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Characterisation and quantification of medium chain and long chain triglycerides and their in vitro digestion products, by HPTLC coupled with in situ densitometric analysis

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Abstract

The development of new and simple high performance thin layer chromatography (HPTLC) assays for the quantification of medium chain triglycerides (MCT, tricaprylin) and long chain triglycerides (LCT, triolein) and their lipolytic products, bile salts (BS) and phospholipids (PL) are described. Different classes of lipids (PL, BS, fatty acids, monoglycerides, diglycerides, and triglycerides) were separated on a single silica gel 60 HPTLC plate by Automated Multiple Development (AMD) methods using a Camag AMD 2. Post-chromatographic staining of long chain lipids (triolein, diolein, monoolein, and oleic acid), PL and BS with a solution of copper sulphate-phosphoric acid and medium chain lipids (tricaprylin, dicaprylin, monocaprylin, and caprylic acid) with a solution of ammonium molybdate-perchloric acid allowed visualisation of the lipids. Lipids were quantified by in situ spectrodensitometric measurements using a Camag TLC scanner 3. The intra- and inter-assay accuracy was between 83 and 115% and the assay was precise to within a CV of less than 20% over a range of 0.1–1 and 5–50 μg for long chain lipids and medium chain lipids, respectively. The methods have been employed to study the kinetics of triolein and tricaprylin lipolysis in an in vitro lipid digestion model commonly used to assess the digestibility of novel oral lipid-based formulations.

Keywords: HPTLC; Densitometry; Triglyceride; In vitro lipolysis; Pancreatic lipase

1. Introduction

Whilst there has been a great deal of interest in the use of lipid-based oral delivery systems as a strategy for bioavailability enhancement for poorly water soluble drugs [1–9], there are relatively few examples of commercial lipid-based formulations. Constraints associated with the development of successful lipid-based formulations include a lack of understanding of the mechanisms by which lipids improve absorption, and the lack of an in vitro model that is predictive of

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bioavailability and therefore able to be used for rapid and systematic assessment of potential lipid-based formulations.

Triglycerides (TG) consisting of medium chain length fatty acids (MCT; C_8–C_{14}) and triglycerides of long chain fatty acids (LCT; C_{16}–C_{22}) are often considered for use in lipidic formulations. MCT and LCT may enhance the absorption of poorly water soluble drugs by their actions on gastric transit, intestinal permeability or drug metabolism, but are perhaps most widely used to enhance drug solubilisation and dissolution in the gastrointestinal tract (GIT) [10,11]. TG lipids and their digestion products may enhance drug solubilisation and dissolution by stimulating bile salt (BS) and phospholipid (PL) secretion into the gastrointestinal (GI) lumen and by enhancing the solubilisation capacity of endogenous BS/PL mixed micelles in the intestine by intercalation into the micelle structures.

After oral administration, hydrolysis of TG by lingual and gastric lipase and most importantly by the pancreatic lipase/colipase complex, proceeds as a two-step reaction. Firstly, hydrolysis of TG yields a single fatty acid (FA) and diglyceride (DG). Secondly, the DG is hydrolysed to produce a second FA and the corresponding 2-monoglyceride (2-MG). Although 2-monoglyceride may undergo isomerisation to 1-monoglyceride (1-MG), which may then be hydrolysed to yield a third FA and glycerol, this process is generally believed to be limited in vivo [12].

Numerous studies of the physicochemical characteristics of lipid digestion products have been conducted in humans [13–20]. However, from a formulation standpoint, the fate of a co-administered drug as digestion of an oily vehicle progresses remains a key issue. The interaction between the co-administered lipid and drug as the formulation is exposed to, and digested and dispersed by the GI environment, will depend on the type of lipid involved and the physicochemical properties of the particular drug. Elucidation of the digestion characteristics of different lipids and lipid vehicles may, therefore, provide a framework for the development of an in vitro model in which the factors that dictate drug distribution into the various phases of the GI contents, and potentially, therefore, dictate the extent of absorption, may be probed. A prerequisite for the establishment of such a model is a reliable, accurate and sensitive analytical method for the quantification of MCT and LCT (and their digestion products), PL and BS in the lipid digest.

The progress of triglyceride lipolysis is generally monitored in vitro using an automated pH-stat titration apparatus [21,22]. A limitation of this methodology is that it quantifies the rate and extent of digestion indirectly via titration of the FA produced. Detection and quantification of the various classes of lipolytic products (i.e. parent TG, DG, MG and FA), however, is complicated by their lack of chromophoric groups. Quantification usually proceeds via extraction from the reaction mixture, separation of lipid classes by thin layer chromatography (TLC), scraping of the adsorbed lipid from the TLC plates, transesterification with methanol, and quantification of FA methyl esters by gas capillary (GC) chromatography [23,24]. Other techniques utilised for the quantification of lipid digestion products include the use of radiolabelled lipids (although lipid extraction from the silica gel is still required for counting by liquid scintillation [25–28]), TLC coupled with flame ionisation detection (FID) and most recently high performance liquid chromatography with mass spectrometry detection (HPLC-MS) [29–31]. HPLC-MS has some advantages, particularly in terms of selectivity, but has not found extensive application in lipid analysis, in part as a result of high cost and limited availability and also as a result of variability in molecular ion fragmentation patterns [31]. As a result, in situ densitometry TLC has increasingly been employed for the quantitation of LCT and its digestion products [16,17,32], however to this point, application to the assay of medium chain lipids has been limited.

The present study describes for the first time, high performance thin layer chromatography (HPTLC) techniques for quantifying both MCT and LCT (and their digestion products), to-

gether with PL and BS. In these studies, triolein, tricaprylin, egg phosphatidylcholine (PC) and Na taurodeoxycholate (NaTDC) were selected as model TG, PL and BS, respectively. The methodology employs a simple acidification and dilution procedure without an extraction step for sample preparation. The method has been employed to study the kinetics of in vitro lipolysis of tricaprylin and triolein by pancreatic lipase.

2. Experimental

2.1. Materials

Oleic acid, 2-monoolein, 1,2-diolein, triolein, and Na taurodeoxycholate (NaTDC) were products from Sigma Chemical Co. (MO, USA). Tributyrin, tricaprylin, 1,3-dicaprylin, 1-monocaprylin and Trizma® maleate were also purchased from Sigma. Two grades of L-α-lecithin (L-α-phosphatidylcholine, PC) were purchased from Sigma. Type X-E (approximately 60% pure PC, from dried egg yolk) was used to prepare mixed micellar PC/NaTDC solutions for digestion experiments and type XI-E (approximately 99% pure PC, from fresh egg yolk in chloroform) was used as a PC standard for HPTLC assays. Analytical grade ammonium molybdate, caprylic acid, 1 M hydrochloric acid, sodium chloride, copper (II) sulphate pentahydrate, ammonia solution (28%, w/w), perchloric acid (70%, w/w) and orthophosphoric acid (85%, w/w) were obtained from Ajax Chemicals (Sydney, Australia). Calcium chloride dihydrate (analytical grade) was purchased from BDH Chemicals (Melbourne, Australia). HPTLC plates (20×10 cm Silica gel 60 F_{254}) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide (1 M) (Titrisol®, Merck) stock solution was used to prepare 0.2 and 0.6 M NaOH titration solutions. Pancreatin (Sigma) from porcine pancreas (activity equivalent to $8 \times$ USP specifications) was used as a source of pancreatic lipase and colipase. All the solvents were of HPLC grade (Mallinckrodt, KY). All material was used as received. Water was obtained from a Milli-Q (Millipore, MA) water purification system.

2.2. Preparation of standard lipid mixture and application onto HPTLC plates

Standard lipid mixtures containing 1 mg/ml of each class of medium chain lipid (caprylic acid, 1-monocaprylin, 1,3-dicaprylin and tricaprylin) or 0.1 mg/ml of each class of long chain lipid (oleic acid, 2-monoolein, 1,2-diolein and triolein), PC and NaTDC were prepared in chloroform-methanol (2:1, v/v). All standard lipid solutions were stored in glass vials with teflon lined caps at −20°C.

Prior to sample application, each HPTLC plate was pre-heated at 100°C for 10 min in a laboratory oven. Plates were placed on a glass slab ($0.5 \times 28 \times 28$ cm) to provide even heat distribution and to prevent overheating of plate regions that were in direct contact with the metal oven racks. An automated sample applicator (Linomat IV, Camag, Muttenz, Switzerland) was used to apply solutions onto the plate. Solutions were applied under a N_2 blanket 15 mm from the side and 8 mm from the lower edge of the plate as 6-mm long bands separated by a 5 mm space. Sixteen tracks were applied to each plate, at a delivery rate of 1 μl/5 s.

Calibration curves for long chain lipids, NaTDC and PC were constructed by applying 1, 2, 5, 8, and 10 μl of standard mixture, corresponding to 0.1, 0.2, 0.5, 0.8 and 1 μg of each lipid class per track, respectively. For medium chain lipid calibration curves, 5, 10, 20, 40, and 50 μl of standard lipid mixture were applied, corresponding to 5, 10, 20, 40, and 50 μg of each lipid class per track, respectively.

2.3. Automated multiple development (AMD) method

The automated multiple development chamber (AMD 2, Camag) allows the multiple development of each HPTLC plate with different mobile phases of decreasing polarity. The mobile phase composition and migration distance of each development step used for the separation of long chain lipids, PC and NaTDC and medium chain lipids are presented in Table 1. For the separation of long chain lipids, PC and NaTDC, the HPTLC

plate was automatically developed six times unidirectionally with different mobile phase compositions and increasing migration distance with each step. Medium chain lipid separation was optimised using four developments. In both cases, the purpose of the first development step (100% hexane) was to elute highly lipophilic contaminants in the sample solution (that were distorting the chromatogram) to the solvent front. Between each development step, the plate was automatically dried for 4 min in vacuo and thereafter conditioned in an N_2/NH_3 atmosphere, obtained by bubbling N_2 through a 1 M NH_3 pre-conditioning solution. The total development times for the separation of long chain, NaTDC, and PC and medium chain lipids were 110 and 58 min, respectively.

2.4. Post-chromatographic derivatisation

The derivatisation solution for the detection of long chain lipids, PC and NaTDC comprised of $CuSO_{4.5}H_2O$-H_3PO_4 (85%, w/w)-Milli-Q water (10:8:82, w/v/v) [17]. Medium chain lipids were visualised by staining with a solution of ammonium molybdate-perchloric acid (70%, w/w)-hydrochloric acid (1 M)-Milli-Q water (6:40:90:75, w/v/v/v) [33]. Immediately after development, HPTLC plates were dipped in the appropriate derivatisation solution, for 8 s using the Camag Immersion Device III. The plates were then drained of excess derivatisation solution and charred by heating at 150°C for 50 min for long chain lipids, NaTDC and PC and at 170°C for 10 min for medium chain lipids. Immediately after charring, long chain lipids, PC and NaTDC appeared as a brown coloured band on a white to faintly blue background. Medium chain lipids appeared slightly yellow in colour immediately after charring and slowly turned blue after approximately 2 h. The background of the plate was white to slightly yellow post charring.

2.5. Densitometry

Densitometric measurements were performed on each plate using the Camag TLC Scanner 3. For each plate 16 tracks were scanned, starting 5 mm from the bottom of the plate to the solvent front (55 mm). The measurement mode was absorption/reflection, with a 20 nm monochromator bandwidth and 20 mm/s scanning speed. Long chain lipids, PC and NaTDC were scanned using a deuterium lamp at 370 nm UV wavelength and 6.0×0.4 mm beam slit. Medium chain lipids were scanned using a tungsten lamp at 800-nm visible wavelength and the beam slit was 10.0×0.4 mm. Long chain lipids, PC and NaTDC were scanned

Table 1
Automated multiple development (AMD) conditions for the separation of long chain lipids (triolein, 1,2-diolein, 2-monoolein, and oleic acid), NaTDC and PC (Panel A) and medium chain lipids (tricaprylin, 1,3-dicaprylin, 1-monocaprylin and caprylic acid) (Panel B)

Step number	%Methanol–water (97:3, v/v)	%Chloroform	%Ether	%Hexane	Migration distance (mm)
A. Separation condition for long chain lipids, NaTDC, and PC					
1	0	0	0	100	55
2	27	73	0	0	22
3	27	73	0	0	25
4	15	85	0	0	35
5	0	0	80	20	38
6	0	0	25	75	55
B. Separation condition for medium chain lipids					
1	0	0	0	100	55
2	15	85	0	0	30
3	0	0	90	10	40
4	0	0	35	65	55

immediately after charring. Medium chain lipids were scanned approximately 12 h after heating to ensure maximal staining of lipids in situ.

2.6. Quantification

The Camag TLC evaluation software (CATS V4.05) was used to analyse the peak height and area of each densitometric chromatogram. Standard calibration curves were prepared by plotting peak area against lipid mass for five different amounts of standards (0.1–1 μg of each class of long chain lipids, PC and NaTDC, and 5–50 μg of each class of medium chain lipids). The relationship between peak area (y) and amount of lipid (x) applied per track was best described by a non-linear saturation equation

$$y = y_0 + \frac{ax}{b + x} \quad (1)$$

where y_0 is the intercept and a and b are constants of the curve.

Unknowns were determined by comparison of peak areas to standard curves run simultaneously on the same plate.

2.7. Assay precision and accuracy

Intra-assay accuracy and precision were determined by replicate analysis ($n = 3$) of quality control (QC) solutions at 0.1, 0.5 and 1 μg for long chain lipids, NaTDC and PC and at 5, 25, and 50 μg for medium chain lipids. Inter-assay accuracy and precision was determined on 3 different days. Since high volumes (up to 160 μl) of sample solution were applied for the quantification of medium chain lipids, the effect of application volume on accuracy and precision was determined by replicate analysis ($n = 3$) of 5 and 50 μg of medium chain lipids in volumes of 5, 80 and 160 μl.

2.8. Application to in vitro lipid digestion

The in vitro lipolysis experiments were conducted using similar methods to those described previously [21,22], which utilised a pH-stat automatic titration unit (Radiometer, Copenhagen, Denmark). Pancreatin extract was prepared by adding 1 g of porcine pancreatin powder (containing pancreatic lipase and colipase) to 5 ml of digestion buffer (Tris-maleate (pH 7.5, 50 mM), 150 mM NaCl, and 5 mM·$CaCl_2 \cdot 2H_2O$) and stirred for 15 min followed by centrifugation at approximately $1600 \times g$ and 5°C for 15 min. The supernatant was collected and stored on ice. Fresh pancreatin extracts were prepared each day. Pancreatic lipase activity determinations were undertaken using 6 ml of tributyrin in 10 ml of digestion buffer at pH 7.5 and 37°C. The activity of pancreatic lipase was expressed in terms of tributyrin units (TBU), where 1 TBU is the amount of enzyme that can liberate 1 μmole of titratable FA from tributyrin per min. The pancreatic lipase activity of the pancreatin employed was 50 TBU per mg of dry pancreatin powder. NaOH (0.2 M) was used as the titration solution for enzyme activity determination and for triolein lipolysis. For tricaprylin lipolysis 0.6 M NaOH was used as the titrant.

The progress of triolein and tricaprylin lipolysis was determined in separate experiments. For each digestion experiment, 250 mg of oil was added to 9 ml of a PC/NaTDC mixed micellar solution in digestion buffer (concentrations were 5 mM PC and 20 mM NaTDC in the 10 ml reaction mixture immediately after enzyme addition). The PC/NaTDC mixed micellar solutions were prepared by dissolving the lecithin (type X-E; approximately 60% pure PC) in chloroform in a 50 ml round bottom flask and evaporating the chloroform off under vacuum (Rotorvapour RE, Buchi, Switzerland), leaving a thin film of lecithin around bottom of the flask. NaTDC and digestion buffer was then added and the solution was stirred and equilibrated for 12 h, forming a clear, slightly yellow solution. The TG lipids (triolein and tricaprylin) were crudely emulsified in the mixed micellar solutions prior to enzyme addition by stirring continuously for 10 min in the reaction vessel, after which time the pH was re-adjusted with NaOH or HCl to 7.500 ± 0.001. Pancreatin extract (1 ml) (containing 10 000 TBU of pancreatic lipase activity) was then added to initiate lipolysis. Lipolysis was allowed to continue for 30 min.

In addition to other unknown components, the lecithin used to prepare PC/NaTDC micelles contained small amounts of TG (~ 1%, w/w), DG (~ 6%, w/w) and FA (< 1%, w/w) which were digested by pancreatic lipase/colipase to produce FA. In addition PC was also hydrolysed by phospholipase A_2 (present in pancreatin) to produce FA and lyso-phosphatidylcholine (lyso-PC). Therefore, in order to compensate for the additional FA produced by these digestion processes (i.e. other than by digestion of added TG), blank or control experiments were performed. Blank digestion experiments were performed in the same manner as the experimental run, except that TG was not added to the PC/NaTDC mixed micellar solutions.

During blank and TG digestion experiments, 100 μl samples were collected at times 0 (before enzyme addition), 1, 2, 3, 4, 5, 10 and 30 min. The 100 μl aliquots of digestion samples were acidified with 50 μl of 1 M HCl and diluted with 2850 μl of chloroform-methanol (2:1, v/v) in polypropylene tubes. The monophasic solution was then centrifuged at approximately $1600 \times g$ for 15 min at 25°C, before application onto the HPTLC plate using the automatic sample applicator.

For the current application, i.e. monitoring lipid concentration during in vitro lipid digestion, the feasibility of reproducible and accurate sampling from the heterogeneous digestion mixture was determined by replicate sampling ($n = 3$) at time 0 (before enzyme addition) and at 30 min post initiation of digestion. Mass balance calculations (Eq. (2)) for samples collected after enzyme addition were based on the assumption that 1 mole of TG equates to 3 moles of FA equivalents, DG to 2 moles of FA equivalents and MG and FA to 1 mole of FA equivalents. Since the total lipid mass is conserved throughout the experiment, the total FA equivalents at any one time derived from the TG remaining ($[TG]_{remaining}$) and FA ($[FA]_{formed}$), MG ($[MG]_{formed}$), and DG ($[DG]_{formed}$) produced, should equal the FA equivalents present in the initial TG added ($[TG]_{initial}$). Since additional FA was produced by the digestion of lecithin, the FA ($[FA]_{blank}$) produced during blank digestion experiments were subtracted from the total FA produced during experimental TG digests, to give true mass balance data. Thus

$$\text{Total lipid mass balance} = \left[\frac{(3 \times [TG]_{remaining}) + (2 \times [DG]_{formed}) + ([MG]_{formed}) + ([FA]_{formed}) - ([FA]_{blank})3 \times [TG]_{initial}}{}\right] \quad (2)$$

where concentrations are expressed in molar terms (Eq. (2)).

3. Results and discussion

3.1. HPTLC assay characteristics

A six-step development program with differing solvent compositions was required to separate the long chain lipids (triolein, diolein, monoolein and oleic acid), PC and NaTDC in a single sample. The separation of medium chain lipids (tricaprylin, dicaprylin, monocaprylin and caprylic acid) was optimised using a 4-step development method. Fig. 1 presents scanned sections of HPTLC plates illustrating the separation of different long chain lipids, PC and NaTDC (panel A) and medium chain lipids (panel B) in standard solutions, and lipid digestion samples. Different lipid classes migrated from the application position in order of decreasing polarity. Triglyceride, being the least polar lipid, migrated furthest up the plate. In the separation of medium chain lipids, PC and NaTDC remained near the origin and were not quantified. Triolein and tricaprylin digestion samples also contained lyso-phosphatidylcholine (lyso-PC) which remained at the origin in both assays and was not quantified. Unidentified bands appeared in chromatograms run using the long chain lipid assay and migrated to a position just before the solvent front. The unknown bands were thought to be contaminants from the polypropylene tubes used during sample preparation, since standards stored in glass did not contain the same band. Other likely contaminating lipids such as phosphatidylethanolamine and lyso-PC, were not responsible for the unknown band and did not elute to this point.

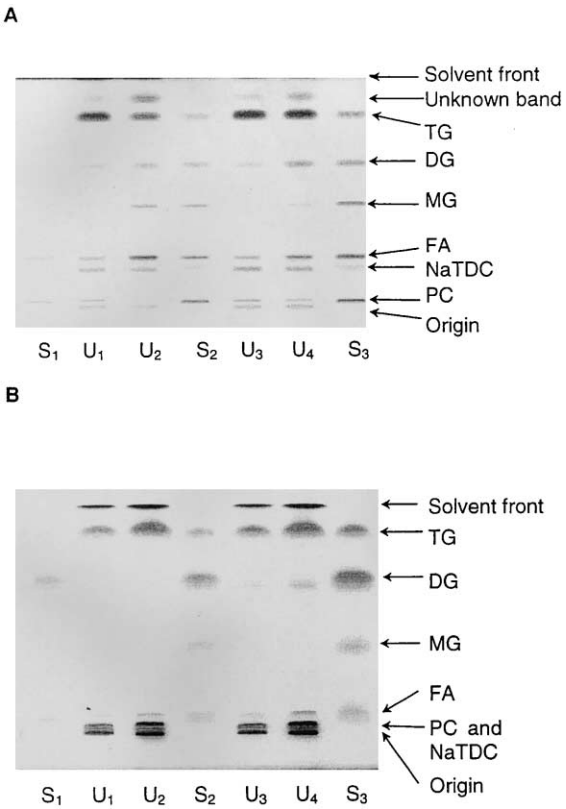

Fig. 1. Sections of scanned HPTLC plates illustrating the separation of phosphatidylcholine (PC), Na taurodeoxycholate (NaTDC), fatty acid (FA), monoglyceride (MG), diglyceride (DG) and triglyceride (TG) present in standard solutions (tracks S_{1-3}) and lipid digestion samples (tracks U_{1-4}). Panel A: Separation of different long chain lipid classes, PC and NaTDC (lyso-phosphatidylcholine (lyso-PC) contained in triolein digestion samples remained at the origin and was not quantified). Panel B: Separation of different medium chain lipid classes (lyso-PC, PC and NaTDC remained near the origin and were not quantified).

In the current methods, distinction between 1,3-diglyceride and 1,2-diglyceride was not made since both isomers migrated the same distance and gave the same densitometric response (data not shown). Consequently either isomer may be used as a standard for the quantification of diglycerides. Similar behaviour was observed for 1-monoglycerides and 2-monoglycerides. 1-monocaprylin and 1,3-dicaprylin isomers were more readily available and therefore were selected for use as standards in the medium chain lipid assay.

Typical densitometric chromatograms of the long chain lipids, PC, and NaTDC (panel A) and medium chain lipids (panel B) are presented in Fig. 2. Fig. 3 shows representative calibration curves for the assay of long chain lipids, PC, and NaTDC (panel A) and medium chain lipids (panel B). The corresponding parameters for each curve are presented in Table 2. The mean relative standard deviation (RSD%) of the calibration curve is calculated from the deviation between each measured standard value and the fitted calibration curve and represents a goodness of fit. The mass

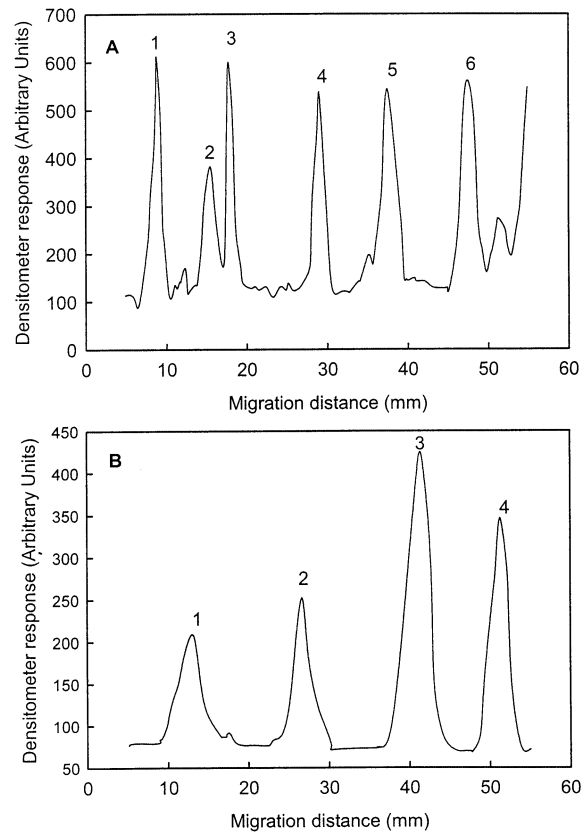

Fig. 2. Representative chromatogram obtained by densitometric scanning of a single standard track on the HPTLC plate. Panel A: mixture containing 1 μg of each of phosphatidylcholine (peak 1), Na taurodeoxycholate (peak 2), oleic acid (peak 3), 2-monoolein (peak 4), 1,2-diolein (peak 5), and triolein (peak 6). Panel B: mixture containing 50 μg of each of caprylic acid (peak 1), 1-monocaprylin (peak 2), 1,3-dicaprylin (peak 3), and tricaprylin (peak 4).

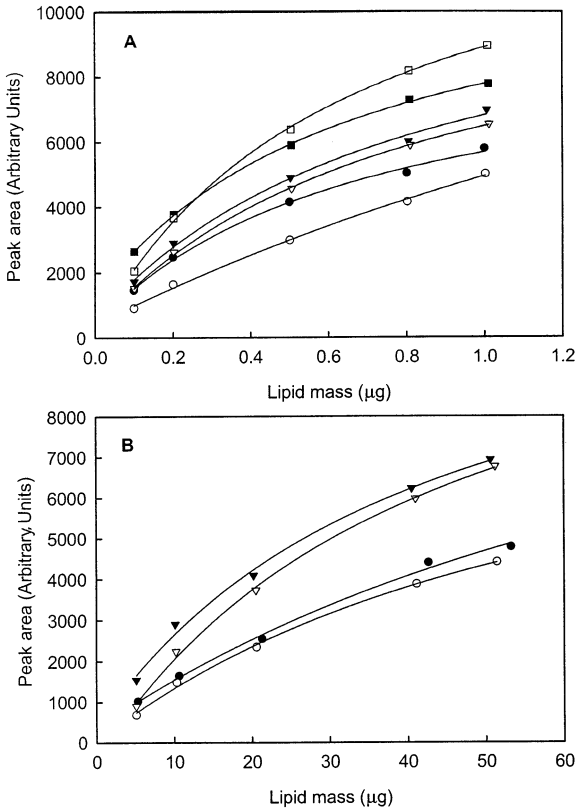

Fig. 3. Standard calibration curves of lipid standards using the saturation equation $y = y_0 + (ax)/(b + x)$. Panel A: Phosphatidylcholine (●), Na taurodeoxycholate (○), oleic acid (▼), 2-monoolein (▽), 1,2-diolein (■), and triolein (□). Panel B: Caprylic acid (●), 1-monocaprylin (○), 1,3-dicaprylin (▼) and tricaprylin (▽). The parameters of each curve are presented in Table 2.

of each lipid class per track in the calibration range for long chain lipids was 0.1–1 μg. A maximum volume of 10 μl of sample solution containing long chain lipids, PC and NaTDC was applied onto the plate. The minimum quantifiable limit (MQL) in term of mass per track was 0.1 μg for each long chain lipid class, PC and NaTDC. Using an application volume of up to 10 μl, this equates to an effective MQL in concentration terms of 0.01 mg/ml. However, whilst not examined here, it may be possible to increase the application volume to increase sensitivity. The mass of each lipid class per track in the calibration range for medium chain lipids was 5–50 μg. A maximum volume of 160 μl of sample solution containing medium chain lipids was applied onto the plate. The MQL in terms of mass per track was 5 μg. Using an application volume of up to 160 μl, this equates to an effective MQL in concentration terms of 0.03 mg/ml for medium chain lipids.

The intra- and inter-assay accuracy and precision data for long chain lipids, PC, NaTDC and medium chain lipids are shown in Table 3. The assay was accurate to within 91–107% of the target and reproducible to less than a CV of 12% for all long chain lipids, PC and NaTDC. The assay was accurate to within 83–115% of the target and reproducible to less than a CV of 19% for the medium chain lipids. To assess the impact of application volume, a separate set of validation runs were performed, for the quantification of 5 and 50 μg of medium chain lipids in application volumes of 5, 80 and 160 μl. In these runs, accuracy (74–124%) and precision (CV of less than 25%) values were slightly wider than those obtained previously but did not show any noticeable trend in assay performance with application volume (data not shown).

Table 2
Non-linear calibration curve parameters for each class of lipid standards (corresponding figures are presented in Fig. 3)[a]

	Parameters			
	a	b	y_0	S.D. (%)[b]
Phosphatidylcholine	8938	687	374	4.0
Na taurodeoxycholate	18922	3172	410	3.9
Oleic acid	11451	828	550	4.3
2-Monoolein	11194	823	339	1.6
1,2-Diolein	10952	665	1207	1.1
Triolein	14472	657	173	1.7
Caprylic acid	12769	97	350	5.1
1-Monocaprylin	9869	64	6	3.8
1,3-Dicaprylin	12144	44	400	4.8
Tricaprylin	13272	44	-412	3.3

[a] Calibration curves were fitted to the saturation equation $y = y_0 + (ax)/(b + x)$.

[b] The mean relative standard deviation (RSD%) of the calibration curve is calculated from the deviation between each measured standard value and the fitted calibration curve and represents a goodness of fit.

Table 3
Intra- and inter-assay accuracy (%) and precision (CV%) for phosphatidylcholine (PC), Na taurodeoxycholate (NaTDC), long chain lipids and medium chain lipids ($n = 3$)

Lipid	Intra-assay accuracy% (precision, CV%)			Inter-assay accuracy % (precision, CV%)		
	Mass per track					
	0.1 μg	0.5 μg	1.0 μg	0.1 μg	0.5 μg	1.0 μg
PC	92.1 (8.8)	98.1 (5.0)	99.7 (1.6)	91.8 (1.0)	98.0 (4.0)	97.3 (4.8)
NaTDC	104.3 (5.0)	101.7 (6.0)	92.7 (8.4)	104.2 (10.1)	98.2 (5.2)	96.5 (4.4)
Oleic acid	103.9 (3.2)	102.0 (0.9)	91.2 (3.3)	106.9 (10.1)	98.9 (2.8)	95.8 (6.8)
2-Monoolein	103.6 (1.5)	96.9 (3.0)	96.0 (8.2)	104.6 (9.8)	95.3 (3.3)	98.7 (3.3)
1,2-Diolein	95.4 (4.0)	98.8 (2.1)	98.4 (11.2)	100.1 (11.1)	95.4 (3.5)	103.1 (3.9)
Triolein	100.6 (0.9)	97.6 (2.1)	99.7 (9.5)	102.5 (5.6)	96.3 (1.5)	100.7 (1.8)
	5 μg	25 μg	50 μg	5 μg	25 μg	50 μg
Caprylic acid	99.4 (12.6)	108.0 (5.8)	98.0 (3.1)	89.3 (14.0)	101.9 (6.9)	97.3 (4.0)
1-Monocaprylin	86.2 (10.0)	90.3 (5.1)	95.0 (4.8)	83.6 (5.1)	95.3 (4.7)	97.7 (8.7)
1,3-Dicaprylin	112.9 (15.0)	102.5 (6.5)	101.5 (5.4)	93.3 (18.3)	99.9 (2.4)	99.1 (4.3)
Tricaprylin	114.1 (10.8)	94.8 (9.7)	100.0 (5.1)	108.1 (6.8)	94.1 (1.6)	94.7 (7.1)

3.2. Application to in vitro lipolysis of MCT and LCT

The HPTLC assays developed were employed to study the kinetics of triolein and tricaprylin in vitro lipolysis by pancreatic lipase/colipase. The changes in lipid concentrations during the 30 min digestion period are shown in Fig. 4 and were qualitatively in agreement with previous studies [22,24,34]. During triolein lipolysis, there was a continuous decrease in triolein, a transient accumulation in diolein and a gradual increase in oleic acid and monoolein. Tricaprylin hydrolysis, however, proceeded more rapidly and was essentially complete after 30 min. Dicaprylin transiently accumulated in the first 5 min, but was further hydrolysed to monocaprylin and caprylic acid. PC concentrations also gradually decreased as hydrolysis by phospholipase A_2 (present in pancreatin extract) occurred, producing a further FA and lyso-PC.

Immediately before enzyme addition (time 0), the emulsion comprising of TG and PC/NaTDC mixed micelles appeared as small oil droplets in a clear slightly yellow solution. However, sampling from the heterogeneous mixture was accurate (90–113% recovery, $n = 3$ samples) and reproducible (CV of less than 20%) due to efficient stirring of the digestion mixture during each kinetic run (data not shown). After enzyme addition, the mixture became white and cloudy as lipolysis proceeded, presumably as a function of the formation of calcium soaps of FA [35]. Mass balance determinations (calculated using Eq. (2)) for samples from the digestion mixture at 30 min gave values between 98 and 109% and the CV of the analysis of replicate samples ($n = 3$) was less than 7% (data not shown).

The HPTLC assays developed may be utilised as an integral part of an in vitro lipid digestion model for assessing the rate and extent of digestion of lipid-based formulations. The model may be further improved by the addition of an ultracentrifugation step to separate the different physical phases of the lipid digest. This allows for investigation of the effects of formulation factors such as TG composition and the inclusion of emulsifiers on both the chemical digestion and physical dispersion of different oily formulations during digestion by pancreatic lipase. Subsequent studies will utilise this in vitro lipid digestion model to investigate the distribution characteristics of a range of lipophilic compounds into the various physical phases formed during lipid digestion. Elucidation of the role which the physicochemical properties of lipophilic drugs play in

terms of their distribution pattern across the lipid digestion products formed within the in vitro lipid digest, will provide a better understanding of the interaction between different lipid vehicles and co-administered lipophilic drugs within the milieu of the GI lumen, and assist in the rational design of new lipid-based formulations.

4. Conclusions

HPTLC analytical methods for the quantification of MCT and LCT (and their digestion products), PC and NaTDC and application of these methods to in vitro lipid digestion studies have been described. The HPTLC assays are simple, rapid and represent an alternative to previous TLC-gas chromatographic methods [23,24], radiolabelled assay techniques [27] and HPLC-MS [31]. Validation experiments have shown the assays to be accurate and precise. Importantly, since an extraction step is not employed, an internal standard is unnecessary. The methods developed may also prove to be useful tools in the development of an in vitro lipid digestion model for assessing the performance of new lipid-based formulations.

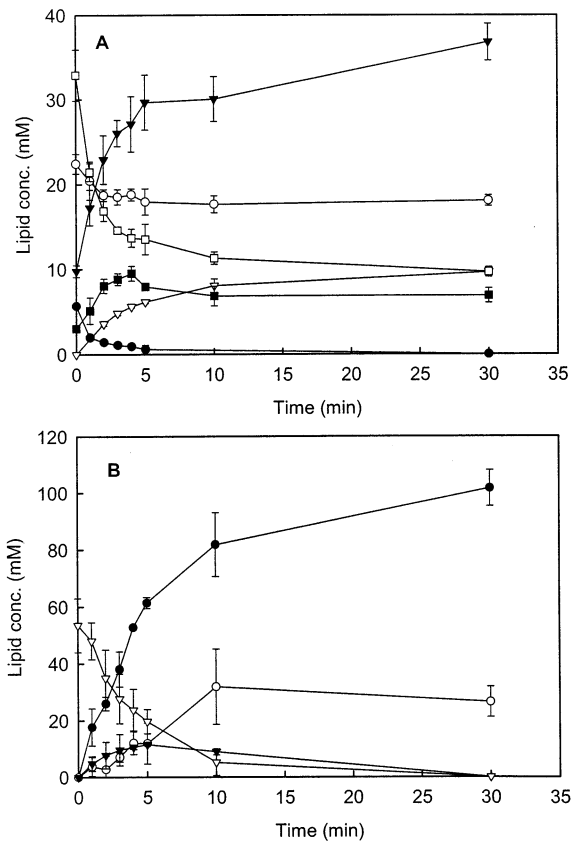

Fig. 4. Changes in lipid concentrations during a 30-min in vitro digestion period. Panel A: Triolein (□), diolein (■), monoolein (▽), oleic acid (▼), phosphatidylcholine (●), and Na taurodeoxycholate (○). Panel B: Tricaprylin (▽), dicaprylin (▼), monocaprylin (○) and caprylic acid (●). Data are mean ± S.D. ($n = 3$).

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