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Intestinal behavior of the ester prodrug tenofovir DF in humans

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ABSTRACT

Tenofovir-disoproxil-fumarate (TDF) is a double ester prodrug which enables intestinal uptake of tenofovir (TFV) after oral administration in humans. In this study, prodrug stability was monitored in situ in the human intestine and in vitro using biorelevant media. In fasted state human intestinal fluids, the prodrug was completely degraded within 90 min, resulting in the formation of the mono-ester intermediate and TFV; in fed state intestinal fluids, the degradation rate of TDF was slightly reduced and no TFV was formed. Intestinal fluid samples aspirated after administration of TDF confirmed extensive intraluminal degradation of TDF in fasted state conditions; a relatively fast absorption of TDF partly compensated for the degradation. Although food intake reduced intestinal degradation, the systemic exposure was not proportionally increased. The lower degradation in fed state conditions may be attributed to competing esterase substrates present in food, lower chemical degradation in the slightly more acidic environment and micellar entrapment, delaying exposure to the "degrading" intestinal environment. The results of this study demonstrate premature intestinal degradation of TDF and suggest that TFV may benefit from a more stable prodrug approach; however, fast absorption may compensate for fast degradation, indicating that prodrug selection should not be limited to stability assays.

1. Introduction

Tenofovir (TFV) is a nucleotide reverse transcriptase inhibitor which is used for the treatment of hepatitis B and HIV-infections. It is a very hydrophilic compound ($\log D_{7.4} = -3.55$) containing two negative charges resulting in low intestinal permeability; therefore, it is classified as a BCS class III compound. (Kearney et al., 2004) One of the strategies used to overcome permeability issues is to increase the lipophilicity of the compound through a prodrug strategy. A prodrug is an inactive form of the drug, which has to be activated once taken up (Jarkko et al., 2008). At this moment, TFV is on the market as the bis-ester prodrug tenofovir disoproxil fumarate (TDF, Viread®). In this prodrug, two isopropyloxycarbonyloxymethyl moieties mask the negative charges of TFV, to form a lipophilic ester prodrug that results in an increased transport across the intestinal epithelium. The prodrug is hydrolyzed in two steps: first, TDF is hydrolyzed by carboxylesterases to the mono-ester intermediate; this intermediate is further hydrolyzed by phosphodiesterases to the active form TFV (Fig. 1). These enzymes are present in the lumen of the intestine, coming from the secretion of the pancreas and from the enterocytes. Only the active form is observed in the systemic circulation. (Fardis and Oliyai, 2007) Compared to direct oral administration of TFV, intake of the prodrug results in a bioavailability increase of tenofovir from 2% to 20% in mice and from 17.1% to 30.1% in dogs (Naesens et al., 1998; Cundy et al., 1998). Although the bioavailability is increased, it remains relatively low. Premature degradation of the ester prodrug in the intestinal tract, either by chemical or by enzymatic hydrolysis, might contribute to the limited efficiency of the prodrug approach.

The intestinal absorption behavior of TDF has been studied in different experimental set-ups including Caco-2 cells (Heimbach et al., 2003; Van Gelder et al., 1999), in situ perfusion systems (Van Gelder, 2000) and cellular incubation systems using CEM and Huh7 cell cultures (Roux et al., 2013). However, to the best of our knowledge, no in vivo data about the intestinal behavior of TDF has been collected yet. When reviewing the available in vitro data about the decomposition of TDF, contradictory observations have been reported. Roux et al. found that decomposition of TDF resulted predominantly from chemical degradation: the first order decomposition rate constant (k_c) amounted to 6.6×10^{-3} min^{-1} for

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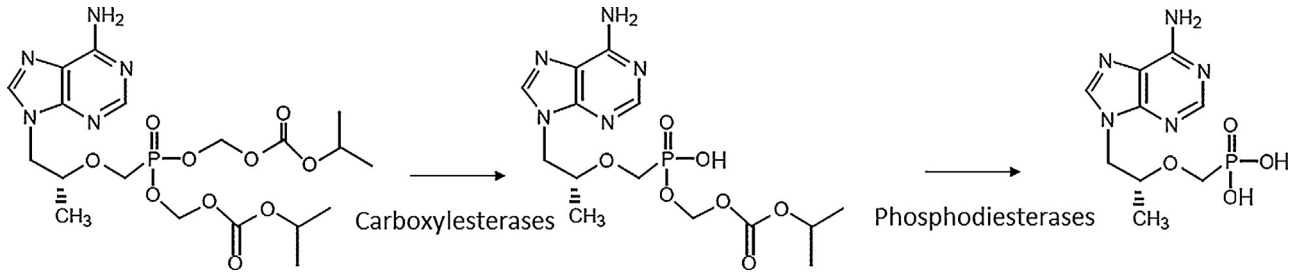

Fig. 1. Degradation profile of TDF.

the chemical degradation as compared to $1.1 \times 10^{-3}\,min^{-1}$ for the enzymatic degradation (Roux et al., 2013). Lung-Chi et al. further explored the influence of pH on the degradation kinetics of ester prodrugs containing an oxycarbonyloxymethyl group (including TDF) and concluded that these prodrugs are most stable in a pH range from 2 to 3. When increasing the pH above 3, they become exponentially more sensitive to chemical hydrolysis (Lung-Chi et al., 2001).

In contrast, Van Gelder et al. attributed TDF degradation during absorption primarily to the enzymatic pathway. By using homogenates of Caco-2 cells and intestinal homogenates from rat, pig and humans, a very fast esterase-mediated degradation of the ester-prodrug was observed. The role of esterases was confirmed by the fact that the degradation was inhibited by co-incubation with esterase inhibitors; degradation was also decreased upon co-incubation with fruit extracts, suggesting competitive inhibition by the esters present in these extracts (Van Gelder et al., 2002).

The data so far suggest that chemical as well as enzymatic degradation may lead to a premature decomposition of TDF in the intestinal tract, resulting in a decreased availability of the prodrug and, eventually, reduced absorption. A drawback of the available literature data is that they provide at best indirect evidence of the intraluminal behavior of TDF in humans. We therefore decided to directly explore the intraluminal behavior of TDF by collecting and characterizing intestinal fluids from healthy volunteers after oral intake of TDF, in both fasting and fed condition. As demonstrated for other drugs, the parallel assessment of intraluminal and systemic concentrations may allow linking the intestinal behavior to the bioavailability of the drug (Brouwers et al., 2007; Brouwers and Augustijns, 2014). The specific aims of this study were (1) to investigate the in vitro degradation profile of TDF in fasted and fed state human intestinal fluids (FaHIF, FeHIF) in order to assess the risk for intestinal decomposition of ester prodrugs prior to absorption, (2) to explore the intraluminal behavior of TDF in healthy volunteers by collecting intestinal fluids after oral intake of TDF and (3) to investigate possible food effects on the intraluminal behavior of TDF.

2. Materials and methods

2.1. Chemicals

TDF was provided by the NIH AIDS Research and Reference Reagent Program (Germantown, MD). TFV was obtained from Watson International (Kunshan City, China). Dimethylsulfoxide (DMSO) and tetrabutylammonium sulfate were obtained from Acros-Organics (Geel, Belgium). Acetic acid was obtained from Chem-lab (Zedelgem, Belgium). Acetonitrile was purchased from Fisher Scientific (Leicestershire, UK). Monobasic potassium phosphate monohydrate ($KH_2PO_4 \cdot H_2O$), bis-4-nitrophenylphosphate, chloroacetaldehyde and pancreatin from porcine pancreas (powder, suitable for cell culture, 4× USP specifications) were obtained from Sigma–Aldrich (St. Louis, MO). Methanol and sodium acetate were purchased from VWR International (Leuven, Belgium). Water was purified with a Maxima system (Elga Ltd., High Wycombe Bucks, UK). All stock solutions were prepared in dimethyl sulfoxide. The mono-ester intermediate of TDF was obtained by degradation of TDF at neutral pH: a 100 μM solution of TDF was made in a phosphate buffer (pH 7.0); following chemical degradation during 7 h at 60 °C, an equal amount of 1 M HCl was added to the solution; quantitative transformation into the intermediate without formation of TFV was confirmed.

2.2. Stabilization mixture

All samples taken during the experiments were diluted in a stabilization mixture to ensure the stability of the ester prodrug and mono-ester intermediate during further processing. The stabilization mixture consisted of MeOH:0.02 N HCl (50:50) containing 400 μM of the esterase inhibitor bis-nitrophenylphosphate.

2.3. In vitro stability studies

2.3.1. Simulated intestinal fluids

The in vitro degradation of TDF was investigated in mixtures of fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) containing 10 mg pancreatin/ml. The inclusion of pancreatin in FaSSIF has been reported by Borde et al. and the United States Pharmacopoeia 2009 to explore the degradation of ester prodrugs (Borde et al., 2012; U.S. Pharmacopeial Convention," 2009). FaSSIF and FeSSIF were made according to the manufacturer's preparation protocol (Biorelevant®, Croydon, UK) by dissolving SIF powder in an aqueous mixture of NaOH/NaH_2PO_4/NaCl for FaSSIF and NaOH/CH_3COOH/NaCl for FeSSIF. The pH was adjusted to 6.5 for FaSSIF and to 5.0 for FeSSIF. After the addition of pancreatin, the solutions were centrifuged and the supernatant was used for stability studies. TDF (stock solution of 5 mM in DMSO) was spiked into 1 ml of FaSSIF and FeSSIF resulting in a start concentration of 50 μM. Samples were taken at predetermined time points and immediately diluted 100 times in the stabilization mixture. The diluted samples were centrifuged and the supernatant was used for analysis.

2.3.2. Human intestinal fluids

Fasted state human intestinal fluids (FaHIF) and fed state human intestinal fluids (FeHIF) were aspirated from four healthy volunteers (two males, two females, between 23 and 27 years old). The study was approved by the Committee of Medical Ethics of the University Hospitals Leuven, Belgium and the procedure followed the tenets of the Declaration of Helsinki. HIF were collected from the duodenum (D2–D3) with a double-lumen polyvinyl catheter according to a previously described protocol. (Clarysse et al., 2011) Samples were collected every 10 min for 120 min in the fasted state and for 90 min in the fed state. In the fed state, the nutritional drink Ensure Plus® was given to the volunteers. One portion of 400 ml has an energy

content of 2526 kJ/600 kcal, of which lipids, carbohydrates, and proteins constitute 29%, 54%, and 17% on energy basis, respectively; the osmolality amounts to 670 mOsm/kg; the pH is 6.6. Pooled samples were made for each nutritional state, by combining equal volumes of the aspirates from all four volunteers. Pooled HIF were stored at −26 °C until further use. The pH amounted to 7.6 for the fasted state pool and to 5.5 for the fed state pool.

For the stability studies, a DMSO stock solution of TDF (5 mM) was spiked into 1 ml of FaHIF/FeHIF generating a concentration of 50 μM; this solution was incubated at 37 °C for 1 h. Every 15 min, a sample was taken and immediately diluted 100 times in the stabilization mixture. These samples were centrifuged and the supernatant was used for analysis. During the stability studies, the pH was measured using a Portamess 911 pH-meter (Knick GmbH & Company, Berlin, Germany).

2.4. Clinical study

To study the intraluminal behavior of TDF in the fasted and the fed state in humans, a crossover study was performed in five healthy volunteers (aged between 22 and 26 years; two men and three women). The procedure followed the tenets of the Declaration of Helsinki and was approved by the Committee of Medical Ethics of the University Hospitals Leuven, Belgium. All volunteers provided written informed consent to participate in this study. After an overnight fast (>12 h), one double-lumen polyvinyl catheter [Salem Sump Tube 14Ch (external diameter 4.7 mm), Sherwood Medical, Petit Rechain, Belgium] was introduced via the nose and positioned into the duodenum (D2/D3). The position of the tube was checked by means of fluoroscopy. It has previously been reported that the presence of a transpyloric tube does not influence gastric emptying or duodenogastric reflux (Müller-Lissner et al., 1982). For the experiments in the fasted state, a single tablet of Viread® (300 mg of TDF) was administered with 250 ml of water. For the experiments in the fed state, 400 ml of a nutritional drink (Fortimel Extra®) was given 20 min prior to intake of the TDF tablet. Fortimel Extra® (Nutricia, Strombeek-Bever, Belgium) was used to simulate a standard meal. One portion of 400 ml has an energy content of 2520 kJ, of which lipids, carbohydrates, and proteins constitute 32%, 41%, and 27% on energy basis, respectively; the osmolality amounts to 470 mOsm/kg; the pH is 6.8. Volunteers were asked to sit in upright position in a bed during the sampling procedure. Samples of human intestinal fluid (sample volume between 1.5 and 4 ml) were aspirated every 15 min up to 4 h for both states. In parallel to the sampling of gastrointestinal fluids, venous blood samples (1 ml) were collected in heparinized tubes (BD Vacutainer systems, Plymouth, UK) at 0, 20, 40, 60, 80, 100, 120, 160, 180, 210, 240, 300, 360, 420 and 480 min after drug intake. These blood samples were centrifuged at 1699 × *g* for 10 min to gain plasma samples which were stored at −26 °C until further analysis. The intestinal samples were stabilized directly after collection by a 100-fold dilution in stabilization mixture (see Section 2.2). After stabilization, the samples were stored at −26 °C until further analysis.

2.5. Analysis of TFV

For the analysis of TFV, both plasma and intestinal samples were processed to obtain a fluorescent derivative of TFV. This derivatization was performed based on a previously described method (Mallants et al., 2005; Sparidans et al., 2003). In 200 μl samples of plasma or stabilized dilutions of intestinal aspirates, proteins were precipitated by adding 600 μl of methanol followed by vortexing (30 s). Supernatant obtained after centrifugation (20,817 × *g*, 10 min) was transferred to new test tubes and evaporated under a gentle stream of air. The residue was redissolved in 200 μl of a buffer solution consisting of 100 mM sodium acetate (pH 4.5) and 3.0% of the derivatizing agent chloroacetaldehyde. This solution was vortexed and kept at 80 °C for 50 min. In this way, tenofovir was converted into its fluorescent derivative (Naesens et al., 1992). The samples were cooled to −30 °C for 5 min. After centrifugation at 20,817 × *g* for 10 min, supernatant was collected and transferred into microvials to be analyzed by HPLC.

After derivatization of the intestinal and plasma samples, concentrations of the fluorescent derivative of TFV were measured by reversed-phase HPLC and fluorescence detection (Mallants et al., 2005). A volume of 30 μl was injected into a Waters HPLC system consisting of a 600 E controller and pump, a 717plus autosampler and a Novapak C-18 column under radial compression (Waters, Milford, MA). Fluorescence signals (excitation 260 nm, emission 425 nm) were monitored with a Jasco FP-1520 fluorescence detector (Tokyo, Japan). Data acquisition and integration were performed using Empower Pro (Empower 2) (Waters, Belgium) software. The mobile phase consisted of a phosphate buffer (10 mM KH_2PO_4, 2 mM tetrabutylammonium sulfate, pH 8.0) (A) and acetonitrile (B). The flow rate amounted to 1.0 ml/min. Separation was carried out with a gradient elution starting with 75% A and 25% B for 3.5 min, followed by a linear gradient from 25% to 90% B over 0.5 min, which was accompanied with a change in flow rate from 1 ml/min to 1.5 ml/min. After 7 min, the mobile phase was changed back to its initial conditions and the column was re-equilibrated during 7 min. The retention time of derivatised TFV was 4.5 min. Calibration curves in plasma and HIF were found to be linear in a concentration range of 0.03–1 μM. The repeatability and precision were tested for the plasma and HIF samples at concentrations of 1 μM and 0.05 μM, respectively. For both concentrations, the mean bias and variability were <10%.

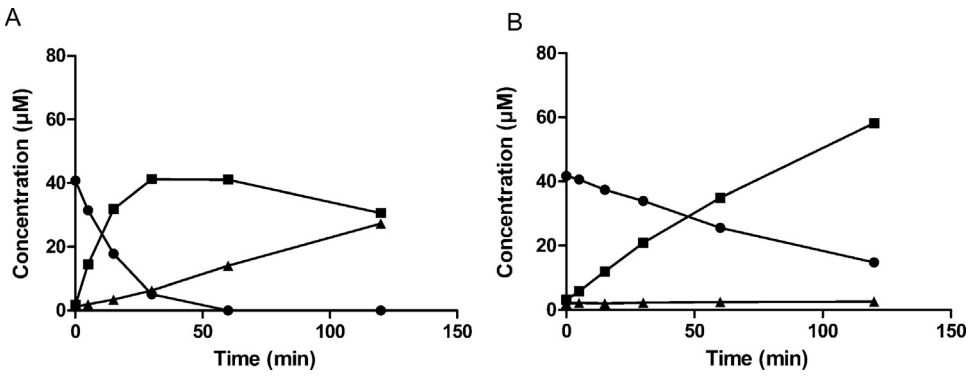

Fig. 2. Time-dependent degradation of tenofovir disoproxil fumarate (●) (at initial concentration of 50 μM) to mono-ester intermediate (■) and tenofovir (▲) in FaSSIF with 10 mg pancreatin/ml (A) and FeSSIF with 10 mg pancreatin/ml (B). Results are expressed as mean ± sd (n = 3).

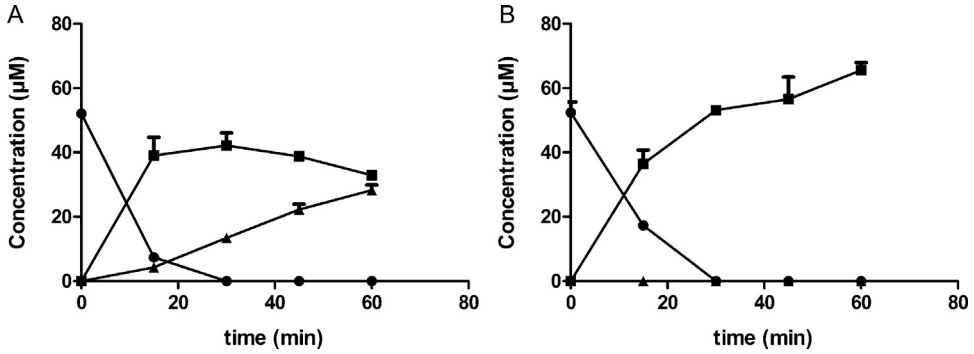

Fig. 3. Time-dependent degradation of tenofovir disoproxil fumarate (●) (at initial concentration of 50 μM) to mono-ester intermediate (■) and tenofovir (▲) in FaHIF (A) and FeHIF (B). Results are expressed as mean ± sd ($n = 3$).

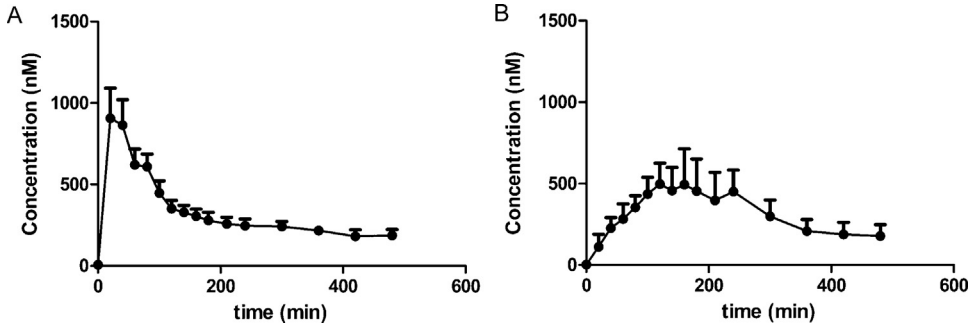

Fig. 4. Plasma concentration-time profiles for TFV in the fasted state (A) and fed state (B) after oral administration of a tablet containing 300 mg TDF. Results are expressed as mean ± sem ($n = 5$).

2.6. Analysis of TDF and mono-ester

Concentrations of TDF and its mono-ester intermediate in diluted intestinal samples were determined using HPLC with MS/MS detection. The Thermo Scientific (San Jose, USA) LCQ Deca XP Max iontrap mass spectrometer system equipped with an electrospray ionization (ESI) source was used. Data acquisition and peak integration were performed with Xcalibur®. A Kinetex® XB-C18 column (length 50 mm × 2.1 mm, particle size 2.6 μm) (Phenomenex, The Netherlands), protected by a Krudkatcher Ultra HPLC In-Line filter (Phenomenex), was used for chromatographic separation. The mobile phases consisted of 0.2% formic acid (A), 5 mM ammonium acetate buffer (pH 4.8) (B) and acetonitrile (C). The flow rate amounted to 200 μl/min. Separation was carried out with a gradient elution starting with 81% A, 9% B and 10% C, changing to 55.5% A, 10.5% B, 34% C after 1 min. After 2 min the composition changed immediately to 30% A, 10% B, 60% C. After 2.5 min, the column was re-equilibrated with the initial conditions for 3 min. The total run time was 8.5 min and the injection volume amounted to 20 μl (full loop mode). The mass spectrometer was operated in the positive electrospray (ESI) mode for TDF and in the negative ESI mode for the mono-ester. The spray voltage, capillary voltage and capillary temperature were 4.50 kV, 12 V and 325 °C, respectively. Nitrogen was used as the sheath gas (59 arbitrary units), ion sweep and auxillary gas (20 arbitrary units). Argon was used as the collision gas at a pressure of 1.5 mTorr. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode. A single precursor–product ion pair was used for detection: m/z_{TDF} 520.20 → 288.0 (collision energy: 32 V) and m/z_{Mono} 402.10 → 328.00 (collision energy: 32 V), both with a scan time of 100 ms. Calibration curves were linear over the concentration range of 0.03–1 μM. The repeatability and precision for TDF and its mono-ester intermediate were assessed at a concentration of 0.3 μM. Repeatability and precision errors were found to be <10% for both compounds.

2.7. In silico profiling

MarvinSketch (ChemAxon, Budapest, Hungary) was used to determine key physicochemical properties including lipophilicity (logD) and dissociation constant (pK_a).

2.8. Data presentation and statistical analysis

All concentration-time profiles obtained from the in vitro studies are presented as mean ± sd for three experiments. All concentration-time profiles from the clinical trial are presented as mean ± sem for five subjects. The reported averages for AUC, C_{max} and t_{max} were calculated based on data from the individual volunteers. A paired *t*-test was used to compare selected parameters ($AUC_{0-4\,h}$ and $AUC_{0-8\,h}$) of the in vivo concentration-time profiles between the fasted and fed state condition. When $p < 0.05$, differences were considered statistically significant.

3. Results and discussion

The in vitro Caco-2 system as well as the rat in situ intestinal perfusion setup have suggested that premature intestinal degradation of TDF into TFV may limit the efficiency of the prodrug approach (Barditch-crovo et al., 2001; Heimbach et al., 2003; Roux et al., 2013; Van Gelder et al., 1999, 2000, 2002; Van Gelder, 2000). In this study, we further explored the intraluminal stability of the ester prodrug using biorelevant approaches, including in vitro as well as in vivo methods. In a first set of experiments, the in vitro stability of TDF in simulated and human intestinal fluids was

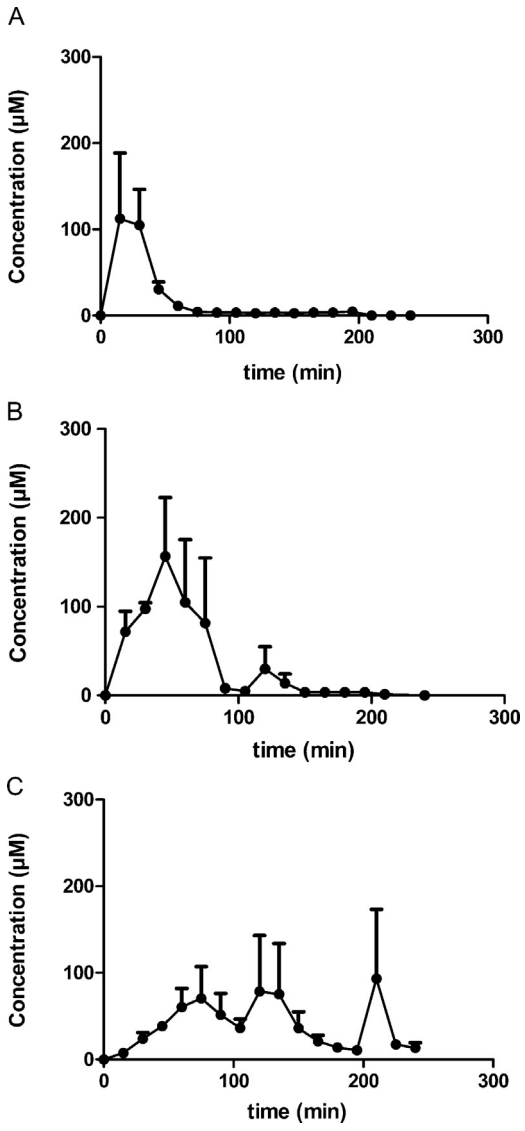

Fig. 5. Duodenal concentration-time profile for TDF (A), mono-ester (B) and TFV (C) in the fasted state after oral administration of a tablet containing 300 mg TDF. Results are expressed as mean ± sem ($n = 5$).

determined; subsequently, the in vivo behavior was explored in humans (Borde et al., 2012).

3.1. In vitro studies

3.1.1. Simulated intestinal fluids

As suggested by United States Pharmacopoeia 2009 and similar to Borde et al., FaSSIF was supplemented with 10 mg pancreatin per milliliter to explore the degradation of ester prodrugs (Borde et al., 2012; U.S. Pharmacopeial Convention," 2009). When TDF was spiked in this solvent system, almost all TDF was degraded within 60 min resulting in the formation of mono-ester intermediate and tenofovir (Fig. 2A). For the fed state, FeSSIF was supplemented with 10 mg pancreatin per milliliter. The pH of FeSSIF was not influenced by the addition of pancreatin powder. When adding TDF to this mixture, TDF slowly degraded, resulting in the formation of the mono-ester intermediate; however, no formation of tenofovir could be observed (Fig. 2B), suggesting less chemical and/or enzymatic degradation in fed state conditions. This reduced degradation might be attributed to the lower pH of FeSSIF (5.0 versus 6.5 in FaSSIF) and/or possible increased micellar entrapment of the hydrophobic TDF ($\log D_{5.5} = 2.29$) in TDF which may result in a shielding effect of the micelles.

3.1.2. Human intestinal fluids

Preliminary experiments showed no degradation of TDF in human gastric fluids obtained in both fasted and fed state conditions; therefore stability was only studied in human intestinal fluids obtained in the fasted (FaHIF) as well as the fed state (FeHIF). Upon addition of TDF to FaHIF, TDF converted rapidly to mono-ester and TFV, resulting in complete degradation within one hour (Fig. 3). In FeHIF, TDF degradation to the mono-ester intermediate was slightly slower than in FaHIF; however, the observed food effect was less pronounced than in simulated fluids. In contrast to the fasted state, the mono-ester intermediate did not further convert to TFV, as also observed in FeSSIF.

From a qualitative point-of-view, the observed TDF degradation in simulated intestinal fluids supplemented with pancreatin (Fig. 2) mimicked TDF degradation in human intestinal fluids. In line with Borde et al. (Borde et al., 2012), however, the degradation rate was higher in human versus simulated fluids, both in fasted and fed conditions.

3.2. In vivo study

In the next step, we wanted to explore whether these in vitro results are representative for the in vivo situation. To the best of our knowledge, no intraluminal in vivo data are available about the intestinal behavior of ester prodrug. Therefore, a clinical study was performed in which one tablet of TDF (Viread®, 300 mg) was given to healthy volunteers in the fasted as well as the fed state. Subsequently, intestinal fluids from the duodenum were aspirated and analyzed. In parallel to the intestinal sampling, blood samples were collected to link the intestinal behavior of TDF to the appearance of tenofovir in the systemic circulation. A similar approach was already successfully applied to investigate the intestinal behavior of fosamprenavir and posaconazole. (Brouwers et al., 2006; Walravens et al., 2011).

3.2.1. Plasma concentration-time profiles

Fig. 4 shows the mean plasma concentration of TFV as a function of time after oral administration of one Viread® tablet containing 245 mg of TDF in fasted and fed state conditions. In plasma, no prodrug nor mono-ester intermediate could be observed (data not shown), suggesting quantitative degradation of TDF during absorption and/or first pass metabolism. In the fasted state, absorption appeared to be very fast with a maximum plasma concentration of 978 ± 183 nM obtained after 28 ± 5 min. The maximum plasma concentration of TFV in the fed state was observed after 132 ± 32 min, with a lower C_{max} of 711 ± 162 nM as compared to fasted state conditions. $AUC_{0-8\,h}$ and C_{max} did not significantly differ between fasted (151 ± 208 μM min; 978 ± 183 nM) and fed (148 ± 394 μM min; 771 ± 162 nM) state conditions; however, t_{max} was significantly increased in fed state conditions. While the increase in t_{max} was in line with literature, our study did not reveal a positive food effect on the bioavailability of TFV (Barditch-crovo et al., 2001). It should be mentioned, however, that the positive food effect reported in literature (25% bioavailability in the fasted state versus 40% in the fed state) was demonstrated after a daily treatment of 23 days, which makes it difficult to compare with our single dose study (Barditch-crovo et al., 2001). To further investigate the difference in concentration-time profile between the fasted and fed state, the intraluminal behavior of TDF was assessed by the simultaneous collection of intestinal fluid samples.

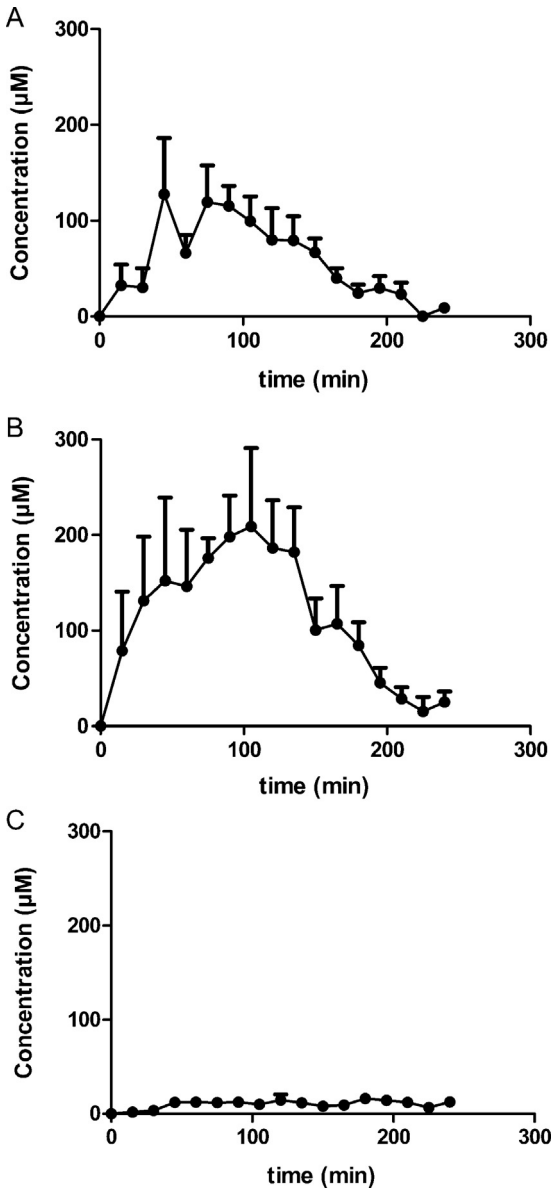

Fig. 6. Duodenal concentration-time profile for TDF (A), mono-ester (B) and TFV (C) in the fed state after oral administration of a tablet containing 300 mg TDF. Results are expressed as mean ± sem ($n = 5$).

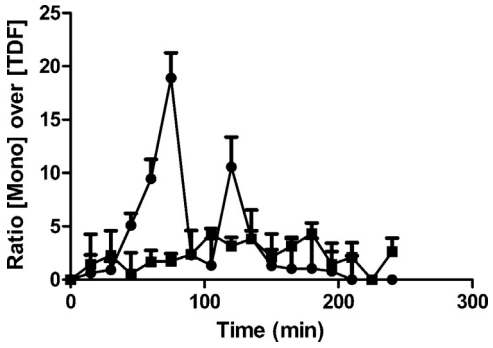

Fig. 7. Ratio of the concentration of mono-ester intermediate over the concentration of TDF in function of time in fasted (●) and fed (■) state condition after oral administration of a tablet containing 300 mg TDF. Results are expressed as mean ± sem ($n = 5$).

3.2.2. Intestinal concentration-time profiles

For the collection of the intestinal fluids, a unique sampling method was used whereby one double-lumen polyvinyl catheter was placed via the nose into the duodenum. After the placement of the catheter and the intake of TDF, intestinal fluids were collected and analyzed for TDF, mono-ester intermediate and TFV.

3.2.2.1. Fasted state condition. The concentrations of TDF, mono-ester intermediate and TFV observed in the duodenum are depicted in Fig. 5 and confirm that rapid degradation is taking place in the intraluminal environment. In the fasted state condition, the mean maximum concentration of TDF (163 ± 71 μM) was reached 24 ± 4 min after the intake of the drug. Similar to the in vitro studies (Figs. 2 and 3), degradation resulted in the formation of both the mono-ester intermediate and tenofovir. The mono-ester intermediate reached a mean maximum concentration of 193 ± 63 μM 33 ± 9 min after intake of the drug. The higher concentration of the mono-ester intermediate compared to the prodrug can probably be attributed to the dual fate of TDF, i.e., the prodrug may be (1) degraded to its mono-ester intermediate, and (2) rapidly absorbed in the non-ionized state (logD7.4 = 2.65, $pK_a = 5.12$). Rapid absorption of TDF is confirmed by the short t_{max} observed for TFV in the plasma profiles (Fig. 4), which corresponds to the relatively short t_{max} of TDF in intestinal samples. In contrast, the intermediate (logD7.4 = −1.54, $pK_{a1} = 0.82$, $pK_{a2} = 5.12$) and tenofovir (logD7.4 = −3.55, $pK_{a1} = 1.35$, $pK_{a2} = 5.12$, $pK_{a3} = 7.91$) are not expected to be absorbed due to their negative charge. In the fasted state, the mean pH of the intestinal fluids was 6.65 ± 0.52 which is in agreement with reported data in the literature (pH between 6.3 and 7.5) (Augustijns et al., 2013). Based on the findings that ester prodrugs are exponentially more sensitive to chemical hydrolysis at a pH > 4, chemical degradation cannot be neglected (Lung-Chi et al., 2001). Also the active compound TFV could be detected in the intestinal samples demonstrating phosphodiesterase activity; no clear C_{max} could be observed, with an average concentration fluctuating around 75 μM.

3.2.2.2. Fed state condition. TDF, the mono-ester intermediate and TFV were also detected in the intestinal samples collected in fed state conditions (Fig. 6). The combination with food induced a delay in gastric emptying, resulting in increased t_{max} values and smoother profiles for TDF and its degradation compounds. The concentration of TDF reached its mean maximum concentration (210 ± 26 μM) 60 ± 7.3 min after drug intake. This differs significantly ($p < 0.05$) from t_{max} of TDF observed in the fasted state condition (24 ± 4 min). The mean maximum concentration of the mono-ester intermediate amounted to 339 ± 40 μM, and was reached 84 ± 14 min after intake of the tablet. The $AUC_{0-4\,h}$ of TDF and the mono-ester were both significantly higher in fed versus fasted state. The 3-fold increase in $AUC_{0-4\,h}$ of TDF (fed: 13.9 ± 2.1 μM min versus fasted: 4.1 ± 1.5 μM min) suggests a slower absorption of TDF in fed state conditions. A food-induced delay in gastric emptying and reduction of the absorption rate is reflected in the plasma concentration-time profiles for TFV (Fig. 4). For TFV, average concentrations fluctuated around 12 μM; the concentration is much lower compared to the fasted state condition indicating a higher intestinal stability of the mono-ester intermediate in the fed state.

When considering the ratio of mono-ester intermediate to prodrug, it appears that there is relatively more mono-ester intermediate present in fasted state conditions than in fed state conditions (Fig. 7). These results indicate slower degradation of TDF in combination with a reduced rate of absorption in postprandial conditions.

Interestingly, the higher intestinal concentrations of TDF in fed state conditions are not accompanied by higher

concentrations in the plasma-concentration profile (Fig. 4), which suggests that the concentrations measured do not reflect the driving force for absorption. It has been mentioned before that micellar entrapment of the prodrug may occur in view of the relatively high lipophilicity of the ester prodrug. Dialysis experiments were performed to explore the association of TDF with micelles; unfortunately, degradation in the media used prevented us to estimate micellar entrapment.

Compared to the observed in vitro degradation profile of TDF, the clinical study confirmed the faster conversion of TDF to mono-ester intermediate in fasted versus fed state conditions. In fasted state conditions, further degradation to TFV was observed both in vitro and in vivo. In fed state conditions, however, the formation of intraluminal TFV in vivo (albeit significantly less than in fasted state) could not be simulated in vitro.

4. Conclusion

The ester prodrug TDF, which has been designed to overcome permeability issues of tenofovir, has a relatively low bioavailability of 25% in man. In the present study we explored whether the limited efficiency of the prodrug is due to premature degradation in the intestinal tract. Based on in vitro studies performed in biorelevant media, the following conclusions could be made: (1) TDF does not degrade in gastric conditions due to the absence of esterases (enzymatic stability) and the acidic pH (chemical stability), (2) in fasted state intestinal conditions, TDF rapidly converts to mono-ester intermediate and TFV, and (3) in fed state intestinal conditions, TDF degradation is slightly reduced compared to the fasted state while the mono-ester intermediate does not further degrade to TFV. The clinical study confirmed extensive intraluminal degradation of TDF in healthy volunteers. Similar to the in vitro experiments, the ester prodrug was more stable in the fed state condition compared to the fasted state condition. However, the increased intraluminal stability of the prodrug in postprandial conditions was not reflected in enhanced systemic exposure of TVF. The results of this study reveal extensive premature degradation of TDF; therefore, tenofovir may benefit from a new prodrug approach. Since fast absorption may (partly) compensate for fast degradation, prodrug selection should be based on (combined) stability and permeability assays.

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