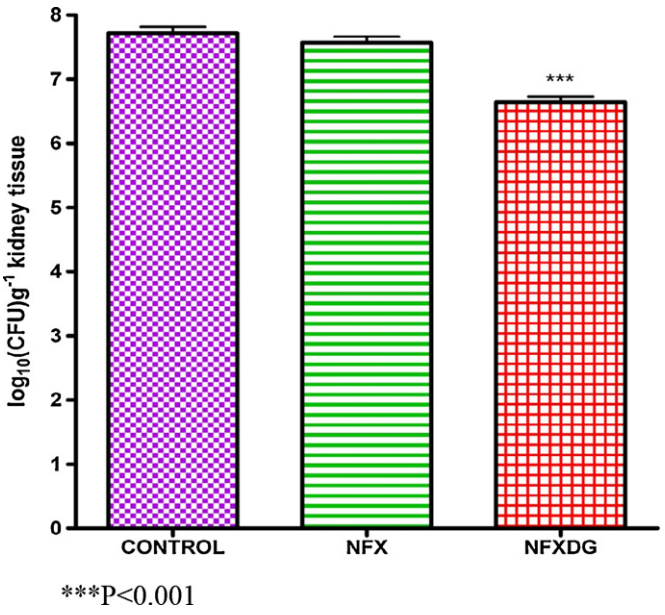

Fig. 6. Log_{10} (CFU) g^{-1} of kidney tissue.

NFXDG followed first order kinetics in aqueous buffers and tissue homogenates. Rate of hydrolysis of NFXDG in small intestinal homogenate was more as compared to phosphate buffer (pH 7.4) owing to presence of lipase in GIT.

In vivo release kinetics was investigated in blood (Fig. 5), feces and urine of Wistar rats. After oral administration, NFX was not detected in the plasma for first 2 h indicating that NFXDG was not hydrolyzed in stomach during that time span. This finding is in accordance with the results of *in vitro* release studies in stomach homogenates where it was observed that the release started after 2 h. NFX was detected in the plasma at 3rd hour and after that, its concentration consistently and slowly increased during 3–6 h attaining a maximum release of 54% at the end of 8 h. The concentration of NFX declined over the period of time and became negligible at the end of 12 h. The release followed zero order kinetics. From the *in vitro* and *in vivo* release studies it can be concluded that minimum hydrolysis (21–32%) occurred in the lumen of upper GIT while maximum concentration of NFXDG that remained intact might have been absorbed into blood owing to its high lipophilicity followed by its hydrolysis in blood corresponding to a total of 54% release of NFX at 8th hour. The slow rate of hydrolysis might be useful in giving longer duration of action. Traces of NFX were found in urine and feces indicating its renal and intestinal excretion.

Norfloxacin is used widely in the treatment of urinary tract infection as it acts against *Escherichia coli* and *S. aureus*. Therefore NFXDG was screened for *in vivo* antimicrobial activity and the extent of protection provided by NFXDG against *S. aureus*-induced renal abscess was studied using mouse renal abscess model and the results were compared with the standard i.e. norfloxacin The number of colonies/200 mg of kidney tissue for NFX treated group were 85 ± 22.74 which were not significantly low as compared to the negative control group (117 ± 47.81). However the number of colonies was significantly reduced to as low as 10 ± 5.01 in NFXDG treated group. The number of colonies/200 mg of kidney tissue was also expressed as logarithm of colony forming units per gram of kidney tissue [Log_{10} (CFU) g^{-1} of kidney; Fig. 6]. At an oral dose of 5.5 mg/kg of NFX thrice a day (at 6 h intervals), the decrease in the Log_{10} (CFU) g^{-1} of kidney was not statistically significant (7.62) in comparison to control group (7.767). Interestingly, equimolar oral doses of NFXDG (14.98 mg/kg; thrice a day) provided statistically significant protection (6.698). Number of microorganisms/g of kidney tissue were also calculated for each group. The control group that did not receive any drug treatment showed extensive growth of 58,500,000 microorganisms while NFX treated group showed 42,500,000 microorganisms at 105 dilution per gram of kidney tissue. NFXDG treated group significantly brought down the number to 5,000,000 (12 times lower than the control). These promising results indicate improved bioavailability of NFXDG compared to NFX as the activities were carried out at equimolar doses.

The histopathology of infected mice kidneys of the negative control group revealed marked inflammatory cell infiltration with microabscess formation in the interstitial tissue. Neutrophils were seen in tubules while some glomeruli appeared inflamed. This histopathology was suggestive of acute superactive interstitial nephritis of kidney with microabscess and focal glomerulitis. NFX-treated kidney exhibited mild, acute interstitial nephritis characterized by inflammatory cell infiltration with few microabscess formations while the glomeruli and tubules appeared unremarkable. NFXDG was able to restore the normal morphology of kidney without any signs of inflammatory cell infiltration, interstitial nephritis, glomerulitis or abscess at the equimolar dose. The results are depicted in Fig. 7. Statistical differences between the groups were calculated by two-way ANOVA followed by Bonfferoni's test. All data are expressed as means $\pm$ SD. Differences were considered at a P value of <0.001 in relation to control.

Survival rate studies were also performed on albino mice infected with *S. aureus* to compare the effects of NFXDG with NFX and negative control over a period of 7 days (Fig. 8). Survival rate in negative control of mice challenged with *S. aureus* infection was only 10% which was brought to 40% by NFX. NFXDG-treated mice exhibited maximum rate of survival (60%) at equimolar dose to

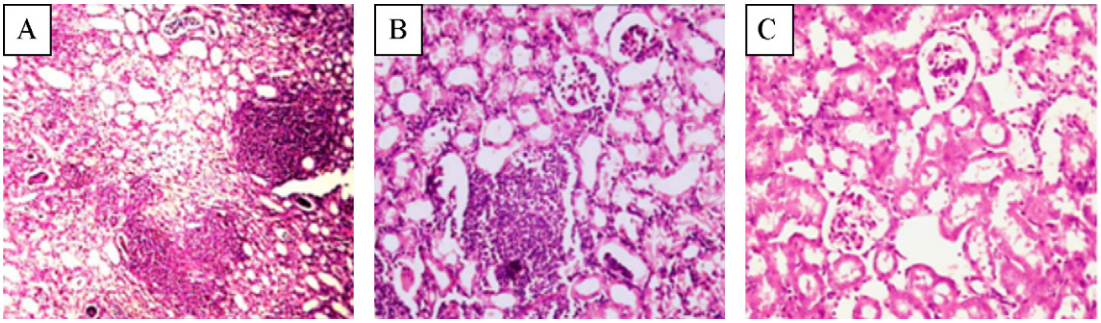

Fig. 7. Photomicrographs of kidney sections infected with *S. aureus*. (A) Negative control showing acute super-active interstitial nephritis with marked inflammatory infiltration/microabccess formation and focal glomerulitis. (B) Infected kidney section treated with NFX showing mild acute interstitial nephritis with interstitial inflammatory cell infiltration/few microabccesses, glomeruli with unremarkable tubules. (C) Infected kidney section treated with NFXDG showing unremarkable glomeruli, tubules, interstitial tissue and blood vessels without evidence of inflammation. Overall histopathology of kidney is unremarkable.

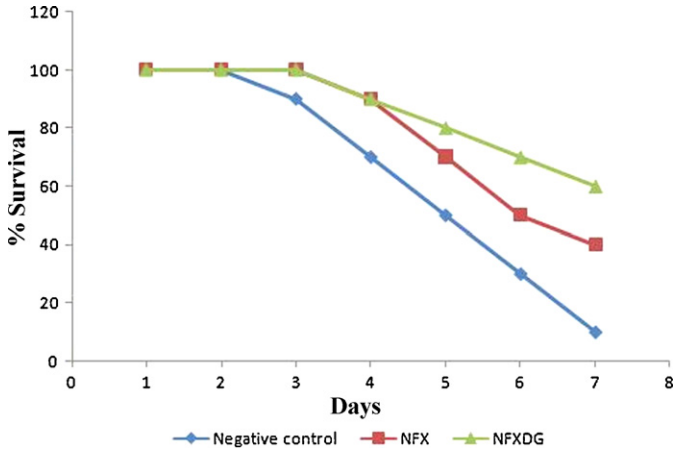

Fig. 8. % survival of mice infected with *S. aureus*.

NFX. These results indicate that NFXDG has enhanced bioavailability than NFX.

5. Conclusion

Dipalmitin prodrug of NFX was successfully synthesized offering improved lipophilicity, better membrane penetration and bioavailability that resulted in improved pharmacological profile than the parent compound at equimolar dose. It can be concluded that diglyceride can serve and be used as a promising carrier to improve the bioavailability of poorly absorbed drugs.

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