

## In vitro hydrolysis of novel gamma-linolenoyloxyalkyl derivatives of theophylline

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### Abstract

A variety of *N*-gamma-linolenoyloxyalkyl derivatives of theophylline were prepared to determine the effect of varying the nature of the alkyl linkage on in vitro rates of hydrolysis catalyzed by using porcine esterase and human plasma. All derivatives displayed first-order hydrolysis kinetics in 80% human plasma. The chiral theophylline derivatives displayed a biexponential hydrolysis profile using porcine esterase suggesting this enzyme is capable of chiral resolution. The susceptibility of the derivatives to undergo hydrolysis varied widely with half-lives ranging from 7.0 to 711 min using porcine esterase and 19.5 min to over 40 h using human plasma. The rate at which these theophylline derivatives were hydrolyzed depended on the nature of the group linking theophylline and gamma-linolenic acid. The rate of hydrolysis occurred in decreasing order with the following linking moieties: methyl > ethyl > propyl > butyl > isobutyl > pentyl > pivalyl. Overall the results demonstrate that the rate of hydrolysis by porcine esterase and human plasma of *N*-gamma-linolenoyloxyalkyl theophylline derivatives can be slowed by increasing the steric hindrance of the alkyl moieties. The rates of hydrolysis of these theophylline derivatives follow a predictable pattern based on the steric hindrance of the linking group. A linear correlation was obtained between  $\log t_{1/2}$  using the effective number of carbons in the linker,  $C_B$  (the number of carbons in the linker plus 0.5 for each branch). A similar trend was observed between  $\log t_{1/2}$  and the standard Charton steric parameter,  $v$ . © 1997 Elsevier Science B.V.

**Keywords:** Theophylline; Prodrugs;  $\gamma$ -linolenic acid; *N*-acyloxyalkyl theophylline derivatives; Enzymatic hydrolysis; Human plasma

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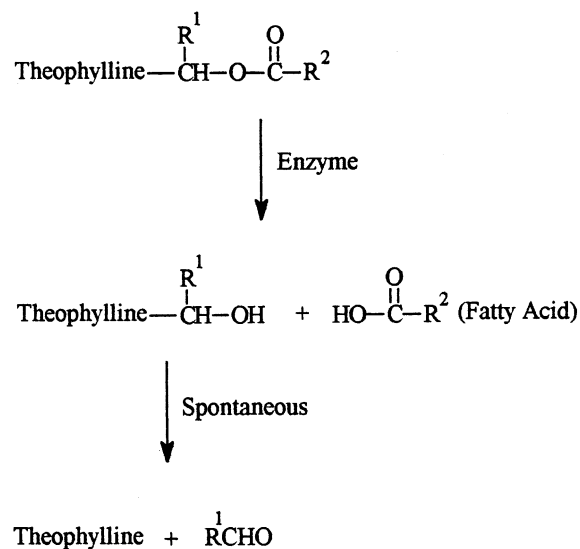
## 1. Introduction

The identification of an effective prodrug for either improved bioavailability upon oral administration or better skin penetration through transdermal application of a parent drug is a challenging exercise. Upon oral administration, the prodrug must have the appropriate lipophilicity and stability in order to be absorbed into the intestinal wall. Once absorbed, the promoiety must be readily cleaved, either in the intestinal wall, in the circulation or at the site of action, to liberate the parent compound (Dickson et al., 1996). For transdermal applications the promoiety must enhance the penetration of the stratum corneum, which is regarded as the main barrier in the penetration process. Once inside the target tissues (epidermis and/or dermis) the prodrug must be readily cleaved by enzymes to regenerate the parent drug (Bonina et al., 1995).

This report describes the design and preparation of polyunsaturated fatty acid (PUFA) promoieties for potential enhanced lipophilic prodrug applications. The development of PUFAs as pharmaceutical drugs is a relatively new concept (Horrobin, 1990) and attaching PUFAs covalently to existing drugs or compounds making lipophilic prodrugs has not been extensively explored. There are a number of advantages in developing PUFA prodrugs. Firstly, PUFA prodrugs would be much more lipophilic than the parent compounds and, as such, may give an improved bioavailability upon oral administration through enhanced absorption via the lymphatic system by bypassing first-pass metabolism (Charman and Porter, 1996). Secondly, PUFAs, have been shown clinically to ameliorate a number of disease conditions. In particular, the fatty acid, gamma-linolenic acid, 6,9,12-octadecatrienic acid (GLA), an 18 carbon PUFA with three double bonds, has a range of desirable anti-inflammatory, anti-cancer and cardiovascular actions (Horrobin, 1992). In fact, GLA in the form of evening primrose oil is marketed as Epogam<sup>TM</sup> for the treatment of atopic eczema and Efamast<sup>TM</sup> for the treatment of mastalgia. Therefore, with the PUFA prodrug approach two different bioactive molecules, with differing modes of action, may be delivered to treat a disease condition simultaneously.

In this study, theophylline was chosen as the model drug to evaluate the PUFA promoieties for the following reasons. Firstly, it is used in the treatment of asthma and its anti-inflammatory mode of action has been proposed as potentially valuable in the treatment of psoriasis (Sloan and Bodor, 1982). However, doses of theophylline required to attain therapeutic blood levels to treat psoriasis result in systemic toxic levels. Therefore, efforts to improve theophylline for topical delivery are required. Secondly, enzymatic hydrolysis assays of measuring half-lives using porcine esterase (Bonina et al., 1991) and human plasma (Bundgaard and Falch, 1985a) have been established using HPLC and since theophylline has a UV active chromophore, the relative hydrolysis rates of the PUFA derivatives can be determined using HPLC with UV detection. Finally, similar derivatives of theophylline using acyl moieties other than PUFAs have previously been examined, hence the general synthetic chemistry was known (Sloan and Bodor, 1982). It is important to note that although theophylline was chosen as the model drug this approach may be applicable to virtually any acidic drug.

Although it is possible to attach PUFAs directly to theophylline through *N*-acyl derivatives (Bodor et al., 1978) we have used the classic



Scheme 1.

*N*-acyloxyalkyl approach as shown in Scheme 1 (Bundgaard, 1985). The advantage is that it gives more flexibility in the design of the prodrug promoieties in that both the alkyl ( $R^1$ ) and acyl portion ( $R^2$ , a fatty acid in our case) of the promoiety can be varied systematically.

In this study, we have varied the alkyl portion to determine what effect this has in terms of porcine esterase and human plasma hydrolysis with these theophylline GLA derivatives. Porcine esterase is regarded as a good model for skin esterases (Bonina et al., 1991) and is, therefore, a model for evaluating prodrugs for topical application. This should lead to better understanding of the fundamental role PUFA promoieties have in terms of drug absorption after oral administration or transdermal applications.

## 2. Materials and methods

### 2.1. Apparatus

The HPLC consisted of a Beckman System Gold™ (Beckman Instruments, Palo Alto, CA) system comprised of a model 126 pump, model 507 autosampler and model 168 variable wavelength diode array detector. The analysis was performed using a reversed-phase ODS column (Beckman, 5  $\mu$ : 25 cm  $\times$  4.6 mm) with methanol–water as the mobile phase at a flow of 1 ml/min and UV detection at 254 nm. The mobile phase was methanol–water (96:4 vol/vol) for the porcine esterase studies and 98:2 (vol/vol) or 96:4 (vol/vol) for the human plasma experiments.

$^1\text{H}$  NMR spectra were recorded with a Bruker AC 250 MHz spectrometer using  $\text{CDCl}_3$  as the solvent. Chemical shifts are given in  $\delta$  (ppm) values measured downfield from the tetramethylsilane internal standard.

Accurate mass measurements were determined by high resolution LSIMS (Liquid Secondary Ion Mass Spectrometry) in positive ion mode using a Micromass AutoSpec Oatof Mass Spectrometer with resolution 8000, voltage scanning and 6 s/scan. The reference compound was polyethylene glycol (PEG) 600 and the matrix was 1:1 glycerol/*meta*-nitrobenzyl alcohol plus 0.1% trifluoroacetic acid.

### 2.2. Chemicals

Theophylline, oxalyl chloride, paraformaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, *sec*-butyraldehyde, pivaldehyde, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (4-DMAP) and zinc chloride were purchased from Aldrich (Milwaukee, WI). GLA was supplied by Callanish Ltd. (Breasclete, Outer Hebrides, UK). Pyridine was obtained from Aldrich and dried over molecular sieves prior to use. All solvents were obtained from Fisher Scientific (Ottawa, Ontario, Canada) and were HPLC grade.

### 2.3. General preparation of $\alpha$ -chloroalkyl esters of GLA

All  $\alpha$ -chloroalkyl esters of GLA were prepared by stirring equimolar amounts of the appropriate aldehyde with the acid chloride of GLA (prepared by refluxing an equal mass of GLA and oxalyl chloride for 1 h and removing the excess oxalyl chloride) in the presence of a catalytic amount of anhydrous  $\text{ZnCl}_2$  at room temperature for 4 h (Bodor et al., 1980). The  $\alpha$ -chloroalkyl esters of GLA were purified by silica gel (200–424 mesh) flash chromatography using hexane–diethyl ether.

### 2.4. 1-(theophyllin-7-yl)methyl gamma-linolenate

7-(hydroxymethyl)theophylline was prepared following the method previously described (Sloan and Bodor, 1982). GLA (1.53 g, 5.5 mmol), 7-(hydroxymethyl)theophylline (1.05 g, 5 mmol) and 4-DMAP (0.61 g, 5 mmol) were added to chloroform (15 ml). A solution of DCC (1.14 g, 5.5 mmol) in chloroform (10 ml) was added slowly over 10 min. The reaction was allowed to stir for 4 h under nitrogen. The precipitated dicyclohexylurea was filtered off, washed and the filtrate evaporated. The residue was purified by silica gel flash column chromatography using a gradient of 20–35% ethyl acetate in hexane to give 1-(theophyllin-7-yl)methyl gamma-linolenate, 1.70 g (73%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.88 (s, 1H, N=CH), 6.24, (s, 2H, N-CH<sub>2</sub>-O), 5.29–5.41 (m, 6H, CH=CH), 3.59 (s, 3H, CH<sub>3</sub>), 3.42 (s, 3H, CH<sub>3</sub>),

2.77–2.81 (m, 4H, CH–CH<sub>2</sub>–CH), 2.35–2.39 (t, 2H, O=C–CH<sub>2</sub>), 2.03–2.08 (m, 4H, CH=CH–CH<sub>2</sub>), 1.59–1.66 (m, 2H, O=C–CH<sub>2</sub>–CH<sub>2</sub>), 1.24–1.39 (M, 8H, CH<sub>2</sub>), 0.86–0.90 (t, 3H, CH<sub>3</sub>). HR-LSIMS MH<sup>+</sup> 471.2959 ± 0.0003, C<sub>26</sub>H<sub>39</sub>N<sub>4</sub>O<sub>4</sub> requires 471.2971.

#### 2.5. (±)-1-(theophyllin-7-yl)ethyl gamma-linolenate

(±)-α-Chloroethyl GLA (0.34 g, 1 mmol), theophylline (0.18 g, 1 mmol) and triethylamine (0.10 g, 1 mmol) were added to anhydrous pyridine (500 μl) in a 4-ml reaction vial and heated to 80°C for 24 h. Excess pyridine was evaporated and the residue was purified by silica gel flash column chromatography using a gradient of 5–30% ethyl acetate in hexane to give (±)-1-(theophyllin-7-yl)ethyl gamma-linolenate: 0.18 g (37%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.82 (s, 1H, N=CH), 7.15–7.30, (q, 1H, N–CH–O), 5.32–5.38 (m, 6H, CH=CH), 3.60 (s, 3H, CH<sub>3</sub>), 3.42 (s, 3H, CH<sub>3</sub>), 2.77–2.82 (m, 4H, CH–CH<sub>2</sub>–CH), 2.33–2.39 (t, 2H, O=C–CH<sub>2</sub>), 2.02–2.06 (m, 4H, CH=CH–CH<sub>2</sub>), 1.59–1.66 (m, 2H, O=C–CH<sub>2</sub>–CH<sub>2</sub>), 1.87–1.90 (d, 2H, CH<sub>3</sub>–CH) 1.26–1.39 (M, 8H, CH<sub>2</sub>), 0.86–0.91 (t, 3H, CH<sub>3</sub>). HR-LSIMS MH<sup>+</sup> 485.3128 ± 0.0003, C<sub>27</sub>H<sub>41</sub>N<sub>4</sub>O<sub>4</sub> requires 485.3128.

The following 1-(theophyllin-7-yl)alkyl gamma-linolenate esters were prepared in a similar manner.

(±)-1-(theophyllin-7-yl)propyl gamma-linolenate: 0.87 g (84%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.77 (s, 1H, N=CH), 7.00–7.06, (t, 1H, N–CH–O), 5.27–5.42 (m, 6H, CH=CH), 3.59 (s, 3H, CH<sub>3</sub>), 3.42 (s, 3H, CH<sub>3</sub>), 2.78–2.82 (m, 4H, CH–CH<sub>2</sub>–CH), 2.34–2.41 (t, 2H, O=C–CH<sub>2</sub>), 2.17–2.29 (m, 2H, O–CH–CH<sub>2</sub>) 2.02–2.10 (m, 4H, CH=CH–CH<sub>2</sub>), 1.57–1.66 (m, 2H, O=C–CH<sub>2</sub>–CH<sub>2</sub>), 1.25–1.41 (M, 8H, CH<sub>2</sub>), 0.93–0.99 (t, 3H, CH<sub>3</sub>), 0.86–0.91 (t, 3H, CH<sub>3</sub>). HR-LSIMS MH<sup>+</sup> 499.3287 ± 0.0004, C<sub>28</sub>H<sub>43</sub>N<sub>4</sub>O<sub>4</sub> requires 499.3284.

(±)-1-(theophyllin-7-yl)butyl gamma-linolenate: 0.80 g (64%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.77 (s, 1H, N=CH), 7.08–7.14, (t, 1H, N–CH–O), 5.27–5.42 (m, 6H, CH=CH), 3.59 (s, 3H, CH<sub>3</sub>), 3.42 (s, 3H, CH<sub>3</sub>), 2.78–2.82 (m, 4H, CH–CH<sub>2</sub>–CH),

2.33–2.40 (t, 2H, O=C–CH<sub>2</sub>), 2.12–2.23 (m, 2H, O–CH–CH<sub>2</sub>) 2.02–2.10 (m, 4H, CH=CH–CH<sub>2</sub>), 1.57–1.66 (m, 2H, O=C–CH<sub>2</sub>–CH<sub>2</sub>), 1.25–1.41 (M, 10H, CH<sub>2</sub>), 0.94–1.00 (t, 3H, CH<sub>3</sub>), 0.86–0.91 (t, 3H, CH<sub>3</sub>). HR-LSIMS MH<sup>+</sup> 513.3441 ± 0.0009, C<sub>29</sub>H<sub>45</sub>N<sub>4</sub>O<sub>4</sub> requires 513.3441.

(±)-1-(theophyllin-7-yl)isobutyl gamma-linolenate: 0.98 g (56%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.73 (s, 1H, N=CH), 6.81–6.85, (d, 1H, N–CH–O), 5.27–5.45 (m, 6H, CH=CH), 3.59 (s, 3H, CH<sub>3</sub>), 3.42 (s, 3H, CH<sub>3</sub>), 2.77–2.82 (m, 4H, CH–CH<sub>2</sub>–CH), 2.52–2.66 (m, 1H, O–CH–CH), 2.35–2.46 (t, 2H, O=C–CH<sub>2</sub>), 2.02–2.10 (m, 4H, CH=CH–CH<sub>2</sub>), 1.57–1.70 (m, 2H, O=C–CH<sub>2</sub>–CH<sub>2</sub>), 1.24–1.42 (M, 8H, CH<sub>2</sub>), 1.05–1.08 (d, 3H, CH–CH<sub>3</sub>) 0.86–0.91 (d and t, 6H, CH<sub>3</sub> and CH–CH<sub>3</sub>). HR-LSIMS MH<sup>+</sup> 513.3438 ± 0.0003, C<sub>29</sub>H<sub>45</sub>N<sub>4</sub>O<sub>4</sub> requires 513.3441.

(±)-1-(theophyllin-7-yl)pentyl gamma-linolenate: 0.21 g (38%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.76 (s, 1H, N=CH), 7.06–7.12, (t, 1H, N–CH–O), 5.27–5.46 (m, 6H, CH=CH), 3.59 (s, 3H, CH<sub>3</sub>), 3.42 (s, 3H, CH<sub>3</sub>), 2.78–2.82 (m, 4H, CH–CH<sub>2</sub>–CH), 2.34–2.40 (t, 2H, O=C–CH<sub>2</sub>), 2.12–2.23 (m, 2H, O–CH–CH<sub>2</sub>) 2.01–2.09 (m, 4H, CH=CH–CH<sub>2</sub>), 1.57–1.66 (m, 2H, O=C–CH<sub>2</sub>–CH<sub>2</sub>), 1.23–1.41 (m, 12H, CH<sub>2</sub>), 0.86–0.92 (t and t, 6H, CH<sub>3</sub> and CH<sub>3</sub>). HR-LSIMS MH<sup>+</sup> 527.3598 ± 0.0003, C<sub>30</sub>H<sub>47</sub>N<sub>4</sub>O<sub>4</sub> requires 527.3597.

(±)-1-(theophyllin-7-yl)pivalyl gamma-linolenate: 0.69 g (40%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.69 (s, 1H, N=CH), 7.21 (s, 1H, N–CH–O), 5.30–5.41 (m, 6H, CH=CH), 3.59 (s, 3H, CH<sub>3</sub>), 3.42 (s, 3H, CH<sub>3</sub>), 2.77–2.82 (m, 4H, CH–CH<sub>2</sub>–CH), 2.36–2.43 (t, 2H, O=C–CH<sub>2</sub>), 2.01–2.12 (m, 4H, CH=CH–CH<sub>2</sub>), 1.58–1.70 (m, 2H, O=C–CH<sub>2</sub>–CH<sub>2</sub>), 1.26–1.44 (M, 8H, CH<sub>2</sub>), 1.06 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>) 0.86–0.91 (t, 3H, CH<sub>3</sub>). HR-LSIMS MH<sup>+</sup> 527.3596 ± 0.0002, C<sub>30</sub>H<sub>47</sub>N<sub>4</sub>O<sub>4</sub> requires 527.3597.

#### 2.6. Apparent lipophilicity indices of theophylline GLA esters 1–7

The lipophilic indices of the theophylline GLA esters were determined using the reversed-phase HPLC method described above for porcine es-

terase hydrolysis experiments. The lipophilic index ( $\log K$ ) is calculated from the equation:

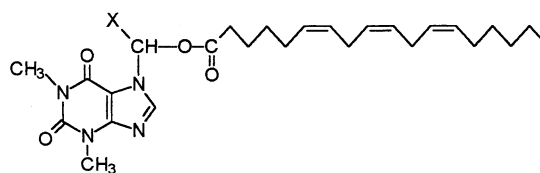
$$\log K = \log[(t_r - t_0)/t_0] \quad (1)$$

where  $t_r$  is the retention time of the ester and  $t_0$  is the retention time of the solvent front.

### 2.7. Determination of porcine esterase and human plasma hydrolysis rates

Porcine esterase (170 units/mg protein) was obtained from Sigma. The esters **1–7** were prepared by dissolving each of the compounds in ethanol to give a 0.01 M concentration. An 80- $\mu$ l aliquot was then added to isotonic phosphate buffer, 19.72 ml, 0.025 M, pH = 7.4 with an ionic strength of 0.5 (KCl) at 37°C. The porcine esterase was diluted 100-fold with the phosphate buffer and 200  $\mu$ l added to the buffered ester solutions to give a final volume of 20.0 ml,  $4 \times 10^{-5}$  M ester concentration and 1.3 units of enzyme per ml. At appropriate time intervals, a 500- $\mu$ l aliquot was removed and added to 500  $\mu$ l of ethanol to quench the reaction. The amount of ester remaining was determined by reversed-phase HPLC as described above. Pseudo-first order rate constants for hydrolysis were determined from either an exponential (Theo-m-GLA, **1**) or biexponential (derivatives **2–7**) fit of the data versus time.

For the hydrolysis experiments in plasma, 100 ml of human plasma from seven volunteers was mixed together, aliquoted into 10-ml vials and frozen until needed. To 4.98 ml of 80% human plasma made up with phosphate buffer, 0.025 M, pH = 7.4 with an ionic strength of 0.5 (KCl) at 37°C was added 20  $\mu$ l of the 0.01 M ester, **1–7**, stock solution to give a final plasma concentration of  $4 \times 10^{-5}$  M. At appropriate time intervals, a 250- $\mu$ l aliquot was removed and added to 500  $\mu$ l of ethanol, vortexed, centrifuged at 2500 rev./min for 2 min and 100  $\mu$ l of the clear supernatant analyzed by HPLC. Pseudo-first order rate constants for hydrolysis were determined from an exponential fit of the data versus time.



<b>1</b>	X = H	Theo-m-GLA
<b>2</b>	X = CH <sub>3</sub>	Theo-e-GLA
<b>3</b>	X = CH <sub>2</sub> CH <sub>3</sub>	Theo-pro-GLA
<b>4</b>	X = CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	Theo-b-GLA
<b>5</b>	X = $\begin{array}{c} \text{CH}_3 \\   \\ \text{CHCH}_3 \end{array}$	Theo-ib-GLA
<b>6</b>	X = CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	Theo-pen-GLA
<b>7</b>	X = $\begin{array}{c} \text{CH}_3 \\   \\ \text{CCH}_3 \\   \\ \text{CH}_3 \end{array}$	Theo-piv-GLA

Fig. 1. Gamma-linolenoyloxyalkyl theophylline esters.

## 3. Results

### 3.1. Synthesis of theophylline GLA esters **1–7**

The general structures of the *N*-gamma-linolenoyloxyalkyl theophylline derivatives prepared are shown in Fig. 1. The objectives of this report were to determine the effect of varying the nature of the alkyl linkage (i.e. varying X) between the drug theophylline and the polyunsaturated fatty acid, GLA, on the rates of hydrolysis, catalyzed by porcine esterase and human plasma. All of the *N*-gamma-linolenoyloxyalkyl theophylline derivatives, **1–7**, were readily prepared from the condensation of the corresponding  $\alpha$ -chloroalkyl ester of GLA and theophylline with the exception of Theo-m-GLA which was prepared by acylation of 7-(hydroxymethyl)theophylline with GLA in the presence of DCC and 4-DMAP.

### 3.2. Kinetics of esters, **1–7**, using porcine esterase

As shown in Fig. 2, Theo-m-GLA is readily hydrolyzed by porcine esterase and follows first-order kinetics over two half-lives indicating the initial concentration was below the saturation level for porcine esterase. Surprisingly, for the derivatives, **2–7**, the hydrolysis data were best fit by the biexponential Eq. (2) as:

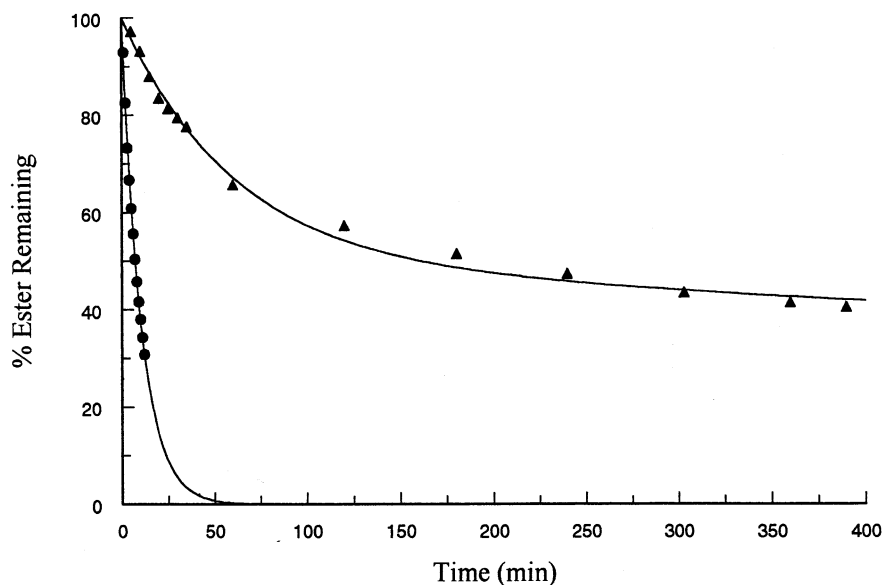


Fig. 2. Exponential fit for the disappearance of Theo-m-GLA, **1**, (●), and biexponential fit for the disappearance of Theo-b-GLA, **4**, (▲) using porcine esterase (pH = 7.4, 37°C). Each time point is an average of two determinations.

%**2–7** remaining

$$= a[\exp(-k_1 t) + \exp(-k_2 t)] \quad (2)$$

illustrated in Fig. 2 for Theo-b-GLA. Since these derivatives were prepared as racemic mixtures the coefficient,  $a$ , should be equal to one-half the initial substrate concentration for this model to fit the two decay regions. As demonstrated for Theo-b-GLA, two distinct hydrolysis regions are seen with the transition occurring when approximately one-half of the starting ester concentration was reached at which time the hydrolysis markedly slowed. The best fit parameters,  $a$  and  $k_1$ , determined from fitting Eq. (2) are given in Table 1 along with the calculated half-lives,  $t_{1/2}$ , and correlation coefficients. The reactions were only monitored for 7 h and there were only a few points describing the second slower hydrolysis region, hence, the rate constants  $k_2$  obtained were not useful as they have a large degree of uncertainty associated with them.

In order to compare the rates of hydrolysis of the various derivatives it is useful to utilize lipophilicity indices (or  $\log K$ ) as determined by reversed-phase HPLC.  $\log K$  is linearly related to  $\log P$ , the  $n$ -octanol/water partition coefficient

and this is a convenient alternative to the task of determining the octanol/water solubility measurements normally required for determining  $\log P$  (Bonina et al., 1995). Fig. 3 shows the graph of  $\log t_{1/2}$  versus  $\log K$  for derivatives **1–7**. As expected, there is a good correlation for the straight chain homologous series **1–4** and **6** in terms of increasing  $\log t_{1/2}$  versus increasing  $\log K$ . However, the branched chain derivatives, **5** and **7** deviate from the line, having shorter retention times compared with their straight chain analogues but having longer half-lives. This indicates that the  $\log K$  method for calculating  $\log P$  values is not useful for these branched chain theophylline derivatives.

A better correlation is obtained simply by adding the number of carbons in the linker of these derivatives. The rationale is that the steric hindrance is proportional to the carbon content of the linking group. Hence a plot of  $\log t_{1/2}$  of the derivatives with straight chain alkyl groups in the linker, **1–4** and **6**, is proportional to the number of carbons in the linker as shown in Fig. 4. The remaining two derivatives, **5** and **7**, which have branching in their linker side chains can be incorporated onto this line if a correction factor is

Table 1

Porcine esterase and 80% human plasma half-lives and lipophilicity indices (log *K*) for *N*-gamma-linolenoyloxyalkyl theophylline esters 1–7

Compound	Porcine esterase (1.3 U/ml) <sup>a</sup>				80% Human plasma <sup>a</sup>	Log <i>K</i> <sup>b</sup>
	<i>a</i> <sup>c</sup>	<i>k</i> <sub>1</sub> <sup>d</sup>	<i>t</i> <sub>1/2</sub> (min)	<i>r</i> <sup>2,e</sup>		
Theo-m-GLA, 1 <sup>f</sup>	100	0.0990	7.0	0.998	19.5	0.144
Theo-e-GLA, 2	50.0	0.0535	13.0	0.992	151	0.161
Theo-pro-GLA, 3	50.0	0.0197	35.2	0.994	206	0.243
Theo-b-GLA, 4	48.9	0.0165	42.1	0.994	365	0.312
Theo-ib-GLA, 5	50.0	0.00659	105	0.990	779	0.299
Theo-pen-GLA, 6	50.0	0.00435	159	0.994	490	0.382
Theo-piv-GLA, 7	50.0	0.000975	711	0.978	2406	0.373

<sup>a</sup>pH = 7.4, 37°C.

<sup>b</sup>Determined by reversed-phase HPLC, see Section 2.

<sup>c,d</sup>Obtained from best fit of the biexponential decay equation, %2–7 Remaining =  $a[\exp(-k_1t) + \exp(-k_2t)]$ . (2)

<sup>e</sup>Correlation coefficient.

<sup>f</sup>Determined from best fit of a single exponential decay equation.

used. Naively, this correction factor takes into account the number of branches in the side chain to transform the branched chain to a theoretical straight chain by adding 0.5 for each branch. So for X equal to CH(CH<sub>3</sub>)<sub>2</sub> the number of carbons in the linker is 4, however, since this group has one branch, the effective number of carbons is increased to 4.5. Similarly for X equal to CH(CH<sub>3</sub>)<sub>3</sub>, the effective number of carbons is corrected to 6 since there are two branches in the linker. When log *t*<sub>1/2</sub> for these two derivatives are added to the plot (Fig. 4), the equation describing the relationship is

$$\log t_{1/2}(\text{esterase}) = 0.36 + 0.38(C_B), \quad r^2 = 0.976 \quad (3)$$

where *t*<sub>1/2</sub> is the theophylline GLA linked derivative half-life from the fast exponential decay region using porcine esterase and *C*<sub>B</sub> is the number of carbons in the alkyl side chain of the linking group plus 0.5 for each methyl branch that occurs in the side chain. We also obtained a good correlation for log *t*<sub>1/2</sub> of these theophylline derivatives versus the Charton steric parameter, *v*, for the various groups in the linker indicating steric hindrance is a major factor. The *v* values refer to the X groups of Fig.

1, i.e. methyl, ethyl, propyl, isopropyl, butyl and t-butyl (Charton, 1977).

### 3.3. Kinetics of esters 1–7 in human plasma

In contrast to the porcine esterase results, all the derivatives including the chiral derivatives displayed strict first-order kinetics in 80% human plasma. This is shown in Fig. 5 for Theo-m-GLA and Theo-pro-GLA. Table 1 shows that *t*<sub>1/2</sub> again increased as the size of the linking group increased in the order methyl < ethyl < propyl < butyl < isobutyl < pentyl < pivalyl indicating that the steric hindrance of the linker also has a marked effect on the plasma enzymes responsible for hydrolysis. It is worth noting that replacing one of the hydrogens in the linker with a methyl group markedly slowed the hydrolysis from *t*<sub>1/2</sub> = 19.5 min for Theo-m-GLA to 2.51 h for Theo-e-GLA.

An analogous relationship between the nature of the linker moiety (i.e. *C*<sub>B</sub>) and log *t*<sub>1/2</sub> in plasma is shown in Fig. 4. The equation fitting this relationship is

$$\log t_{1/2}(\text{plasma}) = 1.18 + 0.36(C_B), \quad r^2 = 0.952 \quad (4)$$

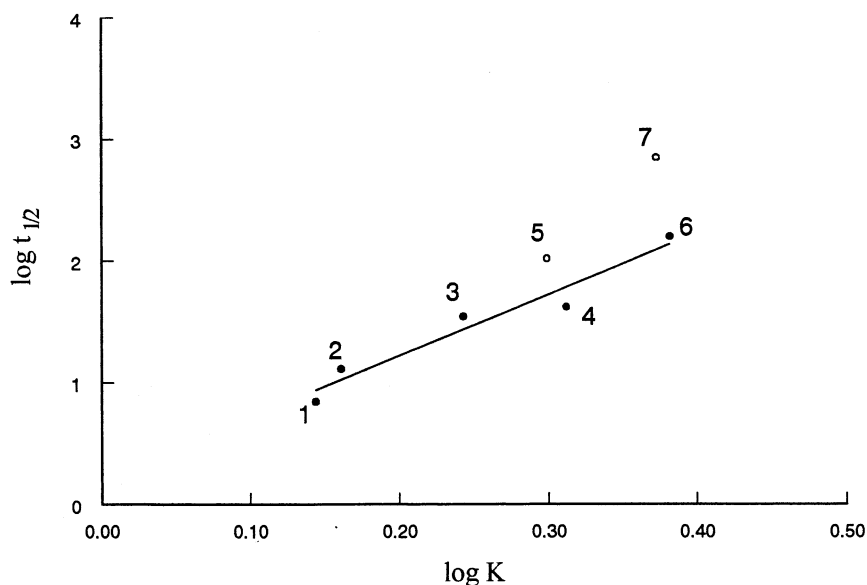


Fig. 3. Plot of  $\log t_{1/2}$  of the fast exponential decay region using porcine esterase (pH = 7.4, 37°C) versus the lipophilicity index,  $\log K$ , for *N*-gamma-linolenoyloxyalkyl theophylline esters 1–7.

#### 4. Discussion

All of the *N*-gamma-linolenoyloxyalkyl theophylline derivatives gave either first-order or biexponential hydrolysis kinetics using either porcine esterase or human plasma. The observation that the hydrolysis experiments using derivatives, 2–7, with porcine esterase were best fit by a biexponential equation can be explained. Theo-m-GLA is achiral and gave strict first-order hydrolysis kinetics whereas the derivatives 2–7 are chiral racemates and gave a second slower hydrolysis region when approximately one-half of the initial substrate concentration disappeared. This suggests that porcine esterase is exhibiting chiral resolution under our hydrolysis conditions. The chiral resolution of porcine esterase has been described (Stoops et al., 1969), namely, the  $k_{cat}$  values for *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester and the corresponding D enantiomer are  $24 \text{ s}^{-1}$  and  $0.9 \text{ s}^{-1}$ , respectively, indicating that the L enantiomer behaves as a hindered substrate. Hence, on this basis, for the chiral derivatives 2–7 in this study, one of the enantiomers is presumably behaving as a more

hindered substrate and being hydrolyzed at a slower rate.

Although the data for the chiral derivatives were best fit by the biexponential Eq. (2), the rate constants,  $k_2$ , obtained were not reliable since we were only able to monitor the hydrolysis until approximately 50% of the substrate remained during the day. Hence the majority of points were described by the fast exponential region, giving  $k_1$ . Autoxidation of the unsaturated fatty acid promoiety and/or chemical hydrolysis from the buffer did not occur since the initial concentration of the Theo-m-GLA derivative remained constant over 7 h. We did not monitor the reaction further than 7 h as we were concerned about eventual autoxidation that would complicate the kinetic picture. It is, however, evident from the data that this second exponential decay is markedly slower with half-lives in the order of several hours. Since we did not see any chiral resolution in the plasma the significance of the relative hydrolysis rates for the slower enantiomer may only be important in topical applications.

To ensure that the biexponential character of the hydrolysis profiles was not a result of precipi-



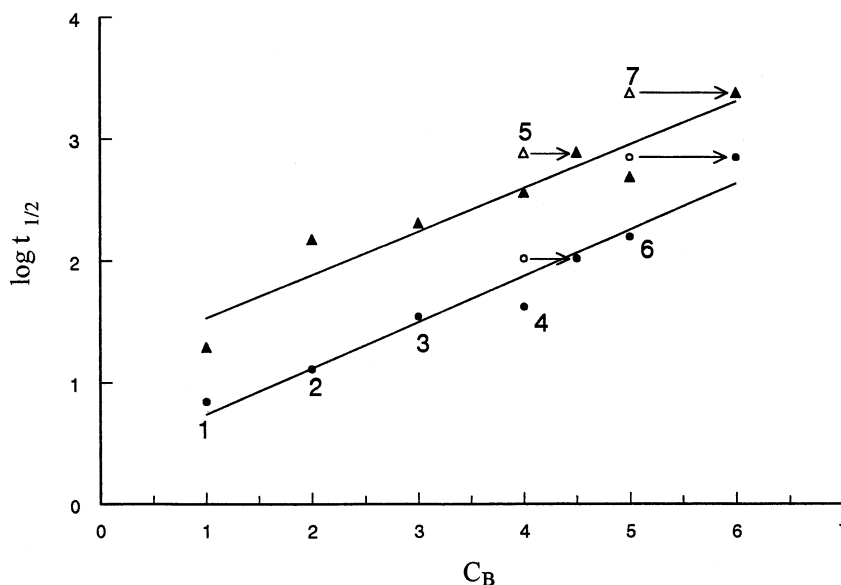


Fig. 4. Plot of  $\log t_{1/2}$  of the fast exponential decay region using porcine esterase (pH = 7.4, 37°C) (●) and 80% human plasma (○) versus  $C_B$  for *N*-gamma-linolenoyloxyalkyl theophylline esters 1–7.  $C_B$  refers to the number of carbons plus 0.5 for each branch in the linker. The arrows show the corrected  $C_B$  values for derivatives 5 and 7.

tation of these lipophilic derivatives from the aqueous buffer, we added Theo-e-GLA to the assay buffer alone and found that the initial concentration remained constant over the sampling time. The fact that these lipophilic GLA derivatives are not precipitating from the buffer is also supported by the observation that Theo-m-GLA gave strict pseudo first-order kinetics over two half-lives.

Theo-m-GLA was quantitatively converted to theophylline indicating that mass balance was achieved throughout the course of the reaction. This is consistent with the previously reported transient very short-lived 7-(hydroxymethyl)-theophylline intermediate formed after initial ester cleavage (Sloan and Bodor, 1982). Although the chiral derivatives were not completely converted to theophylline, mass balance was, nonetheless, achieved as the concentration of Theo-b-GLA plus theophylline was constant throughout the course of the reaction (Fig. 6). Similar mass balances were achieved for the other chiral derivatives.

It is known that *N*-acyloxyalkyl derivatives, formed from aldehydes other than formaldehyde

give *N*- $\alpha$ -hydroxyalkyl intermediates, formed after initial ester cleavage (Scheme 1), and are even more unstable than the corresponding *N*- $\alpha$ -hydroxymethyl intermediate (i.e. 7-(hydroxymethyl)theophylline). Therefore, these data are consistent with the rate of theophylline formation from these *N*-acyloxyalkyl theophylline GLA linked derivatives being based only on the initial ester cleavage.

The results presented here demonstrate that only slight modifications of the alkyl portion of *N*-gamma-linolenoyloxyalkyl theophylline derivatives leads to significant decreases in their rates of hydrolysis. The  $t_{1/2}$  values ranged from 7.0 min for Theo-m-GLA to 711 min for Theo-piv-GLA. A similar finding was observed when the derivatives were hydrolyzed by human plasma,  $t_{1/2}$  = 19.5 min for Theo-m-GLA to over 40 h for Theo-piv-GLA.

Additionally, the rates of hydrolysis of these theophylline GLA derivatives, 1–7, follow a predicted pattern based on a knowledge of the steric hindrance of the linking group. Using both porcine esterase and human plasma we obtained a good correlation (Fig. 4) between  $\log t_{1/2}$  and the

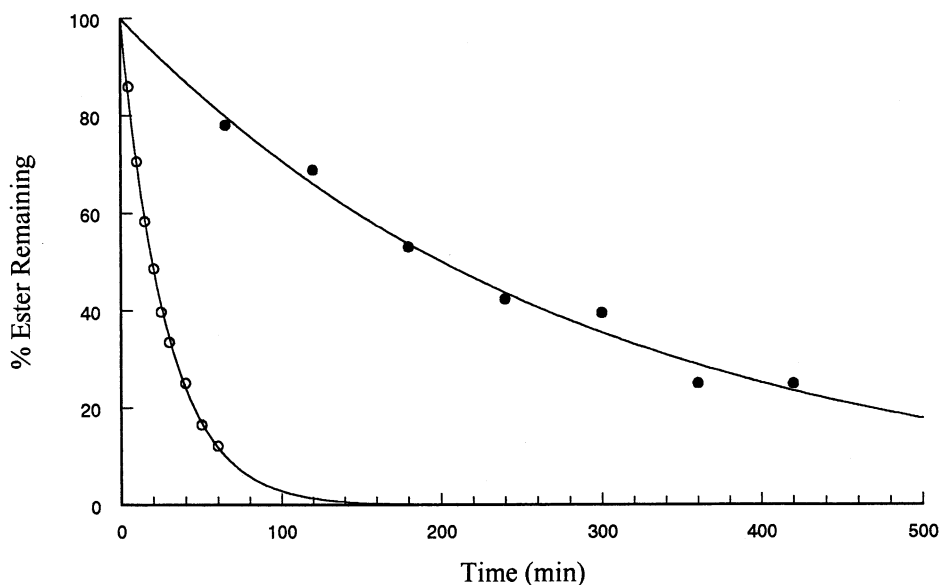


Fig. 5. Rate of disappearance of Theo-m-GLA, 1, (●), and Theo-pro-GLA, 4, (○) using 80% human plasma (pH = 7.4, 37°C). Solid lines are the best exponential fit curves. Each time point is an average of two determinations.

effective number of carbons in the linker,  $C_B$ , defined as the number of carbons in the linker plus 0.5 for each branch. Since the slopes of these two lines were parallel this indicates that one may be able to predict how the other will behave in two biological media.

It has long been known that when utilizing the *N*-acyloxyalkyl prodrug approach both the alkyl and acyl portions of the acyloxyalkyl derivative can be varied systematically. Most work was carried out, however, by only varying the acyl moiety (Bundgaard and Falch, 1985b; Yamaoka et al., 1983; Sinkula and Yalkowsky, 1975). In these cases, the rate of hydrolysis can be either increased or decreased depending on the length of the acyl group. For example, using acyloxymethyl derivatives of phenytoin, it was shown that the butyrate C4 derivative was hydrolyzed faster than the acetate, propionate and C5–10 derivatives. The results presented here demonstrate that the rate of hydrolysis of *N*-gamma-linolenoyloxyalkyl theophylline derivatives can, generally, be decreased by increasing the steric hindrance of the alkyl group. This suggests that with the *N*-acyloxyalkyl approach the rates of hydrolysis may potentially be

fine tuned by varying both the alkyl as well as the acyl moieties.

This study only examined one PUFA, specifically GLA, as this is a fatty acid of significant medical importance. However, the effect of systematically varying the PUFA on the rate of hydrolysis is also of interest. There are numerous PUFAs ranging from 14–22 carbons with varying number as well as position of double bonds present. Substitution of GLA with any of these molecules could alter the hydrolysis rate and work is presently in progress to evaluate different PUFAs.

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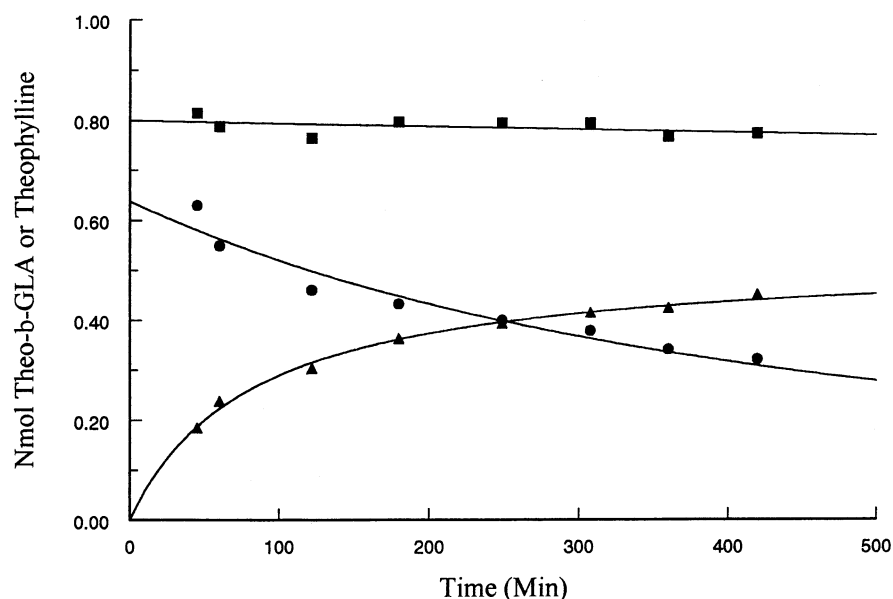


Fig. 6. Quantitative mass balance (nmol) of the disappearance of Theo-b-GLA, 5, (●), appearance of theophylline (▲) and Theo-b-GLA plus theophylline (■) using porcine esterase (pH = 7.4, 37°C). Each time point is an average of two determinations.

## References

- Bodor, N., Kaminski, J.J., Selk, S., 1980. Soft Drugs. 1. Labile quaternary ammonium salts as soft antimicrobials. *J. Med. Chem.* 23, 369–473.
- Bodor, N., Sloan, K.B., Kuo, Y.N., Higuchi, T., 1978. Controlled delivery of theophylline: Chemistry of 7' and 7,7'-acylditheophylline derivatives. *J. Pharm. Sci.* 67, 1045–1050.
- Bonina, F.P., Montenegro, L., Trapani, G., Franco, M., Liso, G., 1995. *In vitro* evaluation of *N*-acyllactam esters of indomethacin as dermal prodrugs. *Int. J. Pharm.* 124, 45–51.
- Bonina, F.P., Montenegro, L., De Capraris, P., Bousquet, E., Tirendi, S., 1991. 1-Alkylazacycloalkan-2-one esters as prodrugs of indomethacin for improved delivery through human skin. *Int. J. Pharm.* 77, 21–29.
- Bundgaard, H. (Ed.), 1985. *Design of Prodrugs*. Elsevier, Amsterdam, pp. 1–92.
- Bundgaard, H., Falch, E., 1985a. Allopurinol prodrugs. I. Synthesis, stability and physicochemical properties of various *N*<sub>1</sub>-acyl allopurinol derivatives. *Int. J. Pharm.* 23, 223–237.
- Bundgaard, H., Falch, E., 1985b. Allopurinol prodrugs. II. Synthesis, hydrolysis kinetics and physicochemical properties of various *N*-acyloxymethyl allopurinol derivatives. *Int. J. Pharm.* 24, 307–325.
- Charman, W.N., Porter, C.J.H., 1996. Lipophilic prodrugs designed for intestinal lymphatic transport. *Adv. Drug Deliv. Rev.* 19, 149–169.
- Charton, M., 1977. The prediction of chemical lability through substituent effects. In: Roche, E.B. (Ed.), *Design of Biopharmaceutical Properties through Prodrugs and Analogs*. American Pharmaceutical Association, Washington, DC, pp. 228–280.
- Dickson, J.K. Jr, Biller, S.C., Magnin, D.R., Petrillo Jr, Hillyer, J.W., Hsieh, D.C., Lan, S.-J., Rinehart, J.K., Gregg, R.E., Harrity, T.W., Jolibois, K.G., Kalinowski, S.S., Kunselman, L.K., Mookhtiar, K.A., Ciosek, C.P. Jr, 1996. Orally active squalene synthase inhibitors: Bis((acyloxy)alkyl) prodrugs of the  $\alpha$ -phosphonosulfonic acid moiety. *J. Med. Chem.* 39, 661–664.
- Horrobin, D.F., 1992. Nutritional and medical importance of gamma-linolenic acid. *Prog. Lipid Res.* 31, 163–194.
- Horrobin, D.F., 1990. Gamma linolenic acid. *Rev. Contemporary Pharm.* 1, 1–41.
- Sinkula, A.A., Yalkowsky, S.H., 1975. Rationale for design of biologically reversible drug derivatives: Prodrugs. *J. Pharm. Sci.* 64, 181–210.
- Sloan, K.B., Bodor, N., 1982. Hydroxymethyl and acyloxymethyl prodrugs of theophylline: enhanced delivery of polar drugs through skin. *Int. J. Pharm.* 12, 299–313.
- Stoops, J.K., Horgan, D.J., Runnegar, M.T.C., de Jersey, J., Webb, E.C., Zerner, B., 1969. Carboxylesterases (EC 3.1.1). Kinetic studies on carboxylesterases. *Biochemistry* 8, 2026–2033.
- Yamaoka, Y., Roberts, R.D., Stella, V.J., 1983. Low-melting phenytoin prodrugs as alternative oral delivery modes for phenytoin: A model for other high-melting sparingly water-soluble drugs. *J. Pharm. Sci.* 72, 400–405.