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Isosorbide-based aspirin prodrugs II. Hydrolysis kinetics of isosorbide diaspirinate

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Abstract

Aspirin prodrugs have been intensively investigated in an effort to produce compounds with lower gastric toxicity, greater stability or enhanced percutaneous absorption, relative to aspirin. This report describes the hydrolysis kinetics and aspirin release characteristics of isosorbide diaspirinate (ISDA), the aspirin diester of isosorbide. ISDA underwent rapid hydrolysis when incubated in phosphate buffered human plasma solutions (pH 7.4) at 37 °C, producing appreciable quantities of aspirin. In 30% human plasma solution the half-life was 1.1 min and 61% aspirin was liberated relative to the initial ester concentration. The hydrolysis kinetics of ISDA were monitored in aqueous solution at 37 °C over the pH range 1.03–9.4. The aqueous hydrolysis followed pseudo-first-order kinetics over several half-lives at all pH values, resulting in a U-shaped pH rate profile. Salicylate esters and salicylic acid were formed during these processes. The hydrolysis characteristics of ISDA were also investigated in pH 7.4 phosphate buffered solutions containing α-chymotrypsin [EC 3.1.1.1] ($t_{1/2}$=200.9 min), carboxyl esterase [EC 3.1.1.1] ($t_{1/2}$=31.5 min), human serum albumin ($t_{1/2}$=603 min), purified human serum butyrylcholinesterase [EC 3.1.1.8] (80 μg/ml; $t_{1/2}$=9.4 min; 55% aspirin), purified horse serum butyrylcholinesterase (100 μg/ml; $t_{1/2}$=1.85 min;11% aspirin) and in 10% human plasma solution in the presence of physostigmine (3 μM). The results indicate that a specific enzyme present in human plasma, probably human butyrylcholinesterase, catalyses aspirin release from isosorbide diaspirinate.
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Keywords: Prodrug; Aspirin; Isosorbide; Hydrolysis; Butyrylcholinesterase

1. Introduction

Aspirin prodrugs have been extensively investigated for many years as a means of depressing gastric toxicity (Jones, 1985) or increasing percutaneous absorption (e.g. Lofttson et al., 1981). The major reason for the lack of progress in this area is that the aspirin *O*-acetyl ester, so essential to its unique pharmacological profile, is rendered highly susceptible to plasma-mediated hydrolysis relative to aspirin itself by esterification of the aspirin carboxylic acid group (Nielsen and Bungaard, 1989). A successful aspirin prodrug must undergo hydrolysis at the carrier ester at a greater rate than at the *O*-acetyl group, whose hydrolysis the carrier group greatly accelerates.

Strategies to overcome this problem may be grouped into those that exploit ester types that are intrinsically chemically unstable, or those that use carrier groups capable of acting as enzyme substrates, thus competing with the rapid *O*-acetyl hydrolysis. Examples of the former approach include aspirin anhydrides (Levy and Gagliardi, 1963), benzodioxinone derivatives (Ankersen et al., 1989; Nielsen and Senning, 1990), acylal derivatives (Hussain et al., 1974, 1979; Truelove et al., 1980), *N*-(hydroxyalkyl) amides (Bundgaard et al., 1988), and 2-formylphenyl derivatives (Abordo et al., 1998). One limitation of this approach is that increasing ester lability diminishes drug stability. The enzyme targeting approach has been more intensively pursued, as successful candidates, although highly susceptible to enzyme-mediated decomposition, might also be chemically stable. Examples in this group include alkyl and aryl esters (Rainsford and Whitehouse, 1976, 1980), triglycerides (Kumar and Billimoria, 1978; Paris et al., 1979, 1980), acyloxyalkyl esters (Los et al., 1982), sulfinyl or sulfonyl esters (Lofttson and Bodor, 1981a,b; Lofttson et al., 1981), phenylalanine derivatives (Banerjee and Amidon, 1981a,b,c; Muhi-Eldeen et al., 1985), amino acid derivatives (Tsunematsu et al., 1991), glycolamide esters (Nielsen and Bungaard, 1989), and

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indolediones as hypotoxic tissue targeting agents (Jaffar et al., 1999). Of these attempts, only some of the glycolamides reported by Nielsen and Bungaard (1989) can be considered successful, as these compounds combine good aqueous stability with the ability to liberate significant amounts of aspirin (50–55%) in human plasma. Interest in the aspirin prodrug area has been renewed with the advent of the so-called NO–aspirins (Del Soldato et al., 1999). These are mutual prodrugs in which aspirin is connected via an ester group to a nitric oxide releasing moiety such as a nitrate ester. The prototype drug in this class, NCX-4016, exhibits greater gastric tolerability than aspirin in several animal models (Takeuchi et al., 1998; Tashima et al., 2000) and appears to depress platelet aggregation, partially through COX-1 inhibition, but also through nitric oxide-dependent mechanisms (Wallace et al., 1999).

We have recently reported on the hydrolysis and anti-platelet effects of ISMNA, the isosorbide mononitrate ester of aspirin (Gilmer et al., 2001). ISMNA undergoes hydrolysis almost exclusively to aspirin in rabbit plasma and is a more potent inhibitor of arachidonic acid-induced platelet aggregation in rabbit platelet-rich plasma than aspirin. The unusually favourable hydrolysis properties of ISMNA in plasma appear to be due to a structural feature of the isosorbide carrier group that promotes hydrolysis through the productive pathway (liberating aspirin), while simultaneously inhibiting hydrolysis at the critical *O*-acetyl group (leading to the salicylate ester). In the light of this discovery it seemed reasonable to speculate that isosorbide might be useful as a building block in the construction of other aspirin prodrug types for potential use in thrombotic or inflammatory disorders. We report here on the synthesis of the isosorbide aspirin diester (ISDA), its ability to liberate aspirin in human plasma and its hydrolysis by other enzyme types. The stability of the prodrug towards aqueous hydrolysis, a critical feature influencing its potential utility, was also investigated. Two previously reported aspirin esters, Benorylate (4-acetamidophenyl acetylsalicylate or paracetamol aspirinate; Williams et al., 1989) and guaiacol aspirinate (Qu et al., 1990) were also prepared and their hydrolysis characteristics in human plasma compared with ISDA.

2. Materials and methods

2.1. Materials

Acetylsalicyloyl chloride (95%) was purchased from Fluka. Aspirin, paracetamol, guaiacol, salicylic acid, rabbit liver carboxyl esterase [EC 3.1.1.1], α-chymotrypsin [EC 3.4.21.1], human serum albumin, human and horse serum butyrylcholinesterase [EC 3.1.1.8], and eserine (physostigmine), were purchased from Sigma. HPLC grade acetonitrile was purchased from Rathburn. All other reagents and chemicals were of analytical grade.

2.2. Human blood collection

Healthy male and female volunteers were consented as blood donors for plasma hydrolysis studies. None of the donors had taken aspirin or NSAIDs in the previous 7 days. All blood samples were collected by venipuncture into 10 ml Sarstedt Monovettes® containing 1:10 vol of 3.2% trisodium citrate solution. Plasma samples were prepared by centrifugation at 4000 rpm for 10 min and refrigerated at 4 °C until required for testing.

2.3. Chemistry

2.3.1. General procedures

Infra-red spectra were obtained using a Nicolet 205 FT infra-red spectrometer. ^{1}H and ^{13}C NMR spectra were obtained using a Brucker 300 MHz FT NMR spectrometer with tetramethylsilane as internal standard. Elemental analyses were performed at the Department of Chemistry, University College Dublin. 4-Acetamidophenyl acetylsalicylate (paracetamol aspirinate or Benorylate) and guaiacol aspirinate (2-methoxyphenyl acetylsalicylate) were prepared by treating acetylsalicyloyl chloride with paracetamol or guaiacol, respectively. Both compounds were characterised by ^{1}H and ^{13}C NMR.

2.3.2. Synthesis of 2,5-diacetyloxysalicyloxy-1,4:3,6-dianhydro-D-glucitol (isosorbide-2,5-diaspirinate) or ISDA (2)

Isosorbide (2.0 g, 13.7 mmol) was suspended in toluene (50 ml) and the suspension cooled to 0 °C. Triethylamine (5 ml) was introduced followed by acetylsalicoyl chloride (6.3 g, 30 mM, 2.2 eq.). The resulting mixture was allowed to warm to 25 °C. After 24 h the mixture was washed with 2 M HCl and saturated aqueous sodium bicarbonate. It was then dried over magnesium sulfate and the solvent removed in vacuo to afford a pale orange oil (5.3 g, 82%). Crystallisation from ethyl acetate/petroleum ether afforded compound **2** as white needles, m.p. 110.5–111.5 °C. IR: 1767, 1727, 1706 cm^{-1}. NMR: δ_H (300 MHz., $CDCl_3$) 2.34 (3H, s, OCOMe), 2.36 (3H, s, OCOMe), 3.94–4.13 (4H, m, IS1-(α+β)H, IS6-(α+β)H), 4.59 (1H, d, *J*4.8, IS3-H), 4.98 (1H, m, IS4-H), 5.35–5.46 (2H, m, IS2-H, IS5-H), 7.07–7.14 (2H, m, ArH-3), 7.27–7.37 (2H, m, ArH-5), 7.53–7.62 (2H, m, ArH-4), 7.95–8.11 (2H, m, ArH-6). δ_C (75.5 MHz., $CDCl_3$) 20.85 (OCOMe), 20.94 (OCOMe), 70.63 (ISC-6), 73.06 (ISC-1), 74.43 (ISC-2), 78.38 (ISC-5), 80.98 (ISC-4), 86.0 (ISC-3), 122.53, 122.65 (ArC-1), 123.77, 123.9 (ArC-3), 126, 126.06 (ArC-5), 131.77, 131. 96 (ArC-4), 134.18, 134.26 (ArC-6), 150.59, 150.68 (ArC-2), 163.5, 163.67 (ArOCOMe), 169.56, 169.66 (ArC(O)OR). C,H requires 61.28%, 4.71%; found 61.46%, 4.72%. FAB^{+} (*m*/*z*) 471.12 (MH^{+}).

2.4. Solubility

The solubility of ISDA was determined in water and in aqueous pH 6.8 buffer. Excess quantities of powdered ISDA were added to screw-capped vials and 5 ml of water or buffer were added. The suspension was vortexed for 5 min and then shaken for 8 h in an incubator maintained at 37 °C. The suspensions were sampled at 1 h intervals into pre-equilibrated syringes and passed through a 0.45 μm membrane filter. The amount of drug in solution was determined by HPLC with external reference standards run on the same day at approximately the expected concentration.

2.5. Hydrolysis experiments

2.5.1. Aqueous buffer kinetics

The hydrolysis of ISDA (**2**) was studied in the pH range 1.03–9.4. A 2 ml aliquot of a 1 mM solution of **2** in acetonitrile was diluted to 10 ml using phosphate, formate or acetate buffers or HCl as appropriate. The buffers were in the concentration range 10–16 mM. A constant ionic strength (μ) of 0.1 was maintained by the addition of an appropriate quantity of NaCl to the solutions. Buffer solutions containing the esters (0.2 mM) were maintained at 37±0.5 °C in screw-capped vials in a water bath and samples were withdrawn at appropriate time intervals. Analyses were performed in triplicate using reverse-phase HPLC.

2.5.2. Plasma hydrolysis studies

Pooled plasma solutions (4 ml) were prepared by centrifugation of citrated human venous blood and dilution of the resultant plasma supernatant with pH 7.4 phosphate buffer as appropriate. A 100 μl aliquot of the test compound (1×10^{-4} M) in acetonitrile was incubated in the preheated solution (37±0.5 °C) and 250 μl aliquots withdrawn at appropriate intervals. Samples were transferred to 1.5 ml Eppendorf tubes containing 500 μl of 2% $ZnSO_4\cdot7H_2O$ in MeCN–H_2O (1:1) solution, vortexed and then centrifuged for 3 min at 10,000 rpm. A 20 μl aliquot of the clear supernatant was analysed by HPLC. The hydrolysis experiment was also performed in the presence of eserine (physostigmine) to confirm the role of esterases in the hydrolysis of ISDA. Eserine (3 μM) was incubated in the buffered plasma solution for 5 min before addition of the ester stock solution. The samples were then processed as above. The cholinesterase activity of all plasma samples was evaluated using a modification of the Ellman approach with butyryl thiocholine as substrate (Chatonnet and Lockridge, 1989). Values were typically between 2200 and 4000 nmol/ml plasma/min.

2.5.3. Enzyme study

Compound **2** (2×10^{-4} M) was incubated at 37 °C in pH 7.4 phosphate buffer containing 10 μg/ml α-chymotrypsin from bovine pancreas [EC 3.4.21.1]. Aliquots were removed at intervals, quenched as described in Section 2.5.2 and analysed by HPLC. The enzyme activity was determined using *N*-acetyl-L-tyrosine ethyl ester as substrate with HPLC as described below to monitor substrate disappearance. The hydrolysis of **2** was evaluated in the presence of human serum butyrylcholinesterase [EC 3.1.1.8] at a concentration of 0.08 mg/ml (9 units/mg protein) in phosphate buffer (pH 7.4) at 37 °C. The hydrolysis was also evaluated in the presence of horse serum butyrylcholinesterase [EC 3.1.1.8] at a concentration of 0.1 mg/ml and at 10 μg/ml (1000 units/mg protein) in phosphate buffer (pH 7.4) at 37 °C. The activity of this preparation was confirmed using a modification of the Ellman assay with butyrylthiocholine as substrate as described in Section 2.5.2. The hydrolysis was evaluated in human serum albumin solution (10 μg/ml) in phosphate buffer (pH 7.4) and in the presence of carboxyl esterase [EC 3.1.1.1] from rabbit liver (0.22 mg/ml).

2.6. Chromatography

High-performance liquid chromatography was performed using a system consisting of a Waters 600 pump and controller, Waters 717 autosampler and a Waters 996 photodiode-array (PDA) detector controlled by Millennium Chromatography Manager. A Waters Nova-Pak® C_8 (4 μm) column 3.9×150 mm was used for the aqueous hydrolysis study samples, whereas a Waters ODS2 4.6×250 mm column was used for the plasma and enzyme study samples. The aqueous kinetics study samples were analysed using an isocratic mobile phase consisting of aqueous 16 mM phosphate buffer 60% (pH 2.4)–acetonitrile 40%. The capacity factors for each analyte in this system were: aspirin 0.5, salicylic acid 1.4, **2** 5.23. The flow rate was 1 ml/min. The enzyme and plasma study samples were analysed using a gradient method employing a mobile phase consisting of 16.2 mM phosphate buffer (pH3.2)–acetonitrile 90:10 grading to 10:90 over the first 10 min then to 65:35 to 12 min and then back to 90:10 to 17 min, at which it was held until the end of the 30 min run. The retention times in this system were: salicylic acid 10.5 min, aspirin 11.5 min and ISDA (**2**) 15.3 min. The eluent in both methods was monitored at 230 nm and peak identity and homogeneity confirmed by photodiode-array analysis. Quantitation was performed by comparison of peak areas with external standards run under the same conditions at about the same concentration. Both methods (gradient and isocratic) were validated for linearity, precision (repeatability), specificity, and sensitivity in accordance with ICH guidelines on analytical validation Q2A and Q2B. A linear response was observed for each analyte (r >0.999) in the range 1–100 μg/ml. The R.S.D. on multiple injections of each analyte at 10 and 100 μg/ml was $<1.5\%$. The limit of quantitation for the relevant analytes in the gradient method was 1 μg/ml. The limit of

quantitation in the isocratic method was 5 μg/ml for aspirin and salicylic acid and 0.5 μg/ml for ISDA.

3. Results and discussion

3.1. Chemistry

ISDA (**2**, Fig. 1) was prepared in good yield by treating a suspension of isosorbide in toluene with two equivalents of acetylsalicoyl chloride in the presence of triethylamine. The product following crystallisation was >99% pure by HPLC and was characterised by NMR, MS and elemental analysis. Two other aspirin esters, 4-acetamidophenyl acetylsalicylate (benorylate) and guaiacol aspirinate (2-methoxyphenyl acetylsalicylate), were prepared by treating paracetamol or guaiacol, respectively, with acetylsalicoyl chloride and triethylamine in toluene. The identity of these compounds was confirmed by ^{1}H and ^{13}C NMR spectroscopy.

3.2. Hydrolysis kinetics

The hydrolysis of ISDA (**2**) was monitored in aqueous solution at 37 °C over the pH range 1.03–9.4. The hydrolysis was observed to follow pseudo-first-order kinetics over several half-lives. The rate of hydrolysis was found to be independent of ionic strength or buffer concentration at pH 2.8, nevertheless a constant ionic strength (μ) of 0.1 was maintained in all solutions. Pseudo-first-order plots for the decomposition of **2** were constructed from the logarithm of remaining ester versus time. The pseudo-first-order rate constants (k_{obs}) appear in Table 1 and the pH rate profile is presented in Fig. 2. The U-shaped pH rate profile indicates the occurrence of specific acid-catalysed (k_H), water catalysed (k_0) or specific base-catalysed (k_{OH}) processes and the overall profile can consequently be accounted for by the expression

$$k_{obs} = k_0 + k_H a_H + k_{OH} a_{OH} \quad (1)$$

where a_H and a_{OH} represent the hydrogen ion and hydroxide ion activity. The hydroxide ion activity at 37 °C

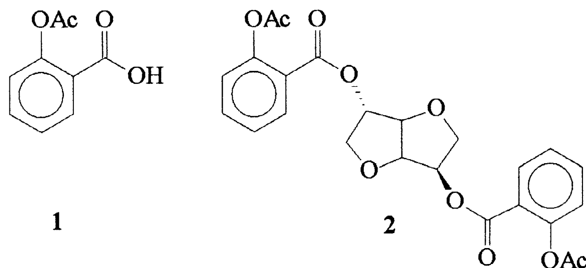

Fig. 1. Chemical structures of aspirin (**1**) and isosorbide diaspirinate (ISDA) (**2**).

Table 1
Pseudo-first-order rate constants and corresponding half-lives for the hydrolysis of **2** in aqueous solution at 37 °C and $\mu = 0.1$

pH	k_{obs} (h^{-1})	$t_{1/2}$ (h)
1.03	48.5×10^{-3}	14.29
1.4	20×10^{-3}	34.65
1.62	11.0×10^{-3}	62.72
1.88	4.8×10^{-3}	144.36
2.24	1.6×10^{-3}	433.13
2.5	2.3×10^{-3}	300.42
4.13	2.3×10^{-4}	3013.04
6.45	6.9×10^{-4}	1004.35
7.29	2.99×10^{-3}	231.77
8.1	15.9×10^{-3}	43.33
9.4	0.29	2.35

was calculated from the expression (Harned and Hamer, 1933)

$$\log a_{OH} = \text{pH} - 13.6 \quad (2)$$

Second-order rate constants for the specific base (k_{OH}) and specific acid (k_H) catalysed reactions were determined from data at high and low pH, respectively. The apparent first-order rate constant for spontaneous decomposition (k_0) was determined from the small plateau region of the pH rate profile at minimum rate of decomposition. The values for the rate constants at 37 °C are

$$k_H = 0.452\ M^{-1}\ h^{-1}$$

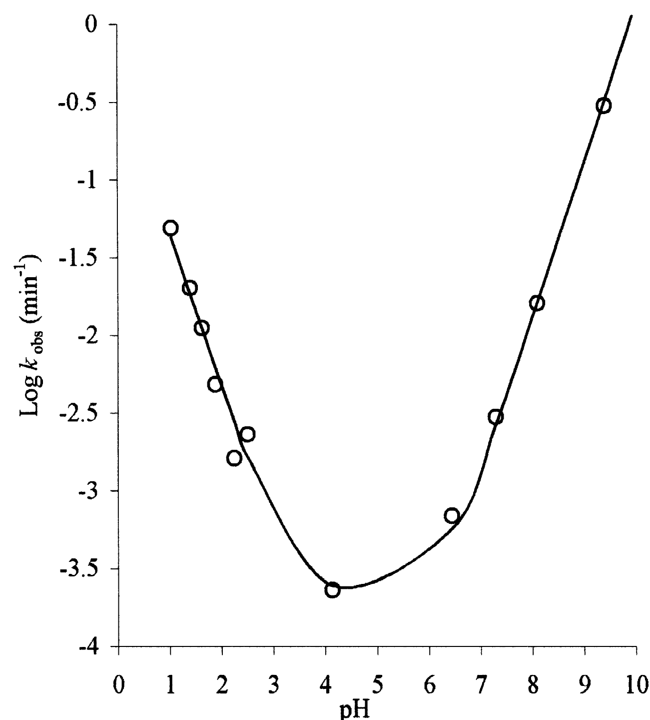

Fig. 2. The pH rate profile for the hydrolysis of **2** in aqueous solution ($\mu = 0.1$) at 37 °C. The points represent log of the pseudo-first-order rate constants determined at each pH from the log of remaining ester versus time, whereas the solid line was constructed using Eq. (1) and the rate constants appearing in the text.

$k_0 = 0.00021\ h^{-1}$

$k_{OH} = 5296\ M^{-1}\ h^{-1}$

The solid line appearing in Fig. 2 was constructed from Eq. (1) using these rate constants.

The half-lives at each pH appearing in Table 1 were calculated using

$$t_{1/2} = 0.693/(k_{obs}) \quad (3)$$

ISDA (**2**) exhibited high aqueous stability at all pH values, but was most stable in the pH range 4–5.5 with optimal measured stability at pH 4.13, at which the half-life was 3013 h (~4 months). At stomach pH (~1.4) the half-life was 62 h, indicating that the ester might be sufficiently stable at low pH to pass through the stomach intact. The high aqueous stability of **2** may be due to steric inhibition by the rather bulky alcohol portion of the molecule. In contrast, aspirin hydrolysis in aqueous solution is autocatalysed by the carboxylate and aspirin has therefore low aqueous stability (St Pierre and Jencks, 1968). Hydrolysis of **2** in aqueous solution proceeded along the k_2 pathway as depicted in Fig. 3 at all pH values with no observable aspirin formation. Instead, a complex mixture of salicylate esters was formed as indicated by their PDA UV spectra (λ_{max} = 295–306 nm).

3.3. Enzyme hydrolysis kinetics

The hydrolysis of ester **2** in phosphate buffered (pH 7.4) human plasma solutions was examined using HPLC with PDA detection (Fig. 4). Rapid hydrolysis was observed in the presence of 10, 30 or 50% buffered human plasma solution. Apparent Michaelis parameters $K_{m,app}$ (Michaelis constant) and $V_{max,app}$ (maximum rate of substrate consumption) for the hydrolysis of **2** were estimated by fitting depletion data to the integrated form of the Michaelis–Menten equation (Eq. (4)) as described by Robinson and Characklis (1984):

$$V_{max}t = So - S + K_m \ln(So/S) \quad (4)$$

In 10% buffered human plasma the $K_{m,app}$ value was 2.33×10^{-4} M and the $V_{max,app}$ value was 4×10^{-5} M/min. The half-lives for the hydrolysis of **2** in plasma presented in Table 2 were calculated from

$$t_{1/2} = 0.693/(V_{max}/K_m) \quad (5)$$

Half-lives calculated in this way showed excellent agreement with values calculated using Eq. (3) and first-order rate constants obtained from the slopes of rectilinear plots such as those appearing in Fig. 6.

Hydrolysis was suppressed when **2** was co-incubated with 3 μM eserine (physostigmine), indicating that hydrolysis of **2** in human plasma is mediated by serine esterases, probably plasma pseudocholinesterase otherwise known as butyrylcholinesterase [EC 3.1.1.8], as this esterase is the most abundant in human plasma and demonstrates broadest specificity. The apparent K_m value for the plasma catalysed hydrolysis of ISDA in the presence of eserine was increased to 6×10^{-4} M.

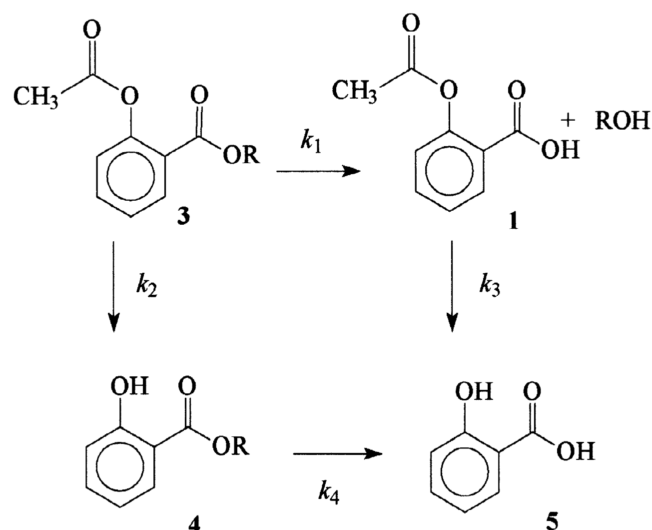

Fig. 3. Scheme showing sequential four-component closed system kinetics for the hydrolysis of aspirin esters (e.g. **3**). In plasma and buffer the k_2 pathway to salicylic acid (**5**) via the salicylate ester (**4**) is usually preferred. ISDA (**2**) is hydrolysed via the favourable k_1 pathway and the k_2 pathway in parallel. Benorylate and guiacol aspirinate undergo hydrolysis exclusively through the k_2 pathway liberating the corresponding salicylate ester **4**.

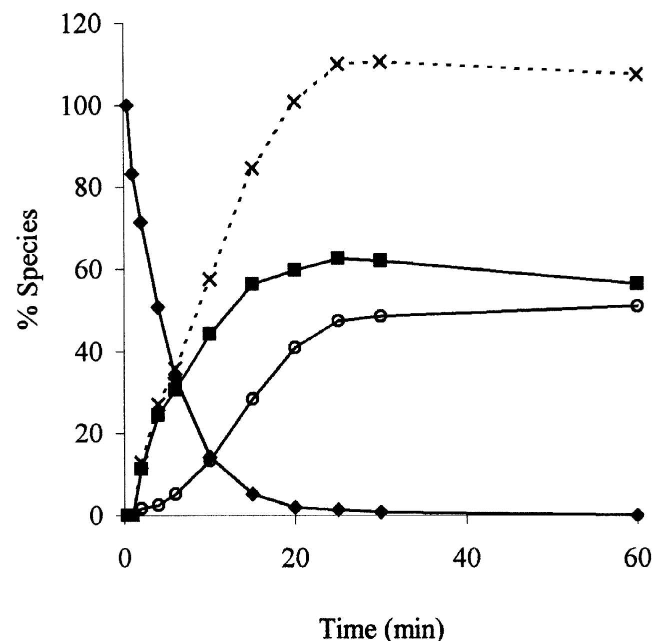

Fig. 4. Plot showing the time course for **2** (♦) in 10% buffered human plasma (pH 7.4) at 37 °C and its hydrolysis products: aspirin (■), salicylic acid (○). The sum of aspirin and salicylic acid is also plotted (×). Other unidentified salicylate metabolites were also formed during this process but were not quantitated.

Table 2
Kinetic data for **2**, benorylate, guaiacol aspirinate in the presence of various enzyme preparations at pH 7.4 and 37 °C

Enzyme solution	k_{obs} (min^{-1})	$t_{1/2}$ (min)	% Aspirin
α-Chymotrypsin (0.1 mg/10 ml)	0.00345	200.87	0
10% Human plasma (**2**) ($n=6$)	0.147	4.7	50.7
Benorylate	1.02	0.7	<0.5
Guaiacol aspirinate	0.77	0.9	<0.5
30% Human plasma	0.626	1.10	61
Human serum BuChE (0.08 mg/ml)	0.074	9.4	55
Human serum albumin (10 μg/ml)	1.15×10^{-3}	603	0
Carboxyl esterase (0.22 mg/ml)	0.022	31.5	0
Horse serum BuChE (0.1 mg/ml)	0.3750	1.85	11
Horse serum BuChE (0.01 mg/ml)	0.0502	13.8	7
10% Human plasma+eserine (3 μM)	0.0235	29.5	0

Of critical importance to the success of an aspirin ester as a potentially useful human prodrug is that it liberates aspirin in human plasma but not in the presence of conditions prevailing before or during the absorption process. Esterification of the aspirin carboxylic acid group dramatically increases the rate of hydrolysis at the *O*-acetyl group such that hydrolysis at the carrier ester does not successfully compete with the acetyl group hydrolysis (Fig. 3: $k_2 \gg k_1$). Aspirin esters do not in general undergo hydrolysis to aspirin in human plasma and most if not all aspirin esters reported thus far should therefore be more appropriately termed salicylic acid prodrugs (Nielsen and Bungaard, 1989). Aspirin is a poor substrate for human plasma butyrylcholinesterase and its half-life in human plasma is about 120 min (Cham et al., 1979), considerably longer than the half-life of related aspirin esters (Nielsen and Bungaard, 1989). The high stability of aspirin towards human plasma catalysed hydrolysis relative to neutral substrates (such as aspirin esters) appears to be due to electrostatic inhibition of Michaelis complex formation rather than substrate repulsion (Masson et al., 1998).

ISDA (**2**) underwent rapid hydrolysis in human plasma solution generating appreciable quantities of aspirin and a complex mixture of aspirinate and salicylate esters which were identified by their characteristic PDA-UV λ_{max} values of 274–278 and 295–305 nm, respectively (Fig. 5). Aspirin and salicylic acid in vitro metabolites were quantitated by external standard and peak homogeneity for all chromatographic components confirmed by PDA analysis. In order to minimize the possibility of coincidental overlap of PDA indistinguishable components, the 10% plasma study samples were analysed using both the gradient and the isocratic HPLC methods. The amounts of aspirin present in the plasma samples were similar using both methods. Hydrolysis in pH 7.4 phosphate-buffered human plasma solutions (10–50%) was associated with the generation of 40–60% aspirin based on the initial molar quantity of **2**, with a mean value of 51% in 10% human plasma ($n=6$). This places the diester **2** among the most successful aspirin prodrugs reported to date and confirms that a feature of the isosorbide group confers extremely rapid hydrolysis on its 2-esters in mammalian plasma. In order to validate this experiment, two putative aspirin prodrugs, Benorylate (paracetamol aspirinate), and Guaiacol aspirinate, were incubated in pH 7.4 buffered human plasma (10%) at 37 °C. Both esters were rapidly hydrolysed with half-lives of 40 and 54 s, respectively. Hydrolysis of these two esters occurred almost exclusively along the k_2 pathway, as depicted in Fig. 3, with the

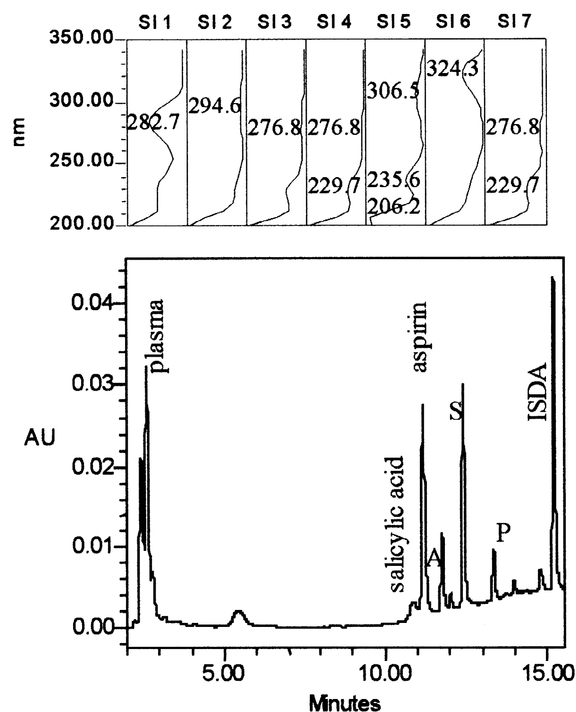

Fig. 5. Chromatogram (230 nm) of a sample obtained following incubation of ISDA in 30% human plasma buffered at pH 7.4 (37 °C) for 3 min. Also shown are PDA spectra of each of the labelled components. Peaks labelled 'plasma' or 'P' were also present in the plasma blank. Peaks labelled 'S' or 'A' have PDA spectra of salicylate esters or aspirinate esters, respectively, and are likely to be in vitro metabolites of ISDA and potential precursors of aspirin or salicylic acid.

formation of negligible quantities of aspirin (<0.5% based on initial ester concentration). The hydrolysis characteristics of Benorylate have been reported previously (Williams et al., 1989; Nielsen and Bungaard, 1989) and results here are in good agreement in terms of both pathway and rate. The comparatively slow hydrolysis of ISDA in 10% buffered human plasma ($t_{1/2}=4.1$ min) relative to these two esters illustrates that the isosorbide group promotes aspirin release by suppressing acetyl group hydrolysis in addition to accelerating hydrolysis at the carrier ester group.

In order to confirm the role of butyrylcholinesterase in the hydrolysis of **2** in human plasma, it was incubated in purified human serum butyrylcholinesterase [EC 3.1.1.8] buffered at pH 7.4. Hydrolysis was associated with the evolution of 55% aspirin based on the initial molar concentration of ISDA. This ratio of k_1 hydrolysis to k_2 hydrolysis (Fig. 3) is similar to that observed during the hydrolysis of **2** in human plasma, indicating that serum butyrylcholinesterase is the principal enzyme hydrolysing **2** in human plasma. Hydrolysis was also examined in the presence of purified horse serum butyrylcholinesterase. This enzyme has high homology with human butyrylcholinesterase, similar catalytic efficiency towards butyrylcholine hydrolysis, and similar substrate specificity. Hydrolysis of **2** in horse serum butyrylcholinesterase occurred through a mixture of the k_1 and k_2 pathways (Fig. 3) generating a complex mixture of products including 11% aspirin based on the initial concentration of the ester. There are three active site exchanges between human plasma butyrylcholinesterase and horse plasma butyrylcholinesterase, which may explain the poorer specificity of the latter towards the k_1 pathway and consequently the lower amount of aspirin produced in this experiment relative to the human plasma experiment. The hydrolysis of **2** was also studied in the presence of carboxylesterase [EC 3.1.1.1] from rabbit liver and in the presence of human serum albumin (HSA), a protein that exhibits esterase-like activity towards some substrates, including aspirin (Williams, 1985), although it has been shown recently that this esterase-like activity may not be intrinsic to HSA (Chapuis et al., 2001). Hydrolysis in the presence of carboxylesterase or HSA was slow relative to diluted human plasma and buffered butyrylcholinesterase solutions (Table 2), with no aspirin liberation, indicating that neither of these proteins has a prominent role in the human plasma-catalysed hydrolysis of **2**.

The potential utility of **2** as an aspirin human prodrug suitable for peroral administration was evaluated by estimating its solubility at several pH values and stability towards hydrolysis by α-chymotrypsin. The aqueous solubility of **2** was rather poor (H_2O, 10 μg/ml; pH 6.8, 10.9 μg/ml), however its stability towards hydrolysis by α-chymotrypsin was high ($t_{1/2}=203$ min), indicating that **2** might survive the absorption process intact and then undergo rapid hydrolysis in plasma, liberating aspirin. The relative stability of **2** in human plasma versus α-chymotrypsin solution is depicted in Fig. 6, showing pseudo-first-order curves for hydrolysis in α-chymotrypsin and in several diluted human plasma solutions.

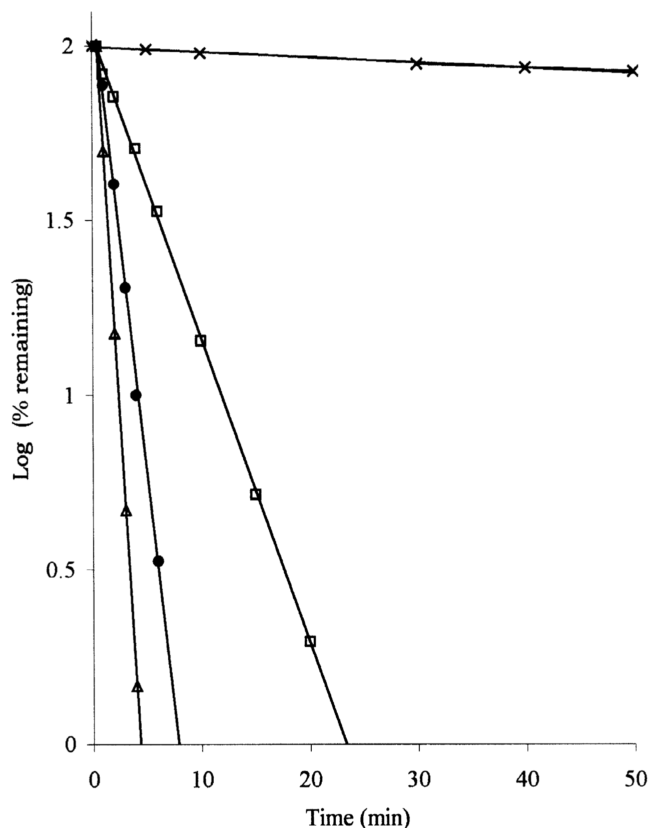

Fig. 6. Plot showing the pseudo-first-order curves for the hydrolysis of **2** in diluted human plasma at pH 7.4 and 37 °C: 10% plasma (□); 30% plasma (●); 50% plasma (△). A pseudo-first-order curve for the degradation of **2** in phosphate buffered (pH 7.4) α-chymotrypsin solution (0.1 mg/ml) at 37 °C is also shown (×).

4. Conclusions

Isosorbide diaspirinate, the aspirin diester of isosorbide, is stable towards aqueous hydrolysis and in the presence of α-chymotrypsin. However, it undergoes rapid hydrolysis in the presence of human plasma solution, liberating significant amounts of aspirin. Besides the isosorbide mononitrate ester of aspirin which we recently reported (Gilmer et al., 2001) and the diaspirinate ester ISDA (**2**) reported herein, only the glycolamide esters of Nielsen and Bungaard (1989) may be regarded as true aspirin esters. The present study confirms that the isosorbide group may be used to construct esters that undergo rapid hydrolysis in human plasma. We are currently designing a new generation of aspirin prodrugs exploiting the unique characteristics of this novel carrier molecule but with enhanced aqueous solubility.

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