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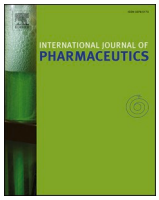

Mini review

Intraluminal enzymatic hydrolysis of API and lipid or polymeric excipients

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A B S T R A C T

The role of intraluminal enzymes for the hydrolysis of active pharmaceutical ingredients (API), prodrugs and pharmaceutical excipients will be reviewed. Carboxylesterases may hydrolyze ester-based API, prodrugs and ester-bond containing polymer excipients, whereas lipases digest lipid formulation excipients, such as mono-, di- and triglycerides. To clarify the conditions that should be mimicked when designing *in vitro* studies, we briefly review the upper gastrointestinal physiology and provide new data on the inter-individual variability of enzyme activities in human intestinal fluids. Afterwards, the methodology for studying enzymatic hydrolysis of API, prodrugs, lipid and polymeric excipients, as well as the main results that have been obtained, are summarized. *In vitro* digestion models used to characterize lipid formulations are well described, but data about the hydrolysis of lipid excipients (including surfactants) has been scarce and contradictory. Data on API and prodrug hydrolysis by esterases is available; however, inconsistent use of enzyme types and concentrations limits structure-stability relationships. Hydrolysis of polymer excipients in the lumen has not been significantly explored, with only qualitative data available for cellulose derivates, polyesters, starches, etc. Harmonization of the methodology is required in order to curate larger enzymatic hydrolysis datasets, which will enable mechanistic understanding and theoretical prediction.

1. Introduction

The gastrointestinal tract (GIT) is designed for enzymatic digestion of ingested nutrients, and secretes enzymes for hydrolysis of e.g. proteins, lipids and starch (Sensoy, 2021). However, components in oral drug products are also substrates for some of these hydrolytic enzymes, which can have implications for the stability of the active pharmaceutical ingredient (API) (Vinarov et al., 2024), but also of the excipients and the formulation itself (Zöller et al., 2022). Intestinal fluids are characterized by an abundance of enzymes that can hydrolyze ester bond-containing API, glyceride-based lipids and even some polymeric excipients (Vinarov et al., 2021).

Hydrolysis-induced changes in the chemical structure of the API can reduce the amount available for absorption (Benet et al., 1996), alter permeability of API or prodrugs by changing lipophilicity (Ohura et al., 2012; Van Gelder et al., 2000), or elicit toxic effects, dependent on the structures formed (Ogiso et al., 1996; Alimonti et al., 2004). Hydrolysis of excipients can affect API dissolution by altering API solubility and may further impact supersaturation and precipitation in enabling oral formulations (Khan et al., 2016; Thomas et al., 2012; Koehl et al., 2020). Hence, pharmacokinetics, and consequently safety and efficacy, may be compromised.

Although the described intraluminal hydrolytic processes are relevant to medicinal chemistry, formulation technology, biopharmaceutics and pharmacokinetics, their potential impact on the final performance of a drug product is often overlooked.

In the current review, we aim to describe the general aspects of intraluminal enzymatic hydrolysis, thus aiding the pharmaceutical scientist in considering and rationalizing its influence on the properties of the drug product. A brief review of GIT physiology will set the stage for three main sections, where the hydrolysis of lipids (in the context of lipid formulations), polymers (amorphous solid dispersions and others) and prodrugs and API in the upper GIT will be discussed. Colonic (Vertzoni et al., 2018; Hammar et al., 2023) and peptide stability (Wang et al., 2015) are not covered in the current review. The state-of-the-art in terms of methodology will be summarized, followed with a short review of the

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main findings, while outlining knowledge gaps. Finally, an outlook for future studies will be presented.

2. GIT physiological conditions

2.1. Enzyme concentrations vs. Activity

One of the critical aspects of studying the enzymatic hydrolysis of API and excipients in the GIT is the activity of the relevant enzymes. However, in contrast to enzymes from bacterial or fungal origin (van Eunen et al., 2010), the activity of digestive enzymes in the different compartments of the GIT is not well characterized. Most studies on luminal enzyme activities are old (Armand et al., 1995; DiPalma et al., 1991; Carriere et al., 1993; Carrière et al., 2000), and the experimental conditions used, such as the assay temperature, are not always reported. Digestive enzymes levels are sometimes expressed as concentrations (μg or mg/L), however, this might lead to a bias since not all of the present enzyme is always active. Therefore, expressing digestive enzymes as activities is more appropriate, but this requires a precise definition and a harmonization of the conditions to use (type of substrate, temperature of the assay, pH, calculation of the data etc.) (van Eunen et al., 2010). In this context, the interplay between pH and enzyme activity must be specifically outlined (Sams et al., 2016; Isaksson et al., 1982; Sky-Peck and Thuvasethakul, 1977). If a study reports the enzymatic activity at a specific pH, the value may be more representative for the enzyme concentration, rather than the biorelevant activity of the enzyme, the reason being that the variation of pH in human GIT fluids (Vinarov et al., 2021) can lead to a similar activity of different levels of enzymes (e.g. lower concentration of enzyme near the pH optimum, compared to a higher enzyme concentration at different pH). Hence, ideally, enzymatic activity should be measured at the pH of the respective GIT fluid sample, in order to provide information on the biorelevant enzyme activity. However, this is especially difficult for human intestinal fluid samples, in which the pH can change quickly after collecting the fluids due to the innate properties of bicarbonate buffer (Litou et al., 2020).

In addition, the activity of the same enzyme can be assessed by several methods using different substrates and the correspondence between the different units is not always established (Hayakawa et al., 1980). For example, trypsin activity is assessed using the p-Toluenesulfonyl-L Arginine Methyl Ester (TAME) or the N_{α}-Benzoyl-L-Arginine Ethyl Ester hydrochloride (BAEE) as substrate (Schwert and Takenaka, 1955; Haverback et al., 1963). In this case, the relation between the two assays has been established (100 TAME U = 5560 BAEE U), but this is not the case for other enzyme assays. Hence, in the following, the level of enzymes in the GIT will be presented in terms of activity, by using substrates and conditions that are currently accepted in the scientific community.

2.2. Stomach

Digestion of lipids and proteins is initiated in the stomach with secretion of gastric lipase and pepsinogen from the chief cells in the gastric glands. Pepsinogen is activated to pepsin by gastric acid, also secreted from the gastric glands. In the fasted state, only around 50 mL of gastric fluid is present in the stomach (Mudie et al., 2014) with a pH between 1 and 4 (Evans et al., 1988; Lindahl et al., 1997). The rheological behavior of gastric fluid is non-Newtonian, with shear-thinning properties, due to the presence of mucins from the gastric mucus (Pedersen et al., 2013).

Another important parameter in GIT physiology is the residence time of an API or formulation in the different segments. In the fasted state, a glass of water (240 mL) which is recommended to aid drug ingestion, will be emptied from the stomach with a half-emptying time of 11 min (Vinarov et al., 2021). The emptying of API to the duodenum will depend on its state in the stomach: dissolved API and excipients will follow the water, whereas larger non-disintegrating dosage forms may remain in the stomach until the housekeeper wave occurs (Vinarov et al., 2021).

In the fed state, the effect of food on API absorption is studied by following a standard clinical protocol such as the one recommended by FDA: the dosage form is taken 30 min after ingestion of the "FDA breakfast", a high-fat and high-calorie meal composed of approximately 150, 250, and 500–600 calories from protein, carbohydrate, and fat, respectively (FDA, 2002). Right after food ingestion, the gastric pH is close to the pH of the food (which can be near neutral), but as gastric acid is secreted, the pH gradually decreases to fasted state values (Pedersen et al., 2013).

Depending on the dosage form and the time for its disintegration, the gastric emptying half-life of Biopharmaceutics Classification System (BCS) class I and II API in the fed state is estimated to be around 40 min for solutions and suspensions (Pentafragka et al., 2020). However, the variation can be very large, with the gastric emptying half-life of disintegrating tablet formulations ranging from 10 min to more than one hour (Kelly et al., 2003; Kelly et al., 2003; Weitschies et al., 2008) and to more than four hours for non-disintegrating formulations (Koziolek et al., 2016).

Pepsin has optimum activity around pH 2 (Campos and Sancho, 2003), and thus will primarily digest proteins after secretion of enough gastric acid to reduce the pH of the digesting food bolus. After arrival in the duodenum, pepsin will be inactivated due to the higher pH. Pepsin primarily hydrolyzes peptide bonds involving aromatic amino acids, such as phenylalanine, tryptophane, and tyrosine. The pepsin content in the stomach exhibits high inter-individual variation and tends to increase upon digestion (Kalantzi et al., 2006). Values found in the literature show large variation partly due to different assays and calculation procedures used. Recently, a consensus on using haemoglobin as a substrate and expressing pepsin activity as "Sigma" or "Anson" units (Anson, 1938; Anson and Mirsky, 1932) has been reached. *In vivo* data showed pepsin activity (measured with haemoglobin) of 1692 U/mL for males and 1534 U/mL for females (Hirschowitz, 1991). A slightly higher activity of 2000 U/mL is used in the final digestion mixtures for static *in vitro* digestion experiments (Minekus et al., 2014). However, new data obtained on human gastric aspirates with more standardized assays which accounts also for intra- and inter-individual variability would be needed to confirm whether the proposed level of pepsin is indeed representative.

Gastric lipase has optimum activity at pH 4.0–5.4 (Sams et al., 2016) and is secreted continuously from the gastric glands. Gastric lipase hydrolyzes the ester bonds in triacylglycerides, with preference for medium chain triacylglycerides (Sassene et al., 2016). The consensus is to determine the gastric lipase activity using the tributyrin assay (TBU) (Grundy et al., 2021). In gastric fluids from fasted volunteers, the activity of gastric lipase was found to be 7.4 ± 4.0 TBU/mL, meaning that partial digestion of lipid-based formulations will occur even in the fasted state (Pedersen et al., 2013). It is estimated that gastric lipase accounts for the hydrolysis of 5–40 % of dietary triglycerides as well as an additional 7.5 % lipolysis in the duodenum (Armand et al., 1999).

2.3. Small intestine

The small intestine is classically divided into three main zones: the duodenum (from the Latin duodecim, i.e. twelve fingers long) which extends from the pylorus to the ligament of Treitz, the jejunum (from the Latin ieiunium, fasting, as it is often empty at autopsy) which corresponds to approximately the first two proximal thirds and the ileum (from the Latin ileus, intestinal obstruction, as it is often full at autopsy).

The duodenum secretes bicarbonate to neutralize gastric acid and provides an appropriate pH for further enzymatic digestion to occur, with median values in the duodenum and proximal jejunum of 6.1 to 7.0 in the fasted state and 4.8 to 6.5 in the fed state (Vertzoni et al., 2019). The duodenum receives secretions of enzymes and bile from the pancreas and gallbladder, respectively (Vasavid et al., 2014). In

response to food ingestion, the exocrine pancreas secretes pancreatic juice containing bicarbonate and diverse enzymes such as lipases, proteases and amylases. Some of these enzymes, like trypsin, are secreted in an inactive form and are activated once delivered into the duodenal lumen (Chandra and Liddle, 2014). Trypsin activity in adults has been found to range between 20 and 50 U/mL (in TAME units) (Bozkurt et al., 1988; Braganza et al., 1978) and even up to 120 U/mL (Dukehart et al., 1989), with a pH optimum of 9 (Gong et al., 2015). Studies reporting chymotrypsin quantification (N-benzoyl-L-tyrosine ethyl ester (BTEE) units) in pancreatic secretions are scarce. Nevertheless, the mean chymotrypsin activity in human intestinal fluid is considered to be around 25 U/mL (Goldberg and Wormsley, 1970), with a slightly lower pH optimum of pH = 8 (Ásgeirsson and Bjarnason, 1991), compared to trypsin.

Bile is produced in the liver, stored in the gallbladder and released into the duodenum. It is a complex mixture of bile acids, cholesterol, pigments, lecithin and mineral salts. In the small intestine, bile acids assist in the emulsification and absorption of fatty acids. They also stimulate lipolysis by emulsifying fats and oils, thus increasing their surface area and increasing the rate of interfacial hydrolysis of the triglycerides by the co-lipase anchored pancreatic lipase (Chandra and Liddle, 2014; Golding and Wooster, 2010).

In healthy adults, pancreatic lipase activity has been shown to fluctuate greatly between 80 and 7000 TBU/mL with a median value around 200 TBU/mL (Carriere et al., 1993; Carriere et al., 2005; Laugier et al., 1991). Its activity is dependent on the protein cofactor colipase that is also secreted from the pancreas (Lowe, 2002). The pH optimum of the pancreatic lipase at biorelevant conditions is around 6.5, due to the effect of bile salts (Borgstrom, 1954), whereas it is higher when measured *in vitro* in the absence of bile salts (7.5 to 8.5) (Borgstrom et al., 1957). Around 40–70 % of the lipolysis of dietary lipids is achieved by pancreatic lipase activity in the small intestine (Armand et al., 1999). Pancreatic lipase is hydrolyzing the ester bond in the 1 and 3 position in acyl glyceride from the surface of lipid droplets and has a higher affinity towards shorter chain acyl glycerides (Benito-Gallo et al., 2015).

2.4. New data on the interindividual variability of lipase, trypsin and chymotrypsin activities in duodenal fluids

The activity of pancreatic lipase, trypsin and chymotrypsin was determined in fasted and fed state duodenal fluids, collected from 11 healthy volunteers. The details of the protocol for fluid collection are described in Goovaerts *et al.* (Goovaerts et al., 2024) and are briefly summarized in the Appendix (section A1), along with the protocols for determination of enzyme activities (section A2).

Fig. 1 shows the evolution of the pancreatic enzyme activities and pH from 30 to 110 min postprandially. All the enzymes showed relatively stable activities over the time period during which samples were collected (differences were not statistically significant). Duodenal fluid samples showed a median pH value of 6.4, with a large dispersion of values between pH 4 and 7, with one sample showing pH of 3.1. A trend of the median pH decreasing with time could be observed, however, the differences from 40 to 110 min were not statistically significant. Significant differences were determined only when comparing the initial pH (at 30 min) with the decreased pH at 70th and 110th min. However, these differences should be interpreted with caution, as they are due to the unusually small dispersion of the pH values at 30 min. The bigger variation of pH which is typically observed (see all other time points) would render the observed changes in pH insignificant.

Trypsin activities (TAME units), measured in duodenal fluids ranged

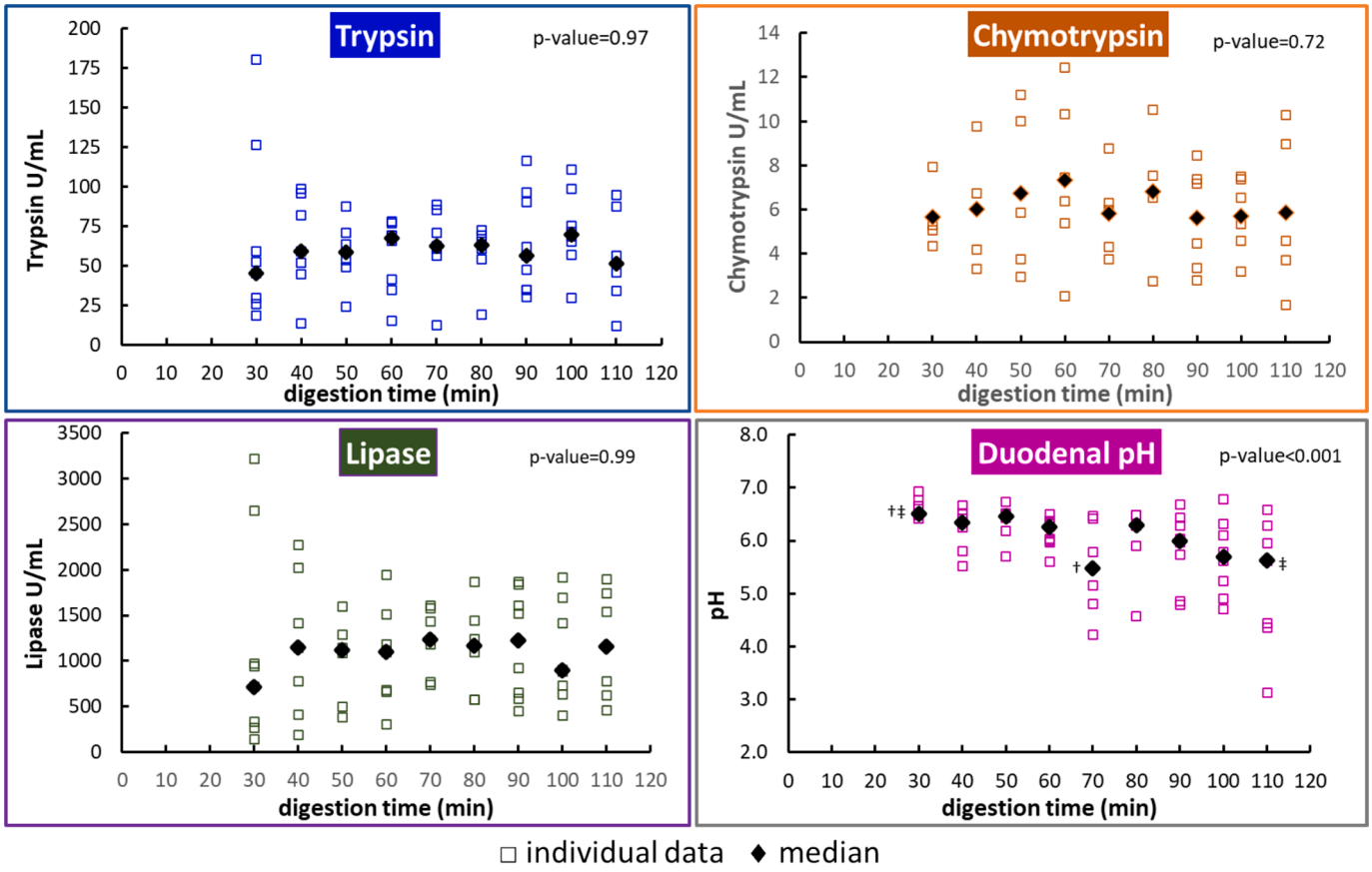

Fig. 1. Trypsin (TAME units), chymotrypsin (BTEE units), lipase (TBU) activities and pH as measured in duodenal fluids from 11 volunteers in the fed state, as a function of time after ingestion of the liquid meal. Statistical significance of the results ($p \leq 0.05$) was tested using ANOVA. Data analyses were performed using the R software, version 4.2.2.

from 12.0 to 180.3 U/mL with an overall median value of 63 U/mL. These values were measured at the physiological temperature of 37 °C, whereas in the literature, trypsin assays are usually performed at 25 °C (Brodkorb et al., 2019), decreasing significantly (e.g. by a factor of two, if the Q10 temperature coefficient or the empirical rule of Van't Hoff is followed) the activities measured. This is in relatively good agreement with the range between 20 and 50 U/mL (in TAME units) (Bozkurt et al., 1988; Braganza et al., 1978) and even up to 120 U/mL (Dukehart et al., 1989) found in clinical samples. Therefore, the mean value of the trypsin activity measured in intestinal fluids appears to be lower than the one recommended by the INFOGEST network method (100 U/mL) (Brodkorb et al., 2019).

Chymotrypsin activities measured at 37 °C, using BTEE as a substrate, ranged from 1.7 to 12.5 U/mL with an overall median value of 6.1 U/mL. The median value obtained in the present study is thus much lower than the value of 25 U/mL found in human duodenal aspirates (Goldberg and Wormsley, 1970), which is also proposed for *in vitro* digestion models such as the INFOGEST model, especially considering that here it was determined at 37 °C, compared to 25 °C in standard assays (Brodkorb et al., 2019).

Finally, pancreatic lipase activities (TBU), ranged from 125 to 3221 U/mL with an overall median value of 808 U/mL. Hence, the activities measured are in coherence with previously reported activities determined in fed state duodenal fluids that ranged from 80 to 7000 U/mL (Carriere et al., 2005; Armand, 2007). The lipase activity recommended in the INFOGEST *in vitro* digestion protocol (2000 U/mL) is at the higher range of the determined values and thus appears to represent the more pronounced enzyme secretions observed in the intestinal fluids of some human volunteers (Minekus et al., 2014; Brodkorb et al., 2019).

Fig. 2 shows the mean values of trypsin, chymotrypsin and lipase activities and the pH measured in the duodenal fluid collected in fed or fasted state averaged from all 11 volunteers. As no time effect was observed in the fed state, average values (n = 2, 5, 7, 8 or 9, as specified in the legend of Fig. 2) were used in Fig. 2. Overall, no effect of the dietary state was observed on the pH or the enzyme activities, except that lipase activity was significantly higher in fed than fasted state ($P < 0.01$) (1093 vs 457 TBU/mL respectively).

The enzymes described above are frequently considered when studying enzymatic hydrolysis in the context of food or pharmaceuticals. In the next section, a family of enzymes which can significantly impact API concentrations in the intestinal lumen, but is sometimes overlooked, is discussed.

2.5. Carboxylesterase characteristics

Carboxylesterases (CES) have essential physiological functions linked to the hydrolysis of xenobiotics containing ester bonds. CES are part of the serine hydrolase superfamily of enzymes and metabolize various API, such as ACE inhibitors, anticoagulants, psychoactives, antivirals etc (Wang et al., 2018). Although CES may play a significant role in the enzymatic hydrolysis of API, their role is frequently overlooked and awareness about their structure and function appears to be limited, in comparison to the well-known GIT enzymes such as pepsin, pancreatic lipase, trypsin etc. Hence, a more detailed overview of CES will be presented below.

The two main isoforms are CES1 and CES2, with CES1 being expressed mainly in the liver and adipocytes, whereas CES2 is mainly expressed in the pancreas and secreted to the duodenum from there (Junge et al., 1979; Wang and Kloer, 1983). CES2 is found in the small intestine and colon (Wang et al., 2018; Hosokawa, 2008). The structure of CES1 has been solved by X-ray crystallography with several ligands (Fig. 3): it consist of a central catalytic domain, an $\alpha\beta$ domain, and an

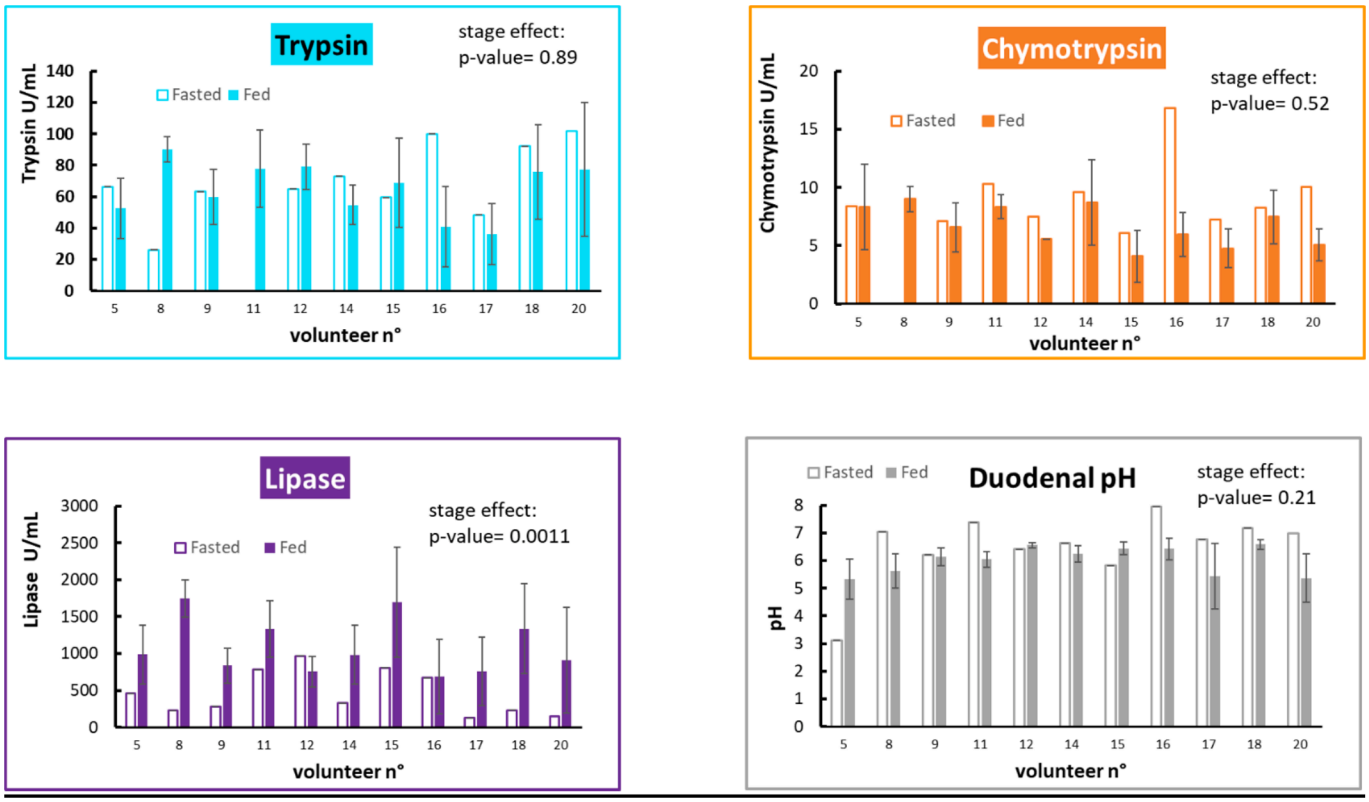

Fig. 2. Trypsin (TAME units), chymotrypsin (BTEE units), lipase (TBU) activities, and pH as measured in duodenal fluids from 11 volunteers in fed (only median + SD is shown) and fasted state. Only one sample was available for each volunteer in the fasted state. For the fed state, n = 2 for volunteer #5, n = 5 for volunteers #1, 2, 3, 4 and 6, n = 7 for volunteer #10, n = 8 for volunteer #8 and n = 9 for volunteers 7, 9 and 11. Statistical significance of the results ($p \leq 0.05$) was tested using a paired *t*-test. Data analyses were performed using the R software, version 4.2.2.

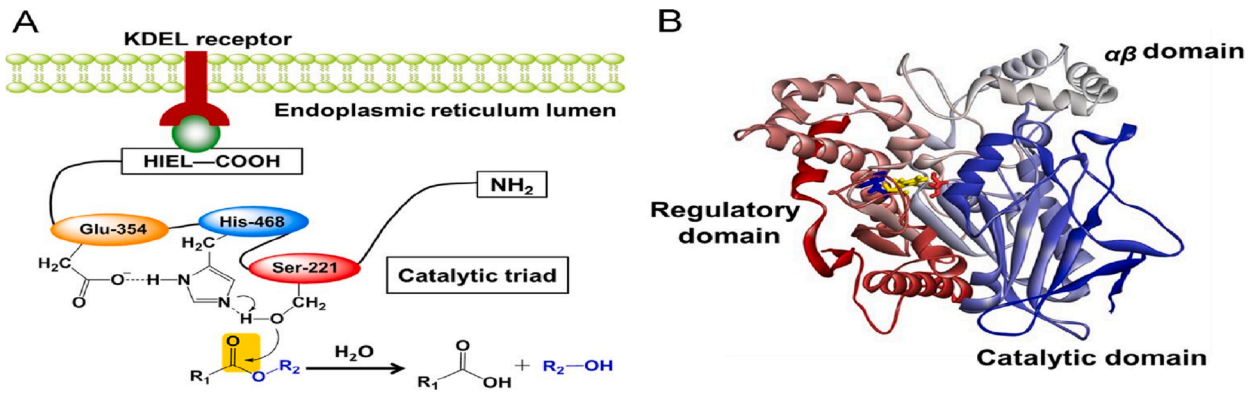

Fig. 3. (A) The scheme for CES2 hydrolysis of ester groups; (B) The 3D structure of CES1. The catalytic triad including Ser221, Glu354 and His468 are colored in red, yellow and blue, respectively. Reprinted from (Wang et al., 2018) under CC BY-NC-ND 4.0 Deed license, https://creativecommons.org/licenses/by-nc-nd/4.0/.

adjacent regulatory domain which contains the low-affinity surface ligand-binding Z-site (Bencharit et al., 2003; Kim et al., 1997; Fleming et al., 2007). According to this crystallographic data, CES1 exists as a monomer, trimer, or hexamer with substrate-dependent equilibrium of homooligomer formation.

Although the structure of CES2 has not been determined yet, it has been established that in contrast to CES1, it only exists as monomers. Recent studies on the structure of a potential mouse ortholog of CES2 (mCES2c (Eisner et al., 2022) showed significant differences in the cap, the flexible lid, and the regulatory domain, compared to human CES1, which could explain the experimentally observed differences in substrate specificity. CES1 metabolizes ester substrates with small alcohol groups and a bulky acyl group (like clopidogrel, oseltamivir, heroin, cocaine, enalapril). CES2 has the opposite preference – it hydrolyzes esters with large alcohol groups and small acyl moieties (like prasugel, flutamide, irinotecan). However, it was recently shown that CES2 can also hydrolyze mono- and diglycerides (Chalhoub et al., 2021).

Similarly to all hydrolases, CES enzymes are also characterized by a pH-dependent activity. The pH optimum of CES2, which operates in the small intestine, is reported to be in the range of 7.5–8.0 (Wang et al., 2018), which is significantly higher than the small intestinal pH (6.1–7.0 and 5.3–6.3, for fasted and fed state conditions, respectively (Vinarov et al., 2021). This mismatch between CES2 pH optimum and intestinal onditions (which is also present for other enzymes such as trypsin and chymotrypsin) must be considered when designing and interpreting results from *in vitro* studies on esterase-mediated API degradation.

A crucial parameter is the activity of CES enzymes in human intestinal environment. Unfortunately, data on CES activity in the small intestine is not yet available and standardized methods for activity determination have not been used consistently in the literature. The latter is critical, as CES activities vary significantly depending on the substrate. For example, an activity of 1543 ± 759 nmol/min/mg protein was determined for the hydrolysis of p-nitrophenylacetate (PNPA, used as a standard substrate), compared to 66 ± 15 nmol/min/mg protein for O-valeryl propranolol when human jejunal S9 fractions were used as an esterase source (Taketani et al., 2007). Data relevant for the human small intestine was reported by van Gelder et al., who showed that PNPA-measured CES activity in intestinal tissue homogenates decreases along the duodenum, ileum and colon with values of 870 ± 600, 600 ± 390 and 414 ± 90 nmol/min/mg protein, respectively (Van Gelder et al., 2000).

3. Methods for studying intraluminal enzymatic hydrolysis

3.1. *In vitro food digestion methods*

The fate of food in the GIT is a key topic in food science and has prompted development of various static and dynamic *in vitro* digestion models. Since these models represent the GI environment, they can also be used to answer questions relevant to oral drug delivery and biopharmaceutics. For example, the dynamic TNO Gastro-Intestinal Model (TIM) (Minekus et al., 1995), and the Dynamic Gastric Model (DGM) developed by the Institute of Food research (UK) (Mercuri, 2009; Wickham et al., 2005), both of which were originally applied mainly in food science research, have currently been adopted in pharmaceutical development.

Dynamic systems are either mono-compartmental (simulating one compartment of the GIT) or multi-compartmental (several compartments). The different systems available have been described (Guerra et al., 2012) and what they are able to mimic was reviewed recently (Dupont et al., 2019). The multi-compartmental models consist of a series of bioreactors (stomach, small intestine) that are interconnected with pumps allowing to simulate the transit of food, regulate the pH and inject the digestive enzymes and bile salts in real time. More recently, a new generation of simulators equipped with in-silicone organs (Morlock et al., 2021; Peng et al., 2021) has been developed, as well as organ-on-a-chip models (de Haan et al., 2021), which open new perspectives for getting closer to a nearly-real human GIT. Although such dynamic models offer very good representation of the *in vivo* reality in the GI tract, their low throughput and considerable initial investment create space for the application of other, more simple models.

Static *in vitro* digestion methods are particularly popular because they are easy to use, cheap and do not require specific equipment. Basically, they consist of one compartment where the conditions of the mouth, stomach and small intestine are consecutively simulated by changing pH, adding enzymes etc. By reproducing the enzymatic and physicochemical conditions in the different compartments of the digestive tract, such models can be used in oral biopharmaceutics to monitor disintegration, dissolution and permeation (when a permeation barrier is included). However, a huge number of protocols differing in the experimental conditions (pH and duration of the different steps, amount of digestive enzymes and bile, etc) have been proposed making the comparison of results between studies impossible (Hur et al., 2011). In 2014, the EU COST Action INFOGEST, after more than 2 years of intense discussions, proposed a "harmonized" static food digestion model based on parameters that were taken from published clinical studies (Minekus et al., 2014). The model was validated towards clinical data (Egger et al., 2017; Egger et al., 2019; Sanchon et al., 2018), was optimized few years later (Brodkorb et al., 2019), and is now used all over the world while in the process of being recognized as an ISO standard. As new data on enzyme activities is being obtained (some of which is presented in the current study), further fine-tuning of the model might be required.

However, static *in vitro* digestion models are a simplistic representation of the physiological reality and cannot answer all research questions. Their advantages, but more importantly their limitations, have

recently been reviewed (Bohn et al., 2018). Overall, they are relevant as screening tools to compare the digestion of different food or food ingredients in identical conditions and have also been successfully used to assess end-points values such as the glycaemic index (Monro and Mishra, 2010) or protein digestibility (Ariëns et al., 2021; Sousa et al., 2023). The model has been extended to simulate the GIT conditions found in infants (Menard et al., 2018) and in elderly (Menard et al., 2023). When static *in vitro* digestion models are too simple to answer more complex research questions, the dynamic *in vitro* digestion models can be a relevant alternative, which has been recently reviewed (Singh, 2024).

3.2. Lipid formulation digestion methods

Lipid-based formulations, and especially self-(nano)emulsifying drug delivery systems (S(N)EDDS), have long been shown to increase the absorption of poorly water-soluble API (Mu et al., 2013). SNEDDS are isotropic mixtures of oils, lipophilic and hydrophilic surfactants and co-solvents that form (nano)emulsion droplets upon dispersion in aqueous media, e.g. GIT fluids. The mechanism for API absorption from nano-emulsions is not fully understood, but as many excipients used in lipid-based formulations are substrate for lipases in the GIT, the digestion of the lipids will inevitably take place.

As a consequence, *in vitro* digestion models, often termed *in vitro* lipolysis models, are used during the development of lipid-based formulations, and are most often carried out in simulated fasted state conditions, i.e. with regard to bile salt concentration. The majority of the described and applied models are static models, focusing on intestinal digestion. In short, the lipid formulation is added to a simulated intestinal medium, containing relevant levels of bile salts and phospholipids. Then the source of pancreatic lipase is added, to initiate the lipolysis. The digestion is run in a thermostated vessel (37 °C) placed in a pH-stat. The pH is kept constant at a relevant pH, often between 6.5 and 7, by titration of NaOH. The NaOH is titrating the free fatty acids generated during hydrolysis of tri- and diglycerides and other digestible excipients in the formulation. As not all fatty acids are protonated at pH 6.5, a back titration to pH 9 is needed to determine the total amount of fatty acids released (Gargouri et al., 1986; Helbig et al., 2012). Calcium chloride is added to the model, either continuously or as an initial concentration. This leads to formation of calcium soaps with the free fatty acids, and thereby they cannot inhibit lipolysis by accumulating on the surface of the digesting lipid droplet (Zangenberg et al., 2001). During digestion, the products formed generate colloidal structures, which can influence the solubilization of the API, and potentially lead to API precipitation. As a measure of the fraction of API available for absorption, the solubilized API is quantified at selected timepoints during digestion (Mu et al., 2013; Feeney et al., 2016).

The activity of pancreatic lipase in the *in vitro* intestinal lipolysis models has generally been around 800 and 1200 U/mL, measured as TBU/mL. Unfortunately, different assays have been used to determine the lipase activity over the years. United States Pharmacopeia (USP) recommend the use of olive oil emulsified by gum arabic as a substrate (USP, 2024), whereas the INFOGEST model applies the TBU assay which uses tributyrin as substrate (Brodkorb et al., 2019).

Most often porcine pancreatin extract is added as source of pancreatic lipase and also provides the colipase, as well as other pancreatic enzymes, including proteases, amylase and carboxyl esterase. For *in vitro* digestion of lipid formulations, where primarily lipase is important, the possibility of using microbial lipases has been assessed. This would simplify the process and enable direct UV measurements, as microbial lipases provide clear solutions, whereas the pancreatic extract forms turbid solutions. Further, when combining *in vitro* lipolysis with permeation through CaCo-2 cell cultures, it is necessary to apply a different kind of lipase, as the CaCo-2 cells are not compatible with pancreatin extract. In this case, an immobilized microbial lipase has been applied and enabled combined *in vitro* lipolysis and permeation through CaCo-2 cells (Keemink et al., 2019). The rate and extent of lipolysis using different microbial lipases on different lipid formulations have recently been compared (Ejskjær et al., 2024). The presently available immobilized lipase has a much lower digestion compared to pancreatic lipase, probably because of lack of specificity. In contrast, some liquid microbial lipases provided almost the same degree of digestion of some lipid formulations, and also same drug solubilization for one model drug. However, more studies are needed to assess whether the microbial lipase has the same affinity towards different fatty acid chain lengths and esters, before recommending to generally use microbial lipases for *in vitro* lipolysis.

Recently, *in vitro* gastric digestion has been added to the intestinal *in vitro* lipolysis models (Bakala-N'Goma et al., 2015; Klitgaard et al., 2020). However, an inherent problem here is the lack of commercial human gastric lipase, or a lipase with the same activity and pH profile. A recombinant human gastric lipase has been used in a few papers (Sassene et al., 2016; Heerup et al., 2021) but is presently not commercially available. Most often, a microbial lipase is used, but recently, a rabbit gastric lipase has become available and is recommended for use in the INFOGEST model (Brodkorb et al., 2019); however, it has not been used for assessment of lipid formulations yet. Gastrointestinal *in vitro* digestion models have primarily been static i.e. conducted in the same vessel, but transfer, or dynamic, models have also been applied (Siqueira Jorgensen et al., 2018; Klitgaard et al., 2017). In these models, the gastric medium is gradually transferred to the intestinal compartment during digestion.

There has been focus on standardizing the *in vitro* lipolysis models used for development of lipid formulations. In the early 2010s, the Lipid Formulation Classification Scheme (LFCS) Consortium, composed of both industry and academia, aimed at standardizing the *in vitro* lipolysis model as a tool for characterizing different kinds of lipid formulations (Williams et al., 2012). These attempts have not yet led to a standardized *in vitro* lipolysis model for evaluating lipid formulations. This is also because there often is a lack of *in vivo in vitro* correlation (IVIVC) between the API solubilisation during *in vitro* lipolysis and the *in vivo* performance (Berthelsen et al., 2019). Several attempts have been made to develop *in vitro* lipolysis models that can display IVIVC. This includes implementing a permeation step in the lipolysis model using different permeation barriers, including both cell cultures such as CaCo-2 and MDCK cells and artificial membranes, as well as phospholipid-based membranes (Permeapad® and PVPA) (Keemink et al., 2019; Falavigna et al., 2021; Keemink et al., 2022; Klitgaard et al., 2021). Another means to obtain IVIVC using *in vitro* lipolysis is to implement a gastric digestion step and/or to simulate the GI physiology of the preclinical species that were used for the *in vivo* study (Berthelsen et al., 2019). Accordingly, simulating the rat GIT, including higher bile salt levels and decreased lipase activity has led to a better correlation with *in vivo* data (Siqueira Jørgensen et al., 2018).

3.3. Methods for studying API hydrolysis by carboxylesterases

Standard methodology for studying API hydrolysis by CES has not yet been established. Hence, studies have been (and continue to be) performed at various conditions. The variety starts at the design of the study, in respect to the degree of biorelevance of the media and the source of the enzymes: (1) human or animal intestinal fluids (highly biorelevant medium and enzymes), (2) cell-based assays and tissue homogenates, in which the medium is usually less biorelevant and enzymes represent not only CES action but CYP metabolism as well, and (3) aqueous-based media combined with commercially available pancreatin or CES enzymes, in which the medium can be biorelevant, depending on inclusion of bile salts and phospholipids, whereas the selection of the enzymes depends on commercial availability.

When considering the choice of assay, one has to take into account several limitations. For example, tissue homogenates will have CYP activity, thus confounding drug hydrolysis by the luminal CES2 with the activity of CES1 which can be present in some of the tissue cells. In

respect to using commercial enzyme sources, one should be careful when selecting pancreatin, as the API may be a substrate of enzymes other than CES (e.g. pancreatic lipase).

Another aspect of designing an *in vitro* luminal esterase degradation study, which should not be overlooked, is the concentration of solubilizing species such as bile salts and phospholipids. A large body of evidence obtained at various conditions indicates that API solubilization can effectively decrease the rate of hydrolysis by esterase enzymes (Vinarov et al., 2024; Hammad and Muller, 1999; Hoppe and Sznitowska, 2014; Bali et al., 2016; Fayed et al., 2016). This underlines the importance of correctly mimicking the composition of fasted- or fed-state human intestinal fluids, especially when the hydrolysis-sensitive API, which are prone to solubilization are considered.

4. Enzymatic hydrolysis of lipids, API and polymeric excipients: Examples

4.1. API hydrolysis by carboxylesterases

Data for the enzymatic hydrolysis of 14 API at intestinal conditions has been collected from the literature (Table 1). However, due to the lack of standardized methodology, direct comparison of literature data is currently not possible. This issue prevents the drawing of any general conclusion about enzyme-substrate specificity or rate of hydrolysis and limits interpretation to a rather descriptive level. In addition, as many of the studies were performed with a cocktail of enzymes (pancreatic extract), rather than purified enzymes, it should be mentioned that other than CES, pancreatic lipase and related enzymes can also contribute to the hydrolysis of some of the API.

The hydrolysis half-lives of some drugs are in the order of several to tens of minutes, which indicates that the hydrolysis product(s) will be

Table 1
Summary of esterase-mediated luminal API degradation studies.

API	Simulated intestinal conditions			Reference conditions	API hydrolysis, D	Ref.
	pH	Bile salts	Enzymes			
Abiraterone acetate	7.5	FaSSIF	20 IU/mL esterase (porcine)	FaHIF, rat perfusion	**$t_{1/2}$ (FaHIF)** < 20 min	(Stappaerts et al., 2015)
Abiraterone acetate	6.5	FaSSIF, FeSSIF, FeSSIF + lipids	80 IU/mL esterase, 0.83 mg/mL pancreatin (porcine, 8x USP)	rat perfusion	D_{120min} (FeSSiF-esterase) ≈ 67 % D_{120min} (FeSSiF-pancreatin) ≈ 15 %	(Braeckmans et al., 2022)
Candesartan cilexetil	5.0–6.5	FaSSIF, FeSSIF	0.1–0.5 % Pancreatin (porcine, 4x)	FaHIF, FeHIF	$t_{1/2}$(FaHIF$_{6.5}$) = 80 min $t_{1/2}$(FeHIF$_{5.0}$) = 137 min	(Vinarov et al., 2024)
Candesartan cilexetil	5.5–7.5	FaSSIF	1–4 % Pancreatin	HIF, DIF	**$t_{1/2}$(5–60 min)** DIF 43.0 ± 2.8 min HIF 49.2 ± 0.5 min	(Borde et al., 2012)
Chloramphenicol succinate	5.5–7.5	FaSSIF	1–4 % Pancreatin	HIF, DIF	$D_{60\ min}$ < 10 %	(Borde et al., 2012)
Cortisone acetate	5.0–6.5	FaSSIF, FeSSIF	0.1–0.5 % Pancreatin (porcine, 4x)	FaHIF, FeHIF	$t_{1/2}$(FaHIF$_{6.5}$) = 121 min $t_{1/2}$(FeHIF$_{5.0}$) = 54 min	(Vinarov et al., 2024)
Danazol	6.8	None	1 % Pancreatin (porcine, 3x)	none	$t_{½}$ = 36.5 h	(Gadkariem et al., 2003)
Desmopressin	rat jejunal fluid and rat jejunal homogenate				**$t_{1/2}$ (rat jejunal fluid) = 31 h** **$t_{1/2}$ (rat jejunal homogenate) = 5h**	(Lepist et al., 1999)
Diloxanide furoate	6.8	None	1 % Pancreatin (porcine, 3x)	none	$t_{1/2}$ = 20 min	(Gadkariem et al., 2004)
Dipropionate ester of DHPG	rat and monkey intestinal homogenates				**$t_{1/2}$ (rat intestinal homogenate) = 21 min**	(Benjamin et al., 1987)
Enalapril	5.5–7.5	FaSSIF	1–4 % Pancreatin	HIF, DIF	$D_{60\ min}$ < 10 %	(Borde et al., 2012)
Ganciclovir prodrugs	Monkey intestinal homogenates				Compound 3, $t_{1/2}$ = 4.076*10^{-4} min Compound 4, $t_{1/2}$ = 3.01*10^{-5} min	(Powell et al., 1991)
Lamivudine derivatives	6.8	None	Pancreatin (porcine)	none	$t_{1/2}$(SIF) = 2.04—–19.4 h	(Gualdesi et al., 2013)
Propranolol prodrugs	7.4	None	Esterase	none	$t_{1/2}$ (esterase): 0.00035 – 0.072 min	(Irwin and Belaid, 1988)
Prednisolone acetate	5.0–6.5	FaSSIF, FeSSIF	0.1–0.5 % Pancreatin (porcine, 4x)	FaHIF, FeHIF	$t_{1/2}$(FaHIF$_{6.5}$) = 110 min $t_{1/2}$(FeHIF$_{5.0}$) = 32 min	(Vinarov et al., 2024)
Tenofovir disoproxil fumarate	6.8–11.0	FaSSIF	480 U/mL pancreatin lipase	Human and mouse intestinal S9 fractions, Caco2- and MDCK-cell permeability, rat pharmacokinetic	$t_{1/2}$ (FaSSIF + PL) = 2.0 ± 2.3 min $t_{1/2}$ (Human Intestinal Extract) = 0.62 ± 1.29 min $t_{1/2}$ (Rat Intestinal Extracts) = 0.58 ± 1.75 min	(Watkins et al., 2017)
Tenofovir disoproxil fumarate	5.0–6.5	FaSSIF, FeSSIF	0.1–0.5 % Pancreatin (porcine, 4x)	FaHIF, FeHIF	$t_{1/2}$(FaHIF$_{6.5}$) = 17 min $t_{1/2}$(FeHIF$_{5.0}$) = 37 min	(Vinarov et al., 2024)
Tenofovir disoproxil fumarate, PNPA	Caco2-cell homogenate			Intestinal homogenates from rat, pig and man	Degradation rate of tenofovir disoproxil in homogenates from Caco-2 monolayers amounted to 0.016 ± 0.003 nmol s^{-1} mg protein^{-1}	(Van Gelder et al., 2000)

*HIF = human intestinal fluids, FaHIF = fasted HIF, FeHIF = fed HIF, DIF = dog intestinal fluids, SIF = simulated intestinal fluids, FaSSIF = fasted state SIF, FeSSIF = fed state SIF, $t_{1/2}$ = half-life, D = degree of API hydrolysis, DHPG = Dihydroxyphenylglycine, PNPA = p-nitrophenyl acetate.

subjected to intestinal absorption, potentially influencing oral pharmacokinetics. Such compounds are abiraterone acetate (Stappaerts et al., 2015), candesartan cilexetil (Vinarov et al., 2024; Borde et al., 2012), diloxanide furoate (Gadkariem et al., 2004), dihydroxyphenylglycine dipropionate ester (Benjamin et al., 1987), ganciclovir prodrugs (Powell et al., 1991), propranolol prodrugs (Irwin and Belaid, 1988) and tenofovir disoproxil fumarate (Vinarov et al., 2024; Van Gelder et al., 2000; Watkins et al., 2017). However, depending on the properties of the prodrug and the hydrolysis products, the prodrug might still play a major role for absorption (Vinarov et al., 2024; Van Gelder et al., 2000; Watkins et al., 2017): this is the case for tenofovir disoproxil fumarate, where the prodrug is the main determinant of intestinal uptake, due to the very poor permeability of the hydrolyzed species. For other API, where hydrolysis kinetics are in the scale of hours, it is expected that hydrolysis will not significantly influence intestinal absorption, especially if the API are highly permeable compounds.

4.2. Lipid excipient hydrolysis

GIT lipases and especially pancreatic lipase are able to hydrolyze excipients used in lipid-based formulations. As mentioned above, medium chain tri- and diacylglycerides are hydrolysed faster than their long-chain counterparts by both gastric and pancreatic lipase (Jandacek et al., 1987; Sek et al., 2001; Sek et al., 2002). Monoacylglycerides are often used as lipophilic cosurfactants in lipid-based formulations, such as SNEDDS (Tran et al., 2016) and are also substrates for pancreatic lipase. For example, Capmul MCM EP (Mono/diglycerides of caprylic acid) was digested up to 20 % after 60 min incubation with lipase from porcine pancreas (Leonaviciute et al., 2016). While some fatty acid ester-based surfactants such as the polysorbates can also be hydrolyzed by pancreatic lipase and CES enzymes (Glucklich et al., 2021; Rasmussen et al., 2024; Christiansen et al., 2010), this is not always the case, as shown for sucrose laurate and tocopheryl polyethylene glycol succinate (TPGS) (Christiansen et al., 2010). The situation is similarly ambiguous when propylene- or PEG-based surfactants used in lipid-based formulations are considered: of the ethoxylated castor oil surfactants, Kolliphor RH40 is not digested, whereas Kolliphor EL is, which is most likely due to Kolliphor RH40 being hydroxylated (Berthelsen et al., 2015; Cuine et al., 2007). However, some studies indicate that Kolliphor RH40 may also be digested, although to a very low extent (<10 %) (Christiansen et al., 2010). A further complication for the assessment of digestion of many pharmaceutical surfactants is that they are not pure, i.e. often contain tri- di- and monoacylglycerides, which are a usual starting point for their synthesis. This includes Labrasol®, where the present tri and di acylglycerides are digested by pancreatic lipase, whereas the PEG-esters and monoglycerides are mainly hydrolyzed by the synergistic action of CES enzymes and pancreatic lipase-related protein 2 (PLRP2) (Fernandez et al., 2007). The extent of enzymatic hydrolysis of lipid excipients and surfactants was recently compared by Zöller et al. (Zöller et al., 2022), who showed highest hydrolysis of medium chain glycerides, followed by long chain glycerides and surfactants. Hydrolysis of surfactants decreased in the following order: glycerol monostearate > polyoxyethylene (20) sorbitan monostearate > PEG-35 castor oil > sorbitan monostearate.

4.3. Polymeric excipient hydrolysis by intestinal enzymes

Polymers are an important class of oral formulation excipients. The GI environment exposes polymers to different stress conditions, including, pH, thermal, enzymatic and mechanical processing. This can lead to the degradation of polymers with susceptible chemical groups (Azevedo and Reis, 2005), unless the respective groups are protected as in the case of cyclodextrins (Buedenbender and Schulz, 2009; Flourié et al., 1993). While the digestion of lipids and proteins in food and pharmaceuticals is extensively studied, this is not the case for the GI digestion of polymeric excipients. The scarce data which is available for the degradation of some pharmaceutically relevant polymers in the GIT is discussed below (see also Table A1 in the Appendix).

4.3.1. Cellulose derivatives

Cellulose and its derivatives were among the first polymers to be used in drug delivery. The conventional nonionic derivatives, for example hydroxypropyl methylcellulose (HPMC), methylcellulose (MC), hydroxypropyl cellulose (HPC) have good chemical stability and provide protection against the strong acidic nature and digestive enzymes in the stomach. However, some cellulose derivatives such as hydroxypropyl methylcellulose acetate succinate (HPMCAS), hydroxypropyl methylcellulose phthalate (HPMCP), and other cellulose esters can undergo chemical or enzymatic hydrolysis, resulting in reaction products such as cellulose, carboxylic acids and glucose (Edgar et al., 2001; Liu et al., 2015).

HPMCAS is a pH-responsive enteric cellulose ester commonly used for coating and as a matrix in amorphous solid dispersions (ASD). In the stomach at low pH (1–3), it is insoluble, but it is ionized in the small intestine at higher pH (5.5–7) (Liu et al., 2015). Different grades of HPMCAS (LF, MF or HF) are designed to trigger API release, depending on their percentage of the substituted contents which includes ester (acetate and succinate) and ether (methyl and hydroxypropyl) moieties. Among these, the ester linkages (highest percentage in HPMCAS-LF) can be enzymatically hydrolyzed, in contrast to the ether bonds (highest percentage in HPMCAS-HF). However, currently there is no direct experimental data on the extent of HPMCAS enzymatic hydrolysis at biorelevant conditions.

The situation is similar with hydroxypropylmethyl cellulose phthalate (HPMCP). The stability of HPMCP was assessed indirectly, by studying the protective and stabilizing behavior of HPMCP coating against digestion of catechin in an *in vitro* digestion model. While HPMCP coating of different thickness was found to increase GI stability of the active, the coating was observed to slightly rupture in the presence of pancreatin, suggesting a possible role of enzymatic hydrolysis of the polymer (Chung et al., 2014).

4.3.2. Polyesters

Poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and their copolymer PLGA are widely used pharmaceutical excipients. Usually, CES are responsible for degradation of PLGA in the intestine by hydrolysis of the ester bonds (Williams and Zhong, 1994). However, a PLA matrix was shown to undergo a rapid degradation in intestinal fluids, under the influence of lipases, indicating that enzymes other than CES may play a role (Landry et al., 1997). According to an *in vivo* study by Chang et al. in rats (Chang et al., 2017), PCL polyester films degraded more via surface erosion, by the action of enzymes in slightly alkaline environment with a significant weight loss of the polymer in intestine when compared to the stomach.

PCL and Poly(l-lactide-co-ε-caprolactone) (PLCL) meshes showed differences in mechanical properties when degraded in phosphate buffer or small intestinal fluids obtained as aspirates collected from patients (Peerlinck et al., 2023). Also, it is noticed that PCL undergoes faster surface degradation under *in vivo* intestinal conditions, which could be attributed to the action of lipase (Bartnikowski et al., 2019).

A pure poly butylene succinate film was found to be less resistant to attack by pancreatic lipase in the intestine as compared to a composite prepared with poly (vinylidene fluoride). This was evidenced by *in vivo* degradation of the pure polymer film implanted in the rat́s duodenum as shown by weight loss of the film and by the appearance of pores and grooves on its surface by erosion governed by pancreatic enzymes (Jeffrey Kuo et al., 2020).

4.3.3. Starch

Generally, starch digestion is initiated in the mouth by saliva amylase, and hydrolysis is continued by pancreatic amylase which is the main enzyme for starch digestion in the GIT. The starch oligomers are

further hydrolyzed into glucose and oligosaccharides by maltase and sucrase. (Boldrini, 2023; Dona et al., 2010).

It has been determined that hydrolysis by α-amylase controls API release from a terpolymeric semi-interpenetrating network composed of starch and polyethylene glycol (Bajpai et al., 2006). According to an *in vitro* release study (Carbinatto et al., 2016) of two different amylose-drug complexes (nimesulide and praziquantel), pancreatin extract was observed to cause a burst-release effect under 30 min of administration, which confirmed the role of the enzymatic degradation in comparison to the control with no pancreatin extract added (Thoma and Bechtold, 1999).

5. Summary and future perspectives

Current understanding and parametrization of GIT physiology has facilitated the development of *in vitro* models of varying complexity which are used for studying enzymatic hydrolysis. However, an important variable, which can severely affect the quantitative results of any study, is the concentration or activity of the used enzymes. The review presents new data on enzyme activity after intake of a liquid meal using standardized analytical methods, however, considering the limited previous data available, and the high interindividual (and possibly even intraindividual) variability, the frequent approach of selecting a single enzyme level is still questionable. In addition, standardization of the methods for studying the enzymatic hydrolysis of API and polymeric excipients is an issue and the INFOGEST model (established for simulating food digestion) is seldom used for lipid formulation hydrolysis where fasted state is the standard reference condition. Hence, the lack of a standard method for studying API or excipient enzymatic hydrolysis in both the fasted and fed state, combined with scarce data available and the frequent use of cocktail of enzymes (obscuring the identity of the active enzyme), hampers mechanistic understanding. As a result, structure-stability relationships of API hydrolysis remain elusive and even less is known about polymer hydrolysis due to lack of data. Understanding of lipid digestion is more advanced, as it has been shown that shorter chain-length glycerides are hydrolyzed faster than their longer-chain counterparts. Therefore, systematic studies of API (including PROTACS and peptides) and polymer hydrolysis at standardized, physiologically relevant conditions are needed to generate a database, that can lead to an increased understanding of how the structure of API and excipients impacts their stability towards enzymatic hydrolysis in the small intestine. In addition, the impact of intra-individual variability, regional differences and how to translate these *in vitro* results to *in vivo* drug exposure *via* physiologically-based biopharmaceutics modelling (PBBM) is of considerable interest.

CRediT authorship contribution statement

Zahari Vinarov: Writing – review & editing, Writing – original draft, Conceptualization. **Anette Müllertzx:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Hristina Mircheva:** Investigation, Data curation. **Yann Le Gouar:** Investigation. **Olivia Menard:** Investigation. **Sharon Pradeep Kumar:** Investigation, Data curation. **Amrit Paudel:** Supervision, Conceptualization. **Didier Dupont:** Writing – original draft, Supervision. **Patrick Augustijns:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

A1. Protocol for collection of human intestinal fluids

Human intestinal fluids were collected at UZ Leuven as part of a study which was approved by the Ethics Committee Research UZ/KU Leuven, Belgium (S53791). The details of the protocol are described in Goovaerts et al. (Goovaerts et al., 2024). The volunteers did not have a history of GIT diseases and did not take any oral medication for two days before participating in the study. The enzyme assays used in the present study for lipase, trypsin and chymotrypsin determination were based on Brodkorb *et al.* (Brodkorb et al., 2019) and are described in the following section.

The protocol for collection of intestinal fluids via a naso-duodenal catheter is summarized in Fig. A1 below. The "fasted" conditions corresponded to the ingestion of 240 mL of (still) water. For the fed state, volunteers ingested 400 mL of Ensure Plus vanilla (600 kcal), followed by 240 mL of water 20 min later. For each volunteer (n = 11), enzyme activities were determined in one fasted state sample (collected between 30 and 60 min after water intake) and in 2–9 fed state samples (collected between 30 and 110 min after meal intake). For lipase determination, protease activities were blocked with Pefabloc (final concentration 5 mM); samples were diluted 1:1 with glycerol in order to preserve enzyme activities during freezing (Roman et al., 2007). For trypsin and chymotrypsin determination, lipolytic activities were blocked by adding 4-bromophenyl boronic acid (final concentration of 5 mM); samples were diluted 1:1 with glycerol before being stored at −20 °C until the analysis.

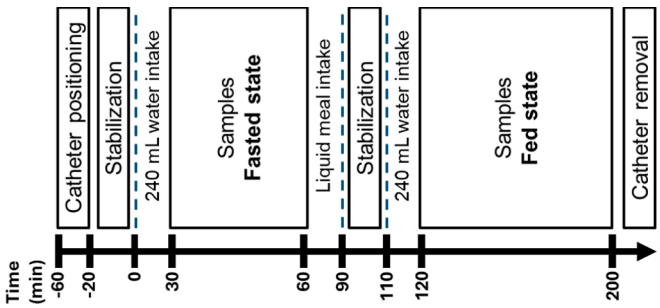

Fig. A1. Experimental design for collecting duodenal fluids in fed and fasted state conditions.

A2. Enzyme assays used for characterization of human intestinal fluids

The digestive enzyme assay protocols are detailed in the supplementary information of Brodkorb *et al.* (Brodkorb et al., 2019) and differ only in the temperature of the assays as all the enzyme activities were measured at 37 °C.

Unless otherwise stated, chemicals were from Sigma-Aldrich (Saint-Louis, USA).

Trypsin activity was assessed by using p-toluene-sulfonyl-L-arginine methyl ester (TAME, ref T4624) as a substrate. TAME plus H_2O produces p-toluene-sulfonyl-L-arginine plus methanol when trypsin is added. One unit of trypsin hydrolyzes 1 μmol of TAME per min at pH 8.1 and 37 °C. TAME was diluted at 5 mM in milli-Q water. Then, 1.3 mL of 46 mM Tris–HCl buffer (pH 8.1) was mixed with 150 μL of substrate and incubated in a spectrophotometer (SAFAS UVmc2, Monaco) at 37 °C at least 5 min to achieve temperature equilibration before adding 50 μL of the human intestinal fluids diluted in cold 1 mM HCl. The increase in absorbance was recorded at 247 nm over 10 min and the slope of the linear part of the curve was calculated.

Chymotrypsin activity was based on the use of N-benzoyl-L-tyrosine ethyl ester (BTEE, ref B6125) as a substrate: one unit of chymotrypsin hydrolyses 1.0 μmol of BTEE per min at pH 7.8 at 37 °C. The substrate, BTEE was dissolved at a concentration of 1.18 mM in methanol/milli-Q water (63.4/36.6: v/v). Then, 0.75 mL of buffer (0.08 M Tris HCl with 0.1 M $CaCl_2$ adjusted to pH 7.8) was mixed with 0.7 mL of BTEE and incubated in a spectrophotometer (SAFAS UVmc2, Monaco) at 37 °C for at least 5 min to achieve temperature equilibration before the addition of 50 μL of the human intestinal fluids diluted in cold 1 mM HCl. The increase in absorbance at 256 nm was recorded over 10 min and the slope of the linear part of the curve was calculated.

Finally, lipase activity was determined using a pH-stat technique (Titrando 842, Metrohm, Villebon-sur-Yvette, France) with tributyrin (TBU, ref T8626) as a substrate (purity $\geq$ 99 %). The pancreatic lipase assay was defined as the release of 1 μmol of butyric acid per min at 37 °C and pH 8.0. The free fatty acids released by the lipase was titrated by 0.1 N NaOH at a constant pH (8.0) using 0.1 N NaoH. The buffer contained 2 mM of $CaCl_2$, 0.3 mM of Tris-HCl, 4 mM of sodium taurodeoxycholate and 150 mM NaCl. The buffer (24 ml) was mixed with TBU (0.828 mL) and equilibrated at 37 °C in the pH-stat vessel. Then,12 μL of duodenal effluent was added and the rate of 0.1 N NaOH was monitored to maintain pH 8 for 8 min.

Table A1
Polymer degradation summary.

Polymer		Gastro-intestinal conditions			*In vitro* Validation	Notes	Ref
		pH	Medium	Enzymes			
Hydroxypropyl methylcellulose acetate succinate.	HPMCAS-MF (Aqoat®) and HPMCAS-MG	6.8 7.0	DAB 10 grade reagents	Pancreatin	Storage: 28 months at 26 °C in closed packaging Automatic titration apparatus	Aqueous coating remained with 50 % Acetyl content Residual lipase activity: Aqueous coating: 13 to 23 % Organic film coating: 5 %	(Thoma and Bechtold, 1999)
	HPMCAS-LF with silicon nanoparticles, (Psi) and Acorbyl palmitate (AP) hydrogel.	6.8	Continuous gradient pH media, Milli-Q water	Thermomyces lanuginosus lipase (100 U/mL)	*Normal conditions*: Shaking at 150 rpm. *Inflamed conditions*: Supernatant from human macrophages cultured with lipopolysaccharide	API release 80 % for 2 h 100 % for 5 h 0.5 μg/mL for 4 h 0.8 μg/mL for 24 h Polymer degradation not measured, focuses on vehicle efficiency for API delivery.	(Li et al., 2018)
	HPMCAS (Aqoat® AS-LF)		human small intestinal fluids		none	Viability: small intestine 2200 CFU/mm^2 Intestinal fluids: 4100 in CFU/mm^2 Polymer degradation not measured, focuses on survival rate of viable bacteria in Probiotics	(Park et al., 2016)
Hydroxypropyl methylcellulose phthalate (HP 55, HP 55-AF and −UF)		6.8		Pancreatin	Storage: 11 months at 26 °C in closed packaging	Aqueous coating left with 70–80 % phtalyl content.	(Thoma and Bechtold, 1999)

(*continued on next page*)

Table A1 (*continued*)

Polymer	Gastro-intestinal conditions			*In vitro* Validation	Notes	Ref
	pH	Medium	Enzymes			
		DAB 10 grade reagents		Automatic titration apparatus	Residual lipase activity: 90 % after 120 min	
Hydroxypropyl methyl cellulose phthalate (HPMCP)	7.0	0.1 M NaOH and 20 mM phosphate buffer	Porcine lipase and pancreatin	Shaking water bath −150 rpm	L-HPMCP (size > 500 μm) 43.8 %,S-HPMCP (size < 500 μm) 33.73 % Polymer degradation not measured, focuses on digestive stability and intestinal transport for the catechin.	(Chung et al., 2014)
Starch (Contramid) with Hydroxypropyl methylcellulose (HPMC) or Poly (vinylalcohol) (PVAL)	6.8	phosphate buffer (50 mM)	α-Amylase from Bacillus species	USP type III dissolution apparatus with phosphate buffer	Starch hydrolysis erosion: 20 % HPMC 15 % after 120 min 20 % PVAL 25 % after 120 min	(Rahmouni et al., 2001)
Phthalate esters and Bisphenol A in low-density polyethylene (LDPE) and polyvinyl chloride (PVC)	6.3	Simulated duodenal juice	Pancreatin, Lipase	In-vitro digestion models with GIT fluids Fed: (Versantvoort et al., 2005) Fasted: (BARGE—INERIS, 2011)	Hydrolysis of DMP and DEP: LDPE (7.2 %, 3.4 %-Fasted) (7 %, 1.6 %-Fed) PVC (6 %, 2.4 %-Fasted) (1.7 %, 0.5 % −Fed)	(Lopez-Vazquez et al., 2022)
Dibutyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP)	7.4	50 mM phosphate buffer	Recombinant human CESs	Pooled microsomes and cytosol of human derived small intestine	DBP hydrolysis Microsomes − 57 % Cytosol − 84 % DEHP hydrolysis microsomes − 2,120 pmol/min/mg protein cytosol- 909 pmol/min/mg protein CES2 activity: 1490 pmol/min/mg protein	(Isobe et al., 2023)
Dimethyl phthalate (DMP), DBP and DEHP.	7.4	0.1 M K,Na-phosphate buffer	Lipase from porcine pancreas Rat cells expressing carboxylesterase isoform	In a suspension of the rat derived small intestine microsomes, with incubation time of 5 min in DBP and DMP, and 20 min for DEHP	DEHPLipase (2.5 nmol/min/mg of monoalkyl protein) DBP-lipase (5.5 nmol/min/mg of monoalkyl protein)	(S.K. Ozaki H, Watanabe Y, , 2017)
Prodan® containing poly(D, L-lactic acid) nanoparticles.	7.5	phosphate buffer	Pancreatin	simulated intestinal fluid under continuous stirring	Lactate formation: Albumin-coated PLA_{50} nanoparticles 60 % after 480 min. PVA-coated PLA_{50} nanoparticles 2.2 % after 480 min	(Landry et al., 1997)
Poly(ε-caprolactone), Poly(l-lactide-co-ε-caprolactone) (PLCL)	6.5	25 mM MES-buffer	Lipase	Small intestinal fluids are freshly collected from patients with (post operative) intestinal probes inside the incubation tubes.	Mechanical degradation: Yield stress-40 % in 70 days & elongation at break Elastic modulus and viscoelastic coefficients −unchanged	(Peerlinck et al., 2023)

(*continued on next page*)

Table A1 (*continued*)

Polymer	Gastro-intestinal conditions			*In vitro* Validation	Notes	Ref
	pH	Medium	Enzymes			
					Polymer degradation not measured, focuses on the mechanical degradation of polymers.	
Starch and Poly(s-caprolactone) (PCL) blend	7.45.5	Phosphate buffer saline solution (0.01 M) Acetate buffer solution (0.1 M)	−Lipase from Thermomyces Lanuginosus. − α-amylase from Bacillus amyloliquefaciens − α-amylase from a genetically modified Bacillus lichenoformis.	constant shaking at 60 rpm for 6 weeks.	Hydrolysis: 5 % enzyme – 100 % (7 days) weight loss-50 %	(Azevedo and Rui, 2005)
Poly (ε-caprolactone)	–	Implants in small intestine of rats	–	–	Degradation Weight loss – ~5mg (28 days) Crystallinity-7 %, Molecular weight-73 %	(Chang et al., 2017)
Poly (vinylidene fluoride) (PVDF), Polybutylene Succinate		Implants in the duodenum	Pancreatic lipase		Degradation (1 month) Weight loss-2.51 % Tensile strength loss-21.52 % Release of acidic by-products	(Jeffrey Kuo et al., 2020)
Poly(ε-caprolactone) (PCL) and cellulose acetate (CA	6.0 7.4	0.05 M phosphate buffer 0.05 M acetate buffer	Lipase	The vials were incubated in a thermostatted oven	Degradation (250 h) Pure PCL mass retention 50 % Pure CA mass retention 105 %	(Gan et al., 1997)
			α-amylase	The vials were placed in a water bath	Degradation (250 h) Pure PCL mass retention 100 % Pure CA mass retention 108 %	
Starch crosslinked with other polymers.	6.8	phosphatebuffer	α-amylase fromBacillus Subtilis	Flow throughdiffusion cell, under constant stirring at 50 rpm	Degradation (10 h) Released sugar – 125 μmol (g gel)$^{-1}$	(Bajpai et al., 2006)
Starch (70 % amylose, 30 % amylopectin)	6.9	Phosphate buffer	Pancreatin	paddle apparatus(USP II) at 50 rpm	API release 100 % in 120 min & for few at 240 min. Polymer complex degradation not measured, focuses on accelerated API release rates.	(Carbinatto et al., 2016)
High amylose starch	6.8	phosphate buffer solution	Pancreatinfrom porcine pancreas	In simulated medium of the smallintestine under slow stirring (50 rpm)	Hydrolysis (120 min) Amylose complexes-100 % High amylose complex-80 %	(Yang et al., 2013)
Starch	8.1	Duodenal-bile juice	α-amylase	Simulated duodenal juice	Degradation Number of pores ~8.5 (16 h), ~13 (20 h). Amylose content 27.38 % (16 h) 28.66 % (20 h)	(Leyva-López et al., 2019)

Data availability

Data will be made available on request.

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