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Effect of aluminium ion on bioavailability of levofloxacin following oral administration of cilexetil ester of levofloxacin as prodrug in rats

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A prodrug of levofloxacin (LVFX), cilexetil ester of LVFX (LVFX-CLX), was synthesized to examine whether the prodrug can avoid chelate formation with metal cations in the gastrointestinal tract. LVFX-CLX exhibited a 10-times higher partition coefficient than LVFX. In vitro, LVFX was precipitated by 76.1% in the presence of a 10-times higher concentration of aluminium chloride (Al^{3+}), but LVFX-CLX was not. LVFX-CLX was rapidly hydrolyzed enzymatically by rat plasma, intestinal mucosal and liver homogenates at 37 °C, but not by pancreatic enzymes and luminal fluid. The minimum inhibitory concentration values of LVFX-CLX against *S. aureus, E. coli* and *P. aeruginosa* were far higher than that of LVFX. In rats, area under the plasma concentration-time curve from zero to 4 h ($AUC_{0\text{-}4h}$) of LVFX after oral administration of LVFX-CLX was 1.34-fold higher than that after LVFX, though it did not reach significance level. Co-administration of Al^{3+} with LVFX and LVFX-CLX in rats decreased $AUC_{0\text{-}4h}$ of plasma LVFX by 75% and 60%, respectively, however, the $AUC_{0\text{-}4h}$ of plasma LVFX after co-administration of LVFX-CLX and Al^{3+} was 2.2-times higher than that after co-administration of LVFX and Al^{3+}. These results suggested that the use of LVFX-CLX may reduce the modulation of intestinal microflora caused by LVFX and the suppressive effect of Al^{3+} on intestinal absorption of LVFX.

1. Introduction

Levofloxacin (LVFX), the levo-isomer of ofloxacin, displays wide antibacterial activity against most strains of bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal and abdominal infections, including gram-positive, gram-negative and atypical bacterial pathogens. Compared to earlier antibiotics of the new quinolone antimicrobial family such as ciprofloxacin, LVFX strongly inhibits the growth of gram-positive bacteria than gram-negative bacteria, especially of *Pseudomonas aeruginosa*. It is also known that co-administration of aluminium hydroxide reduces the oral bioavailability of LVFX by 50% or more due to the formation of insoluble chelate complexes by interacting with carboxyl and keto groups of the quinolone (Tanaka et al. 1993; Trivedi 2007; Uivarosi 2013; Pitman et al. 2019). To avoid the interaction between LVFX and metal cations in clinicals, it is recommended that LVFX should be taken 2 h after metal cation containing drugs such as antacids (Parpia et al. 1989; Miyazaki et al. 2002).

Previously, to avoid the chelate formation of ofloxacin (OFLX) with metal cations, we examined the usefulness of prodrug formation by synthesizing the pivaloyloxymethyl ester (PVM) of OFLX (OFLX-PVM) (Maeda et al. 1993). In that study, the area under the plasma concentration-time curve from zero to 24 h ($AUC_{0\text{-}24h}$) of OFLX was decreased by 47.6% when OFLX was co-administered with aluminium hydroxide in rabbits. However, the $AUC_{0\text{-}24h}$ of OFLX in plasma after oral administration of OFLX-PVM was not suppressed by the co-administration of aluminium hydroxide, and plasma levels were comparable to that after oral administration of OFLX alone. That results implied that the use of certain prodrugs could be useful in preventing the chelate formation with metal-containing drugs and suppression of oral bioavailability of quinolone antibiotics. However, it is also known that the PVM moiety yields formalin and pivalic acid by hydrolysis, and the pivalic acid depletes carnitine and it may cause hypoglycemia such as consciousness decrease and convulsions with low carnitine blood disease (Ito 1995; Nakajima 2010; Nakazaki 2018). Based on these reports, PVM was considered to be undesirable as masking moiety of carboxyl groups.

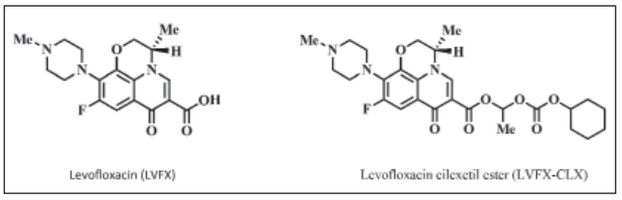

Fig.1: Chemical structures of levofloxacin and Levofloxacin cilexetil ester.

In the present study, we again examined the usefulness of prodrug formation in preventing chelate formation of LVFX by synthesizing the cilexetil (CLX) ester of LVFX (LVFX-CLX) as a model prodrug of LVFX (Fig. 1). CLX has been used as masking agent for the carboxyl group of candesartan, an angiotensin II receptor antagonist, and candesartan CLX is now widely used in pharmacotherapy to treat essential hypertension (Sever 1997; Hoy 2010; Yan 2016). CLX was also used in a prodrug of sanfetrinem, a carbapenem antibiotic (Iavorone 1997). The CLX moiety is not reported to produce hazardous decomposition products (Yamamoto 2005). Using LVFX and LVFX-CLX, we examined the lipophilicity (partition coefficient), interaction, or chelate formation, with aluminium ion *in vitro*, enzymatic stability, minimum inhibitory concentrations (MIC) for some bacteria, and effect of aluminium chloride on oral bioavailability of LVFX from LVFX-CLX prodrug in rats.

2. Investigations and results

2.1. Partition coefficients

Partition coefficients (PC) of LVFX and LVFX-CLX determined in a chloroform/pH 6.5 phosphate buffer partition system were 5.27 (log 0.72) and 56.1 (log 1.75), respectively. LVFX-CLX exhibited approximately 10-times higher PC than LVFX.

2.2. Chelate formation with aluminium ion

LVFX and LVFX-CLX solutions were mixed with aluminium chloride solution dissolved in pH 6.5 Tris-HCl buffer, respectively. A cloudy precipitate was observed immediately after the mixing of LVFT and aluminium chloride solutions. When 5- and 10-times higher concentrations of aluminium chloride than LVFT were added, the precipitation percentages of LVFX were 68.0% and 76.1% of initial LVFX concentrations, respectively. In contrast, no precipitation of LVFX-CLX was observed even after addition of a 10-times higher concentration of aluminium chloride. These results indicate that aluminium ions do not bind to LVFX-CLX, different from the case of LVFX, *in vitro*.

2.3. Enzymatic stability of LVFX-CLX

The enzymatic stability of LVFX-CLX was determined in rat plasma (20%), small intestinal mucosal homogenates (5%), and liver homogenates (5%) at 37.0 °C. LVFX-CLX was hydrolyzed rapidly in these biological fluids and converted to LVFX at a rate of 100% within 0.25 h after the start of incubation. In contrast, LVFX-CLX was not hydrolyzed in pancreatic enzyme solution (1 mg/mL) and luminal fluid during 1-h incubation. These results suggested that orally administered LVFX-CLX is stable in the gastro-intestinal lumen, but rapidly hydrolyzed and converted to LVFX in the intestinal mucosa, circulating blood and also in the liver.

2.4. Determination of MIC values

MIC values of LVFX and LVFX-CLX against *S. aureus, E. coli,* and *P. aeruginosa* were determined. LVFX-CLX exhibited far higher MIC values than LVFX in each bacterium (Table 1). These results suggested that LVFX-CLX given orally may cause less damage on intestinal microflora in the gastrointestinal tract compared to LVFX.

Table 1: Comparison of antibacterial activity of LVFX-CLX (prodrug) and LVFX

Microorganism	MIC (μg/mL)	
	LVFX-CLX	LVFX
Staphylococcus aureus (ATCC29213)	50.0	0.39
Escherichia coli (ATCC25922)	25.0	0.05
Pseudomonas aeruginosa (ATCC27853)	50.0<	3.13

2.5. Effect of aluminium chloride on oral bioavailability

The effect of aluminium chloride on oral bioavailability of LVFX and LVFX-CLX was examined in rats. The concentration-time profiles of LVFX in plasma following oral administration of LVFX and LVFX-CLX with and without aluminium chloride are shown in Figs. 2 and 3, respectively. Intact LVFX-CLX was not detected in plasma even after 0.25 h when LVFX-CLX was administered orally. Co-administration of aluminium chloride significantly decreased plasma concentrations of LVFX after oral administration of LVFX and LVFX-CLX, respectively (Figs. 2 and 3). Some pharmacokinetic parameters are summarized in Table 2. Oral administration of LVFX-CLX exhibited comparable C_{max} and 1.34-fold higher $AUC_{0\text{-}4h}$ values of plasma LVFX as compared with LVFX administration, though a significant difference was not detected in $AUC_{0\text{-}4h}$ values between LVFX-CLX and LVFX groups. Co-administration of aluminium chloride with LVFX and LVFX-CLX, respectively, reduced C_{max} and $AUC_{0\text{-}4h}$ values of plasma LVFX by 86% and 76%, and by 57% and 60%, respectively. The averaged $AUC_{0\text{-}4h}$ value of plasma LVFX of LVFX-CLX and Al^{3+} co-administration group was 2.2-times higher than that of LVFX and Al^{3+} co-administration group, though a significant difference was not detected between the two groups.

Table 2: Pharmacokinetic parameters of LVFX following oral administration of LVFX or LVFX-CLX with or without aluminium chloride in rats

Drug	C*max*	t*max*	$AUC_{0\text{-}4h}$
	(μg/mL)	(h)	(μg · h/mL)
LVFX	12.2±3.0	0.5±0.4	19.3±7.9
LVFX+Al^{3+}	1.7±1.1*	1.6±1.0	4.7±3.7*
LVFX-CLX	12.0±6.0	1.0±0.0	25.9±5.6
LVFX-CLX+Al^{3+}	5.1±0.9*	1.5±1.3	10.3±1.0*

Each value represents the mean±S.D. (n= 4 for LVFX and LVFX+Al^{3+}, n=3 for LVFX-CLX and LVFX-CLX+Al^{3+}). Doses of LVFX, LVFX-CLX and $AlCl_3$ (Al^{3+}) were 30 mg/kg, 44 mg/kg and 30 mg/kg, respectively. * p<0.05, significantly different from corresponding LVFX or LVFX-CLX alone, respectively.

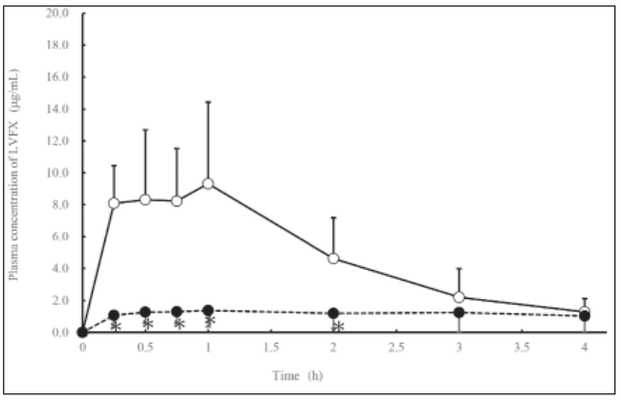

Fig. 2: The concentration-time profiles of LVFX in plasma following oral administration of LVFX with (●) or without $AlCl_3$ (○) in rats. Doses of LVFX and $AlCl_3$ were 30 mg/kg and 30 mg/kg, respectively. Error bar represents the standard deviation (n=4). *P<0.05, Significantly different from without $AlCl_3$.

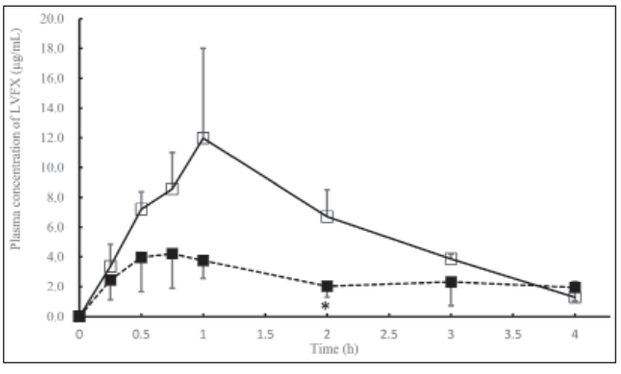

Fig. 3: The concentration-time profiles of LVFX in plasma following oral administration of LVFX-CLX with (■) or without $AlCl_3$ (□) in rats. Doses of LVFX-CLX and $AlCl_3$ were 44 mg/kg and 30 mg/kg, respectively. Error bar represents the standard deviation (n=3). *P<0.05, Significantly different from without $AlCl_3$

3. Discussion

Most quinolone antibiotics form poorly soluble chelate complexes with metal cations, as well as tetracyclines, and their oral bioavailabilities are significantly suppressed when a chelate was formed. For example, the co-administration of antacids such as aluminium hydroxide with LVFX has been reported to reduce the blood concentrations of LVFX by about 50% due to the formation of an insoluble chelate (Hoo et al. 1974; Tanaka et al. 1993; Trivedi 2007; Uivarosi 2013; Pitman et al. 2019), and the potential of *in vitro* chelate formation of metal cations was in the following order: $Fe^{3+} > Al^{3+} > Cu^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+}$ (Hoo et al. 1974).

Currently, it is recommended that quinolone antibiotics and antacids should be taken at least 2 h apart. However, if prodrug can avoid chelate formation in vivo, the use of prodrugs would have much clinical significance. Previously, a prodrug of OFLX, OFLX-PVM, was examined whether it avoids the chelate formation with aluminium hydroxide in rabbits (Maeda et al. 1993), because PVM ester masks the metal cation binding site on the carboxyl group. In the present study, we further evaluated the usefulness of the CLX ester prodrug of LVFX, or LVFX-CLX, in preventing chelate formation, because the CLX moiety is considered to be safe, different from the PVM moiety (Ito 1995; Yamamoto 2005; Nakajima 2010; Nakazaki 2018). Actually, the CLX moiety is used as clinically available ester prodrug of candesartan (Sever 1997; Hoy 2010; Yan 2016, Iavorone 1997). The efficacy of a prodrug in preventing chelate formation has also been reported using ethoxycarbonyl 1-ethyl hemiacetal ester of levofloxacin (LVFX-EHE) (Otori et al., 2016). In that study, the AUC_{0-4h} value of LVFX in plasma after oral co-administration of LVFX-EHE and aluminium hydroxide was similar to that after administration of LVFX alone in rats.

In the present study, we the examined lipophilicity, chelate formation with aluminium ion, enzymatic stability of LVFX-CLX (Fig. 1) in various biological samples, MIC values for some bacteria, and the effect of aluminium chloride on the oral bioavailability of LVFX from LVFX-CLX prodrug in rats (Tables 1 and 2, Figs. 2, 3). In these studies, LVFX-CLX exhibited approximately 10-times higher PC than LVFX. This result indicated the increase in lipophilicity of LVFX-CLX compared to LVFX. The solubility of LVFX-CLX *in vitro* was not affected at all by the presence of aluminium ions, different from the case of LVFX, indicating that chelate formation with aluminium ion did not occur on LVFX-CLX at least *in vitro*. In the enzymatic stability study, LVFX-CLX was rapidly hydrolyzed enzymatically, but not by chemically. The MIC values of LVFX-CLX against intestinal bacteria were far higher compared those of to LVFX. The less antibacterial activity and stability in intestinal luminal fluid of LVFX-CLX may reduce the disruption of intestinal bacteria possibly caused by LVFX. It is reported that prodrugs are designed to overcome pharmaceutical and/or pharmacokinetically based problems associated with the parent drug molecule, and they are pharmacologically inactive chemical derivatives of a drug molecule that require a transformation within the body in order to release the active drug (Stella et al. 1985). The higher lipophilicity of LVFX-CLX compared to LVFX, the less antibacterial activity and rapid transformation of LVFX-CLX to LVFX in intestinal mucosa should indicate that LVFX-CLX fits appropriately to the concept of prodrugs proposed by Stella et al. (1985). When LVFX-CLX was administered orally in rats, the AUC_{0-4h} of plasma LVFX was higher by 1.34-fold compared with LVFX administration group, though it was not of significant difference (Figs. 2 and 3, Table 2). The slightly higher AUC of LVFX-CLX than LVFX may be due to the higher lipophilicity of LVFX-CLX than LVFX. The C_{max} and AUC_{0-4h} values of plasma LVFX after oral administration of LVFX were significantly decreased by 86% and 76%, respectively, by co-administration of aluminium chloride in rats (Table 2). This result was in agreement with that reported by Shiba et al. (1992). They reported that the C_{max} of LVFX following its oral administration at a dose of 100 mg in humans was decreased significantly by 35.2%, the urinary excretion rate over the next 24 h was decreased significantly by 71.6%, and the AUC_{0-24h} value was decreased significantly by 56.3% by the co-administration of 1 g of aluminium hydroxide. In the present study, C_{max} and AUC_{0-4h} values of plasma LVFX after oral administration of LVFX-CLX were also decreased by 57% and 60%, respectively, by co-administering aluminium chloride (Fig. 3). However, the averaged AUC_{0-4h} value of plasma LVFX of LVFX-CLX and Al^{3+} co-administration group was 2.2-times higher than that of LVFX and Al^{3+} co-administration group. In addition, there were no significant differences in AUC_{0-4h} values of plasma LVFX among LVFX alone, LVFX-CLX alone, and LVFX-CLX+Al^{3+} co-administration groups. These statistical analyses may suggest that the suppressive effect of aluminium chloride on

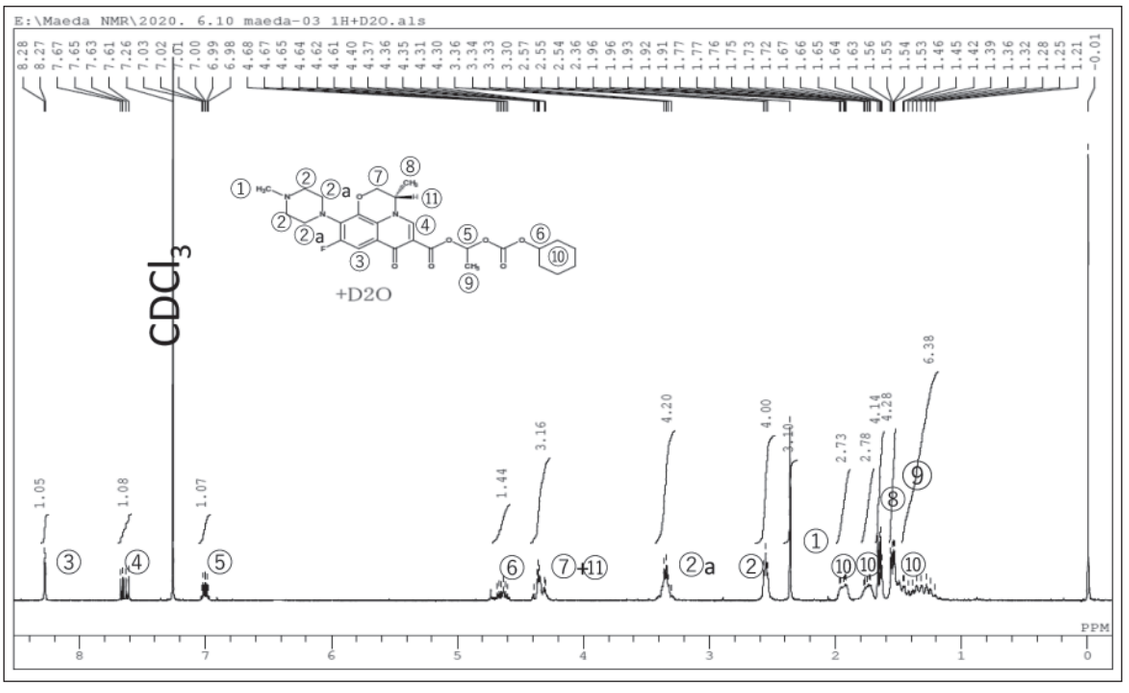

Fig.4: 1H-NHR Chart of LVFX-CLX.

intestinal absorption of LVFX from LVFX-CLX prodrug is lower compared with LVFX administration. In the *in vitro* study, chelate formation of LVFX-CLX was not observed at all. However, *in vivo*, the intestinal absorption of LVFX from LVFX-CLX prodrug was suppressed by the co-administration of aluminium chloride (Fig. 3). Regarding the membrane transport mechanism of LVFX-CLX, it will be generally considered that LVFX-CLX, a neutral and lipophilic compound, is absorbed by simple diffusion due to its high lipophilicity. In contrast, regarding LVFX, the contribution of some specific transport systems including influx and efflux transporters has been suggested. For example, Maeda et al. (2007) reported that plural transporters are functional for the transport of LVFX in Caco-2 cells, and OATP1A2 is likely to function as a high-affinity transporter. Takaai et al. (2007) reported that some transporters are responsible not only for the efflux but also for the influx of LVFX-CLX at the apical membrane of Caco-2 cells. In addition, Fukumori et al. (2009) reported that the specific uptake system for LVFX-CLX in LS180 cells is identical/similar to that in Caco-2 cells, but that OATPs and OCTNs contribute little to levofloxacin uptake in the human intestinal epithelial cells. Further study is necessary to clarify why the intestinal absorption of LVFX from LVFX-CLX prodrug was suppressed by co-administered aluminium chloride, though LVFX-CLX could avoid the chelate formation with aluminium ions *in vitro*. The study on the transport mechanisms of LVFX and LVFX-CLX in the small intestine will be important. In conclusion, obtained results suggested that the use of LVFX-CLX may reduce the modulation of intestinal microflora caused by LVFX and the suppressive effect of metal cations including Al^{3+} on intestinal absorption of LVFX.

4. Experimental

4.1. Materials

The following compounds were used: LVFX (Apollo Scientific, UK), ciprofloxacin (LKT Laboratories, Inc.), 1-chloro ethyl cyclohexyl carbonate (Toronto Research Chemicals Inc.), pancreatin (from porcine pancreas) and anhydrous aluminium chloride (Wako Pure Chemical Ind, Ltd, Japan). All other chemicals and solvents were analytical grade.

4.2. Synthesis of levofloxacin cilexetil ester

LVFX-CLX was synthesized by the modified method of Daehne et al. (1970), who reported a method to synthesize acyloxymethyl esters of ampicillin. Anhydrous potassium carbonate 553 mg (4 mmo1) was added to the levofloxacin 722 mg (2 mmo1) solution dissolved in *N,N*-dimethylformamide (DMF) (25 mL) with vigorous stirring in round bottom flask. Then, 1-chloro ethyl cyclohexyl carbonate 1.2 mL (8 mmol) was added and stirred for 2 h at 70 °C under argon atmosphere. Crushed ice and water (60 mL) were slowly added to the mixture after cooling. The reaction mixture was extracted three times with ethyl acetate (50 mL x 3). The extract was washed with distilled water and dehydrated with anhydrous sodium sulfate. Then, the organic solvent was gradually evaporated to dryness. Recrystallization of the precipitate was made from chloroform and cyclohexane. This method gave an 89.3% yield of LVFX-CLX. The chemical structures of LVFX-CLX is shown together with LVFX in Fig. 1. Melting point of LVFX-CLX determined on Yanagimoto micro melting point apparatus (MP-S3, Yanaco, Kyoto, Japan) was 124-126 °C(dec.). LVFX-CLX was dissolved in $CDCl_3$ and proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL AL-300 at 300 MHz (JEOL Ltd., Tokyo, Japan). ^{1}H NMR chart of LVFX-CLX is shown in Fig. 4. Chemical shifts relative to Me_4Si (δ 0.00) were as follows: ^{1}H-NMR δ: 1.29-1.39 (6H, m), 1.53-1.56 (3H, m), 1.63-1.67 (3H, m), 1.73-1.76 (2H, m), 1.91-1.96 (2H, m), 2.36 (3H, s), 2.55-2.56 (4H, m), 3.30-3.40 (4H, m), 4.30-4.41 (3H, m), 4.65 (1H, td, *J* = 9.1, 5.1 Hz), 7.00 (1H, dq, *J* = 10.8, 2.7 Hz), 7.64 (1H, dd, *J* = 12.5, 6.2 Hz), 8.27 (1H, d, *J* = 2.2 Hz). As ^{1}H NMR spectral data only displayed the specific signals of LVFX-CLX, the purity might be more than 99%. The purity of the compound was also examined by thin layer chromatography (Kieselge1 60 F254 plates, Merck, USA) using chloroform-methanol-acetic acid-distilled water (l5:5:2:1, v/v/v/v) as a developing solvent. Detection of LVFX and LVFX-CLX were made by short wave ultraviolet light (254 nm). The *Rf* values of LVFX and LVFX-CLX were 0.45 and 0.72, respectively. Taken together, the synthesized material was particularly pure, being completely uncontaminated by LVFX. Mass spectra were recorded on JEOL JMS-700 spectrometers by direct inlet system, and it was *m/z*: 531(M^+).

4.3. Partition coefficient

As organic phase, chloroform was used. Chloroform and 0.l M phosphate buffer (pH 6.5) were mutually saturated before starting the experiments. LVFX and LVFX-CLX were dissolved in phosphate buffer at a concentration of approximately l00 μg/mL, respectively. Four milliliters of drug solution were added to 4 mL of chloroform, and the mixture was vigorously shaken for 30 min at 25 °C. After centrifugation at 3,000 rpm for 10 min, the concentration of the compound in both phases was determined by HPLC. Partition coefficient (PC) was expressed as the concentration ratio between chloroform and pH 6.5 phosphate buffer, and its logarithm value (log PC).

4.4. Chelation with aluminium ion in vitro

LVFX and LVFX-CLX were, respectively, dissolved in pH 6.5 Tris-HCl buffer at a final concentration of 20 μg/mL. Aluminium chloride was also dissolved in pH 6.5 Tris-HCl buffer at final concentrations of 0, 20, 100 or 200 μg/mL. The drug solution and aluminium chloride solution were mixed at an equal volume, and the mixture solution was stand for 30 min at a room temperature (24 °C). The mixture was centrifuged at 3,000 rpm for 10 min, and the supernatant was filtered through a syringe filter with 0.22 μm of pore size (Millipore, Tokyo, Japan). The filtrates (each 1 mL) containing LVFX-CLX was mixed with 0.5 mL of 1 mol/L NaOH and stand for 1 h at 37 °C to hydrolyze LVFX-CLX to LVFX. The concentrations of LVFX in each sample were determined by HPLC.

4.5. Enzymatic stability

Enzymatic stability of LVFX-CLX was determined in rat plasma, rat small intestinal mucosal homogenates, rat liver homogenates, pancreatin, and rat luminal fluid containing intestinal contents at 37 °C for l h in the same manner as described previously (Maeda et al. 1989). Briefly, LVFX-CLX was dissolved in 0.025M Tris buffer (pH 7.4) at a concentration of 0.5 mM. Heparinized plasma was freshly obtained from male Sprague-Dawley rats. The rat small intestinal mucosa and liver homogenates were prepared in 3-fold volume of ice-cold, 0.0l M phosphate buffer (pH 7.4) containing l.l5 % KCl, and the supernatants were obtained after centrifugation at 3,000 rpm for 10 min, respectively. Pancreatin from porcine pancreas was dissolved in pH 7.0, 0.05 M 3-morpholinopropane-sulfonate buffer at a concentration of 1.0 mg/mL. Luminal fluid was prepared by washing whole small intestinal lumen with 10 ml of pH 6.5, 0.l M phosphate buffer. Plasma, supernatants of intestinal mucosa and liver homogenates, pancreatin solution, and luminal fluid were prewarmed at 37 °C, and these biological samples were mixed with LVFX-CLX solution prewarmed at 37 °C to start the metabolic stability study of LVFX-CLX. In this study, the final concentrations of LVFX-CLX in the reaction medium was adjusted at 0.1 mM. The final concentrations of plasma, supernatants of intestinal mucosa and liver homogenates, and pancreatin were 20%, 5% and 1.0 mg/mL, respectively. Concentrations of LVFX from LVFX-CLX hydrolysis in the reaction mixture were determined at 0.25 and 1.0 h after the start of incubation at 37 °C, in which the further metabolic reaction after sampling was stopped by adding methanol at a volume ratio of 1:2.

4.6. Determination of MIC values

Determination of MIC values of LVFX and LVFX-CLX were made by agar plate dilution method using Mueller-Hinton broth (Nippon Becton Dickinson Co., Ltd, Tokyo, Japan). Briefly, LVFX (10 mg) or LVFX-CLX (10 mg) was dissolved in 1 mL of 0.l mol/L hydrochloric acid, and 4 mL of 0.l mol/L phosphate buffer (pH7.4) was added to the solution. The final concentrations of LVFX and LVFX-CLX in medium were adjusted to 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, 0.1, 0.05, and 0.025 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 the sterilized isotonic sodium chloride solution. These bacterial solutions were inoculated in Mueller-Hinton broth containing LVFX or LVFX-CLX. Facultative anaerobic and aerobic bacteria were cultured under aerobic condition at 37 °C for 20 h.

4.7. Absorption study in rats

The animal study was performed in compliance with the Care and Use of Laboratory Animals of the Committee for Animal Experiments of Fukuyama University. Male Sprague-Dawley rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and on the day before animal experiments, rats weighing 250-300g were subjected to jugular cannula indwelling operation. Rats were anesthesized with a mixture of medetomidine (0.15 mg/kg), midazolam (2 mg/kg) and butorphanol (2.5 mg/kg) and laid on a water bed kept at 37.0 °C. After operation, rats were fasted overnight with free access to water. On the day of the experiments, rats were divided into the following four groups: LVFX (30 mg = 0.083 mmol/1 mL/kg) with or without aluminum chloride (30 mg = 0.226 mmol/1 mL/kg), and LVFX-CLX (44 mg = 0.083mmol/1 mL/kg) with or without aluminum chloride (30 mg = 0.226 mmol/1 mL/kg). Drugs were administered orally by gastric intubation. Blood (100 μL each) was collected via cannula inserted into jugular vein at 0.25, 0.5, 0.75, 1, 2, 3, and 4 h after administration. The number of rats used for LVFX, LVFX+$A1^{3+}$, LVFX-CLX, and LVFX-CLX+Al^{3+} groups were 4, 4, 3, and 3, respectively. This study was approved by the ethics committee of Faculty of Pharmacy & Pharmaceutical Science, Fukuyama University (2019-A-2). At the end of absorption study, rats were killed by injection of extra-amount of sodium pentobarbital followed by decapitation.

4.8. Pharmacokinetic analysis

As pharmacokinetic parameters, peak plasma concentration (C_{max}) of LVFX, time to reach C_{max} (t_{max}), and $AUC_{0\text{-}4h}$ of plasma LVFX were estimated. $AUC_{0\text{-}4h}$ values of plasma LVFX were calculated according to the trapezoidal rule (Ritchel 1976).

4.9. Analysis of LVFX

Concentrations of LVFX in various biological samples were determined by using HPLC according to the modified method of Gao el al. (2007). Briefly, each biological sample (50 μL) was mixed with 100 μL of internal standard solution containing

10.0 µg/mL ciprofloxacin in methanol. The mixture was vortex-mixed for 30 s and centrifuged at 15,000 × g for 5 min at 4 °C, and a 20 µL aliquot of the supernatant was injected into HPLC column. The HPLC system (Shimazu, Kyoto, Japan) used was consisted of a model LC-20AD pump, a fixed injection loop of 20 µL, and a model SPD-10Avp UV detector. Data acquisition was performed with the Sepu3000's processor (Hang Zhou, China). The column used was a 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 containing 0.3% triethylamine (pH 3.3 adjusted with phosphoric acid) at a volume ratio of 16 : 84. The flow rate of mobile phase was 1 mL/min. Detection was made at 295 nm.

4.10. Statistical analysis

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

Conflict of Interest. The authors declare no conflict of interest.

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