Pharmaceutical Sciences, 2023, 29(1), 65-74
doi:10.34172/PS.2022.15
https://ps.tbzmed.ac.ir/

Research Article

Ester Prodrugs of Levofloxacin to Prevent Chelate Formation in Presence of Aluminium Ion

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Article Info

Article History:
Received: 22 Feb 2022
Accepted: 28 Mar 2022
ePublished: 3 Apr 2022

Keywords:
-Levofloxacin
-Oral bioavailability
-Prodrug

Abstract

Background: Intestinal absorption of levofloxacin (LFX) is decreased by the concomitant administration of antacids due to the formation of insoluble chelate complexes with various metal cations.

Methods: The following four ester prodrugs of LFX—cilexetil ester (LFX-CLX), medoxomil ester (LFX-MDX), ethoxycarbonyl 1-ethyl hemiacetal ester (LFX-EHE) and pivaloyloxymethyl ester (LFX-PVM)—were synthesized. Then, the lipophilicity, *in vitro* chelate formation with aluminum chloride ($AlCl_3$), chemical and enzymatic stability, minimum inhibitory concentrations (MICs) against some bacteria, and the efficacy in preventing chelate formation of prodrugs with aluminum hydroxide ($Al(OH)_3$) in rabbits were evaluated.

Results: The synthesized ester prodrugs of LFX exhibited high purity and higher lipophilicities than LFX depending on the ester moieties. MICs of the prodrugs against *S. aureus*, *E. coli*, and *P. aeruginosa* were more than 10 times higher than those of LFX. Prodrugs were stable chemically but unstable enzymatically and generated LFX in biological specimens. When $AlCl_3$ solution was mixed with LFX solution *in vitro*, insoluble chelate complex was formed immediately. In rabbits, co-administration of $Al(OH)_3$ with LFX reduced the oral bioavailability of LFX by approximately 40%. In contrast, no precipitation was observed when $AlCl_3$ solution was mixed with each prodrug solution *in vitro*, and co-administration of $Al(OH)_3$ exerted no significant effect on the oral bioavailability of LFX when each prodrug was administered in rabbits.

Conclusion: The ester prodrug approach of LFX could be a feasible strategy for avoiding chelate formation with aluminum ion *in vivo*.

Introduction

Fluoroquinolones (FQs), including new ones such as ciprofloxacin, ofloxacin (OFX), levofloxacin (LFX), and sparfloxacin, are an important group of synthetic antibacterial compounds with a fluorine atom at position 6 and a piperazine ring at position 7 of quinolone-3-carboxylic acid.[1] FQs are prescribed widely as the first line of defense against various bacterial infections.[2,3] New FQs exhibit the longer half-lives *in vivo* and an extended spectrum of antibacterial activity compared with the older-generation FQs. For example, LFX, a levo-isomer of D, L-racemate OFX, has improved activity against gram-positive bacteria and excellent activity against gram-negative bacteria and the atypical organisms compared to ciprofloxacin and OFX.[4,5] The oral bioavailability of LFX is high,[3,6] and LFX was classified as a Biopharmaceutics Classification System (BCS) Class I drug with high solubility and high permeability.[7]

Orally administered FQs including LFX, however, form insoluble chelate complexes with various metal cations such as aluminum ion (Al^{3+}), magnesium ion, and calcium ion. Moreover, the oral bioavailability of FQs is greatly reduced when FQs are administered concomitantly with metal-containing drugs.[2,3,8-11] It was reported that co-administration of 1g aluminum hydroxide ($Al(OH)_3$), a widely used antacid in clinical practice, with 100 mg LFX decreased the peak plasma concentration (Cmax) of LFX by 35.2%, the urinary excretion rate by 71.6%, and the value of the area under the concentration-time curve from 0 to 24 h ($AUC_{0\text{-}24h}$) by 56.3% in humans.[9] Another study reported that co-administration of $Al(OH)_3$ reduced the oral bioavailability of LFX by approximately 60% in humans.[6] In various experimental animals, antacids and metal cation-containing drugs also decreased the oral absorption of LFX. For example, the pretreatment of dogs with 1g sucralfate containing $Al(OH)_3$, which is

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used to treat and prevent gastrointestinal ulcers, decreased the Cmax by 70.8% and the oral bioavailability of orally administered LFX (5 mg/kg dose) by 55.6%.[12] The binding of metal ions to the 4-keto- and 3-carboxyl-groups of FQs to form nonabsorbable chelates has been suggested as the possible mechanism responsible for the reduced absorption of FQs.[1-3] To avoid chelate formation of LFX in clinical, staggered dosing was suggested, in which antacids taken 2 h after LFX ingestion.[13,14] However, staggered dosing of antacids would be inconvenient in actual clinical and daily life situations. Previously, the efficacy of ester prodrugs in avoiding chelate formation was examined using the pivaloyloxymethyl (PVM) ester prodrug of OFX because the addition of an ester moiety at the 3-position carboxyl acid was thought to inhibit the binding of metal cations by steric hindrance.[15] The PVM ester moiety has been used in prodrugs such as pivampicillin, pivmecillinam, and cefetamet pivoxil.[16-18] In rabbits, co-administration of $Al(OH)_3$ with OFX decreased the $AUC_{0\text{-}24h}$ of plasma OFX by 47.6% compared with the administration of OFX alone, but co-administration of $Al(OH)_3$ with OFX-PVM had no significant effect on the plasma OFX concentrations. The results suggested that the use of a certain ester prodrug can avoid the chelate formation of FQs *in vivo*.[15] The usefulness of the prodrug approach in preventing chelate formation was also reported using the ethoxycarbonyl 1-ethyl hemiacetal ester prodrug of LFX (LFX-EHE) in rats. The co-administration of $Al(OH)_3$ with LFX-EHE had no significant effect on plasma LFX levels, although co-administration of $Al(OH)_3$ with LFX decreased the $AUC_{0\text{–}4\ h}$ of plasma LFX by about 50% of LFX alone. Based on these results, it was stated that LFX-EHE avoids insoluble chelate formation with metal-containing drugs in the intestinal tract and is rapidly hydrolyzed to the parent drug.[19,20] Recently, the efficacy of the cilexetil ester prodrug of LFX (LFX-CLX) was examined.[21] In *in vitro* binding study, LFX was precipitated by 76.1% immediately after mixing with aluminum chloride ($AlCl_3$) solution, but LFX-CLX was not. However, the co-administration of $AlCl_3$ with LFX-CLX decreased the AUC of plasma LFX by approximately 60% compared with that after oral administration of LFX alone in rats,[21] different from the cases of OFX-PVM[15] and LFX-EHE.[19,20] The significant difference in the chelate formation between $AlCl_3$ and $Al(OH)_3$ with ester prodrugs was thought to be derived from the cytotoxicity of $AlCl_3$. $AlCl_3$, an antiperspirant salt used to treat hyperhidrosis, may have induced barrier dysfunction and inflammation of the intestinal epithelium,[22,23] which would induce enzymatic degradation of LFX-CLX and release LFX in the intestinal lumen. Further study is necessary to clarify the efficacy of ester prodrugs of FQs in avoiding chelate formation in vivo using $Al(OH)_3$.

In this study, four ester prodrugs of LFX—LFX-CLX, LFX-EHE, medoxomil ester (LFX-MDX), and PVM ester (LFX-PVM)—were synthesized (Figure 1). CLX ester moiety has been used in prodrugs of sanfetrinem[24] and candesartan;[25] the EHE ester moiety, in a prodrug of ampicillin;[26] and the MDX ester moiety, in prodrugs of azilsartan,[27] olmesartan,[28] and ertapenem.[29] We then evaluated the lipophilicity (the partition coefficient at pH 6.5), *in vitro* chelate formation with $AlCl_3$, chemical and enzymatic stability, minimum inhibitory concentrations (MICs) against some bacteria, and *in vivo* efficacy in preventing chelate formation with $Al(OH)_3$ in rabbits using each LFX prodrug.

Materials and Methods

Materials

The following materials were used: LFX (Apollo Scientific, UK); ciprofloxacin (LKT Laboratories, Inc., USA); 1-chloro ethyl cyclohexyl carbonate (Toronto Research Chemicals Inc., Canada); 1-chloroethyl ethyl carbonate (Tokyo Chemical Industry Co., Ltd., Japan); 4-(chloromethyl)-5-methyl-1,3-dioxol-2-one (BLDpharm, China); chloromethyl pivalate (FUJIFILM Wako Pure Chemical Corporation, Japan); dried $Al(OH)_3$ gel (Pfizer Japan Inc., Tokyo); and pancreatin (from porcine pancreas) and anhydrous $AlCl_3$ (Wako Pure Chemical Industries, Ltd., Japan). All other chemicals and solvents were of analytical grade.

Synthesis of levofloxacin prodrugs

The LFX ester prodrugs were synthesized according to the following modified method reported by Daehne *et al.*[30] To synthetize LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM, 1-chloro ethyl cyclohexyl carbonate (24 mmol); 1-chloroethyl ethyl carbonate (24 mmol); 4-(chloromethyl)-5-methyl-1,3-dioxol-2-one (24 mmol); or chloromethyl pivalate (24 mmol), respectively, added to an N,N-dimethylformamide solution that contained anhydrous potassium carbonate (12 mmo1) and LFX (6 mmo1). The mixture solution was stirred for 2 h under argon atmosphere. In the case of LFX-CLX, LFX-EHE, and LFX-MDX, the product was extracted with ethyl acetate, washed with distilled water, dehydrated with anhydrous sodium sulfate, and evaporated until dry. Recrystallization

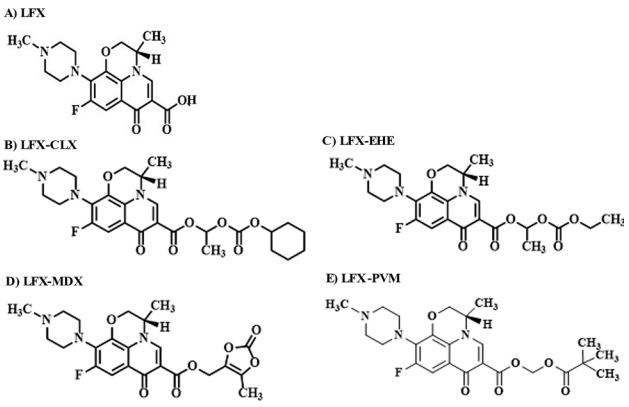

Figure 1. Chemical structures of levofloxacin and its ester prodrugs. (A) LFX, levofloxacin; (B) LFX-CLX, levofloxacin-cilexetil ester; (C) LFX-EHE, levofloxacin-ethoxycarbonyl 1-ethyl hemiacetal ester; (D) LFX-MDX, levofloxacin-medoxomil ester; and (E) LFX-PVM, levofloxacin-pivaloyloxymethyl ester.

of LFX-CLX, LFX-EHE, and LFX-MDX was made from chloroform and hexane, from diethyl ether, and from ethyl acetate, respectively. In the case of LFX-PVM, crushed ice and distilled water (360 mL) was slowly added to the mixture after it was stirred for 2 h and then cooled. Crystal precipitated was filtered with filter paper (ADVANTEC®, Toyo Roshi Kaisha, Ltd., Japan) and dried at 40 ˚C for 5 h. The melting points were determined on Yanagimoto micro melting point apparatus: Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-400S at 400 MHz (JEOL Ltd., Tokyo, Japan), and chemical shifts relative to Me_4Si (δ 0.00) were estimated. NMR spectra were measured in $CDCl_3$ (δ = 7.26 ppm). The carbon nuclear magnetic resonance (^{13}C- NMR) spectroscopic data were recorded with a JEOL JNM-400S at 101 MHz (JEOL Ltd., Tokyo, Japan), and chemical shifts relative to $CDCl_3$ (δ = 77.0 ppm) were estimated. The mass spectra of the prodrugs were recorded on JEOL JMS-700 spectrometers (JEOL Ltd., Tokyo, Japan) through the direct inlet system.

Partition coefficients

Partition coefficients of LFX and the synthesized prodrugs (LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM) were determined in a partition system of chloroform and pH 6.5, 0. l M Tris-HCl buffer at 25 ˚C, in which the initial concentration of each compound in a buffer solution was approximately l00 μg/mL. Concentrations of each compound in both solvents after vigorous shaking were determined by high-pressure liquid chromatography (HPLC). Calculated log P was estimated using ChemDraw, a chemical drawing tool.

Chelation with Al^{3+} ion in vitro

LFX and the LFX ester prodrugs were dissolved in pH 6.5 Tris-HCl buffer solutions at an initial concentration of 20 μg/mL. Separately, $AlCl_3$ was dissolved in the same buffer at concentrations of 100 and 200 μg/mL. Each drug solution and $AlCl_3$ solution were mixed at equal volumes, and the mixture was left to stand for 30 min at 25°C. In the chelate formation *in vitro* study, $AlCl_3$ was used instead of $Al(OH)_3$, because $AlCl_3$ is a water-soluble compound, but $Al(OH)_3$ is not. Also, $Al(OH)_3$ is converted to $AlCl_3$ in the acidic condition like gastric juice. The mixture solution (or suspension) was centrifuged at 3,000 x g for 10 min, and the supernatant was filtered through a syringe filter with a 0.22 μm pore size (Millipore, Tokyo, Japan). The concentration of LFX in each filtrate was determined by HPLC after the prodrug was hydrolyzed with 1 mol/L of sodium hydroxide (NaOH).

Chemical and enzymatic stability

To evaluate the chemical (non-enzymatic) stability of prodrugs, pH 6.5 and pH 7.4 phosphate buffer solutions (PBS) used. As preliminary experiments of enzymatic stability, LFX ester prodrugs (initial concentration: 0. 1 mM) was incubated for 2 h at 37 ˚C in the following specimens: plasma (male Sprague-Dawley rats), small intestinal mucosal homogenates, liver homogenates, pancreatin solution (1.0 mg/mL), and luminal fluid with intestinal contents of rats. Each biological specimen including pancreatin solution was prepared using pH 7.4, 25 mM Tris-HCl buffer.[31] Based on the results of preliminary study, the enzymatic stability of the LFX ester prodrugs in rat 10% plasma and 2% small intestinal mucosal homogenates was determined for 15 min at 37 ˚C to compare the enzymatic stability among ester prodrugs. Concentrations of generated LFX from each LFX ester prodrug in the reaction mixture were determined periodically, in which the further metabolic reaction after the sampling was stopped by adding methanol at a volume ratio of 1:2.

Determination of the minimum inhibitory concentration (MIC) values

MIC values of LFX and LFX prodrugs were determined by the agar plate dilution method using Mueller-Hinton broth (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) in the same manner as reported previously.[15] Briefly, LFX (10 mg) or each LFX ester prodrug (corresponding to 10 mg LFX) was dissolved in 1 mL of 0. l mol/L hydrochloric acid, and 4 mL of 0. l mol/L PBS (pH 7.4) was added to the solution. The final concentrations of LFX and each LFX ester prodrug in the incubation medium were adjusted to 62.50, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49, 0.24, 0.12, 0.06, and 0.03 μg/mL. The bacterial numbers of *Staphylococcus aureus* (ATCC29213), *Escherichia coli* (ATCC25922), and *Pseudomonas aeruginosa* (ATCC27853) were adjusted to McFarland 0.5 (1.5×10^6 cells/mL) with a sterilized normal saline. These bacterial suspensions were inoculated into Mueller-Hinton broth that contained LFX or LFX ester prodrugs. Facultative anaerobic and aerobic bacteria were cultured under aerobic conditions at 37 ˚C for 20 h.

The absorption study in rabbits

Male albino rabbits purchased from Japan SLC, Inc. (Hamamatsu, Japan) weighing 2.0–3.0 kg were fasted overnight with free access to water before each experiment. The following drug was suspended in 3 mL water containing 0.2 mL of 1.0 mol/L HCl and was administered orally to rabbits using gastric intubation: LFX (20 mg/kg, 0.055 mmol/kg); LFX-CLX (29.4 mg/kg, 0.055 mmol/kg); LFX-EHE (26.4 mg/kg, 0.055 mmol/kg); LFX-MDX (26.2 mg/kg, 0.055 mmol/kg); and LFX-PVM (26.4 mg/kg, 0.055 mmol/kg) with or without $Al(OH)_3$ (100 mg/kg, 1.282 mmol/kg). Then, blood (100 μL each) samples were periodically collected from the ear veins of rabbits. The following pharmacokinetic parameters estimated: Cmax of LFX, the time to reach the Cmax (Tmax), and the $AUC_{0\text{-}6h}$ of LFX in plasma. The $AUC_{0\text{-}6h}$ value of plasma LFX was estimated by the trapezoidal rule.[32]

Analysis of LFX

The concentrations of LFX in various biological samples

were determined using ciprofloxacin as an internal standard by HPLC in the same manner as reported previously[21] according to the reported modified method.[33] Briefly, to 0.1 mL sample containing LFX prodrug. 0.05 mL of 1.0 mol/L NaOH was added and incubated for 30 min and then neutralized with 0.05 mL of 1.0 mol/L HCl solution. The HPLC system (Shimazu, Kyoto, Japan) used consisted of a model LC-20AD pump, a 20μL fixed injection loop, and a model SPD-10Avp UV detector. Data were acquired with the Sepu3000's processor (Hang Zhou, China). The column used was an L-column 2 ODS (150 mm × 4.6 mm, i.d., 5 μm, CERI Co., Ltd., Saitama, Japan). The mobile phase consisted of acetonitrile and water that contained 0.3% triethylamine (pH 3.3 adjusted with phosphoric acid) at a volume ratio of 16:84. The flow rate of the mobile phase was 1 mL/min. The detection was made at 295 nm.

Statistical analysis

The measurement data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the unpaired Student's *t*-test. P-values less than 0.05 were statistically significant.

Results

Synthesis of LFX-ester prodrugs: LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM

The purity of each prodrug that was synthesized in this study was high, since the lH-NMR spectral data displayed only specific signals of each prodrug. The purity of each prodrug was also evaluated by thin layer chromatography (TLC) using chloroform-methanol-acetic acid-distilled water (15:5:2:1, v/v/v/v) as a developing solvent. Each spot was detected using shortwave ultraviolet light (254 nm). The Rf values of LFX, LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM in TLC were 0.45, 0.78, 0.67, 0.60, and 0.75, respectively. The chemical structures of LFX and its ester prodrugs that were synthesized in this study are shown in Figure 1. The 1H NMR chart, 13C NMR chart, and mass spectrum of each prodrug are shown as supplementary data in Figures S1-S4.

LFX-CLX

The synthetic yield of LFX-CLX was 89.3% (2,845 mg). The melting point was 124–126°C (dec.). The chemical shifts relative to Me_4Si (δ 0.00) were as follows: ^{1}H-NMR ($CDCl_3$) δ: 1.22-1.47 (6H, m), 1.51 (3H, q, *J* = 3.4 Hz), 1.64-1.65 (3H, m), 1.73 (2H, m), 1.92-1.95 (2H, m), 2.54 (3H, s), 2.80 (4H, m), 3.48 (4H, m), 4.36 (2H, d, *J* = 9.6 Hz), 4.44-4.47 (1H, m), 4.64 (1H, td, *J* = 4.5, 8.9 Hz), 6.95-7.01 (1H, m), 7.46 (1H, dd, *J* = 12.1, 15.3 Hz), 8.22 (1H, d, *J* = 10.5 Hz). ^{13}C-NMR ($CDCl_3$) δ: 18.3, 19.8, 23.6, 25.1, 31.4, 31.5, 35.5, 45.4, 49.3, 54.7, 55.1, 68.1, 91.4, 104.8, 104.9, 105.0, 105.2, 107.9, 108.2, 123.4, 123.5, 130.7, 130.8, 130.9, 130.9, 139.7, 139.8, 139.9, 145.4, 145.8, 152.4, 152.5, 154.2, 154.3, 156.7, 156.8, 162.2, 162.9, 172.3, 172.5. and mass spectra *m/z*, 531 (M^+). Calculated for $C_{27}H_{34}FN_3O_7$, molecular weight 531.58.

LFX-EHE

The synthetic yield of LFX-EHE was 85.5% (2,447 mg). The melting point was 167–173°C (dec.). The chemical shifts relative to Me_4Si (δ 0.00) were as follows: ^{1}H-NMR ($CDCl_3$) δ: 1.32 (3H, t, *J* = 7.3 Hz), 1.48 (3H, dd, *J* = 3.4, 6.6 Hz), 1.64 (3H, t, *J* = 5.5 Hz), 2.36 (3H, s), 2.55 (4H, m), 3.34 (4H, m), 4.20-4.27 (2H, m), 4.32-4.40 (2H, m), 4.52 (1H, t, *J* = 8.9 Hz), 6.94-6.99 (1H, m), 7.35 (1H, dd, *J* = 12.8, 22.0 Hz), 8.19 (1H, d, *J* = 11.4 Hz). ^{13}C-NMR ($CDCl_3$) δ: 14.1, 18.4, 19.7, 46.3, 50.4, 50.5, 54.7, 55.7, 64.4, 68.0, 91.5, 91.6, 105.0, 105.1, 105.2, 105.4, 107.8, 108.0, 123.0, 123.0, 123.4, 123.4, 131.7, 131.7, 131.9, 131.9, 139.6, 139.6, 145.4, 145.8, 153.0, 153.1, 154.4, 154.6, 156.9, 157.0, 162.4, 163.2, 172.4, 172.6. and mass spectra *m/z*, 477 (M^+). Calculated for $C_{23}H_{28}FN_3O_7$, molecular weight 477.4894.

LFX-MDX

The synthetic yield of LFX-MDX was 75.4% (2,140 mg). The melting point was 185–187°C (dec.). The chemical shifts relative to Me_4Si (δ 0.00) were as follows: ^{1}H-NMR ($CDCl_3$) δ: 1.56 (3H, d, *J* = 6.4 Hz), 2.24 (3H, s), 2.35 (3H, s), 2.54 (4H, m), 3.30-3.38 (4H, m), 4.35-4.41 (3H, m), 5.05 (2H, s), 7.59 (1H, d, *J* = 12.3 Hz), 8.26 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 9.5, 18.2, 46.4, 50.5, 50.6, 53.7, 55.0, 55.7, 68.1, 105.7, 105.9, 108.3, 123.2, 123.2, 123.6, 131.9, 132.0, 133.7, 139.5, 139.5, 140.2, 145.4, 152.3, 154.6, 157.0, 164.2, 172.7. and mass spectra *m/z*, 473 (M^+). Calculated for $C_{23}H_{24}FN_3O_7$, molecular weight 473.1598.

LFX-PVM

The synthetic yield of LFX-PVM was 89.0% (2,538 mg). The melting point was 214–216°C (dec.). The chemical shifts relative to Me_4Si (δ 0.00) were as follows: ^{1}H-NMR ($CDCl_3$) δ: 1.22 (9H, s), 1.55 (3H, d, *J* = 6.9 Hz), 2.36 (3H, s), 2.55 (4H, m), 3.33-3.39 (4H, m), 4.33-4.40 (3H, m), 5.96 (2H, s), 7.65 (1H, d, *J* = 12.8 Hz), 8.30 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 18.2, 26.9, 38.8, 46.4, 50.6, 54.9, 55.7, 68.1, 79.8, 105.7, 105.9, 108.1, 123.2, 123.3, 123.5, 131.8, 132.0, 139.5, 145.5, 154.6, 157.0, 163.2, 172.7, 177.5. and mass spectra *m/z*, 475 (M^+). Calculated for $C_{24}H_{30}FN_3O_6$, molecular weight 475.5174.

Partition coefficients

Calculated log P values and the apparent partition coefficients (PCs) of LFX and LFX ester prodrugs determined at pH 6.5 using chloroform as an organic phase are listed in Table 1. The PCs of LFX and the LFX ester prodrugs at pH 6.5 increased in the following order: LFX < LFX-CLX < LFX-MDX < LFX-EHE < LFX-PVM, in which the PCs of the ester prodrugs were approximately 3.0, 4.3, 13.4, and 22.6 times higher, respectively, than that of LFX (Table 1).

The chelate formation with aluminum ion (Al^{3+}) in vitro

When the LFX (20 μg/mL) solution was mixed with $AlCl_3$ solution (100 μg/mL and 200 μg/mL), a cloudy precipitate was observed immediately after mixing, in which the

Table 1. Partition coefficients of LFX and the LFX ester prodrugs at pH 6.5.

Compound	Calculated log P[a]	PC[b]
LFX	1.35	5.6
LFX-CLX	3.98	16.9
LFX-EHE	2.77	75.2
LFX-MDX	0.88	24.1
LFX-PVM	3.53	125.8

[a]Calculated log P was estimated using ChemDraw, a chemical drawing tool.
[b]PC: Partition coefficients were determined in a chloroform / pH 6.5, 0.1 M Tris-HCl buffer partition system at 25°C.

precipitation percentage of LFX within 30 min was 68.0% and 76.1% of the initial LFX concentration, respectively. In contrast, the ester prodrugs examined showed no cloudy precipitation after mixing them with the $AlCl_3$ solution, and LFX was recovered at a concentration of 100% of the initial concentration from the mixture solution (Table 2). These results indicated that the examined ester prodrugs of LFX are stable chemically and can avoid the chelate formation with Al^{3+} ion.

Chemical and enzymatic stability of the prodrugs

To evaluate the chemical stability of ester prodrugs of LFX, the effect of pH was examined using pH 6.5 and pH 7.4 PBS. As shown in Table 3, examined prodrugs were stable chemically. In a preliminary study of enzymatic stability of prodrugs in biological specimens, prodrugs were hydrolyzed by almost 100% within 15 min and generated LFX in the intestinal mucosal homogenates, liver homogenates, and plasma, in which prodrugs were stable in the pancreatin solution (1.0 mg/mL). These results indicated that the synthesized ester prodrugs of LFX are rapidly hydrolyzed enzymatically. To compare the effects of different ester moieties on enzymatic stability, the stability of the prodrugs was determined periodically for 15 min using diluted plasma (10%) and intestinal mucosa homogenates (2%).

The enzymatic stability of different ester prodrugs was evaluated by determining the hydrolyzed amounts of LFX during 1-min incubation. The increasing order of enzymatic stability of ester prodrugs was in the following order: LFX-CLX < LFX-EHE < LFX-MDX, LFX-PVM in the 2% intestinal homogenates, and LFX-EHE, LFX-PVM < LFX-CLX, LFX-MDX in the 10% plasma (Table 3). These results indicated that some differences exist in the enzymatic stability among different prodrugs, and between intestinal homogenates and plasma. However, these results also suggested that all four prodrugs examined are hydrolyzed rapidly in the intestinal membrane without showing any difference in their enzymatic stability in *in vivo* conditions (intact tissues), different from the cases in *in vitro* condition.

Determination of MIC values

The MIC values of the LFX ester prodrugs against *S. aureus, E. coli,* and *P. aeruginosa* were more than 10 times higher than that of LFX (Table 4). These data indicated that the LFX ester prodrugs themselves are pharmacologically inactive or have low activity levels as antibiotics, which agrees well with the prodrug concept.[34]

Effect of $Al(OH)_3$ on the oral bioavailability of LFX after administration of LFX and LFX ester prodrugs in rabbits

The concentration-time profiles of LFX in plasma following oral administration of LFX, LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM (20 mg/kg as LFX) with or without $Al(OH)_3$ (100 mg/kg) in rabbits are shown in Figure 2(A–E), respectively. Intact LFX ester prodrugs were not detected in plasma even at 0.25 h after oral administration of prodrugs. Pharmacokinetic parameters such as Cmax, Tmax, and $AUC_{0\text{-}6h}$ of LFX in plasma are summarized in Table 5. The co-administration of $Al(OH)_3$ with LFX significantly reduced the Cmax and $AUC_{0\text{-}6h}$ of LFX by approximately 47% and 40% of LFX alone, respectively. The administration of prodrugs, except for LFX-CLX,

Table 2. Precipitation percentages of LFX and the LFX ester prodrugs in the presence of 5- and 10-times higher concentrations of aluminum chloride.

Compound	$Al(Cl)_3$ concentration	
	50 µg/mL	100 µg/mL
LFX	68.0%	76.1%
LFX-CLX	0	0
LFX-EHE	0	0
LFX-MDX	0	0
LFX-PVM	0	0

Each sample was prepared with pH 6.5 Tris-HCl buffer. LFX and the LFX ester prodrugs solution (20 µg/mL) were mixed with $Al(Cl)_3$ solution (100 or 200 µg/mL) at a volume ratio of 1 : 1, respectively. The mixture solution was left to stand for 30 min at 24°C.

Table 3. Stability of LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM in buffer solution (pH 6.5 0.1-M phosphate buffer; pH 7.4 0.1-M phosphate buffer), and enzymatic solution (2% intestinal mucosal homogenates; 10% plasma) at 37 ˚C.

Compound	Extent of hydrolysis (%)		
	1 min	5 min	15 min
	0.1 M PBS (pH 6.5)		
LFX-CLX	0	0	0.5
LFX-EHE	0	0	0.1
LFX-MDX	0	0	0.6
LFX-PVM	0	0	0.1
	0.1 M PBS (pH 7.4)		
LFX-CLX	0	0	0
LFX-EHE	0	0	0
LFX-MDX	0	0	0.1
LFX-PVM	0	0	0
	Mucosal homogenates		
LFX-CLX	16.9±17.0	56.2±37.1	79.6±26.6
LFX-EHE	26.2±24.4	62.7±38.8	83.6±28.3
LFX-MDX	64.9±12.5	77.4±1.50	91.8±7.60
LFX-PVM	68.0±39.5	98.1±1.90	100±0.00
	Plasma		
LFX-CLX	10.1±4.1 0	39.8±1.80	72.1±4.30
LFX-EHE	4.8±0.8 0	22.6±2.60	47.2±17.9
LFX-MDX	37.6±14.7	53.3±13.0	75.2±5.40
LFX-PVM	7.9±1.40	41.4±8.50	81.6±5.90

Each value represents the mean±S.D. (n=3).
The initial concentration of LFX and its prodrugs were 0.1 mM in each incubation medium.
Intestinal mucosal homogenates (2%) and plasma (10%) were prepared using pH 7.4, 25 mM Tris-HCl buffer.

showed a significantly higher $AUC_{0\text{-}6h}$ of LFX than the administration of LFX alone, and co-administration of $Al(OH)_3$ with prodrugs had no significant effect on $AUC_{0\text{-}6h}$ of LFX in plasma. In the case of LFX-CLX, $AUC_{0\text{-}6h}$ of the plasma LFX was comparable with that of LFX alone. However, the co-administration of $Al(OH)_3$ with LFX-CLX did not reduce $AUC_{0\text{-}6h}$ of plasma LFX, different from the case of LFX. Among all prodrugs, LFX-EHE showed a significantly higher Cmax and $AUC_{0\text{-}6h}$ and a shorter Tmax of plasma LFX even after co-administration with $Al(OH)_3$ compared with the LFX alone (Table 5).

Discussion

Prodrugs are designed to overcome the limitations and enhance their pharmacokinetics and/or pharmacological profiles, such as low water solubility, disagreeable taste, poor lipophilicity, low absorption or distribution, and bacterial resistance of parent drugs.[35-38] In this study, four ester prodrugs of LFX, LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM, were synthesized to avoid chelate formation by masking the binding site of metal cations on quinolone-3-carboxylic acid, because staggered dosing is required when both LFX and antacids or metal cation-containing drugs are ingested in clinicals.[1,3,13,14] Ester moieties such as

Table 4. Comparison of the antibacterial activity or minimum inhibitory concentrations (μg/mL) of LFX and the LFX prodrugs.

Compound	MICs (μg/mL)		
	Staphylococcus aureus **(ATCC29213)**	***Escherichia coli*** **(ATCC25922)**	***Pseudomonas aeruginosa*** **(ATCC27853)**
LFX	0.34±0.09	0.06±0.04	1.860±1.32
LFX-CLX	40.10±28.66	13.86±12.10	58.37±7.25
LFX-EHE	40.10±28.66	5.69±8.58	44.33±31.46
LFX-MDX	30.47±29.68	7.45±7.63	35.43±25.25
LFX-PVM	30.47±29.68	9.53±7.98	39.60±20.08

MIC, minimal inhibitory concentration. MIC was determined via the agar plate dilution method using Mueller-Hinton broth.

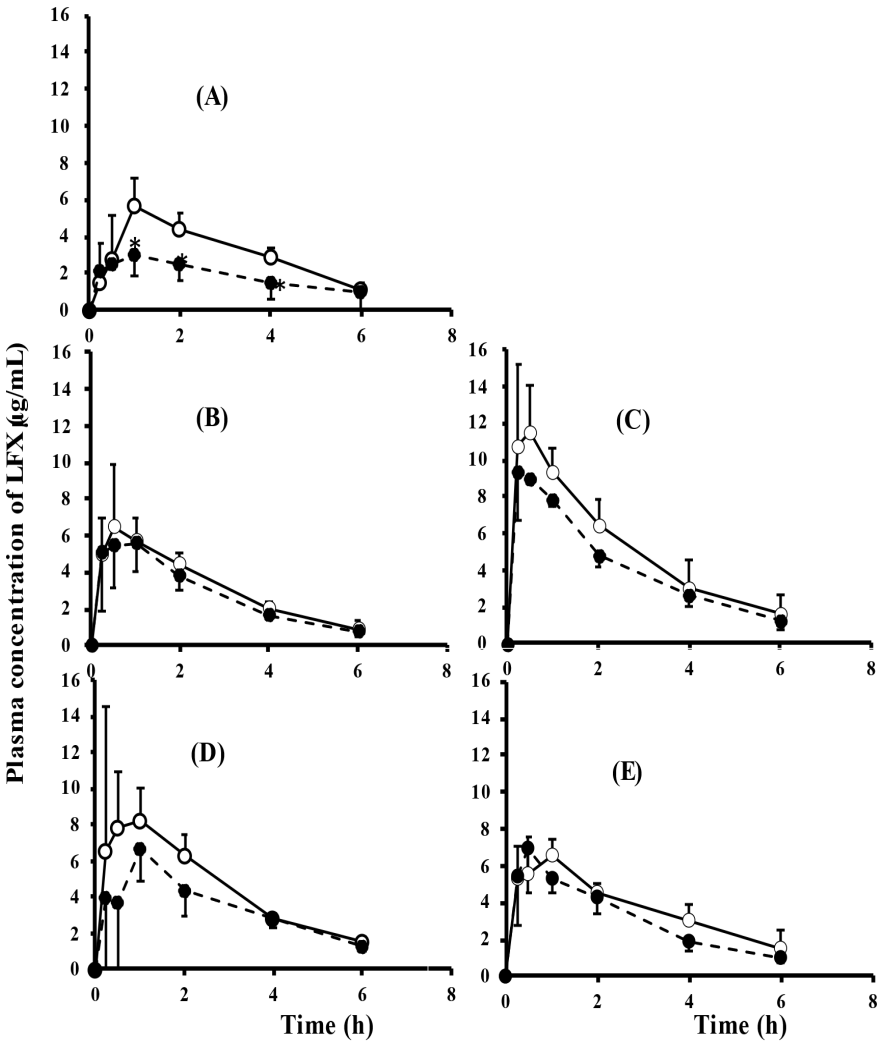

Figure 2. The concentration-time profiles of LFX in plasma following oral administration of LFX or its ester prodrug without (open circle) or with $Al(OH)_3$ (solid circle) in rabbits. Each value represents the mean ± SD. (A) LFX (trial number, n = 4); (B) LFX-CLX (n = 3); (C) LFX-EHE (n = 3); (D) LFX-MDX (n = 3); and (E) LVX-PVM (n = 3). The doses of LFX and the ester prodrugs were 20 mg/kg as LFX, and the dose of $Al(OH)_3$ was 100 mg/kg. *p < 0.05.

Table 5. Pharmacokinetic parameters of LFX following oral administration of LFX and the LFX ester prodrugs with or without $Al(OH)_3$ in rabbits.

Compound	Cmax (μg/mL)	Tmax (h)	$AUC_{0\text{-}6h}$ (μg × h/mL)
LFX	6.0±1.1	1.3±0.5	19.1±0.5
+ $Al(OH)_3$	3.2±1.1*	0.9±0.8	11.2±3.5**
LFX-CLX	7.0±2.9	0.6±0.4	19.5±2.7
+ $Al(OH)_3$	6.1±2.0	0.6±0.4	17.9±2.7
LFX-EHE	12.3±2.6**	0.4±0.1*	31.3±7.8*
+ $Al(OH)_3$	10.1±1.2	0.3±0.1*	25.5±2.4**
LFX-MDX	10.0±5.0	0.8±0.4	27.5±6.5*
+ $Al(OH)_3$	7.9±3.2	1.1±0.9	21.1±1.8
LFX-PVM	6.8±1.2	0.8±0.3	22.7±0.8*
+ $Al(OH)_3$	7.0±2.4	0.4±0.1	19.1±4.1

Each value represents the mean ± SD. The doses of LFX, LFX-CLX, LFX-EHE, LFX-MDX, LFX-PVM, and $Al(OH)_3$ were 20 mg/kg, 29.4 mg/kg, 26.4 mg/kg, 26.2 mg/kg, 26.4 mg/kg, and 100 mg/kg, respectively. *p < 0.05 and **p < 0.01 vs. LFX alone. In the case of the prodrugs, there was no significant difference between those with and without $Al(OH)_3$.

CLX, EHE, MDX, and PVM have been used in clinically available prodrugs of antibiotics, as described in the introduction.[6,24-29] LFX is a zwitterionic compound with a carboxylate (pK_a = 5.7) and an amine (pK_a = 7.9)[39] that has been reported to be freely soluble in glacial acetic acid and chloroform but sparingly soluble in water. As for the membrane permeability of LFX, its oral bioavailability has been reported to be almost 100% in humans.[3,6] Thus, it is classified as a BCS Class 1 drug with high solubility and high permeability.[7] However, FQs, including LFX, form an insoluble chelate complex, and their oral bioavailability is greatly reduced when they are administered with drugs that contain metal cations.[2,8,40]

This study compared four LFX ester prodrugs (LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM) in terms of their PCs, *in vitro* chelate formation with Al^{3+} ion, enzymatic stability in various biological specimens, MIC values for some bacteria, and the effect of $Al(OH)_3$ on the oral bioavailability of LFX from LFX ester prodrugs in rabbits. The prodrugs had higher PCs than LFX at pH 6.5 (Figure 1 and Table 1), and all of them avoided chelate formation with Al^{3+} ion completely *in vitro* (Table 2), possibly due to the masking of the metal cation-binding site on quinolone 3-carboxylic acid.[1] All prodrugs examined were stable chemically, and rapidly hydrolyzed and generated LFX in the intestinal mucosal homogenates, liver homogenates, and plasma (Table 3). Some difference was observed in the enzymatic stability among four different ester moieties when the stability was evaluated at the relatively small number of enzymes and between the intestinal mucosal homogenates and plasma. However, all the four ester prodrugs examined would be hydrolyzed rapidly mostly in the small intestinal membrane during the absorption process. The MIC values of the ester prodrugs against some bacteria were much higher than that of LFX, and there was no difference between the MIC values of the four examined ester prodrugs (Table 4). Based on these *in vitro* studies, it was speculated that LFX ester prodrugs can avoid hydrolysis and chelate formation with metal cations in the gastrointestinal lumen at least partly and that the absorbed ester prodrugs are rapidly converted into the parent drug, LFX, in the intestinal membrane.

Next, we examined the oral bioavailability of LFX from the ester prodrugs after their administration with and without $Al(OH)_3$ in rabbits (Figure 2 and Table 5). When prodrugs alone were administered orally, the $AUC_{0\text{-}6h}$ values of plasma LFX after the oral administration of prodrugs, except of LFX-CLX, were significantly higher than that of LFX. The higher plasma AUC of LFX after prodrug administration would be due to the higher lipophilicity in the luminal fluid of the prodrugs (Table 1). In humans, however, the oral bioavailability of LFX in the absence of metal cations is reportedly almost 100%.[3,6] However, the bioavailability of LFX in rabbits might be lower compared to that in humans because the prodrugs increased the Cmax and the $AUC_{0\text{-}6h}$ values of LFX. The lower oral bioavailability of LFX in rabbits than in humans may be at least partly due to the coprophagy of rabbits. Rabbits perform coprophagy to obtain many essential nutrients given the structure of their digestive system,[41] and the stomach contents of rabbits are not emptied completely even in untreated normal conditions.[42] Orally administered LFX may bind to metal cations in gastric contents, and the binding, or chelate formation, of LFX in the stomach may reduce the intestinal absorption of LFX at least partly in rabbits. In contrast, different from the case in rabbits, there was no difference in $AUC_{0\text{–}4\,h}$ value of plasma between LFX and LFX-EHE in rats.[19] Further study will be necessary regarding the interspecies difference in oral bioavailability of FQs, including LFX, by focusing on the effect of coprophagy. Co-administration of $Al(OH)_3$ with LFX significantly reduced the Cmax and the $AUC_{0\text{-}6h}$ values of the plasma LFX. In contrast, co-administration of $Al(OH)_3$ with the LFX ester prodrugs had no significant effect on the intestinal absorption of LFX (Table 5). There are marked interspecies differences between various metabolic enzymes, including carboxylesterases (CESs). CESs play a critical role in catalyzing hydrolysis of esters, amides, carbamates, and thioesters, as well as in bioconverting prodrugs and soft drugs, and CES1 was found to be most abundant in the liver while CES2 was primarily expressed in the intestine in the species, with notable species differences.[43,44] Similarly, it was reported that, in preclinical animal models, CES2 isozymes were also major intestinal enzymes, but they have different substrate specificities to human CES2. Additionally, although Caco-2 cells are frequently used in absorption studies, they mainly express the human CES1 isozyme, hCE1, with a quite different substrate specificity from hCE2. Caco-2 cells are reportedly unsuitable for predicting human intestinal absorption of prodrugs.[45] All these show that it is important to consider the contribution of interspecies' substrate specificity and expression sites, and the extent of activity of CESs, to the pharmacokinetics and pharmacology of ester prodrugs in preclinical studies.

Conclusion

The effectiveness of four ester prodrugs of LVFX in avoiding the chelate formation in the presence of aluminum ion were investigated. Without chelation, the oral bioavailability of LFX has been reported to be virtually 100% in humans. Thus, the target prodrugs should have high oral bioavailability and avoid chelate formation with metal cations *in vivo*. The four examined ester prodrugs of LFX—LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM—exhibited higher lipophilicity than LFX, less antibacterial activity than LFX, chemically stable but enzymatically unstable, and avoidance of chelate formation *in vitro*. In rabbits *in vivo*, co-administration of $Al(OH)_3$ with LFX significantly reduced plasma LFX levels. However, co-administration of $Al(OH)_3$ exerted no significant effect on the intestinal absorption of LFX when administered as prodrugs. In conclusion, the ester prodrug approach of LFX could be a feasible strategy for avoiding chelate

formation with Al^{3+} *in vivo*.

Ethical Issues

The animal study was performed in compliance with the Care and Use of Laboratory Animals of the Committee for Animal Experiments of Fukuyama University. This study was approved on March 17, 2021 by the Ethics Committee of the Faculty of Pharmacy & Pharmaceutical Science of Fukuyama University. The license number of this animal study is 2021-A-12.

Author Contributions

YM contributed to the research policy, experiment design, data analysis, and writing and editing of the manuscript. All the authors except for TMu participated in the experiments. TMu contributed to the experiment design, data analysis, and writing of the manuscript. All the authors have read and agreed to publish the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Data

Supplementary data related to this article (Figures S1-S4) available at https://doi.org/10.34172/PS.2022.15.

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