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Research Paper

Chain length affects pancreatic lipase activity and the extent and pH–time profile of triglyceride lipolysis

Paloma Benito-Gallo [a], Alessandro Franceschetto [a,b], Jonathan C.M. Wong [a], Maria Marlow [a], Vanessa Zann [c], Peter Scholes [c], Pavel Gershkovich [a,*]

[a] School of Pharmacy, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom
[b] School of Pharmacy, University of Padua, Via Marzolo 5, Padua 35131, Italy
[c] Quotient Clinical Ltd., Mere Way, Ruddington Fields, Nottingham NG11 6JS, United Kingdom

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ABSTRACT

Triglycerides (TG) are one of the most common excipients used in oral lipid-based formulations. The chain length of the TG plays an important role in the oral bioavailability of the co-administered drug. Fatty acid (FA) chain-length specificity of porcine pancreatic lipase was studied by means of an *in vitro* lipolysis model under bio-relevant conditions at pH 6.80. In order to determine the total extent of lipolysis, back-titration experiments at pH 11.50 were performed. Results suggest that there is a specific chain length range (C2–C8) for which pancreatic lipase shows higher activity. This specificity could result from a combination of physicochemical properties of TGs, 2-monoglycerides (2-MGs) and FAs, namely the droplet size of the TGs, the solubility of 2-MGs within mixed micelles, and the relative stability of the FAs as leaving groups in the hydrolysis reaction. During experimentation, it was evident that an optimisation of lipolysis conditions was needed for tighter control over pH levels so as to better mimic *in vivo* conditions. 1 M NaOH, 3.5 mL/min maximum dosing rate, and 3 μL/min minimum dosing rate were the optimised set of conditions that allowed better pH control, as well as the differentiation of the lipolysis of different lipid loads.

1. Introduction

Since the advent of high throughput techniques and development of combinatorial chemistry in the early 1990s, the number of potential drug candidates has significantly increased [1]. Physical properties of the new chemical entities have changed towards higher molecular weight, higher melting point, increased H-bonding capacity, and increased lipophilicity, leading to poorer solubility in aqueous media [2]. Indeed, it was estimated that in 2005 40% of the top 200 oral marketed oral drugs were poorly water-soluble [3]. Latterly in 2007, it was reported that up to 70% of the new active molecules in the development pipeline exhibited poor aqueous solubility [4]. Since low aqueous solubility can be associated with poor absorption and hence poor bioavailability, it is clear that one of the main challenges for pharmaceutical scientists is finding novel formulations capable of improving the intraluminal solubility of poorly soluble drugs.

The co-administration of hydrophobic drugs with dietary or formulation lipids in many cases results in improved oral bioavailability. Different proposed mechanisms by which lipidic formulations increase oral bioavailability include the following: (a) promoting drug solubilisation in the gastrointestinal tract, by providing lipidic components that increase the inherent solubilisation capacity of the intestinal fluids [5], (b) delaying gastric emptying and transit time [6], (c) increasing apparent drug permeability through inhibition of efflux transporters such as P-glycoprotein [7,8], (d) changing the membrane fluidity of enterocytes [9], and (e) reducing hepatic first-pass metabolism if lymphatic transport is involved [10].

Recently, there has been a growing interest in oral lipid-based drug delivery systems (LBDDSs) as a formulation strategy for efficient delivery of poorly water-soluble compounds [11,12–14]. Marketed formulations such as Marinol® (dronabinol) [15],

Abbreviations: DLS, dynamic light scattering; FA, fatty acid; LBDDS, lipid based drug delivery system; LCT, long-chain triglyceride; MCT, medium-chain triglyceride; MG, monoglyceride; SCT, short-chain triglyceride; TBU, tributyrin unit; TG, triglyceride; Tri-C2, glyceryl triacetate; Tri-C4, glyceryl tributyrate; Tri-C8, glyceryl trioctanoate; Tri-C10, glyceryl tridecanoate; Tri-C18, peanut oil.

* Corresponding author at: Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom. Tel.: +44 0115 84 68014.
E-mail address: pavel.gershkovich@nottingham.ac.uk (P. Gershkovich).

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Neoral® (cyclosporine A) [16], Rocaltrol® (calcitriol) [17], Agenerase® (amprenavir) [18] and Accutane® (isotretinoin), have demonstrated that LBDDSs are an accepted and successful commercially viable formulation strategy for sparingly soluble compounds.

In vitro lipolysis is capable of mimicking the intestinal lipid digestion process, and therefore is a suitable method to trace the fate of drugs delivered by means of LBDDSs. The *in vitro* lipolysis model (extensively reviewed elsewhere [19–21]) has been previously developed and utilised by different research groups (including the University of Copenhagen [22,23], Monash University [24], Gattefossé [25] and The Hebrew University of Jerusalem [26]). Although the concept and fundamental principles of the model are similar between groups, experimental conditions and parameters vary among them. In this regard, the Lipid Formulation Classification System Consortium has published a number of studies aimed to reduce the variability in the experimental approach between different groups [7,27,28]. Briefly, the protocol for *in vitro* lipolysis consists of the dispersion of the LBDDS in the experimental medium consisting of simulated intestinal fluids. The addition of pancreatic lipase to the medium initiates the lipolysis process. The digestive enzyme hydrolyses triglycerides (TGs) in the formulation, releasing fatty acids (FAs) and inducing a drop in pH. In order to keep the pH at a constant value throughout the experiment (to mimic *in vivo* conditions), a pH-stat titrator is used. The instrument continuously measures and controls this transient drop in pH by equimolar titration of NaOH. The University of Copenhagen has used a slightly different approach, a dynamic *in vitro* lipolysis model, in which the rate of hydrolysis is controlled by continuous addition of calcium chloride [22,23]. Once the process is finished (or deliberately stopped by addition of an inhibitor), the resulting solution is ultracentrifuged and separated into three distinct layers: (i) an upper undigested lipid phase, (ii) a middle aqueous phase, containing colloidal structures within which poorly-water soluble drug molecules are solubilised, and (iii) a lower sediment phase, comprising FAs calcium soaps. It is assumed that drug molecules solubilised in the aqueous micellar phase are most readily available for absorption. After density-gradient separation, each phase is analysed for drug content. Finally, the percentage of drug dose solubilised in the aqueous phase *in vitro* is then compared with the *in vivo* pharmacokinetic data obtained following oral administration (to an animal or a human) of the LBDDS.

TGs are the main constituents of dietary lipids [29] and one of the most common excipients used in LBDDSs [12]. TG-based drug delivery systems, which belong to Type I formulations according to Lipid Formulation Classification System [30], are the most basic LBDDSs since they include neither surfactants nor co-solvents. The FA chain length of the TG in the formulation is an important factor in the oral bioavailability of the co-administered poorly water-soluble drug [31]. In general, following absorption into the enterocyte, lipolysis products derived from short-chain TGs (SCTs, <C6) and medium-chain TGs (MCTs, C6–C12) diffuse across the cell gaining access to the portal vein. However, FAs and MGs derived from long-chain triglycerides (LCTs, >C12) are re-esterified, incorporated into chylomicrons, and enter the lymphatic system, bypassing the hepatic first-pass metabolism [32]. SCTs are known to induce tight junction permeability changes [33], while the micellar solubilisation capacity of MCTs and LCTs has been proven to be higher than that of SCTs [26,34]. Although the assessment of the performance of TGs with different chain lengths has been carried out before, these studies have only focused on the end result, i.e. drug solubilisation across lipolysis phases [7,24,26,35–37]. Limited attention has been drawn to the causes for substrate specificity of the pancreatic lipase [38,39]. A better knowledge of the lipolysis process itself, and the factors governing lipase activity, would help to rationalise the performance of LBDDSs and eventually aid in the development of optimised lipidic formulations.

Accordingly, the first objective of this study was to gain a deeper understanding of the mechanism behind pancreatic lipase activity, by evaluating the *in vitro* lipolysis of equimolar amounts of TGs with different chain lengths.

Because different pH–time profiles were observed during the lipolysis of TGs with different chain lengths, it was evident that an optimisation of lipolysis conditions was needed for tighter control over pH levels so as to better mimic *in vivo* conditions. Therefore, the second aim of the study was to find an optimised set of conditions (in terms of titrant concentration and maximum and minimum titrant addition rates) capable of maintaining the pH environment within the physiological range (6.75–6.85) during the hydrolysis of TGs with different carbon chain lengths. The hydrolysis of different volumes of oil was also evaluated to assess a variety of possible scenarios in the intestine, from the ingestion of an oil-containing capsule in fasting conditions to the consumption of a high-fat meal.

2. Materials and methods

2.1. Materials

Sodium hydroxide solutions (NaOH, 0.5 M and 1 M), Trizma® maleate, sodium taurocholate hydrate (98% w/w), L-α-lecithin (~60% pure L-α-phosphatidylcholine, from egg yolk), pancreatin powder from porcine pancreas (8 × United States Pharmacopeia specifications activity), glyceryl triacetate (≥99.9%), glyceryl trioctanoate (≥99%), and peanut oil were all purchased from Sigma–Aldrich (Dorset, UK). Sodium chloride (99.5% w/w) was a product from Fisher Scientific (Leicester, UK). Calcium chloride anhydrous (93% w/w), and glyceryl tributyrate (98%) were purchased from Alfa Aesar (Heysham, UK). Glyceryl tridecanoate (≥98%) was obtained from TCI (Tokyo, Japan). The standard buffer solutions (pH 4, 7, 10 and 12), utilised for calibration of the pH-electrode, were purchased from YSI Incorporated (Ohio, USA) and Hanna Instruments (Rhode Island, USA). Water was obtained from a Purelab Ultra Genetic purification system (Elga LabWater, Illinois, USA).

2.2. Lipidic formulations

Glyceryl triacetate (tri-C2) and glyceryl tributyrate (tri-C4) served as model molecules for SCTs (<C6). Glyceryl trioctanoate (tri-C8) and glyceryl tridecanoate (tri-C10) represented MCTs (C6–C12). In a similar manner to previous publications [26,40], peanut oil (tri-C18) was chosen as the prototype for LCTs (>C12). Peanut oil contains mainly LCTs (C16 and C18), the vast majority of which is triolein [29].

2.3. Preparation of simulated digestion buffers

The preparation of the bio-relevant digestion buffer simulating the contents of the jejunum in the fasted state was based on previous reports [26,41] with a minor modification. This change consisted in decreasing the pH of the buffer from 7.40 to 6.80 to achieve maximum pseudo-physiological conditions [18]. The lipolysis medium contained 50 mM Trizma® maleate [35,36,42,43], 150 mM sodium chloride, 5 mM calcium chloride, 5 mM sodium taurocholate, and 1.25 mM L-α-lecithin. The pH of the medium was adjusted to 6.80 ± 0.05 at 37 °C using 1 M NaOH solution as titrant, and a pH-stat titrator unit (T50 Graphix, Mettler Toledo Inc., Leicester, UK) coupled to a pH-electrode (DGi111-SC, Mettler Toledo Inc., Leicester, UK).

The incomplete digestion buffer used for the preparation of the enzyme extract was prepared in a similar manner, although it did not include bile salts or phospholipids to prevent the deactivation of the lipase prior to the lipolysis experiments.

2.4. Preparation of lipase/co-lipase extract

Porcine pancreatin powder, containing equimolar amounts of lipase and co-lipase [23], was prepared as described by Sek et al. [44]. Briefly, one gram of pancreatin powder was added to 5 mL of incomplete digestion buffer and vortex-mixed for 15 min at room temperature. After centrifugation at ~1200*g* (Harrier 18/80 centrifuge, swing-out rotor, MSE, London, UK) and 4 °C for 15 min, the supernatant was collected and stored on ice to avoid denaturation. The activity of the lipase/co-lipase extract used in this study was 42 tributyrin units (TBU) per milligram of dry pancreatin powder (735 TBU per millilitre of digest), where 1 TBU is the amount of enzyme that can release 1 μmol of butyric acid from tri-C4 per minute.

2.5. Experimental procedure: lipolysis of equimolar amounts of different triglycerides

The procedure of the *in vitro* lipolysis was similar to that described previously [7,24,26,36,37]. A fixed molar amount of oil (860 μmol) was added to 35.5 mL of digestion buffer dispersed in a reaction vessel with continuous stirring and kept at 37 °C. After 15 min of equilibration, 3.5 mL of lipase/co-lipase extract was added to the mixture to initiate the enzymatic hydrolysis. A pH-stat titrator unit was used to keep experimental pH under control (6.75–6.85) by titrating the released ionised FAs with 0.5 M NaOH solution. The maximum and minimum rates of titrant addition were set up through the instrument control software (LabX light v3.1) at 1 mL/min and 10 μL/min, respectively. The experiments were considered to be completed when the dosing rate of NaOH was lower than 10 μL/min. Each experiment was repeated five times.

Control experiments ($n = 5$) were performed without any formulation, to correct for the amount of NaOH solution needed to neutralise the acids released as a consequence of the lipolysis of phospholipids, or arising from the lipolysis of impurities in the bile and pancreatin extracts.

The extent of digestion was expressed as percentage of the maximum theoretical quantity of lipid susceptible to hydrolysis. Accordingly, it was assumed that one TG initially released two FAs and one 2-monoglyceride (2-MG). It has been reported that 2-MGs can isomerise to 1/3-monoglyceride (1/3-MG) and be subsequently lipolysed releasing a third FA and glycerol, as depicted in Fig. 1 [45–48]. The apparent extent of lipolysis at pH 6.80 was calculated from the volume of titrant consumed during the *in vitro* digestion, as expressed in Eq. (1):

$$\text{Extent of lipolysis } (\%) = \frac{V \cdot 0.5 \cdot \text{MW}}{3 \cdot \rho \cdot v} \cdot 100 \qquad (1)$$

where V is the volume (L) of titrant consumed during the digestion at pH 6.80, 0.5 M is the concentration of the titrant, MW is the molecular weight (g/mol) of the oil under investigation, 3 is the maximum quantity of FAs than can be released from one TG, ρ is the density (g/mL) of the oil, and v is the volume (mL) of oil dispersed in the lipolysis medium.

2.6. Experimental procedure: back-titrations

Based on their apparent pK_a, FAs released as a consequence of enzymatic hydrolysis at pH 6.80 may be only partially ionised. As a result of this titration by NaOH, lipase activity determination can be underestimated in direct titration experiments. In order to calculate the total extent of lipolysis, back-titrations [7,49] were performed. In these experiments, the pH of the medium was elevated to pH 11.50 ± 0.05 by quick addition of 0.5 M NaOH. Control experiments without any triglyceride were performed to correct for the amounts of NaOH needed to raise the pH of the medium up to 11.50.

The total extent of lipolysis was calculated using Eq. (1), where V represented the volume of NaOH added originally at pH 6.80 (titration of ionised FAs) plus the volume of NaOH added during the back-titration experiments (titration of unionised FAs).

2.7. Solubility effect of glyceryl triacetate on the extent of lipolysis

As opposed to the other model triglycerides, tri-C2 was completely soluble in the bio-relevant media due to its high water solubility (58 g/L at 25 °C, [50]). In order to determine whether this factor would affect pancreatic lipase activity, additional lipolysis experiments ($n = 3$) with higher amounts of tri-C2 were performed. 1500 μL and 2100 μL of tri-C2, representing values slightly below (49 g/L) and above (68 g/L) the solubility limit, respectively, were lipolysed under the same conditions described in Sections 2.5 and 2.6.

2.8. Measurement of droplet size and total surface area of the equimolar triglyceride emulsions following dispersion in the lipolysis buffer

Dynamic light scattering (DLS) was used to determine the mean droplet size (d_H) of the emulsions in the digestion medium before the addition of pancreatic lipase, just after the equilibration period. DLS measurements were carried out at a scattering angle of 173° and 37 °C, using a Zetasizer Nano ZS ($\lambda = 633$ nm, Malvern Instruments, Malvern, UK). As the emulsions were too turbid, they were diluted with incomplete lipolysis buffer to $5 \cdot 10^{-2}$% *v*/*v* to avoid multiple scattering effects. Size determinations were performed for all TG emulsions at least 8 times. Diluted digestion buffer was also analysed to account for any contribution of bile salts and phospholipids to DLS measurements. As expected, droplet size of digestion buffer particles was below the detection limit of the instrument [51], and their size could not be determined.

Droplet size measurements were used to calculate the specific surface area (S_S, surface area per unit volume [52]) of the emulsions formed prior to enzyme addition. Assuming emulsions were

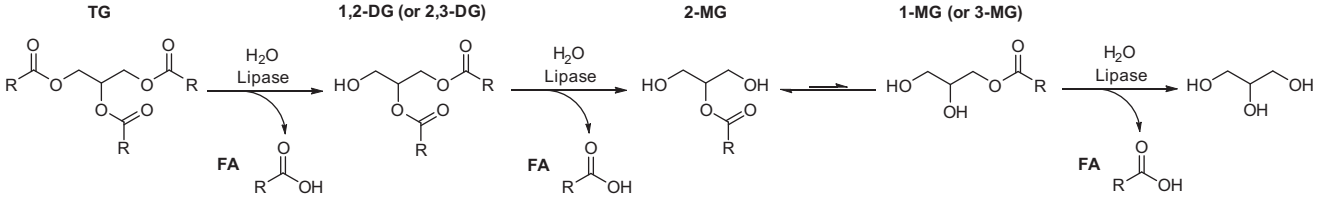

Fig. 1. Lipolysis of triglyceride by pancreatic lipase. Pancreatic lipase shows the same selectivity towards the hydrolysis at positions *sn-1* and *sn-3* of the triglyceride when the fatty acid side chains are identical.

formed by spherical droplets, the surface was determined using the following equation:

$$S_S = \frac{S_T}{V_T} = \frac{n \cdot S_i}{V_T} = \frac{V_T/V_i \cdot S_i}{V_T} = \frac{S_i}{V_i} = \frac{\pi d_H^2}{1/6\pi d_H^3} = 6\frac{1}{d_H} \quad (2)$$

where S_T is the total surface area of lipid, n is the number of lipid droplets, S_i is the surface area of a single lipid droplet, V_T is the total volume of lipid, and V_i is the volume of a single lipid droplet.

2.9. Optimisation of the in vitro lipolysis model

The experimental conditions described in Section 2.5, where equimolar concentrations of oil were used, served as a starting point for the optimisation of the lipolysis model. Here, the lipolysis model was optimised to be able to analyse different volumes of oil and the lipolysis of TGs with different chain lengths (short, medium and long) with one set of conditions. The titrant concentration and the maximum and minimum rate of addition were varied in order to find a set of conditions that maintained the pH between 6.75 and 6.85 during lipolysis. The sets of conditions evaluated during the optimisation of the model are listed in Table 1. Each set of conditions was assessed for SCTs, MCTs and LCTs, and with oil volumes of 200, 500 and 1000 μL, five times. The dispersion of 200 μL of TG in the model (~40 mL) would be equivalent to a 1000 μL lipid-containing capsule in the human gastrointestinal tract (~250 mL [53]). Similarly, 1000 μL of oil dispersed in the lipolysis medium would be comparable to a high-fat meal in the *in vivo* situation [26]. 500 μL was chosen as a value in between the previous two conditions.

2.10. Statistical data analysis

All presented data are expressed as mean ± standard deviation (SD). A one way ANOVA (followed by post hoc Tukey–Kramer multiple comparison test) or an unpaired *t*-test, where appropriate, was used for determining statistically significant differences among the experimental groups. A p value of 0.05 was considered the minimal level of significance. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. In vitro lipolysis of equimolar amounts of different triglycerides

The changes in pH over time during the *in vitro* lipolysis of equimolar quantities of selected TGs are depicted in Fig. 2. Regardless of carbon chain length, all pH–time profiles showed an initial drop of pH as a result of the delay between the pH-stat titrator detecting the first ionised FAs and the subsequent addition of NaOH solution for the titration. The initial burst of hydrolysis has already been reported by other authors [23]. Since transit time along the gastrointestinal tract is known to be variable [54], experiments were not performed for a fixed period of time, but were allowed to proceed until the titrant addition rate was low (10 μL/min), indicating the absence of any FAs to titrate, i.e. absence of TG hydrolysis. As a result, the digestion of each lipid took different times, with the hydrolysis of tri-C8 being the longest process (~80 min), followed by tri-C18, tri-C10 and tri-C2 (~35 min). The lipolysis of tri-C4 took the shortest time (~20 min).

The cumulative volumes of 0.5 M NaOH solution required over time during the *in vitro* digestion of equimolar amounts of the selected TGs are represented in Fig. 3. The amount of titrant consumed was used in Eq. (1) to calculate the apparent extent of lipolysis at different time-points, which is also shown in Fig. 3. All lipids showed a fast initial increase in hydrolysis rate, which subsequently decreased and stayed almost constant for the rest of the process. The lipolysis of tri-C8 resulted in the highest consumption of titrant, and thus in the highest apparent extent of lipolysis by direct titration (93 ± 2%). Tri-C4 was hydrolysed to a lower extent (62 ± 6%), but the process was completed one hour earlier. The apparent extents of lipolysis of tri-C10 (43 ± 2%), tri-C2 (33 ± 0%) and tri-C18 (12 ± 3%) were lower than that of tri-C4, despite the longer durations of the reaction.

Table 1
Sets of conditions assessed during the optimisation of the lipolysis model (n = 5).

Concentration of titrant (M)	Maximum dosing rate (mL/min)	Minimum dosing rate (μL/min)[a]
0.5	1	10
1	1	10
1	1	3
1	3.5	3

[a] The minimum dosing rate and the termination rate were set to coincide in all experiments.

3.2. Back-titration studies

The results from the back-titration experiments showed that the extent of lipolysis at pH 6.80 was underestimated by direct titration for all lipids except for tri-C8 (Fig. 4). Based on the cumulative titrant volumes of both direct and back titrations, the lipolysis of tri-C2, tri-C4 and tri-C8 was almost complete (98 ± 2%, 91 ± 9% and 96 ± 5%, respectively), and not statistically different from each other ($p < 0.001$). The total extent of hydrolysis of tri-C10 was 67 ± 3%, whereas that of tri-C18 was only 31 ± 6%.

3.3. Solubility effect of glyceryl triacetate on the extent of lipolysis

The apparent and total extent of lipolysis of tri-C2 in volumes below and above its solubility limit is shown in Supplementary Table 1. No statistically significant differences were found among groups. This result suggests that the lipolysis of 860 μmol of tri-C2 could be compared with that of the other triglycerides even when this oil was completely solubilised in the bio-relevant media and the others were not.

3.4. Droplet size and total surface of the equimolar triglyceride emulsions following dispersion in the lipolysis buffer

The particle size and the specific surface area of the equimolar emulsions are shown in Table 2. All emulsions showed one population and tight peak widths. Tri-C4 had the smallest droplet size (124 ± 6 nm) and the highest specific surface area ($436 \cdot 10^{-3} \pm 12 \cdot 10^{-3}$ nm^{-1}), followed by tri-C2, tri-C8, tri-C10, and tri-C18. The relatively large droplet sizes are consistent with the poor dispersion properties of Type I lipidic formulations [11].

3.5. Optimisation of the in vitro lipolysis model

The effect of the concentration of titrant, and maximum and minimum titrant dosing rates on the control over the lipolysis process was investigated to find an optimised set of conditions capable of keeping the pH environment within the physiological range (6.75–6.85), during the hydrolysis of TGs with different carbon chain lengths. Also the lipolysis of different TG volumes was evaluated in order to assess a variety of possible scenarios in the intestine.

3.5.1. 0.5 M NaOH, 1 mL/min maximum rate, 10 μL/min minimum rate

The initial set of conditions was characterised by a prolonged time to gain control over pH during the lipolysis of tri-C4, and by

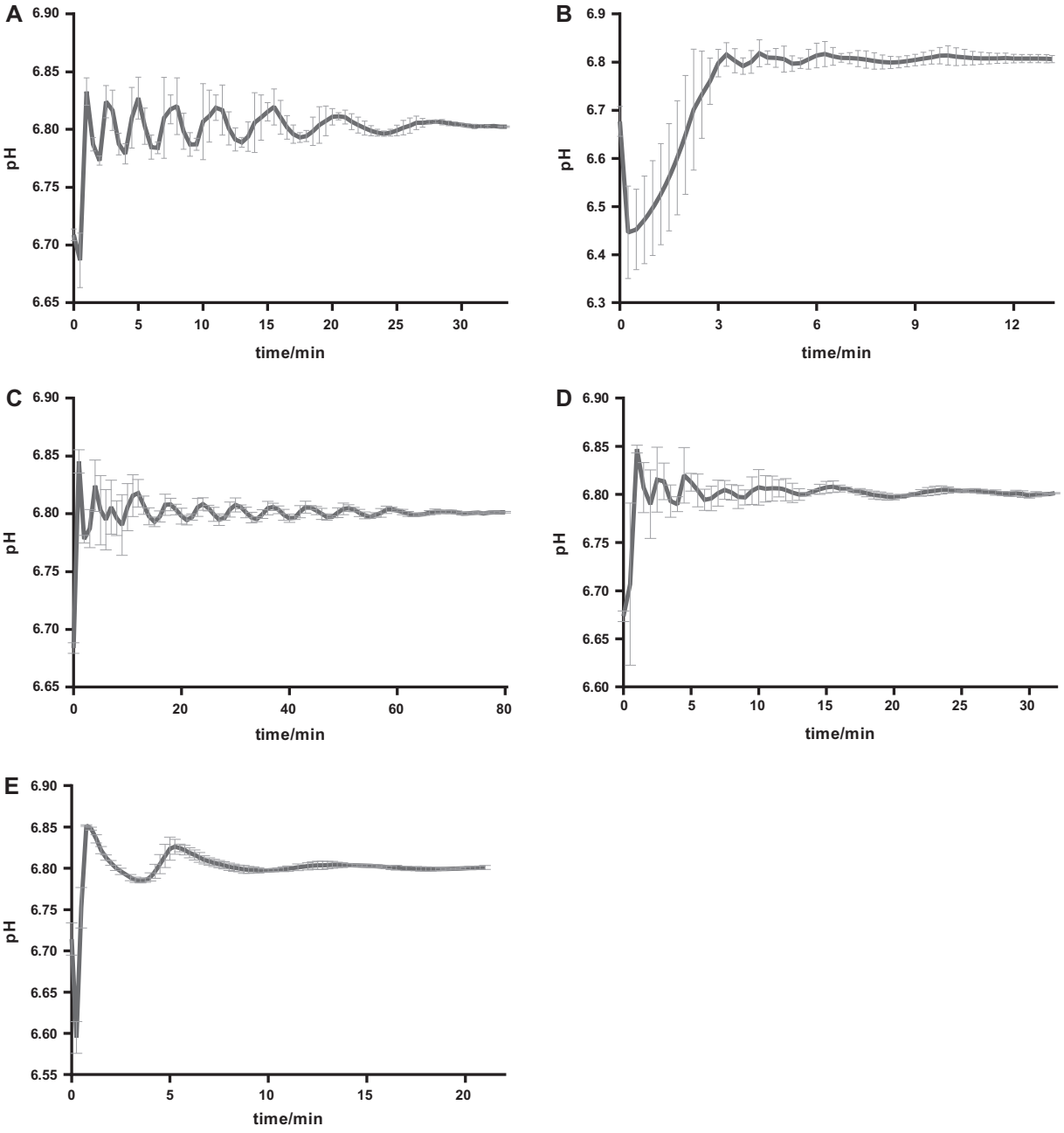

Fig. 2. pH–time profiles obtained during the *in vitro* lipolysis of equimolar amounts of: (A) glyceryl triacetate (tri-C2), (B) glyceryl tributyrate (tri-C4), (C) glyceryl trioctanoate (tri-C8), (D) glyceryl tridecanoate (tri-C10) and (E) peanut oil (tri-C18). The pH of the medium was kept constant at 6.80 ± 0.05 by addition of 0.5 M NaOH solution at 1 mL/min and 10 μL/min maximum and minimum dosing rate, respectively. The process was considered to be completed when the titrant addition rate was slower than 10 μL/min. Values are expressed as mean ± SD (n = 5).

a transient drop of pH during the hydrolysis of 500 and 1000 μL of tri-C8 (Supplementary Fig. 1). In addition, high volumes of titrant were required during the lipolysis of 1000 μL of tri-C2, tri-C4 and tri-C8 which lead to dilution (approximately 25%) of the experimental medium. Prolonged times to complete the process (e.g. over two hours for 1000 μL of tri-C4) were additional issues encountered while assessing the set of conditions.

3.5.2. 1 M NaOH, 1 mL/min maximum rate, 10 μL/min minimum rate

Titration with 1 M NaOH considerably reduced the time needed to gain initial control over the pH for the lipolysis of tri-C2 and tri-C4, and avoided or decreased the transient loss of control during the lipolysis of tri-C8 (Supplementary Fig. 2). Despite improvements, these conditions caused a premature cessation of the process for the lipolysis of 200 μL of tri-C18. A marked elevation of the pH above the pre-determined threshold at the beginning of the process led to very slow titrant dosing rate that was recognised by the titrator as lower than the termination rate and the process was terminated after just 90 s.

3.5.3. 1 M NaOH, 1 mL/min maximum rate, 3 μL/min minimum rate

Reducing the minimum rate of addition from 10 to 3 μL/min enabled the continuation of the lipolysis of 200 μL of tri-C18 (Supplementary Fig. 3). Nevertheless, the loss of control over pH (1000 μL of tri-C8), the sharp drop of pH and the prolonged time to reach the control band (500 and 1000 μL of tri-C4) were still unresolved issues.

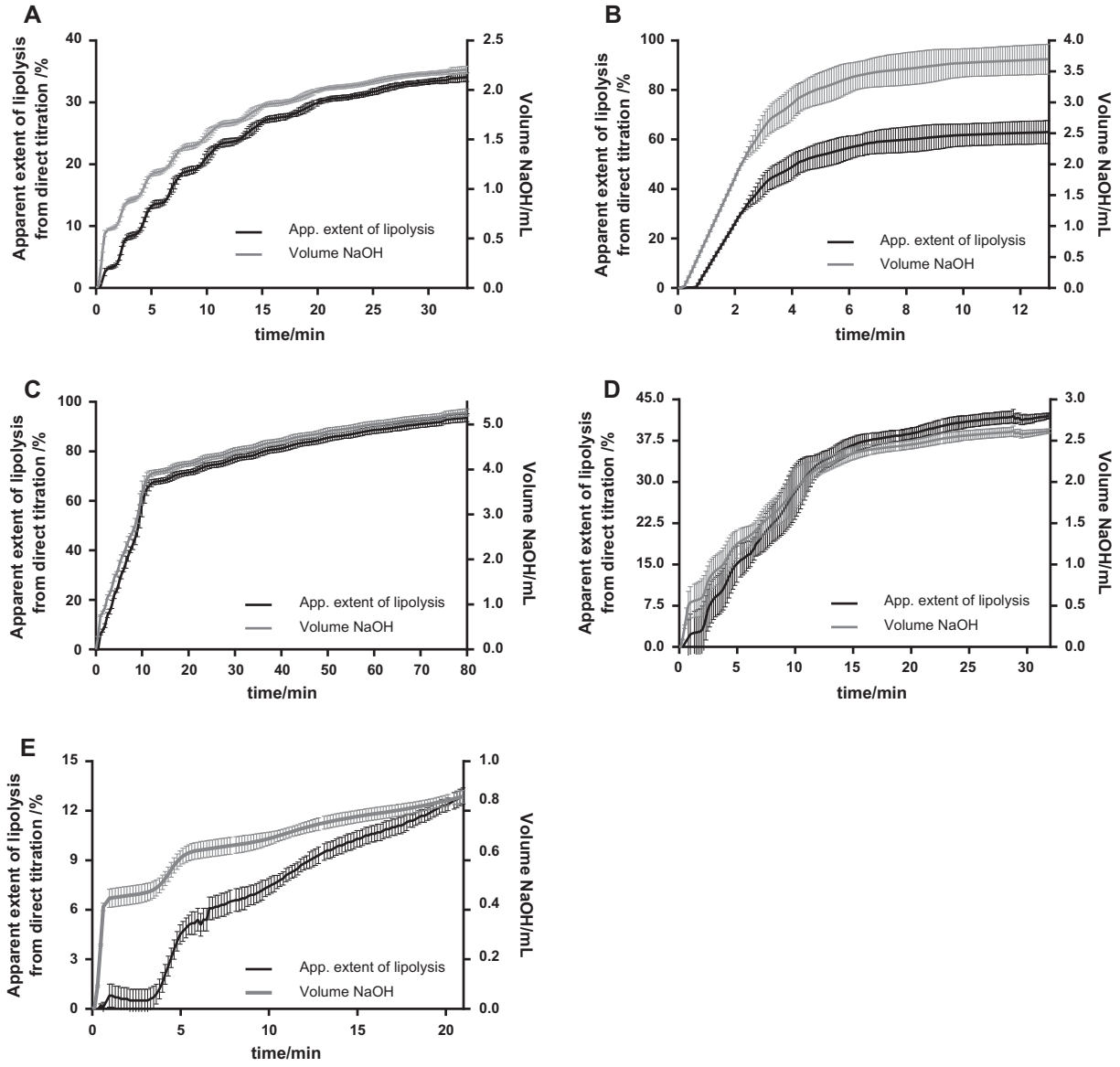

Fig. 3. Apparent extent of lipolysis (black) and volume of 0.5 M NaOH solution consumed over time (grey) during the direct *in vitro* lipolysis at pH 6.80 ± 0.05 of equimolar amounts of: (A) glyceryl triacetate (tri-C2), (B) glyceryl tributyrate (tri-C4), (C) glyceryl trioctanoate (tri-C8), (D) glyceryl tridecanoate (tri-C10) and (E) peanut oil (tri-C18). The extent of lipolysis was calculated using Eq. (1), assuming that the digestion of one molecule of triglyceride released three molecules of fatty acid and one molecule of glycerol (with isomerisation of 2-monoglyceride to 1/3-monoglyceride in between). Values are expressed as means ($n = 5$) ± SD.

3.5.4. 1 M NaOH, 3.5 mL/min maximum rate, 3 μL/min minimum rate

The increment of the maximum addition rate from 1 to 3.5 mL/min achieved the control over pH throughout the lipolysis of all evaluated TGs and volumes (Supplementary Fig. 4). In terms of reaction time, lipolysis of short- and medium-chain TGs lasted less than 30 min. Lipolysis of tri-C18 came to an end before reaching 45 min. Statistically significant differences ($p < 0.05$) in NaOH consumption were observed during lipolysis of different volumes of the same TG (except for 500 and 1000 μL of tri-C18).

4. Discussion

4.1. In vitro lipolysis of equimolar amounts of different triglycerides and back-titration studies

In this work, the extent of lipolysis of lipidic Type I formulations, based on TGs, has been evaluated by means of an *in vitro* lipolysis model, to better understand the mechanisms behind pancreatic lipase activity. The assessment of the lipolysis process by direct titration at pH 6.80 showed there are significant differences in the pH–time profiles (Fig. 2) and the amount of titrant consumed (Fig. 3) for each TG. In addition and in agreement with previous studies, there is also more extensive lipolysis (Fig. 4) of medium-chain TGs by pancreatic lipase when compared with long-chain TGs. Most of previous *in vitro* lipolysis reports have compared formulations with the same volume [39] or same mass [7,35,36,45,55] of lipid. However, to compare pancreatic lipase activity on different TG substrates, the assessment is more informative mechanistically when performed with equimolar amounts as reported here.

Another consideration in the experimental procedure is that the lipolysis of Type I formulations, results in lipolytic products that have low degree of ionisation at physiologically relevant pH (e.g. pH 6.80). Some authors [7,39,49,55–58] have partially resolved this, by performing back-titrations and defining a correction factor to determine the real extent of lipolysis. In the light of this,

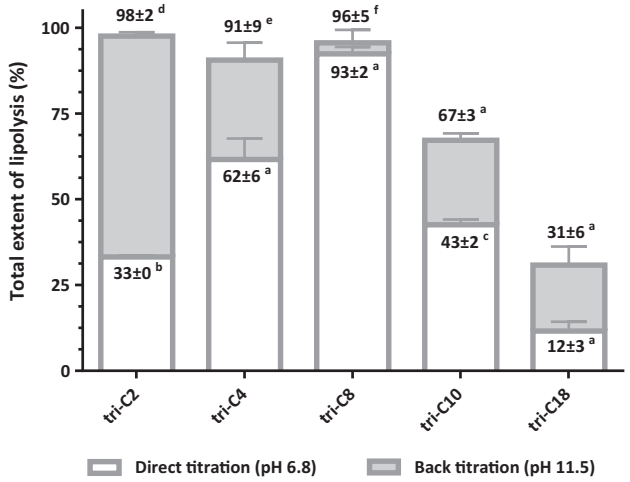

Fig. 4. Comparison of the total extent of lipolysis for the *in vitro* lipolysis of equimolar amounts of different triglycerides: glyceryl triacetate (tri-C2), glyceryl tributyrate (tri-C4), glyceryl trioctanoate (tri-C8), glyceryl tridecanoate (tri-C10) and peanut oil (tri-C18). White colours represent the apparent extent of lipolysis calculated when the pH of the medium was kept at 6.80 ± 0.05 (direct titration). Grey-shade areas represent the underestimated extent of lipolysis calculated after back-titration experiments, when the pH of the medium was elevated to 11.50 ± 0.05. Values are expressed as means (n = 5) ± SD. One-way ANOVA followed by post hoc Tukey–Kramer test was used for statistical analysis. [a] Statistically significantly different from all other TGs ($p < 0.001$). [b] Statistically significantly different from tri-C4, tri-C8 and tri-C18 ($p < 0.001$), and from tri-C10 ($p < 0.01$). [c] Statistically significantly different from tri-C4, tri-C8 and tri-C18 ($p < 0.001$), and from tri-C2 ($p < 0.01$). [d] Statistically significantly different from all other TGs ($p < 0.001$), except for tri-C4 and tri-C8 ($p < 0.05$). [e] Statistically significantly different from all other TGs ($p < 0.001$), except for tri-C2 and tri-C8 ($p < 0.05$). [f] Statistically significantly different from all other TGs ($p < 0.001$), except for tri-C2 and tri-C4 ($p < 0.05$).

Table 2
Hydrodynamic droplet size (d_H) and specific surface area (S_S) of the diluted ($5 \cdot 10^{-2}$% v/v) triglyceride emulsions formed upon dispersion of equimolar amounts of oil in the digestion buffer after the equilibration period, prior to enzyme addition (mean ± SD, $n \geqslant 8$). One way ANOVA followed by post hoc Tukey–Kramer test was used for statistical analysis.

Triglyceride	d_H (nm)	S_S (nm^{-1}) · 10^3
Glyceryl triacetate (tri-C2)	138 ± 4[a]	436 ± 12[a]
Glyceryl tributyrate (tri-C4)	124 ± 6[a]	485 ± 25[a]
Glyceryl trioctanoate (tri-C8)	155 ± 7[b]	388 ± 19[b]
Glyceryl tridecanoate (tri-C10)	162 ± 7[c]	371 ± 17[c]
Peanut oil (tri-C18)	189 ± 7[a]	318 ± 12[a]

[a] Statistically significantly different from all other TGs ($p < 0.001$).
[b] Statistically significantly different from all other TGs ($p < 0.001$), except for tri-C10 ($p < 0.05$).
[c] Statistically significantly different from all other TGs ($p < 0.001$), except for tri-C8 ($p < 0.05$).

back-titration experiments were undertaken at pH 11.50, immediately after direct titrations had been performed. The pH value of 11.50 was chosen to guarantee both complete FA ionisation and pancreatic lipase inhibition [59].

For tri-C2, the apparent extent of lipolysis was approximately 33%. This value suggests that only triglycerides were hydrolysed. However, back-titration results indicate 66% of the lipolysis extent was underestimated and thus diglycerides and MGs were lipolysed as well. Similarly, the calculated extent of lipolysis at pH 6.80 of tri-C4 was 66%, indicating that all TGs and diglycerides were lipolysed. Subsequent titrations at pH 11.50 revealed that 33% of the extent of the process had been underestimated in direct titrations. Interestingly, pK_a values of acetic and butyric acid are 4.74 and 4.82 [60] respectively, and therefore all acid molecules should have been ionised at pH 6.80. However, it has been suggested previously that the apparent pK_a of FAs within the aqueous micellar solution is higher than that calculated in standard conditions [7], which could explain the incomplete ionisation. Another possible explanation for this phenomenon is that the lipase was still active, and therefore catalysed the release of one more FA during the time taken (60 s) for the increase of pH levels from 6.80 to 11.50.

For tri-C8 the apparent extent of lipolysis calculated indirectly from the NaOH volume data showed that almost complete hydrolysis was achieved. Fig. 3C shows that for tri-C8 the apparent extent of the lipolysis-time profile is characterised by two distinct slopes, i.e. two different lipolysis rates. The inflection point of this graph falls almost exactly at the 66% value of the lipolysis extent. It could be assumed that the first part of the profile (from 0% to 66%) represents the lipolysis of TGs and diglycerides, and the second part of the profile (the remaining 33%) represents the isomerisation of 2-MG to 1/3-MG and subsequent lipolysis to glycerol and one FA. It is conceivable that the second stage of the process (characterised by the least steep slope) was the slowest, since it involved two steps (isomerisation and hydrolysis), and because the affinity of pancreatic lipase towards monoglycerides is lower than towards TGs and diglycerides [38]. Back-titration data demonstrated that almost all released FAs during the lipolysis of tri-C8 were ionised at pH 6.80 ± 0.05, which is in agreement with the pK_a of octanoic acid: 4.89 [60].

Back-titration results for tri-C10 revealed that the total extent of hydrolysis was around 66%; thus, pancreatic lipase catalysed the lipolysis of all TGs and diglycerides, but not monoglycerides. Although the pK_a of decanoic acid (4.90, [60]) is higher than that of octanoic acid, the unionised to ionised FA ratio (~0.5) did not follow theoretically predicted values. However, similar results have been found in other laboratories. Williams et al. [7] reported a ratio of 0.43 after the lipolysis of a mixture of tri-C8 and tri-C10 at pH 6.5. Likewise, Fernandez et al. [55] determined a ratio of 0.33 while assessing the lipolysis of Gelucire® 44/14 at different pH values.

Finally, the total extent of lipolysis of LCT tri-C18 indicates that lipase acted on half of the TGs to release two FAs per one molecule of tri-C18. In this case the incomplete ionisation of oleic acid at pH 6.80 was expected since its pK_a is 9.85 [61]. Accordingly, around 20% of the extent of the process was undetected by direct titration.

Overall, the trend in extent of lipolysis, and thus lipase activity (tri-C2, tri-C4, tri-C8 > tri-C10 > tri-C18) correlates with results observed by the only two other authors who have undertaken these equimolar lipolysis comparisons. Firstly, Dicklin et al. [62] incubated the TGs with pancreatic tissue homogenate for a fixed period of time without titrating the released FAs. In this study, no statistical differences were found among the specific activities that porcine pancreatic tissue homogenates showed towards tri-C4, tri-C6 (glyceryl trihexanoate) and tri-C8, although they were all higher than the lipase activity demonstrated by tri-C10. While, Ciuffreda et al. [63] assessed the *in vitro* lipolysis of different TGs by direct titration at pH 8 and reported an ascending order of lipase activity from tri-C18 and tri-C10 to tri-C4, but no lipolytic activity was detected for tri-C2.

A theory as to the increased pancreatic lipase activity for the shorter TG chain lengths could be explained based on a two-step process as described by Lengsfeld et al. [64], whereby adsorption at the oil–water interface is followed by a catalysis reaction. Therefore, substrate specificity of lipase could arise from any of these two steps, and could be due to the ability of the lipase to adsorb at the interface, as well as to the chemical affinity the binding site shows towards the TG acyl chain.

Binding site affinity could explain the lower activity observed for tri-C10 and tri-C18 when compared to tri-C2, tri-C4 and tri-C8. X-ray crystallographic studies have shown that the active site of pancreatic lipase is formed by three residues: serine 153 (Ser153), histidine 264 (His264) and aspartate 177 (Asp177) [65].

The catalytic triad is pulled together through hydrogen bonds between the hydroxyl group of Ser153 and one imidazole nitrogen of His264, and between the other imidazole nitrogen and the carboxylic group of Asp177 (Fig. 5). It is under this conformation that the hydrolysis reaction can take place. The hydroxyl group of Ser153 is thought to initiate the reaction through a nucleophilic attack to the first (or third) glyceryl carbon, with the fatty carboxylate being the leaving group [66]. Consequently, the reaction would become faster the more electrophilic the glyceryl carbon is and the better leaving group (more stable) the carboxylate is. In terms of electrophilicity, all TGs are analogous. However, in terms of the leaving group, carboxylates of shorter chain length are better candidates (the stronger the acid, the weaker the conjugate base, the better the leaving group), and accordingly tri-C2, tri-C4 and tri-C8 were lipolysed to the greatest extent.

Regarding the lipase adsorption to the interface, the difference in activity could be attributed to the size of the oil droplets and/or to the inhibitory effects of the lipolysis products. Since pancreatic lipase carries out interfacial catalysis, the higher the substrate surface is, the more extensive the lipolysis becomes. Therefore, in theory, the TGs with smaller oil droplets, are supposed to be lipolysed to a greater extent. Indeed, results derived from DLS measurements showed the extent of lipolysis was directly proportional to the specific surface area (Table 2). Alternatively, the lipolysis process could be inhibited by the interfacial activity of amphiphiles such as diglycerides, unionised FAs and, mainly, 2-MGs [67–69]. Unless incorporated within mixed micelles, 2-MGs could form a layer at the droplet surface that efficiently blocks the access of the lipase [69]. Therefore, it could be hypothesised that 2-MGs derived from tri-C10 and tri-C18 are the least solubilised and inhibited the process to a greater extent.

In summary, the results suggest that there is a specific chain length range (C2–C8) for which pancreatic lipase shows higher activity. We hypothesise that this specificity could result from a combination of physicochemical properties of TGs, 2-MGs and FAs, namely the droplet size of the TGs, the solubility of 2-MGs within mixed micelles, and the relative stability of the FAs as leaving groups in the hydrolysis reaction.

4.2. Optimisation of the in vitro lipolysis model

The role of the concentration of titrant and maximum and minimum titrant addition rates, in the control of the lipolysis process, was investigated to find an optimised set of conditions capable of maintaining the pH environment within physiological range (6.75–6.85) during the hydrolysis of TGs with different carbon chain lengths. The hydrolysis of different volumes of oil (200, 500 and 1000 μL) was evaluated to assess a variety of possible scenarios in the intestine, from the ingestion of an oil-containing capsule in fasting conditions to the consumption of a high-fat meal.

The first set of conditions evaluated (0.5 M NaOH with 1 mL/min maximum and 10 μL/min minimum dosing rates, Supplementary Fig. 1) was found to be suitable for tri-C10 and tri-C18, but not for tri-C2, tri-C4 and tri-C8. The high activity that pancreatic lipase showed towards tri-C4 – translated into a large amount of liberated ionised FAs – presented a problem for the

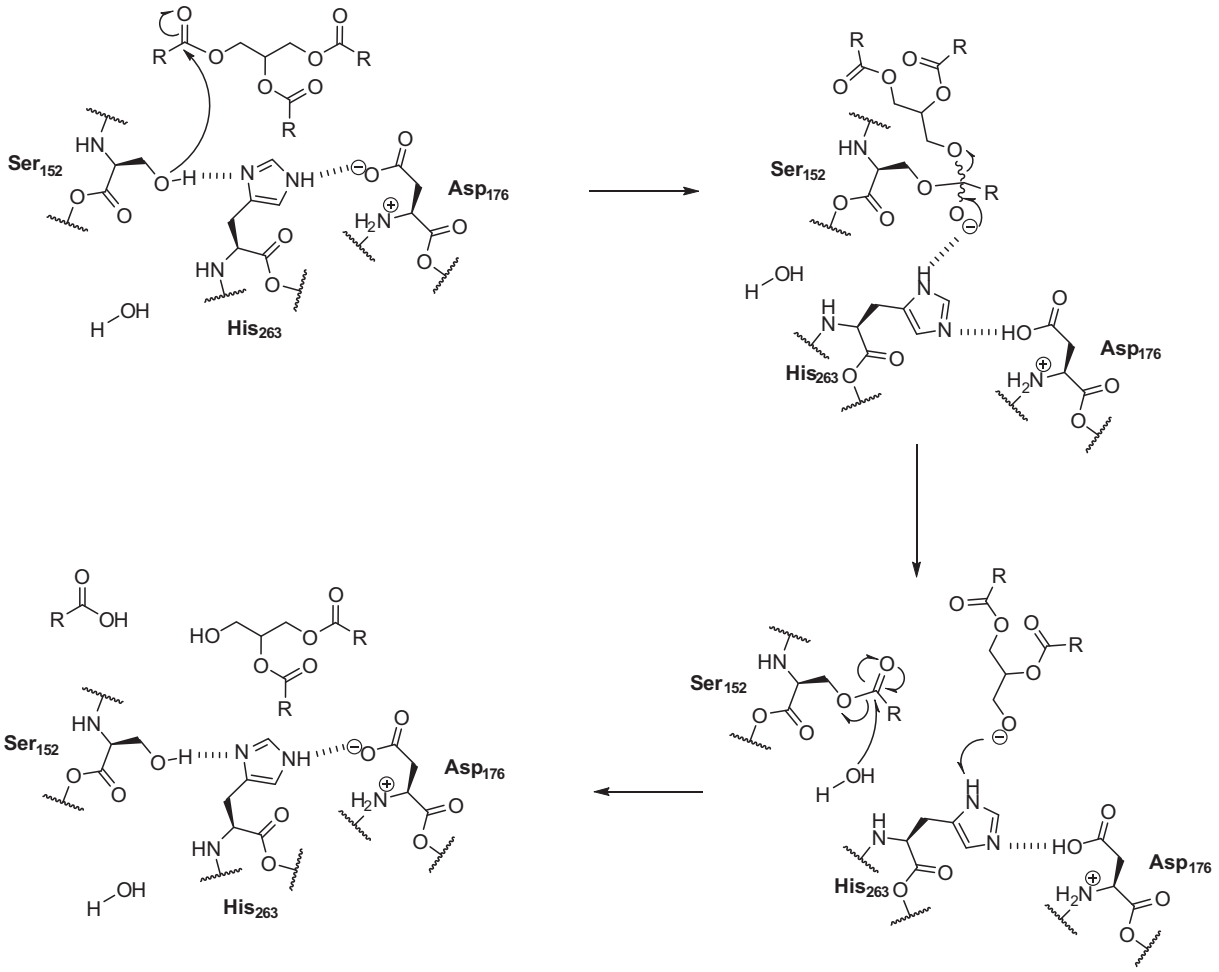

Fig. 5. Proposed molecular mechanism of triglyceride lipolysis by pancreatic lipase focused on the catalytic triad.

titrator when trying to regain control over pH during the initial stages of the process. Most importantly, during the "delayed" periods, pH of the medium dropped to acidic values. If the fate of an ionisable drug across lipolysis phases had been assessed under these conditions, such low pH values could have affected the distribution of the compound, leading to incorrect interpretations of the performance of the lipidic formulation. Regarding the lipolysis of tri-C8, the drawback was not the initial drop of pH, but the loss of control over pH at a later point in the reaction. Apart from pH control, other reasons to disregard this set of conditions were dilution of the medium due to large volumes of titrant needed during the lipolysis of 1000 μL of tri-C2, tri-C8 and tri-C10, and prolonged times to complete the process. Based on these results, it was decided to increase the concentration of the titrant up to 1 M but maintain the same maximum and minimum rates of addition of NaOH (Supplementary Fig. 2). Despite improvements, the new conditions introduced a problem of premature stop of titration with small volumes of tri-C18. To avoid reaching the termination rate at initial stages of the process, it was decided to reduce the minimum rate to 3 μL/min (Supplementary Fig. 3). This new set of conditions enabled the continuation of the lipolysis of 200 μL of tri-C18, but was still suboptimal due to the loss of control over pH during the lipolysis of 1000 μL of tri-C8. There was also a sharp drop of pH and prolonged time to reach control band at initial stages of the lipolysis of 500 and 1000 μL of tri-C4. Finally, by increasing the maximum addition rate up to 3.5 mL/min, all previous issues (premature stop of titration, loss of control over pH, and prolonged time to reach control band) were avoided and the control over pH throughout the lipolysis of all evaluated TGs and volumes was achieved (Supplementary Fig. 4). The implementation of this method resulted in shorter reaction times, which allows the assessment of several formulations on the same day. Statistically significant differences ($p < 0.05$) in NaOH consumption were observed during lipolysis of different volumes of the same TG indicating the optimised conditions were capable of distinguishing among the different fat-digesting situations that were mimicked.

5. Conclusions

In these studies, the *in vitro* lipolysis by pancreatic lipase under bio-relevant conditions at physiological pH of equimolar amounts of TGs with different chain lengths has been evaluated for the first time. The assessment of the process by direct titration at pH 6.80 showed there are significant differences in the pH–time profiles and the amount of titrant consumed for each TG. The combined results of direct and back-titration studies proved there is a specific chain length range (C2–C8) for which pancreatic lipase showed higher activity. Based on the obtained results, it is hypothesised that the specific surface area of the dispersed oil droplets, the solubility of 2-MGs within mixed micelles, and the relative stability of the FAs as leaving groups in the hydrolysis reaction, are the physicochemical properties which could determine the total extent of lipolysis.

1 M NaOH titrant concentration, 3.5 mL/min maximum titrant dosing rate and 3 μL/min minimum titrant dosing rate, were found to be the conditions that better maintain pH environment within physiological range (6.75–6.85) during the hydrolysis of TGs with different carbon chain lengths. This optimised set of conditions also allowed the differentiation of the lipolysis of different lipid loads.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2015.04.027.

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