Journal of Animal and Feed Sciences, 34, 1, 2025, 20–28, https://doi.org/10.22358/jafs/190440/2024
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SHORT COMMUNICATION

Isolated digesta as an effective and reliable *ex vivo* system to study the release profile of drug formulations: study on *Candida utilis* urate oxidase

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KEY WORDS: digestion model, isolated chyme, oral drug formulations, urate oxidase

Received: 4 April 2024
Revised: 27 May 2024
Accepted: 25 June 2024

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ABSTRACT. Many artificial systems simulate the physiological state of the human gastrointestinal tract (GIT) and its separate compartments. These systems have the biorelevant media and imitate the physical forces and transit times of the GIT compartments, however, they lack the food-related and within-compartmental regulations and thus issues with translation of the data obtained to clinics arise. We aimed to introduce an alternative, simple and reliable *ex vivo* system which can be used in a laboratory setting, using fresh chyme from fed or fasted animals (pigs) to study the release profile of various drugs. For the present study we used porcine chyme collected from different gut compartments (stomach, duodenum and ileum) of six cross-bred male pigs in the fed state. Five different formulations of urate oxidase from *Candida utilis* were used as examples of tested drug substances. The performance of each formulation was tested by incubation in chyme at 37 °C for up to 4 h in the presence of uric acid. Samples were taken during the whole incubation time and the uric acid levels were estimated. The proposed *ex vivo* system provides information about the stability and performance of active drug substances in different gut compartments and can be used to test different formulations, assess possible drug-drug interactions, and the effects of fed and fasted conditions on the test substance of interest in the small and large intestine, taking into consideration diet-related changes in GIT secretions and intercompartmental regulation.

Introduction

In vitro models for digestion are widely used and have a broad range of applications, including studies on specific receptor binding, cell penetration, modulation of the activity of P-glycoprotein (PgP), breast cancer resistant protein (BCRP), organic anion transporters (OAT), cytochrome P450 (CYP 450), drug-drug interactions, as well as the digestibility of individual nutrients, bioaccessibility of environmental contaminants and the dissolution/release of new pharmaceutical products and formulations (Verhoeckx et al., 2015). These models vary from simple beakers with artificial biorelevant media, to sophisticated, dynamic models that simulate part of or the entire gastrointestinal tract (GIT) (Verhoeckx et al., 2015). Despite the physiology of the GIT being well understood and all the stages of digestion thoroughly characterized, both in terms of conditions and regulation of digestion, none of the currently available models can successfully depict the digestion process and thus provide researchers with precise information concerning the gut survival of ingested components.

Various artificial GIT models have been developed over the last few decades. They include complex systems reflecting the function of several GIT compartments (such as the TNO gastrointestinal model (TIM) (Bellman et al., 2016), the engineered stomach and small intestinal system (ESIN) (Guerra et al., 2016), the *in vitro* dynamic system (DIDGI®) (de La Pomelie et al., 2019), a GIT simulator (Simgi®) (e.g., Miralles et al., 2018) reflecting the interactions between the stomach and small intestine, a dynamic stomach model (dynamic gastric model (DGM) (Wickham et al., 2012), the human gastric simulator (HGS) (Kong and Singh, 2010), the artificial gastric digestive system (AGDS) (Liu et al., 2019) and the near real dynamic *in vitro* human stomach (new DIVHS) system (e.g., Wang et al., 2019). However, all the above-mentioned models are mainly used for the investigation of feed digestion mechanisms, since it's been recognized that the digestion and performance of pharmaceuticals and drugs employs different digestion mechanisms (Sensoy, 2021). Even though artificial GIT models are used during the initial steps of drug development, further progress undoubtedly requires the use of animal models.

Moreover, data obtained using the dynamic GIT models often requires a complicated analytical tool, such as physiologically based pharmacokinetic (PBPK) modelling, which involves the integration of mathematical modelling and simulations to predict the clinical pharmaco-kinetic profile of a drug. In fact, PBPK modelling extrapolates *in vitro* to *in vivo* data, which is useful in predicting how new formulations of known substances are dissolved and how the food components interact with any drugs administered *per os*. However, PBPK modelling has its own limitations and unfortunately can't be used when describing new drug substances and their formulations. Even properly developed PBPK models, for known drug substances, do not model the mechanisms involved in switching between the fasted and fed state, as well as any food-related and 'within compartmental' digestive regulation (Sager et al., 2015; Li et al., 2018; Tistaert et al., 2019). These limitations and requirements for data analysis undoubtedly limit the use of *in vitro* models of the GIT.

In the present work we used different oral formulations of *Candida utilis* urate oxidase as an example of a tested substance. Urate oxidase was chosen as an enzyme model since a lot of the studies related to urate oxidase of different origins and its' effectiveness in reducing blood uric acid levels are currently taking place around the world, with moderate success with regards to implementation of the enzyme as a drug (Louyot et al., 1970; Sherman et al., 2008; Szczurek et al., 2017; Pierzynowska et al., 2020; Yip et al., 2023). Chyme from the small intestine of juvenile pigs was chosen as a test system, since the characteristics of the gastrointestinal tract and digestion properties (structure, length, food retention time and food digestion/absorption etc.) are very similar between humans and pigs (Tajima and Aminov, 2015; Roura et al., 2016; Bergen, 2022).

The aim of the current study was to introduce isolated porcine digesta as a simple, efficient, and relevant *ex vivo* test system which could be used for fast and reliable estimation of the dissolution and release profile of oral drug formulations and comparison of their stability and biological activity.

Material and methods

Animals

The present study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All efforts were made to minimize animal suffering. The study was approved by the Second Local Ethics Committee for Animal Experimentation in Warsaw, Poland (approval no. WAW2/088/21).

The experiment was performed on crossbred ((Polish Landrace × Yorkshire) × Hampshire)) pigs (*Sus scrofa domesticus*) purchased from a local herd (Karniewek, Poland). Six, male pigs, aged 16 ± 2 weeks and weighing 35 ± 3 kg, were used in the study. Pigs were fed a high-fat diet, with 18% fat content, mimicking the human diet (Morawski, Żurawia, Poland), and sacrificed 3 h after feeding (animals were euthanized by intravenous injection of sodium pentobarbiturate (100 mg/kg), Morbital (Biowet, Puławy, Poland)). Digesta from the duodenum, jejunum and ileum was collected, pooled and immediately frozen in aliquots at −80 °C for further experiments.

Test articles

Five different formulations of urate oxidase from *Candida utilis* (cat #U2625, Merck, Darmstadt, Germany) were used in the study. The main features of the formulations used are provided in the Table 1.

Table 1. Urate oxidase formulations from *Candida utilis* used in the study

Formulation	Specific activity, units/mg	Activity per tablet	Form
F1	15	275	tablet
F2	26	500	tablet
F3	25	475	tablet
F4	25	470	tablet
F0 (drug substance)	26	N/A	powder

Test procedure

Experimental conditions and set-up are provided in Table 2. Chyme samples were thawed, and all tubes and jars were labelled appropriately. Fifteen ml of chyme from the duodenum, jejunum and ileum were taken from previously defrosted chyme samples and placed into 100 ml plastic jars to warm up for ~15 min at 37 °C, with shaking. Simulated intestinal fluid (SIF) (pH 6.8), in the same volume, was used as a comparator. To prepare the SIF, potassium phosphate monobasic (10.2 g) and sodium dodecyl sulfate (3.75 g) were dissolved in a 1000 ml of deionized water and then pH adjusted to 6.8 ± 0.1 with 1 N NaOH. The pH of each sample was recorded at the end of incubation using a pH meter, Jenway 370 (Keison Products, Essex, England). A 0.5 ml sample of chyme from the duodenum, jejunum and ileum was taken as a negative control.

Table 2. Experimental set-up

Ingredients	Test system	Test formulation	No. of tablets/ weight of substance	Urate oxidase, units
1	Duodenal chyme	F0	53 mg	500
2	Jejunal chyme			
3	Ileal chyme			
4	Duodenal chyme	F1	2 tablets	546
5	Jejunal chyme			
6	Ileal chyme			
7	Duodenal chyme	F2	1 tablet	506
8	Jejunal chyme			
9	Ileal chyme			
10	Duodenal chyme	F3	1 tablet	474
11	Jejunal chyme			
12	Ileal chyme			
13	Duodenal chyme	F4	1 tablet	471
14	Jejunal chyme			
15	Ileal chyme			
16	SIF, pH 6.8	F0	53 mg	500
17	SIF, pH 6.8	F1	2 tablets	546
18	SIF, pH 6.8	F2	1 tablet	506
19	SIF, pH 6.8	F3	1 tablet	474
20	SIF, pH 6.8	F4	1 tablet	471

SIF – simulated intestinal fluid; formulations F0–F4 – see Table 1

After warming up and pH measurement, the appropriately weighed uric acid from *Candida utilis* sample was added to each jar to achieve a final concentration of 1.7 mg/ml. Then all the jars were incubated at 37 °C for ~30 min with shaking to allow the urate to be well mixed. At the end of the incubation period, 0'+ chyme sample (0.4 ml) was collected and frozen at −20 °C.

The appropriate test article (F0 (powder) or tablets) was added to the experimental jars based on Table 2.

The content of the jars was mixed well and at time 0 (0'+346, reaction start), as well as at 15, 30, 60, 90, 120, 180 and 240 min after the start of the incubation period, two samples of 0.4 ml were collected from each jar in two empty pre-labelled 1.5 ml Eppendorf tubes (one for urate measurement and one for urate oxidase estimation). Samples were placed in a heating block (VWR International, Radnor, Pennsylvania, USA) warmed at 90 °C to inactivate and stop the urate oxidase reaction for accurate urate measurement. The tubes were then frozen until further analysis of uric acid concentration.

Urate measurements

The concentration of urate in samples was measured spectrophotometrically, using an enzymatic Uric Acid Assay Kit, Liquick Cor-UA 60 PLUS (cat. # 2-258, P.Z. Cormay S.A., Lomianki, Poland), according to the manufacturer's protocol with modifications for a microplate reader. Prior to analysis samples were thawed, extracted with 0.068M Li_2CO_3 (pH 11.5) (1:3, weight: volume, incubation 30 min on shaker at room temperature) and, following gentle mixing, were incubated in boiling water for 10 min, followed by 15 min centrifugation at 13 000 g at 4 °C. The resultant supernatant was then filtered through the Centrifugal Concentrator Vivaspin 500, 10 kDa (Vivaspin R, Sartorius, Göttingen, Germany) at 15 min at 13 000 g and 4 °C. The filtrate was used to determine urate concentrations. Optical density was measured using a Spectra Max i3x Multi-Mode detection platform (Molecular Devices, LLC, Sunnyvale, CA, USA)

Statistical analysis

Data is expressed as mean ± standard deviation (SD), from three independent experiments. The data distribution was checked using a Shapiro-Wilk normality test. A one-way ANOVA was used to estimate differences, data was not corrected for multiple comparisons. In all statistical analyses, $P \leq 0.05$ was considered significant. All analyses were carried out using Prism, version 10 (GraphPad Software, Inc., San Diego, CA, USA).

Results

pH values

Data on mean pH values in different test systems are provided in Figure 1. The highest pH values were observed in the SIF system (6.44 ± 0.07) and the lowest pH was observed in the ileal chyme test system (5.14 ± 0.03). The pH values obtained from the duodenal and jejunal test systems were 5.9 ± 0.08 and 6.28 ± 0.08, respectively. All differences described above were significant ($P < 0.05$).

Changes in urate content

The curves of uric acid degradation and within-system differences of urate degradation by different formulations are shown in Figure 2 A–H.

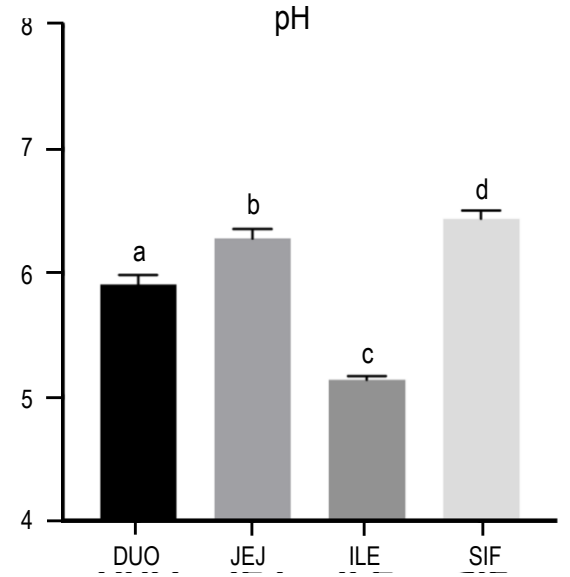

Figure 1. pH values in the different test systems after 15 min of incubation

DUO – duodenal chyme samples, JEJ – jejunal chyme samples, ILE – ileal chyme samples, SIF – simulated intestinal fluid samples. Data was analysed using an ordinary one-way ANOVA test and presented as mean ± SD. Small letters given with result bars describe significant differences between groups when $P < 0.05$

Urate degradation, which reflects enzyme activity, was significantly different between formulations within the same experimental system. For example, the most active formulations in the SIF system were F0 and F1 (lowest AUCs of UA concentration), while the F2 formulations demonstrated the lowest activity in the same incubation system (Figures 2A,B). At the same time, in the duodenal chyme test system the activities of F0, F1 and F2 formulations were not significantly different (Figures 2C,D). In the jejunal chyme test system, the F1 formulation appeared to be the most efficient in terms of urate degradation (Figures 2E,F), while in the ileal test system, the F1 and F0 formulations' activity was the highest, while the F2, F3 and F4 formulations demonstrated significantly lower abilities to degrade urate (Figures 2D,H).

The between-system differences in urate degradation by urate oxidase formulations are shown in Figures 3A–E. No differences were observed between the activity profiles of urate oxidase in the SIF test system and the ileal test system for any of the formulations (Figures 3A–E), but there were significant differences in the urate degrading ability of the F0 and F2 formulations in the SIF test system, when compared to that of the duodenal test system (Figures 3A,C).

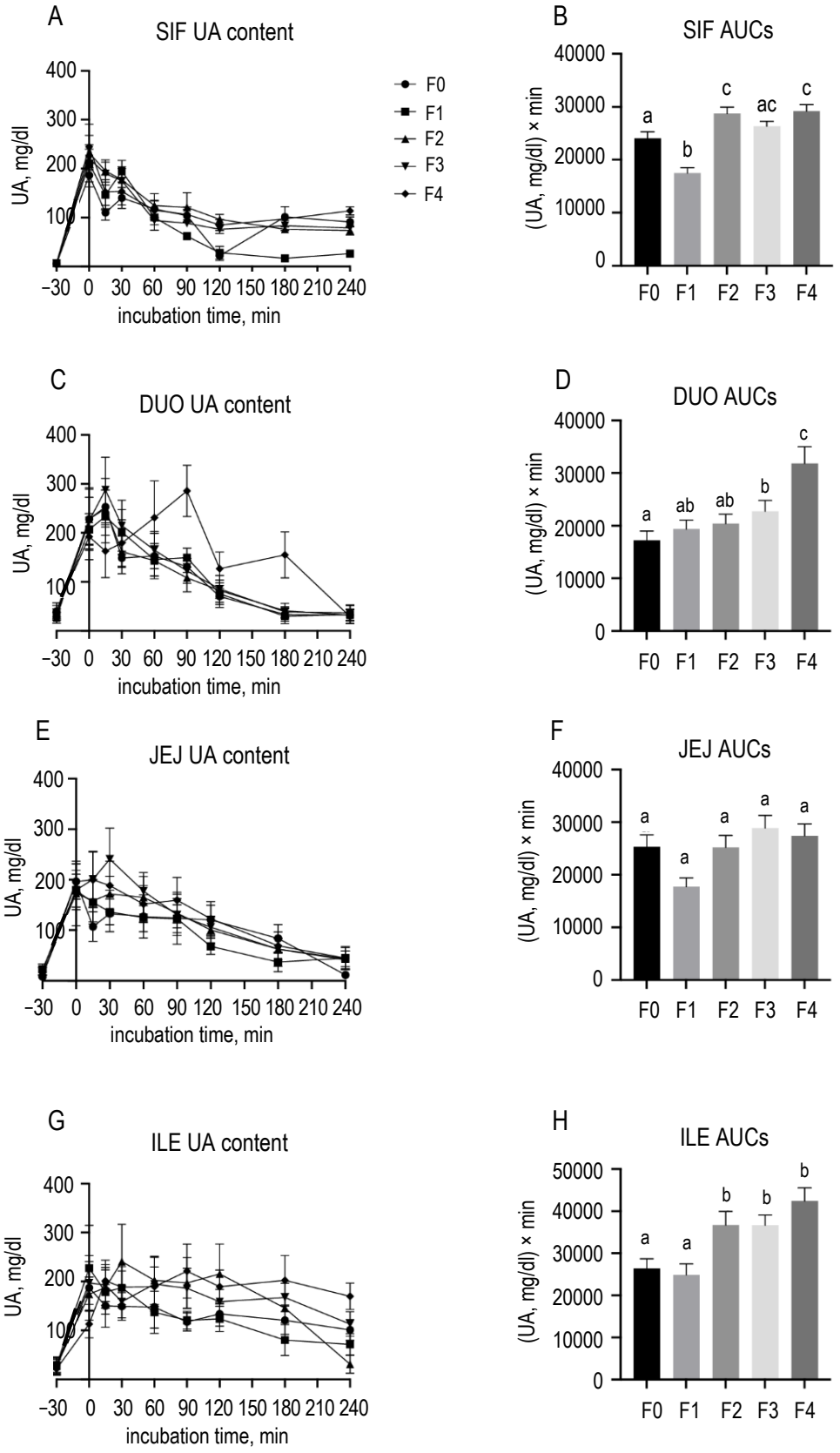

Figure 2. Uric acid degradation profiles in the different test systems

DUO – duodenal chyme samples, SIF – simulated intestinal fluid samples, JEJ – jejunal chyme samples, ILE – ileal chyme samples, UA – uric acid, AUC – area under the curve; formulations F0–F4 – see Table 1. Data was analysed using an ordinary one-way ANOVA test. Data on uric acid content is presented as mean ± SD, data on AUCs is presented as mean ± SEM. Small letters given with result bars describe significant differences between groups when $P < 0.05$

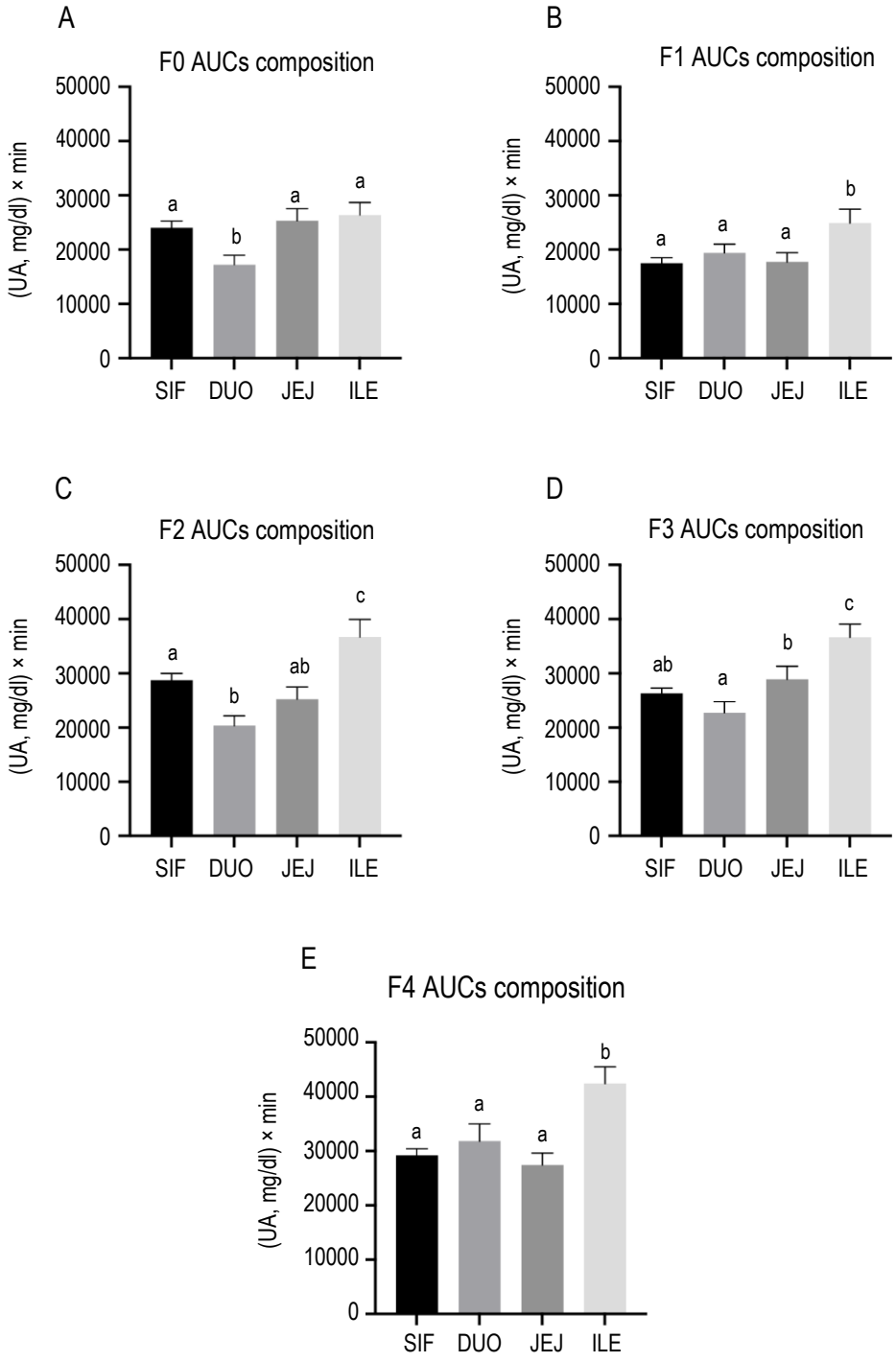

Figure 3. Performance of urate oxidase formulations in different test systems

SIF – simulated intestinal fluid samples, DUO – duodenal chyme samples, JEJ – jejunal chyme samples, ILE – ileal chyme samples, UA – uric acid, AUC – area under the curve; formulations F0–F4 – see Table 1. Data was analysed using an ordinary one-way ANOVA test. Data is presented as mean ± SEM. Small letters given with result bars describe significant differences between groups when $P < 0.05$

Discussion

The *ex vivo* system described above enables the use of less animals during drug development and results achieved are closely mimic the 'fate' of the drug of interest in the GIT. The use of such a system significantly reduces the number of animals used in drug testing experiments, eliminating their potential suffering during the study. Thus, animal studies used in conjunction with the *ex vivo* system described in the current study would allow us to run the studies on digestion according the 3 R principle (Diaz et al., 2020).

There are many aspects of digestive physiology which are not represented in the currently available *in vitro* models of digestion. Majority of digestive models, even the most relevant and complicated, do not reflect diet-dependent changes in intestinal gland secretion, changes in viscosity of intestinal content and the duration of its processing in all separate GIT compartments, nutrient-dependent absorption in the small intestine, etc. (Lex et al., 2022; Staniszewska et al., 2023). It is possible however, to take into account and measure all of the above-mentioned parameters *in vivo*, but these studies require large numbers of experimental animals, which could be an ethical issue. *In vitro* studies, in turn, introduce a number of simplifications e.g., composition of gut chyme vs. media used in *in vitro* experiment and approximations which undoubtedly influence the relevance of the data obtained (Lex et al., 2022).

The present study demonstrated a simple, but relevant *ex vivo* GIT compartment, sensitive enough to study enzymatic effectiveness, using the example of urate oxidase in chyme from fed and fasted, young, healthy pigs, which reflects the properties of the stomach and intestinal tract, includes the major and minor factors involved in digestion and allows for the fast and efficient estimation of dissolution, biological activity, and release profile of drug substances in different formulations. This model not only gives an understanding of the environmental surrounds in the case of the ingestion of the drug urate oxidase, but fully represents this environment and at the same time makes it possible to perform a huge range of modifications (meal composition, dietary additives, time of fasting and after meal conditions, etc.).

Considering the above-mentioned environmental influences in the gut lumen, one should mention that pH values measured in the presented test system have been shown to be a very representative and sensitive parameter. There are a lot of investigations and publications on pH values of different GIT compartments and one of most recent studies (Henze et al., 2021) shows a gradual pH change in the porcine small intestine, from 6.7–7.5 in the duodenum to 7.6–8.0 in the ileum. pH values in the present study were generally lower (5.9 ± 0.08 (duodenal chyme), 6.28 ± 0.08 (jejunal chyme) and 5.14 ± 0.08 (ileal chyme)), which could be explained by both dietary influence (meal composition) and cecoileal reflux, both of which could significantly reduce pH (Cuche et al., 1998; Hăbeanu et al., 2022). Thus, drug formulations for various diseases could be tested together with meals recommended for each particular sickness (macro- and micronutrients, fiber content, etc.), which undoubtedly could bring more relevance to the test outcome.

The observation of various patterns of drug formulation activity between the incubation systems with intestinal content reflect the physiological differences between GIT compartments and provides sufficient and important information on the stability of formulations, not only in the gut compartment of interest (e.g., substrate digestion, site of absorption), but in other parts of the GIT as well. For example, it was clearly shown that the activity of the enzyme responsible for urate degradation is very dependent on the milieu of the different gut compartments (Figures 2A–H), thus enabling researchers to choose an optimal drug formulation, as well as indicating that the stability and activity is dependent on the GIT compartment.

At the same time, comparison of the same drug formulation activity in the diverse experimental systems (Figures 3A–E) confirms that the artificial incubation system (SIF) often does not reflect conditions created in the compartment of interest (duodenal compartment), but rather creates an environment similar to the one observed in jejunal and ileal compartments.

Of course, the described model of digestion has its limitations and confines. The most sensitive and most important, however usually neglected limitation of the present study, as well as all other *in vitro* models, is the lack of product elimination from the reaction environment, e.g., central absorption. However, it looks like the products of urate oxidase, CO_2 and H_2O, are more effectively neutralized in the gut chyme than in any other artificial buffer. Unfortunately, the pH values of chyme at the end of the reaction cannot be reported, but the relatively high temperature of incubation (37 °C) decreased the dissolution ability of CO_2 and in this way pH levels should be stabilized.

Currently, this system has only been tested for microbial urate oxidase, which is not intended to enter the circulation (Szczurek et al., 2017; Pierzynowska et al., 2020), thus, the measurement of absorption of a drug substance could be challenging. Moreover, any facility willing to introduce this technique should have allowance for work with large laboratory animals, which are close to humans in terms of metabolism e.g., pigs, and for the processing of biological samples and all protocols involving animals should be approved by local ethical authorities. However, obtaining gut digesta from local small slaughterhouses could also be considered, providing that the facility possesses such a license.

Conclusions

Even though there are many artificial models of digestion available to date, both dynamic and static, there is still an increasing demand in simple and apt tools which can be easily operated, are reliable and provide fast and relevant data. None of the currently available models, even the most advanced ones, depict the entire process of digestion which begins in the oral cavity and ends in the colon. The lack of food-related and within-compartmental regulation specificity in the artificial models of digestion necessitates the need for animal studies to be performed. The present study describes the use of isolated porcine digesta which appeared to be a simple, efficient, and relevant *ex vivo* model. The artificial model of digestion based on the isolated porcine chyme could be used to estimate the dissolution and release profile of enzymatic drugs and compare their stability and biological activity when active substances are used in different formulations and in doing so substantially decrease the number of animals required for the testing of oral drug formulations.

Conflict of interest

The Authors declare that there is no conflict of interest.

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