# Enzymatic prodrug degradation in the fasted and fed small intestine: *in vitro* studies and interindividual variability in human aspirates

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## ABSTRACT

The aim of the current study was (1) to develop an automation-based protocol for *in vitro* assessment of enzymatic drug stability at fasted- and fed-state intestinal conditions, (2) to characterize the inter-individual variability of drug degradation in fasted- and fed-state human intestinal fluids, and (3) to compare the obtained *in vitro* results to drug degradation in human intestinal fluids by taking variability into account. In human intestinal fluids, drug degradation displayed large inter-individual variability, with coefficients of variance generally ranging between 30 and 70 %. The effect of food on the inter-individual variability was highly dependent on the type of drug. The increase of pH in the range between 5.0 and 7.0 significantly accelerated the degradation rate of the studied drugs both in the *in vitro* and *ex vivo* experiments. In contrast, the increase of bile salt and phospholipid concentrations in the *in vitro* screen decreased strongly the degradation rate of the hydrophobic drugs. The developed automated *in vitro* screen mimicked relatively well the *ex vivo* degradation of all drugs in the fasted state, whereas in the fed state the degradation of only one of the drugs was adequately reproduced.

**Keywords**

drug stability; prodrug; ester hydrolysis; human intestinal fluids;

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## 1. Introduction

The interactions of oral drugs with the complex environment of the gastrointestinal tract (GIT) may cause issues with drug stability, due to large pH shifts and various enzymes. Ester-based prodrugs can be especially sensitive to GIT conditions, as the ester bond in the drug structure can be cleaved (Lavis, 2008). Whether this event is desirable or not depends on the prodrug strategy (Beaumont et al., 2003; Rautio et al., 2018): for example, if the aim is to increase aqueous solubility, the conjugated acidic or basic moiety should be hydrolyzed in the intestine (preferably near the enterocytes) in order to decrease drug polarity thereby enhancing permeation and absorption (*e.g.* irinotecan hydrochloride, prednisolone sodium succinate). In contrast, some prodrugs are engineered to enhance the passive permeability of otherwise hydrophilic compounds by conjugation with lipophilic counterparts, with the aim to improve oral absorption and intracellular delivery (Beaumont et al., 2003). In this case, hydrolysis in the intestinal lumen (prior absorption) can be a hazard. Hence, the rate and extent of drug degradation at GIT conditions is of considerable interest in oral drug development.

In terms of the enzymatic drug degradation, the carboxyl esterase (CES) family of enzymes drive the hydrolysis of ester-based oral drugs in the intestinal lumen (Di, 2019; Wang et al., 2018). These enzymes have two main isoforms (CES-1 and CES-2) that are characterized by differences in both abundance and substrate specificity. The main isoform found in the GIT is CES-2, which selectively degrades drugs with relatively large alcohol moiety and compact acyl group (*e.g.* flutamide, prasugrel, irinotecan)(Wang et al., 2018). Other enzymes such as phosphodiesterases, carboxypeptidases, phospholipase C etc. can also cause (pro)drug degradation (Wiemer, 2020). Hydrolysis can have a significant impact on the solubility and permeability of the drug, which can in turn affect oral absorption and bioavailability (Geboers et al., 2015; Stappaerts et al., 2015). Hence, significant efforts have been dedicated to study drug stability and degradation in the GIT. The enzymatically driven intestinal drug degradation is usually studied by using *in vitro* methods with varying level of complexity and biorelevance (Borde et al., 2012; Fayed et al., 2016; Gadkariem et al., 2004; Geboers et al., 2015; Gupta et al., 2009; Hoppe and Sznitowska, 2014; Nielsen et al., 2005; Singh et al., 2017; Stappaerts et al., 2015; Wang et al., 2015; Xiang et al., 2017; Yadav et al., 2016). Factors such as the type of the used enzymes, the presence of bile salts and phospholipids, pH and electrolyte concentrations should be carefully selected as they could significantly impact the outcome of the experiments.

The conditions that have been used to study enzymatic drug degradation *in vitro* are summarized in Table 1. Stomach stability has usually been studied in simulated gastric fluids according to USP: pH = 1.2, pepsin from gastric mucosa at 3.2 mg/mL and 34 mM NaCl. In contrast, a variety of conditions have been employed to investigate drug stability in the small intestine. In terms of pH, most studies fall within the range from 5.5 to 7.5, with pH = 6.8 being the most common, which represents well the pH values encountered in the fasted and fed small intestine. Porcine pancreatin was the first choice as a source of enzymes, with concentrations generally ranging between 0.3 and 1 %, with one study going up to 4 % (Borde et al., 2012).

Selecting a biorelevant enzyme concentration is still a hurdle for every *in vitro* enzymatic assay, since the data about CES activity in human intestinal fluids is scarce and official guidelines are lacking. Mucosa or tissue homogenates have also been used in drug degradation assays. Surprisingly, bile salts and phospholipids were usually not included in the composition of the biorelevant medium ($n$ = 24, Table 1), although they may solubilize poorly water-soluble drugs, which can in turn affect drug degradation (Bali et al., 2016; Fayed et al., 2016; Hammad and Muller, 1999; Tirucherai and Mitra, 2003). Only a handful of studies ($n$ = 8, Table 1) have included a bile salt or a bile salt/phospholipid mixture together with an enzyme source, in order to provide a reaction medium composition which is closer to the reality of the human intestine.

Considering the various simplifications that accompany the study of drug degradation at *in vitro* conditions, it is natural to question the scope of validity of the obtained results. One way to address this issue is to provide a consistent comparison between the *in vitro* data and an *in vivo* model: *e.g.* by the *ex vivo* study of drug degradation in human or animal intestinal fluids (Berardi et al., 2018; Borde et al., 2012; Hu et al., 2013; Wang et al., 2015; Yadav et al., 2016). In all cases, the validation set consisted of pooled intestinal fluids from different origins (dog, rat, human), which could not account for the inherent (and possibly substantial) interindividual compositional variability of the fluids (Vinarov et al., 2021). In addition, the *in vitro* enzymatic assays were usually characterized by a relatively low throughput, which limits their application in industrial settings.

The aim of the current study was (1) to develop an automation-based protocol for *in vitro* assessment of enzymatic drug stability at fasted- and fed-state intestinal conditions, (2) to characterize the inter-individual variability of drug degradation in fasted- and fed-state human intestinal fluids (HIF), and (3) to compare the obtained *in vitro* results to drug degradation in HIF by taking variability into account.

**Table 1**. Summary of the conditions used to study the enzymatic drug degradation at stomach and intestinal conditions and if *in vitro*-in vivo correlation (IVIVC) was evaluated.

| API | Gastric conditions | | Intestinal conditions | | | IVIVC | Reference |
|---|---|---|---|---|---|---|---|
| | pH | Enzymes | pH | Bile salts | Enzymes | | |
| Clopidogrel.HSO4 | not tested | | rabbit mucosa (small intestinal and colonic) | | | none | (Bali et al., 2016) |
| 17 peptide drugs | 1.2 | pepsin | 6.8 | none | 1 % Pancreatin (porcine, 3x) | HGF, HIF, PGF, PIF | (Wang et al., 2015) |
| Cowpea virus | 1-4 | pepsin | 6.8 | none | 1 % Pancreatin (porcine, 3x) | PGF, PIF | (Berardi et al., 2018) |
| Gingerol, shogaol | 1.2 | pepsin | 6.8 | none | 1 % Pancreatin (porcine, 3x) | none | (Bhattarai et al., 2007) |
| Chloramphenicol succinate | not tested | | 5.5-7.5 | FaSSIF | 1-4 % Pancreatin | HIF, DIF | (Borde et al., 2012) |
| Enalapril | not tested | | 5.5-7.5 | FaSSIF | 1-4 % Pancreatin | HIF, DIF | (Borde et al., 2012) |
| Candesartan cilexetil | not tested | | 5.5-7.5 | FaSSIF | 1-4 % Pancreatin | HIF, DIF | (Borde et al., 2012) |
| Peptide D3 | 1 | pepsin | 6.8 | none | 1 % Pancreatin (porcine, 8x) | microsomes | (Elfgen et al., 2017) |
| Candesartan cilexetil | not tested | | rabbit mucosa (small intestinal and colonic) | | | none | (Fayed et al., 2016) |
| Danazol | 1.2 | pepsin | 6.8 | none | 1 % Pancreatin (porcine, 3x) | none | (Gadkariem et al., 2003) |

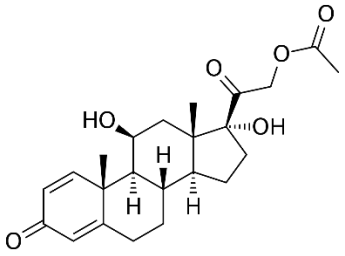
| API | Gastric conditions | | Intestinal conditions | | | IVIVC | Reference |
|---|---|---|---|---|---|---|---|
| | pH | Enzymes | pH | Bile salts | Enzymes | | |
| Diloxanide furoate | 1.2 | pepsin | 6.8 | none | 1 % Pancreatin (porcine, 3x) | none | (Gadkariem et al., 2004) |
| 7 lamivudine derivatives | 1.2 | pepsin | 6.8 | none | Pancreatin (porcine) | none | (Gualdesi et al., 2013) |
| SGC-liposomes | 1.2 | pepsin | 5.0-6.8 | FaSSIF, FeSSIF, FeSSIFv 2, others | 1 % Pancreatin (porcine, 8x) | rat gastric and intestinal fluds | (Hu et al., 2013) |
| D-allulose | 1.6 | pepsin | 6.5 | FaSSIF | 1 % Pancreatin | PK in rats; hepatocytes (rat and human) | (Maeng et al., 2019) |
| Mycophenolate mofetil | not tested | | 6.5 | FaSSIF v2 | none | HIF, hepatocytes, liver S9 fraction, blood, PBPK modeling | (Malmborg and Ploeger, 2013) |
| Midodrine | not tested | | 6.5 | FaSSIF v2 | none | HIF, hepatocytes, liver S9 fraction, blood, PBPK modeling | (Malmborg and Ploeger, 2013) |
| Bambuterol | not tested | | 6.5 | FaSSIF v2 | none | HIF, hepatocytes, liver S9 fraction, blood, PBPK modeling | (Malmborg and Ploeger, 2013) |
| Bupivacaine and lidocaine analogues | not tested | | 7.5 | none | 1 % Pancreatin (porcine, 3x) | human plasma | (Nielsen et al., 2005) |
| Liposomes | not tested | | 7.4 | 10 mM NaTC (no pancreatin) | 0.3 % Pancreatin (porcine, 8x) | none | (Parmentier et al., 2011) |
| Albumin (BSA) | not tested | | 7.5 | 10 mM NaTC | 1 % Pancreatin | none | (Ramaldes et al., 1996) |
| Collagen tripeptide | not tested | | 6.8 | none | 1 % Pancreatin (porcine, 3x) | rat absorption; rat gastric fluid, rat plasma; Caco-2 permeation | (Ramaldes et al., 1996) |
| Abiraterone acetate | not tested | | 7.5 | FaSSIF | 20 U/mL esterase (porcine) | FaHIF, rat perfusion | (Stappaerts et al., 2015) |
| Myricetin, myricitrin | 1.2 | pepsin | 6.8 | none | 1 % Pancreatin (porcine) | none | (Xiang et al., 2017) |
| Liraglutide | 1.2 | pepsin | 6.8 | none | 1 % Pancreatin (porcine, 3x) | none | (Ismail et al., 2019) |
| Bergenin prodrugs | 1.2 | pepsin | 6.8 | none | 1 % Pancreatin (porcine); esterase from porcine liver | rat plasma | (Singh et al., 2017) |
| Peptide conjugates | not tested | | mouse small intestinal homogenate | | | mouse plasma, ileal gut sacks, Caco2 cells | (Dong et al., 2012) |
| Milk fat globule membrane proteins | 2 | pepsin | 6.5 | none | trypsin and α-chymotrypsin | none | (Le et al., 2012) |
| | | | 6.5 | 30 g/L bile salts | 0.45% Pancreatin (porcine, 4x) | none | (Le et al., 2012) |
| Amino Acid Prodrugs | not tested | | rat intestinal perfusate | | | Caco2 cells, Valacyclovirase | (Gupta et al., 2009) |
| Hydrophilic peptides | not tested | | Brush Border Membrane Vesicles from rat small intestine | | | Caco2, permeation | (Hess et al., 2007) |
| l-Glu-l-Trp-OH | not tested | | Caco2 cell homogenate | | | Caco2 permeation | (Bergeon and Toth, 2007) |
| Cytotoxic T-lymphocyte-epitope model peptide | not tested | | 6.8 | none | trypsin, chymotrypsin and elastase | porcine intestinal mucosa, aminopeptidase, carb. peptidase A | (Marschutz et al., 2002) |
| Leu-enkephalins and ester prodrugs | not tested | | human plasma, porcine gut homogenate, aminopeptidase, carboxypeptidase A, Caco-2 cell homogenate | | | none | (Fredholt et al., 2000) |
| Indomethacin esters | not tested | | rat plasma | | | none | (Bonina et al., 1997) |
| Ganciclovir Prodrugs | not tested | | Monkey intestine, liver and skin homegenates | | | none | (Powell et al., 1991) |
| Propranolol prodrugs | not tested | | 7.4 | none | esterase | none | (Irwin and Belaid, 1988) |
| Infliximab, adalimumab | 1.2 | pepsin | 6.8 | none | Pancreatin, trypsin, chymotrypsin or elastase | HGF, HIF | (Yadav et al., 2016) |

## 2. Materials and methods

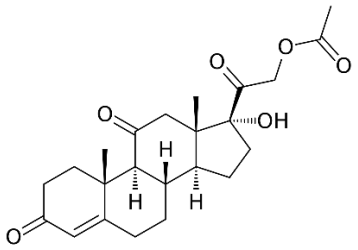
### *2.1. Materials*

Candesartan cilexetil (> 98.0 %, TCI), prednisolone acetate (>97 %, TCI), cortisone acetate (> 99 %, TCI) and tenofovir disoproxil fumarate (> 98 %, Sigma-Aldrich) were used as model drugs for the enzymatic degradation studies (Figure 1). The four studied APIs were selected for several reasons. Candesartan cilexetil was chosen for the purpose of comparison with other studies on enzymatic drug degradation (see Table 1). Prednisolone acetate and cortisone acetate were selected because they have small differences in their molecular structure, which facilitates the interpretation of the link between structure and degradation rate. Finally, tenofovir disoproxil fumarate was chosen because it has very high aqueous solubility, in contrast to the other three APIs, which widens the chemical space of the studied molecules.

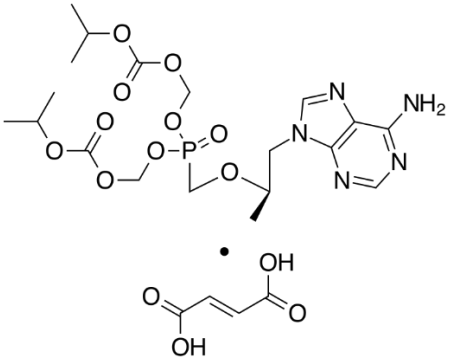
**Prednisolone acetate (PA)**



**Cortisone acetate (CA)**



**Tenofovir disoproxil fumarate (TDF)**



**Candesartan cilexetil (CC)**

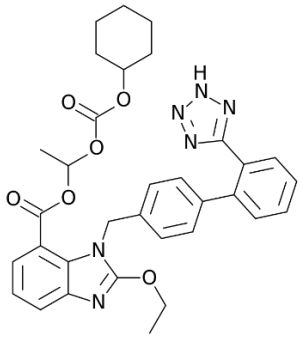


**Figure 1**. Chemical structures of the studied drugs.

Candesartan (98 %, AlfaAesar) and prednisolone (> 98 %, TCI) were used as standards to identify and quantify enzymatic hydrolysis products. Pancreatin from porcine pancreas (4x USP specifications, Sigma-Aldrich) was used as an enzyme source. FaSSGF/FaSSIF/FeSSIF powder composed of sodium taurocholate and soy lecithin was obtained from Biorelevant.com Ltd. and was used to prepare biorelevant media for the *in vitro* degradation studies. Sodium acetate trihydrate (99 %, Sigma-Aldrich), 2-[N-morpholino]-ethanesulfonic acid (MES) monohydrate (ultrapure) and sodium chloride (99 %, VWR International) were used for buffer preparation,

whereas hydrochloric acid (0.1 N, Merck & Co.) and sodium hydroxide solutions (0.1 N, Merck & Co. ) were used for pH adjustment. DMSO (min. 99.9 %, ACS reagent grade, AlfaAesar) was used to prepare stock solutions of the drugs. Acetonitrile for liquid chromatography (gradient grade, Merck & Co) and trifluoroacetic acid (TFA, 99.9 %, Supelco) were used for chromatographic analysis. Deionized water from a Milli-Q (Merck & Co.) or a Maxima system (Elga, Ltd., High Wycomb Bucks, UK) was used for solution and mobile phase preparation.

### *2.2. Drug degradation protocols*

#### 2.2.1. *In vitro* screening of drug degradation

Pancreatin powder (6, 12 or 30 mg) was weighted in 10 mL screw-cap vials by an automated solid dosing system (ChemSpeed®). The accuracy of each weighing was checked and vials in which the weighing error was bigger than 10 % were replaced by manually weighed samples. Afterwards, the vials were arranged in aluminum holders and placed on a Hamilton® liquid pipetting robot at $T$ = 37 °C.

Three biorelevant media were used to study drug degradation *in vitro*: one fasted state medium at pH = 6.5 and two fed state media at pH = 6.5 and 5.0. Fasted state medium was prepared by using the FaSSIF/FeSSIF/FaSSGF powder to yield a solution containing 3 mM sodium taurocholate and 0.75 mM phospholipids in 20 mM MES buffer set at pH = 6.5. To match the ionic strength of the phosphate buffer-based FaSSIF solution, 150 mM NaCl was also dissolved in the medium. MES buffer was used in place of the standard phosphate buffer, as the latter is known to interfere with the action of phosphatase enzymes that are responsible for the degradation of phosphate ester prodrugs (Brouwers et al., 2007). Fed state media were also prepared by using FaSSIF/FeSSIF/FaSSGF powder, but at five times higher concentration, yielding 15 mM sodium taurocholate and 3.75 mM phospholipids at pH = 6.5 (20 mM MES buffer + 150 mM NaCl) or pH = 5.0 (20 mM acetate buffer + 300 mM NaCl). The salt added to the pH = 5.0 buffer was introduced in order to match the ionic strength of the standard FeSSIF 5.0 medium, whereas the salt added at pH = 6.5 corresponded to the FaSSIF 6.5 medium. After addition of the buffer to the FaSSIF/FeSSIF/FaSSGF powder, the media were stirred at $T$ = 37 °C for at least 60 min before use.

The media were transferred to the Hamilton® liquid pipetting robot in 50 mL tubes and the automated protocol for media dosing was initiated. The protocol consisted in adding 6 mL medium to each vial, after which shaking at 300 rpm at $T$ = 37 °C was started. Then, a protocol for addition of the drug solution was initiated, which consisted in the pipetting of 60 µL of drug stock solution in DMSO by using an 8-channel automated pipettor. The stock solution concentration was selected in order to produce the following drug concentrations after dilution: 41.7 µg/mL PA and CA, 250 µg/mL TDF and 10 µM CC. The final drug concentrations of PA, CA and TDF were selected by considering therapeutically-relevant single doses (25 mg for PA and CA, 150 mg for TDF)(Sweetman, 2009), whereas solubility was the main determinant for CC. Fasting gastric fluid volume of 50 mL was assumed (Vinarov et al., 2021) and a volume of 250 mL was added to account for intake of one glass of water, making a total of 300 mL at gastric conditions. To

calculate intestinal concentrations, 1:1 dilution of the concentration calculated in the gastric environment was assumed, yielding luminal concentrations of 41.7 µg/mL for PA and CA, and 250 µg/mL for TDF.

After the addition of the drug stock solution, time dependent 250 µL samples were taken by an 8-channel automated pipettor at t = 0.4, 5, 15, 45, 90 and 120 min by the Hamilton® robot. Each sample was added to 750 µL acetonitrile in 96 deep well UPLC plates in order to precipitate the enzymes and stop the enzymatic degradation (Borde et al., 2012; Gupta et al., 2009). The deep well plates were covered with a plastic lid to prevent evaporation. The lid was automatically manipulated during sampling.

The generated 192 samples (two 96-well UPLC plates) were then subjected to centrifugation at 3000 rpm (Megafuge 1.0R, Heraeus), in order to induce sedimentation of any remaining enzyme precipitates. The samples were then introduced to a UPLC instrument for quantitative analysis of drug degradation.

A set of 32 samples was analyzed in each experiment, which allowed the simultaneous screening of 4 pancreatin concentrations (0, 1, 2 and 5 mg/mL) in 3 biorelevant media ($fasted_{6.5}$, $fed_{6.5}$ and $fed_{5.0}$) in duplicate per drug.

2.2.2. *Ex vivo* human intestinal fluid experiments

Experiments with human intestinal fluids were performed with previously collected aspirates (Riethorst et al., 2016), which were stored at a -25 °C freezer. Pools from aspirates collected as a function of time (each 10 min for 90 min, see Riethorst et al. 2016 for more details) from the same individual were used. Due to the very limited sample volumes, a small-volume protocol for studying drug degradation was developed. Adjustment of pH was also performed in order to: (1) fix the pH at a constant value, solving the issue with the endogenous bicarbonate buffer which causes a gradual increase of pH (due to the loss of $CO_2$ from the solution), (2) remove the uncertainty originating from interindividual differences in pH, which may impact strongly the enzymatic degradation and (3) perform the tests at pH values that have been used to study fasted (pH = 6.5) and fed state conitions (pH = 5.0). All experiments were performed in duplicate.

For each experiment, the intestinal fluid (either fasted- or fed-state) of a specific healthy volunteer was thawed and 2.5 mL were pipetted to a plastic tube. Afterwards, the pH was adjusted to 5.0 with glacial acetic acid (for the fed state samples) or to 6.5 with 2 M MES buffer solution (for the fasted state samples). The dilution of the samples caused by the pH adjustment protocol was < 10 %. Then, 200 µL of the intestinal fluids were pipetted to 24 wells in a low-evaporation 96 well plate (360 µL total well volume). The empty space between the wells was filled with water to increase the relative humidity inside the plate and further reduce evaporation. Then, 2 µL drug stock solution in DMSO was added to each well, the plate lid was put on top and shaking at 600 rpm at $T$ = 37 °C was started on a Thermostar microplate shaker (BMG Labtech GmbH). The same concentrations of drug stock solutions and the same dilution factor as in the *in vitro* screening

experiment were used, yielding the same final drug concentrations of 41.7 µg/mL PA and CA, 250 µg/mL TDF and 10 µM CC.

Sampling was performed at $t$ = 15, 45, 90 and 120 min, by aspirating 20 µL from each well by a 12-channel micropipette and transferring the samples to 12 eppendorf tubes, which were pre-filled with 180 µL of solvent (83 % acetonitrile, 17 % water). Hence, the final composition of the sample matrix was equivalent to the *in vitro* screening (75 % acetonitrile, 25 % water). Afterwards, the samples were centrifuged for 5 min at 20000×$g$ to remove the precipitated enzymes, then 150-200 µL of the supernatant were transferred to HPLC vials with 200 µL glass inserts and the samples were analyzed by HPLC.

### *2.3. Analytical methods*

#### 2.3.1. UPLC analysis after *in vitro* drug degradation

The samples were analyzed by using an Acquity UPLC H-Class System (Waters, USA). The system comprised of a quaternary solvent manager, a sample manager with flow-through needle (SM-FTN) design, column heater, mobile phase pre-heater and a photodiode array (PDA) detector. An Acquity UPLC® BEH C18 column with pore size of 130Å, particle size of 1.7 µm, inner diameter of 2.1 mm and 50 mm length was used for analysis. Injection volume of 2 µL was used. All studied drugs and enzymatic degradation products were analyzed by using a binary gradient of 0.1 % TFA and acetonitrile at a 0.6 mL flow rate. For PA, P, CA, TDF and the tenofovir monoester, the gradient started from 5 % of acetonitrile at $t$ = 0 min, then hold at initial conditions until 0.8 min, increased to 100 % acetonitrile at 2.8 min, hold at 100 % acetonitrile until 3.1 min, followed by a decrease to 5 % acetonitrile at 3.2 min and hold until 4.0 min (total run duration of 4.0 min). The PDA detector was set to measure the absorbance between 210 and 400 nm and the wavelength of maximum absorbance was selected for quantitation of each compound (245 nm for PA and P; 242 nm for CA; 258 nm for TDF and the tenofovir monoester). A slightly different protocol was used to determine CC and C: the gradient started from 30 % of acetonitrile at $t$ = 0 min, then hold at initial conditions until 0.1 min, linearly increased to 100 % acetonitrile at 5.3 min, hold at 100 % acetonitrile until 6.3 min, followed by a decrease to 30 % acetonitrile at 6.4 min and hold until 7.5 min (total run duration of 7.5 min). Quantitation of both CC and C was obtained by using the data at 254 nm. All drugs were quantified by preparing a standard calibration curve in the same solvent mixture (75 % acetonitrile + 25 % water) as the analyzed samples. All calibration curves were linear with $R^2 \geq 0.99$.

#### 2.3.2. HPLC analysis after drug degradation in human intestinal fluids

The samples from the human intestinal fluid drug degradation experiments were analyzed by using a Hitachi Chromaster HPLC system (VWR International) consisting of a 5160 pump, a 5260 autosampler, 5440 FL fluorescence detector and 5410 UV detector. A Novapak C18 column under radial compression (4 µm, 8 × 100 mm, Waters, Milford, MA, USA) was used to separate the injected analytes (sample injection volume of 20 µL). TDF was analyzed by using gradient elution: start at 15 % acetonitrile and 85 % 0.1 % trifluoroacetic acid (TFA) in water, hold until

0.5 min, then increase to 80 % acetonitrile until 0.7 min, hold until 4.0 min, revert to 15 % acetonitrile until 4.2 min and hold until 7.0 min. The details for the chromatographic analysis of the other drugs studied by isocratic elution are described in Table 2. All drugs were quantified by preparing a standard calibration curve in the same solvent mixture (75 % acetonitrile + 25 % water) as the analyzed samples. There were no peak overlaps from the matrix and the peak shape, as well as the retention time of the standards and the analyzed samples were the same. All calibration curves were linear with $R^2 \geq 0.99$.

**Table 2**. Isocratic analysis conditions of human intestinal fluid samples by HPLC.

| Drug | Acetonitrile | 0.1 % TFA | *t*, min | Detection |
|---|---|---|---|---|
| Candesartan cilexetil & candesartan | 45 % | 55 % | 10 | Fluorescence, $\lambda_{EX}$: 250nm, $\lambda_{EM}$:380 nm |
| Cortisone acetate | 45 % | 55 % | 10 | UV, $\lambda_{ABS}$: 242 nm |
| Prednisolone acetate & prednisolone | 45 % | 55 % | 10 | UV, $\lambda_{ABS}$: 245 nm |

## 3. Results

### *3.1. In vitro screening of enzymatic degradation*

#### 3.1.1. Effect of substrate and enzyme concentration

Classical enzyme kinetics analysis requires an understanding of the link between the reaction rate and the enzyme and substrate concentrations. CC was selected as model drug for this part of the study and its degradation was assessed at concentrations of 10, 20 and 30 µM. Due to the very poor aqueous solubility of CC, high bile salt and phospholipid concentrations were required to keep the drug in solution. Hence, fed-state conditions (FeSSIF 6.5) were selected as a biorlevant medium. The concentration of enzymes was varied by studying CC degradation at pancreatin concentrations of 1, 2 and 5 mg/mL.

CC degradation was governed by the enzyme concentration, whereas the substrate concentration had no significant effect (Figure 2). Chemical degradation (base or acid-catalyzed hydrolysis) did not occur, as evidenced by constant concentration of the blank (no pancreatin) samples. The reaction followed first-order kinetics at all conditions studied, as evidenced by the linear dependence of the logarithm of drug concentration scaled with the initial drug concentration ($C/C_0$) on time. Therefore, the reaction rate constant and degradation half-life could be extracted from the slope of each curve (Table S1 in the Supporting information). Analysis of these results in the framework of the Michaelis-Menten description of enzyme kinetics indicates that the experimental conditions used in the study are representative for the substrate-rich region of the general enzyme kinetics curve (Michaelis et al., 2011): hence, the reaction kinetics of CC is determined primarily by the concentration of enzyme, whereas it is insensitive to the substrate concentration.

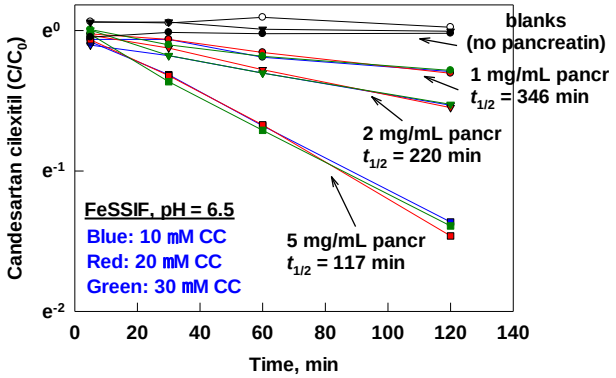



**Figure 2**. Enzymatic degradation of Candesartan cilexetil as a function of reaction time at 10 µM (blue line), 20 µM (red line) and 30 µM (green line) drug, and at 1 mg/mL (circles), 2 mg/mL (triangles) and 5 mg/mL (squares) pancreatin. Blank samples (no pancreatin added) for 10, 20 and 30 µM are also shown (black lines). Each drug concentration is scaled with the corresponding initial drug concentration ($C_0$) to allow proper analysis of the degradation kinetics ($n$=2). The error bars can be smaller than the symbols.

Therefore, the systematic experiments with all drugs that are described in the following section were performed at a constant drug concentration and different enzyme (pancreatin) concentrations.

3.1.2. Effect of biorelevant medium type and drug structure

The enzymatic degradation of the studied drugs at 1, 2 and 5 mg/mL pancreatin at fasted (pH = 6.5) and fed state conditions (pH = 6.5 or 5.0) is described in the current section. Similarly to the case of CC, the rate of drug degradation followed a first-order reaction kinetics which allowed the extraction of the degradation rate constant. The interpretation of the decrease of drug concentration as enzymatic hydrolysis was confirmed by comparing drug disappearance with the appearance of the reaction products (candesartan for CC and prednisolone for PA, see Figure S1 in the Supporting information). The contribution of chemical degradation (non-enzymatic hydrolysis) was negligible for all studied drugs, as determined from experiments in absence of pancreatin (Figure S2 in the Supporting information). The results showed that the degradation rate increased linearly with the increase of pancreatin concentration at all conditions, for PA, CA and TDF (Figure 3). Exception from this rule was CC at fed conditions at pH = 5.0: in this case, there was no effect of the pancreatin concentration and a plateau was reached at around 40 % degradation (Figure S3 in the Supporting information).

Comparison of the degradation rate at fasted and fed state conditions (FaSSIF 6.5 vs. FeSSIF 6.5) showed that the higher bile salt and phospholipid concentrations in the fed state slow down the enzymatic drug degradation. The relative decrease in the degradation rate was highest for PA (two-fold decrease), followed by CA and CC, whereas the effect was much smaller for TDF.

To evaluate the effect of pH, the enzymatic drug degradation at pH = 5.0 and pH = 6.5 was compared at fed state conditions (FeSSIF 5.0 vs. FeSSIF 6.5). In this case, only TDF degradation

was significantly affected: a four-fold increase in the rate constant was observed at 5 mg/mL pancreatin. In contrast, the pH had negligible effect on the degradation of PA and CA. Experiments at pH = 5.0 were not performed with CC.

In order to directly compare the degradation stability of the different drugs the degradation half-lives ($t_{1/2}$) were calculated from the rate constants. In the fasted state, TDF was degraded by the enzymes very quickly, with $t_{1/2}$ varying from 11.1 to 71.6 min depending on the pancreatin concentration (Table 3). In contrast, the enzymatic degradation of PA and CC was significantly slower, as evidenced by the $t_{1/2}$ = 33.5-149.1 min and 48.6-109.7 min for PA and CC, respectively. The degradation of CA was the slowest, with $t_{1/2}$ = 93.8-677.6 min. Hence, at fasted state conditions, the enzymatic degradation rate of the studied drugs decreased in the order TDF >> PA > CC > CA. This ranking was confirmed also by the fed state data at pH = 6.5 and 5.0. The reproducibility of the reported data was good, as demonstrated by the calculated median and mean coefficient of variation of the half-lives (3.3 % and 5.1 %, respectively).

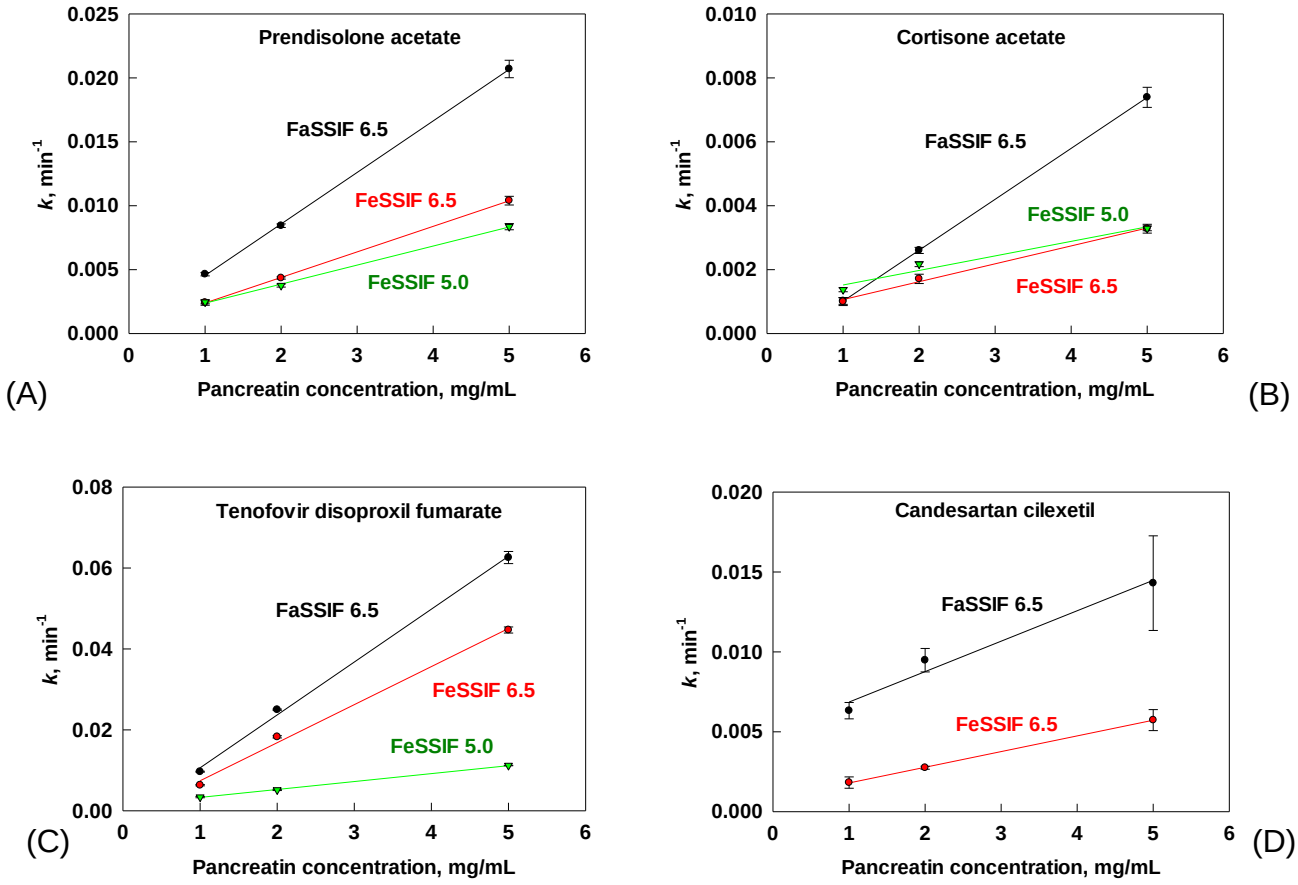



**Figure 3**. Degradation rate constants as a function of pancreatin concentration for (A) prednisolone acetate, (B) cortisone acetate, (C) tenofovir disoproxil fumarate and (D) candesartan cilexetil in $FaSSIF_{6.5}$ (black), $FeSSIF_{6.5}$ (red) and $FeSSIF_{5.0}$ (green).

The reported half-lives can be put into perspective if the timescale of degradation is compared to the average residence time of drugs in the small intestine, which is in the order of 180-240 min (Vinarov et al., 2021). As the degradation half-lives at higher pancreatin concentrations are less than 180 min for all drugs studied, this indicates that their degradation may occur at small intestinal conditions, affecting drug absorption in cases where the physicochemical properties (*e.g.* lipophilicity, solubility) of the product(s) are significantly different from the parent compound. However, one could question whether the high pancreatin concentrations where the short half-lives were observed are biorelevant. To investigate this issue, experiments with human intestinal aspirates were performed and the results are described in the following section.

**Table 3**. Enzymatic degradation half-life of the studied drugs in biorelevant media.

| | **Half-life ($t_{1/2}$), min** | | | | | |
|---|---|---|---|---|---|---|
| **Pancreatin** | **FaSSIF 6.5** | | **FeSSIF 6.5** | | **FeSSIF 5.0** | |
| **(mg/mL)** | **AVG** | **SD** | **AVG** | **SD** | **AVG** | **SD** |
| | | | **Prednisolone acetate** | | | |
| 1 | 149.1 | 4.2 | 285.2 | 24.9 | 280.5 | 16.0 |
| 2 | 82.2 | 1.4 | 159.3 | 3.3 | 184.2 | 5.9 |
| 5 | 33.5 | 1.1 | 66.6 | 2.2 | 82.8 | 2.4 |
| | | | **Cortisone acetate** | | | |
| 1 | 677.6 | 71.4 | 695.1 | 84.2 | 506.2 | 23.2 |
| 2 | 267.0 | 9.8 | 404.4 | 34.2 | 319.4 | 11.3 |
| 5 | 93.8 | 4.0 | 211.3 | 8.9 | 210.6 | 4.8 |
| | | | **Tenofovir disoproxil fumarate** | | | |
| 1 | 71.6 | 0.8 | 109.2 | 2.7 | 204.3 | 9.6 |
| 2 | 27.7 | 0.2 | 37.9 | 0.6 | 133.8 | 4.3 |
| 5 | 11.1 | 0.3 | 15.5 | 0.3 | 61.8 | 0.3 |
| | | | **Candesartan cilexetil** | | | |
| 1 | 109.7 | 8.9 | 345.5 | 30.8 | nd | |
| 2 | 73.2 | 5.6 | 219.9 | 27.6 | nd | |
| 5 | 48.6 | 10.1 | 117.1 | 3.4 | nd | |

*nd: not determined*

### *3.2. Degradation kinetics in human intestinal fluids*

The results for the enzymatic degradation kinetics of PA, CA, TDF and CC in human intestinal fluid aspirates are presented in the current section. The impact of fasted vs. fed state, along with the effect of pH are described first (section 3.2.1), after which the interindividual variability of drug degradation in fasted and fed state conditions is presented (section 3.2.2).

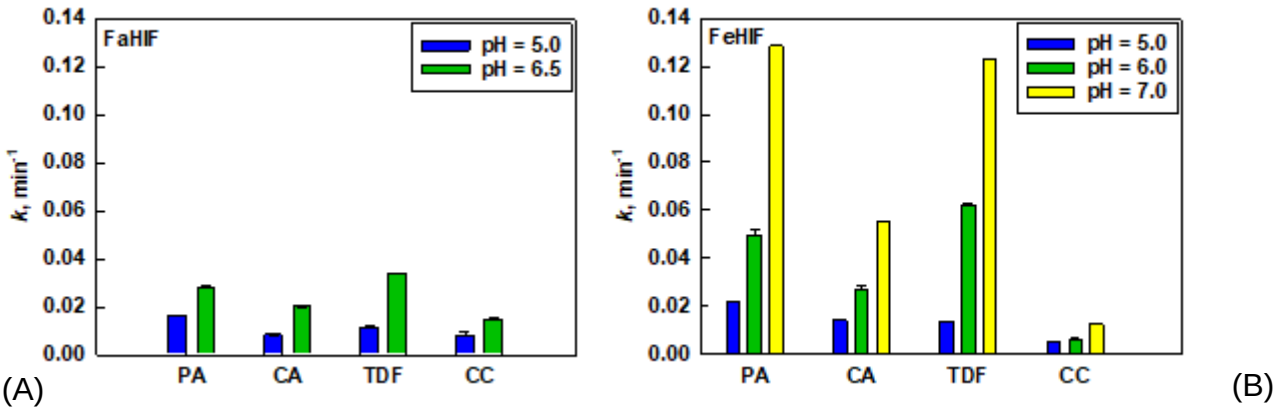
#### 3.2.1. Effect of pH and fasted vs. fed state

The experiments in simulated intestinal fluids containing pancreatic enzymes (section 3.1.2) showed that in some cases pH can impact the rate of drug degradation. To check if this is also the case *ex vivo*, the pH of pooled human intestinal fluid aspirates was adjusted and degradation experiments were performed at fasted (pH = 5.0 and 6.5) and fed state conditions (pH = 5.0, 6.0

and 7.0). The obtained degradation profiles followed first order kinetics (similarly to the experiments with simulated intestinal fluids), allowing us to extract the rate constant of the reaction.

The increase of pH significantly accelerated the enzymatic degradation of all drugs studied (Figure 4). A linear dependence between the logarithm of the rate constant in fed state intestinal aspirates and pH was established, indicating that the drug degradation rate increases exponentially with the increase of pH in the range of 5.0 to 7.0 (Figure S4 in the Supporting information). The reproducibility of the reported data was good, as demonstrated by the calculated median and mean coefficient of variation of the rate constants (2.8 % and 5.0 %, respectively).

The experiments performed with pancreatin in biorelevant media suggested another important effect: a decrease in the drug degradation rate when switching from fasted to fed state. To check if this is also confirmed *ex vivo*, drug stability in human aspirates collected in the fasted and fed state were compared. The pH of both pools was adjusted to 5.0. In contrast to *in vitro* data, drug degradation was significantly faster in fed state aspirates for PA and CA, with a modest effect for TDF, whereas a slight decrease (close to the experimental error) was observed for CC (Figure 4A and B). Head-to-head comparison of the effect of fasted/fed state on degradation is presented also in Figure S5 in the Supporting information.




(A) (B)

**Figure 4**. Degradation rate constant of the studied drugs in (A) fasted and (B) fed state human intestinal fluids at different pH.

3.2.2. Interindividual variability

The composition of biological fluids varies significantly between individuals, implying functional differences that may impact oral absorption and bioavailability. To check to what extent the variable composition of human intestinal fluids can influence drug degradation, aspirates from nine fasted state and eight fed state volunteers were evaluated. To account for the different pH of the samples, the fasted state aspirates were set to pH = 6.5, whereas the fed state aspirates were set to pH = 5.0.

(A) Fasted state

The time-dependent drug degradation in fasted state human intestinal fluids is presented in Figure 5. The biggest interindividual variability was observed for PA and CA degradation (Figure 5A and 5B): the intact drug after 120 min incubation varied between 80 % and 0 %, depending on the individual. However, the individuals could be separated in two subgroups: a slow-metabolism group (70-85 % intact drug at $t$ = 120 min), comprised of healthy volunteer (HV) 5 and HV9, while the rest of the individuals formed a subgroup with faster metabolism (0-25 % intact drug at $t$ = 120 min).

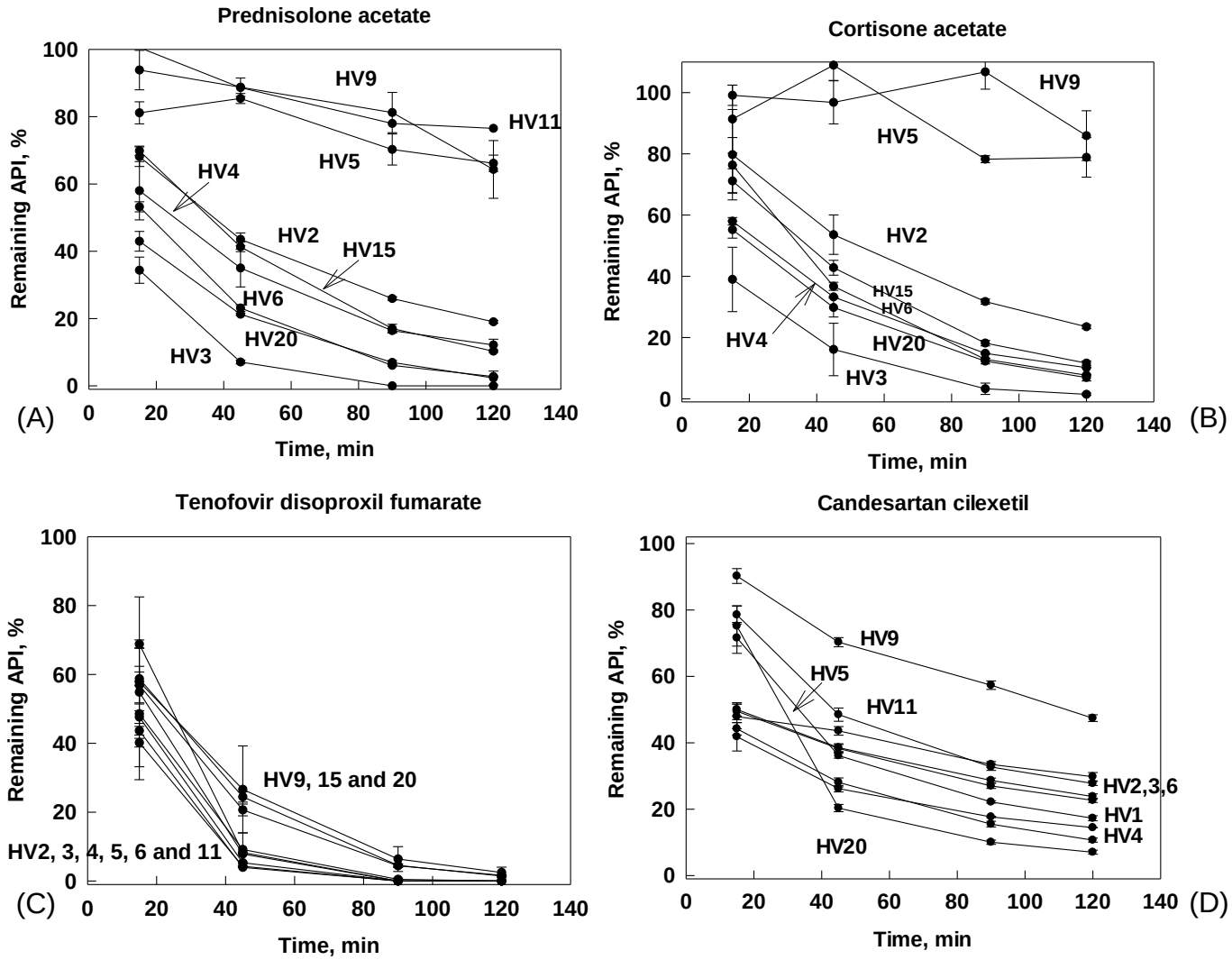



**Figure 5**. Interindividual variability of drug degradation in fasted state human intestinal fluids for (A) prednisolone acetate, (B) cortisone acetate, (C) tenofovir disoproxil fumarate and (D) candesartan cilexetil. The pH of all fasted state aspirates was set to 6.5. For each individual, the intestinal fluids were pooled from time-dependent aspirates. $n$ = 2.

In contrast, the degradation of TDF showed much lower variability, with the drug being almost completely degraded in all studied fasted aspirates after 120 min incubation (Figure 5C). By using the data obtained at $t$ = 45 min, the individuals could again be separated in a slow- (≈ 25 % intact drug, HV9, HV15 and HV20) and fast-metabolism subgroups (≈ 5 % intact drug, all other HVs).

CC showed an intermediate variability profile, with the percentage of intact drug varying between 10 and 50 % after 120 min incubation (Figure 5D). One of the studied aspirates (HV9) showed considerably slower degradation of CC (50 % intact CC at $t$ = 120 min), compared to the rest of the set (10-30 % intact CC at $t$ = 120 min). The obtained results were in agreement with the degradation profile of CC in pooled fasted state human intestinal fluids as determined by Borde et al (Borde et al., 2012).

(B) Fed state

The degradation of the studied drugs in fed state human aspirates is presented in Figure 6. Similarly to the fasted state, the interindividual variability at $t$ = 120 min was dependent on the type of drug with the percentage of intact drug varying as follows: 0 to 20 % for PA, 0 to 40 % for CA, 0 to 35 % for TDF and 30 to 75 % for CC. However, much bigger variability was observed at shorter timescale ($t$ = 15 min), where the remaining drug varied between 10 and 80 % for PA, 20 to 95 % for CA, 35 to 90 % for TDF and 55 to 100 % for CC.

The dataset could also be studied for potential grouping (fast/slow metabolism) of the studied HVs. However, only two major observations could be reported: (1) all studied drugs were degraded very quickly in the aspirated fluids of one of the studied individuals (HV2) and (2) for TDF, HVs 1, 10 and 14 showed slower TDF degradation, whereas HV 3, 6, 8 and 9 metabolized TDF significantly faster.

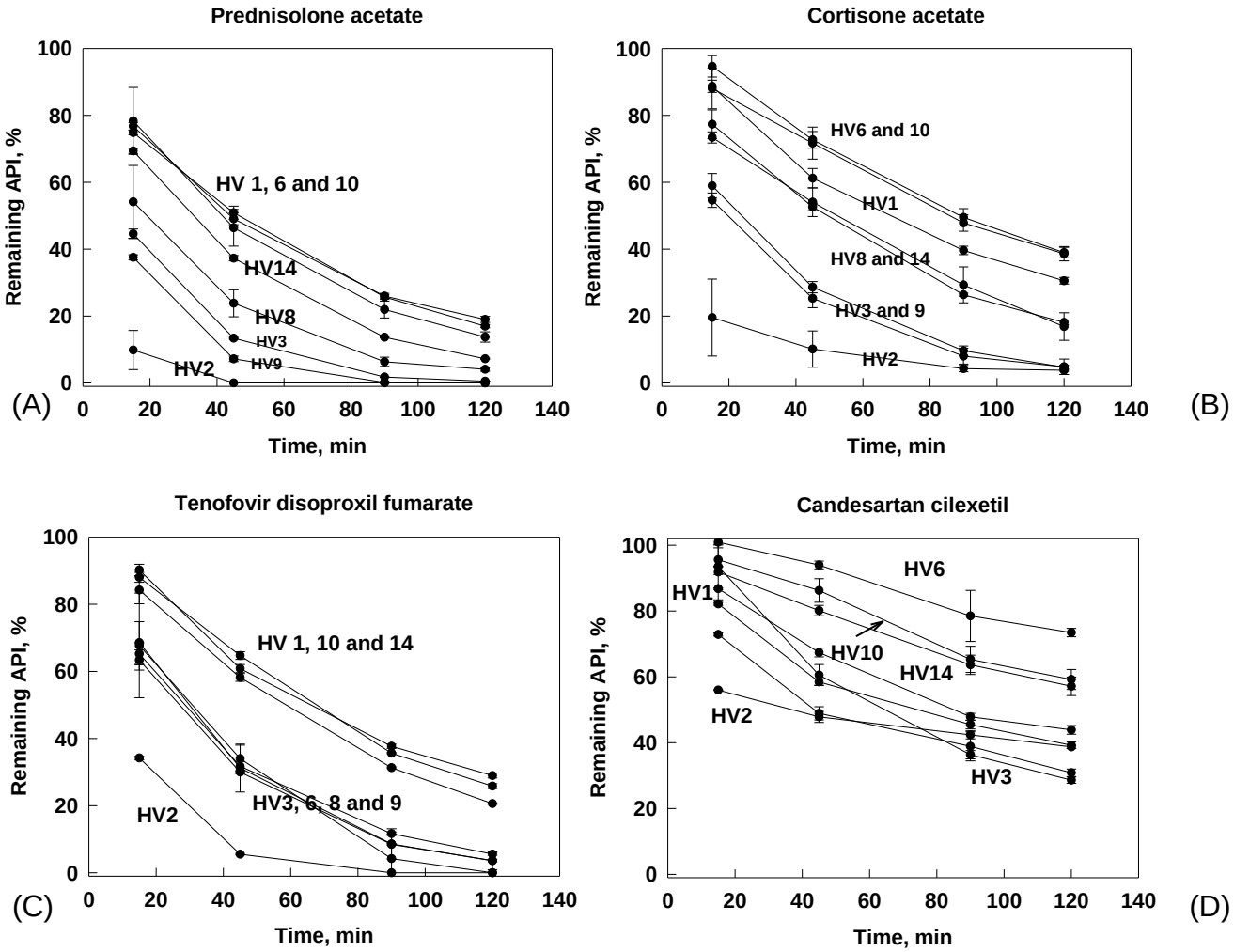



**Figure 6**. Interindividual variability of drug degradation in fed state human intestinal fluids for (A) prednisolone acetate, (B) cortisone acetate, (C) tenofovir disoproxil fumarate and (D) candesartan cilexetil. The pH of all fed state aspirates was set to 5.0. For each individual, the intestinal fluids were pooled from time-dependent aspirates. $n$ = 2.

(C) Variability analysis of human aspirates data and comparison to *in vitro* results

The obtained data for the drug degradation in individual human aspirates can be used to both assess the interindividual variability *in vivo* and to check if it can be encompassed by the used *in vitro* screening protocol. The drug half-life ($t_{1/2}$) was extracted from the kinetic data gathered in fasted and fed aspirates based on the first-order reaction kinetics model and statistical analysis was performed (Table 4). The large dispersion of the data and the non-normal distribution indicated that the parameters usually used to compare variance between datasets (*e.g.* the coefficient of variance) cannot be applied in this case. Alternatively, an analogue of the coefficient of variance which provides better description of such datasets can be used (Arachchige et al., 2022). Instead of the ratio of the standard deviation to the mean (STDEV/mean), the authors show that more robust results are obtained when dividing the interquartile range (IQR) by the median, yielding

$RCV_Q$ = 0.75×IQR/median. The numerical coefficient is used to standardize the obtained values to the normal distribution (Arachchige et al., 2022).

**Table 4**. Statistical analysis of the enzymatic degradation half-life of the studied drugs in fasted (pH = 6.5) and fed state (pH = 5.0) human aspirates.

| Enzymatic degradation half-life in aspirated human intestinal fluids, min | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Prednisolone acetate** | | **Cortisone acetate** | | **Tenofovir disoproxil fumarate** | | **Candesartan cilexetil** | |
| | **$FaHIF_{6.5}$** | **$FeHIF_{5.0}$** | **$FaHIF_{6.5}$** | **$FeHIF_{5.0}$** | **$FaHIF_{6.5}$** | **$FeHIF_{5.0}$** | **$FaHIF_{6.5}$** | **$FeHIF_{5.0}$** |
| **MED** | 45.7 | 32.0 | 40.1 | 53.0 | 18.0 | 30.7 | 71.3 | 124.0 |
| **AVG** | 109.6 | 32.3 | 120.6 | 53.9 | 17.2 | 36.5 | 80.4 | 137.1 |
| **GM** | 62.5 | 28.7 | 64.2 | 49.2 | 16.6 | 30.6 | 71.8 | 125.8 |
| **IQR** | 166.3 | 24.8 | 83.4 | 40.7 | 7.6 | 28.1 | 47.8 | 67.6 |
| **$RCV_Q$, %** | 272.8 | 58.1 | 156.0 | 57.6 | 31.6 | 68.8 | 50.3 | 40.9 |

MED – median; AVG – average; GM – geometric mean; IQR – interquartile range;
$RCV_Q$ – coefficient of variance, 0.75*IQR/MED;

For most of the studied systems, the coefficient of variance $RCV_Q$ falls in the range from 31.6 to 68.8 %, with the exception of PA and CA in the fasted state (272.8 and 156.0 %, respectively). As shown in Figure 5A and 5B in the previous section, two groups of drug metabolizers could be clearly identified for PA and CA in the fasted state, yielding a bimodal distribution of the degradation rate constants. Hence, the extremely slow PA and CA degradation measured for the slow metabolizers leads to a large increase of the IQRs, which results in significantly increased $RCV_Q$ values.

In the fasted state, TDF showed the smallest half-life variability with an $RCV_Q$ of 31.6 % and very similar values of the average, median and geometric mean (16.6 to 18.0 min), whereas PA and CA displayed much bigger variation (272.8 and 156.0 %, respectively) due to the slow and fast metabolizer groups. The variation for CC ($RCV_Q$ of 50.3 %) was closer to the values measured for TDF. The variation decreased in the fed state, with values ranging between 40.9 and 68.8 % for all drugs studied. Smallest variation was measured for CC ($RCV_Q$ of 40.9 %), followed by PA and CA ($RCV_Q \approx 58$ %) and TDF ($RCV_Q$ of 68.8 %).

Switching from fasted to fed state had a different effect on the degradation rate, depending on the studied drug (Figure 7). Large decrease in variability was observed for PA and CA, with $RCV_Q$ decreasing from 272.8 and 156.0 % (respectively) to values around 58 %. The opposite was observed for TDF, for which $RCV_Q$ increased more than two-fold. Weak effect of the fasted vs. fed state was measured for CC, for which $RCV_Q$ decreased slightly from 50.3 to 40.9 %.

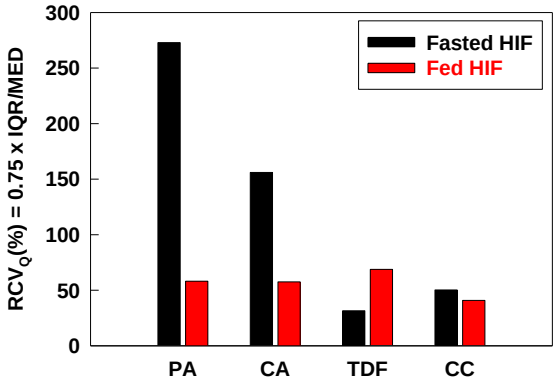



**Figure 7**. Effect of the fasted- and fed-state on the variability of drug degradation kinetics in human intestinal fluids for prednisolone acetate, cortisone acetate, tenofovir disoproxil fumarate and candesartan cilexetil, displayed as the coefficient of variation $RCV_Q$ = 0.75×IQR/median (Arachchige et al., 2022).

Apart from the interindividual variability, the fasted/fed state can also impact the rate of drug degradation itself. Such analysis of the obtained dataset showed that the switch from fasted to fed state decreased the median, average and geometric mean half-life of PA and CA, whereas the opposite was observed for TDF and CC (Table 3). Hence, fed state degradation was quicker for PA and CA and slower for TDF and CC. To assess the impact of drug structure on degradation, we should then consider separately the results in the fasted and the fed state. Due to the relatively smaller data variability in the fed state, the obtained results showed a clear picture: the degradation half-life increased in the order TDF ≈ PA < CA < CC. However, the large dispersion of the results for CA and PA degradation in the fasted state led to significant differences between the calculated median, average and geometric mean half-life, confounding the interpretation. When the average values are considered (which places a bigger weight on the few very slow CA and PA metabolizers), the degradation half-life increased in the order TDF < CC < PA ≈ CA, whereas if the median is considered (bigger weight on the most frequent half-life values), then the order shifted to TDF < PA ≈ CA < CC. A general conclusion that emerged for the presented analysis was the TDF was degraded very quickly (compared to the other drugs) at both the fasted and the fed state.

After characterizing drug degradation in human intestinal fluids, it is interesting to check if the observed degradation rates and variability (visualized by the half-life) can be encompassed by the enzymatic *in vitro* screening assay used in the current study (Figure 8). The used range of pancreatin concentrations (from 1 to 5 mg/mL) covers relatively well the *in vivo* variability in the degradation rate of all drugs in the fasted state, whereas only the degradation of TDF was completely covered in the fed state. In all cases but one (CC-fed state), the drug degradation rate measured *in vitro* was slower, compared to the results obtained in human intestinal fluids.

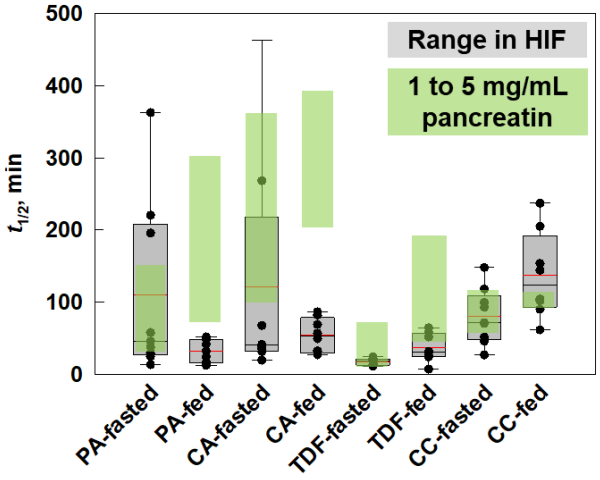



**Figure 8**. Comparison between the enzymatic degradation half-lives measured in human intestinal fluids (in grey) and in pancreatin-based biorelevant media (in green). The pH is 6.5 for the fasted state and 5.0 for the fed state for both the intestinal fluid aspirates and the biorelevant media.

## 4. Discussion

### *4.1. Screening of drug degradation in pancreatin-based media: scope and limitations*

A major question which appears when studying enzymatic drug degradation by *in vitro* methods relates to their bio-relevance: how close is the observed drug degradation profile to the real situation *in vivo*? One of the possible ways to address this is by comparing the pancreatin-based *in vitro* results to *ex vivo* measurements in human intestinal fluids. However, such studies use pooled intestinal fluids (Borde et al., 2012; Malmborg and Ploeger, 2013; Stappaerts et al., 2015; Wang et al., 2015; Yadav et al., 2016) – hence, the information about the interindividual variability of drug degradation is lost, which oversimplifies the data interpretation.

By complementing the characterization of interindividual variability of drug degradation in human intestinal fluids with results for the pancreatin concentration-dependent drug degradation in biorelevant media (Figure 8), the usefulness of the commonly applied *in vitro* screening methods can be evaluated. The presented data shows that due to the drug-to-drug differences in both the rate of degradation and the extent of variability in human intestinal fluids, it is difficult to define a "biorelevant" range of pancreatin concentrations that will adequately reproduce the *in vivo* degradation profile of all drugs. In addition, further insight is needed to understand better the reasons for the large interindividual variability in the degradation profile of some of the drugs and not others. Therefore, in the context of pharmaceutical development it could then be more useful to define a pancreatin concentration which is representative for the "worst-case scenario", *viz.* a concentration which matches the "quick metabolizers" in the 25$^{th}$ percentile of the obtained half-lives.

At the same time, the cases of slower drug degradation by pancreatin, as compared to human intestinal fluids (*e.g.* CA degradation), suggest the use of higher pancreatin concentrations. For the

same reason, concentrations of 40 mg/mL pancreatin have been used *in vitro*, without reproducing the fast degradation rate measured in human aspirates (Borde et al., 2012). However, pancreatin does not dissolve well in aqueous medium even at the highest concentration used in the current study (5 mg/mL). Hence, the slower degradation in pancreatin might not be due to its concentration, but to the lack of enzymes that are otherwise present in the intestinal fluids. Such enzymes may originate from the shedding of the intestinal walls/mucosa – a factor, which is not modelled in standard pancreatin-based drug degradation studies. Hence, the option to combine pancreatin with additional enzymes that represent mucosal and cellular metabolism when performing *in vitro* studies of drug degradation should be considered in the future.

### *4.2. Effect of bile salts/phospholipids on drug degradation*

The increase of bile salt and phospholipids concentration (FaSSIF vs. FeSSIF) in the pancreatin-based biorelevant media decreased strongly the degradation rate of the studied poorly water-soluble drugs (CA, PA and CC), whereas the effect was smaller for TDF (section 3.1.2). The opposite trend was observed regarding the effect of pH: biggest impact was measured for TDF (faster degradation at higher pH), whereas the effect was negligible for the rest of the studied drugs.

The described results demonstrate a possible link between the physicochemical properties of the drug molecules (aqueous solubility/lipophilicity) and the drug degradation rate. The decreased degradation rate of poorly water-soluble drugs in presence of higher concentrations of bile salts and phospholipids could be explained by their micellar solubilization: once the drug molecules are incorporated in the micelles, they are protected from the enzyme action due to decreased chemical potential and the more difficult formation of the enzyme-substrate complex. Very similar effect of solubilization-mediated protection of prodrugs (ganciclovir esters) from chemical and enzymatic hydrolysis via complexation with hydroxypropyl-β-cyclodextrin was reported by Tirucherai & Mitra (Tirucherai and Mitra, 2003). The authors also found that the degradation rate decreased with increasing solubilizer concentration, and that the effect was more pronounced for the more hydrophobic prodrugs, further corroborating the results in the current study. Fayed et al. showed that the stability of CC to enzymatic degradation by rabbit intestinal mucosa was enhanced in a concentration-dependent manner by the Tween 80 surfactant (Fayed et al., 2016). Therefore, *in vitro* experiments indicate that the solubilization of drug molecules in self-assembled aggregates (micelles, vesicles) or molecular containers (*e.g.* cylcodextrins) suppresses intestinal drug degradation. However, experiments with more drugs that encompass a wider chemical space are needed to define a quantitative link between the drug physicochemical properties and the degradation rate constant.

Although results obtained in simple aqueous media (Hammad and Muller, 1999; Hoppe and Sznitowska, 2014), enzyme-based biorelevant media (Tirucherai and Mitra, 2003) and mucosal models (Bali et al., 2016; Fayed et al., 2016) indicate that solubilization decreases the drug degradation rate, this has not been confirmed yet with human intestinal fluids. The major issue is the lack of data: the results for drug degradation in human aspirates are not accompanied by chemical analysis of the concentration of bile salts, phospholipids and enzymes. In the current

study, the concentration of bile salts and phospholipids in the used intestinal fluids was previously characterized (Riethorst et al., 2016). However, no direct correlation with the drug degradation rate was found (data not shown), most likely because a key factor – the concentrations of the enzymes – was missing. Hence, human intestinal fluids have to be thoroughly characterized in order to study the effect of solubilization on drug degradation *ex vivo*.

### *4.3. Effect of pH on drug degradation*

The faster degradation at higher pH, which was visualized very well in the experiments with human intestinal fluids, could be explained with the pH optimum of the carboxyl esterase (CES): pH = 6.5 for CES 1 and pH = 7.5 – 8.0 for CES2 (Wang et al., 2018). Tissue distribution studies of CES in humans show that CES2 is found at high concentrations in the small intestine, whereas CES1 is almost completely missing and is instead found in the liver (Di, 2019; Wang et al., 2018). Therefore, CES2 which has higher optimal pH is the main enzyme responsible for the hydrolysis of ester prodrugs in the human intestinal fluids. The latter is in excellent agreement with the strong increase of the drug degradation rate constant when increasing the pH from 5.0 to 7.0 in human intestinal fluids observed in the current study (Figure 4). The contribution of chemical degradation to the observed enzymatic hydrolysis is negligible, as determined by simulated intestinal fluid experiments in absence of pancreatin (Figure S2 in the Supporting information). The differences in the effect of pH between the pancreatin-based *in vitro* screening and the results from the human intestinal fluids could then be explained by considering that the pH optima and relative concentration of CES1 and CES2 in the porcine pancreatin are not known. Hence, the presence of CES1 or CES2 in porcine pancreatin with lower pH optimum (compared to human CES2) could account for the smaller effect of pH on drug degradation *in vitro*.

### *4.4. Potential impact of degradation variability on drug pharmacokinetics*

The results presented in section 3.2.2 showed a considerable inter-individual variability in the enzymatic degradation rate of the studied drugs. This is also illustrated by the coefficient of variation of the drug half-life, which ranges from 31.6 to 272.8 %, depending on the type of drug and fasted- vs. fed-state conditions (Table 3). An important question which arises in this context is whether the individual differences in drug degradation may lead to clinically relevant changes in drug pharmacokinetics (PK).

Based on first principles, one can imagine that impact on PK may be expected for prodrugs, which have been specifically engineered to enhance oral absorption. This is the case for TDF, which has extremely low permeability in its non-esterified form (tenofovir) obtained by enzymatic degradation (Geboers et al., 2015; van Gelder et al., 2002). Physiologically-based biopharmaceutics modelling has been used to explore the impact of TDF intestinal degradation on its PK and the results have clearly showed that TDF oral absorption is limited by the action of the intestinal enzymes (Moss et al., 2017). Thus, the observed relative coefficients of variance of TDF half-life (68.8 and 31.6 % in the fed and fasted state, respectively) could be identified as a primary driver for the interindividual variability in oral PK studies of TDF, where coefficients of variance around 20 % have been established for AUC and $C_{max}$ (Chittick et al., 2006).

## 5. Conclusions

The enzymatic degradation of four ester-type drugs was studied at fasted and fed conditions by an automated, pancreatin-based *in vitro* screen and by *ex vivo* experiments in human intestinal fluids. The *in vitro* screen was successfully used to generate time-dependent profiles of drug degradation, which followed first-order reaction kinetics and allowed the extraction of the kinetic rate constant and half-life. The strong dependence of the rate constant on the enzyme concentration indicated that the reaction takes place in the substrate-rich region of the Michaelis-Menten enzyme kinetics analysis. Drug degradation in human intestinal fluids displayed large inter-individual variability, with coefficients of variance $RCV_Q$ generally ranging between 30 and 70 %, but increasing above 150 % in two cases. The fasted versus fed state was observed to increase, decrease or to have no effect on the inter-individual variability depending on the type of drug. The increase of pH in the range between 5.0 and 7.0 significantly accelerated the degradation rate of the studied drugs both in the *in vitro* and *ex vivo* experiments, in agreement with the higher pH optimum of the CES-2 enzyme. In contrast, the increase of bile salt and phospholipid concentrations in the *in vitro* screen decreased strongly the degradation rate of the more hydrophobic drugs, which may be solubilized in the micelles and thus partially protected from the enzymes. The developed *in vitro* degradation screening methodology covers relatively well the *in vivo* variability in the degradation rate of all drugs in the fasted state, whereas only the degradation of TDF was completely covered in the fed state. Hence, the *in vitro* screening can be used to generate a database of drug degradation rate constants in the fasted state, bridging the knowledge gap between the drug molecular structure and enzymatic stability. In addition, the revealed large interindividual variability of drug degradation will be essential to further refine and develop existing *in vitro* methodologies.

**Acknowledgements**

The authors thank Tom Ooms, Tom de Waal, Marlies Braeckmans, Désirée Beyens and Glenn Exelmans for the useful discussions and technical assistance. The financial support of VLAIO project HBC.2018.2293 is acknowledged. Z.V. gratefully acknowledges the support of the Bulgarian Ministry of Education and Science, under the National Research Program "VIHREN-2021", project 3D-GUT (№ KP-06-DV-3/15.12.2021).

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