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Research paper

Assessment of enzymatic prodrug stability in human, dog and simulated intestinal fluids

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ABSTRACT

The aim of this study was to determine the stability of three ester prodrugs, chloramphenicol succinate, enalapril and candesartan cilexetil, in human proximal small intestinal fluid (HIF), dog proximal small intestinal fluids (DIF) and simulated intestinal fluid (FaSSIF), with the addition of pancreatin. The total protein content in the proximal jejunal fluids was determined in HIF and DIF, respectively. Candesartan cilexetil was significantly degraded in HIF (initial $t_{1/2(0-5min)}$ = 5.4 ± 0.5 min) and in DIF (initial $t_{1/2(0-5min)}$ = 5.7 ± 0.1 min), while chloramphenicol succinate and enalapril were stable in both media. The degradation of candesartan cilexetil was shown to be mediated by enzymes following Michaelis–Menten enzyme kinetics and was inhibited by addition of esterase inhibitors. The enzymatic capacity reflected by V_{max} was 4-fold higher in DIF than in HIF and correlated to its 2-fold higher protein concentration. The degradation of candesartan cilexetil in the FaSSIF-pancreatin solution was slower ($t_{1/2}$ = 207 ± 34 min) than the degradation in both HIF and DIF. Changing the pH to the enzyme optima or increasing the amount of pancreatin, increased the degradation rate of candesartan cilexetil, but not in the magnitude as in HIF. As a result, two *in vitro* models, based on *in vivo* intestinal fluids, were developed using candesartan cilexetil as a model drug. The DIF seems to be a reasonably good model for HIF, although the degradation capacity seems to be somewhat higher, possibly due to the higher enzyme concentration in DIF. Future investigations will develop novel enzymatic based *in vitro* models for rapid assessment and biopharmaceutical screening tools for prodrugs.

1. Introduction

Oral drug delivery is the dominating route used in medical treatment and prediction of the rate, extent of drug absorption and bioavailability are crucial processes for a successful pharmaceutical formulation development. During the last two decades there has been a substantial increase in the understanding of the mechanisms of the relevant absorption processes such as dissolution, transit, permeability and first-pass metabolism [1–5]. However, there has been less focus on the stability of drugs and prodrugs in the intestinal lumen. Drug degradation is important to investigate at an early stage of drug development in order to understand limitations in drug absorption, which is necessary for accurate predictions of clinical doses in man, development of speciality delivery systems and administration through alternative routes. Luminal stability issues become especially important for prodrugs, since this is a drug delivery approach often used to enhance membrane permeation of hydrophilic and poorly absorbed drugs by increasing the lipophilicity of the parent compound and/or target a certain membrane transporter. Application of this drug design approach intends to increase the transmembrane transport by passive diffusion and/or carrier-mediated transport [6,7]. Instability in the intestinal lumen is often associated with chemical (pH-dependent) instability and/or enzymatic degradation in the upper gut, where the latter is the focus of the current work. In the small intestine enzymes such as amylases, peptidases, proteases and esterases are secreted into the lumen. Carboxyl esterases secreted from the pancreas into the intestinal lumen are most often associated with enzymatic hydrolysis of prodrugs [8]. Stoeckel et al. showed that poor bioavailability of cephalosporins may be due to enzymatic hydrolysis of the prodrugs in the intestinal juice [9]. Degradation in intestinal fluid has also been shown for the prodrugs of acyclovir and danazol [10,11]. Brouwers et al. showed that fosamprenavir was rapidly degraded in human intestinal fluid (HIF) [12]. Enzymatic cleavage of prodrugs before intestinal absorption could be avoided by investigating the stability of prodrugs and drugs in HIF. If this fluid is not available, animal intestinal fluid and/or simulated intestinal fluid may be used as surrogate models. For instance, the stability and *in vitro* metabolism of peptide model drugs have been studied in gastrointestinal

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fluids from humans and rats [13,14]. However, more comparative studies for degradation of drugs in real and simulated intestinal media are needed to increase the knowledge of different enzymatic *in vitro* models.

The objective of this work was to investigate and compare the stability of three ester prodrugs in HIF, dog intestinal fluid (DIF) and simulated human intestinal fluid (FASSIF), with the addition of pancreatin (FaSSIF-P). Further, a characterisation of the protein content, and identification of proteins, was done in HIF and DIF in order to evaluate the observed enzymatic activity in the investigated intestinal fluids.

2. Materials and methods

2.1. Model drugs

Three model ester prodrugs were used to develop an *in vitro* model to investigate the stability of prodrugs in intestinal fluids (Fig. 1) and to make a species comparison regarding degradation rate in intestinal fluid from dog and man. The model drugs were chloramphenicol succinate (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), candesartan cilexetil (AstraZeneca R&D, Mölndal, Sweden) and enalapril (Sigma–Aldrich Chemie GmbH, Steinheim, Germany, the maleate salt of enalapril was used). All the substances selected contained an ester bond between active drug and prodrug moiety. Following oral administration, they are bioactivated by hydrolysis of the ester groups during the first-pass process to enalapril, chloramphenicol and candesartan (Fig. 1). Chloramphenicol succinate is an antibiotic produced by Streptomyces venezuele and acts as an inhibitor of proteins synthesis in bacteria and to a lesser extent in eukaryotic cells. Because of its serious side-effects it is now reserved to life-threading infections. Enalapril is a highly potent inhibitor of angiotensin-converting enzyme (ACE) and it is used in the therapy for heart failure and hypertension. Candesartan cilexetil is a nonpeptide angiotensin II receptor antagonist and is used in the treatment of hypertension [15].

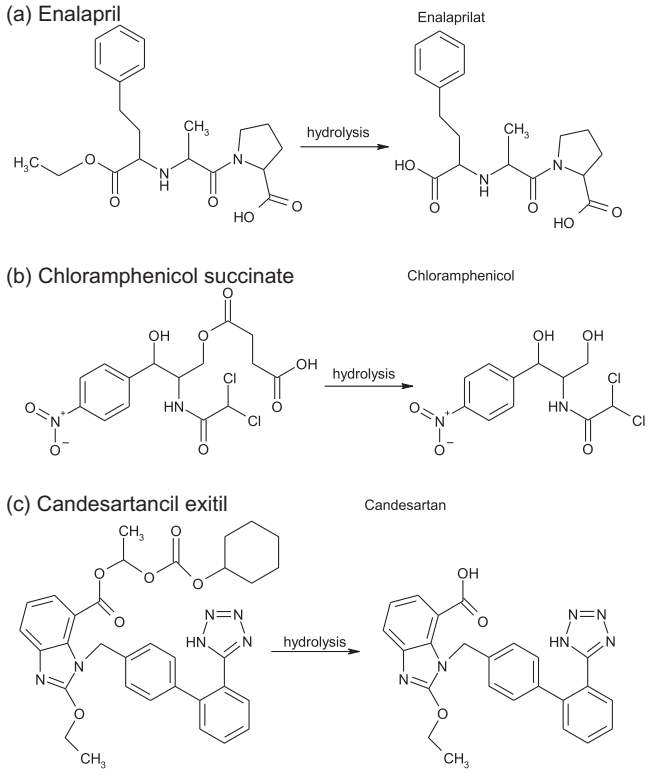

Fig. 1. Chemical structure and degradation pattern of (a) enalapril, (b) chloramphenicol succinate and (c) candesartan cilexetil.

2.2. In vivo collected and simulated intestinal fluids

The sampling of the HIF was performed at the Clinical Research Department of the University Hospital in Uppsala, Sweden, and was approved by the Ethics Committee of the Medical Faculty at Uppsala University. HIF was sampled from 15 healthy individuals, aged 18–40 years. All the subjects had normal clinical and laboratory values and none of the subjects received any medication before or on the day of fluid collection. Before the collection, the subjects had fasted for at least 10 h. An intestinal intubations device (Loc-I-Gut®) was used when collecting intestinal fluid from upper jejunum [16,17]. In Loc-I-Gut®, a tube is introduced through the mouth and once the perfusion tube is in place, two balloons are inflated, creating a 10-cm-long jejunal segment. A vacuum pump is connected to the proximal drainage of the channel of the perfusion tube to drain HIF above the perfused segment. The perfusate (HIF) leaving the jejunal segment was collected in 10-min fraction on ice and immediately frozen at −70 °C. Three batches, sampled with a 2 years interval, were used for the stability experiments in this study.

Fasting DIF was collected from four male Labrador dogs having a chronic fistula at mid-jejunum (approx 76 cm below pylorus). The dogs had fasted from 9 a.m., the day prior to collection. Before DIF collection, all dogs were given 75 ml of a 0.9% NaCl-solution to stimulate the production of intestinal fluid. The fluids were pooled, centrifugated at 4 °C and 3000 rpm for 10 min and stored at −70 °C. The sampling was approved by the Animal Ethics Committee in Gothenburg, Sweden. Three pooled batches ($n = 4$), sampled with a 4 weeks interval between them, and one batch ($n = 1$) sampled and immediately used, were used for the experiments in this study.

FaSSIF was prepared according to the method presented by Galia and coworkers [18]. Pancreatin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was added to the FaSSIF in concentrations of 10 mg/ml and 40 mg/ml respectively, to obtain a simulated media for stability testing (FaSSIF-P). The former pancreatin concentration is suggested by United States Pharmacopoeia 2009 in simulated intestinal fluid [19]. Since pilot testing indicated rather low solubility of pancreatin (the solution was opaque even at low concentrations), 40 mg/ml was used as a 'suspension media'. To investigate whether different pH values affect the enzyme activity, a FaSSIF-P solution with the same amount of pancreatin, 10 mg/ml, but at different pH values were prepared. The pH was adjusted to pH 5.5, 6.5 or 7.5 with 1 M sodium hydroxide or 1 M hydrochloric acid.

2.3. Study design

2.3.1. Preparation of stock solutions

The stock solutions of chloramphenicol succinate (50 μM) and enalapril (200 μM) were prepared in FaSSIF. The stock solutions of candesartan cilexetil was prepared in ethanol (Kemetyl AB Haninge, Sweden) and diluted with FaSSIF (1:9) to a concentration of 10–500 μM. Only ethanol was used in the concentration range 500–20,000 μM for making stock solution (used for the concentration–degradation graph). No effect on the enzymatic degradation in HIF was observed using either only FaSSIF or pure ethanol for the preparation of stock solutions of candesartan cilexetil (data not shown).

2.3.2. Drug stability testing in intestinal fluids

HIF, DIF and FaSSIF were taken from the freezer and preincubated (900 μl) in an Eppendorf thermomixer comfort (Eppendorf AG, Hamburg, Germany) at 37 °C and 650 rpm, for 45 min ($n = 2$). The incubation was started by adding 100 μl stock solution of one of the model prodrug. Samples of 100 μl were withdrawn after 0.5, 5, 15, 30 and 60 min and 100 μl of ice cold acetonitrile containing 0.5 vol.% formic acid, to stop the enzymatic activity, was added [20]. The samples were vortexed and centrifuged (Rotina 46R, Hettich, Tuttlingen, Germany) at 2°C and 10 000 rpm, for 10 min. The prodrug, and drug for candesartan, content in the supernatant was analysed by HPLC. The stability of candesartan cilexetil was also investigated in FaSSIF-P.

The degradation of candesartan cilexetil in HIF and DIF, in the concentration interval 1–2000 μM, was investigated in order to determine the enzymatic kinetic parameters V_{max} and K_M. The experiments were performed as described above.

2.3.3. The effect of enzyme inhibitors

Experiments to inhibit the degradation were carried out with candesartan cilexetil in DIF ($n = 2$). Two esterase inhibitors, ethylenediaminetetra acetic acid (EDTA, 5 mM in water, Merck GR Analyse, Darmstadt, Germany) and phenylmethanesulfonylfluoride (PMSF, 0.2 mM in ethanol, Sigma–Aldrich Chemie GmbH, Steinheim, Germany), were added (10 μl) to 900 μl DIF five minutes prior to the start of the incubation [21].

2.3.4. The effect of freezing, thawing and long time storage

The effect of short-term freezing and thawing on the enzyme activity in the intestinal fluids was investigated by determining the stability of candesartan cilexetil in completely fresh DIF and in DIF that had been stored at −70 °C for one and four weeks, respectively ($n = 2$). The effect of long-term storage of intestinal fluid at −70 °C was investigated by determining the stability of candesartan cilexetil in three different batches of HIF, ba1 (four years old), ba2 (2 years old) and ba3 (sampled just prior to the study and frozen at −70 °C).

2.4. Identification of proteins in HIF and DIF

2.4.1. Separation of proteins by gel electrophoresis

Three different batches of DIF (baA, baB and baC) and three different batches of HIF (ba1, ba2 and ba3) were investigated for their respective content of total and individual proteins. The total protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, US), in accordance with the description provided by the manufacturer. The individual proteins were separated under reducing conditions with SDS–polyacrylamide gel electrophoresis (SDS–PAGE). NuPAGE® Bis-Tris gradient gels (4–12%, Novex, San Diego, CA, US) was used with NuPAGE® MES as running buffer.

The gels were stained with SyproRUBY® protein gel stain and scanned using a Molecular Imager FX system and Quantity One software (Bio-Rad, München, Germany). Protein bands were excised from the DIF and HIF gels, de-stained and in-gel digested with porcine trypsin, which cleaves proteins specifically after lysine and arginine sites. Digestion with trypsin was performed over night and tryptic peptides were extracted using 1% acetonitrile and 1% formic acid. The resulting peptides were analysed on an Applied Biosystem 4700 Proteomics Analyser MALDI-TOF/TOF mass spectrometer in reflector mode (Applied Biosystems, CA, US). Precursor ions for MS/MS analysis were automatically selected by the 4700 software, and MS/MS was performed with collision-induced dissociation. MS/MS data analysis was performed by the use of MASCOT software (www.matrixsciences.com) through the GPS Explorer™ Software (Applied Biosystems, CA, US).

2.5. Specific enzyme activity

2.5.1. Protease activity

EnzChek® protease assay kit (Molecular probes, Eugene, OR, US) was used to investigate the protease activity in HIF, DIF and in FaSSIF-P solution with two different concentrations of pancreatin at 10 mg/ml and 40 mg/ml. EnzChek® protease assay kit contains casein derivates labelled with pH-insensitive red-fluorescent BODIPY® TR-X dye-labelled peptides with an excitation/emission maxima of 589/617 nm. A 100 μl of the casein solution from the assay kit, 2–15 μl DIF, HIF or FaSSIF-P solution (10 mg/ml and 40 mg/ml pancreatin) and FaSSIF was dispersed to each well of a microplate reader to receive a final volume of 200 μl. The microplate was immediately placed in a Spectramax Gemini fluorometer (Molecular Devices, CA, US) preheated to 37 °C. The protease activity was measured for one hour, and the software SoftMax Pro (Molecular Devices, CA, US) was used to calculate the activity (V_{max}).

2.5.2. Carboxyl-ester lipase activity

Para-nitrophenyl acetate (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was used as substrate when investigating carboxyl-ester lipase activity in HIF, DIF and FaSSIF-P solution with 10 mg/ml and 40 mg/ml pancreatin [22]. Para-nitrophenyl acetate was dissolved in methanol and then diluted with FaSSIF (1:50) to receive a 2 mM stock solution. To a 100 μl stock drug solution, 10–30 μl HIF¸ DIF or FaSSIF-P solution and FaSSIF were added to each wells of a micro plate reader to receive a final volume of 150 μl. The micro plate was immediately placed in a spectrophotometer (Spectramax Gemini, Molecular Devices, CA, US) preheated to 37 °C. The absorbance was measured at 400 nm during five minutes, and the software SoftMax Pro was used to calculate the activity (V_{max}).

2.6. Drug assay

Candesartan cilexetil, chloramphenicol succinate, enalapril (and their corresponding drug form) samples from the stability experiments were immediately analysed with an Alliance 2690 separations module connected to a 2487 Dual λ Absorbance Detector (Waters, Milford, MA, US). Chromatographic data were collected and evaluated by the software Millennium (Waters, Milford, MA, US). All samples were quantified with external standards. The injection volume varied between 10 and 80 μl dependent on the concentration of the drug in the sample.

In the analysis of candesartan cilexetil and its acid candesartan (AstraZeneca R&D, Mölndal, Sweden), an XTerra™ RP 8 column (3.5 μm, 100 ∗ 3.9 mm (Waters)) was used and the flow rate was set to 0.8 ml/min. The mobile phase used was a mixture of acetonitrile/0.4 mM acetic acid (50:50 v/v). The detector wavelength was set to 254 nm. For the analysis of chloramphenicol succinate and chloramphenicol, a Zorbax Eclipse XDB C_{18} column (5 μm, 150 ∗ 4.6 mm (Agilent)) was used and the flow rate was set to 1.0 ml/min. Detection was done at 278 nm and the mobile phase consisted of a mixture of acetonitrile/phosphate buffer, pH 6.2 (25:75 v/v). Enalapril and enalaprilat were analysed at 215 nm using a Zorbax Eclipse XDB C_{18} column (5 μm, 150 ∗ 4.6 mm (Agilent)). The column was kept at 60 °C by a column heater (Croco-cil, Scantec Lab AB, Sweden). The flow rate was set to 1.0 ml/min, and a binary mobile phase, consisting of phosphate buffer, pH 3, (A) and acetonitrile (B), was used for the analysis. The gradient started at 78% A, at which it was held for 4 min, and thereafter decreased to 50% A in 11 min.

The limit of quantification (LOQ) for candesartan cilexetil and candesartan was 0.1 μM, and the standard curves were linear in the range 0.5–100 μM. Samples with a higher concentration than 100 μM were diluted with mobile phase. The reproducibility, ex-

pressed as the percentage relative standard deviation (%RSD) for the standards used in the analysis sequence, was always below 5%. The standards were prepared in its, respectively, mobile phase.

2.7. Data evaluation

All results are presented as means from duplicate ($n = 2$) experiments if not stated differently. The degradation rate was characterised as degradation half-lives calculated by linear regression of the % remaining parent drug vs incubation time.

The maximum degradation rate (V_{max}) and the drug concentration providing half maximum degradation rate (K_M), were calculated from the Michaelis–Menten equation (Eq. (1)):

$$v_0 = \frac{V_{max} \cdot [S]}{K_M + [S]} \quad (1)$$

there the degradation rate (v_0) was determined from the first 5 min of incubation in the experiments with candesartan cilexetil using different drug concentrations (S).

3. Results

3.1. Degradation of model drugs in HIF

Both chlorampenicol succinate and enalapril were almost stable in HIF (<10% degraded in 60 min), whereas there was an initial (first 5 min) rapid degradation of candesartan cilexetil ($t_{1/2(0–5\ min)} = 5.4 \pm 0.5$ min) followed by a slower degradation phase ($t_{1/2(5–60\ min)} = 49.2 \pm 0.5$ min) (Fig. 2a). The degradation of candesartan cilexetil in HIF was accompanied by the formation of candesartan, which demonstrated mass-balanced degradation on a molar basis (Fig. 2b). Since only candesartan cilexetil was found to be significantly degraded in the HIF, only this drug was used in the subsequent experiments in this study.

3.2. Stability of candesartan cilexetil in real intestinal and simulated fluids

The rate of *in vitro* metabolism of candesartan cilexetil in HIF, DIF, FaSSIF and FaSSIF–P is shown in Fig. 3. There was no degradation of candesartan cilexetil in FaSSIF, while there was a clear degradation in both HIF and DIF (Fig. 3, Table 1). The degradation in HIF and DIF was characterised by two phases, one initial phase (the first 5 min) with a rapid degradation ($t_{1/2(0–5\ min)} = 5.4 \pm 0.5$ and 5.7 ± 0.1 min, respectively) followed by a slower phase of degradation ($t_{1/2(5–60\ min)} = 49 \pm 0.5$ and 43 ± 2.8 min, respectively). The degradation rate calculated for phase I and II was similar in HIF and DIF (Table 1). The initial degradation rate, calculated during the first five minutes, for different start concentrations of candesartan cilexetil in HIF and DIF, plotted against the concentration of candesartan cilexetil in HIF and DIF, is shown in Fig. 4. The calculated V_{max}- and K_M-values were 4–5 times higher ($V_{max} = 95 \pm 17$ nmol/min (DIF), 22 ± 3 nmol/min (HIF) and $K_M = 660 \pm 287$ μmol/l (DIF), 141 ± 65 μmol/l (HIF), respectively, in DIF compared to HIF.

Addition of 10 and 40 mg/ml pancreatin to FaSSIF, lead to a $t_{1/2(0–5\ min)} = 119 \pm 6.0$ and 54 ± 5.6 min, respectively, of candesartan cilexetil (Fig. 3, Table 1). Only one phase was observed for the degradation of candesartan cilexetil in FaSSIF-P. The degradation rate calculated from this phase (0–60 min) was significantly slower compared to HIF and DIF (Table 1). Investigation of the effect of changing the pH (from 5.5 to 7.5) in pancreatin containing solutions (10 mg/ml) on the degradation of candesartan cilexetil showed that the degradation of candesartan cilexetil was higher in a FaSSIF-P solution with pH 5.5 than in a solution with a higher pH (Fig. 5).

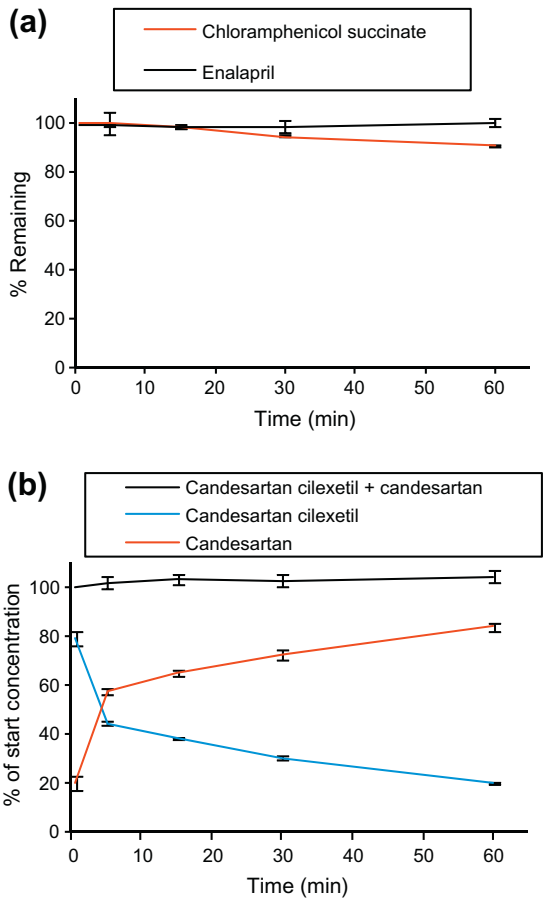

Fig. 2. Stability of the model drugs (a) chloramphenicol succinate and enalapril and (b) candesartan cilexetil and the formation of candesartan expressed as mean per cent remaining (±SD) during 60 min of incubation in human intestinal fluid ($n = 2$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

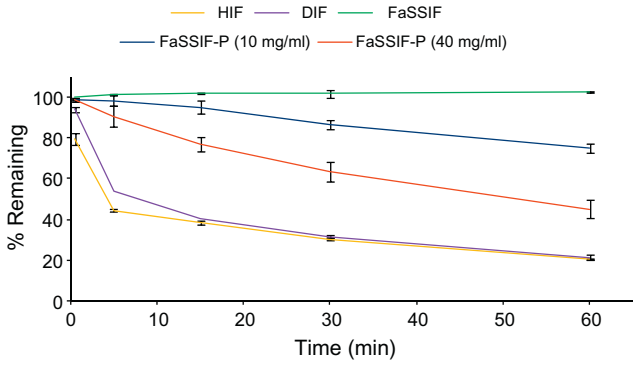

Fig. 3. Stability of candesartan cilexetil expressed as mean per cent remaining (± SD) during 60 min of incubation in human intestinal fluid (HIF), dog intestinal fluid (DIF), FaSSIF and FaSSIF-P containing 10 or 40 mg/ml pancreatin, respectively ($n = 2$). The concentration of candesartan cilexetil in the stability experiments in HIF, DIF, FaSSIF and FaSSIF-P was 50 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Effect of enzyme inhibition

The degradation of candesartan cilexetil in DIF containing the esterase inhibitors, PMSF and EDTA was almost inhibited. A 84 ± 4% of the start concentration was left after 60 min (Table 1).

Table 1
The mean (±SD) degradation rate ($t_{1/2}$) and the percentage remaining after 60 min for human intestinal fluid (HIF), dog intestinal fluid (DIF) with and without the enzyme inhibitors (PMSF and EDTA) and fasted simulated small intestinal fluid (FaSSIF) without and with the addition of pancreatin (FaSSIF-P) at 10 mg/ml or 40 mg/ml. The initial degradation phase is based on the values from the first 5 min and phase II on the values from 5 to 60 min ($n = 2$).

Media	% Remaining after 60 min	$t_{1/2}$ (min), Initial phase	$t_{1/2}$ (min), Phase II
FaSSIF	100 (0.2)	–	–
FaSSIF-P (10 mg/ml)	75.0 (2.2)	119 (6.0)	–
FaSSIF-P (40 mg/ml)	45.3 (4.5)	54 (5.6)	–
DIF	21.4 (1.2)	5.7 (0.1)	43.0 (2.8)
HIF	20.5 (0.5)	5.4 (0.5)	49.2 (0.5)
DIF + PMSF and EDTA	84.0 (3.6)	366 (27)	–

3.4. The effect of long- and short-term storage on enzyme activity

The effect of long- and short-term storage of intestinal fluid in freezer on the degradation of candesartan cilexetil is shown in Fig. 6. The results showed that there was no effect on the degradation rate of candesartan cilexetil in HIF stored for 2 or 4 years in the freezer compared to HIF that only had been frozen for some weeks (Fig. 6a). The degradation rate of candesartan cilexetil in DIF stored for 1 and 4 weeks in the freezer was slower, especially during the first five minutes, compared to the degradation in DIF that had not been frozen. The degradation rate in DIF stored in the freezer also appeared to be independent of the storage time (Fig. 6b, Table 2). The fresh DIF was not centrifuged before the experiment was carried out since it should be as fresh and unaffected as possible. However, the fluid was centrifuged to remove solid material before it was frozen. Consequently, the content in the frozen DIF was not

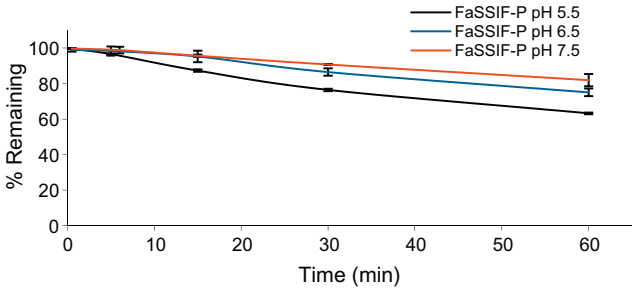

Fig. 5. Stability of candesartan cilexetil expressed as mean per cent remaining (±SD) during 60 min of incubation in FaSSIF-pancreatin solutions (10 mg/ml) with three different pH values, pH5.5, 6.5 and 7.5 ($n = 2$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

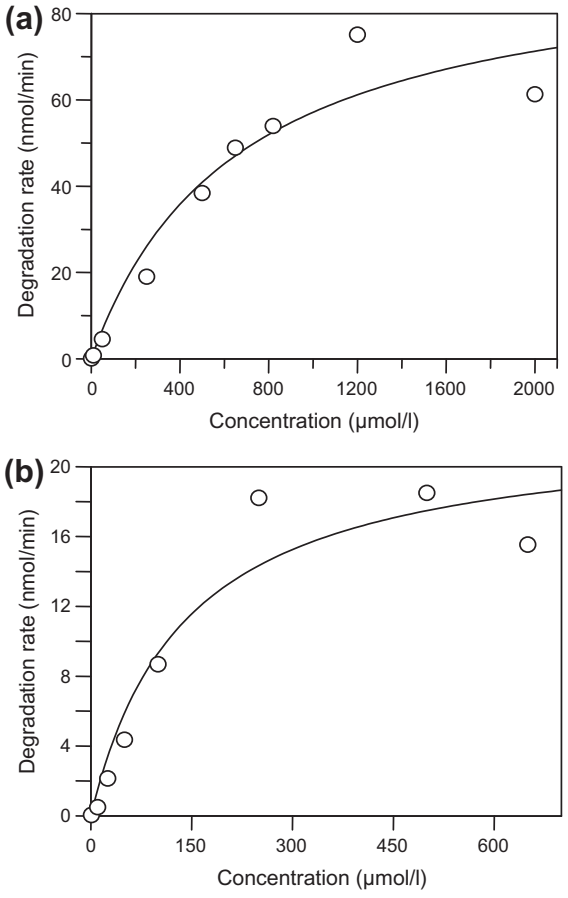

Fig. 4. Michaelis–Menten plots of degradation rate vs. drug concentration for the first 5 min of candesartan cilexetil in (a) dog intestinal fluid (DIF) and (b) human intestinal fluid (HIF).

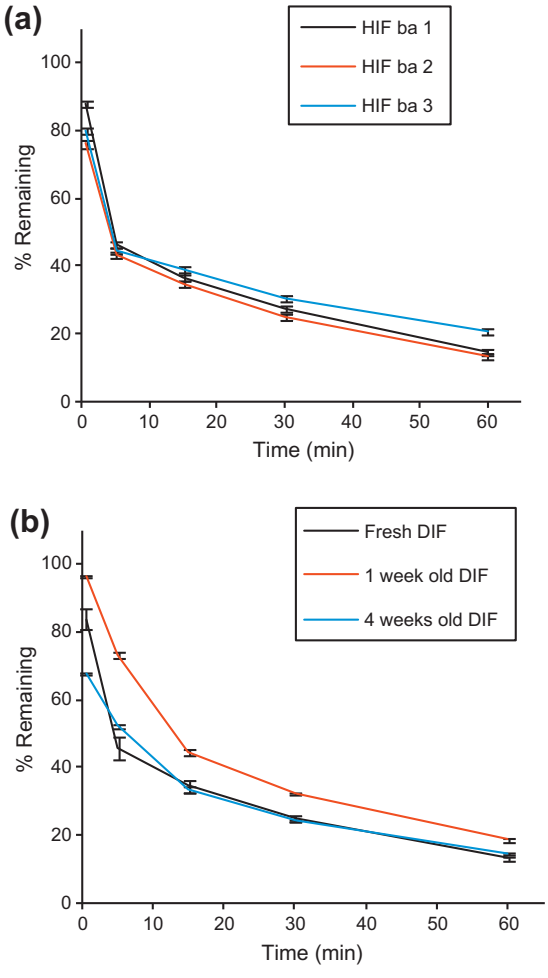

Fig. 6. Degradation of candesartan cilexetil during 60 min of incubation in (a) three different batches of human intestinal fluid (HIF) (ba1 was 4 years old, ba2 was 2 years old and ba3 was fresh, but had been frozen) and (b) dog intestinal fluid (DIF) stored for 4 weeks, 1 week and fresh (immediately used after collection). The values are presented as mean ± SD ($n = 2$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The mean (±SD) degradation rate ($t_{1/2}$) in three different batches of human intestinal fluid (HIF) and dog intestinal fluid (DIF). The initial degradation is based on the values from the first 5 min and phase II on the values from 5 to 60 min ($n = 2$).

Intestinal fluid	$t_{1/2}$ (min), Initial phase	$t_{1/2}$ (min), Phase II
HIF, fresh	5.4 (0.5)	49.2 (0.5)
HIF, 2 years	5.9 (0.3)	32.3 (0.5)
HIF, 4 years	4.8 (0.0)	32.7 (0.7)
DIF, fresh	5.1 (0.4)	30.7 (1.1)
DIF, 1 week	11.4 (0.8)	29.4 (0.2)
DIF, 4 weeks	12.1 (0.4)	31.3 (0.2)

exactly the same as in the fresh DIF and the potential difference in the content might have affected the result.

3.5. Identification of proteins in HIF and DIF

The total protein concentration was approximately twice as high in DIF as in HIF. The mean values (±SD) were 5.6 ± 1.8 and 2.4 ± 0.3 mg/ml ($p < 0.05$, $n = 3$), respectively. pH for the HIF batches were 6.7 ± 0.2 and in DIF 7.1 ± 0.4 ($n = 3$). Both the results

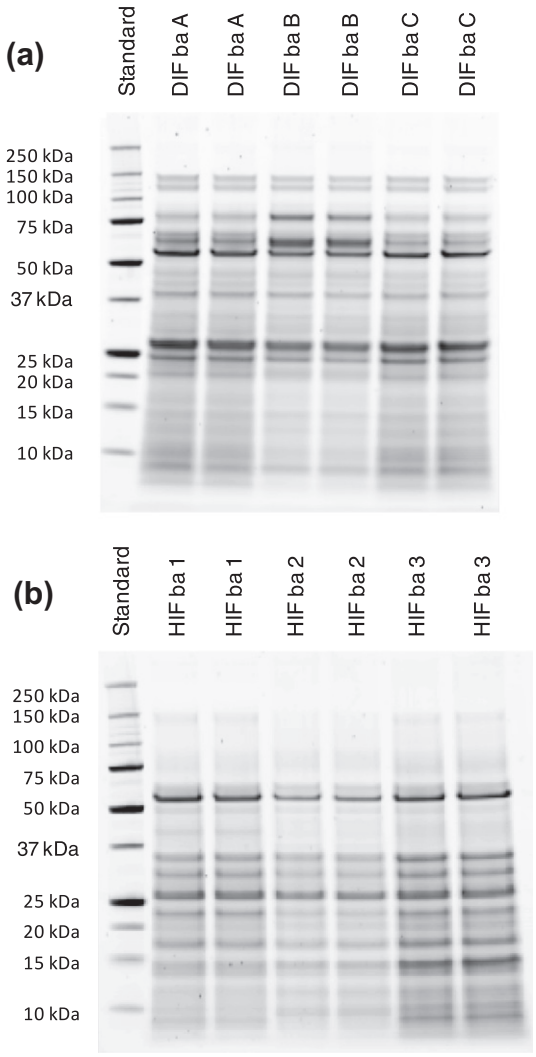

Fig. 7. Electrophoretic patterns of three different batches (a) dog intestinal fluid (DIF) and (b) human intestinal fluid (HIF) obtained by NuPAGE® Bis-Tris gradient gels and stained with SyproRUBY® protein gel stain.

Table 3
Protease and carboxyl-ester lipase activity in human intestinal fluid (HIF) and dog intestinal fluid (DIF) and fasted simulated small intestinal fluid containing 10 or 40 mg/ml pancreatin, respectively (FaSSIF-P). Protease activity was measured using an EnzChek® protease assay kit. Carboxyl-ester lipase activity was measured at 400 nm with p-nitrophenyl acetate as substrate ($n = 2$).

Media	V_{max} (abs unit/time)	
	Protease	Carboxyl-ester lipase
HIF	0.139	0.003
DIF	0.177	0.002
FaSSIF-P (10 mg/ml)	0.071	0
FaSSIF-P (40 mg/ml)	0.111	0.001

from protein concentration determination and pH measurement are similar to previous reported values [1,22–24]. From the SyproRUBY® stained SDS–PAGE-gels, several distinct protein bands were visualised for both DIF and HIF samples, with protein molecular weights up to 150 kDa, but a high background was also observed (Fig. 7). The image analysis showed little variations between the three replicates for each species. Apparently DIF ba B and HIF ba 2 appear to be slightly reduced compared to others of unknown reasons.

Six bands were excised from the DIF gel for identification by MS analysis, ranging from 20 to 80 kDa. These were identified as IgA heavy and light chains, secretory component of polymeric Ig receptor and pancreatic enzymes such as alpha-amylase, caldecrin and anionic trypsin. When comparing the gel bands in DIF and HIF gels, only two protein bands were found to correspond partly to the same proteins: alpha-amylase around 56 kDa and IgA light chain around 27 kDa. Several protein bands were found to correspond to a mixture of proteins, and several proteins were present in a partly degraded form, especially in HIF. The HIF bands were more easily identified due to sequence availability in well-annotated databases such as Uniprot (www.uniprot.org). Among the proteins identified in HIF were Ig A heavy and light chain, alpha-amylase, carboxypeptidase A1, carboxypeptidase B, elastase IIA, elastase IIIA, anionic trypsin, phospholipase A2, triacylglycerol lipase and lithostathine 1 alpha.

3.6. Investigation of specific enzyme activity in intestinal fluids

The V_{max} for proteases and carboxyl-ester lipase in HIF, DIF and the two FaSSIF-P solutions are shown in Table 3. The proteases in DIF showed the highest V_{max} followed by the proteases in HIF. The 10 mg/ml FaSSIF-P solution showed the lowest V_{max}, while the FaSSIF-P solution with a higher pancreatin concentration (40 mg/ml) had a V_{max} close to the one in HIF.

The carboxyl-ester lipase activity showed almost the same trend as for protease activity, but HIF had a higher activity compared to DIF (Table 3). In the FaSSIF-P solution with a low pancreatin concentration (10 mg/ml), there was hardly any activity at all.

4. Discussion

One way to increase the intestinal absorption and bioavailability for some poorly absorbed drugs is by administration as prodrugs. The success of the prodrug ester approach critically depends on the solubility and permeability of the prodrug ester as well as its stability to chemical and enzymatic ester cleavage. It is important in drug development to design a prodrug that is degraded at the right site and rate. The cleavage of the ester bond is often supposed to take place in the enterocytes, liver and/or plasma after the drug has been absorbed. Instability in the gastrointestinal fluid may therefore jeopardise the successful transmucosal transport of the prodrug designed for permeability enhancement.

The present study was carried out to investigate the stability of ester prodrugs in HIF and to compare it with the stability found in DIF and simulated media (FaSSIF-P).

In this study, candesartan cilexetil was the only model drugs that was degraded in the HIF while both chloramphenicol succinate and enalapril were stable in the HIF. Interestingly, this shows that there is difference in substrate specificity between luminal and enteric/hepatic esterases since all drugs mainly appears as the active drug in plasma. Thus, by rational design of prodrugs, cleavage to active drug can be obtained at the desired location.

A clear degradation of candesartan cilexetil was observed in HIF and DIF; however, it was stable in FaSSIF, containing no enzymes. In addition, the degradation of candesartan cilexetil was accompanied with a mass-balanced formation of candesartan. This metabolic step was also inhibited by the addition of the esterase inhibitors, PMSF and EDTA. This indicates that the degradation of candesartan cilexetil was due to enzymatic cleavage of the ester bond and not by changes in pH, light or temperature. PMSF and EDTA inhibit carboxyl esterases, cholin esterases and A-esterases, which may be responsible for the degradation of candesartan cilexetil in intestinal fluid. While Taketani et al. have shown that carboxyl esterase shows only a low hydrolase activity in microsomes from dog small intestine compared to human [25]. The results in this study imply that there is a hydrolase activity in both human and dog small intestinal fluid. Thus, in evaluating small intestinal stability, it is important to include both experiments performed in small intestinal fluid as well as in microsomes. The enzymes in the intestinal fluid could also come from dead gut wall cells, which will be abraded into the intestine. But since this is the same for both *in vivo* and in collected fluid, this reflects almost the same environment for *in vitro* drug degradation testing as in the body (oxygen level, etc. is of course different *in vivo* compared to *in vitro* testing with collected fluid). Improved understanding of the esterases in humans and relevant species, such as tissue localisation, substrate specificity and enzymatic capacity, will increase the successful development design of new prodrugs.

The recommendation today is that drug degradation should be determined in HIF, and where not available, in animal intestinal fluid or in USP simulated intestinal fluids, at 37 °C with an exposure time representative of *in vivo* [26,27]. The present study showed that the degradation pattern of candesartan cilexetil was qualitative similar in HIF and DIF, but the maximum rate of degradation (V_{max}) was approximately four times higher in DIF compared to HIF. This may be explained by the higher concentration of proteins that is enzymes found in DIF. The amount of proteins in the collected DIF could also be even lower compared to intestinal fluid in the dog intestine (*in vivo*), since water was added prior collecting of the DIF. In FaSSIF-P, the degradation of candesartan cilexetil was much lower compared to the degradation in HIF. The degradation rate of candesartan cilexetil in FaSSIF-P increased with an increased concentration of pancreatin and at lower pH in the solution, but did not reach the same level as observed in HIF. The latter may simply be an effect of pH, but higher enzyme activity at this pH cannot be ruled out, as shown for trypsin, which has higher activity at pH 5.0 than at pH > 5 [28]. A plausible explanation of the low degradation in the FaSSIF-P solutions compared to HIF could be the low activity of carboxyl-ester lipase measured in pancreatin solutions [29]. The enzyme activity of carboxyl esterase and protease was more similar in HIF and DIF, than in simulated intestinal fluid containing pancreatin.

Another difference between the simulated and collected intestinal fluids is that the degradation in HIF (and also in DIF) is divided into two phases, while only one phase of degradation could be seen in the FaSSIF-P solutions (Fig. 3). This has been observed in other studies as well and can possibly be explained by enzymatic inactivation of the enzymes responsible for the degradation of candesartan cilexetil, as observed in similar studies with peptides [30,31]. Thus, the results in the present study support that DIF can be used as an alternative to HIF for degradation studies of prodrugs and drugs. However, the use of simulated intestinal fluids for stability predictions may underestimate the *in vivo* degradation of drugs/prodrugs and overestimate their *in vivo* absorption and bioavailability.

Freeze storage of pancreatic juice and intestinal fluid is often required due to technical and logistical limitations. Repeated freezing and thawing of samples may be necessary, but few studies have been conducted to examine the effect of storage conditions on enzyme activities in pancreatic juice or intestinal fluid [28,32]. The investigation of the effect of short- and long-term storage of the intestinal fluid in freezer (−70 °C) in the present study showed that the degradation of candesartan cilexetil was approximately the same in fresh intestinal fluid as in fluid stored in the freezer for up to 4 years. The same batch in this long-term test was not used, which is needed to perform a correct stability testing. However, since pooled HIF was used, the batch to batch variation is limited and a comparison could be done. This is in accordance with earlier results showing that storage of pancreatic juice for 3 weeks at −20 or −80 °C did not affect the activity of the enzymes trypsin, chymotrypsin or lipase. A second freezing and thawing resulted in a decreased trypsin activity of 40% and a decreased lipase activity of 83% [32]. Thus, it is recommended that stability determination of prodrugs in intestinal fluid is performed in fluid that has only been frozen and thawed once.

5. Conclusions

Enzymatic-mediated degradation in the intestinal lumen is a valuable information in drug development and is possible to determine by using *in vitro* testing in intestinal fluids collected from both humans and dogs. Intestinal fluid from dog appears to be a reasonably good model for predicting intestinal stability in corresponding fluid from man, although the degradation capacity seems to be somewhat higher, possibly due to a higher enzyme concentration. Finally, testing with normal pancreatin levels in simulated intestinal fluids seems to underestimate the degradation rate and is therefore not recommended for quantitative assessment of drug absorption and/or BCS assessments. This study also showed that the enzymatic activity was well preserved when the intestinal fluids are stored in freezer at −70 °C.

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