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# Studies in the synthesis and *in vitro* hydrolysis of novel aspirin prodrugs

Louise Moriarty, B. Sc. (App. Chem. & Q. Mgt.)

A thesis presented to the University of Dublin
for the degree of
Doctor of Philosophy in Pharmaceutical Chemistry

Based on research carried out under the supervision of
John Gilmer
B.A. (Mod.), Ph.D
at the
Department of Pharmaceutical Chemistry,
School of Pharmacy,
Trinity College Dublin.

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# Abstract

Prolonged aspirin use has been associated with an increased risk of gastrointestinal bleeding, leading in some instances to haemorrhage and death. This thesis describes the design of aspirin prodrugs, from which aspirin might be regenerated after passage through the GI tract, thereby improving the side-effect profile of the agent.

Numerous strategies investigated for the formation of true aspirin prodrugs are reviewed in Chapter 1. Of these attempts, only some of the glycolamides reported by Bundgaard et al. can be considered successful, since these compounds combine good aqueous stability with the ability to liberate aspirin (50%) in human plasma.

Chapter 2 describes the design of a nitric oxide-releasing aspirin prodrug - isosorbide mononitrate aspirinate (ISMNA) - by integrating aspirin with isosorbide-5-mononitrate (IS-5-MN), which is a clinically successful long-acting nitrate. ISMNA is an effective anti-platelet agent in human, dog and rabbit. ISMNA liberated up to 78.7% aspirin in rabbit plasma.

Isosorbide diaspirinate (ISDA) is an also effective anti-platelet agent in dog, rabbit and human. Chapter 3 describes the investigation of the potential of ISDA for use as an aspirin prodrug. ISDA was discovered to be an effective aspirin prodrug in human plasma. From this work we identified an *in vitro* metabolite of ISDA, which contributes to its aspirin release characteristics. This *in vitro* metabolite (isosorbide-2-aspirinate-5-salicylate) is converted to 98% aspirin by human plasma butyrylcholinesterase, making this compound the most effective aspirin prodrug reported to date.

Based on the remarkable discovery of isosorbide-2-aspirinate-5-salicylate as an aspirin prodrug in Chapter 3, the investigation continued with the design of a new generation of aspirin prodrugs. This is described in Chapter 4 where a study of the structure-activity relationship (SAR) of isosorbide-based aspirin esters, which can assist in their design, is also presented. Other approaches to nitric oxide-releasing aspirin prodrugs are described in Chapter 5, based on existing aspirin esters.

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# Abbreviations

| | |
|---|---|
| **AA** | arachidonic acid |
| **AChE** | acetylcholinesterase |
| **ADP** | adenosine diphosphate |
| **AsCl** | acetylsalicyloyl chloride |
| **Asp** | aspirin |
| **Bn** | benzoyl |
| **BNPP** | bis-4-nitrophenylphosphate |
| **BuChE** | butyrylcholinesterase |
| **BW284C51** | 1:5-bis(4-allyl-dimethyl) ammoniumphenyl-pentan-3-one |
| **cGMP** | cyclic guanine monophosphate |
| **ChE** | cholinesterase |
| **CNS** | central nervous system |
| **COX** | cyclooxygenase |
| **CPA** | carboxypeptidase A |
| **CT** | α-chymotrypsin |
| **DBU** | 1,8-diazabicyclo-undec-7-ene |
| **DCC** | dicyclohexylcarbodiimide |
| **DCM** | dichloromethane |
| **DFP** | diisopropylfluorophosphate |
| **DMAP** | dimethyl aminopyridine |
| **DTNB** | 5,5-dithiobis-(2-nitrobenzoic acid) |
| **EC** | International Enzyme Commission Classification |
| **EDTA** | ethylenediamine tetraacetic acid |
| **ES** | enzyme-substrate |
| **$Et_3N$** | triethylamine |
| **GI** | gastro-intestinal |
| **GMP** | guanine monophosphate |
| **GTN** | glyceryl trinitrate |
| **HPLC** | High Performance Liquid Chromatography |
| **HSA** | human serum albumin |
| **I** | ionic strength |
| **IEC** | International Enzyme Commission |
| **IL** | interlukin |
| **iNOS** | inducible nitric oxide synthase |
| **IR** | Infra-Red |
| **IS** | isosorbide |
| **ISDA** | isosorbide diaspirinate |
| **ISDN** | isosorbide dinitrate |
| **ISMNA** | isosorbide mononitrate aspirinate |
| **IS-5-MN** | isosorbide-5-mononitrate |
| **iso-OMPA** | tetraisopropylpyrophosphoramide |
| **$k^1$** | capacity factor |
| **$k_{obs}$** | pseudo first-order rate constant |
| **LRMS** | low resolution Mass Spectrophotometery |
| **MDA** | malondialdehyde |
| **MI** | myocardial infarction |
| **mg** | milligramme |
| **ml** | millilitre |
| **mm** | millimetre |
| **MS** | Mass Spectrophotometry |
| **NCX** | NicOx |
| **nM** | nanomolar |

| | |
|---|---|
| **NMR** | Nuclear Magnetic Resonance |
| **NO** | nitric oxide |
| **P** | partition coefficient |
| **OA** | osteo-arthritis |
| **PDA** | photodiode array |
| **PDB** | protein database format |
| **PFP** | pentafluorophenol |
| **PG** | prostaglandin |
| **$PGD_2$** | prostaglandin $D_2$ |
| **$PGE_2$** | prostaglandin $E_2$ |
| **$PGG_2$** | prostaglandin $G_2$ |
| **$PGH_2$** | prostaglandin $H_2$ |
| **PGHS** | prostaglandin endoperoxide synthase |
| **$PGI_2$** | prostacyclin |
| **PRP** | platelet rich plasma |
| **RA** | rheumatoid arthritis |
| **RBC** | red blood corpuscle |
| **RSD** | relative standard deviation |
| **RT** | room temperature |
| **Sal** | salicylic acid |
| **SAR** | structure activity relationship |
| **s.d.** | standard deviation |
| **SMILES** | Simplified Molecular Input Line Information System |
| **TEPP** | tetrakisethoxy dehydroxypyrophosphate |
| **THF** | tetrahydrofuran |
| **TLC** | Thin Layer Chromatography |
| **TNB** | 5-thio-2-nitrobenzoic acid |
| **$TXA_2$** | thromboxane $A_2$ |
| **$t_{1/2}$** | half-life |
| **UV** | Ultra-Violet |
| **µg** | microgramme |
| **µM** | micromolar |

# Chapter 1

## Overview to aspirin and potential aspirin prodrugs

## 1.1 Introduction

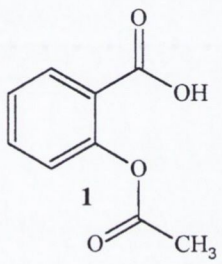
Aspirin (**1**) has become one of the most heavily consumed of the 'over the counter' drugs, since its development over 100 years ago. In the United States alone, approximately 20 to 30 billion tablets containing aspirin are purchased annually [1, 2]. It is well established that aspirin is a highly effective anti-inflammatory, analgesic and anti-pyretic agent. More recently, aspirin has become an important agent in the fight against heart disease, stroke and thrombosis, not only in those patients with an established condition but also in primary prevention [3]. The widespread use of aspirin has been predicted to increase even further, since there is new evidence to suggest this agent is associated with a reduction in the risk of colorectal cancer and a protective effect against Alzheimer's disease and other forms of dementia [4,5]. However, prolonged aspirin use has been associated with a number of adverse effects, mostly affecting the upper gastrointestinal tract and the kidney [6]. For example, it has been reported that 10% of patients on low dose aspirin (10 mg daily) for 12 weeks developed endoscopic gastric ulcers [7]. The problem of aspirin-induced gastrointestinal (GI) damage has been the focus of intense pharmaceutical attention for many years however, conventional solutions such as enteric coating have been found to be inadequate. One intensively investigated strategy has been the development of aspirin prodrugs, from which the agent is regenerated after passage through the GI tract. The major problem in the development of aspirin prodrugs is that hydrolysis in plasma tends to occur *via* a pathway, which liberates salicylic acid and not aspirin.

This chapter gives a brief overview of aspirin, including its history, pharmacokinetics, mechanism of action, therapeutic and non-beneficial effects. The latter section of this chapter contains a review of previously reported strategies directed at the development of true aspirin prodrugs. The objective of this thesis was to investigate the development of novel aspirin prodrugs, particularly aspirin prodrugs bearing a nitric oxide releasing moiety.

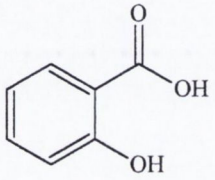
## 1.2 Aspirin overview

### 1.2.1 History of aspirin

Aspirin (acetylsalicylic acid) (**1**) is derived from salicylic acid (2-hydroxybenzoic acid) (**2**), whose medicinal properties originate in the traditional treatments that used plants (e.g. willow bark, poplar and beech leaves) in which salicylates occur naturally [8]. The Assyrians used the extract of willow leaves for painful muscloskeletal conditions, while the Egyptians employed myrtle and willow leaves for joint pain and the relief of pain and inflammation [9]. Hippocrates used the juices of the poplar tree for treating eye diseases and those of the willow bark for pain in childbirth and for fever [10].




**1**




**2**

The Reverend Edmund Stone made the first recorded description of the use of willow bark as an anti-inflammatory agent in 1763. He wrote of the successful treatment of patients with fever, using about 1 g of powdered willow bark, taken in a dram of water every four hours. In 1828, Johann Buchner, Professor of Pharmacy at Munich, successfully extracted salicin from willow bark. The following year, Henri Leroux obtained crystalline salicin. Salicylic acid was first derived from salicin by the Italian chemist Raffaelle Piria; its chemical structure was elucidated in Marburg, Germany, by Hermanne Kolbe, who synthesised salicylic acid in 1859 [8]. By 1874 industrial production was well under way [9]. This led to the liberal use of pure salicylic acid, which reduced fever immediately and dramatically relieved pain associated with rheumatism, neuralgia and headaches. The first clinical trial was carried out by Dr. Thomas MacLangan, a Scottish physician, who in 1876 self-administered 2 g of salicin. Subsequently he used it on patients with acute rheumatism, resulting in complete relief of fever and joint inflammation.

However, its sharp bitter taste and side effects of gastric irritation were problematic for chronic users. Felix Hoffman, a chemist with Bayer and Company, Germany, developed a new form of salicylic acid to overcome his father's difficulty with taking this medicine to relieve symptoms of rheumatism. On August 10th, 1897, Hoffman synthesised a new substance, through the acetylation of salicylic acid. The resulting product was named acetylsalicylic acid. The next step, in the development of acetylsalicylic acid was a pharmaceutical trial of this remedy. Heinrich Dreser, a pharmacist at Bayer, by testing the new substance on animals and on himself, showed it to have analgesic, anti-pyretic and anti-inflammatory properties [11]. On 23rd January 1899, this substance was given the brand name of 'Aspirin' (**1**). The name contains the root of spiric acid from *Spirea Ulmera*, chemically identical to salicylic acid, together with 'A' as an abbreviation for acetyl [10]. It was officially entered on the trademark list as number 36433 of the Imperial Patent Office in Berlin on 6th March 1899 [9]. In the first decade following its launch, a variety of publications appeared recommending aspirin for the treatment of rheumatism of joints and muscles, and for other conditions where pain was mainly caused by inflammation.

Today, aspirin is most commonly used in the treatment of heart disease, arthritis and headache. Since its launch, several other drugs were discovered which shared some or all of these effects [12]. Amongst these were acetaminophen (paracetamol) and indomethacin. Because of the similarity of their therapeutic actions, these drugs became collectively known as the non-steroidal anti-inflammatory drugs (NSAIDs). Despite the diversity of chemical structure of these drugs, they possess similiar therapeutic properties and side-effects [13].

### 1.2.2 Pharmacokinetics of aspirin

#### 1.2.2.1 Absorption

The major systemic transport pathway after gut absorption for most orally administered drugs is *via* the hepatic portal vein, since it has a high capacity to transport both water soluble and poorly water soluble compounds [14]. Aspirin and salicylic acid are rapidly and extensively absorbed by a first-order process following oral administration. The absorption half-life of aspirin is less than that of salicylic acid and the differences in the rate and extent of absorption are influenced by factors such as pH, pre-absorptive hydrolysis and first-pass effects [10]. Aspirin and salicylic acid are both weak acids with pKa values of 3.6 and 3.0 respectively. Consequently, both demonstrate a pH dependence on absorption, being most poorly absorbed from alkaline conditions [15]. The passive absorption of aspirin occurs in both the stomach and small intestine, with greater absorption of aspirin and salicylic acid from the duodenum than from the stomach. The intestinal absorption of both compounds is dependent upon the rate at which the stomach empties [16].

#### 1.2.2.2 Distribution

Aspirin and salicylic acid are extensively distributed throughout the body fluids following oral administration. Salicylates are distributed across membranes primarily by passive diffusion and can be detected in most body tissues and fluids. Since salicylic acid in plasma is largely ionised, only a small amount crosses the blood-brain barrier [17]. The binding of aspirin and salicylic acid to serum proteins is an important factor in their distribution. In the normal therapeutic concentration range the protein binding of salicylic acid is particularly high (80-90%). The binding of aspirin to plasma proteins is more complex and is possibly due to the ability of aspirin to acetylate various proteins, including glycoproteins and lipids in the stomach, kidney, liver and bone marrow, as well as haemoglobin [10].

### 1.2.2.3 Metabolism

Aspirin undergoes rapid hydrolysis to salicylic acid *in vivo*. The pre-absorptive hydrolysis of aspirin is most likely due to enzymatic hydrolysis, which can occur in the lumen, or which may be associated with brush-border esterase activity [10]. Following oral administration, aspirin undergoes pre-systemic metabolism in the liver and gut. Aspirin reaching the systemic circulation is hydrolysed in plasma and red blood cells. The enzymes capable of hydrolysing aspirin in plasma and other tissues are generally referred to as 'aspirin esterases', since they catalyse the hydrolysis of the acetyl ester moiety of aspirin to liberate salicylic acid and free acetate [18]. Cholinesterase accounts for up to 90% of the enzymatic degradation of aspirin in serum [19]. Numerous studies, including disc electrophoresis experiments [20] and enzyme inhibition studies [21], have identified the primary enzyme in plasma responsible for the hydrolysis of aspirin as butyrylcholinesterase (EC 3.1.1.8, also known as plasma cholinesterase or pseudocholinesterase).

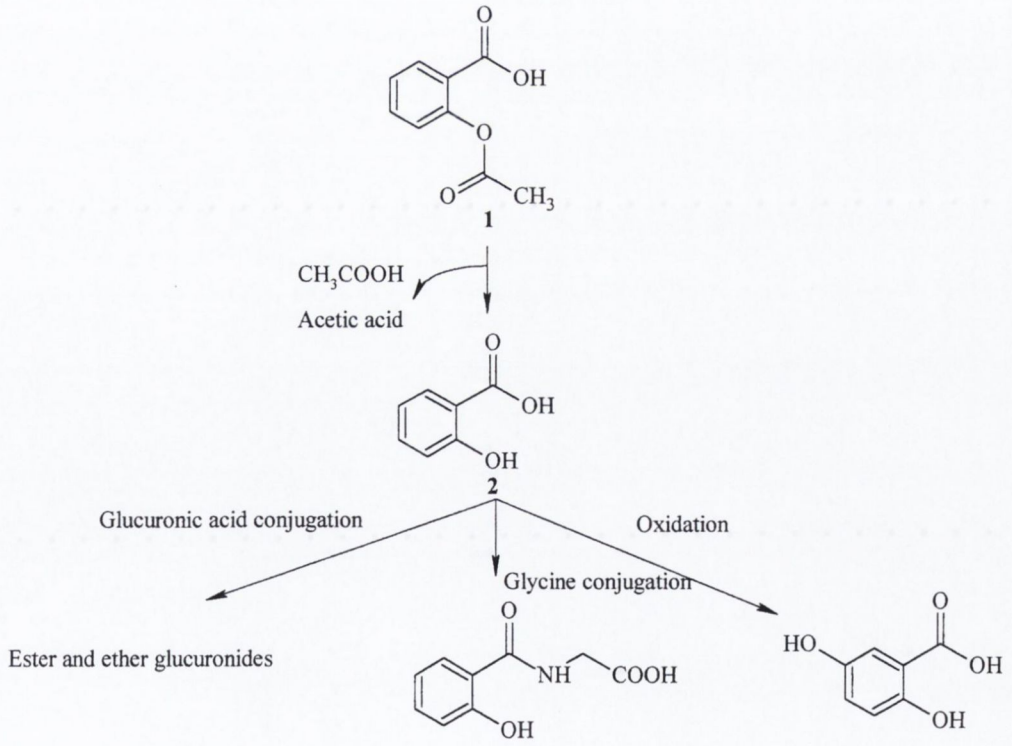



*Figure 1.1 The metabolic pathways of aspirin and salicylic acid [10].*

The major *in vivo* metabolic pathways for aspirin are depicted in *Fig. 1.1.* Aspirin is rapidly hydrolysed to salicylic acid with a half-life of 15 to 20 minutes in plasma, whereas salicylic acid can be detected in the blood for several hours [22]. The rate of conversion of aspirin to salicylate may be important since aspirin has different properties to salicylate. There may be inter-subject variability in the relative contribution of the five metabolic pathways to the metabolism of salicylic acid [10].

Salicylic acid is also liberated from aspirin through the acetylation of cyclooxygenase-1 (COX-1) in blood cells such as platelets and leukocytes, which are exposed to aspirin in the pre-systemic portal circulation. This is one of the means by which aspirin achieves its anti-platelet effect. Although salicylic acid can be detected in the blood for several hours after aspirin ingestion, it does not inhibit platelet COX-1.

#### 1.2.2.4 Elimination

The elimination half-lives of aspirin and salicylic acid range from 14 to 19 minutes and 230 to 300 minutes respectively. It is possible to monitor blood levels of salicylates or urinary metabolites to obtain a measure of aspirin ingestion and metabolism. The renal clearance of salicylic acid (a first-order process) involves glomerular filtration, active tubular secretion and partial passive tubular back-diffusion. Only the non-ionic form of salicylic acid undergoes tubular re-absorption and therefore, the renal clearance of salicylic acid (pKa of 3.0) is sensitive to the pH of urine [10].

### 1.2.3 Mechanism of action of aspirin

#### 1.2.3.1 Aspirin and inhibition of COX

Before 1971 very little was known about the mechanism of action of aspirin and aspirin-like drugs. In the 1960s Harry Collier, a pharmacologist, had termed aspirin as an 'anti-defensive' drug because of its ability to prevent the physiological defense mechanisms of pain, fever and inflammation from normal function. He wrote that aspirin-like drugs act by 'inhibiting some underlying cellular mechanism that takes part to different extents in different responses mediated by different endogenous substances' [10]. It was following the work of Collier that prostaglandin researchers began to consider its mechanism of action. In 1971, three papers appeared in Nature

demonstrating that aspirin and several other aspirin-like drugs blocked prostaglandin synthesis: in a cell-free system (Vane) [23], in an isolated perfused organ (Ferreira et al.) [24] and in human platelets following oral administration (Smith and Willis) [25]. This research proved significant as it became apparent that prostaglandins were involved in the pathogenesis of inflammation, fever and pain [10].

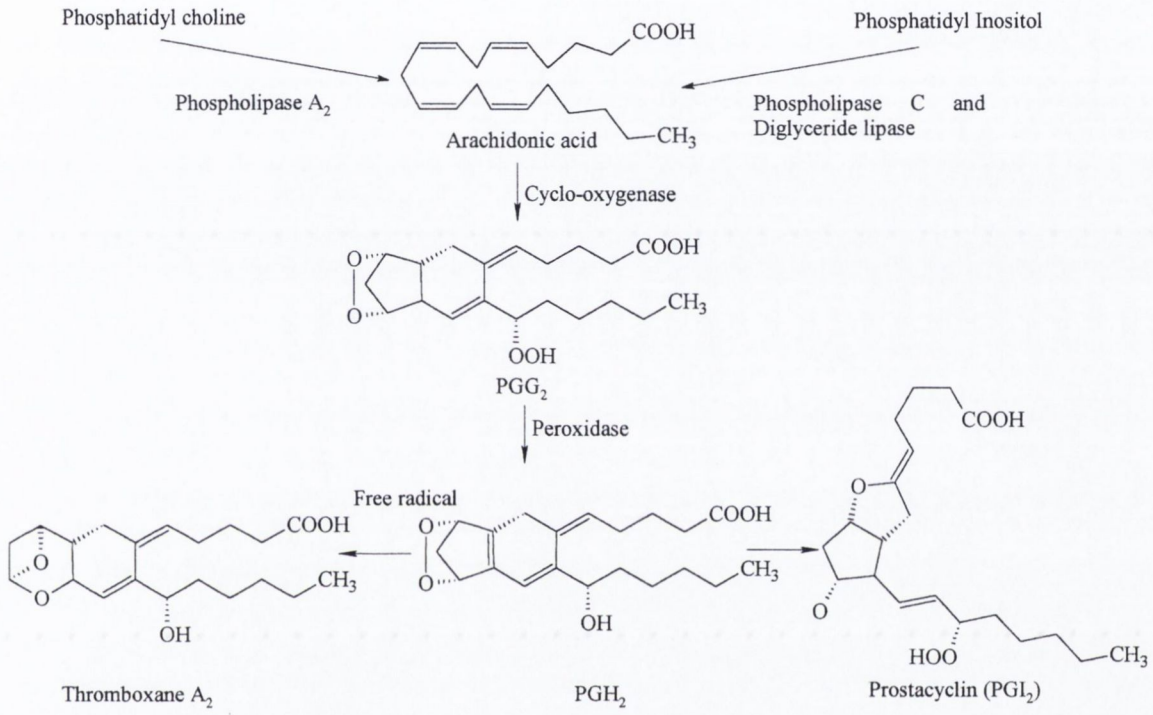



*Figure 1.2 The metabolism of arachidonic acid* [10].

Prostaglandins are a group of pharmacologically active lipid substances, which have a wide variety of physiological and pathophysiological effects [26] and are involved in a number of homeostatic processes [27]. The prostaglandins belong to the family of eicosanoids, which are formed initially by the conversion of arachidonic acid (AA) to an unstable endoperoxide intermediate, $PGH_2$, by the enzyme prostaglandin endoperoxide synthase (PGHS) also known as cyclo-oxygenase (COX) [28]. Arachidonic acid does not exist in free form but rather as an integral part of endogenous cell phospholipids. COX is the rate-limiting enzyme in the conversion of AA to $PGH_2$, which is the starting material for a wide group of biologically active

mediators such as $PGE_2$, prostacyclin ($PGI_2$) and thromboxane $A_2$ ($TXA_2$); generally referred to as cyclic prostanoids. Their formation depends on the actions of specific isomerases acting upon $PGH_2$ [29] as presented in *Fig. 1.2.*

Aspirin acts by selectively inhibiting prostaglandin synthesis through acetylation of the hydroxyl group of a specific amino acid (a single serine residue at position 529 in human COX-1) within the polypeptide chain of platelet prostaglandin endoperoxide synthase, causing the irreversible loss of its cyclo-oxygenase activity. The acetyl group blocks an important structural channel, required for arachidonic acid to reach the enzyme's active site. The result is the reduced conversion of arachidonic acid to $PGG_2$, $PGH_2$ and $TXA_2$ [30]. Roth et al. have reported that aspirin produces maximal inhibition of arachidonic acid-stimulated platelet aggregation at a concentration of 10 μM [31]. Aspirin acetylation results in irreversible COX inhibition, since platelets being anucleic cannot synthesise new COX-1. Thus new enzyme must be synthesised before prostanoids are again produced. Prostaglandins are not stored within cells and so their release is dependent upon their biosynthesis [32].

### 1.2.3.2 COX isoforms

Recent studies have revealed the existence of at least two isoforms of COX. In 1989, Simmons et al. identified a second isoform of the enzyme and named it COX-2 (PGHS-2), distinguishing it from the original enzyme, COX-1 [33]. Subsequently Guiliano and Warner reported that the two enzymes have a similar size (71 KD) and almost identical enzyme kinetics [28]. Comparison of COX-1 with COX-2 leads to a sequence identity of approximately 60% [34]. Studies of the tertiary structures of COX-1 and COX-2 have however, demonstrated key differences such as the exchange of ILE [I] in COX-1 for VAL [II] in COX-2 at positions 434 and 523. These substitutions result in a larger and more flexible substrate channel in COX-2 than in COX-1. Consequently, the inhibitor-binding site in COX-2 is approximately 25% larger than in COX-1 [27].

The inhibition of prostaglandins produced from COX-2, which are involved in the inflammatory process, explains some of the therapeutic effects of aspirin.

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[I] Isoleucine
[II] Valine

Alternatively, the inhibition of prostaglandins produced from COX-1, which are involved in normal 'housekeeping' functions, may disrupt normal physiology and elicit undesirable effects, such as gastric lesions and increased gastric bleeding. In this regard, selective inhibition of COX-2 could provide an anti-inflammatory action without the side effects of the currently available NSAIDs, including aspirin [35, 36, 37, 38]. Mitchell et al. have shown that NSAIDs have different profiles of inhibition for COX-1 and COX-2 in a range of models [39]. The relative activity of NSAIDs in inhibiting COX-1 and COX-2 is an important parameter that correlates with the adverse effects of these drugs. Selectivity for the COX-2 enzyme is estimated by taking the ratio of the concentration of the NSAID producing 50% inhibition of COX-2 to that producing the same degree of inhibition of COX-1 ($IC_{50}$). Those NSAIDs with high ratios, such as aspirin, show high GI toxicity. Several *in vitro* PGHS-1 or -2 expression systems have been developed and used to study the isoform selectivity of various NSAIDs. Chulada and Langenbach have shown that aspirin is 5- to 10-fold more active towards COX-1 than COX-2 [40].

### 1.2.4 Therapeutic applications of aspirin

Aspirin is the most extensively used agent worldwide for its analgesic, anti-inflammatory and anti-pyretic properties. However, today, the major use of aspirin is in the prevention of heart disease, an unknown indication until the middle of the last century. Studies carried out over the last 20 years have changed the perception of the therapeutic profile of this agent. Aspirin has been associated with a reduction in the risk of colorectal cancer and a protective effect against Alzheimer's disease and other forms of dementia [41]. The optimum dose of aspirin used for therapeutic purposes varies according to the clinical conditions diagnosed, the length of treatment and the desired benefits.

#### 1.2.4.1 Analgesic actions of aspirin

$PGE_2$ and $PGI_2$ cause an increased susceptibility to the effects of chemical and mechanical stimulation on the afferent nerve endings. Aspirin and other NSAIDs, function locally to block pain rather than to affect its recognition in the brain [17].

Aspirin-like drugs are effective as analgesics under conditions of low to moderate pain intensity but not of high intensity. However, aspirin is effective against any pain with an inflammatory component, such as advanced cancer, which can be treated with aspirin in combination with morphine. However, aspirin is also extremely effective, at low doses, against the pain of childbirth, surgery and headache [10].

### 1.2.4.2 Anti-pyretic actions of aspirin

Fever has been defined as the disturbance of deep body temperature following infection and inflammation [10]. Aspirin-like drugs reduce fever depending on their ability to inhibit $PGE_2$ biosynthesis within the pre-optic hypothalamic region, which serves to regulate body temperature. Circulating $PGE_2$ enters the central nervous system (CNS) activating heat gain pathways and inhibiting heat loss pathways leading to a rise in body temperature. Agents that inhibit COX maintain the body at a lower temperature by preventing prostaglandin synthesis [17].

### 1.2.4.3 Anti-inflammatory actions of aspirin

Inflammation is the natural response of living tissue to injury. $PGE_2$ is produced in excessive amounts at sites of inflammation and is a potent vasodilator of vascular smooth muscle resulting in the characteristic signs of vasodilation and erythema (redness) seen in inflammation. Many other products of COX have been detected in areas affected by inflammation including $PGD_2$, prostacyclin and $TXA_2$. However, they are present at less than a quarter the concentration of $PGE_2$ [1]. Aspirin and other NSAIDs have offered symptomatic relief to patients with painful inflammatory or arthritic conditions. Two of the most prevalent conditions involving inflammation and pain are rheumatoid arthritis (RA) and osteoarthritis (OA) [42]. To achieve an anti-inflammatory action in RA, aspirin must be given in large doses [10].

It remains unclear whether the anti-inflammatory activity of aspirin is intrinsic to the drug or due to the biological effects of its metabolite, salicylic acid.

### 1.2.4.4 Aspirin in cardiovascular disease

Cardiovascular disease is the primary cause of morbidity in the adult Western population [43]. Aspirin is now known to be one of the safest, most important and cost effective agents in the fight against cardiovascular and cerebrovascular disease and it is believed that regular intake of aspirin could prevent as many as 100,000 deaths worldwide arising from stroke and heart attack each year [44]. The recommended dose of aspirin for the prevention of occlusive vascular disease is between 75 and 300 mg daily, although taking into account the benefits and risks, the optimal dose is more likely to be 100 mg daily. It is possible that lower doses may be equally effective, however such doses (30 and 50 mg daily) have had only limited evaluation [45]. Buring and Hennekens have reported that aspirin is not only effective in the treatment of those patients with established cardiovascular disease but also in the primary prevention of cardiovascular disease in healthy subjects [46].

Aspirin is a powerful anti-thrombotic agent. Clinical thrombosis generally occurs at sites of pathological damage to blood vessels due to the presence of atherosclerotic lesions, necrotic tissue or inflammatory cells. Platelets form the core of a developing thrombus to which are added erythrocytes, neutrophils and monocytes. As previously described, platelets are anucleate, have a life span of 8-11 days and do not usually adhere to each other or to other blood cells. Thromboxane $A_2$ is synthesised and released by platelets in response to a variety of stimuli (thrombin, collagen and adenosine phosphate) and in turn induces irreversible platelet aggregation [32]. The biochemical rationale for the anti-thrombotic action of aspirin is based on the ability of aspirin to inhibit COX-1 [47]. Since platelets are anucleate, they are unable to generate new COX enzyme and are therefore, unable to synthesise new $TXA_2$ so aspirin inhibition of COX is effective for the life span of the cell [48]. This profound selective irreversible inhibition of platelet function is typical of aspirin but not the non-aspirin NSAIDs.

Aspirin does not inhibit, to the same extent, platelet aggregation induced by adenosine diphosphate (ADP), collagen or low levels of thrombin [49].

### 1.2.4.5 Aspirin and cancer

Colon cancer is the second most common malignancy in the developed world with an estimated 94,100 new cases and 46,600 deaths in the United States in 1997. A retrospective study found that patients taking relatively low doses of aspirin (maximum effect seen with a dose of four to six 325 mg tablets weekly) over long periods of time has considerably reduced the risks of developing colon cancer [50]. In 1975 Bennett and Del Tacca found that human colorectal cancer cells produced more $PGE_2$ than the surrounding mucosa. They hypothesised that cancers, which produce excessive amounts of $PGE_2$, might promote their own growth and/or spread, a theory that was subsequently supported by animal experiments [51]. Clues to aspirin's ability to decrease the incidence of colon cancer were initially obtained from case-controlled and observational epidemiological studies [52,53,54,55]. Most published studies show a 40-50% reduction in the incidence of colon cancer in subjects taking aspirin. Little epidemiological evidence is available on other NSAIDs and colon cancer risk reduction [56]. Several other tumours, including head and neck, breast and oesophageal cancers produce excessive levels of PG due to increased COX-2 activity [57, 58].

### 1.2.4.6 Aspirin and Alzheimer's disease

Increasing evidence indicates that inflammation is involved in the pathogenesis of Alzheimer's disease. If inflammation is part of the pathway leading to Alzheimer's disease, anti-inflammatory agents may be effective in slowing disease progression or preventing the onset of the disease. While many publications have found evidence for a role of COX-2 in this disease [27], several studies, including that of Stewart et al., have shown a reduced incidence of Alzheimer's disease among NSAID users [59].

### 1.2.5 Non-beneficial effects of aspirin therapy

Each member of the NSAID family has its own side-effect profile, however, the underlying mechanism is the inhibition of the COX enzyme. Overall, studies have shown that the ability of a given NSAID to inhibit COX-1 is related to the degree of its side-effect profile. In the 21st century we are faced with the challenge of balancing the benefits and adverse effects of these agents and developing new, safer agents with similar efficacy.

#### 1.2.5.1 Aspirin and the gastrointestinal (GI) tract

In the GI tract, the adverse effects of aspirin are ascribed to both direct and indirect mechanisms [60]. The direct effect is related to the acidic nature of aspirin and aspirin-like NSAIDs. These acids become lipid soluble at low pH and following oral administration can cross the lipid membrane barrier into the gastric mucosal cells. Within these cells the acids become trapped as they lose their lipid solubility due to a change in the pH of their surroundings. This disrupts normal cell function and damage to the surface mucosal cells compromises the normal protective mechanisms. The indirect effect is due to inhibition of COX-1 activity, as described previously (Section 1.2.3) [61]. COX-1 is involved in the production of prostaglandins, which have a vital protective role in the GI tract, such as maintaining an effective mucous-bicarbonate barrier, submucosal blood flow, more rapid and effective mucosal changes to tissue damage and more rapid recovery after such damage.

Lanas has categorised aspirin users into three main types: those who do not bleed (approximately 10%), those who are sensitive to aspirin and may lose two to ten millilitres of blood daily (approximately 80%) and finally, those who are particularly susceptible to gastric irritation (approximately 10%) [63]. Factors, which increase the risk of NSAID related GI damage include age, a previous history of peptic ulceration and those patients who are in their first three months of NSAID treatment. Other high risk factors include the use of anti-coagulants and underlying cardiovascular disease [62]. Factors influencing the occurrence of GI side-effects during aspirin therapy include the dose and dosing interval, the duration of treatment and the type of formulation used.

Even with low doses (325 mg) or very low doses (75 mg) a risk may still be detected [63].

NSAIDs can cause unwanted effects, including bleeding, perforation, ulceration and stenosis, in all parts of the GI tract and can also interfere with the healing of damaged mucosa [62]. NSAID-induced ulcers are large and multiple and are more commonly found in the stomach than the duodenum. They are relatively 'painless', an effect thought to be related to the analgesic effects of NSAIDs [37]. Croft et al. reported that 1 g of aspirin taken daily causes about 1 millilitre of blood loss [64]. Endoscopically controlled studies have shown that ingestion of aspirin, 80-300 mg daily for 14 days, results in gastric and duodenal erosions and endoscopic ulcers, which may be present in more than 60% of patients after one month of therapy [65]. Lanas has shown that 75 mg of aspirin daily causes gastric mucosal bleeding [66]. Cryer and Feldman have shown that as little as 10 mg aspirin daily induces significant gastric injury [7]. Slattery et al. claim that the relative risk of bleeding ranges from 2.3 to 6.4 after the administration of a 75 mg dose to a 1200 mg dose of aspirin daily [67]. The risks of hospitalisation for serious upper GI complications, including bleeding and perforation, from NSAID use are one to two per cent per year. Ray and Griffin reported that in the United States as many as 41,000 hospitalisations and 3,300 deaths occur each year among elderly NSAID users [68].

#### 1.2.5.2 Aspirin and other unwanted side effects

Aspirin may decrease the production of renal prostaglandins, which play an important role in tissue homeostasis and function [10]. Asthmatics can suffer from a particular type of asthma known as aspirin-induced asthma and it is thought that inhibition of COX triggers specific biochemical reactions that lead to open asthma attacks [10]. Reye's syndrome is a rare, acute and sporadic liver disorder, which arises due to an unusual response to viral infection and is modified by a range of exogenous agents such as aspirin [10]. Some patients are hypersensitive to aspirin and may develop a rash or experience an anaphylactic reaction [17]. A prostaglandin-dependent effect of NSAIDs is impaired platelet aggregation, which may explain the risk of bleeding from pre-existing

lesions early in the course of NSAID therapy [69]. Some patients develop aspirin resistance over time, even with high doses of aspirin [70].

### 1.2.6 Stability of aspirin

Aspirin undergoes decomposition in the solid-state form [71, 72] and is affected not only by temperature and humidity but also by numerous chemical agents with which it may be combined. In the presence of moisture, aspirin hydrolyses at such a rate that the given pharmaceutical formulation does not have a practical shelf life. The poor aqueous stability of aspirin is related to intramolecular nucleophilic catalysis, induced by the carboxylic acid group, as presented in *Fig. 1.3*. The decomposition of solid aspirin (in powder form) in the absence of water is insignificant relative to hydrolysis in the presence of water [73].

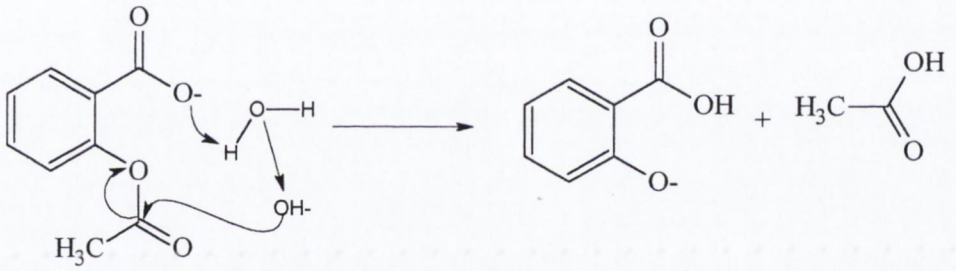



*Figure 1.3 The intramolecular nucleophilic catalysis of aspirin.*

The extent of the problem involved in the preparation of a liquid dosage form of aspirin was summed up when Friedlander and Feinburg recommended that 'aspirin should never be used in aqueous solution because of its instability' [74]. Various methods for preparation of a stable aspirin solution have been extensively studied, including the use of organic solvents such as ethanol, propylene glycol and glycerin. These were found not to be palatable for oral administration [75]. Shwartz et al. developed an aspirin elixir, in the hope it would be suitable as a paediatric medicine. The formula recommended the use of ethanol and polyethylene glycol as solvents. However, after 24 days 17.7% of the drug had hydrolysed in this solvent, presumably through a mixture of transesterification and hydrolysis [76].

### 1.2.7 Formulations of aspirin

Many preparations of aspirin are available in pure form or in combination with other anti-platelet, anti-coagulant, sedative or buffering agents in order to improve its side-effect profile while maintaining adequate blood levels after oral administration. These are similar in their metabolism and elimination but differ in their rates of disintegration, dissolution and absorption and in their site of absorption. These differences are of clinical importance, since the performance of aspirin can be vital in the treatment of acute MI and other forms of thrombotic arterial diseases, where the risk of mortality following MI is reduced significantly by starting aspirin therapy immediately [77].

It has been postulated that enteric-coated tablets may have an impact in reducing the GI effects of aspirin. This strategy is based on the assumption that these are mainly due to a local effect [16]. However, it has been found that enteric-coated aspirin does not protect from aspirin-induced risk of upper GI bleeding, irrespective of the dose [78, 79]. This is consistent with COX-1 inhibition being the underlying mechanism for the adverse effects of aspirin. Sagar and Smith compared the bioavailability of aspirin in pure aspirin tablets, chewed tablets, effervescent tablets and enteric-coated tablets, with respect to peak concentration and time of appearance of the drug in plasma after an oral dose. Aspirin availability was found to decrease in the following order: soluble tablets > chewable tablets > pure tablets > enteric-coated tablets [80]. The use of aspirin in topical form was studied for its dose dependent block of low cutaneous pain. Steen et al. prepared aspirin in a mixed ointment of white petroleum jelly and liquid paraffin. Results from the study indicate that aspirin dose dependently blocks cutaneous pain, induced by a low pH buffer. However, this analgesic effect was due to local and not systemic effects [81]. McAdam et al. developed a patch application in which aspirin was found to be stable with less than 2% salicylic acid detected after six months at 25°C. However, no aspirin was detected in plasma at any time, since much of the applied aspirin was converted to salicylic acid by the skin; most likely by enzymatic hydrolysis [82].

Since the development of various dosage forms of aspirin proved inadequate, a more recent approach involved the chemical transformation of aspirin, in an attempt to

improve the stability and therefore, extend its means of formulation while also improving the side-effect profile of the drug.

## 1.3 Chemical Derivatisation of aspirin

A prodrug has been described as the chemical modification of a biologically active compound to form a new inactive derivative, which upon either *in vivo* enzymatic or non-enzymatic attack will liberate the parent drug [83]. Prodrugs consist of two parts, namely the drug and the promoiety, which is the modifying unit of the structure attached covalently to the drug. The prodrug must regenerate the parent drug *in vivo* while at the same time be sufficiently stable *in vitro* so that a suitable pharmaceutical product may be developed [84]. Prodrugs can improve the physical characteristics of a drug such as taste and smell or increase the concentration of the active at its site of action, thereby increasing its efficiency and eliminating its adverse effects [84]. NSAID prodrugs have been extensively investigated for many years as a means of depressing gastric toxicity or increasing percutaneous absorption [85]. NSAID esters generally exhibit depressed gastric toxicity relative to the parent compound [86, 87, 88, 121]. Aspirin is a carboxylic acid and the temporary masking of its acidic function is a promising means of reducing or eliminating the GI toxicity due to the direct action of aspirin on the mucosa. The capacity of aspirin esters to exhibit therapeutic effects comparable to aspirin is dependent upon their ability to release aspirin following absorption through the gastric mucosa. Some groups may not be sufficiently labile *in vivo* to ensure a high rate of prodrug conversion.

A recent strategy has been the design of aspirin prodrugs as substrates for butyrylcholinesterase (EC 3.1.1.8), an esterase abundant in plasma (Bundgaard and Nielsen) [89]. Many enzymes are present in the organism that are capable of hydrolysing esters.

### 1.3.1 Stability of aspirin prodrugs

Protection of the carboxylic acid group of aspirin, through the design of prodrugs, should enhance the stability of aspirin, especially in aqueous solutions. It has been established that aspirin, when in the ionised form, can undergo intramolecular and aqueous catalysed nucleophilic hydrolysis, as presented in *Fig. 1.3* [90]. Masking of the carboxylic acid functionality should prevent nucleophilic hydrolysis from occurring and consequently result in improved stability.

It should be appreciated that in order for prodrugs of aspirin and other NSAIDs to be useful they must satisfy a number of criteria. These include: an ability to be readily hydrolysed following absorption to release the parent drug; adequate aqueous solubility and lipophilicity to ensure absorption following oral administration, suitable physico-chemical characteristics for formulation and adequate stability towards GI enzymes [91, 92].

### 1.3.2 Typical hydrolysis pathways of aspirin prodrugs

The hydrolysis of aspirin esters may occur simultaneously through two distinct routes (*Fig 1.4*) according to a competitive sequential four-component closed system [93]. Hydrolysis can occur along the $k_1$ pathway at the carboxylic ester bond liberating aspirin (**1**) or along the $k_2$ pathway at the *O*-acetyl group liberating salicylic acid (**2**) *via* the salicylate ester (**3**). A prerequisite for any true aspirin prodrug is that the hydrolytic rate constant $k_1$ should be greater than the rate constant associated with deacetylation, $k_2$.

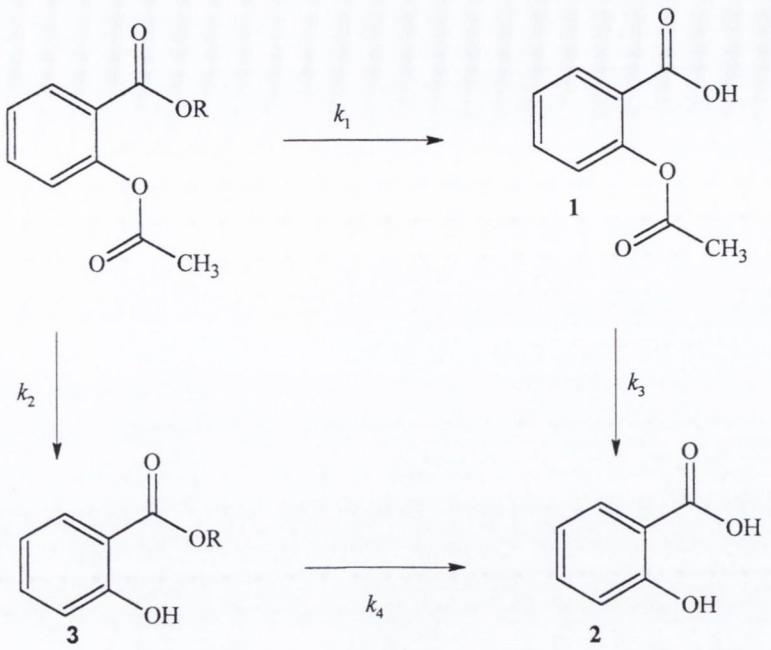



*Figure 1.4 The hydrolytic pathways of aspirin prodrugs* [83].

However, a major problem in the design of aspirin prodrugs is the enzymatic lability of the acetyl ester group in aspirin. Esterification and consequent neutralisation of the carboxyl group renders the acetyl group highly susceptible to plasma-mediated hydrolysis relative to aspirin itself. Aspirin, which is negatively charged is a poor substrate for plasma esterases and its half life in human plasma is about 120 minutes [94], considerably longer than the half life of related aspirin esters [96]. A successful aspirin prodrug must undergo hydrolysis at the carboxylic ester group at a greater rate than at the *O*-acetyl group ($k_1 > k_2$), whose hydrolysis the carboxylic ester group greatly accelerates. Otherwise the derivatives will behave as prodrugs of salicylic acid and not as true aspirin prodrugs [83].

### 1.3.3 Design of aspirin prodrugs

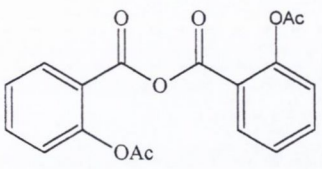
Attempts to design aspirin prodrugs have generally furnished aspirin esters that undergo hydrolysis at the *O*-acetyl moiety at a greater rate than at the carboxylic ester group ($k_2 > k_1$).

Aspirin prodrugs have been classified into two groups: those derivatives that undergo enzymatic cleavage to regenerate the parent drug and those that hydrolyse non-enzymatically. The former group includes triglycerides, thio esters and phenylalanine esters while the latter group includes benzodioxanones and some acylal derivatives [95]. The following section illustrates the difficulty in designing aspirin prodrugs since the majority of esters investigated were found to act as prodrugs of salicylic acid. The major breakthrough in this area involved work reported by Bundgaard et al. who designed aspirin esters as substrates for butyrylcholinesterase (EC 3.1.1.8), an esterase abundant in plasma. A good fit to the active site of this enzyme appeared to enhance hydrolysis towards aspirin liberation [96]. There follows a review of the strategies directed at the development of aspirin prodrugs.

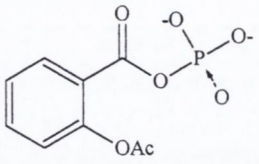
#### 1.3.3.1 Aspirin anhydride

One of the first attempts to develop an aspirin prodrug involved the synthesis of aspirin anhydride (**4**). However, problems arising from its poor aqueous solubility (3.2 mg per 100 ml at 37°C), slow dissolution rate and immunogenicity prevented its use as a derivative of aspirin. Ester **4** was less soluble in water than aspirin and was only slowly hydrolysed by neutral or dilute acid solutions [97]. Levy and Gagliardi found that aspirin anhydride (**4**) was absorbed more slowly than aspirin. Furthermore, the total amount of apparent salicylate excreted following administration of ester **4** was considerably less than after aspirin intake, which indicates that aspirin anhydride is not absorbed completely [98]. These findings correlate with the results obtained by Wood and Harvey-Smith who reported that administration of aspirin anhydride (**4**) did not produce beneficial effects comparable to aspirin [97]. A study, carried out by De Weck, of the immunological effects of ester **4** found that it is a potent immunogen capable of reacting with molecules carrying amino groups. Ester **4** was also found to be capable of sensitising experimental animals in a variety of ways, forming conjugates with

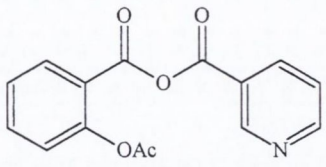
proteins *in vitro*, which are capable of inducing the formation of anti-aspirin antibodies in patients ingesting aspirin and of eliciting reactions of aspirin specificity in sensitised animals [99].




**4**




**5**




**6**

Following the work described above, Loftsson et al. reported the investigation of mixed anhydrides, including a phosphoric acid mixed anhydride (**5**) and pyridine containing anhydrides (**6**) in an attempt to improve the physicochemical properties of aspirin anhydride. However, the synthesis of these compounds proved problematic, since they could not be easily obtained in pure form. Aspirin anhydride (**4**) was formed from their synthesis as a major impurity[100].

It would appear, therefore, that aspirin anhydrides possess unsuitable characteristics, notably undesirable immunogenicity, for them to be considered as potential aspirin prodrugs.

### 1.3.3.2 Benorylate

Benorylate (7), 4-acetamidophenyl 2-acetoxybenzoate, was first synthesised by Robertson in 1963 as a potential mutual prodrug of aspirin and paracetamol [101]. Robertson found ester **7** had a slower absorption rate compared to aspirin and paracetamol, which may be due to its low aqueous solubility [102].

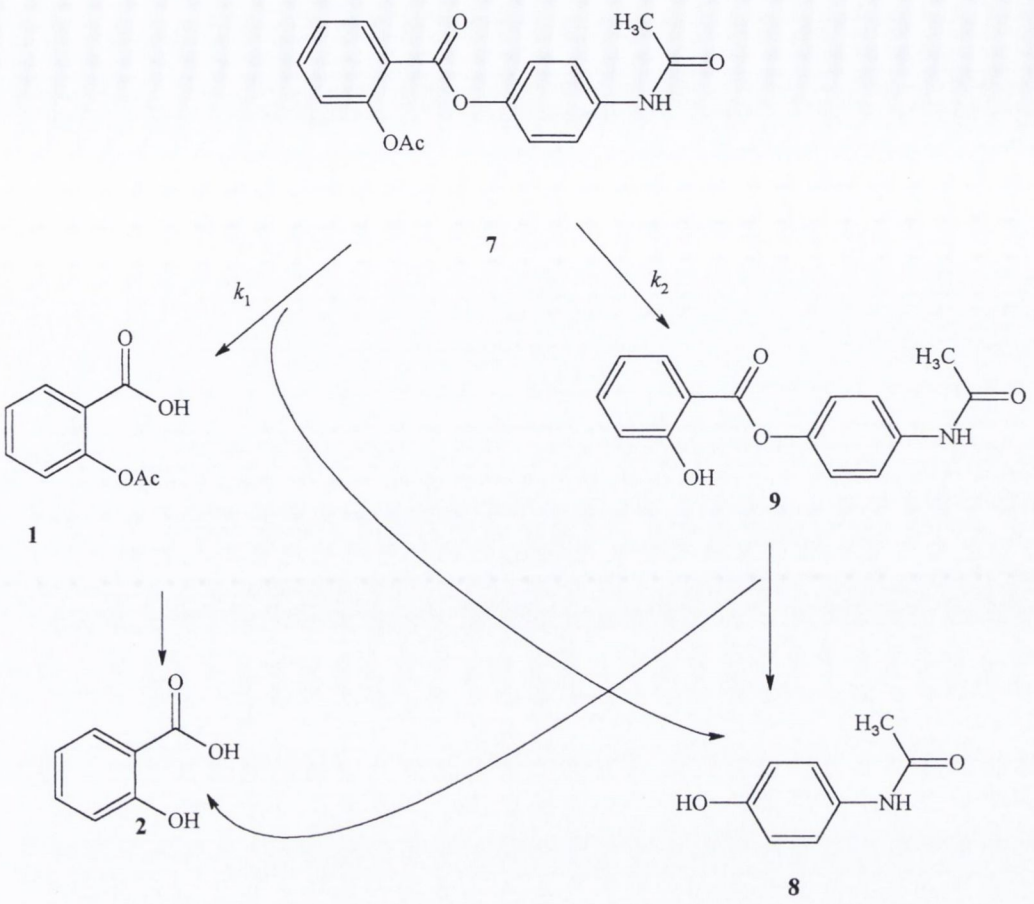



*Figure 1.5 The metabolic pathway of benorylate (7) in man* [102].

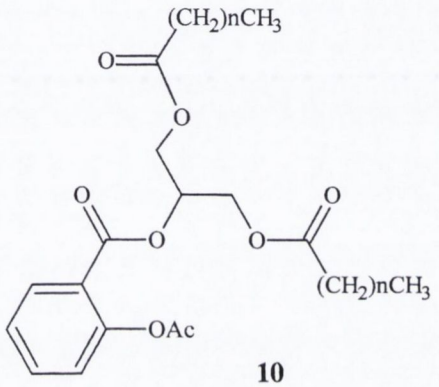
The potential hydrolysis pathways of benorylate (**7**) are presented in *Fig. 1.5*. When incubated in human plasma, its rapid hydrolysis was associated with the liberation of paracetamol (**8**) and salicylic acid (**2**). Hydrolysis *via* the aspirin route was minor. Williams reported that following a single oral dose (4 g) of ester **7** *in vivo*, only salicylate and paracetamol (**8**) were detected in the plasma [103]. Clinically, benorylate (**8**) is comparable to aspirin in the treatment of rheumatoid arthritis and osteo-arthritis [104]. Animal studies have shown that it has anti-inflammatory, anti-pyretic and analgesic properties comparable with those of aspirin [101]. The anti-inflammatory effects are most likely mediated by both salicylic acid and paracetamol.

The success of chemically combining aspirin and paracetamol is rather limited, since negligible levels of aspirin were liberated *in vitro*. The addition of paracetamol did not improve the analgesic effect of aspirin. The lack of gastric toxicity of ester **7**

may be due to its retarded absorption. However, this is achieved at the expense of a delayed onset of action, one of the major merits of aspirin.

### 1.3.3.3 Triglycerides

Rainsford et al. prepared a series of aspirin esters incorporating a triglyceride structure, an approach based on known lipid metabolic pathways. The idea behind the development of aspirin triglycerides was that aspirin would be released by lipases prior to or following absorption of the drug [10].




**10**

Paris et al. reported on a series of the aspirin triglycerides (1,3-bis (alkanoyl)-2-(*O*-acetylsalicyloyl) glycerides) (**10**) having aspirin at the two-position and glycerol and fatty acids at the one-position and three-position respectively. The compounds were administered orally and tested for efficacy using the rat paw oedema test and the stomachs were examined for lesions. The results of this study suggested that these esters induce a low degree of gastric ulceration [105]. Similar results were obtained from a study of cyclic aspirin triglycerides [106]. However, it has not been proven that the aspirin triglycerides (**10**) are in fact true aspirin prodrugs. Recent work showed only their blood salicylate levels and the pharmacological testing involved only anti-inflammatory studies [107]. Further work would need to be carried out including *in vitro* and *in vivo* studies to determine the actual pathway of hydrolysis and whether the lack of gastric irritation is due to the natural pathways of hydrolysis of triglycerides or the lack of aspirin as a hydrolytic product. Biological testing for aspirin release should

involve the measurement of $TXA_2$ levels and platelet aggregation, since these effects are not connected to salicylic acid, which is in contrast to the anti-inflammatory effects.

### 1.3.3.4 Thiomethyl esters

Loftsson et al. reported the investigation of methylthiomethyl (**11**), methylsulphinylmethyl (**12**) and methylsulphonylmethyl (**13**) esters of aspirin as potential true aspirin prodrugs, whose possible degradation pathways are presented in *Fig 1.6.* These esters could potentially hydrolyse to liberate aspirin or the corresponding salicylate ester. Alternatively, the thiomethyl esters (**11**) could undergo reversible oxidation to the sulfinyl esters (**12**) and subsequently to the sulfonyl esters (**13**). The sulfur containing esters hydrolysed much more rapidly in plasma than, for example, the simple methyl ester of aspirin [100]. While the pH 7.96 hydrolysis of the methylthiomethyl ester (**11**) liberated 90% aspirin, the same compound in plasma hydrolysed *via* the aspirinate and salicylate pathways. In contrast, ester **12** hydrolysed almost exclusively to aspirin, in plasma, while at pH 7.96 hydrolysis occurred *via* both pathways. Oral administration of the methylsulfinylmethyl derivative (**12**) to beagle dogs was associated with the generation of higher levels of aspirin than a similar oral dose of aspirin [108]. The results of this study indicate that these esters may have potential as aspirin prodrugs, especially the methylsulfinylmethyl ester (**12**). However, following the work described above, Nielsen and Bundgaard demonstrated that only 30% and 20% aspirin was liberated from esters **12** and **13** respectively, following hydrolysis in 10% human plasma [96]. There appears to be no obvious explanation for this discrepancy.

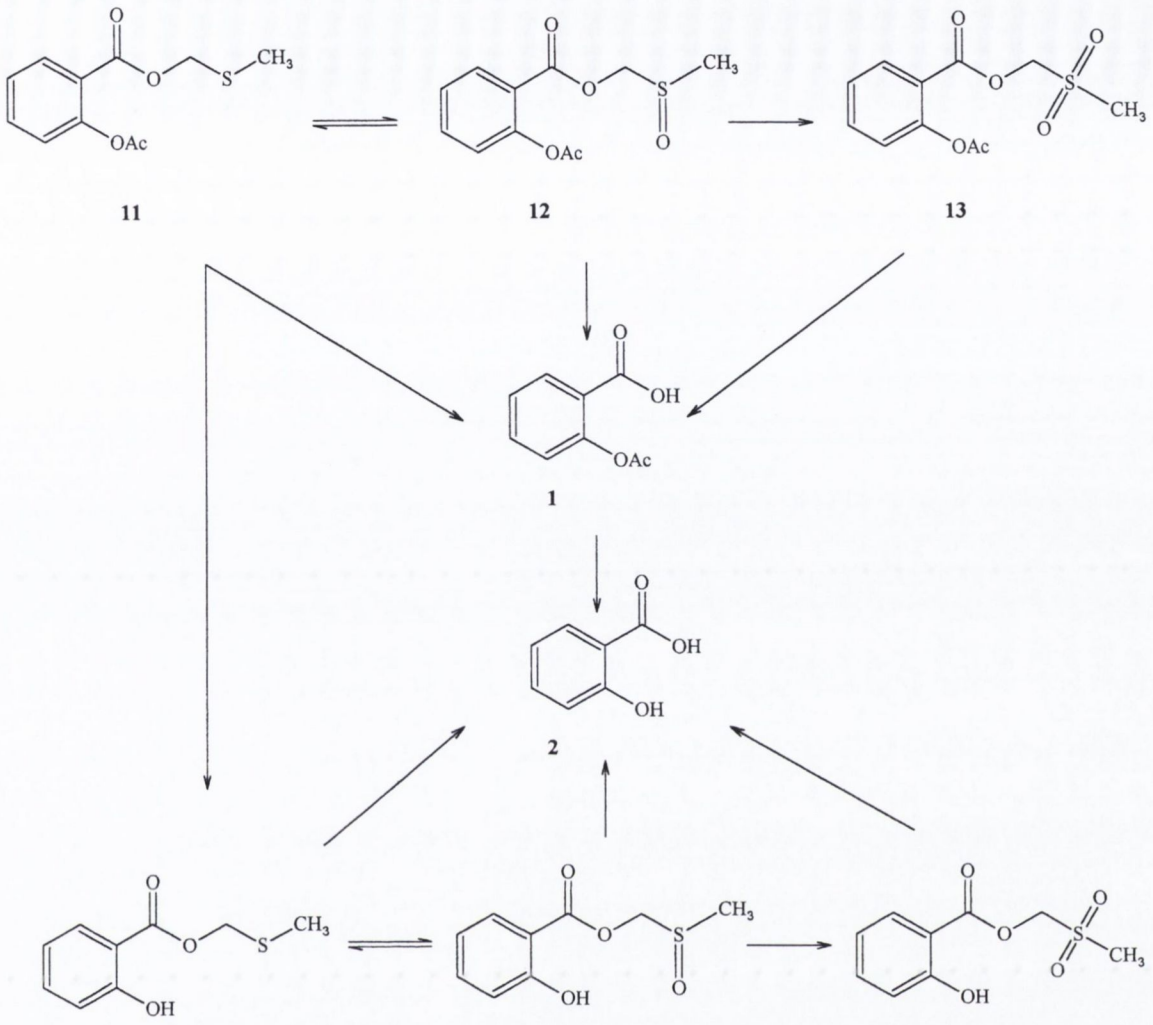


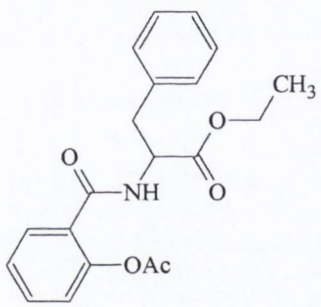

*Figure 1.6 The potential degradation pathways of aspirin thioesters* [10].

While this strategy has potential as a basis for the development of aspirin prodrugs, there has been no report of the anti-thrombotic effect or toxicity profile of these esters. A study of their physico-chemical characteristics, susceptibility towards aqueous hydrolysis and platelet aggregation characteristics would be useful in this regard.

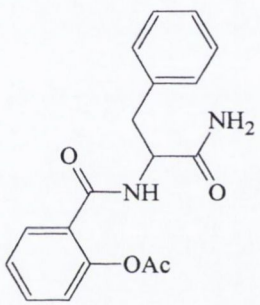
A study by Venuti et al. on thioesters of NSAIDs other than aspirin found that esters of this general class might offer a means to manipulate the physico-chemical characteristics and hydrolysis pathways of the parent NSAIDs. This class of esters may improve the therapeutic profile and reduce the gastrointestinal erosive activities of the parent NSAIDs [109].

1.3.3.5 Phenylalanine esters

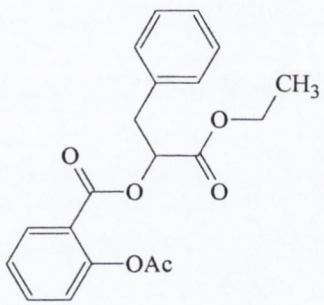
Banarjee and Amidon investigated aspirin phenylalanine ethyl ester (**14**) and phenylalanine esters, including aspirin phenylalanine amide (**15**) and aspirin phenylacetic ethyl ester (**16**) as potential substrates of digestive enzymes. The use of amino acid residues could allow for the synthesis of non-toxic drugs with diverse properties. These prodrugs were tested for their stability *in vitro* towards carboxypeptidase A and α-chymotrypsin. It was anticipated that ester **14** would undergo initial cleavage by α-chymotrypsin to liberate aspirin phenylalanine while subsequent hydrolysis by carboxypeptidase could liberate aspirin. The reactions were followed by measuring the consumption of sodium hydroxide using a pH-stat titrino while aspirin was detected by TLC [110, 111, 112].




**14**




**15**




**16**

Based on this system it was initially proposed that aspirin phenylalanine ethyl ester (**14**) liberated aspirin in response to hydrolysis by carboxypeptidase. However, a subsequent study carried out by Muchi-Eldeen and Hussain, using a specific HPLC and TLC assay, determined that the original work was in error. The aspirin detected was not released from the aspirin ester, but was formed as a hydrolysis product of aspirin anhydride (**4**), which was itself present as an impurity. This is perhaps predictable, given the high stability of carboxamides relative to phenol esters. The proposed hydrolytic pathway of ester **14** is presented in *Fig. 1.7* [113].

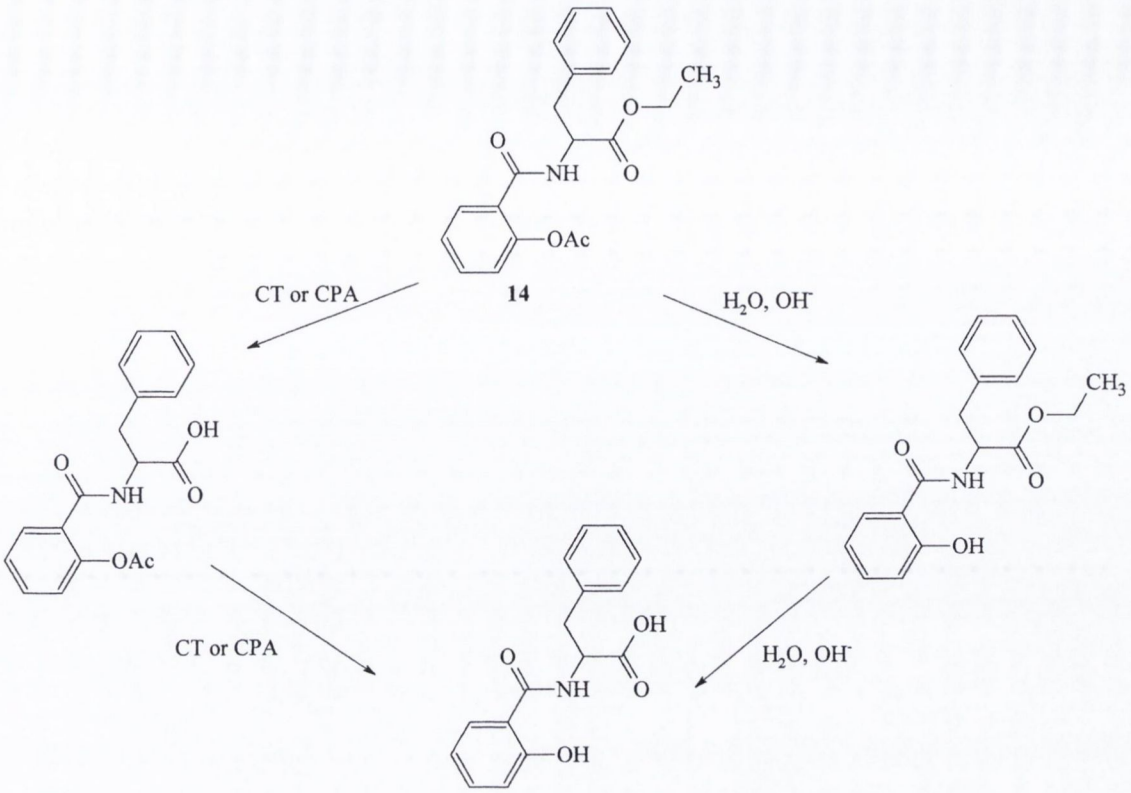



*Figure 1.7 Proposed hydrolysis pathways of aspirin phenylalanine ethyl ester (**14**); where CPA = carboxypeptidase A and CT = α-chymotrypsin* [113].

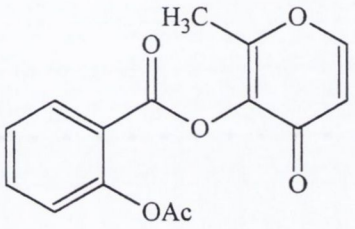
Following the work described above, Tsenematsu et al. investigated the potential of amino acid esters as aspirin prodrugs through the synthesis of aspirin-L-arginine and aspirin *p*-guanidino-L-phenylalanine. Both are water-soluble and were designed as substrates for trypsin and carboxypeptidase B [114]. However, despite the esters being good substrates for trypsin, hydrolysis occurred ultimately *via* deacetylation to liberate salicylic acid, in the presence of carboxypeptidase B. Aspirin was not liberated during their hydrolysis.

### 1.3.3.6 Aspalatone

Aspalatone (**17**) (3-(2-methyl-4-pyronyl)-2-acetoxybenzoate), the maltol ester of aspirin, was developed by Suh et al. as a possible anti-platelet agent and substitute for aspirin in the treatment of such cardiovascular effects as MI [115]. It has been reported that anti-oxidants, such as maltol, may be beneficial in preventing platelet aggregation,

since they are associated with an increase in anti-aggregatory $PGI_2$ production in arterial walls [116].

In rat serum, intestinal fluid and liver and gastric mucosal homogenates the exclusive metabolic product of ester **17** was its salicylate ester, salicylic acid maltol ester. Studies carried out *in vivo* found that after the administration of 80 mg/kg of ester **17**, salicylic acid maltol ester was liberated and this was subsequently hydrolysed to salicylic acid. It appears that aspalatone (**17**) is therefore, a prodrug of salicylic acid and not aspirin [115].

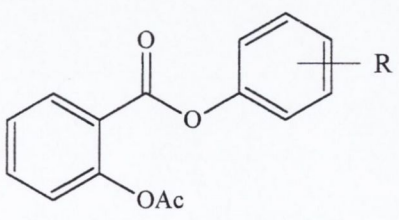



**17**

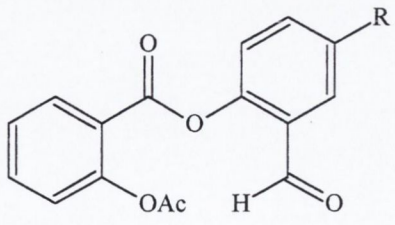
Han et al. have also demonstrated that aspalatone (**17**) inhibits collagen-induced platelet aggregation in rodents *in vitro*, *ex vivo* and *in vivo* [117]. However, this effect appears to be related to maltol (3-hydroxy-2-methyl-γ-pyrone), which has been previously isolated as an active antioxidant component [118].

### 1.3.3.7 Formylphenyl esters of aspirin

Bowden et al. studied the synthesis and non-enzymatic hydrolysis of a series of 2-, 3- and 4-formylphenyl aspirins (**18**) (R = CHO) as well as a series of 4-substituted 2-formylphenyl aspirins (**19**), as potential aspirin prodrugs [83].




**18**



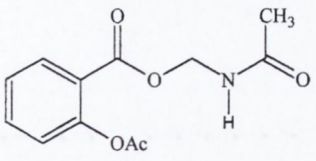

**19**

The hydrolysis of the esters of type **18** and **19** under alkaline conditions resulted in the liberation of aspirin and the formyl phenol. The hydrolysis of the substituted formyl esters and the 2-formyl phenyl aspirin was dependent on intramolecular catalysis. This was apparent from the rates of alkaline hydrolysis and activation parameters, with half-life data ranging from 15 seconds to 15 minutes. Esters **18** and **19** were more lipophilic than aspirin, which might enhance their oral absorption. These potential aspirin prodrugs were also tested for their anti-inflammatory activity in the rat paw oedema model. The 4-substituted 2-formylphenyl aspirins did not elicit a response whereas the 2- and 3-formylphenyl aspirin appeared to elicit a similar response to aspirin [83, 119].

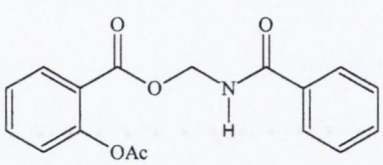
Although these esters hydrolysed to aspirin under alkaline conditions, the rates of hydrolysis indicate their poor stability characteristics. It is not clear from this work that the specific base hydrolysis would occur faster than plasma esterase-mediated hydrolysis at the *O*-acetyl group.

### 1.3.3.8 2-Acetoxybenzoate esters of *N*-(hydroxyalkyl) amides

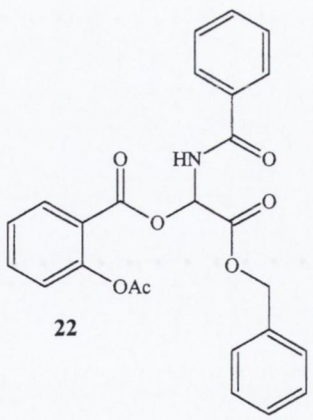
Bundgaard et al. reported that various *N*-acyloxyalkyl derivatives of primary amides are highly unstable under aqueous conditions. This work led to the development of a series of aspirin esters derived from *N*-(hydroxymethyl) acetamide (**20**), *N*-(hydroxymethyl) benzamide (**21**) and α-hydroxy-*N*-benzoylglycine benzyl ester (**22**) [95].




**20**




**21**




**22**

These esters were found to undergo rapid spontaneous hydrolysis at pH 7.4 with the liberation of in excess of 80% aspirin. Compounds **20** and **21** hydrolysed less rapidly in the pH range of 2 to 5.5 ($t_{1/2}$ <10 minutes) than under more acidic and basic

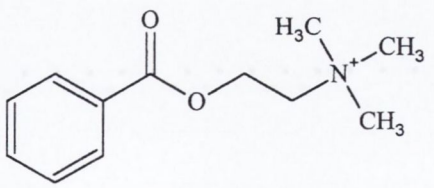
conditions, while ester **22** displayed the highest stability at lower pH ($t_{1/2}$ of 70 to 200 minutes). Esters **20**, **21** and **22** cleaved predominantly to aspirin in the presence of 80% human plasma with half-lives of 1.1 minute, 8 seconds and 0.3 seconds respectively. However, given their lability at pH 7.4, any aspirin release in plasma would be attributable to spontaneous decomposition. For ester **20**, the enzyme-mediated and spontaneous hydrolysis may compete to some extent [95].

The implication of these results is that esters of this type might decompose in the gut with increasing pH, which is not a desirable outcome. The non-enzymatic instability of these esters would also create potential difficulties in finding a suitable pharmaceutical formulation in which they are stable.

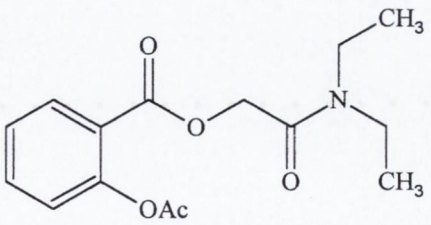
### 1.3.3.9 Glycolamides

Following the work described above, Bundgaard et al. published work, which led to the major breakthrough in the design of true aspirin esters. This involved the design of aspirin esters as substrates for butyrylcholinesterase (EC 3.1.1.8), an enzyme abundant in plasma. This enzyme-targeting approach has since been intensively pursued, as successful candidates, though highly susceptible to enzyme-mediated hydrolysis, might also be chemically stable.

Bundgaard et al. developed a series of aspirin esters of certain 2-hydroxyacetamides, also known as glycolamides, in an attempt to find novel esters, which are highly susceptible to enzymatic hydrolysis *in vivo* and *in vitro* [95].




**23**




**24**

Using benzoic acid as a model, a series of benzoate esters of various *N*-substituted glycolamides were synthesised. All esters possessed high stability towards hydrolysis in aqueous solutions. The esters hydrolysed rapidly in 50% human plasma, with

different rates and benzoic acid was produced in quantitative amounts [120]. Evidence suggested the enzyme in human plasma that was responsible for more than 90% of the observed hydrolysis was butyrylcholinesterase (EC 3.1.1.8). It was of particular interest to note the similarity between the prototypical substrate for butyrylcholinesterase (EC 3.1.1.8) - benzoyl choline (**23**) - and the glycolamide esters, of which the *N,N*-disubstituted esters was the best fit for the trimethyl binding site of the enzyme [89].

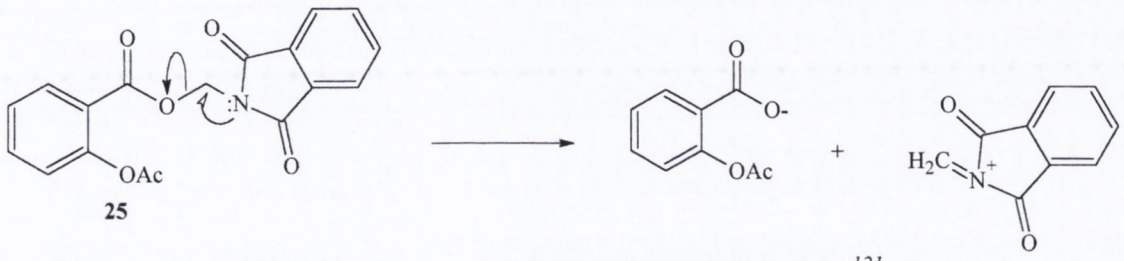
The high reactivity of these glycolamides was transferred to other acids, including aspirin. The unsubstituted or monosubstituted esters of aspirin hydrolysed through both pathways with the salicylate pathway dominant. The *N,N*-diethylglycolamide ester (**24**) and the *N,N*-dimethylglycolamide ester liberated the most aspirin, 55% and 50% respectively. These compounds behaved similarly in blood, liberating 48% and 46% of aspirin respectively. The enzyme responsible for their hydrolysis, butyrylcholinesterase (EC 3.1.1.8), is present in approximately the same concentration in both plasma and whole blood. Apart from their enzymatic hydrolysis to aspirin, both esters demonstrated suitable chemical stability, aqueous solubility and lipophilicity, which augments their potential as aspirin prodrugs [96]. Therefore, the dimethyl and diethyl glycolamide esters (**24**) of aspirin may be considered as true aspirin prodrugs. It may be possible using this approach to improve the ratio of aspirin versus salicylic acid release. Bundgaard anticipated that it might be possible to obtain any desired lipophilicity and solubility, by modifying the *N*-alkyl groups, while retaining a lability of the ester towards enzymatic hydrolysis. However, the structure activity relationship in this class appears to be rather intolerant.

An extension of this novel approach to NSAIDs, other than aspirin, was studied and positive results were obtained, with respect to the route of enzymatic hydrolysis and stability in aqueous conditions [89]. This demonstrates the flexibility of designing prodrug esters for aspirin and other NSAIDs, as substrates for esterases.

1.3.3.10 *N*-Hydroxymethyl imides

Subsequent to the development of the glycolamide aspirin esters by Bundgaard et al., a study by Omar was carried out on the applicability of *N*-hydroxymethyl imides as aspirin esters. This strategy involved the replacement of the *N,N* disubstituted amide group with an imide moiety [121].

Ester **25** was more lipophilic than aspirin, with a high stability at pH 1.3. However, at pH 7.4 and in rabbit plasma ester **25** was found to undergo rapid hydrolysis to aspirin. The suggested hydrolysis pathways are shown in *Fig. 1.8* [121].




**25**

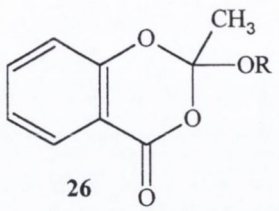
*Figure 1.8 The possible hydrolysis pathway of* ***25*** [121].

Although ester **25** liberated aspirin in 80% rabbit plasma, its instability towards non-enzymatic hydrolysis, at physiological pH, renders it unsuitable for use as an aspirin prodrug and also poses problems for formulation. However, its rapid hydrolysis in rabbit plasma confirms that the imide moiety might afford a good fit to the trimethylamino site of butyrylcholinesterase (EC 3.1.1.8) in plasma.

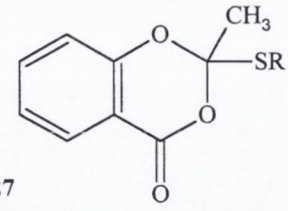
## 1.3.4 Miscellaneous aspirin prodrug strategies

### 1.3.4.1 Benzodioxanones

A novel approach to the design of potential aspirin esters was the incorporation of a common ortho ester function (2-substituted 2-methyl-4H-1,3-benzodioxin-4-one) of the general type **26** and **27** shown below.




**26**




**27**

Depending on the substituent, R, the cyclic *ortho* ester can hydrolyse to liberate aspirin, salicylic acid or both, according to *Fig. 1.9*. The strength of the bonds to the chiral carbon is an important factor in the hydrolysis pathways of these esters [122].

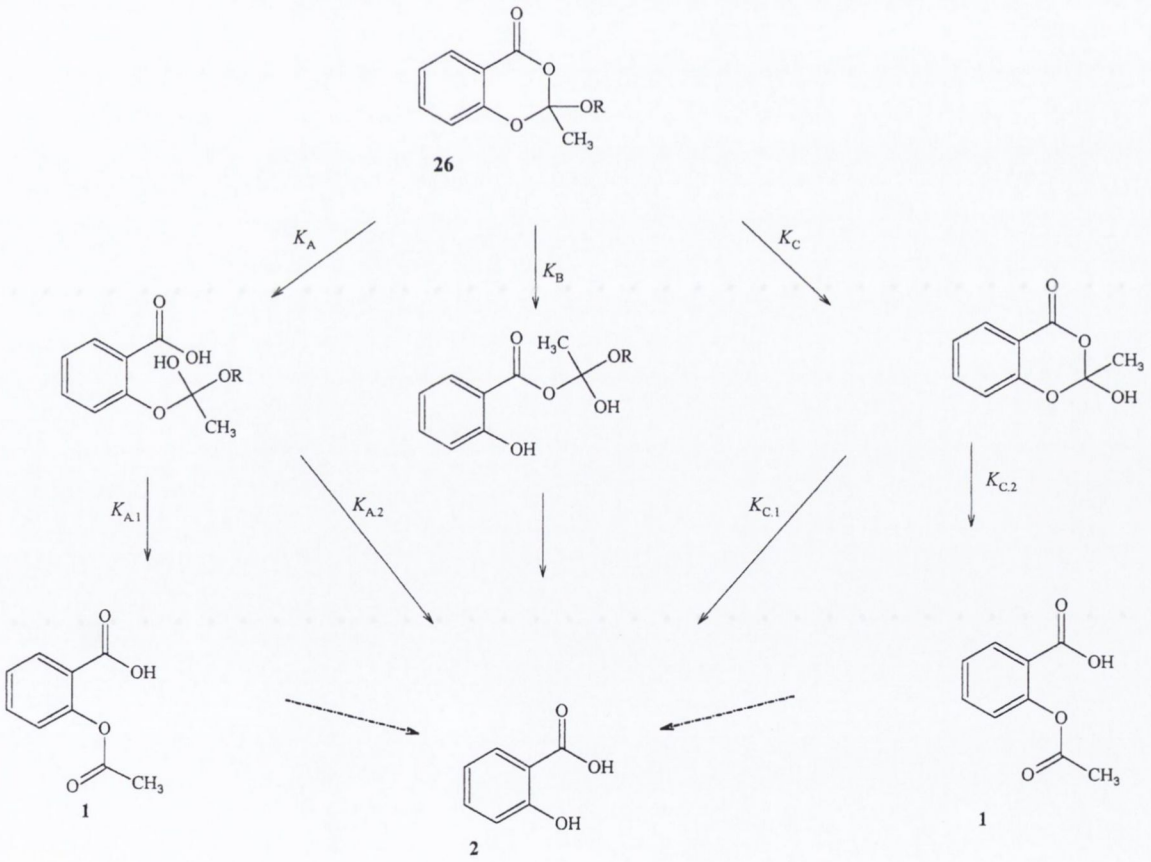
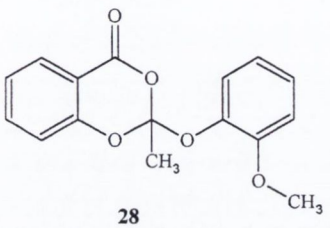
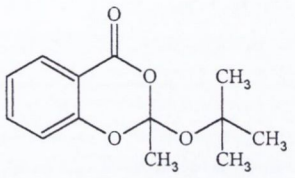



*Figure 1.9 The possible degradation pathways of cyclic ortho aspirin esters* [122].

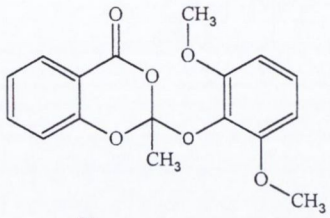
Ankerson et al. investigated an extensive series of aspirin esters of the type **26** and **27**, without success [123]. Hundewadt and Senning determined that ester **28**, (2-[2-methoxy phenoxy]-2-methyl-4*H*-1,3-benzodioxin-4-one) hydrolysed in 10% plasma to liberate aspirin (70%), with a half-life of 80 minutes [124]. Hansen and Senning determined that the rapid hydrolysis of esters **29** and **30**, in 10% plasma, was associated with the liberation of aspirin [125].



**28**



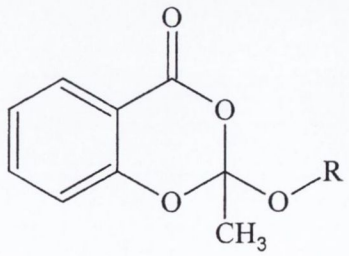
**29**



**30**

A series of esters (type **30**) with different substituents on the benzyl alcohol group was investigated. At pH 7.4, the esters hydrolysed exclusively to salicylic acid. However, at pH 10.0 ester **30** was the only ester found to liberate aspirin (82.2%), although its half-life was shorter than desired (value not reported) [126]. Further *in vitro* studies on this ester are required to determine its potential as a true aspirin prodrug, since aqueous hydrolysis studies are not necessarily a good indication of the routes of hydrolysis of aspirin esters *in vivo*.

As an extension of the work reported above Nielsen and Senning investigated a series of 15 esters in which the *tert*-butyl moiety was replaced with other tertiary aliphatic alkyl substituents. Of the nine esters that were sufficiently stable to be considered as potential aspirin prodrugs, only four esters of type **31** hydrolysed to liberate aspirin in plasma and under non-enzymatic conditions. However, in plasma the hydrolysis was too rapid for them to be considered as potential aspirin prodrugs [127].



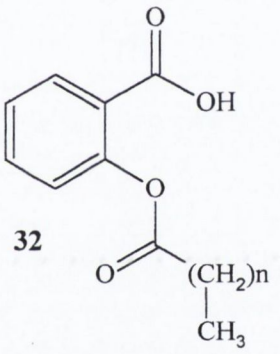
**31**

where R = *tert*-butyl,
3-methyl-2-hexyl,
2-(4-chlorophenyl)-1,1-dimethylethyl,
1,1-dimethyl-2-phenylethyl.

Kahns and Bundgaard also investigated esters of this type in an attempt to develop prodrugs of salicylamide to improve its bioavailability characteristics. However, their rates of hydrolysis were only slightly greater under enzymatic conditions than aqueous hydrolysis [128].

Despite their intensive investigation, many of the benzodioxan-4-one type esters appear to be too unstable for use as aspirin prodrugs or were determined to be prodrugs of salicylic acid. The most promising cyclic ortho ester was ester **28**. From a study of its stability in the pH range 3.0 to 7.4, the half-life data indicates this ester may be too unstable to be considered as an aspirin prodrug, ($t_{1/2}$ in the range of 66 and 139 minutes). Esters of this class appear to be too intrinsically unstable to allow them to reach the portal circulation intact.

### 1.3.4.2 *O*-acyl derivatives of aspirin




**32**

n = maximum of 8 carbon chain

A different strategy to enhance the anti-platelet action of aspirin while reducing adverse GI effects involved the design of compounds with increased lipophilicity, by extending the carbon chain length of the *O*-acyl group. This was intended to confine the drug to the portal circulation, resulting in reduced $TXA_2$ production, while at the same time decreasing the adverse effects in the systemic circulation, including inhibition of prostacyclin synthesis. This may be achieved by increasing the hepatic extraction of an aspirin compound with increased chain length.

Hung et al. found that compounds of type **32** caused less GI damage than aspirin, following oral administration to disease stressed rat. All compounds of type **32** exhibited a significant anti-platelet effect, equivalent to aspirin in the same concentration range (600 μM), against arachidonate-induced aggregation. However,

the availability of these compounds in the systemic circulation was less than aspirin and decreased with increased lipophilicity. Therefore, a suitable balance of therapeutic and non-beneficial effects of aspirin and other NSAIDs may be achieved by varying the physicochemical characteristics of the agents [129, 130].

In conclusion, despite the extensive search for potential aspirin prodrugs, only certain glycolamides hydrolysed at the carboxylic ester group to liberate aspirin. The majority of aspirin esters reported to date hydrolyse at the labile acetyl group, liberating salicylic acid.

## 1.4 Nitric oxide-releasing prodrugs

An area of much current interest is the design of nitric oxide-releasing aspirin esters, termed NO-aspirins, in which aspirin is linked to a carrier bearing a nitrate group. These molecules are designed to cross the GI barrier and undergo cleavage by plasma esterases, liberating aspirin and the nitric oxide donor. Nitric oxide esters of NSAIDs other than aspirin have also been investigated [131]. Nitric oxide donors liberate nitric oxide *in vivo*, which has important effects on the circulation such as inducing vasodilation, diminishing myocardial contractile force, inhibiting platelet aggregation and counteracting the effects of $TXA_2$ [132]. Nitric oxide has also been shown to impart beneficial effects on the gastric mucosa. Mahmood et al. have suggested that the inhibition of prostaglandin synthesis by COX inhibitors, such as aspirin, results in increased NO production. The ability of NO to inhibit the primary step in inflammation (leukocyte adhesion) may enhance the anti-inflammatory effects of these agents [133].

### 1.4.1 NO-aspirins

NO-aspirin esters incorporate a NO-releasing moiety, which is linked *via* an ester bond to the carboxylic acid of the parent compound and the NO released from these compounds is due to this nitrate group ($R\text{-}O\text{-}NO_2$). NO release counteracts the effects of aspirin, which inhibits the production of prostacyclin (*Fig. 1.10*). The physiological

functions of prostacyclin include protection of the gastric mucosa, dilatation of the coronary arteries and inhibition of platelet aggregation [134]. Since all NO-NSAIDs are nitric acid esters it is assumed that NO formation from these compounds occurs through similar pathways to those responsible for NO generation from glyceryl trinitrate (GTN) and other organic nitrates [145].

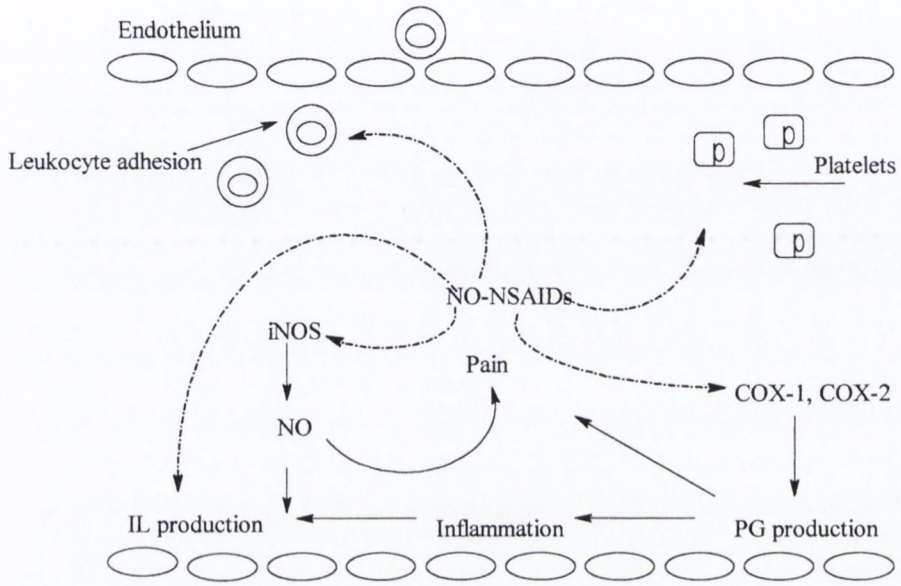



*Figure 1.10 The cellular events underlying inflammatory and thrombogenic processes. The continuous line represent the cell target or a pathway, the dotted line represents sites of NO activity e.g. platelet aggregation and leukocyte adhesion. Abbreviations: iNOS, inducible nitric oxide synthase; IL, interlukin* [135].

Essential to the success of the nitro-aspirin approach is that the drug liberates aspirin rather than salicylic acid *in vivo*, since the latter is a weak and reversible COX inhibitor [136]. A second and potentially more critical design criterion is that nitric oxide release is carefully modulated *in vivo* to avoid some of the problems associated with nitrate administration, such as headache or nitrate tolerance [137].

### 1.4.1.1 Biological effects of NO

Nitric oxide (NO) is synthesised *in vivo* from the amino acid L-arginine by an enzyme known as NO synthase [138]. In the cardiovascular system the release of NO allows the vascular endothelium to respond to changes in its environment and regulates blood

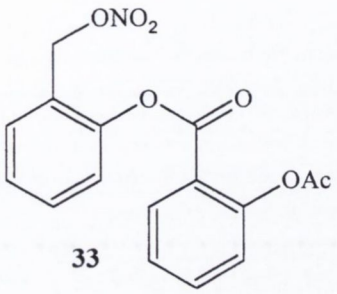
flow and blood pressure through an action on the vascular smooth muscle. In addition, NO plays a role in balancing the interaction between the endothelium and platelets and it may also be involved in the regulation of vascular smooth muscle proliferation [138]. NO acts on vascular smooth muscle and platelets through the stimulation of soluble guanylate cyclase and the elevation of cyclic GMP [138]. Cyclic GMP (cGMP), formed from the activation of soluble guanylate, is a cellular second messenger that mediates vasodilation in response to a variety of drugs and endogenous substances such as nitroso agents. It is also involved in the inhibition of platelet activation [139].

In 1977, Shultz et al. and Katsuki et al. demonstrated independently that the release of NO from vasodilators, either following metabolic transformation, as with glyceryl trinitrate (GTN) or spontaneously as with sodium nitroprusside, produced a dose-dependent increase in the levels of cGMP in smooth muscle [140,141]. It was subsequently shown that the existing nitrovasodilators and NO activate soluble guanylate cyclase [142]. Nitrovasodilators have been used clinically for over 100 years and are still widely used in conditions such as angina pectoris, congestive heart failure, hypertensive emergencies, pulmonary hypertension and fibrinolysis [138]. Nitroglycerin has been used for the treatment of angina pectoris for the last century and several other nitro compounds with similar chemical properties have since been introduced, for example, isosorbide dinitrate (ISDN), and isosorbide-5-mononitrate (IS-5-MN). These compounds all contain a nitrate ester bond (R-O-$NO_2$), which distinguishes the organic esters from nitro compounds, which possess a carbon-nitrogen bond (R-C-$NO_2$).

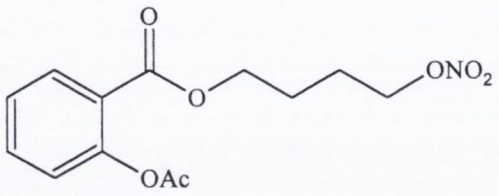
NO is also active in the production of prostanoids with important pharmacological effects on the cardiovascular system. NO released from NO donors activates COX, resulting in an increase in prostanoid production (prostacylin and $TXA_2$) in infarcted heart muscle. NO and prostacylin partially inhibit the actions of $TXA_2$ [143]. NO exerts many of the same actions in the stomach as the prostaglandins [144]. In particular, NO relaxes smooth muscle and is thus thought to counteract the reduction in gastric blood flow caused by inhibitors of prostaglandin synthesis, such as NSAIDs [145]. In the gastric mucosa, NO exerts protective effects against NSAID-induced damage. Lanas et al. carried out a case-control study that determined the

nitrovasodilators are associated with a decreased risk of upper gastrointestinal bleeding [146].

A series of NO-releasing derivatives of aspirin (**33** and **34**) have been developed, which appear to have a better safety profile than aspirin in terms of gastric toxicity [147,135].




**33**




**34**

1.4.1.2 NCX 4016 and NCX 4215

Lechi studied the anti-platelet effect of NCX 4016 (**33**) *in vitro*, and found maximum inhibition of arachidonic acid-induced platelet aggregation at a concentration of 100 μM, whereas aspirin produces the same effect at 10 μM [148]. Fiorucci et al. observed that this aspirin ester **33** stimulated a significant increase in cGMP levels within platelets over a ten-minute incubation period, consistent with the generation of NO [149]. Wallace et al. found that NCX 4215 did not inhibit COX activity, but had significant inhibitory effects on platelet aggregation induced by thrombin (in human platelets), collagen or ADP (in rat platelets) [150]. This pattern indicates that the observed inhibitory effects of esters **33** and **34** occurred by nitric oxide release rather than aspirin release.

NCX 4016 (**33**) (65 mg/kg daily) has been reported by Yamamoto et al. to increase prostacyclin production in infarcted heart muscle, overcoming the inhibitory effect of aspirin [143]. Ester **33** has been reported to inhibit prothrombotic activities in human monocytes [151] and to reduce infarct size caused by myocardial ischemia-repurfusion in the anaesthetised rat [152]. NCX 4016 (**33**) has also been shown to reduce blood pressure in hypertensive rats [153] and to exhibit chemopreventative effects in a rat model of colonic adencarcinoma by a COX-independent pathway [154]. Numerous

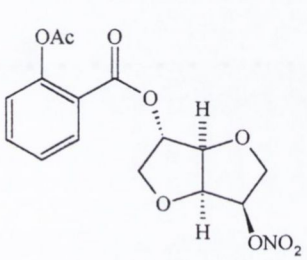
studies [155, 156, 157, 158] including that of Kato et al. [159] who studied the gastric toxicity of this nitroaspirin ester **33** in normal rat stomachs, found that NCX 4016 did not induce damage whereas aspirin, at an equimolar dose, produced haemorrhagic lesions on the gastric mucosa. Al Swayeh et al. have shown that while the anti-oedema activity of NCX 4016 and aspirin are similar, the nitroaspirin appeared to be more potent than aspirin [160]. Ester **34** also appears to impart beneficial effects on the gastric mucosa. Wallace et al. studied the toxicity profile of NCX 4215 (**34**), and found it did not produce macroscopically visible damage in the rat stomach after administration of 300 mg $kg^{-1}$ was administered. An equimolar dose of aspirin however, caused widespread hemorrhagic damage [150].

In summary, NCX 4016 (**33**) was shown to have reduced COX inhibitory potency relative to aspirin while NCX 4215 (**34**) was shown to lack a COX inhibition activity. The pharmacokinetics of these nitro-aspirins has been evaluated and revealed both salicylate and NO-releasing moieties [135]. There is no evidence, such as *in vitro* hydrolysis studies, to show that aspirin is released from the NO-aspirin esters. Therefore, it appears that NCX 4016 (**33**) and NCX 4215 (**34**) are useful NO-releasing esters with little gastric toxicity. However, they cannot be considered as true aspirin prodrugs at this time.

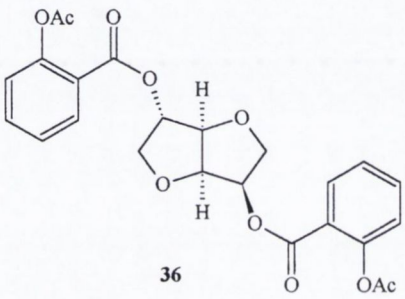
NCX 4016 has most recently been shown to be safe for the gastrointestinal tract in humans, following Phase I clinical endoscopy studies. NCX 4016 has also shown positive Phase I/IIa clinical results, following novel inhibition of vascular inflammation and platelet activation. In light of these results NicOx also announced the initiation of a Phase II trial in symptomatic peripheral aerterial disease [161].

## 1.5 Objectives

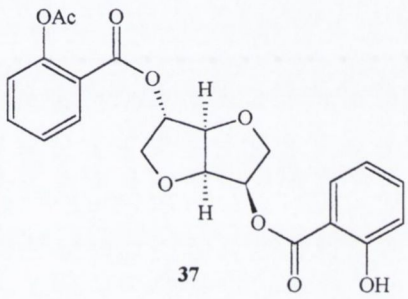
The purpose of our study was to develop prodrug derivatives of aspirin that would be readily absorbed and which would then be rapidly hydrolysed in blood and other tissues to release aspirin. The following chapters describe the development of isosorbide-based aspirin esters. These include isosorbide mononitrate aspirin (ISMNA) (**35**) and isosorbide diaspirinate (ISDA) (**36**). Both aspirin esters were previously synthesised in this laboratory and shown to exhibit an anti-thrombotic response in human, rabbit and dog.




**35**

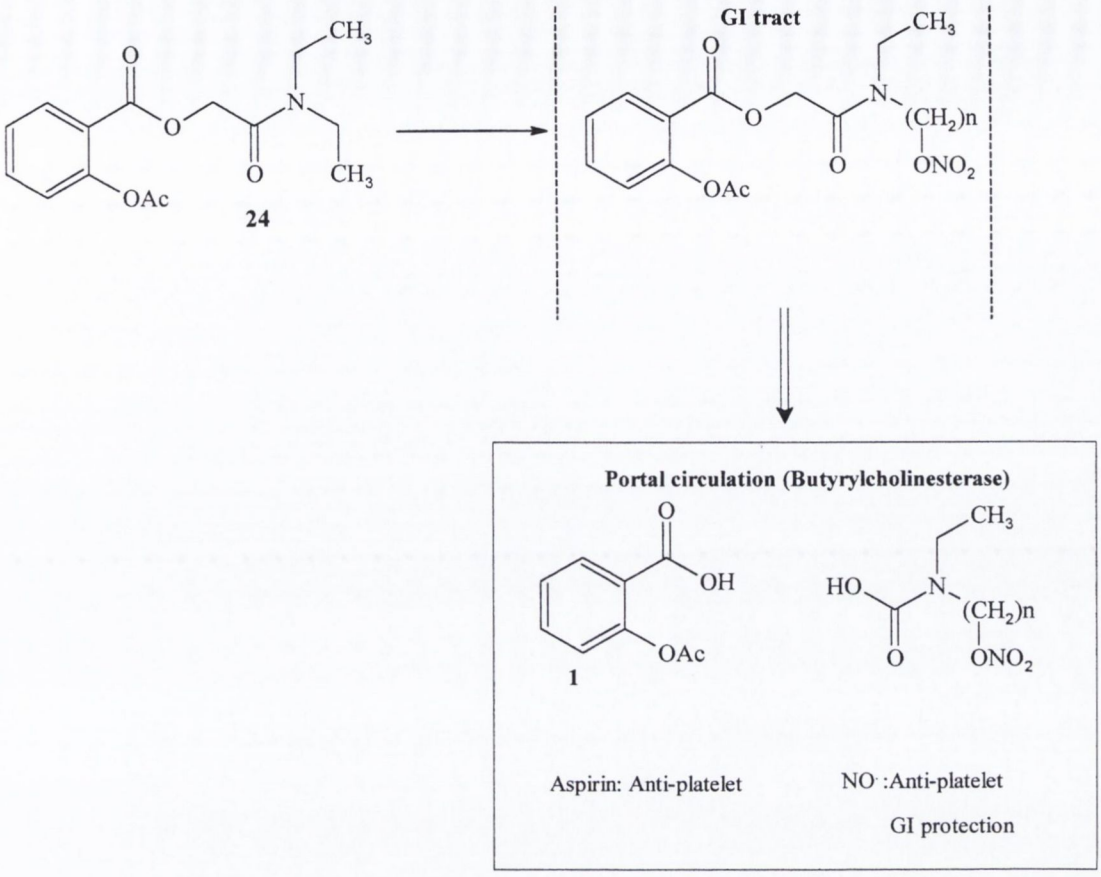



**36**




**37**

The actual pathways of hydrolysis of the aspirin esters **35** and **36** were established following *in vitro* studies. From this work it became apparent that an *in vitro* metabolite of ISDA (**36**), isosorbide-2-aspirinate-5-salicylate (**37**), was a potentially useful aspirin ester. The potential of isosorbide-2-aspirinate-5-salicylate (**37**) as a substrate for butyrylcholinesterase, an enzyme abundant in plasma, was determined. Following this work, modifications were made to ester **37** in an attempt to incorporate a nitric oxide-releasing moiety into its structure and a structure activity relationship (SAR) of isosorbide-based aspirin esters was established.




**24**

**1**

*Figure 1.11 Scheme illustrating the potential of nitric oxide-releasing aspirin esters.*

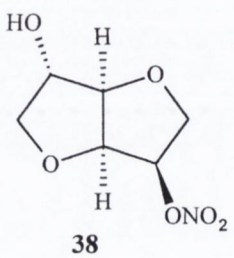
A secondary goal of this thesis was to develop aspirin prodrugs based on known true aspirin prodrugs, such as **24**, with the incorporation of a nitric oxide-releasing functionality (*Fig. 1.11*). These esters were originally designed as substrates for butyrylcholinesterase and are anticipated to cross the GI barrier and undergo cleavage by plasma esterases to liberate aspirin and the nitric oxide donor. The *in vitro* hydrolysis pathways of these esters were examined and their potential as aspirin prodrugs established.

# Chapter 2

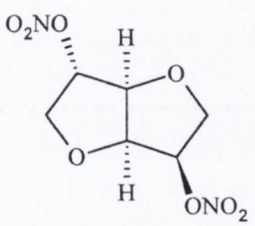
## Synthesis and *in vitro* hydrolysis kinetics of isosorbide mononitrate derivatives of aspirin

## 2.1 Introduction

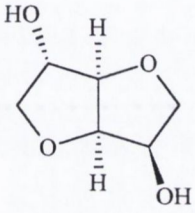
Aspirin prodrugs have been extensively investigated over the years as a means of depressing gastric toxicity or increasing percutaneous absorption. A more recent approach has been the development of the so-called NO-aspirins in which aspirin is connected *via* the ester group to a nitric oxide-releasing moiety (Chapter 1). The approach presented in the current chapter involves the combination of a known organic nitrate with aspirin.




**38**




**39**

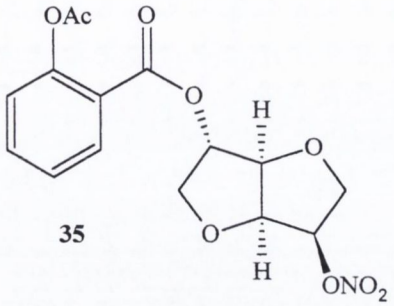



**40**

IS-5-MN (**38**) (isosorbide-5-mononitrate) and ISDN (**39**) (isosorbide dinitrate) are organic nitrate esters used extensively for the treatment and prevention of cardiovascular conditions [162]. They are prepared from isosorbide (1,4-3,6-dianhydro-D-glucitol) (**40**), a carbohydrate with two sterically and electronically different hydroxyl groups [163]. IS-5-MN (**38**) is a metabolite of ISDN (**39**) [164] and the two share similar pharmacological profiles. In order to exert their pharmacological activities they, and all organic nitrate esters, must undergo enzyme-mediated hydrolysis to liberate NO. Clinically, IS-5-MN (**38**) and ISDN (**39**) are used in the treatment of angina pectoris, MI and congestive heart failure [165]. Numerous *in vivo* studies have found that both **38** and **39** elicit a marked decrease in platelet aggregation [166, 167].

ISMNA (**35**) (isosorbide mononitrate aspirin ester) was developed as a potential aspirin prodrug through the combination of IS-5-MN (**38**) with aspirin (**1**). This approach seemed promising since aspirin and IS-5-MN share similar therapeutic effects in the treatment of angina and are frequently co-prescribed. Previously reported NO-aspirins, have exploited novel nitrate groups with unknown nitric oxide release characteristics (Chapter 1) [147]. One of the advantages of the approach presented here is

that IS-5-MN is a clinically successful long-acting nitrate and should therefore, augment aspirin's anti-platelet effects, without producing nitric oxide-associated side effects [137].




35

ISMNA (**35**) was a potent inhibitor of *ex vivo* $TXB_2$ synthesis following oral administration (4 mg $kg^{-1}$) to groups of six beagles [168]. $TXB_2$ is a more stable metabolic product of $TXA_2$ and is therefore, a more suitable index of cyclooxygenase activity *in vivo* [169]. ISMNA (**35**) administration was also associated with reduced aggregation to arachidonic acid *ex vivo* in dog whole blood [168]. Gilmer et al. evaluated ISMNA (**35**) for its ability to inhibit platelet aggregation in rabbit platelet rich plasma induced by the following agonists: arachidonic acid (100 μM), ADP (1.2 μM), phorbol ester (0.5 μM), platelet activating factor (5 nM) and the thromboxane mimic U46619 (1.5 μM). ISMNA (**35**) suppressed platelet response to arachidonic acid at 1 μM whereas 10 μM aspirin showed no inhibitory effects [170]. ISMNA was also administered transdermally in a pilot human study (n = 8) and was associated with marked anti-platelet effects [171].

In order to determine the mechanism of the anti-thrombotic action of ISMNA (**35**), its kinetic pathway was investigated *in vitro* in the present work. Aspirin liberation from ester **35** was not expected during hydrolysis, since there is a well-established route of hydrolysis of aspirin esters (Chapter 1), which involves deacetylation to ultimately liberate salicylic acid and not aspirin. The combination of aspirin and IS-5-MN (**38**) was investigated using two distinct methods to afford ester **35** and a cyclic *ortho* ester (**41**). The potential of both esters for use as aspirin prodrugs was evaluated.

## 2.1.1 Synthesis of ISMNA and ISMN-2-benzodioxin-4-one

Two potentially bioreversible modes of chemically combining aspirin (**1**) and IS-5-MN (**38**) were investigated: orthoester or benzodioxin-4-one formation (**41**) and direct esterification of the aspirin carboxylic acid with IS-5-MN (**38**) [172].

ISMNA was obtained by treating IS-5-MN (**38**) with acetylsalicyloyl chloride in toluene in the presence of triethylamine (*Fig. 2.1(i)*). The method used to prepare the ISMN-2-benzodioxin-4-one (**41**) was based on the synthesis of benzodioxinones reported by Hundewadt and Senning [124]. IS-5-MN (**38**) was stirred with acetylsalicyloyl chloride in anhydrous chloroform for one hour at room temperature, followed by a brief reflux (*Fig. 2.1(ii)*). Both esters were isolated as colourless crystalline material.

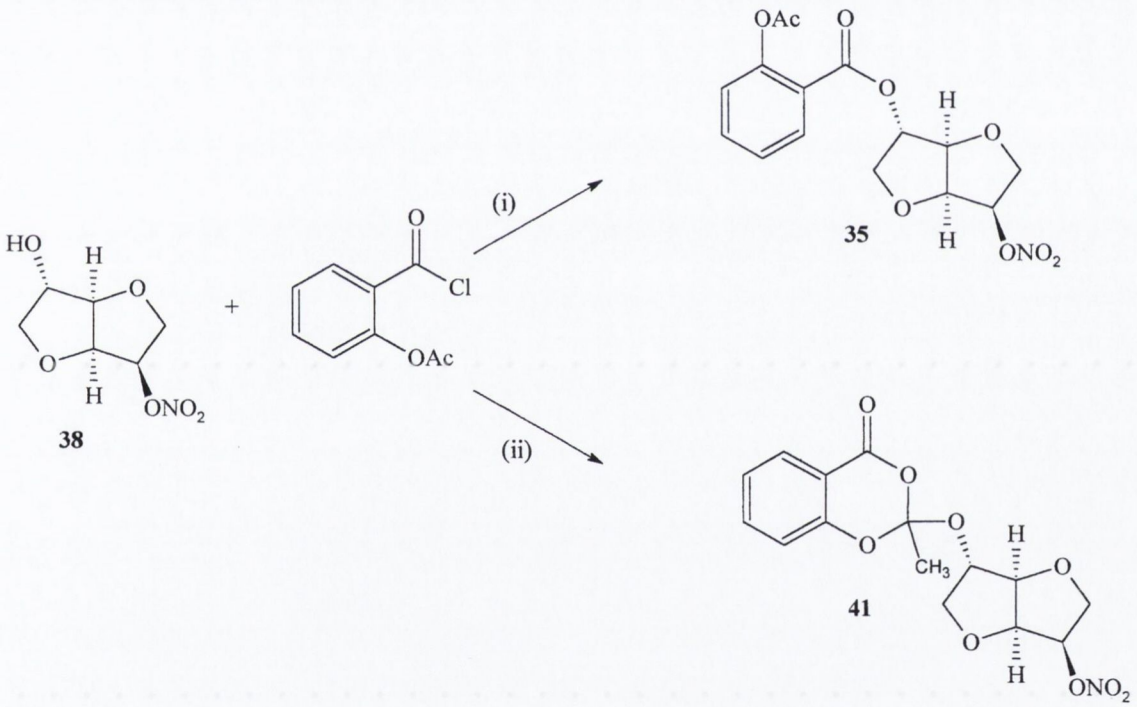



*Figure 2.1 The synthesis of ISMNA and ISMN-2-benzodioxin-4-one: (i) $Et_3N$, 24 hr, 0-15°C, toluene, (ii) 1 hr, chloroform, 25°C followed by reflux for 10 minutes.*

Previous studies on the synthesis and analysis of cyclic *ortho* type esters such as ISMN-2-benzodioxin-4-one (**41**) are described in Chapter 1. It is generally accepted that benzodioxinones are the kinetic product of nucleophilic attack on acetylsalicyloyl chloride, whereas esters are the thermodynamic products [125]. The predominant

influence on these reactions appears to be the presence or absence of base but they are also dependent on solvent, substrate and temperature [127]. In the reaction between acetylsalicyloyl chloride and IS-5-MN (**38**), the role of base was pivotal in determining whether ISMNA (**35**) or ISMN-2-benzodioxin-4-one (**41**) was formed. The temperature and solvent described were determined by trial and error to be optimal for reaction efficiency (chemical yield and time).

Spectroscopic data indicated the presence of the main functional groups of both esters, which were shown to be pure by HPLC. Certain distinguishing features allowed for the comparison of esters **35** and **41**. The IR spectra confirmed the presence of two carbonyl groups in the spectrum of ISMNA (**35**) while one was found in the spectrum of ester **41**. Both the $^{1}H$ and $^{13}C$ NMR spectra showed the acetyl methyl group of ISMNA (**35**). HPLC retention times differed for both esters (10.38 and 11.55 minutes) while the PDA UV data of the isomers also differed: $\lambda_{max}$ of 228 and 274 nm; 238 and 299 nm for esters **35** and **41** respectively. The melting points of esters **35** and **41** were also distinguishable: 80-84°C and 122-125°C respectively.

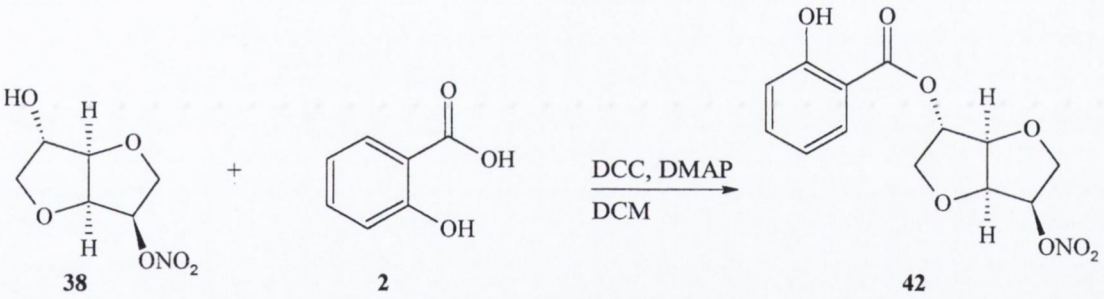



*Figure 2.2 The synthesis of ISMN-salicylate.*

ISMN-salicylate (**42**), a potential metabolite of ISMNA (**35**), was synthesised for use as an external standard in kinetics studies of its parent **35**. The synthesis involved the coupling of IS-5-MN (**38**) and salicylic acid (**2**), in the presence of dicyclohexylcarbodiimide (DCC) catalysed by dimethylaminopyridine (DMAP), as presented in *Fig. 2.2*. In order to prevent self-esterification of salicylic acid (**2**), this reagent was introduced dropwise. Alternatively, a higher equivalence of IS-5-MN (**38**) could be introduced into the reaction vessel, as this excess may be removed by

washing. IR, MS and NMR confirmed the presence of the expected functional groups and the ester was homogenous by HPLC.

## 2.2 Physico-chemical studies

Lipid solubility influences the gastric absorption of acidic NSAIDs and the gastric mucosal impact following oral administration [173]. Prodrugs of aspirin and other NSAIDs are expected to overcome many of the properties responsible for GI damage. The lipophilicity and aqueous solubility of ISMNA (**35**) and ISMN-benzodioxin-4-one (**41**) were studied to determine their potential usefulness as aspirin esters.

### 2.2.1 Lipophilicity

The absorption, distribution and metabolism of a drug involves its passage across the phospholipid membrane of various cells throughout the body. The pharmacological effectiveness of an agent depends on its ability to cross these membranes as well as its ability to impart physiological actions [174]. Lipophilicity is a measure of the extent to which a drug dissolves in a lipid phase relative to an aqueous phase. Ideally, lipophilicity is measured as the partition coefficient between a phospholipid bilayer and water. However, the bilayer is usually replaced, for practical reasons, with a suitable organic solvent such as octanol. The partition coefficient, P, is an equilibrium constant, which may be described by Eq. (2.1), where $k_1$ is the rate constant of drug transport from the aqueous phase to the organic phase and $k_2$ is the rate constant of the reverse process. The partition coefficient (P) is derived from the observed value ($P_{app}$), which can be measured according to Eq. 2.2: $C_o$ and $C_w$ are the concentrations in the organic and aqueous phases while $V_o$ and $V_w$ are the respective volumes [96].

$$P = \frac{k_1}{k_2} \tag{2.1}$$

$$P_{app} = \frac{C_o V_w}{C_w V_o} \tag{2.2}$$

Values are usually reported as the $\log_{10}$ of the partition coefficient (P). A drug that distributes 10:1 in organic medium and water has a log P of 1. It is generally accepted that adequate absorption of orally administered drugs correlates with a log P value of greater than or equal to 2 [121]. If the lipophilicity of a drug is too low, the rate of passive diffusion will be low. However, too high a lipophilicity may result in the majority of the drug remaining in the lipid phase, away from its site of action.

### 2.2.2 Determination of lipophilicity

The lipophilicities of ISMNA (**35**), ISMN-2-benzodioxin-4-one (**41**) and potential metabolites were estimated using SMILES, a computer-based chemical notation system designed for modern chemical information processing. The estimation is based on the calculation of *c*log P values for each fragment in the molecule. The estimated log P values of ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**), aspirin (**1**) and salicylic acid (**2**) are presented in *Table 2.1*. The results suggest that esters **35** and **41** possess optimum lipophilicity required for oral absorption. The results were correlated to standard literature *c*log P values for aspirin (**1**) and benzoic acid, which were 1.19 [119] and 1.87 [175] respectively. The SMILES generated *c*log P of benzoic acid was 1.89.

| Compound No. | *c*Log P | $t_r$ min | $k^1$ | Log $k^1$ |
|---|---|---|---|---|
| **35** | 1.20 | 10.38 | 4.93 | 0.69 |
| **42** | 2.00 | 14.54 | 7.31 | 0.86 |
| **1** | 1.02 | 2.47 | 0.41 | -0.39 |
| **2** | 1.18 | 2.76 | 0.58 | -0.24 |
| **41** | 1.93 | 11.55 | 5.60 | 0.75 |

*Table 2.1 Lipophilic and chromatographic data for ISMNA, ISMN-2-benzodioxin-4-one and potential metabolites.*

Numerous studies have demonstrated a relationship between partition coefficient data and reverse phase HPLC retention [175]. The chromatographic lipophilicity parameter was determined by means of the reverse phase HPLC capacity factor ($k^1$). The capacity factor is taken as a measure of the relative lipophilicity and calculated

according to Eq. (2.2), where $t_r$ is the retention time of the solute and $t_o$ describes the elution time of the system.

$$k^1 = \frac{(t_r - t_o)}{t_o} \qquad (2.2)$$

An isocratic reverse-phase HPLC chromatographic system was employed, which involved elution on a Nova-Pak C8 column (3.9 x 150 mm) at 1 ml/minute, with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%). The retention time and corresponding capacity factor were determined for the analytes, as presented in *Table 2.1*.

A linear relationship was expected between the lipophilicity parameters log $k^1$ and *c*Log P. The departure of ISMNA (**35**) (not shown) from linearity may reflect a different retention mechanism for this compound. A plot for aspirin (**1**), salicylic acid (**2**), ISMN-2-salicylate (**42**) and ISMN-2-benzodioxin-4-one (**41**) appears in *Fig. 2.3* (r = 0.9984).

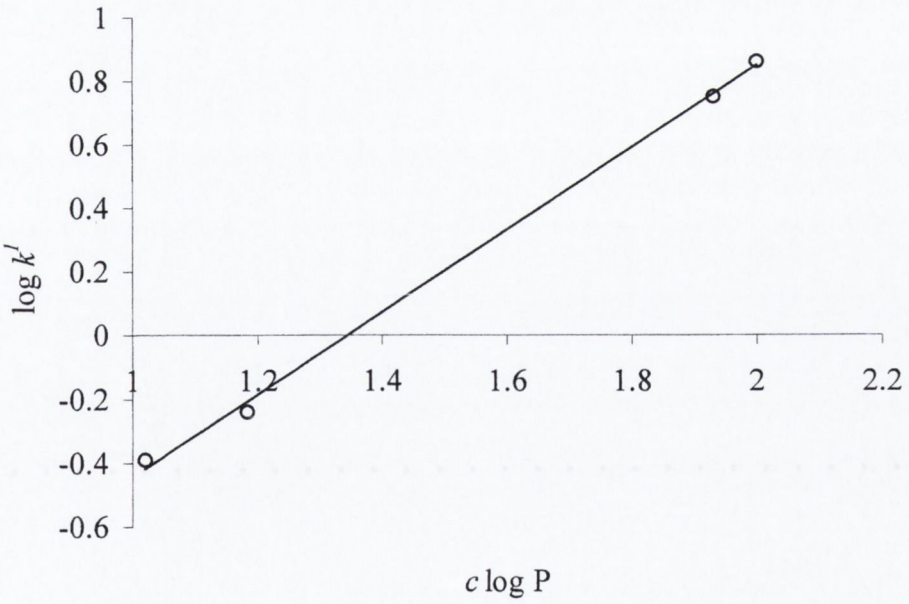



*Figure 2.3 Plot of clog P against log $k^1$ for ISMN-benzodioxinone, aspirin, salicylic acid and ISMN-salicylate.*

The aqueous solubility of ISMNA (**35**) in water at 37°C was 90 µg/ml. Aqueous solubility is important both for formulation and optimal absorption following oral

administration. Attempts to determine the aqueous solubility of ISMN-2-benzodioxin-4-one (**41**) were unsuccessful due to its rapid hydrolysis under aqueous conditions.

## 2.3 Aqueous buffer hydrolysis

For ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**) to be considered as potential aspirin prodrugs, they should reach the pre-systemic circulation intact and subsequently undergo enzyme-mediated hydrolysis to liberate aspirin. The stability of esters **35** and **41** over a range of pH values typically encountered *in vivo* is a critical feature influencing their potential viability. Although it was expected that acid- and base-catalysed hydrolysis might occur to some extent, the rate was predicted to be negligible compared to enzyme-mediated hydrolysis. Their rate and hydrolysis pathways (*Fig. 2.5*) were determined at several pH values.

### 2.3.1 pH 7.4 hydrolysis studies

Beside usage of the various enzyme systems in the body to carry out necessary activation of prodrugs, the buffered and relatively constant physiological pH (pH 7.4) may be useful in triggering the release of a drug from its prodrug form [176]. If so, the prodrug would display a high degree of chemical lability at this pH. In this regard, the stability of ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**) towards hydrolysis at pH 7.4 (37°C) was studied. The hydrolysis was monitored by HPLC on a Nova-Pak C8 (3.9 x 150 mm) column eluted at 1 ml/minute with phosphate buffer pH 2.5 (60%) and acetonitrile (40%). The reactions were monitored for a decrease in ester concentration over time and followed pseudo first-order kinetics. The concentration of ester remaining in solution was calculated from the measured peak areas by reference to those of standards chromatographed under the same conditions at approximately similar concentrations. Pseudo first-order rate constants were calculated from the slope of linear plots of the logarithm of remaining ester concentration versus time according to Eq. (2.3) and the half-lives were calculated according to Eq. (2.4) as presented in *Table 2.2*.

$$k_{obs} = \text{slope x } 2.303 \tag{2.3}$$

$$t_{1/2} = \frac{0.693}{k_{obs}} \tag{2.4}$$

| Compound No. | $k_{obs}$ $h^{-1}$ | $t_{1/2}$ h |
|---|---|---|
| **35** | 0.01 | 48.50 |
| **41** | 0.02 | 32.10 |

*Table 2.2 Kinetic data for the hydrolysis of ISMNA and ISMN-2-benzodioxin-4-one at pH 7.4 (37°C).*

The reported value for the half-life of aspirin at pH 7.4 (37°C) is approximately 18.5 hours [170, 177], which is significantly faster than ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**). The higher aqueous stability of esters **35** and **41** is presumably due to protection of the carboxylic acid functionality, since in aspirin this group is thought to induce intramolecular nucleophilic catalysis when ionised (*Fig. 2.4*) [90]. The carboxylate acts as a general base and generates hydroxide. Hydrolysis may also occur by intermolecular hydroxide attack on the acetyl carbonyl of aspirin. ISMNA (**35**) was more stable then ISMN-2-benzodioxin-4-one (**41**) ($t_{½}$ of 48.5 and 32.1 hours respectively) at pH 7.4 and 37°C. No aspirin was liberated during the hydrolysis of esters **35** and **41**.

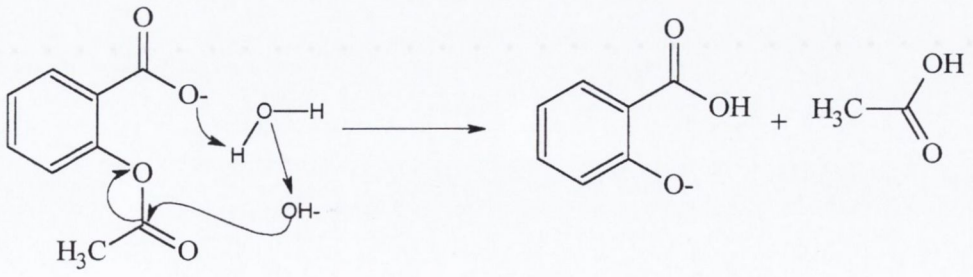



*Figure 2.4 The intramolecular nucleophilic catalysis of aspirin [90].*

### 2.3.2 pH rate profile of ISMNA

The hydrolysis kinetics of ISMNA (**35**) decomposition was monitored in aqueous solution at 37°C over the pH range 1.10-12.0. Typical pH values a drug may encounter after oral administration include pH 6.4 (saliva), pH 7.39 (venous blood), pH 7.40 (arterial blood), pH 5.5 (duodenum) and pH 1.5 (stomach) [178]. Therefore, in order for a drug to reach its target tissue *in vivo* it must be stable over a wide pH range. Stability at low pH is an important aspect of aspirin prodrug design since they are required to pass through the stomach intact.

Buffers, including phosphate, acetate and formate, were prepared with the assistance of the Phenomenex HPLC training program (Calculation Assistant). Total salt concentration was kept constant at 0.0162 M by the addition of sodium chloride. The ionic strength (I) of the buffers was calculated according to the equation derived by Lewis and Rendall (Eq.(2.5)), where $c_i$ is the concentration of the ions present and $z_i$ is the charge [179]. Pseudo first-order rate data for the decomposition of ISMNA (**35**) were calculated from plots of the logarithm of remaining ester versus time. The pseudo first-order rate constants ($k_{obs}$) appear in *Table 2.3*.

$$I = \tfrac{1}{2} \Sigma c_i z_i^2 \qquad (2.5)$$

The rate data indicates the occurrence of specific acid- and base-catalysed hydrolysis. The relative stability of ISMNA (**35**) at pH 1.1, 8.25 and 11.1 is depicted in *Fig. 2.5*. At pH 12.0, ester **35** was subject to rapid hydrolysis with a half-life of 1.5 hours. In comparison, at pH 6.8 the half-life of ester **35** was 301 hours. It can be concluded that ISMNA (**35**) is stable at pH values of between 4 and 6.8. A similar study of aspirin hydrolysis would reveal a significant rate increase in the neutral pH region, as previously described. Ester **35** displayed high stability at pH 1.1, which indicates that this ester might pass through the acidic conditions of the stomach intact.

| pH | I | $k_{obs}$ $min^{-1}$ (x $10^{-2}$) | $t_{1/2}$ min |
|---|---|---|---|
| 1.1 | 0.074 | 0.09 | 753.26 |
| 4.2 | 0.008 | 0.02 | 3013 |
| 6.8 | 0.083 | 0.23 | 301.30 |
| 7.4 | 0.086 | 1.43 | 48.50 |
| 8.25 | 0.088 | 2.46 | 28.17 |
| 9.3 | 0.095 | 21.65 | 3.20 |
| 11.1 | 0.066 | 352.96 | 0.20 |
| 12.0 | 0.092 | 3003.3 | 0.02 |

*Table 2.3 Kinetic data for the hydrolysis of ISMNA over a range of pH, I = ionic strength.*

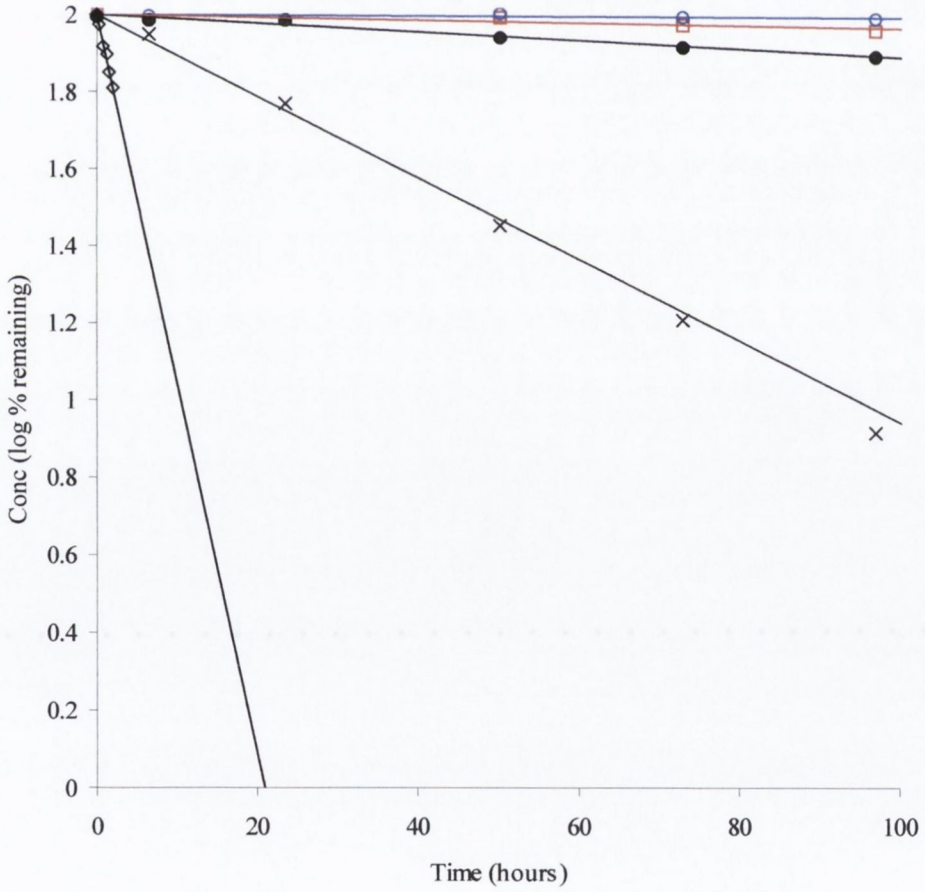



*Figure 2.5 Plot of logarithm of % ISMNA remaining against time, at pH 1.1 (□), pH 4.2 (o), pH 6.8 (●), pH 8.25 (x) and pH 9.3 (◊).*

## 2.4 Plasma hydrolysis studies

For ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**) to possess clinical efficacy, a considerable fraction must be hydrolysed *in vivo* to liberate aspirin as the aspirin ester itself, and its potential metabolites, are expected to be inactive against COX-1. Conversion of prodrugs to the parent drug *in vivo* can take place through a variety of mechanisms. Prodrugs containing carboxyl-protecting functionalities, such as ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**), can be hydrolysed to aspirin by esterases. In the present work, the hydrolysis of esters **35** and **41** was studied in plasma, since it is known to be a rich source of esterases. The following sections contain a brief overview of esterases, determination of enzyme kinetics, possible hydrolysis pathways of esters **35** and **41** and plasma hydrolysis studies of esters **35** and **41**.

### 2.4.1 Esterases

Esterases are a large and diverse group of enzymes present in vertebrates, which are capable of hydrolysing esters, peptides and amides. Ester prodrugs are hydrolysed by esterases in the intestinal mucosa, liver, blood and locally at their site of action [180]. A drug may be hydrolysed by more than one esterase at different sites. In general, esterases have been characterised by their substrate specificity and their sensitivity to a variety of inhibitors [181]. However, esterases can show common properties in relation to substrates and inhibitors (*Fig 2.6*) [182]. With the IEC (International Enzyme Commission) classification (*Table 2.4*), three separate esterase classes are identified: carboxylesterases, arylesterases and cholinesterases. Cholinesterases are primarily involved in drug hydrolysis in plasma; arylesterases in plasma and red blood cells and carboxylesterases in the liver, gut and other tissues [180]. It was anticipated that the esterase responsible for the hydrolysis of esters **35** and **41** in plasma was butyrylcholinesterase (EC 3.1.1.8), an enzyme abundant in plasma. Therefore, the following sections focus on a discussion of the cholinesterases, cholinesterase inhibitors and determination of butyrylcholinesterase activity in the plasma of relevant species.

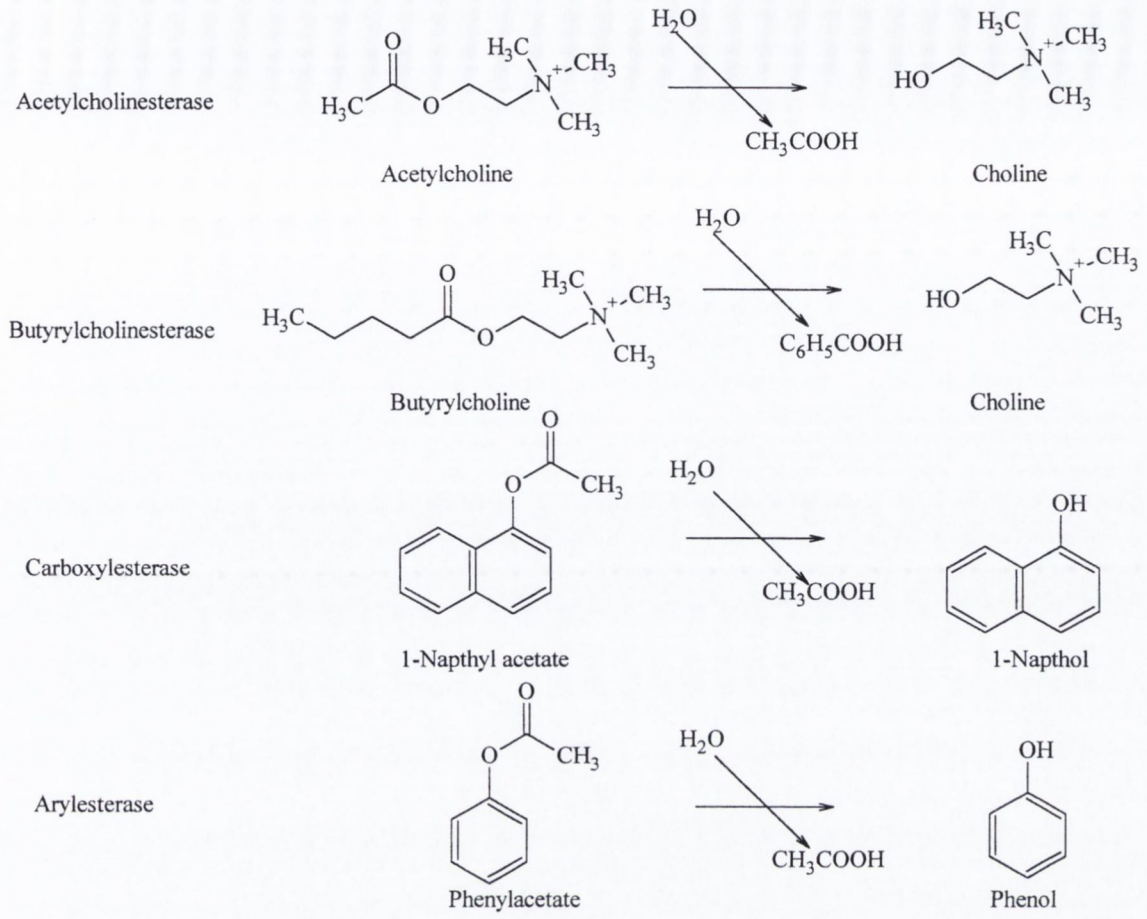



*Figure 2.6 The hydrolysis of model substrates by esterases* [180].

| EC no. | Systematic name | Trivial name | Typical substrate |
|---|---|---|---|
| 3.1.1.1 | Carboxylic ester hydrolase | Carboxylesterase | Aliphatic esters |
| 3.1.1.2 | Aryl ester hydrolase | Arylesterase | Aromatic esters |
| 3.1.1.3 | Glycerol ester hydrolase | Lipase | Triglycerides |
| 3.1.1.6 | Acetic ester hydrolase | Acetylesterase | Acetic esters |
| 3.1.1.7 | Acetylcholine acetyl hydrolase | Acetylcholinesterase (true ChE) | Acetylcholine |
| 3.1.1.8 | Acylcholine acyl hydrolase | Butyrylcholinesterase (pseudoChE) | Acylcholine |

*Table 2.4 Classification of esterases* [180].

### 2.4.2 Cholinesterases

Cholinesterases are present in the circulatory systems of most animals, as well as in the liver, intestinal mucosa and other tissues [180]. Acetylcholinesterase (EC 3.1.1.7) has been detected in the nervous tissue of all animals and in the red blood cells (RBCs) of most species. Silk et al. reported that butyrylcholinesterase (EC 3.1.1.8) is present in smaller amounts in the nervous system of all animals and in the plasma of most species [183]. In humans the highest concentration of BuChE is found in plasma [184]. Several molecular forms of BuChE have been identified in human serum. These forms can be separated into a number of bands using electrophoresis, gel filtration, column chromatography or ultrafiltration [185].

Cholinesterases are unusual among enzymes, since they have a broad specificity towards both charged and uncharged substrates. However, they have a preference for the acylcholine configuration [196]. Sir Henry Dale (1914) first suggested that an esterase capable of hydrolysing acetylcholine exists in blood [186]. Stedman et al. applied the generic name 'cholinesterase' in 1932 [187]. Alles and Hawes (1940) [188] showed that at least two enzymes capable of hydrolysing choline esters exist within the blood of mammals. Subsequently, numerous studies [189,190,191,192] led to their nomenclature as acetylcholinesterase (EC 3.1.1.7, AChE) and butyrylcholinesterase (EC 3.1.1.8, BuChE). The identification of AChE and BuChE was based on their activity against acetylcholine, butyrylcholine and related compounds and on their relative susceptibility towards different inhibitors. AChE is capable of hydrolysing acetylcholine while BuChE hydrolyses acetylcholine but also hydrolyses larger choline esters such as butyrylcholine (from where it takes its name) and benzoylcholine, suggesting that its active site is larger than AChE [193]. While its biological function is unknown, BuChE hydrolyses many drugs including heroin [194], cocaine [195] and aspirin [19] suggesting that it may have a role as a general scavenger of xenobiotic hydrophobic esters [196].

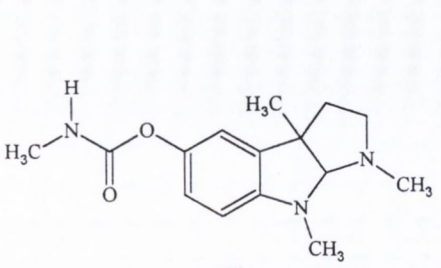
To explain the specificity of the cholinesterases for cationic substrates and inhibitors (particularly those containing a positively charged nitrogen atom linked to methyl or ethyl groups), it was hypothesised that the E-S (enzyme-substrate) complex was formed at two different sites at the active centre of the enzyme: the anionic site and

the esteratic site [197]. The anionic site plays an important role in determining enzyme specificity while the esteratic site is involved in the hydrolytic process. However, Hasan et al. have suggested that the 'anionic site' of the enzyme may not contain a specific negative charge but may be better considered complementary to a trimethyl functionality [198]. Once the substrate is reversibly bound, hydrolysis takes place in two stages. Firstly, the acetyl group (of acetyl choline) is transferred to the serine residue of the esteratic site and choline is released. Secondly, the intermediate acetylated enzyme reacts with water to yield acetic acid and regenerate the active enzyme [180].

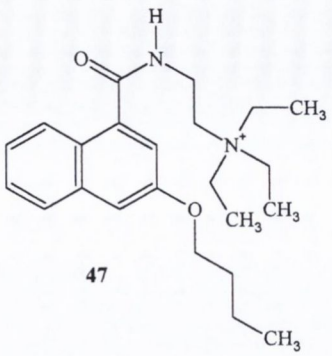
BuChE and AChE show considerable similarity in their protein sequences and in molecular forms. However, differences exist in their expression during tissue differentiation and development [193]. The dimensions and environs of the active site gorge of the cholinesterases [199,200] are important in determining the selectivity of substrate and inhibitors. Saxena et al. have reported that the volume of the BuChE active site gorge is approximately 200 $Å^3$ larger than that of the AChE gorge [201].

### 2.4.3 Cholinesterase inhibitors

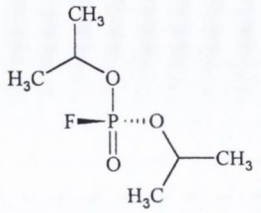
Acetylcholinesterase and butyrylcholinesterase can be distinguished by their substrate specificity and by their reactions with specific inhibitors. Quaternary ammonium bases are classified as strong inhibitors of cholinesterases. Acetylcholine itself is a reversible inhibitor of AChE, since excessive amounts of this substance reduce enzyme activity: this is generally not seen with other esterases. AChE is irreversibly inhibited by organophosphate compounds, such as DFP (**43**) (diisopropylfluorosphosphate), TEPP (tetrakisethoxy dehydroxypyrophosphate) and paraoxon, which bind with the enzyme at the esteratic site. BuChE is much less sensitive to inhibition by excess substrate than AChE, but organophosphates, carbamates, quaternary ammonium salts and eserine are effective. BNPP (bis-4-nitrophenylphosphate) at concentrations of approximately 100 μM, and EDTA (ethylenediamine tetraacetic acid) do not inhibit cholinesterases [202].



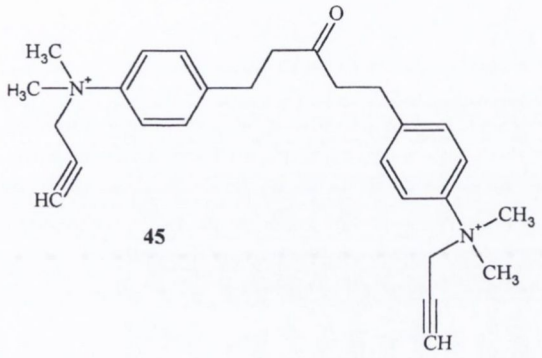
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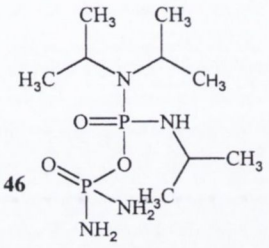
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Of all the agents that inhibit cholinesterases, none can compare in potency with eserine (physostigmine) (**44**) or DFP (**43**). DFP (**43**) inhibits cholinesterases at a concentration of $10^{-11}$ M while cholinesterases are completely inhibited by eserine at a concentration of $10^{-5}$ M [203]. For other esterases significantly higher concentrations of eserine (**44**) are required for inhibition. Specific inhibitors of BuChE and AChE have been found. BW284C51 (1:5-bis (4-allyl-dimethyl) ammoniumphenyl-pentan-3-one) (**45**) is a specific inhibitor of AChE [204] while iso-OMPA (tetraisopropylpyrophosphoramide) (**46**) is a specific inhibitor of BuChE [205]. Dibucaine (**47**) is a unique inhibitor of butyrylcholinesterase [182], since it inhibits an atypical form of BuChE to a different extent than the typical BuChE. This atypical BuChE can also be distinguished by its degree of inhibition by fluoride [180].

## 2.4.4 Determination of plasma cholinesterase activity

Due to the potential importance of butyrylcholinesterase (EC 3.1.1.8) in the hydrolysis of ester **35** we decided to monitor the BuChE activity of donor plasma solutions used in the current work. Numerous procedures are available for the estimation of cholinesterase activity and the result obtained is dependent on the type of assay procedure followed and the substrate used [206]. The most generally successful has been

the method of Ellman et al., which involves the reaction of thiocholine (**48**) with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (**49**) [207, 208]. The liberation of 5-thio-2-nitrobenzoic acid (TNB) (**50**) (yellow colour) is monitored at 412nm *(Fig. 2.7)*. This assay is based upon the measurement of reaction product, not remaining substrate. Butyrylthiocholine is the typical substrate used, since it is the least labile thiocholine ester and is relatively specific for butyrylcholinesterase (EC 3.1.1.8). At pH 7.4, undesirable non-enzymatic hydrolysis is negligible and the assay is linear. A true estimation of cholinesterase activity can be obtained if the assay is carried out at 37°C: physiological temperature. A unit of cholinesterase activity is that producing 1 μmol of choline per minute from substrate [209].

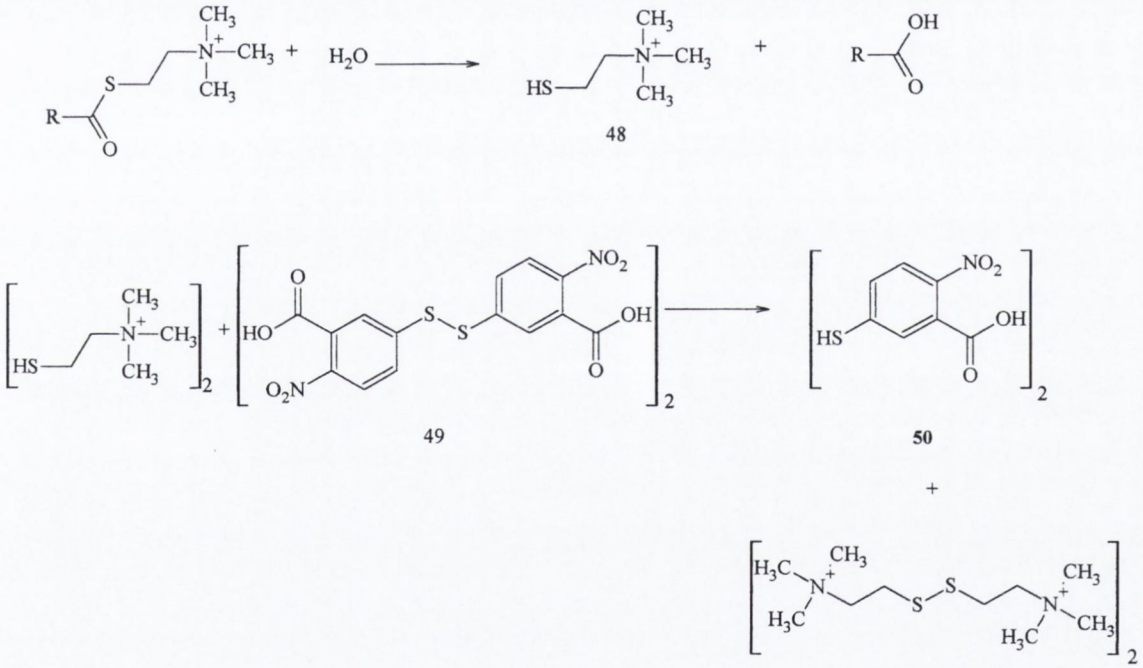


*Figure 2.7 Reactions involved in estimating thiocholine ester hydrolysis [209]. The sulfhydryl groups, liberated by the hydrolysis of thiocholine, react with DTNB to liberate the yellow-coloured 5-thio-2-nitrobenzoate ion (TNB), which can be monitored spectrophotometrically.*

There exists a marked inter-species variation in butyrylcholinesterase activity [210, 211, 212, 213]. *Table 2.5* presents literature values for the BuChE activity of human, rabbit and dog plasma, which were comparable with measured activities (n = 3) in this laboratory.

| Species | Literature BuChE activity (nM/ml of plasma/minute) | Measured BuChE activity (nM/ml of plasma/minute) mean (range) |
|---|---|---|
| Dog | 2360 [214] | 3973 (2122-5828) |
| Rabbit | 750 [205] | 1734 (1656-1812) |
| Human | 3300 [193] | 3474 (1705-5242) |

*Table 2.5 Butyrylcholinesterase activity of dog, rabbit and human plasma.*

### 2.4.5 Determination of Michaelis-Menten kinetics

Kinetic parameters that may be obtained from hydrolysis studies in plasma include $k_{obs}$ and $t_{1/2}$ as well as those parameters relating to the fit of the data to Michaelis-Menten kinetics. Silverman has given a detailed description of these parameters and the influence of the substrate and enzyme on the rate of enzyme-catalysed reactions [215], which is described below.

The simplest form of an enzyme-catalysed reaction is shown in Eq. (2.6), where $k_s$ is the dissociation constant for an ES (enzyme-substrate) complex. The rates of enzyme-catalysed reactions are dependent upon substrate concentration. At low substrate concentration the intial rate is proportional to both $[E]_0$ (the total enzyme concentration) and [S] - a second order reaction. As the substrate concentration increases it becomes easier for the enzyme to find the substrate until all of the enzyme active sites are bound with substrate. At this point the enzyme is saturated and any increase in substrate concentration will not increase the reaction rate. This rate is the maximum rate, $V_{max}$, which is only attained at infinite substrate concentration. At this point the rate is zero order with respect to [S] and is dependent only on [E].

$$E + S \overset{k_s}{\longleftrightarrow} ES \longleftrightarrow EP \longleftrightarrow E + P \qquad (2.6)$$

The $K_m$ is the concentration of substrate that produces half the $V_{max}$. The $K_m$ is a dissociation constant and the smaller the $K_m$ value, the stronger the interaction (the tighter the binding) between E and S and therefore, the higher the concentration of the ES complex. The $K_m$ and $V_{max}$ for an enzyme-catalysed reaction are expressed

quantitatively in the Michaelis-Menten equation (Eq. 2.7), the basic equation of enzyme kinetics.

$$v = \frac{[E]_0 [S] k_{cat}}{K_m + [S]} \tag{2.7}$$

The term $K_{cat}$, the turnover number, is the rate constant for conversion of the ES complex to product (Eq. (2.8)). It is a measure of the rate at which the enzyme catalyses the reaction.

$$K_{cat} [E]_0 = V_{max} \tag{2.8}$$

This equation for $K_{cat}$ (Eq. (2.8)) can now be substituted into Eq. (2.7), which can be further reduced to Eq. (2.9), an alternative form of the Michaelis-Menten equation.

$$v = \frac{[V_{max}] [S]}{K_m + [S]} \tag{2.9}$$

At high [S], the enzyme becomes saturated and is present almost completely in the ES form. Under such conditions, $K_m$ is said to be negligible compared to [S]. Eq. (2.10) describes this and shows that the rate is at its maximum under such conditions.

$$v = \frac{[V_{max}] [S]}{[S]} = V_{max} \tag{2.10}$$

As previously stated $V_{max}$ is independent of [S]. When [S] = $K_m$ Eq. (2.10) can be rewritten as Eq. (2.11), which shows that the rate is at half the maximum value.

$$v = \frac{V_{max} K_m}{K_m + K_m} = \frac{V_{max}}{2} \tag{2.11}$$

At low [S], where S < < $K_m$, [S] is negligible relative to $K_m$, so Eq. (2.9) becomes Eq. (2.12), which shows that the rate of the reaction is now at half the maximum rate.

$$v = \frac{[V_{max}]\,[S]}{K_m} \qquad (2.12)$$

From this equation it can be seen that at low [S], the rate is proportional to [S], where $V_{max}/K_m$ is the proportionality constant.

In typical Michaelis-Menten kinetics, the rate of hydrolysis initially follows zero-order kinetics, and as the substrate concentration is decreased, the rate becomes first-order. This correlates with the initial substrate concentration being higher than the $K_m$. For determinations of $K_m$ and $V_{max}$ values for an enzyme with one substrate, the range of substrate concentrations should be at least 10-fold [216]. In practice the concentration range may be limited at the lower end by sensitivity of the assay method and at the upper end by substrate solubility.

The progression curve of an enzyme-catalysed reaction starts off linear in the initial-rate phase, but falls off with time. Assuming the only cause of this loss of linearity is the decrease of substrate, an integrated form of the initial-rate equation (Michaelis-Menten equation, Eq. (2.7)) can be used to describe the entire progress curve and to obtain a straight-line graph [217, 218].

The computer analysis of progress curves for enzyme-catalysed reactions involves a series of mathematical and computational tasks, which include the derivitisation of an integrated rate equation and the use of this equation in a non-linear regression computer program, where $K_m$ and $V_{max}$ are the parameters to be estimated. The computer program employed in the current work for estimation of Michaelis-Menten parameters was the 'Scientist' Micromath Scientific software [220]. The integrated form of the Michaelis-Menten equation, as reported by Robinson and Characklis, was employed, as shown in Eq. (2.13), where T is an independent variable and S a dependant variable [219]. The unknown parameters are $S_0$, $V_{max}$, and $K_m$. The former was estimated from the intercept of a plot of log remaining compound versus

time. The $V_{max}$ and $K_m$ were given initial estimate values on which the fit was based [220].

$$V_{max} \times T = (S_0 - S) + K_m \times \ln\frac{(S_0)}{S} \qquad (2.13)$$

In the current work, the rates of enzymatic hydrolysis ($k_{obs}$) were obtained from the slope of a linear plot of ln (ester concentration) against time. However, the rates of hydrolysis ($k$) were also estimated from the $K_m$ and $V_{max}$ values (Eq. 2.14), which were obtained by fitting the depletion data of the appropriate ester to the integrated form of the Michaelis-Menten equation (Eq. 2.13). Eq. 2.14 is derived from Eq. 2.12 and Eq. 2.15.

$$k = \frac{[V_{max}]}{[K_m]} \qquad (2.14)$$

$$v = k[S] \qquad (2.15)$$

In general, there was good agreement between the rate constants obtained from the linear plot of the degradation data and from the fit of the degradation data to Eq. 2.13. This validated our methods for estimating the required kinetic parameters.

### 2.4.6 Potential hydrolysis pathways of ISMNA and ISMN-2-benzodioxin-4-one

As previously described, hydrolysis of aspirin esters may occur at the carboxylic acid ester bond ($k_1$) liberating aspirin or at the acetyl group, leading ultimately to salicylic acid *via* the salicylate ester ($k_2$). Evidently, a necessary criterion for any aspirin prodrug is that it undergoes hydrolysis at least partially through the $k_1$ pathway, since salicylic acid is a weak reversible inhibitor of platelet COX-1. The potential hydrolytic pathways of ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**) are presented in *Fig. 2.8* and *Fig 2.9* respectively.

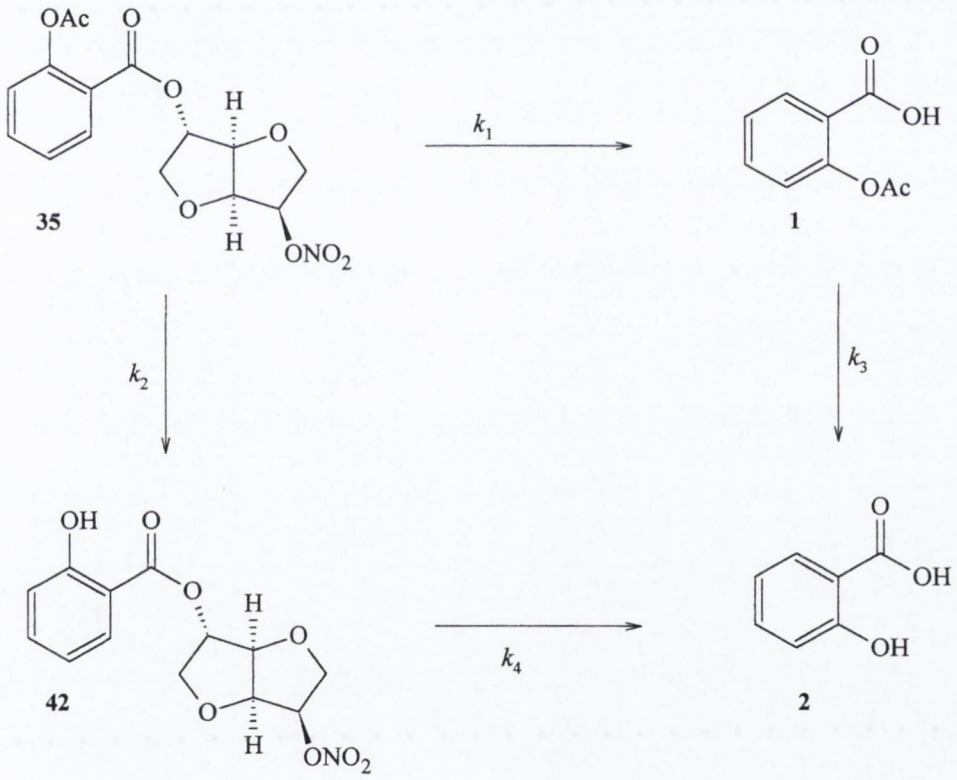



*Figure 2.8 The potential hydrolysis pathways of ISMNA.*

The hydrolysis characteristics that can be envisaged for ortho esters are more complicated than for simple aspirin esters (*Fig. 2.9*). Many of the ortho esters investigated appear to furnish insignificant amounts of aspirin, as hydrolysis occurs *via*

the routes $k_B$ and $k_C$ to liberate salicylic acid. However, some sterically bulky groups appear to promote hydrolysis through the $k_A$ pathway to liberate aspirin [172]. In route $k_B$ the intermediate shows only a transitory existence.

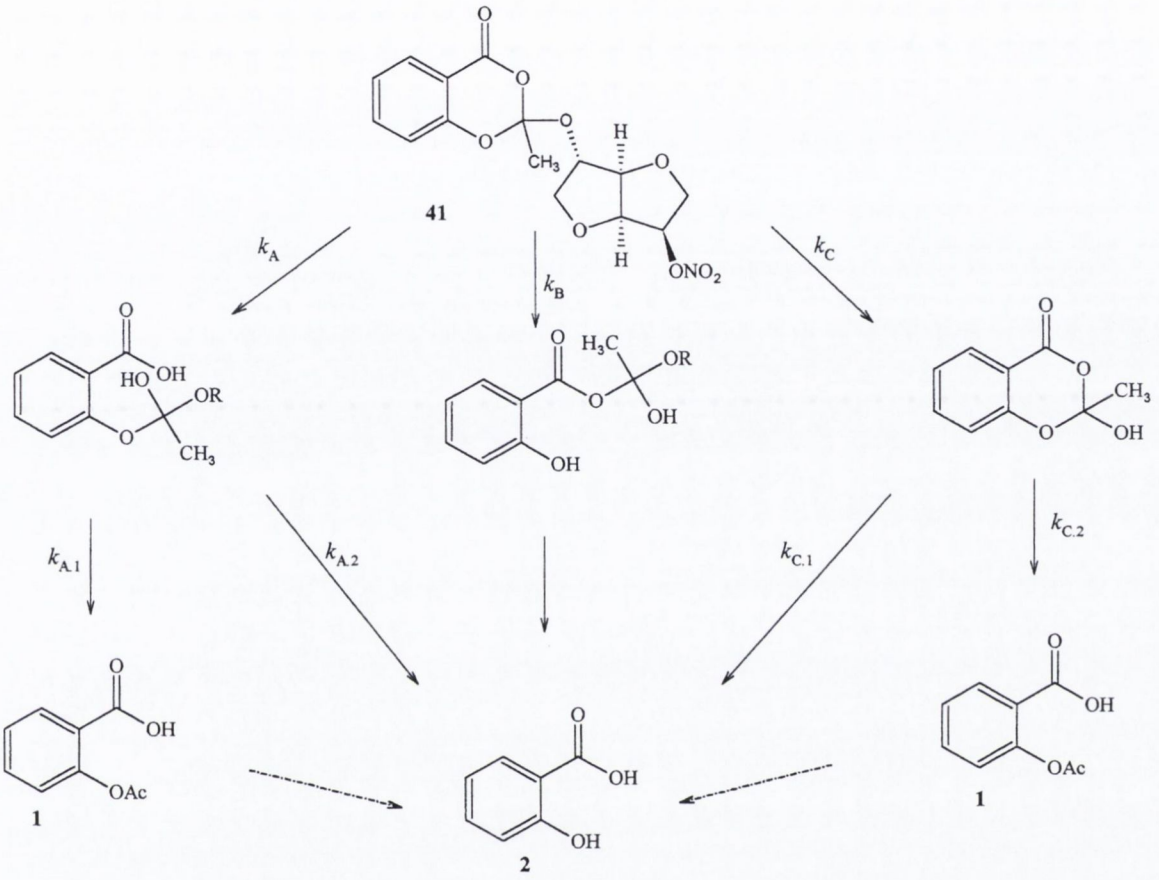



*Figure 2.9 The potential hydrolysis pathways of ISMN-2-benzodioxin-4-one, R = IS-5-MN* [99].

## 2.4.7 Procedures in plasma hydrolysis studies

The hydrolysis of esters **35** and **41** was studied in plasma solution to determine their potential for aspirin release. The hydrolysis of ester **35** was studied in dog plasma to determine whether aspirin release in that species could explain its observed anti-platelet effect. Similarly, the hydrolysis of ester **35** was studied in rabbit plasma to determine if aspirin release could explain its anti-thrombotic effect in this species [170]. The anti-platelet effect in dog and rabbit had provided encouragement for the development of ester **35** as an anti-platelet agent in humans. The hydrolysis of ISMNA (**35**) in human plasma was studied in this context.

Plasma was obtained by centrifugation of whole blood, which had been collected by venipuncture and treated immediately with anti-coagulant (sodium citrate). The ratio of blood to anti-coagulant used varied between species studied, as presented in *Table 2.6*.

| **Species** | **Anti-coagulant** | | **Volume blood** | **Total volume** |
|---|---|---|---|---|
| | Conc. (%w/v) | Volume (ml) | ml | ml |
| Dog | 3.8 | 13 | 8 | 21 |
| Human | 3.8 | 1 | 9 | 10 |
| Rabbit | 3.8 | 13 | 8 | 21 |
| Rat | 3.8 | 2 | 6 | 8 |
| | 3.8 | 1 | 9 | 10 |

*Table 2.6 Preparation of blood samples.*

The final samples were analysed by reverse phase HPLC using a mobile phase that afforded good separation of the esters and potential metabolites. The method involved isocratic elution on a Nova-Pak C8 column (3.9 x 150 mm) with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) at 1 ml/minute. Interferences from the sample treatment step did not impact on the separation. The concentration of analytes in the plasma was calculated from the measured peak areas by reference to those of standards chromatographed under the same conditions. The chromatographic method was validated for linearity, precision, specificity and sensitivity. A linear response was observed for each analyte ($r > 0.999$) in the range 1-100 μg/ml. The RSD on multiple injection of each analyte at 10 μg/ml and 100 μg/ml was <1.5%. The limit of quantitation for the relevant analytes was 1 μg/ml.

The efficiency of the method (recovery and stability) was determined using toluic acid as an internal standard [221]. The recovery of toluic acid from plasma was 86.81% ± 1.65%. Abu-Qare and Abou-Donia have reported that the recovery of toluic acid from rat plasma, following a solid extraction sample treatment step, was 82.8 ± 3.9% [222]. Due to the more complex nature of biological fluids such as plasma, the RSD of biological assays is typically higher than for chemical assays. The incomplete

recovery of toluic acid may be attributed to protein binding of the acid prior to the centrifugation step and also incomplete removal of proteins from the plasma solutions. The assay is comprised of many steps, which may each have a contributory factor to the reduced recovery. However, some compounds are more susceptible to protein binding than others (Section 1.2.2).

### 2.4.8 Dog plasma hydrolysis studies

The hydrolysis of ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**) was studied in 10% buffered dog plasma (pH 7.4) at 37°C. The hydrolysis was observed to follow pseudo first-order kinetics over several half-lives. The half-life and rate constant ($k_{obs}$) data were determined from a plot of the logarithm of remaining ester concentration against time and are presented in *Table 2.7*. The hydrolysis proceeded rapidly with the half-life of ester **35** being considerably shorter than that for ester **41**. The $V_{max}$ was similar for both esters while the $K_m$ value for ISMNA (**35**) was lower than ISMN-2-benzodioxin-4-one (**41**). This indicates a stronger affinity of ISMNA (**35**) for the enzyme in dog plasma responsible for the hydrolysis, presumably BuChE. No significant inter-breed differences were observed when the experiment was performed in Labrador or Beagle plasma.

| **Compound No.** | $K_m$ | $V_{max}$ | $t_{1/2}$ | $k_{obs}$ | |
|---|---|---|---|---|---|
| | M (x $10^{-4}$) | M $min^{-1}$ (x $10^{-4}$) | min | $min^{-1}$ | |
| | | | | mean | (s.d, n) |
| **35** | 1.260 | 0.101 | 10.81 | 0.07 | (± 0.005, n = 2) |
| **41** | 5.530 | 0.102 | 42.39 | 0.02 | |

*Table 2.7 Kinetic data for the hydrolysis of ISMNA and ISMN-2-benzodioxin-4-one in 10% dog plasma at pH 7.4 and 37°C.*

A typical progression curve for the hydrolysis of ISMNA (**35**) in 10% dog plasma appears in *Fig. 2.10*. The predominant route of hydrolysis was *via* deacetylation, to liberate ISMN-salicylate (**42**). Aspirin (**1**) and salicylic acid (**2**) were minor products of the hydrolysis (<0.5%). ISMN-salicylate (**42**) was stable towards enzymatic hydrolysis, remaining essentially unchanged when liberated. This observation may be

due to a structural feature of ester **42**, which reduces its fit to the active site of dog plasma BuChE. Hydrolysis of ISMN-2-benzodioxin-4-one (**41**) in dog plasma was associated with the liberation of salicylic acid (**2**) and ISMN-acetate.

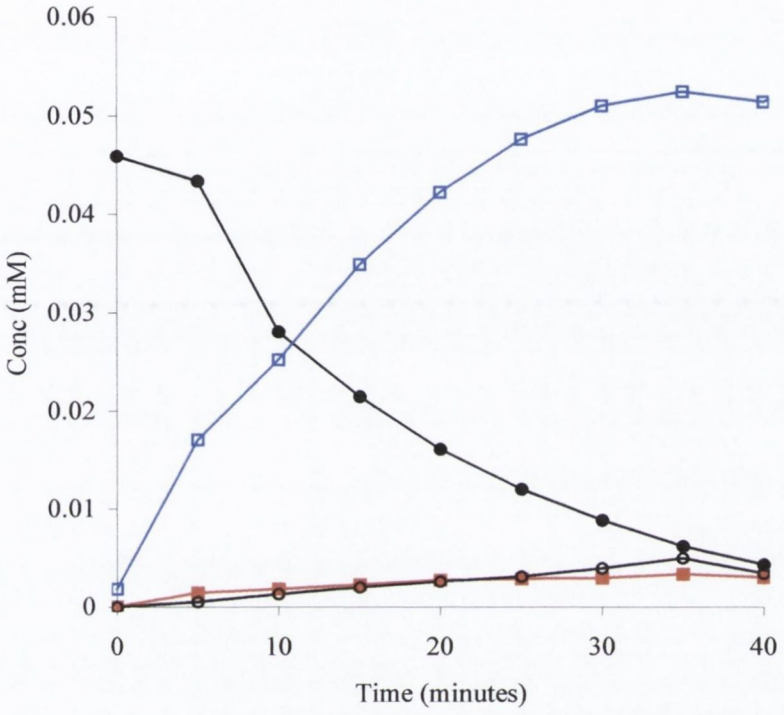



*Figure 2.10 Typical progression curve for the hydrolysis of ISMNA in 10% dog plasma at pH 7.4 and 37°C: ISMNA (●), ISMN-salicylate (□), aspirin (■) and salicylic acid (o).*

The results of this study do not explain the anti-platelet effects of ISMNA (**35**), observed in an earlier study, following oral administration to beagles. A possible explanation of these results is that other enzymes in the dog that possess different catalytic activity to butyrylcholinesterase, may be responsible for aspirin release. Experiments are planned to test this hypothesis using dog gut or liver homogenate.

### 2.4.9 Human plasma hydrolysis studies

The hydrolysis of ISMNA (**35**) and ISMN-2-benzodioxin-4-one (**41**) was studied in 10% buffered human plasma (pH 7.4) at 37°C. The hydrolysis of both esters followed pseudo first-order kinetics as shown in the kinetic data presented in *Table 2.8*.

The slower hydrolysis of ester **41** implies that ISMN-2-benzodioxin-4-one (**41**) is more stable towards enzymatic hydrolysis in human plasma solution than ISMNA (**35**), presumably due to a poorer fit to the active site of human plasma cholinesterase.

| Compound No. | $K_m$ | $V_{max}$ | $t_{1/2}$ | $k_{obs}$ | |
|---|---|---|---|---|---|
| | M (x $10^{-4}$) | M $min^{-1}$ (x $10^{-4}$) | min | $min^{-1}$ | |
| | | | | mean | (s.d, n) |
| **35** | 11.45 | 11.66 | 0.90 | 0.78 | (± 0.099, n = 3) |
| **41** | ____ | ____ | 19.25 | 0.04 | |

*Table 2.8 Kinetic data for the hydrolysis of ISMNA and ISMN-2-benzdioxin-4-one in 10% human plasma at pH 7.4 and 37°C.*

A typical progression curve for the hydrolysis of ester **35** in 10% human plasma is presented in *Fig. 2.11*. The hydrolysis was associated with the liberation of a mixture of ISMN-salicylate (**42**), salicylic acid (**2**) and aspirin (**1**). Ester **35** released 6.59-8.43% aspirin during its hydrolysis with a mean value of 7.13% (n=3), based on initial ester concentrations. Nielsen and Bundgaard tested a number of compounds in dilute plasma and found that in most instances <0.5% aspirin was liberated, due to very rapid hydrolysis at the acetyl group [96]. The unusual preference for hydrolysis at the carboxylic ester group of ester **35** is most likely due to a structural feature of the ISMN group, which promotes enzymatic attack at the carboxyl carbon and suppresses attack at the acetyl group.

A number of studies have shown that ISMNA possesses a weak anti-platelet effect in humans. McCafferty and Malcom observed a weak anti-thrombotic effect when ISMNA was tested in a human pilot transdermal trial with eight patients [171]. In human PRP it was determined that the $IC_{50}$ of ISMNA was approximately 85 μM whereas aspirin possesses an $IC_{50}$ of approximately 8 μM, under similar conditions [223]. The observed weak anti-thrombotic effect of ISMNA may be attributed to the 7.13%

aspirin released in dilute human plasma. However, this concentration of aspirin might not be sufficient for ISMNA (**35**) to be clinically useful.

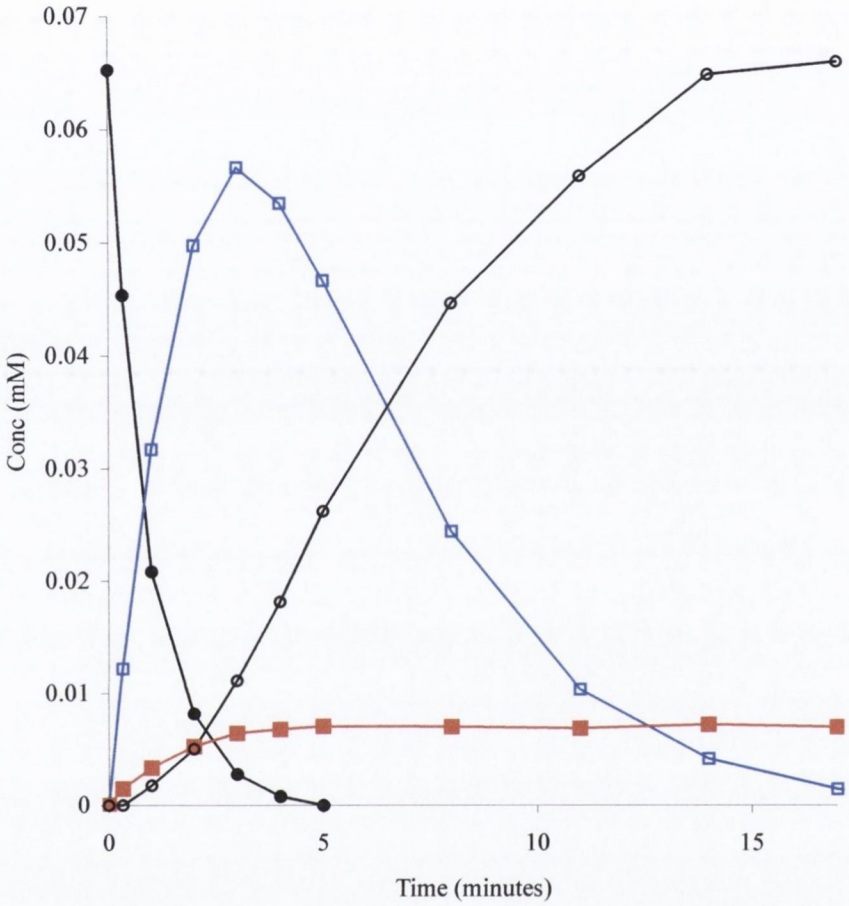



*Figure 2.11 Typical progression curve for the hydrolysis of ISMNA in 10% human plasma at pH 7.4 and 37°C: ISMNA (●), ISMN-salicylate (□), aspirin (■) and salicylic acid (○).*

In human plasma, the salicylate ester **42** (blue line in *Fig. 2.11*) showed only a transitory existence due to its rapid hydrolysis to salicylic acid (**2**). This contrasts with the stability of ester **42** in dog plasma solution (*Fig. 2.10*). Typically, the rate of plasma-mediated hydrolysis of salicylate esters is slow. For example, methyl salicylate hydrolyses in 80% human plasma with a half-life of 17.6 hours [89]. It would appear that the ISMN group promotes rapid hydrolysis of the salicylate ester in human plasma solution.

The hydrolysis of ester **42** was studied in 10% buffered human plasma (pH 7.4) at 37°C, to confirm its susceptibility to enzyme-mediated hydrolysis. The study indicated that ISMN-salicylate (**42**) was itself highly susceptible to hydrolysis in human plasma solution. The reaction followed pseudo-first order kinetics with a rate constant of 0.055 $min^{-1}$ and a corresponding half-life of 12.6 minutes. However, the degradation data displayed a poor fit for the Michaelis-Menten model.

The hydrolysis of ISMNA (**35**) was studied over a range of plasma concentration (37°C) to determine the influence of esterases on its half-life and hydrolytic product distribution. Plasma solutions were prepared by the appropriate dilution in phosphate buffer pH 7.4. The pseudo first-order rate parameters for the hydrolyses are presented in *Table 2.9*. As expected, the more rapid hydrolysis was observed when ISMNA was incubated at the higher plasma concentrations, presumably due to the presence of higher concentrations of plasma butyrylcholinesterase.

| **% Plasma** | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ mean (s.d., n) | **% aspirin formed** mean (range) |
|---|---|---|---|
| 2 | 2.55 | 0.27 | 9.78 |
| 5 | 2.13 | 0.33 (± 0.006, n = 2) | 9.70 (7.26-12.15) |
| 10 | 0.90 | 0.78 (± 0.099, n = 3) | 7.13 (6.59-8.43) |
| 15 | 0.59 | 1.20 (± 0.156, n = 3) | 10.95 (8.9-11.88) |
| 20 | 0.50 | 1.35 (± 0.074, n = 2) | 7.80 (7.1-8.5) |
| 30 | 0.22 | 3.16 (± 0.114, n = 2) | 5.89 (5.17-6.6) |

*Table 2.9 Kinetic data for the hydrolysis of ISMNA in human plasma at pH 7.4 and 37°C.*

As expected, a plot of the logarithm of the rate constant for hydrolysis against percent plasma was linear (*Fig. 2.12*, r = 0.9788), which indicates enzyme-mediated hydrolysis. The slight deviation from linearity observed may be attributed to the sample treatment step. At higher plasma concentrations the samples contain more proteins, which might not be completely removed by centrifugation. Consequently, higher protein binding of

the substrate might lead to an underestimation of analyte detected.

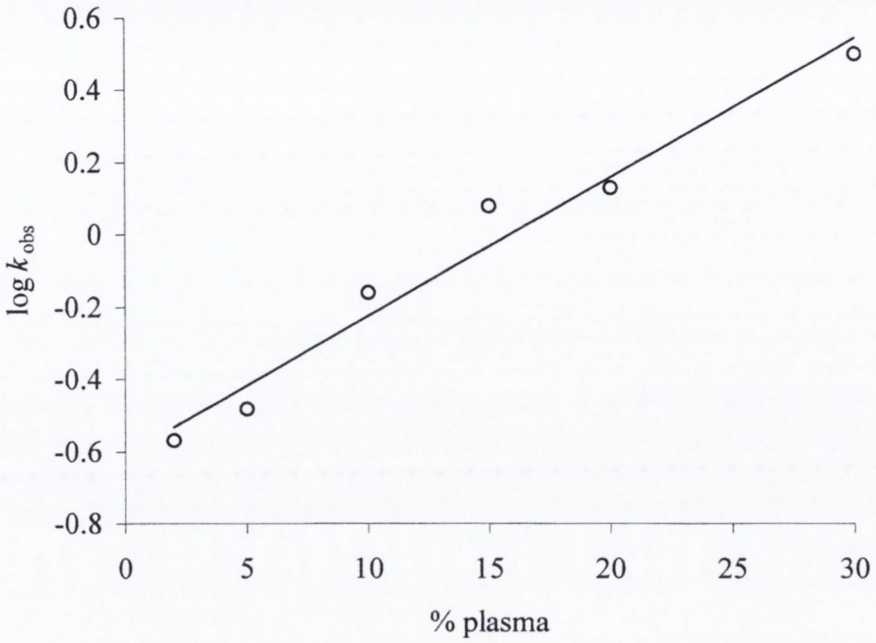



*Figure 2.12 Plot showing correlation between % plasma (human) and $K_{obs}$.*

The influence of human plasma BuChE activity on the hydrolysis of ester **35** was investigated. The butyrylcholinesterase activity of plasma from a number of healthy human volunteers was measured according to the method described in Section 2.4.4 and subsequently the hydrolysis was followed in 10% buffered plasma (pH 7.4) at 37°C. The hydrolyses followed pseudo first-order kinetics, as shown in the kinetic data presented in *Table 2.10*.

| Source | Plasma BuChE activity nM/ml plasma/min | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ | % Aspirin formed |
|---|---|---|---|---|
| A [I] | 3443 | 0.75 | 0.92 | 7.88 |
| B [II] | 2738 | 1.05 | 0.66 | 8.96 |
| C [II] | 3912 | 0.68 | 1.03 | 6.51 |

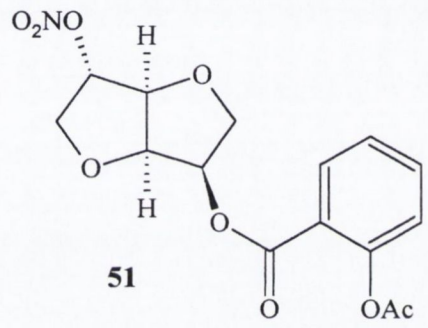
*Table 2.10 Kinetic data for the hydrolysis of ISMNA in three sources of human plasma at 37°C: [I] = female, [II] = male.*

Chatonet has reported that adult human plasma contains an average of 3300 ng of BuChE/ml and 8 ng of AChE/ml as measured by immunosorbent assay [193]. The variation in cholinesterase activity observed in the present study (*Table 2.10*) was not surprising since it has been well established that a significant variation exists in the blood plasma cholinesterase activity of normal human subjects exists. In general, the percent variation of butyrylcholinesterase (EC 3.1.1.8) activity is significantly greater than that of acetylcholinesterase (EC 3.1.1.7) [224]. Vorhaus and Kark claimed that although the cholinesterase activity varied widely from one individual to another, the level of each individual was 'remarkably constant' from day to day and from month to month [225]. The inter- and intra-individual variation in the butyrylcholinesterase activity of plasma from a number of healthy volunteers is presented in *Table 2.11*. The RSD of the method for determination of BuChE activity (n = 3) was 1.81%.

| **Plasma source** | **Duration of plasma storage** | **BuChE activity** | | |
|---|---|---|---|---|
| | days | nM/ml plasma/min | | |
| $A^{I}$ | 1 day | 3043[a] | 2668[a] | 2269[a] |
| | 7 days | 2777 | | |
| | 21 days | 2895 | | |
| $B^{I}$ | 1 day | 2879[a] | 2504[a] | |
| | 7 days | 2652 | | |
| | 21 days | 2817 | | |
| $C^{II}$ | 1 day | 3364[a] | 3372[a] | |
| | 7 days | 3020 | | |
| | 21 days | 3443 | | |
| $D^{II}$ | 1 day | 3599[a,b] | 2730[a] | |
| | | 3521[b] | | |
| | | 3543[b] | | |
| | 21 days | 3286 | | |

*Table 2.11 Plasma cholinesterase activities of a number of human plasma sources; I = female, II = male, [a] = determinations repeated from the same source using plasma sampled at different times, [b] = determinations on the same plasma sample as a test for precision of the method, RSD = 1.81 %.*

In order to gain a better insight into the true hydrolytic picture of ISMNA (**35**) *in vivo*, its hydrolysis in human whole blood was investigated. A higher concentration of the protein-precipitating agent ($ZnSO_4$) in the sample treatment step was required, since blood is a more complex biological medium than plasma. The modified sample treatment step was considered successful, since no matrix peaks co-eluted with the peaks of interest. The rapid hydrolysis ($t_{1/2}$ of 13.37 seconds; $k_{obs}$ of 0.052 $sec^{-1}$) occurred *via* deacetylation. The observation that the hydrolytic rate in whole blood was more rapid than in plasma solution suggests the presence of higher concentrations of the hydrolysing enzyme or additional enzymes in the red blood cells. Red blood cells possess a high content of acetylcholinesterase (EC 3.1.1.7) [196].




**51**

Isosorbide-5-aspirinate-2-mononitrate (**51**) is an isomer of ISMNA (**35**), which was synthesised as detailed in Chapter 3 (Section 3.1.1). The hydrolysis of ester **51** was studied in 10% buffered human plasma (pH 7.4) at 37°C to determine whether a structure activity relationship may be observed between isosorbide mononitrate aspirin esters. The hydrolysis proceeded with a pseudo first-order rate constant of 0.52 $min^{-1}$ and a corresponding half-life of 1.33 minutes, with the exclusive liberation of ISMN-5-salicylate. It appears that the position of the aspirinate group on the isosorbide ring dictates the pathway of hydrolysis for aspirin esters of this type.

It was of interest to note that ISMN-5-salicylate, liberated during the hydrolysis, remained essentially unchanged. In contrast, ISMN-2-salicylate (**42**) underwent rapid hydrolysis in dilute human plasma, as previously described (*Fig. 2.11*). A rapid hydrolysis of the 2-salicylate was expected since the 2-position (*exo*) is more exposed than the sterically hindered *endo* 5-position. This explains why IS-5-

MN (**38**) is a clinically more useful organic nitrate than IS-2-MN [165]. The rapid release of NO from IS-2-MN has been associated with some of the adverse effects of nitrate administration including headache and nitrate tolerance [137]. However, such a rapid hydrolysis of the 2-ester is more likely related to its fit in the active site of human BuChE and not its *exo* conformation.

### 2.4.10 Rabbit plasma hydrolysis studies

The hydrolysis of ISMNA was studied in 10% buffered rabbit plasma (pH 7.4) at 37°C, in triplicate (RSD: 0.023%), the results for which are presented in *Table 2.12*.

| $K_m$ M (x $10^{-4}$) | $V_{max}$ M $min^{-1}$ (x $10^{-4}$) | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ | % Aspirin formed |
|---|---|---|---|---|
| 0.333 | 0.156 | 3.30 | 0.21 | 78.69 |
| 0.198 | 0.143 | 3.21 | 0.22 | 76.5 |
| 0.356 | 0.160 | 3.85 | 0.18 | 71.35 |

*Table 2.12 Kinetic data for the hydrolysis of ISMNA in 10% rabbit plasma at 37°C.*

The hydrolysis of ISMNA (**35**) in 10% rabbit plasma is presented in *Fig. 2.13* and *Fig. 2.14*. Remarkably, the hydrolysis liberated up to 78.7% aspirin, based on initial ester concentrations. This is surprising since, in dog and human plasma solution, deacetylation was the predominant route of hydrolysis. This is the most favourable aspirin release reported from any aspirin ester to date.

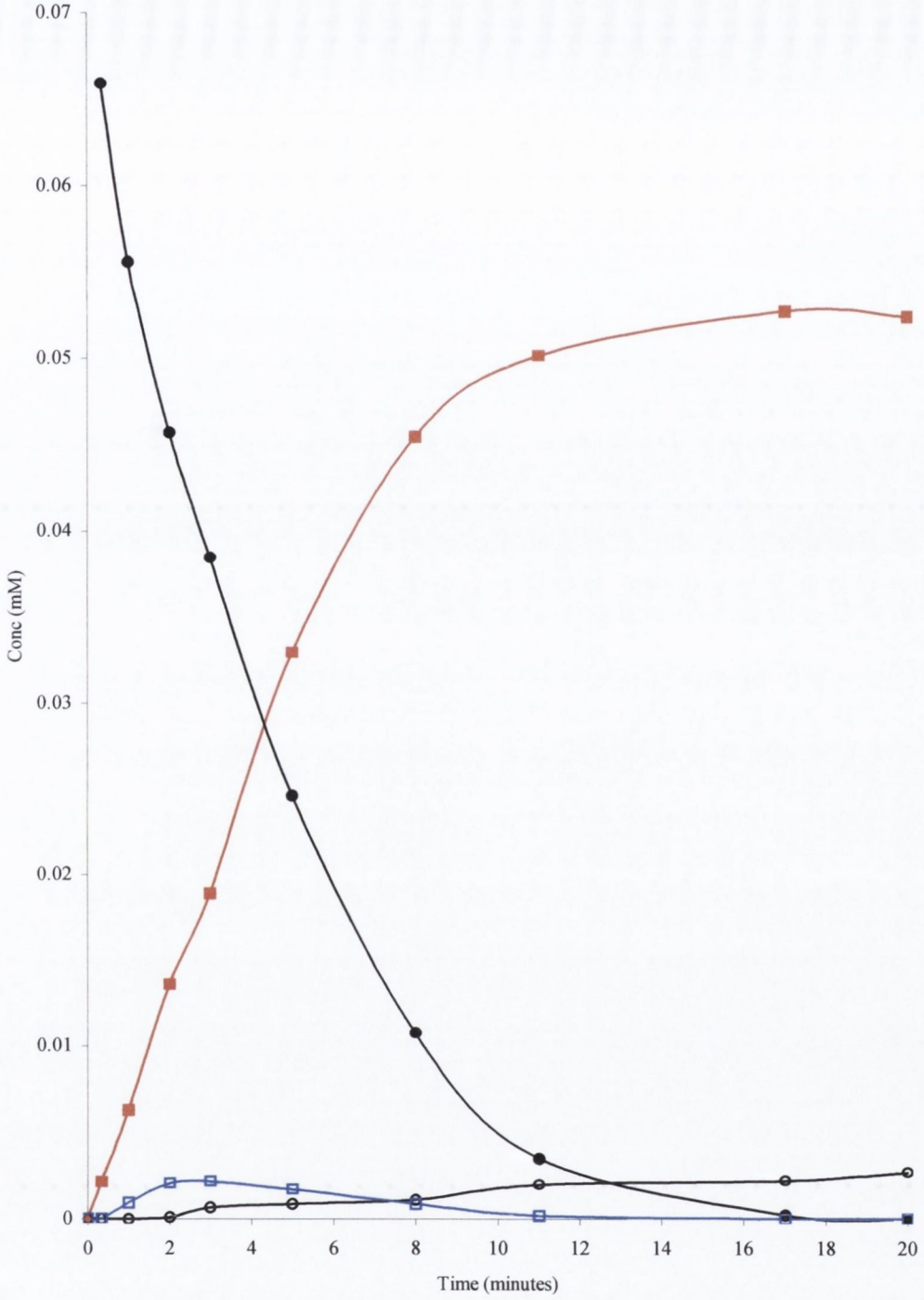



*Figure 2.13 Typical progression curve for the hydrolysis of ISMNA in 10% rabbit plasma at pH 7.4 and 37°C: ISMNA (●), ISMN-salicylate (□), aspirin (■) and salicylic acid (○).*

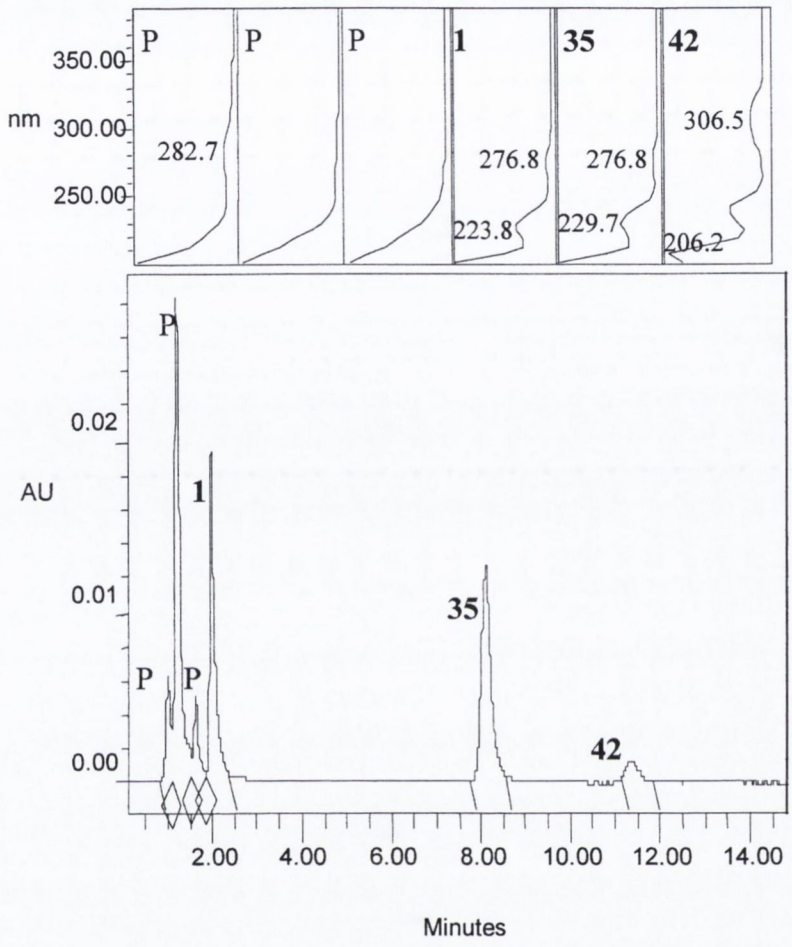



*Figure 2.14 Typical chromatogram (230 nm) of a sample obtained following incubation of ISMNA in 10% rabbit plasma at pH 7.4 and 37°C for 4 minutes. Also shown are PDA spectra of each of the labelled components.* ***P*** *represents plasma peaks present in the plasma blank. Aspirin (**1**), ISMNA (**35**) and ISMN-2-salicylate (**42**).*

The $K_m$ and $V_{max}$ presented in *Table 2.12* are an indication of the excellent fit of ester **35** to that enzyme in rabbit plasma responsible for its hydrolysis, presumably butyrylcholinesterase. However, these results perhaps indicate the presence of a unique BuChE type in rabbit plasma for which ester **35** is an excellent fit. Consequently, hydrolysis is promoted at the carboxylic ester group, to liberate aspirin. The observed aspirin release was not related to the BuChE activity of rabbit plasma, since it has been established that both dog and human plasma possess higher BuChE activities (*Table 2.5*) but do not generate comparable concentrations of aspirin.

Numerous studies have shown that rabbit plasma is a rich source of specific esterases [226], which are known to have broad substrate specificities. Tomic et al. have intensively investigated the specificity of rabbit esterases for sugar substrates. The authors have reported the selective hydrolysis of various acylated sugars - including esterified monosaccharides - using rabbit serum or esterases isolated from the rabbit [227, 228, 229, 230, 231, 232]. More recently, the authors have reported on the characterisation of esterases with specificity for carbohydrate substrates isolated from rabbit serum [233]. The authors revealed the presence of a serine esterase in rabbit serum and more specifically revealed the presence of several BuChE isoenzymes in rabbit serum, which may be active in the hydrolysis of acylated sugar substrates [233].

Similar hydrolysis studies of ISMNA (**35**) in 10% rabbit plasma (pH 7.4, 37°C), carried out at a later date were associated with the liberation of a mixture of ISMN-salicylate (**42**) and aspirin (**1**). This is in contrast to the results described above, where aspirin was the predominant *in vitro* metabolite. An investigation into any possible causes of this variation (BuChE activity, temperature, diet [234] and history of anaesthetic administration in the rabbits [235]) yielded no obvious reasons for the unexpected hydrolysis pattern.

A possible explanation for the anomalous results is that rabbit plasma BuChE varies between sources. Main et al. observed a typical butyrylcholinesterase but also a subtype butyrylcholinesterase, of lower molecular weight [236]. It is known that 75% of the total cholinesterase activity of rabbit serum may be attributed to this smaller BuChE form. This cholinesterase is resistant to inhibition by eserine whereas the larger BuChE is completely inhibited at very low concentrations of eserine ($1 \times 10^{-5}$ M) [236]. If the smaller BuChE is a subunit of the larger esterase, then it may be possible that any variation in ester hydrolysis by rabbit sera could be related to the different states of aggregation of the subunit.

The hydrolysis of ISMN-2-benzodioxin-4-one (**41**) was also studied in 10% buffered rabbit plasma (pH 7.4) at 37°C. However, hydrolysis proceeded slowly (half-life of 25.76 minutes) and did not appear to obey Michaelis-Menten kinetics. It is presumed that ester (**41**) is a poor fit for rabbit plasma butyrylcholinesterase (EC 3.1.1.8). No aspirin was generated during its hydrolysis.

The unique hydrolysis of ISMNA (**35**) in rabbit plasma, with subsequent aspirin release, might explain the anti-platelet effect of ISMNA in rabbit PRP [170]. Further work will be required to investigate the observed inter-species variation for the hydrolysis of ISMNA. In our laboratory Meadhbh Lally studied the hydrolysis of ISMNA in a number of species including rat, guinea pig and hamster. Significant aspirin release was observed only in hamster plasma [237]. Since both rabbit and hamster are herbivores it may be of interest to study the hydrolysis of ISMNA (**35**) in human plasma of vegetarians. Gilmer at al. also studied the hydrolysis of ISMNA in rat liver homogenate and human saliva [238]. However, no aspirin was liberated during its hydrolysis.

## 2.5 Enzyme inhibition studies

Enzyme inhibition studies are useful, since it is well established that the various classes of esterases are inhibited to a varying extent by specific inhibitors [196]. The enzyme presumed responsible for the hydrolysis of ester **35** in plasma solution was butyrylcholinesterase (EC 3.1.1.8). The hydrolysis of ester **35** was studied in buffered human and rabbit plasma (pH 7.4) at 37°C, co-incubated with eserine (3 μM, 1 mg /10 ml), a potent cholinesterase inhibitor [239]. The rate of hydrolysis was suppressed in both species in the presence of eserine (*Table 2.13*). The result of this study clearly indicates that it is a cholinesterase, which is responsible for the rapid hydrolysis of ISMNA (**35**) in dilute plasma.

| **Plasma** | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ | **% Aspirin formed** |
|---|---|---|---|
| Human 10% | 0.90 | 0.78 | 8.00 |
| Human 10% with eserine | 64.17 | 0.01 | 0.00 |
| Rabbit 10% | 3.45 | 0.20 | 75.5 |
| Rabbit 10% with eserine | 91.18 | 0.01 | 0.40 |

*Table 2.13 Kinetic data for the plasma hydrolysis of ISMNA in the presence of eserine (pH 7.4, 37°C).*

## 2.6 Hydrolysis studies with purified enzyme preparations

The hydrolysis of ester **35** was studied with purified horse serum butyrylcholinesterase (EC 3.1.1.8) and other enzyme preparations to confirm the role of butyrylcholinesterase in the plasma-mediated hydrolysis of ester **35**. Horse serum BuChE has high homology with human serum butyrylcholinesterase (EC 3.1.1.8), similar catalytic efficiency towards butyrylcholine hydrolysis and similar substrate specificity [268]. Incubation in the presence of BuChE (pH 7.4, 37°C) was expected to give an indication of the susceptibility of ISMNA (**35**) to hydrolysis by this enzyme *in vitro*. The activity of the BuChE preparation (1,000 units/mg protein) was confirmed using a modification of the Ellman method with butyryl thiocholine as substrate [207]. The hydrolysis followed pseudo first-order kinetics over several half-lives, as shown in the kinetic data presented in *Table 2.14*.

| **Enzyme source** | **EC number** | **Enzyme conc.** mg/ml | $k_{obs}$ $min^{-1}$ | $t_{1/2}$ min mean (s.d., n) |
|---|---|---|---|---|
| Horse serum BuChE | EC 3.1.1.8 | 0.01 | 0.13 | 6.78 (±0.056, n=3) |
| Rabbit carboxylesterase | EC 3.1.1.1 | 0.028 | 0.02 | 35.0 |
| Human serum albumin | | 0.01 | 0.001 | 603 |
| α-chymotrypsin | EC 3.4.21.1 | 0.1 | 0.004 | 167 |

*Table 2.14 Kinetic data for the hydrolysis of ISMNA in a number of purified enzyme preparations (pH 7.4, 37°C).*

A progression curve for the hydrolysis of ester **35** with purified horse serum butyrylcholinesterase (EC 3.1.1.8) is presented in *Fig. 2.15*. The hydrolysis was associated with the generation of ISMN-salicylate (**42**) and subsequent hydrolysis to salicylic acid (**2**), with no aspirin (**1**) being liberated.

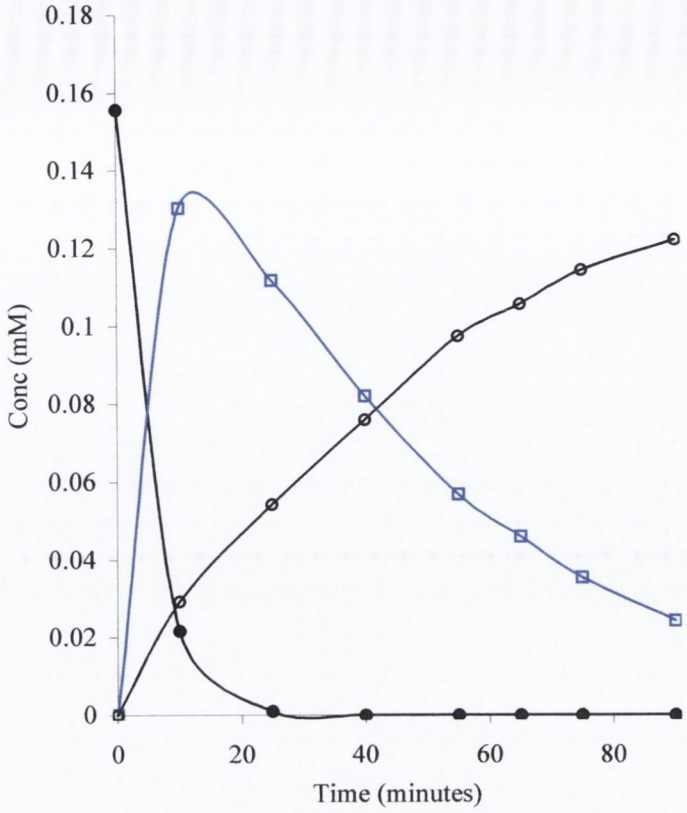



*Figure 2.15 Progression curve for the hydrolysis of ISMNA with purified horse serum butyrylcholinesterase at 0.1mg/10 ml (pH 7.4, 37°C): ISMNA (●), ISMN-salicylate (□) and salicylic acid (o).*

This result indicates that butyrylcholinesterase (EC 3.1.1.8) is responsible for the rapid plasma-mediated hydrolysis of ISMNA (**35**). However, the specificity of this enzyme in rabbit plasma solution is clearly different, since it is directed towards predominant hydrolysis at the carboxyl-protecting moiety to liberate aspirin. Since aspirin (up to 8.43%) was generated from the hydrolysis of ISMNA (**35**) in 10% human plasma, it was expected that some aspirin would be liberated from horse serum BuChE-mediated hydrolysis of ester **35**. Perhaps one explanation for the observed result is that horse serum BuChE possesses three amino acid differences in the active site gorge compared with human BuChE [268]. These differences might significantly alter the pathway of hydrolysis.

Inoue et al. have indicated that aspirin is hydrolysed by a purified carboxylesterase from human intestinal mucosa [240]. An esterase, with some features of

carboxylesterases (EC 3.1.1.1) has been isolated from rabbit liver [205]. In this regard, the degradation of ester **35** was studied in the presence of a carboxylesterase from rabbit liver (EC 3.1.1.1) at pH 7.4 and 37°C. The hydrolysis was slower than butyrylcholinesterase-mediated hydrolysis. The degradation followed pseudo first-order kinetics (*Table 2.14*) and generated the salicylate ester **42** exclusively. The results of this study indicate that rabbit liver carboxylesterase (EC 3.1.1.1) does not contribute significantly to the rapid hydrolysis of ester **35** in rabbit plasma solution. The hydrolysis of ISMNA in horse serum BuChE was approximately 18 times faster than in rabbit liver carboxylesterase, based on intrinsic enzyme activities.

Human serum albumin (HSA) is the most abundant protein found in plasma and serves as a transport mechanism for a number of endogenous and exogenous compounds. Much attention has been paid, therefore, to the study of drug interactions with HSA [241]. Furthermore, investigations have revealed 'enzyme-like' activity of albumin preparations. The participation of albumin in the hydrolysis of aspirin has been described (1968) [242]. The catalytic effect of serum albumin on the hydrolysis of *p*-nitrophenyl esters was first described in 1951 [243, 244]. In several cases, the esterase activity of HSA could be attributed to low levels of contamination of albumin by one or more enzymes [245]. The possibility of a potential contamination of serum albumin by butyrylcholinesterase (EC 3.1.1.8) was first investigated by Tove [246] and more recently by Whelpton and Hurst [247]. Chapuis et al. found that the apparent esterase-like activity of a number of commercially available HSA preparations was in fact due to contamination by butyrylcholinesterase [241].

The hydrolysis of ISMNA was studied in the presence of HSA at pH 7.4 and 37°C and was slower than butyrylcholinesterase-mediated hydrolysis (*Table 2.14*). ISMN-salicylate (**42**) was the exclusive degradation product with no aspirin being detected. Therefore, it can be concluded that the observed hydrolysis of ISMNA in dilute plasma (**35**) is not mediated by HSA.

Oral delivery and subsequent intestinal absorption is a preferred route for drug administration. Intestinal absorption can be considered as the amount of unchanged drug absorbed from the intestinal lumen, which appears in the portal circulation (or intestinal lymph) [248]. The extent of absorption of a drug is a consequence of its

physico-chemical properties, such as lipophilicity and aqueous solubility. Resistance to enzymatic degradation is also an important factor relating to the intestinal absorption of a drug. The pancreatic serine endopeptidases (chymotrypsin, trypsin and elastase) present in high concentrations in the lumen of the small intestine represent a major metabolic barrier to the delivery of certain drugs [249, 250, 251]. α-Chymotrypsin (EC 3.4.21.1) is an enzyme present in the lumen of the gastrointestinal tract [252]. In this regard, it was decided to determine the stability of ISMNA (**35**) towards enzymatic degradation in the lumen by performing hydrolysis studies in the presence of α-chymotrypsin. α-Chymotrypsin could potentially hydrolyse ISMNA, since it has been found to exhibit some esterase activity [253]. It has been discovered that this enzyme hydrolyses several acylated α-amino acid esters including acetyl-, chloroacetyl and benzoyl-L-valine methyl esters [254], acetyl-L-phenylalanine glycolamide esters [255] and acylated glycine methyl esters [256, 257].

The stability of ISMNA (**35**) towards α-chymotrypsin (EC 3.4.21.1) was determined at pH 7.4 and 37°C. The enzyme activity was determined using *N*-acetyl tyrosine ethyl ester as substrate [258] with the reaction monitored (230 nm) on an isocratic HPLC system using a Spherisorb C18 (4.6 x 250 mm) column eluted with water and acetonitrile (1:1) at 1 ml/minute.

The hydrolysis of ISMNA followed pseudo first-order kinetics with a $V_{max}$ of 8.69 x $10^{-4}$ M $min^{-1}$ and a corresponding $K_m$ of 9.50 x $10^{-4}$ M, as presented in *Table 2.14*. The salicylate ester (**42**) was the exclusive product generated. Although the hydrolysis was more rapid than in aqueous buffer, the rate was significantly slower than in the presence of dilute plasma. The results of this study indicate that ISMNA (**35**) is a poor substrate for α-chymotrypsin. Therefore, ester **35** might be stable against degradation by this and other pancreatic serine endopeptidases.

The hydrolysis of ISMN-2-benzodioxin-4-one (**41**) was also studied in the presence of α-chymotrypsin (EC 3.4.21.1, 1 mg/10 ml). The pseudo first-order rate constant was 0.008 $min^{-1}$ with a corresponding half-life of 86.60 minutes. The hydrolysis was associated with the liberation of ISMN-acetate and salicylic acid (**2**). The results of this study suggest that ISMN-2-benzodioxin-4-one (**41**), like ISMNA

(**35**), is more stable under these conditions than in dilute plasma. Therefore, it can be concluded that ester **41** is a poor substrate for α-chymotrypsin.

## 2.7 Conclusion

The design of an aspirin prodrug has been investigated, based on the inclusion of a well-known organic nitrate, IS-5-MN (**38**). Aspirin prodrugs are designed to cross the gastro-intestinal barrier and undergo hydrolysis by plasma esterases, liberating aspirin and the nitric oxide donor. Hydrolysis of aspirin esters may occur simultaneously through two distinct routes: at the carboxylic acid ester functionality to liberate aspirin or at the labile acetyl group to liberate the salicylate ester and ultimately salicylic acid.

The mononitrate aspirin ester of isosorbide, ISMNA (**35**), inhibits platelet aggregation in rabbit platelet rich plasma (at 1 μM) [170] and after oral administration to beagles (4 mg $kg^{-1}$) [168]. These results suggested that ester **35** might be capable of liberating aspirin in rabbit and dog. ISMNA (**35**) and ISMN-benzodioxin-4-one (the cyclic ortho ester of IS-5-MN and aspirin) were synthesised and their potential as aspirin prodrugs evaluated.

Ester **35** possesses suitable lipophilicity and stability at physiological pH to be absorbed intact after oral administration. The observed stability of ISMNA to α-chymotrypsin (EC 3.4.21.1) may be important for the applicability of ISMNA as an agent with decreased GI toxicity.

In dilute human plasma, the rapid hydrolysis of ester **35** was associated with the liberation of up to 8.1% aspirin. The extent of aspirin release from human plasma might explain the weak anti-platelet activity of ISMNA in humans but is not thought to be clinically useful. In dog plasma solution, the slower hydrolysis was not associated with aspirin liberation. The results obtained in dilute human and dog plasma indicates the susceptibility of ISMNA (**35**) to enzymatic degradation *in vivo*. However, questions still arise regarding the anti-thrombotic effect of ester **35** in dogs. It is possible that other enzymes exist in the dog, which may release aspirin. Future experiments are planned, to test this possibility, using dog gut or liver homogenate.

In rabbit plasma solution, ISMNA (**35**) hydrolysed rapidly, generating up to 78.7% aspirin. This is a remarkable result, since aspirin esters typically hydrolyse at

the labile *O*-acetyl group and not at the carboxylic ester bond. The implication of these results is that ISMNA (**35**) is a uniquely good fit for rabbit plasma butyrylcholinesterase (EC 3.1.1.8). Evidence that butyrylcholinesterase is the enzyme responsible for the hydrolysis of ISMNA was obtained from hydrolysis studies in a purified horse serum BuChE preparation and almost complete inhibition of plasma-catalysed hydrolysis by eserine ($10^{-3}$ M).

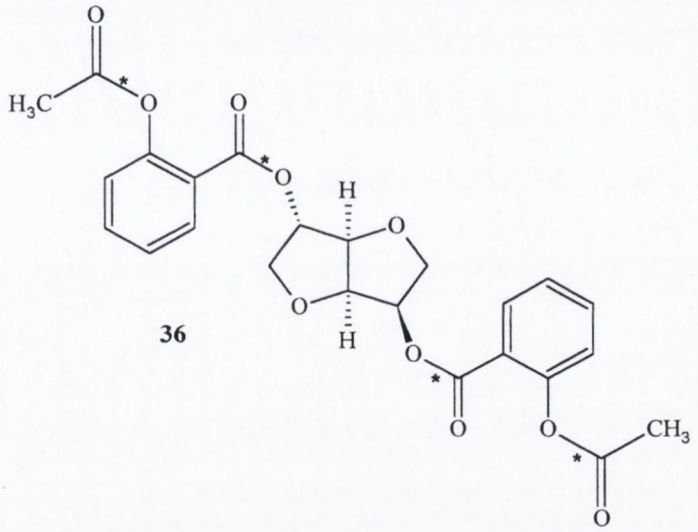
In conclusion, ISMNA is a potentially useful biolabile prodrug for aspirin. The ester combines a high susceptibility to undergo enzymatic hydrolysis in plasma with a high stability in aqueous solution. The results also show that ISMN-2 esters generally undergo rapid hydrolysis in plasma. Future work regarding ISMNA (**35**) should involve a more extensive study of the apparent interspecies variation of its hydrolysis *in vitro*.

# Chapter 3

## Investigation of isosorbide diaspirinate as a potential aspirin prodrug: Identification of a novel isosorbide-based aspirin prodrug

## 3.1 Introduction

Interest in the development of novel aspirin prodrugs has been renewed with the advent of nitric oxide-releasing aspirins [131]. The previous chapter described the hydrolysis of ISMNA (**35**), a nitro-aspirin ester, which is a more potent inhibitor of arachidonic acid-induced platelet aggregation than aspirin in rabbit platelet rich plasma [170]. ISMNA is susceptible to rapid hydrolysis in rabbit plasma, liberating aspirin (up to 78.7%). The unusually favourable hydrolysis of ISMNA to aspirin in rabbits appears to be related to the structural properties of isosorbide (**40**). Therefore, we investigated a potential aspirin prodrug based on the core structure of isosorbide (**40**) for possible use in thrombotic or inflammatory disorders.



**36**

ISDA (**36**) has been shown to possess significant anti-platelet properties in several species. Oral administration of ester **36** at 2 mg $kg^{-1}$ to a group of six beagles was associated with greater than 90% inhibition of *ex vivo* $TXB_2$ production. Ester **36** inhibits arachidonic acid-induced platelet aggregation in rabbit PRP at a concentration of 1 μM whereas 10 μM aspirin showed no inhibitory effects [168]. In our laboratory, it was shown that ester **36** is a more potent inhibitor of arachidonic acid-induced aggregation *in vitro* in human whole blood ($IC_{50}$ = 35 μM), human PRP ($IC_{50}$ = 4-5 μM) and platelet malondialdehyde (MDA) synthesis than aspirin ($IC_{50}$ in human whole blood of 40 μM and in human PRP of 8-9 μM) [259].

These results suggested that ISDA (**36**) might be capable of liberating aspirin (**1**) in human, rabbit and dog plasma. It was decided to investigate the potential of

ester **36** as an aspirin prodrug in the light of these results and the rapid hydrolysis of the related ISMNA (**35**) in various plasma types. It was expected that ISDA would be a substrate for butyrylcholinesterase-mediated hydrolysis.

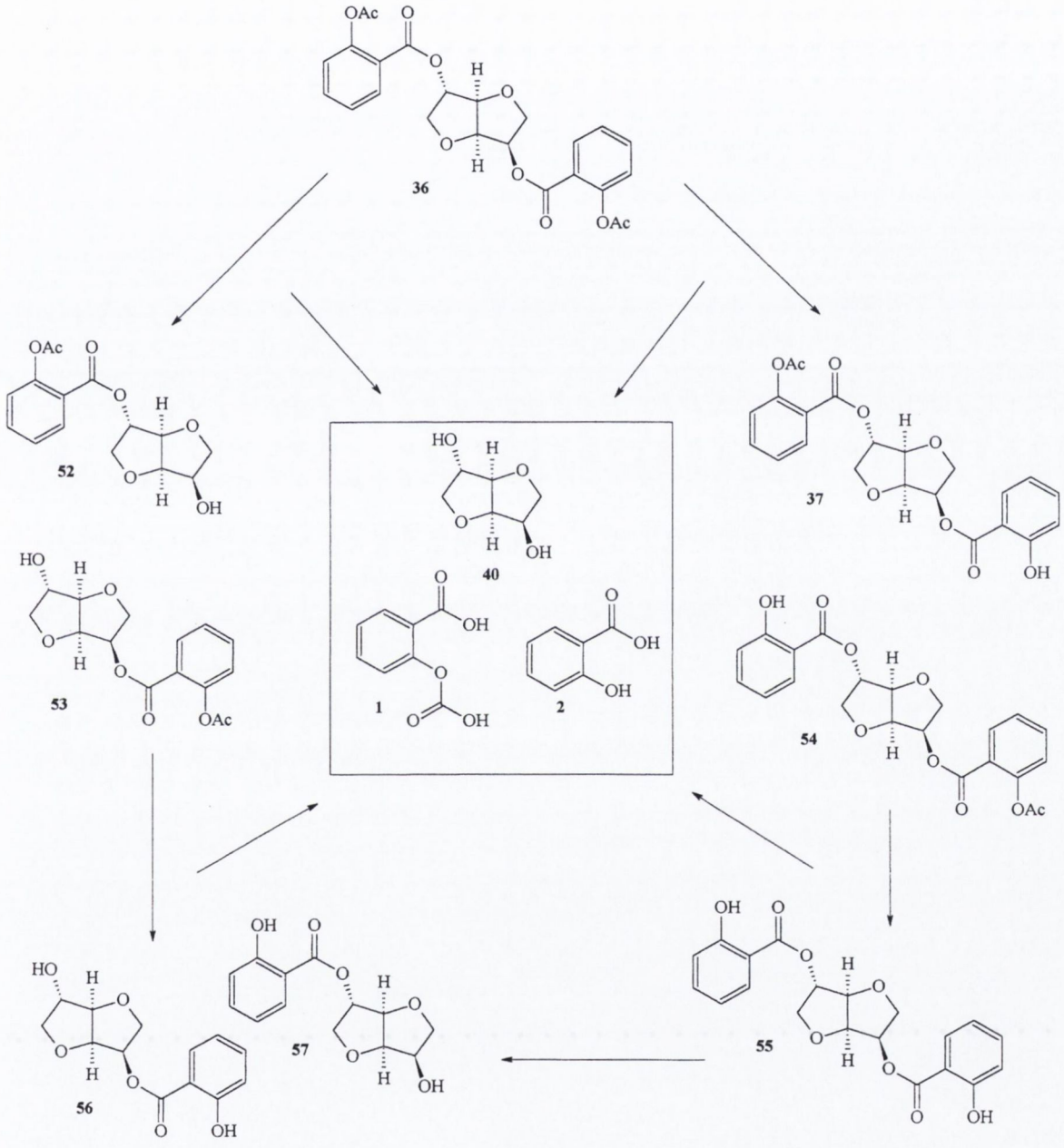



*Figure 3.1 The potential hydrolysis pathways of ISDA.*

The potential hydrolysis pathways of ester **36** are illustrated in *Fig 3.1* and are complex due to the presence of four ester groups in the molecule (*) - at one or both *O*-acetyl or carboxylic ester groups - to liberate an extensive range of potential metabolites. The ultimate products of hydrolysis are aspirin (**1**), salicylic acid (**2**) and isosorbide (**40**). The elucidation of the complex kinetic pathways of ISDA required the synthesis of ester **36** and its potential metabolites.

### 3.1.1 Synthesis of ISDA and potential metabolites

The potential metabolites of ISDA (**36**) were synthesised according to well-established chemical reactions. Esterification of isosorbide-type compounds formed the basis of the synthesis and was carried out by direct nucleophilic substitution or *via* coupling in the presence of DCC and DMAP [260]. In certain instances, the use of a protected form of salicylic acid (**2**) was required to prevent self-esterification of the phenol group. Benzyloxy benzoic acid (**58**) the benzyl protected salicylic acid, was chosen since the benzyl group is readily removed by catalytic hydrogenation under conditions that were expected to leave the hydrolysis-sensitive groups intact. Compound **58** was synthesised according to the scheme illustrated in *Fig. 3.2*.

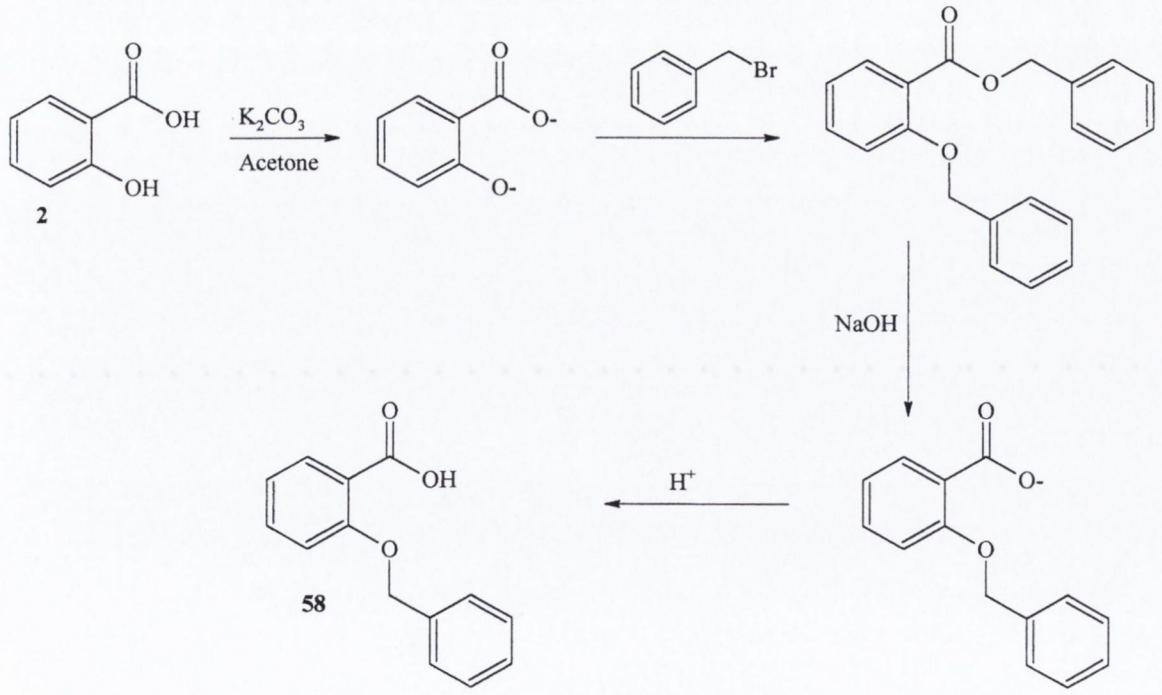



*Fig. 3.2 The synthesis of benzyloxy benzoic acid.*

### 3.1.1.1 Synthesis of ISDA

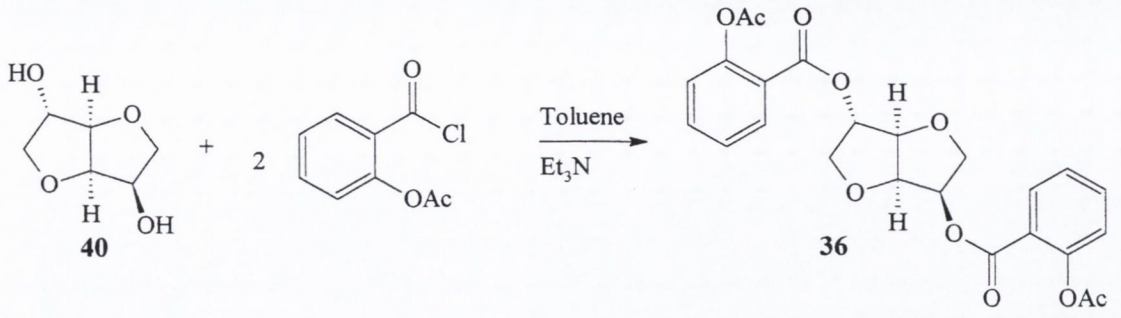



*Figure 3.3 The synthesis of ISDA.*

Isosorbide diaspirinate (**36**) was synthesised according to the scheme presented in *Fig. 3.3*. The reaction was found to go to completion although TLC analysis identified the presence of a significant impurity in the crude product. This impurity was aspirin anhydride (**4**), formed by attack of the nucleophilic aspirin (present as an impurity of acetylsalicyloyl chloride) on the carbonyl of the acid chloride (*Fig. 3.4*). Aspirin anhydride (**4**) has previously been investigated as an aspirin prodrug (Chapter 1) but was found to possess unsuitable characteristics, such as its instability towards random acylation and associated mutagenesis.

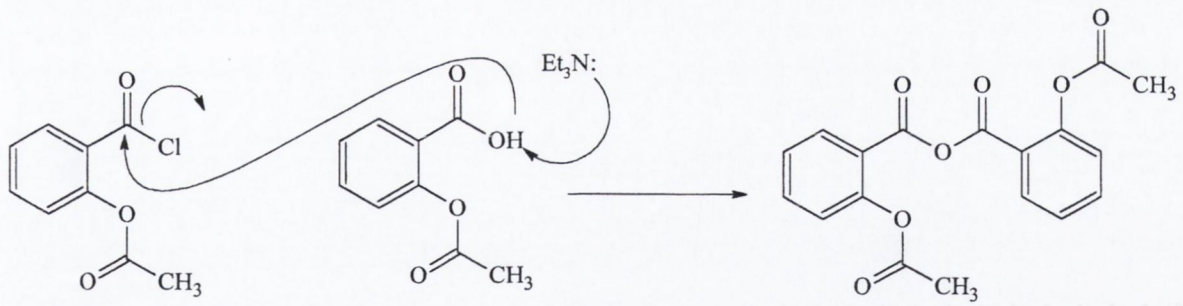



*Figure 3.4 Formation of aspirin anhydride as an impurity in the synthesis of ISDA.*

The diaspirinate was recrystallised from ethanol to yield a white crystalline material (65.7%), which was unambiguously identified by IR, MS and NMR. The ester (**36**) was homogenous by TLC and two different HPLC methods.

### 3.1.1.2 Synthesis of isosorbide-5-aspirinate

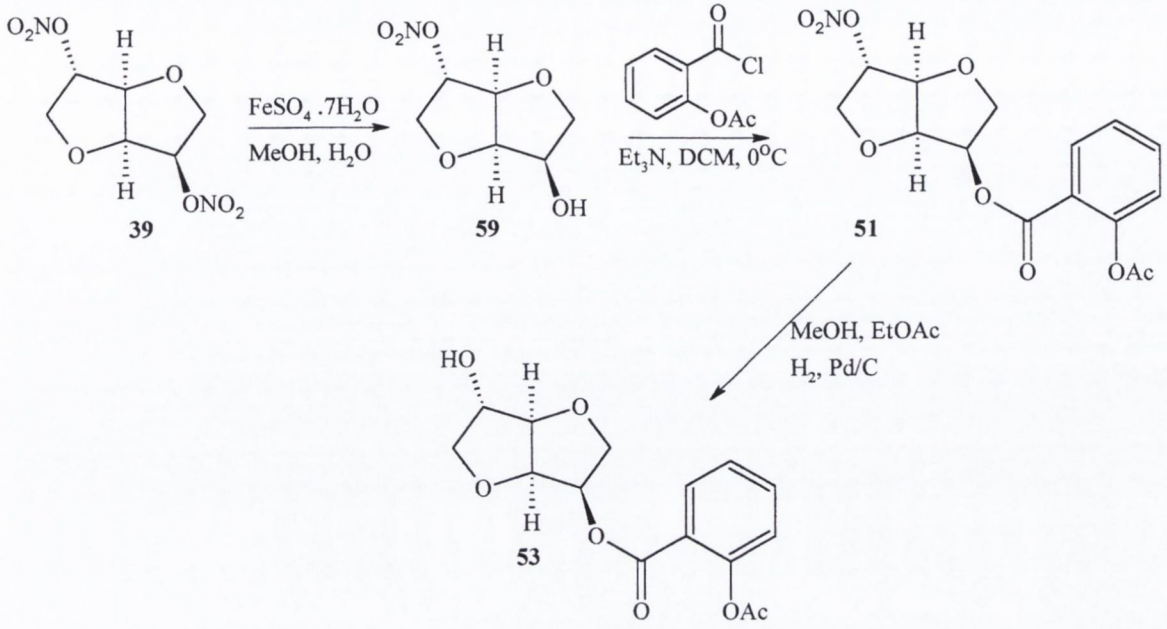



*Figure 3.5 The synthesis of isosorbide-5-aspirinate.*

Isosorbide-5-aspirinate (**53**) was synthesised according to a three-step route as presented in *Fig. 3.5*. Since ISDN (**39**), the organic nitrate used in the prevention and treatment of cardiovascular diseases such as angina, is explosive it is available only as a lactose blend (60% lactose). ISDN (**39**) was isolated after partitioning between water and dichloromethane to give complete recovery from the organic layer. The initial step involved selective reduction of the isolated **39**, at the 5-$ONO_2$ position. This reaction required careful control to prevent complete reduction to isosorbide (**40**). Attempts to selectively reduce compound **39** have been described in the literature. However, most furnish mixtures of the 2- and 5-nitrates or isosorbide itself [261]. De Luchi reported that the 2-nitrate may be obtained in good yield using iron (II) sulfate in methanol and water under reflux conditions [262]. It would appear that reducing metal salts have the ability to interact with the *endo* part of the substrate (at the 5-position) although it is more sterically hindered. Other reducing agents, such as zinc in acetic acid lead to the preferential reduction of the more exposed *exo* nitrooxy (2-nitrooxy) position [262]. The

selective reduction of ISDN (**39**) in the presence of iron (II) sulfate afforded isosorbide-2-mononitrate (**59**) as a white crystalline material in high yield (79.6%), which was characterised by IR, NMR and MS.

The acylation of compound **59** by acetylsalicyloyl chloride, in the presence of triethylamine and toluene at 0°C, afforded isosorbide-5-aspirinate-2-mononitrate (**51**) as a white crystalline material, its purity being determined by NMR, IR, and MS. Reduction of the nitro group of compound **51** by catalytic hydrogenation afforded isosorbide-5-aspirinate (**53**), which was recrystallised from ethanol to afford a white crystalline material (68%). Ester **53** was characterised by IR, NMR and MS and was homogenous by TLC and by two different HPLC methods.

### 3.1.1.3 Synthesis of isosorbide-2-aspirinate

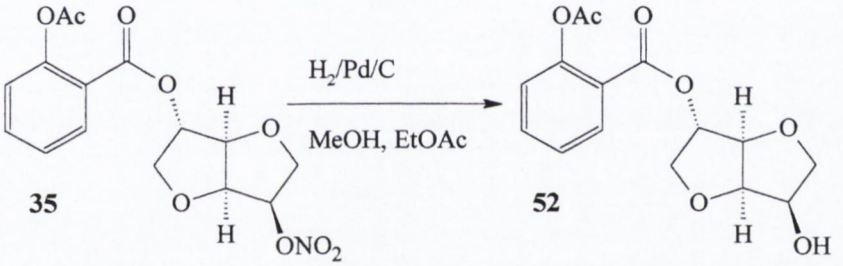



*Figure 3.6 The synthesis of isosorbide-2-aspirinate.*

Isosorbide-2-aspirinate (**52**) was synthesised according to the scheme presented in *Fig 3.6.* The synthesis involved catalytic hydrogenation at the 5-nitrooxy position of ISMNA (**35**). The reaction product was purified by column chromatography to yield ester **52** as a white crystalline material (40.26%). IR, NMR and MS characterised the product with additional analysis by TLC and two different HPLC methods.

### 3.1.1.4 Synthesis of isosorbide disalicylate

Isosorbide disalicylate (**57**) was synthesised according to the scheme presented in *Fig. 3.7.* The two-step synthesis involved diesterification followed by reduction. Benzyl-protected salicylic acid (**58**) was used to prevent self-esterification of salicylic acid. The benzyl-protected disalicylate ester (**60**) was deprotected using hydrogen and

palladium on charcoal to afford product (42%) as a white crystalline material, which was characterised by NMR, MS and IR. Ester **57** was homogenous by TLC and by two different HPLC methods.

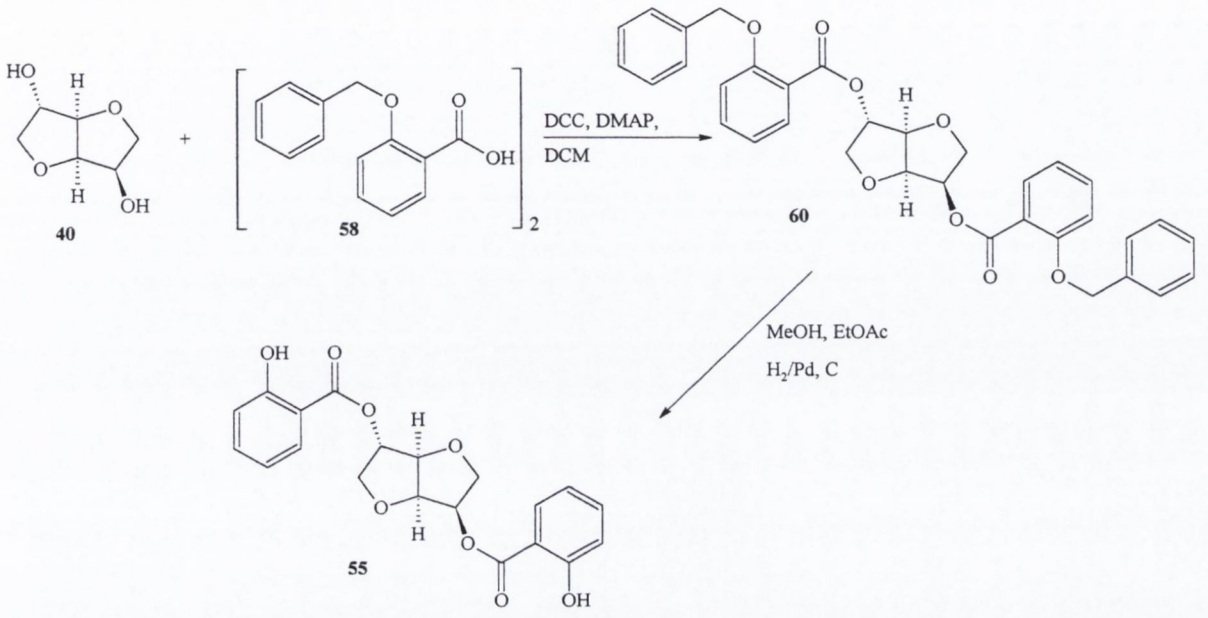



*Figure 3.7 The synthesis of isosorbide disalicylate.*

### 3.1.1.5 Synthesis of isosorbide-2-aspirinate-5-salicylate

The multi-step synthesis of isosorbide-2-aspirinate-5-salicylate (**37**) is presented in *Fig. 3.8*. Esterification of ester **52** at the reactive 5-hydroxy position was performed in the presence of DCC and DMAP. The protected form of salicylic acid (**58**) was again employed in the synthesis to prevent self-esterification. The intermediate product was obtained after column chromatography as a yellow oil, which was characterised by IR, NMR and MS. Ester **37** was homogenous by TLC and by two different HPLC methods.

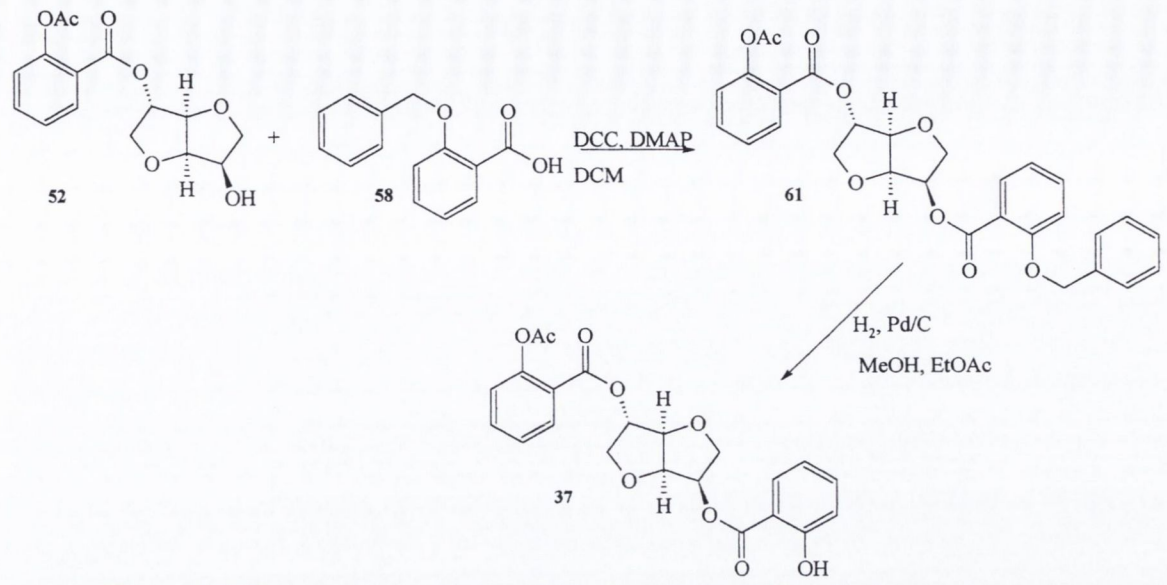



*Figure 3.8 The synthesis of isosorbide-2-aspirinate-5-salicylate.*

Isosorbide-2-aspirinate-5-benzyloxy benzoate (**61**) was deprotected using 10% palladium on charcoal under hydrogen. The crude product was further purified by column chromatography to yield ester **37** as a white crystalline material (25.5%). The product was found to be pure by IR, NMR and MS and was homogenous by TLC and by two different HPLC methods.

### 3.1.1.6 Synthesis of isosorbide-2-salicylate-5-aspirinate

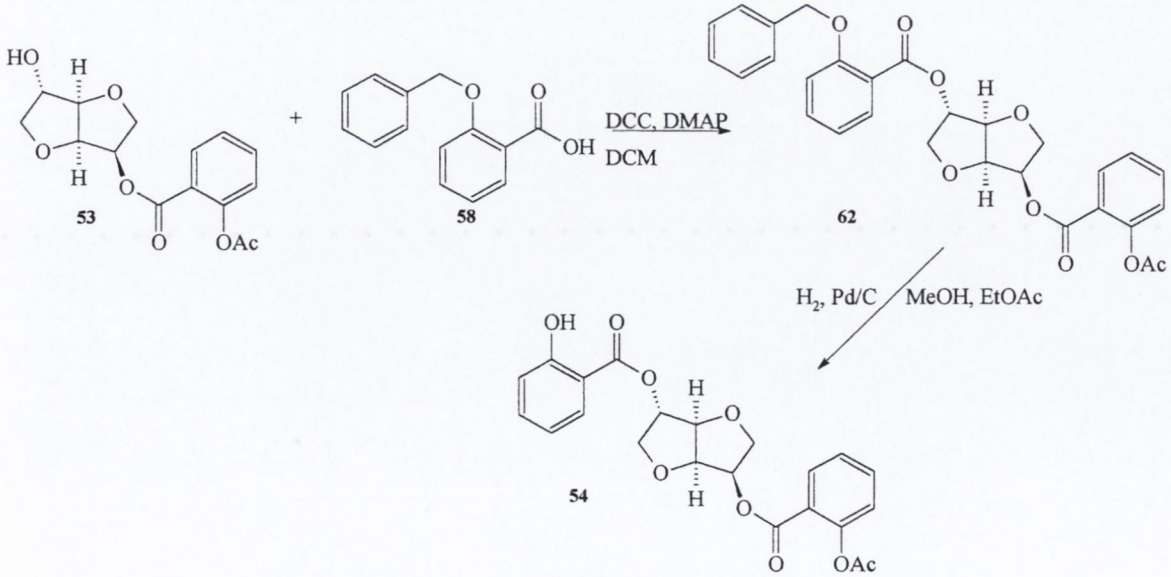



*Figure 3.9 The synthesis of isosorbide-2-salicylate-5-aspirinate.*

Isosorbide-2-salicylate-5-aspirinate (**54**) was synthesised according to the scheme presented in *Fig. 3.9*. The synthesis involved esterification of isosorbide-5-aspirinate (**57**) followed by deprotection. Compound **53** was obtained as described in Section 3.1.1.2. The product (**54**) was purified by column chromatography to yield product (20%) as white crystalline material. The poor yield was due to incomplete reduction of the starting material. The final product was characterised by NMR, IR and MS and determined to be pure by TLC and by two different HPLC methods.

### 3.1.1.7 Isosorbide-5-salicylate

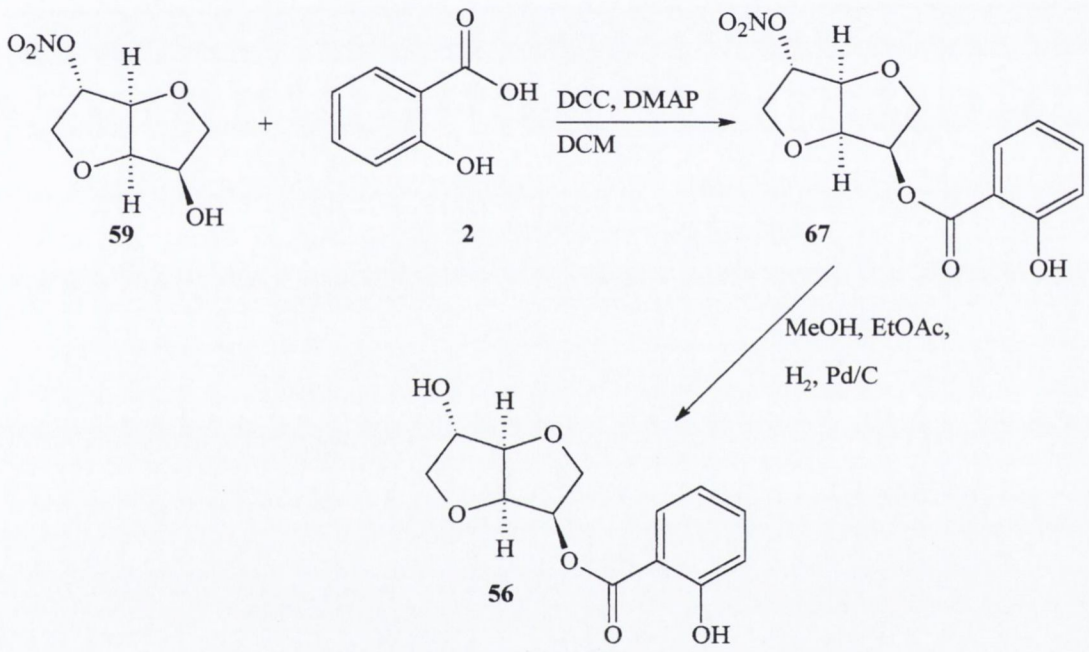



*Figure 3.10 The synthesis of isosorbide-5-salicylate*

Isosorbide-5-salicylate (**56**) was synthesised according to the scheme presented in *Fig. 3.10*. The synthesis involved esterification of compound **59** followed by removal of the nitrate group. The reaction product was purified by column chromatography to yield product as crystalline material (69%). Recrystallisation in ethanol afforded isosorbide-5-salicylate (**56**) as colourless needles, which were characterised by NMR, IR and MS and determined to be pure by HPLC and TLC.

## 3.1 Development of a reverse phase HPLC method for the separation of ISDA and potential metabolites

As previously described, our objective was to determine the potential usefulness of ISDA (**36**) as an aspirin prodrug. This led us to investigate the hydrolysis of ester **36** under aqueous and enzymatic conditions. Since all potential metabolites of ester **36** had been synthesised, it was desirable to develop a chromatographic method capable of their separation.

Initially, samples from the *in vitro* studies of ISDA were monitored by an isocratic reverse phase HPLC method, which involved elution on a Nova-Pak C8 column (3.9 x 75 mm) with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) at 1ml $min^{-1}$. This method was validated for linearity, precision, specificity and sensitivity. A linear response was observed for each analyte ($r > 0.999$) in the range 1-100 μg/ml. The RSD on multiple injection of each analyte at 10 μg/ml and 100 μg/ml was <1.5%. The limit of quantitation was 5 μg/ml for aspirin and salicylic acid and 0.5 μg/ml for other possible degradation products.

While the above isocratic HPLC method was initially employed for the assay of plasma samples (from which relevant kinetic data was obtained) (*Fig. 3.11*), this system was not capable of the complete separation required. The least retained potential metabolites, including aspirin (**1**), isosorbide-2-aspirinate (**52**), isosorbide-5-aspirinate (**53**) and salicylic acid (**2**) were found to co-elute in some assays, particularly aspirin (**1**), isosorbide-5-aspirinate (**53**) and isosorbide-2-aspirinate (**52**), which were not distinguishable by PDA spectra. Attempts were made to improve the retention characteristics of the separation by altering the mobile phase composition, flow rate and stationary phase dimensions. However, these attempts did not give the desired separation. It was decided to alter the selectivity of the retention mechanisms by changing to a gradient method.

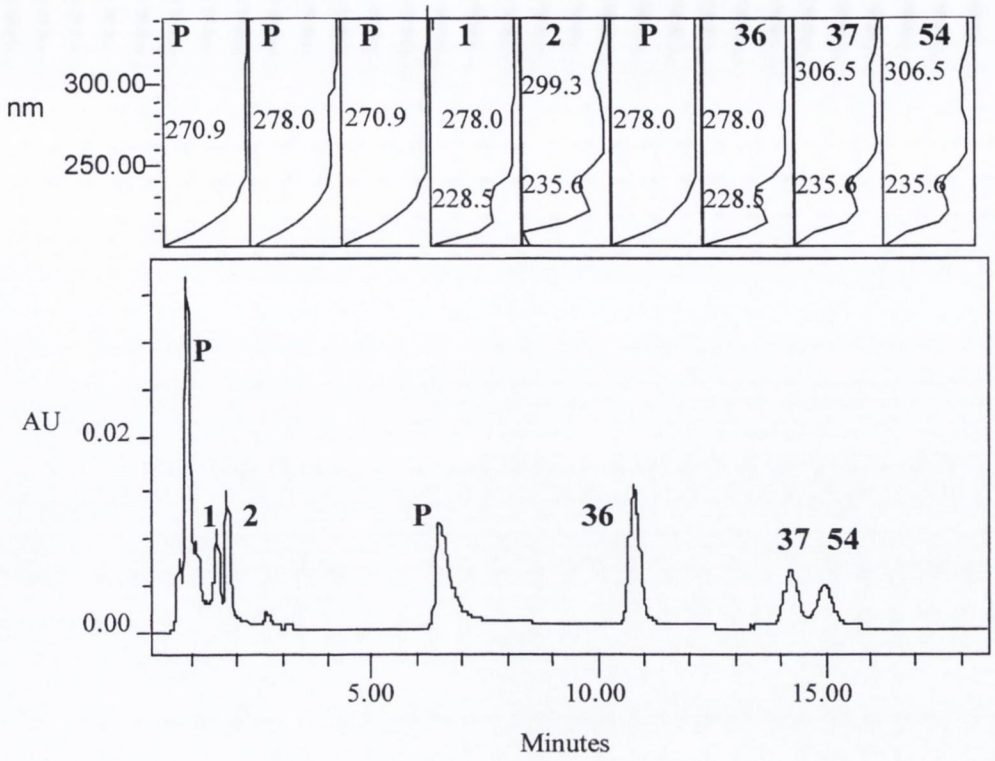



*Figure 3.11 Chromatogram (230 nm) of a sample obtained following incubation of ISDA in 50% buffered human plasma (pH 7.4) for 2 minutes. Also shown are PDA spectra of each of the labelled components. Peaks labelled* ***P*** *represent plasma peaks, which were present in the plasma blank: Aspirin (**1**), salicylic acid (**2**), ISDA (**36**), isosorbide-2-aspirinate-5-salicylate (**37**) and isosorbide-2-salicylate-5-aspirinate (**55**).*

### 3.2.1 Gradient elution

The technique of gradient elution allows for additional mobile phase control and is defined as the use of two or more solvents composing a mobile phase whose concentration ratio is varied with time [263]. In developing a gradient HPLC method sample components must be retained sufficiently on the column, sufficiently resolved from one another to allow for their quantitation and possess relatively good peak characteristics (band broadening characteristics). Parameters influencing these chromatographic characteristics are initial and final solvent composition, run time, gradient shape (linear, concave or convex), flow rate and equilibration period. A run time of no greater than 25 minutes (including a five minute re-equilibration period) and a linear gradient shape were also selected as criteria. Gradient elution of biological

samples involves the separation and detection of analytes at low concentrations (in the μg/ml range). Therefore, any resolution achieved at high concentrations must also be retained at low concentrations. The chromatographic conditions must be adjusted to allow for elution of the peaks of interest in a region free of interfering plasma peaks.

Initially, the mobile and stationary phase were chosen based on the isocratic system described above: that is elution on a Nova-Pak C8 column with a mobile phase consisting of phosphate buffer pH 2.5 and acetonitrile. The least retained components, including aspirin (**1**), isosorbide-2-aspirinate (**52**), isosorbide-5-aspirinate (**53**) and salicylic acid (**2**) were poorly resolved under isocratic conditions. Therefore, the separation of these analytes was closely monitored during method development.

| **Time** mins | **Flow rate** ml $min^{-1}$ | **% A** | **% B** | **Curve** |
|---|---|---|---|---|
| | 0.75 | 75 | 25 | |
| 5 | 0.75 | 90 | 10 | 6 |
| 10 | 1 | 35 | 65 | 6 |
| 15 | 1 | 90 | 10 | 6 |
| 25 | 1 | 60 | 40 | 6 |

*Table 3.1 Reverse phase HPLC gradient method for the separation of aspirin ($t_r$ 4.78 min), isosorbide-5-aspirinate ($t_r$ 5.02 min), isosorbide-2-aspirinate ($t_r$ 5.68 min) and salicylic acid ($t_r$ 6.13 min). A = pH 2.5 phosphate buffer and B = acetonitrile, on a Nova-Pak C8 3.9 x 150mm column.*

*Table 3.1* presents the gradient method, which at pH 2.5 phosphate buffer and on a Nova-Pak C8 column gave the optimum separation, with respect to resolution, selectivity and efficiency. The retention times of aspirin (**1**), isosorbide-5-aspirinate (**53**), isosorbide-2-aspirinate (**52**) and salicylic acid (**2**) were 4.78, 5.02, 5.68 and 6.13 minutes respectively (*Fig. 3.12(i)*). Although good selectivity was afforded from this method, aspirin (**1**) and isosorbide-5-aspirinate (**53**) were not baseline separated (*Fig. 3.12(i)*).

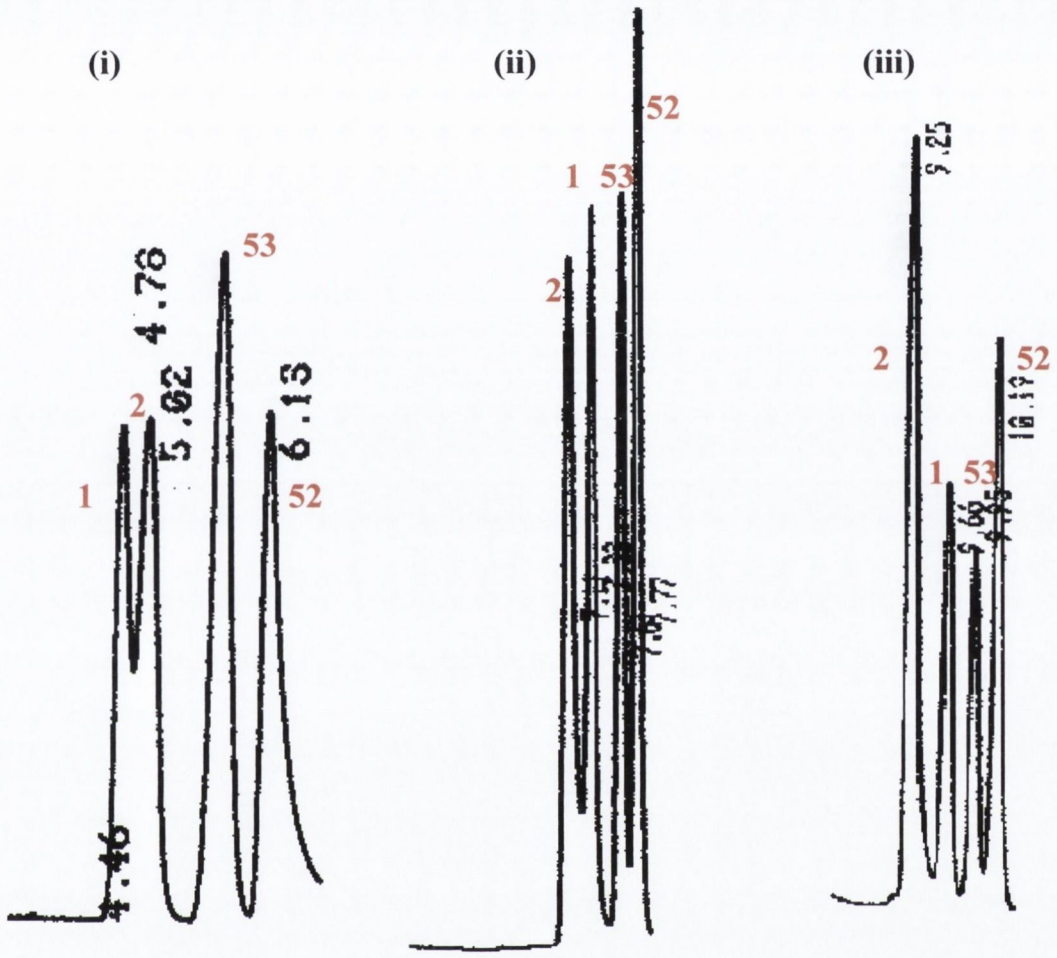



*Figure 3.12 A series of chromatograms for the separation of aspirin (**1**), salicylic acid (**2**) isosorbide-2-aspirinate (**52**) and isosorbide-5-aspirinate (**53**) according to the gradient methods presented in: (i) Table 3.1 (ii) Table 3.2 and (iii) Table 3.3.*

### 3.2.2 pH effects

We investigated the possibility of improving the separation of aspirin (**1**) and isosorbide-5-aspirinate (**53**) with a suitable adjustment of mobile phase pH. Aspirin and other weak acids are more hydrophobic at pH values less than their pKa and are consequently retained longer, due to ion supression. The retention of neutral compounds, such as isosorbide-2-aspirinate (**52**) and isosorbide-5-aspirinate (**53**) should not vary significantly, if at all, when the mobile phase pH is changed. The percent ionisation of aspirin and salicylic acid over a pH range slightly above and

below their pKa was determined. The percent ionisation of acids and bases may be calculated according to Eq. (3.1) and (3.2) respectively [264].

$$\% \text{ ionization for HA} = \frac{100}{1 + 10^{(\text{pKa - pH})}} \quad (3.1)$$

$$\% \text{ ionization for } BH^{+} = \frac{100}{1 + 10^{(\text{pH - pKa})}} \quad (3.2)$$

The pKa of compounds **1** and **2** are 3.5 and 2.97 respectively [265]. At pH 2.5, aspirin (**1**) and salicylic acid (**2**) are 9.1% and 25.3% ionised respectively. At pH 3.0, aspirin and salicylic acid are 24.0% and 48.3% ionised respectively. Therefore, the retention of salicylic acid was expected to decrease at the higher pH. The mobile phase pH was consequently changed to pH 3.19 by adjusting the phosphate buffer composition.

| **Time** mins | **Flow rate** ml $min^{-1}$ | **% A** | **% B** | **Curve** |
|---|---|---|---|---|
| | 1.25 | 90 | 10 | |
| 10 | 1 | 25 | 75 | 6 |
| 15 | 1 | 60 | 40 | 6 |
| 20 | 1 | 90 | 10 | 6 |
| 25 | 1 | 90 | 10 | 6 |

*Table 3.2 Reverse phase HPLC gradient method for the separation of salicylic acid ($t_r$ 7.89 min), aspirin ($t_r$ 8.09 min), isosorbide-5-aspirinate ($t_r$ 8.37 min) and isosorbide-2-aspirinate ($t_r$ 8.51 min. A = pH 3.19 phosphate buffer and B = acetonitrile, on a Nova-Pak C8 3.9 x 150mm column.*

*Table 3.2* presents the gradient method, which at pH 3.19 and on a Nova-Pak C8 column (3.9 x 150 mm), gave the optimum separation of salicylic acid (**2**), aspirin (**1**), isosorbide-5-aspirinate (**53**) and isosorbide-2-aspirinate (**52**) (retention times: 7.89, 8.09, 8.37 and 8.51 minutes respectively). Altering the mobile phase pH from pH 2.5

to pH 3.19 interchanges the retention times of salicylic acid and aspirin (*Fig. 3.12(ii)*). However, this did not result in the desired baseline separation.

### 3.2.3 Stationary phase effects

The possibility that altering the stationary phase would improve the separation was investigated. A less polar Spherisorb C18 bonded phase (4.6 x 250 mm) was employed and the mobile phase pH was maintained at pH 3.19.

| **Time** mins | **Flow rate** ml $min^{-1}$ | **% A** | **% B** | **Curve** |
|---|---|---|---|---|
| | 1 | 90 | 10 | |
| 10 | 1 | 10 | 90 | 6 |
| 15 | 1 | 65 | 35 | 6 |
| 17 | 1 | 90 | 10 | 6 |
| 25 | 1 | 90 | 10 | 6 |

*Table 3.3 Reverse phase HPLC gradient method for the separation of salicylic acid ($t_r$ 8.51min), aspirin ($t_r$ 8.95 min), isosorbide-5-aspirinate ($t_r$ 9.33 min) and isosorbide-2-aspirinate ($t_r$ 9.59 min). A = pH 3.19 phosphate buffer and B = acetonitrile, on a Spherisorb C18 4.6 x 250 mm column.*

*Table 3.3* presents the gradient method, which at pH 3.19 phosphate buffer and on a Spherisorb C18 column (4.6 x 250 mm) gave the optimum separation. The resulting retention times of salicylic acid (**2**), aspirin (**1**), isosorbide-5-aspirinate (**53**) and isosorbide-2-aspirinate (**52**) were 8.51, 8.95, 9.33 and 9.59 minutes respectively (*Fig. 3.12(iii)*). This separation gave the desired resolution for the respective analytes. However, to complete the method development a mixture of ISDA (**36**) and all potential metabolites was injected (*Fig. 3.13*).

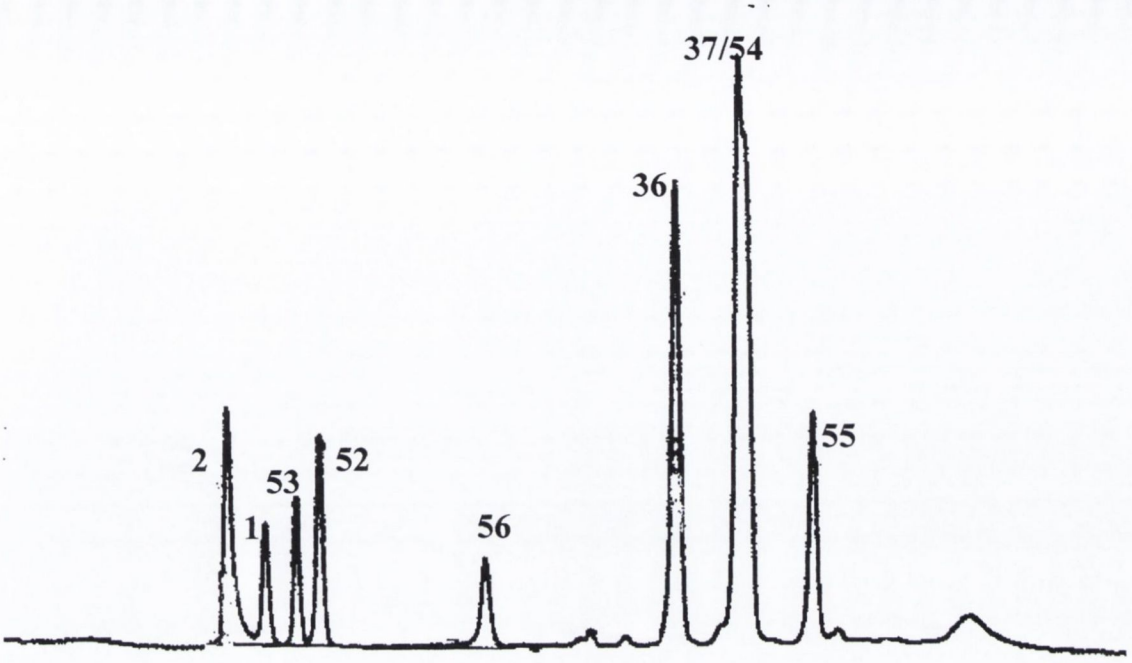



*Figure 3.13 Chromatogram (230 nm) of a sample obtained following HPLC analysis of a mixture of ISDA and its potential metabolites according to the gradient table presented in Table 3.3. Salicylic acid (**2**), aspirin (**1**), isosorbide-5-aspirinate (**53**), isosorbide-2-apsirinate (**52**), isosorbide-5-salicylate (**56**), ISDA (**36**), isosorbide-2-aspirinate-5-salicylate (**37**), isosorbide-2-salicylate-5-aspirinate (**54**) and isosorbide disalicylate (**55**).*

The later eluting salicylate metabolites, isosorbide-2-aspirinate-5-salicylate (**37**) and isosorbide-2-salicylate-5-aspirinate (**54**), were not sufficiently separated for quantitative purposes under gradient conditions. Any improvement in their separation was counterbalanced by an increase in analysis time and a decrease in peak efficiency. However, under isocratic conditions the later eluting components in the mixture were completely separated. Therefore, the ratio of ester **37** and **54**, released during plasma hydrolysis of ISDA, was obtained under isocratic conditions (~ 2:1, **37**:**54**). The retention times of each component in the mixture are presented in *Table 3.4* along with their corresponding capacity factors.

| Compound No. | $t_r$ min | $k^1$ |
|---|---|---|
| **2** | 8.53 | 3.87 |
| **1** | 8.93 | 4.10 |
| **53** | 9.31 | 4.32 |
| **52** | 9.58 | 4.47 |
| **56** | 9.96 | 4.69 |
| **36** | 13.58 | 6.76 |
| **37/54** | 14.29 | 7.17 |
| **55** | 15.11 | 7.63 |

*Table 3.4 Chromatographic data for the separation of ISDA and potential metabolites according to the reverse phase HPLC gradient method presented in Table 3.3.*

A typical ISDA plasma sample was also analysed under the conditions developed. The plasma peaks did not co-elute with the peaks of interest. The plasma peaks were identified by their characteristic UV spectrum ($\lambda_{max}$ in the range 280.0 to 280.2). A typical chromatogram is presented in *Fig. 3.14*, along with their PDA spectra. Potential aspirinate metabolites of ISDA (**36**) have characteristic UV spectrum with $\lambda_{max}$ at approximately 225 nm and 276 nm. Potential salicylate metabolites of ISDA also have characteristic UV spectrum with $\lambda_{max}$ at approximately 230 nm and 306 nm.

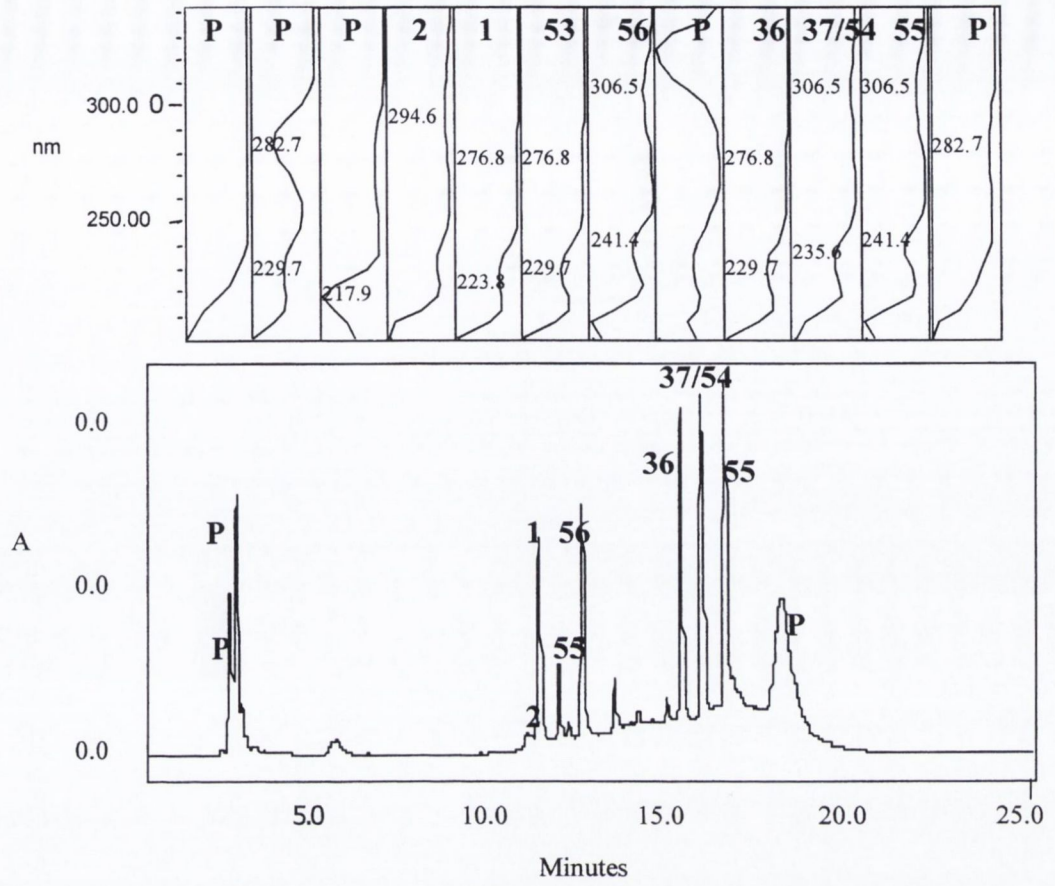



*Figure 3.14 A typical chromatogram (230 nm) of a sample obtained following the incubation of ISDA in 30% human plasma at pH 7.4 and 37°C. Peaks labelled* ***P*** *represent plasma peaks present in the plasma blank. Also shown are PDA spectra of each of the labelled components: Salicylic acid (**2**), aspirin (**1**), isosorbide-5-aspirinate (**53**), isosorbide-2-aspirinate (**52**), isosorbide-5-salicylate (**56**), ISDA (**36**), isosorbide-2-aspirinate-5-salicylate (**37**), isosorbide-2-salicylate-5-aspirinate (**54**) and isosorbide disalicylate (**55**).*

The method was validated for linearity, precision, specificity and sensitivity. A linear response was observed for each analyte ($r > 0.9999$) in the range 1-100 μg/ml. The RSD on multiple injection of each analyte at 10 μg/ml and 100 μg/ml was <2.1%. The limit of quantitation for the relevant analytes was 1 μg/ml. The gradient method developed was successfully employed in the assay of plasma samples for the determination of the hydrolysis pathways of ISDA (**36**).

## 3.2 Physico-chemical studies

It was decided to determine the lipophilicity and aqueous solubility of ISDA (**36**), since these are useful indicators for the extent of absorption of a drug following oral administration.

### 3.3.1 Lipophilicity studies

The lipophilicities of ISDA (**36**) and its potential metabolites were estimated using the SMILES computer program as described in Chapter 2 (*Table 3.5*). The lipophilicity of ester **36** was estimated to be 2.24, which is greater than that of aspirin and ISMNA (**35**). The salicylate metabolites of ester **36**: isosorbide disalicylate (**55**); isosorbide-2-aspirinate-5-salicylate (**37**) and isosorbide-2-salicylate-5-aspirinate (**54**) have significantly higher lipophilicities, since they are less polar. The products of hydrolysis at the carboxylic ester group: isosorbide-5-aspirinate (**53**) and isosorbide-2-aspirinate (**52**) have lower lipophilicities. This effect may be due to the influence of the hydroxyl group at the isosorbide ring of ester **52** and **53**.

| Compound No. | *c*log P | $t_r$ min | $k^1$ | Log $k^1$ |
|---|---|---|---|---|
| **36** | 2.24 | 9.66 | 4.52 | 0.655 |
| **55** | 3.84 | 20.32 | 10.61 | 1.026 |
| **37** | 3.04 | 16.61 | 8.49 | 0.929 |
| **54** | 3.04 | 15.55 | 7.89 | 0.897 |
| **52** | 0.33 | 4.89 | 1.79 | 0.254 |
| **53** | 0.33 | 5.44 | 2.11 | 0.324 |
| **35** | 1.20 | 6.41 | 2.66 | 0.425 |

*Table 3.5 Lipophilic and chromatographic data for ISDA and potential metabolites.*

In order to validate the estimated lipophilicity data, the results were correlated with capacity factors calculated from HPLC retention times (*Fig. 3.15*, r = 0.9799). The HPLC method involved the isocratic elution on a Nova-Pak C8 (3.9 x 75 mm) column

with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) at 1 ml/minute to give the desired retention characteristics (*Table 3.5*). Gradient elution was not required since it was the retention time of each metabolite, not concentration, which was the desired parameter.

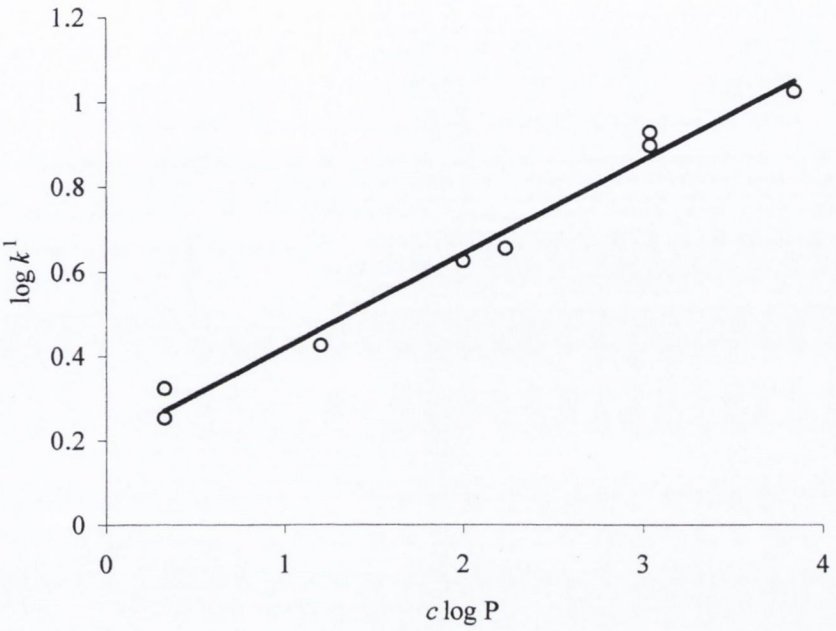



*Figure 3.15 Plot of clog P against log $k^1$ for ISDA and potential metabolites.*

### 3.3.2 Aqueous solubility studies

The solubility of ISDA (**36**) was determined over a period of one week in water and in phosphate buffer at pH 2.5, pH 6.8 and pH 9.74. Saturated solutions of the ester were shaken at 37°C and analysed by isocratic elution on a reverse phase Nova-Pak C8 column (3.9 x 75 mm) with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%). Since it was the concentration of ISDA in solution that was required and not its degradation pathway, gradient elution was not necessary. Quantitation was achieved by chromatographing external standards on the same day, under the same conditions and in a similar concentration range.

| **Time** hours | **Water (pH 7)** μg/ml | **pH 2.5** μg/ml | **pH 6.80** μg/ml | **pH 9.74** μg/ml |
|---|---|---|---|---|
| 1 | 9.39 | 11.53 | 7.02 | 2.81 |
| 7 | 19.12 | 19.12 | 13.78 | 13.30 |
| 1 week | 15.67 | 16.76 | 10.9 | 9.74 |

*Table 3.6 Solubility data for ISDA in water, pH 2.5, pH 6.8 and pH 9.74 at 37°C.*

*Table 3.6* presents the solubility of ISDA (**36**) in water (pH 7), pH 2.5, pH 6.8 and pH 9.74. The concentrations of ISDA in solution were measured as μg/ml. The solubility of ester **36** in water and at pH 2.5 was similar after seven hours, while at higher pH the solubility was lower. The solubility of ISDA at each pH value decreased after one week. This effect may be due to the aqueous hydrolysis of ester **36**. The aqueous solubility of ester **36** was unexpectedly low (approximately 10-16 μg/ml). However, the dose of ISDA (**36**) required to affect platelet function following oral administration is difficult to predict.

## 3.4 pH rate profile of ISDA

Stability towards aqueous hydrolysis is an important consideration for potential aspirin prodrugs. Typical pH values a drug may encounter after oral administration include pH 6.4 (saliva), pH 1.5 (stomach) and pH 5.5 (duodenum) [178]. Stability at low pH is an integral part of the design of aspirin esters, to prevent the direct contact irritation associated with aspirin on the gastric mucosa. Furthermore, the pharmaceutical dosage forms of many drugs incorporate a range of pH values to which that drug must be stable. In order to predict the stability of ester **36** in aqueous solution, the decomposition kinetics were studied in detail as a function of pH.

The *in vitro* hydrolysis of ester **36** was monitored in aqueous solution at 37°C and over a pH range of 1.03 to 9.40. A range of buffers, including phosphate, formate and acetate were employed, which were maintained at approximately constant ionic strength by the addition of a calculated amount of NaCl ($I \sim 0.10$, Chapter 2). The rate of hydrolysis was independent of ionic strength, as determined at pH 2.8. The buffer concentration was in the range 0.01 M to 0.06 M. Since it was the concentration of

ester **36** remaining and not appearance of metabolites that was being monitored, gradient elution was not required. The isocratic system described in Section 3.3 was employed. Quantitation was achieved by measuring the peak areas in relation to those of external standards chromatographed under the same conditions and in a similar concentration range. The hydrolyses followed pseudo first-order kinetics over the pH range studied. Pseudo first-order plots of the logarithm of remaining ester concentration against time were constructed from which the pseudo first-order rate constant and half-life were determined. The pseudo first-order rate parameters appear in *Table 3.7* while the pH rate profile is presented in *Fig. 3.16*.

| **pH** | **I** | $k_{obs}$ hours $^{-1}$ | $t_{1/2}$ hours |
|---|---|---|---|
| 1.03 | 0.13 | 0.05 | 14.28 |
| 1.40 | 0.11 | 0.02 | 34.65 |
| 1.62 | 0.11 | 0.01 | 62.72 |
| 1.88 | 0.11 | 0.005 | 144.38 |
| 2.50 | 0.28 | 0.002 | 300.39 |
| 4.13 | 0.11 | 0.0002 | 3013.04 |
| 6.45 | 0.08 | 0.007 | 1004.34 |
| 7.29 | 0.09 | 0.003 | 231.77 |
| 8.10 | 0.09 | 0.02 | 43.22 |
| 9.40 | 0.10 | 0.29 | 2.348 |

*Table 3.7 Kinetic data for the hydrolysis of ISDA over a pH range of 1.03-9.40 at 37°C.*

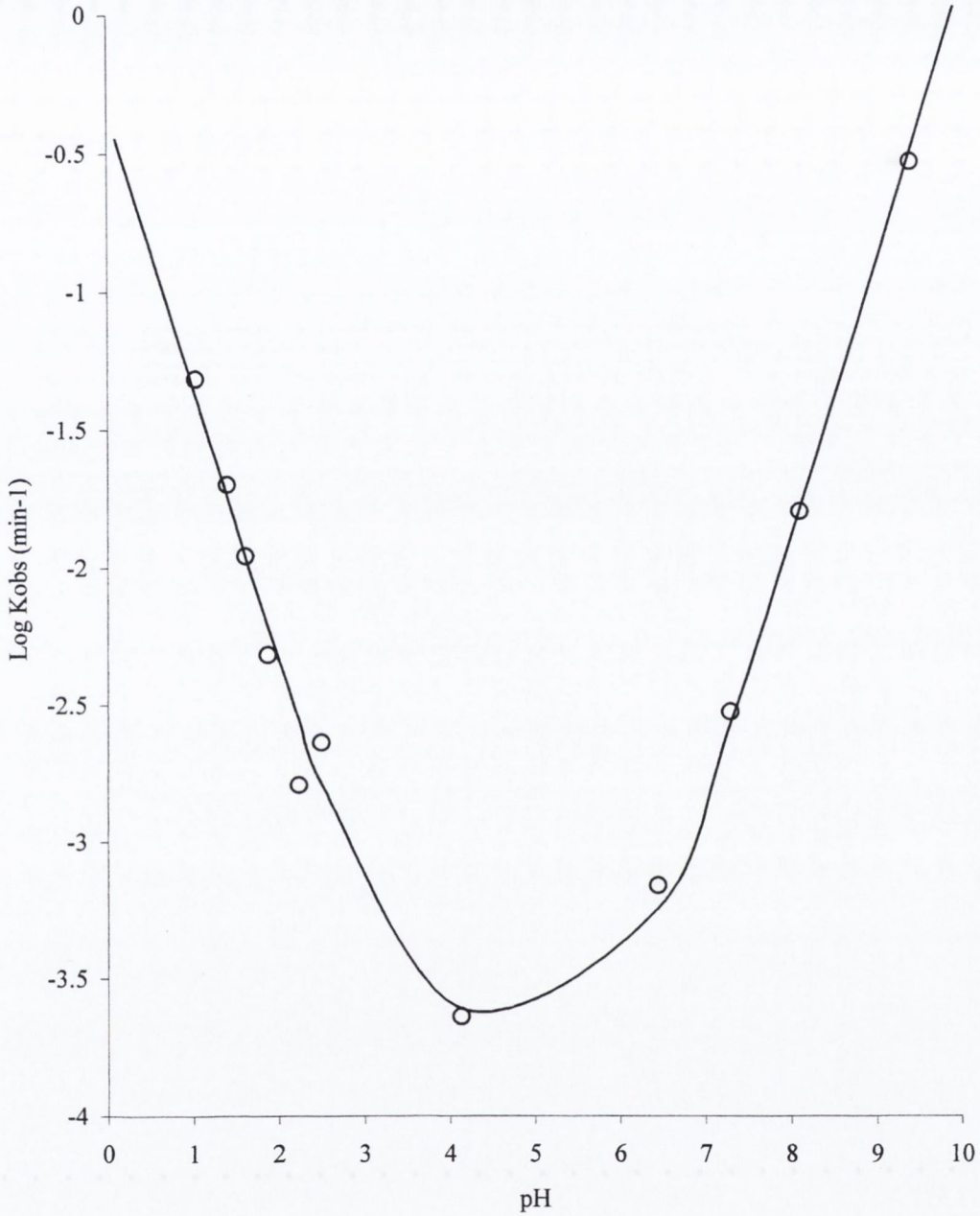



*Figure 3.16 pH rate profile for the hydrolysis of ISDA in aqueous solution at 37°C. The points represent log of the pseudo first-order rate constants determined at each pH. The continuous line represents the theoretical pH hydrolysis curve, constructed using Eqn. 3.3 and the rate constants presented in Table 3.7.*

The influence of pH on the degradation of ISDA (**36**) (U-shaped profile) is presented in *Fig. 3.16* from which it is apparent that the degradation is subject to acid- and base-catalysed hydrolysis. The overall hydrolytic reaction can be described in terms of a water-catalysed or spontaneous reaction as well as acid- and base-catalysed reactions according to Eq. (3.3): $k_O$ describes the spontaneous hydrolysis, $k_H$ describes the acid-catalysed hydrolysis and $k_{OH}$ describes the base-catalysed hydrolysis [266].

$$k_{obs} = k_O + k_H a_H + k_{OH} a_{OH} \qquad (3.3)$$

The terms $a_H$ and $a_{OH}$ refer to the hydrogen ion and hydroxide ion activity respectively. The latter were calculated from the measured pH at 37°C according to the equation of Harned and Hamer (Eq. (3.4)) [267].

$$\text{Log } a_{OH} = \text{pH} - 13.6 \qquad (3.4)$$

Values of the second-order rate constants for the apparent acid ($k_H$) and base ($k_{OH}$) catalysed decomposition were determined from the straight line portions of the pH rate profile at low and high pH values respectively. The apparent first-order rate constant for spontaneous decomposition ($k_O$) was obtained from the plateau region of the pH rate profile. The values for the first-order rate constants were: $k_H = 0.452\ M^{-1}\ min^{-1}$, $k_O = 0.00021\ M^{-1}\ min^{-1}$ and $k_{OH} = 5296\ M^{-1}\ min^{-1}$. The solid curve shown in *Fig. 3.16* was constructed from these constants and Eq. (3.3).

The rate data for ISDA implies that overall its stability is maximal in the pH range 4-6. However, ester **36** is most stable at pH 4.13, undergoing very slow hydrolysis with a half-life of approximately 3013 hours. The stability of ISDA (**36**) in the pH range 4 to 6 indicates the aspirin ester might be stable in dosage forms at this pH range. At pH 1.4 (pH of the stomach) the hydrolysis of ester **36** proceeded with a half-life of 62 hours. This indicates that ester **36** may be sufficiently stable to pass through the stomach intact, thereby reducing or eliminating the direct contact effect of acidic aspirin on the stomach mucosa. ISDA also appears to possess suitable stability at approximate physiological pH (pH 7.4).

Over the pH range studied, the hydrolysis was associated with the liberation of a mixture of *in vitro* salicylate metabolites, including salicylic acid (**2**), isosorbide disalicylate (**55**), isosorbide-2-aspirinate-5-salicylate (**37**) and isosorbide-2-salicylate-5-aspirinate (**54**). Although quantitation was not achieved for the degradation products, their liberation was supported by PDA detection. The high aqueous stability of ISDA (**36**) may be due to the bulky nature of the molecule. As described in Chapter 2, aspirin hydrolysis in aqueous solution is autocatalysed by the carboxylate. Since this group is protected in ester **36**, intramolecular nucleophilic catalysis is not possible. It has been reported that there is a one hundred-fold rate enhancement for the hydrolysis of aspirin compared to phenyl acetate due to the intramolecular effects of aspirin [177].

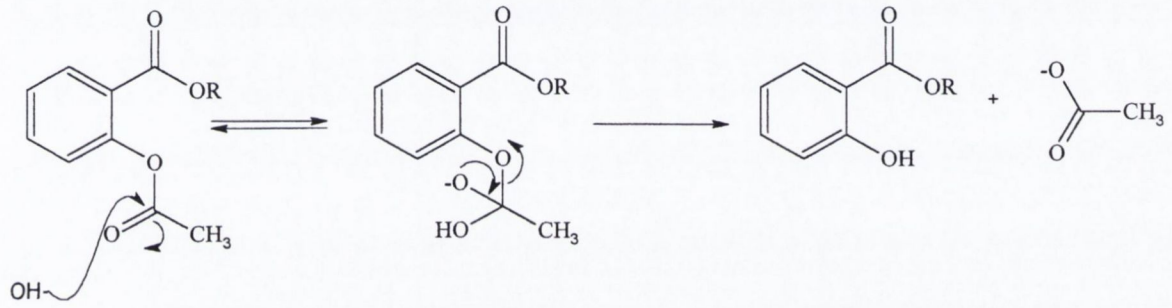



*Figure 3.17 Hydroxide catalysed hydrolysis of ISDA; R = isosorbide monoaspirinate.*

The hydrolysis of ester **36** under alkaline conditions is depicted in *Fig. 3.17*, where the R group represents the remaining monosubstituted isosorbide-aspirinate. The hydrolysis involves acyl transfer, which occurs by both bond formation between the acyl carbon and the hydroxide ion and in addition, bond breakage at the acetyl moiety. Due to the bulky nature of the substituted aspirin, a reduced reactivity results by sterically hindering the approach of the incoming hydroxide nucleophilic species. The hydrolysis of ester **36** under acidic conditions may occur through protonation of the acyl oxygen (further polarising the carbonyl group), hence aiding attack at the acyl carbon.

## 3.3 Enzyme hydrolysis studies

The potential of ISDA to release aspirin *in vivo* was studied in plasma, since this system mimics conditions found *in vivo*. The hydrolysis of ISDA was also studied in a number of purified enzyme preparations to confirm that butyrylcholinesterase (EC 3.1.1.8) is the enzyme responsible for its hydrolysis. Further evidence for the BuChE hydrolysis of ester **36** was obtained from inhibition studies.

### 3.5.1 Plasma hydrolysis studies

The hydrolysis of ISDA (**36**) was studied in dog plasma to determine whether aspirin release in that species could explain the anti-platelet effects of ISDA observed following oral administration to beagles. Similarly, the hydrolysis of ISDA (**36**) was studied in rabbit plasma to determine whether aspirin liberation could explain the observed anti-thrombotic effects in rabbit platelet rich plasma. The results in these species had provided encouragement for the development of ester **36** as an anti-platelet agent in humans. The hydrolysis characteristics in human plasma were evaluated in this context.

The *in vitro* plasma samples were initially monitored by isocratic elution on a Nova-Pak C8 (3.9 x 75 mm) column with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) at 1 ml/minute. However, this system did not afford the desired separation of all potential metabolites. Therefore, the gradient method developed in Section 3.2 was employed in the subsequent analysis of plasma samples (*Table 3.3*). The concentration of ISDA (**36**) and its degradation products were calculated from the measured peak areas by reference to those of standards chromatographed under the same conditions.

#### 3.5.1.1 Hydrolysis studies in dog and rabbit plasma

As previously described, the hydrolysis of ISDA (**36**) was studied in dog and rabbit plasma solution to determine whether aspirin release is responsible for its anti-platelet effect in these species. The hydrolysis studies were replicated. The variation between days and between plasma samples was insignificant.

| **HPLC conditions** | $K_m$ M (x $10^{-4}$) | $V_{max}$ M $min^{-1}$ (x $10^{-4}$) | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ mean | (s.d., n) |
|---|---|---|---|---|---|
| Isocratic | 2.450 | 0.092 | 19.80 | 0.04 | |
| Gradient | 1.598 | 0.050 | 24.95 | 0.03 | (± 0.003, n = 3) |

*Table 3.8 Kinetic data for the hydrolysis of ISDA in 10% dog plasma (pH 7.4, 37°C).*

*Table 3.8* presents the kinetic data for the pseudo first-order hydrolysis of ester **36** in 10% buffered dog plasma (pH 7.4) at 37°C. The rates of hydrolysis were rapid in comparison to hydrolysis under aqueous conditions (*Table 3.7*). The Michaelis-Menten parameters appearing in *Table 3.8* were calculated using the Robinson and Characklis equation (Eq. 2.14) described in Chapter 2. A typical progression curve for the hydrolysis is presented in *Fig. 3.18*.

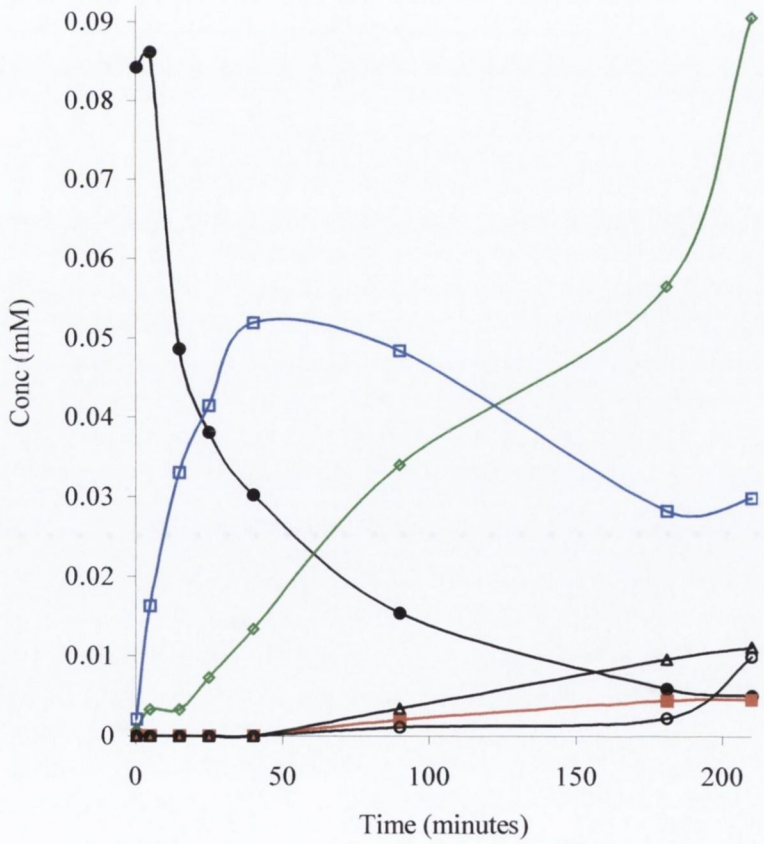



*Figure 3.18 Typical progression curve for the hydrolysis of ISDA in 10% dog plasma at pH 7.4 and 37°C: ISDA (●), aspirin (■), salicylic acid (o), isosorbide-2/5-aspirinate-2/5-salicylate (□), isosorbide disalicylate (◊) and isosorbide-5-salicylate (Δ).*

The hydrolysis was associated with the liberation of isosorbide-2-aspirinate-5-salicylate (**37**) and isosorbide-2-salicylate-5-aspirinate (**54**), which subsequently hydrolysed to isosorbide disalicylate (**55**). This is the typical pathway of hydrolysis for aspirin esters. Interestingly, the acetyl group at the 5-position appears to be more susceptible to attack than at the 2-position, since a greater concentration of ester **37** was liberated. Only minor quantities of aspirin, isosorbide-2-aspirinate (**52**) and isosorbide-5-aspirinate (**55**) were liberated. Esters **52** and **53** are omitted from *Fig. 3.18* for sake of clarity. ISMNA (**35**) demonstrated a similar pattern of hydrolysis in dog plasma.

The results of this study do not explain the observed anti-platelet effects of ISDA (**36**) following oral administration to beagles [168]. These results suggest that aspirin may be released by other enzymes in the dog, which could possess different catalytic activity to butyrylcholinesterase (EC 3.1.1.8). Experiments are planned using dog gut or liver homogenate to test this possibility. Work is also ongoing to test the intrinsic anti-platelet activity of the salicylate metabolites in the dog.

| **HPLC conditions** | $K_m$ | $V_{max}$ | $t_{1/2}$ | $k_{obs}$ | |
|---|---|---|---|---|---|
| | M (x $10^{-4}$) | M $min^{-1}$ (x $10^{-4}$) | min | $min^{-1}$ | |
| | | | | mean | (s.d., n) |
| Isocratic | 5.183 | 1.578 | 2.10 | 0.33 | |
| Gradient | 3.866 | 1.398 | 2.43 | 0.29 | (± 0.013, n = 4) |

*Table 3.9 Kinetic data for the hydrolysis of ISDA in 10% rabbit plasma at pH 7.4 and 37°C.*

The kinetic data for the hydrolysis of ISDA (**36**) in 10% buffered rabbit plasma (pH 7.4) at 37°C is presented in *Table 3.9*. The rate of hydrolysis was faster than in dog plasma (*Table 3.8*). Since it has been established that the BuChE activity of dog plasma is greater than rabbit plasma [205, 214] this observation must be due to the presence of a unique BuChE type in rabbit plasma, which promotes the hydrolysis of ester **36**. It is also possible that the hydrolysis is influenced by the presence of esterases with specificity for sugar compounds in rabbit plasma [227] (Chapter 2).

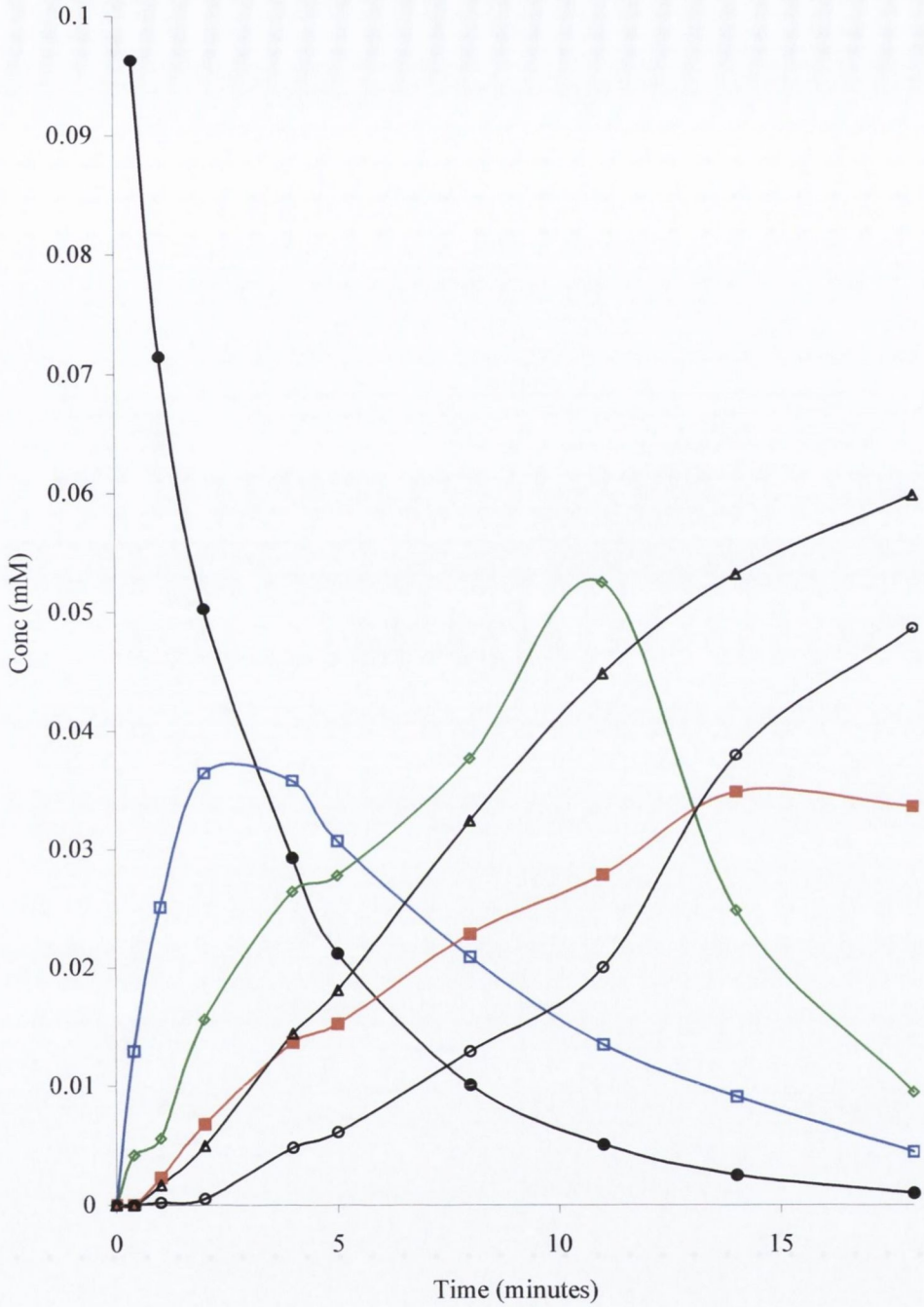



*Figure 3.19 Typical progression curve for the hydrolysis of ISDA in 10% rabbit plasma at pH 7.4 and 37°C: ISDA (●), aspirin (■), salicylic acid (o), isosorbide-2/5-aspirinate-2/5-salicylate (□), isosorbide disalicylate (◊), isosorbide-5-salicylate (Δ).*

A typical progression curve for the hydrolysis of ISDA (**36**) in rabbit plasma solution is presented in *Fig. 3.19*. Hydrolysis occurred at both the *O*-acetyl and carboxyl ester sites, generating a mixture of *in vitro* metabolites. Minor amounts of isosorbide-2-

aspirinate (**52**) and isosorbide-5-aspirinate (**53**) were released but are omitted from *Fig. 3.19* for clarity. Isosorbide-2-aspirinate-5-salicylate (**37**) and isosorbide-2-salicylate-5-aspirinate (**54**) were liberated from ester **36** and were rapidly hydrolysed to isosorbide disalicylate (**55**). The hydrolysis was also associated with the generation of 27.6%-44.33% aspirin, with a mean value of 36.6% (n = 3). This is somewhat less than the amount generated following incubation of ISMNA in rabbit plasma solution (up to 78.7%) but substantially greater than the amount of aspirin generated when ISDA was incubated in dog plasma solution. This confirms that rabbit plasma contains an enzyme specific for isosorbide ester derivatives. Interestingly, ISDA was shown in our laboratory to be more potent than aspirin in the inhibition of arachidonic acid-induced platelet aggregation in rabbit PRP despite liberating only 36% aspirin in the present study [238].

### 3.5.1.2 Hydrolysis studies in human plasma

The hydrolysis of ISDA (**36**) was studied in 10% buffered human plasma (pH 7.4) at 37°C and followed pseudo first-order kinetics as described in *Table 3.10*. The observed hydrolysis was more rapid than under aqueous conditions and was comparable to its hydrolysis in rabbit plasma solution, despite rabbit plasma having a lower BuChE activity than human plasma (*Table 2.5)*. This is further evidence for the existence of a unique BuChE in rabbit plasma, which promotes hydrolysis of ISDA.

| **HPLC conditions** | $K_m$ | $V_{max}$ | $t_{1/2}$ | $k_{obs}$ | |
|---|---|---|---|---|---|
| | M (x $10^{-4}$) | M $min^{-1}$ (x $10^{-4}$) | min | $min^{-1}$ | |
| | | | | mean | (s.d., n) |
| Isocratic | 3.825 | 0.598 | 5.35 | 0.13 | (± 0.008, n = 2) |
| Gradient | 1.595 | 0.302 | 4.43 | 0.16 | (± 0.005, n = 3) |

*Table 3.10 Kinetic data for the hydrolysis of ISDA in 10% human plasma at pH 7.4 and 37°C.*

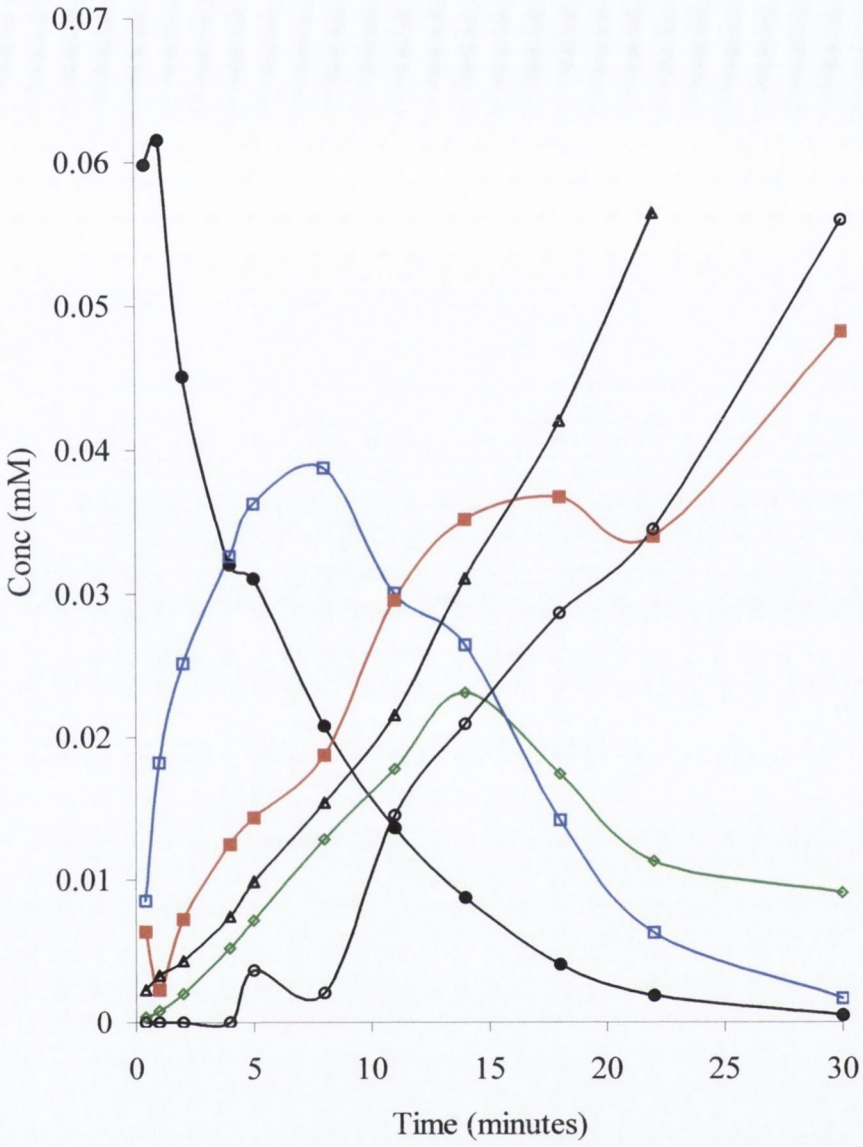



*Figure 3.20 Progression curve for the hydrolysis of ISDA in 10% human plasma (pH 7.4, 37°C): ISDA (●), aspirin (■), salicylic acid (o), isosorbide-2/5-aspirinate-2/5-salicylate (□), isosorbide disalicylate (◊) and isosorbide-5-salicylate (Δ).*

The progression curve for the hydrolysis of ISDA (**36**) in 10% human plasma (*Fig. 3.20*) represents the average degradation data of three hydrolysis studies. Remarkably, the hydrolysis was associated with the generation of 40%-60% aspirin, with a mean value of 51% (n = 6). The observed aspirin release correlates with the anti-platelet effect of ISDA in human whole blood and human PRP, as previously described. Interestingly, it appears from the progress curve that there is a late burst of aspirin release, which occurs when ISDA has almost completely metabolised. This perhaps

indicates a release of aspirin from one of the *in vitro* metabolites of ISDA. A mixture of the salicylate esters: salicylic acid (**2**); isosorbide-2-aspirinate-5-salicylate (**37**), isosorbide-2-salicylate-5-aspirinate (**54**) and isosorbide disalicyate (**55**) were also generated. These were identified by PDA detection and by reference to external standards. However, as previously described, the ratio of esters **37** and **54** liberated during human plasma hydrolysis was 2:1. Therefore, we consider the liberation of ester **37** only. Isosorbide-2-aspirinate (**52**) and isosorbide-5-aspirinate (**53**) were subsequent products of the hydrolysis, however, they are omitted from *fig. 3.20* for clarity.

The possibility that an *in vitro* metabolite of ISDA releases aspirin in human plasma can be illustrated with the following picture (*Fig. 3.21*).

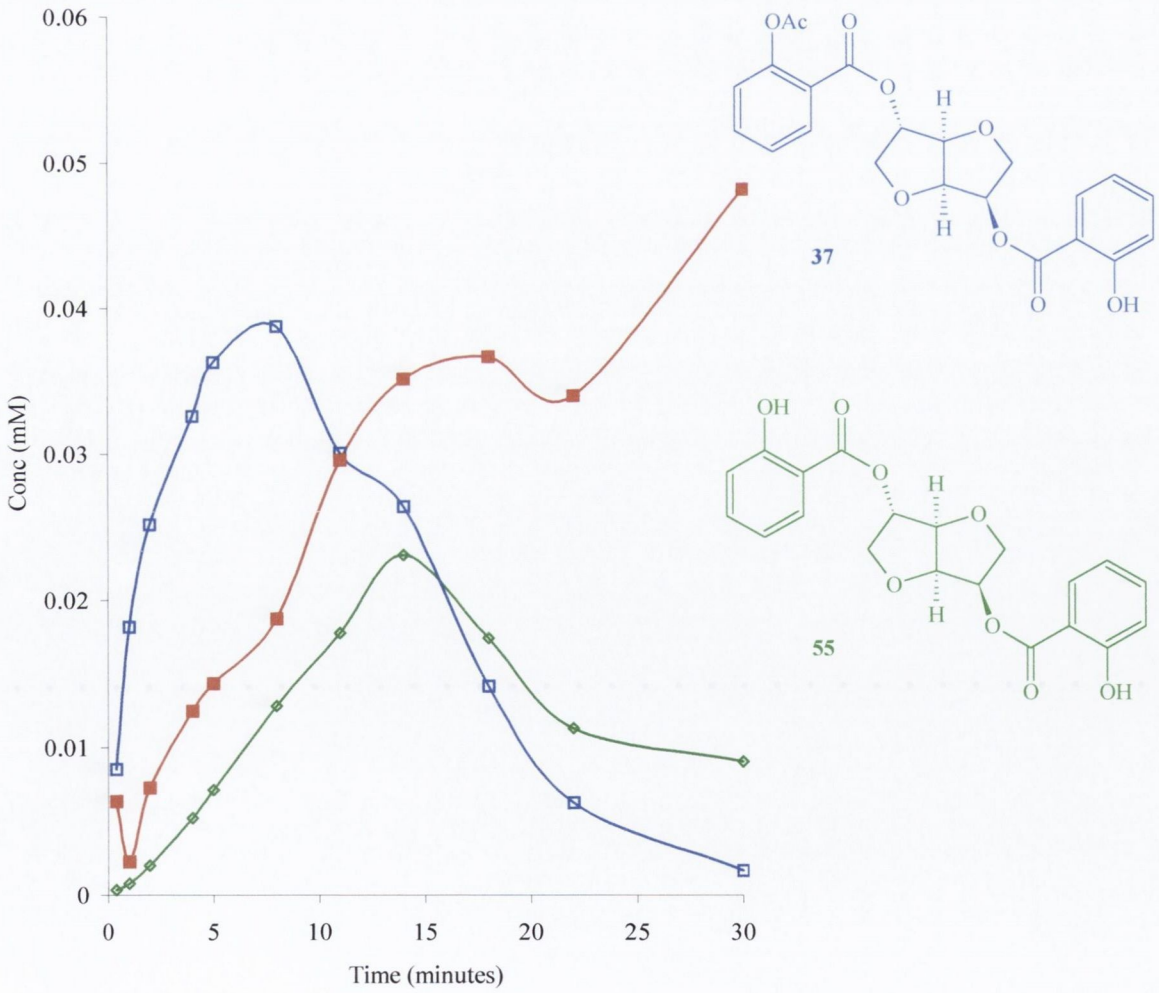



*Figure 3.21 Progression curve for the hydrolysis of isosorbide-2-aspirinate-5-salicyate (liberated from ISDA) in 10% human plasma: Aspirin (■), isosorbide-2-aspirinate-5-salicylate (□) and isosorbide disalicylate (◊).*

Isosorbide-2-aspirinate-5-salicylate (**37**) (blue line in *Fig. 3.21*) was liberated from the hydrolysis of ISDA. Surprisingly, it was observed that the hydrolysis of ester **37** corresponds with a significant release of aspirin (red line in *Fig. 3.21*). This was not the expected pathway of hydrolysis for ester **37**, since in dog and rabbit plasma solution its hydrolysis was associated with the exclusive generation of isosorbide disalicylate (**55**) (green line). It seems that ester **37** is capable of releasing aspirin in human plasma solution. Investigations into this observation are described later in this chapter.

| **% Plasma** | $K_m$ | $V_{max}$ | $t_{1/2}$ | $k_{obs}$ | **% Aspirin formed** |
|---|---|---|---|---|---|
| | M (x $10^{-4}$) | M $min^{-1}$ (x $10^{-4}$) | min | $min^{-1}$ | |
| 10% | 1.595 | 0.302 | 4.43 | 0.16 | 51.0 |
| 30% | 1.264 | 1.015 | 1.11 | 0.63 | 63.95 |
| 50% | 9.791 | 12.560 | 0.68 | 1.02 | 60.47 |

*Table 3.11 Kinetic data for the hydrolysis of ISDA in 10%, 30% and 50% human plasma at 37°C.*

The hydrolysis of ISDA was studied in 30% and 50% human buffered plasma (pH 7.4) at 37°C. Results of this study were expected to give an indication of the anti-platelet potential of ISDA in humans as well as the influence of esterases on its rate and hydrolytic product distribution. The hydrolyses followed pseudo first-order kinetics as described in *Table 3.11*.

At a higher plasma concentration, the hydrolysis of ISDA was more rapid, presumably due to the presence of higher concentrations of butyrylcholinesterase. The hydrolysis of ISDA in 30% human plasma is presented in *Fig 3.22*. At each plasma concentration, there was a significant increase in aspirin release corresponding to the hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**). The concentration of aspirin liberated at 30% and 50% plasma was 63.9% and 60.47% respectively. In 30% human plasma, isosorbide-5-salicylate (**56**) and aspirin (**1**) were stable up to 60 minutes, at which point the analysis was stopped. However, it was anticipated that salicylic acid (**2**) would be the ultimate product of their hydrolysis.

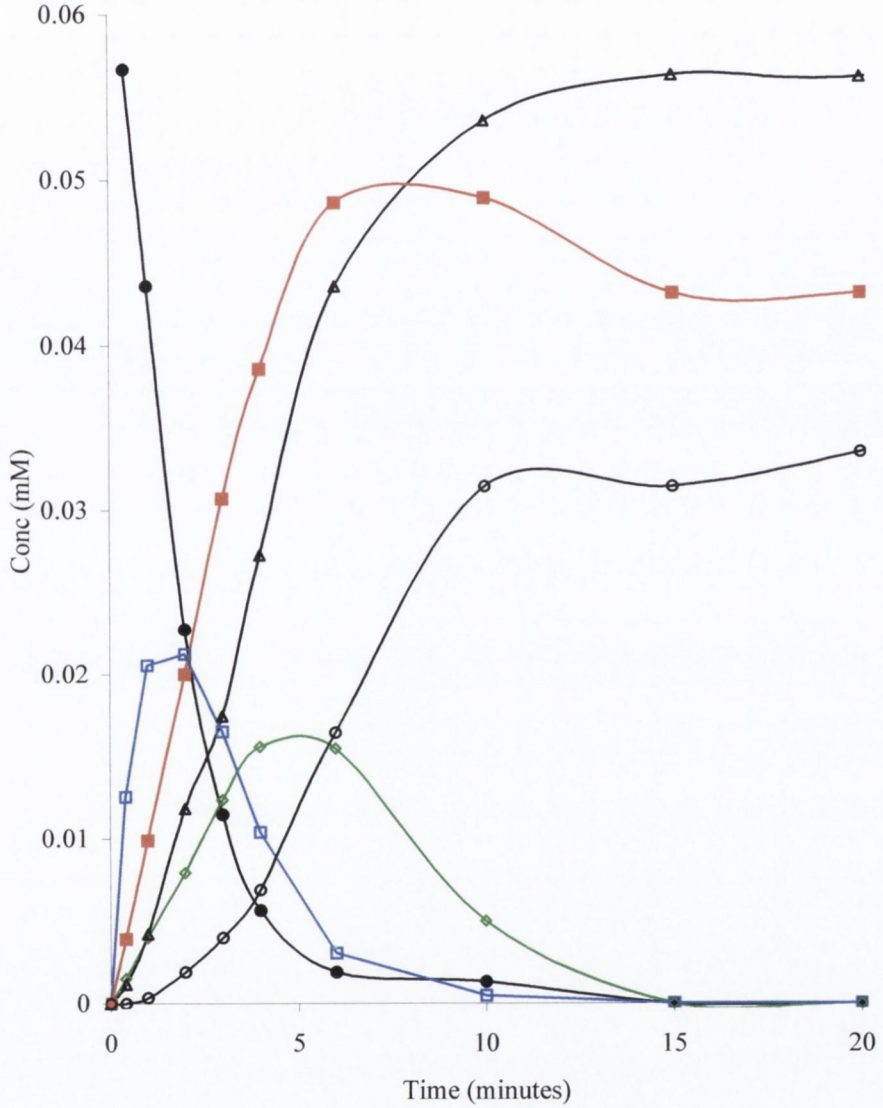



*Figure 3.22 Progression curve for the hydrolysis of ISDA in 30% human plasma: ISDA (●), aspirin (■), salicylic acid (o), isosorbide-2-aspirinate-5-salicylate (□), isosorbide disalicylate (◊) and isosorbide-5-salicylate (Δ).*

A plot of log $k_{obs}$ against percent plasma (*Fig. 3.23*, r = 0.9788) was linear, which indicates that the observed hydrolysis of ISDA is enzymatic.

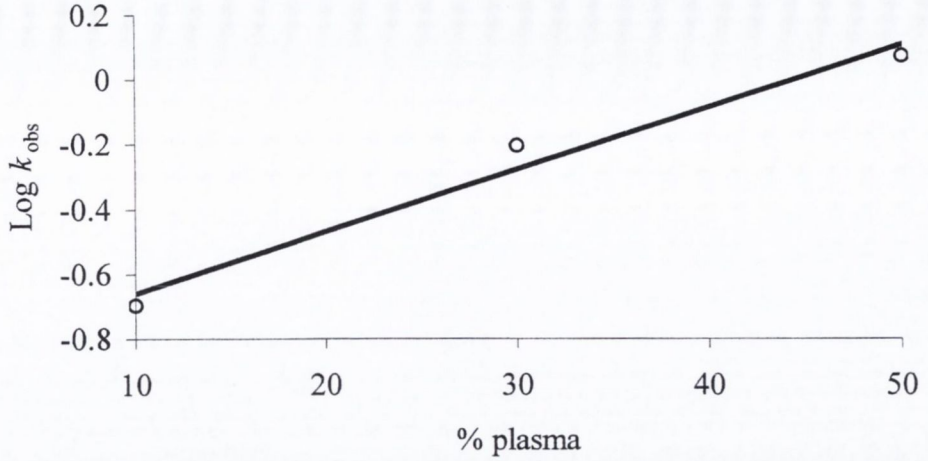



*Figure 3.23 Plot showing the correlation between percent plasma (human) and $K_{obs}$*

The hydrolysis studies of ISDA (**36**) in human plasma solution places it among the most successful aspirin prodrugs reported to date (Chapter 1) and confirms that a feature of the isosorbide group confers the ability to rapidly hydrolyse i*n vitro* to release aspirin. It would also appear that an *in vitro* metabolite of ISDA (isosorbide-2-aspirinate-5salicylate) has the potential to release aspirin in human plasma.

### 3.5.2 Enzyme inhibition studies

We determined that significant concentrations of aspirin were liberated during hydrolysis of ISDA in rabbit and human plasma solution. The enzyme presumed responsible for the hydrolysis of ISDA (**36**) in plasma was butyrylcholinesterase (EC 3.1.1.8). To confirm this, its hydrolysis was studied in rabbit and human plasma solution co-incubated with eserine; a potent esterase inhibitor (Chapter 2) [203]. The study was carried out by incubating plasma with eserine at a concentration of 3 μM (1 mg/10ml) while the progress of the reaction was monitored by gradient elution using the reverse phase LC method described previously (*Table 3.3*). Kinetics for the plasma hydrolysis of ISDA with eserine are presented in *Table 3.12*. Hydrolysis was suppressed when ISDA was co-incubated with eserine.

| Enzyme Source | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ |
|---|---|---|
| 10% Human plasma | 4.43 | 0.16 |
| 10% Human plasma inhibited with eserine | 29.49 | 0.02 |
| 10% Rabbit plasma | 2.43 | 0.29 |
| 10% Rabbit plasma inhibited with eserine | 20.50 | 0.03 |

*Table 3.12 Kinetic data for the hydrolysis of ISDA in plasma, co-incubated with eserine.*

In both rabbit and human plasma solution co-incubated with eserine, no aspirin was liberated during the hydrolysis. This is in contrast to the results obtained in plasma solution without eserine, where 36% and 51% aspirin was liberated respectively. It would appear, therefore, that the rate and pathway of hydrolysis is significantly altered by the presence of a cholinesterase inhibitor.

It may be concluded that the major enzyme present in plasma, which is responsible for the hydrolysis of ISDA (**36**) is BuChE (EC 3.1.1.8). Although eserine is known to be a potent inhibitor of both acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8), insignificant concentrations of the former are found in plasma [203].

### 3.5.3 Hydrolysis studies with purified enzyme preparations

In addition to inhibition studies, the hydrolysis of ISDA (**36**) was studied in the presence of a number of purified enzyme preparations, to confirm that butyrylcholinesterase (EC 3.1.1.8) was the enzyme responsible for its hydrolysis *in vitro*.

ISDA (**36**) was incubated in the presence of purified horse serum butyrylcholinesterase (EC 3.1.1.8), buffered at pH 7.4 (37°C), at a concentration of 0.1 mg/10 ml and 1.0 mg/10 ml. As previously discussed, this enzyme has high homology with human serum butyrylcholinesterase (EC 3.1.1.8), similar catalytic efficiency towards butyrylcholine hydrolysis, and similar substrate specificity [268]. Therefore, incubation in horse serum butyrylcholinesterase should indicate the potential of ISDA

as a substrate for this enzyme. The activity of the enzyme preparation (1,000 units/mg protein) was confirmed using a modification of the Ellman method as described in Chapter 2, with butyryl thiocholine as substrate [207]. The hydrolyses were monitored using the isocratic HPLC method detailed in Section 3.2. Kinetic data for the pseudo first-order hydrolysis is presented in *Table 3.13*.

| **Purified enzyme source** | **Enzyme conc.** mg/ml | $k_{obs}$ ($min^{-1}$) | $t_{1/2}$ (min) | **% Aspirin formed** |
|---|---|---|---|---|
| Horse serum BuChE | 0.10 | 0.38 | 1.85 | 11.0 |
| | 0.01 | 0.05 | 13.80 | 7.0 |
| Human serum BuChE | 0.08 | 0.074 | 9.36 | 55.3 |
| Rabbit liver carboxyl esterase | 0.22 | 0.02 | 31.50 | 0 |
| Human serum albumin | 0.01 | 0.001 | 603.0 | 0 |
| α-Chymotrypsin | 0.01 | 0.003 | 200.9 | 0 |

*Table 3.13 Kinetic data for the hydrolysis of ISDA in the presence of purified enzyme preparations at pH 7.4 and 37°C.*

As expected, a more rapid rate of hydrolysis was observed when ISDA was incubated in the presence of a higher concentration of enzyme. A typical progression curve for the hydrolysis of ISDA (**36**) in the presence of purified horse serum butyrylcholinesterase (EC 3.1.1.8, 0.1 mg/ml) is presented in *Fig. 3.24*.

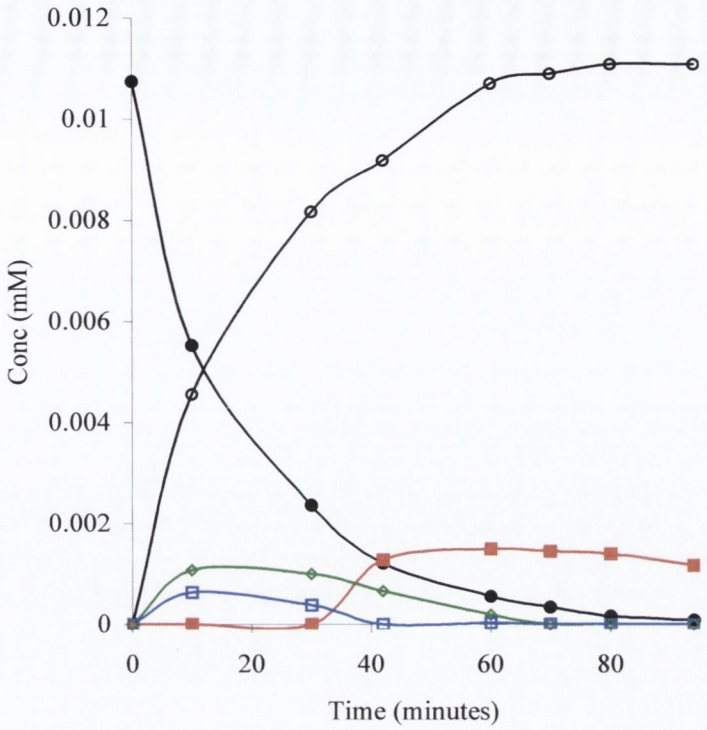



*Figure 3.24 Typical progression curve for the hydrolysis of ISDA with purified horse serum butyrylcholinesterase at pH 7.4 and 37°C: ISDA(●), aspirin (■), salicylic acid (o), isosorbide disalicylate (◊) and isosorbide-2-aspirinate-5-salicylate (□).*

The products of hydrolysis were identified by HPLC as aspirin (**1**), salicylic acid (**2**), isosorbide-2-aspirinate-5-salicylate (**37**) and isosorbide-2-salicylate-5-aspirinate (**54**). Approximately 11% aspirin was liberated, based on initial ester concentrations. This is somewhat less than the amount generated when ISDA was incubated in human plasma solution. However, as already discussed (Chapter 2), horse serum BuChE has three amino acid differences in the active site gorge compared with human serum BuChE [268]. These differences might alter the extent of aspirin liberation from horse serum BuChE.

Following the work described above, the hydrolysis of ISDA was studied in purified human serum BuChE (EC 3.1.1.8) (*Table 3.13*), monitored according to the gradient HPLC method developed in Section 3.2 (*Table 3.3*). The hydrolysis followed pseudo first-order kinetics and was more rapid than hydrolysis in horse serum BuChE at similar enzyme concentrations. The hydrolysis was associated with the liberation of approximately 55.3% aspirin. A mixture of isosorbide disalicylate (**55**), isosorbide 2-aspirinate-5-salicylate (**37**), isosorbide 2-salicylate-5-aspirinate (**54**) and salicylic acid

(**2**) were also liberated. These were identified by PDA detection and by reference to external standards. These results confirm that the fit of ISDA to the active site of human BuChE promotes hydrolysis at the carboxylic ester moiety and confirms ISDA as one of the most successful potential aspirin prodrugs investigated to date.

As discussed in Chapter 2, carboxylesterases have been found in rabbit liver that catalyse the hydrolysis of aspirin esters [205]. Furthermore, ester-type drugs are hydrolysed by intestinal esterases belonging to the class of B-esterases, which include carboxylesterases [269]. As previously discussed, aspirin prodrugs must be stable under conditions found in the intestinal mucosa but not under plasma hydrolysis conditions. The hydrolysis of ISDA (**36**) was studied in the presence of rabbit liver carboxylesterase (EC 3.1.1.1, 0.22 mg/ml). The hydrolysis was significantly slower than BuChE-mediated hydrolysis, despite a higher concentration of carboxylesterase used. The hydrolysis was 10 times faster in human BuChE than in carboxylesterase, based on intrinsic enzyme activities, albeit toward different substrates.

The disappearance followed pseudo first-order kinetics, as presented in *Table 3.13*. The hydrolysis was associated with the exclusive liberation of salicylate esters. No aspirin was liberated from the substrate. This result suggests that carboxylesterase does not contribute significantly to the hydrolysis of ISDA, which might be sufficiently stable towards B-esterases present in the intestinal mucosa to be absorbed intact.

As described in the previous chapter, human serum albumin (HSA) is a protein abundant in plasma, which displays some esterase activity [246]. The hydrolysis of ISDA (**36**) was studied in the presence of human serum albumin (0.01 mg/ml) at pH 7.4 and 37°C. The hydrolysis followed first-order kinetics as presented in *Table 3.13*. The rate of hydrolysis was significantly slower than in the presence of butyrylcholinesterase, at the same concentration (half-life of 603 and 13.86 minutes respectively). In the presence of HSA, ISDA metabolism was associated with the liberation of a mixture of the salicylate esters **37** and **54**. No aspirin (**1**) or salicylic acid (**2**) was detected. It can be concluded that ISDA is not hydrolysed by human serum albumin.

As described in Chapter 2, the pancreatic serine endopeptidases, which include α-chymotrypsin, are present in the lumen of the small intestine and may reduce the intestinal absorption (oral bioavailability) of a drug. This enzyme has also been shown

to rapidly hydrolyse a variety of esters (Chapter 2). We have previously established that ISDA (**36**) is stable towards carboxylesterase, which is present in the intestinal mucosa. The stability of ISDA towards α-chymotrypsin should give an indication of the enzymatic stability of ester **36** in the intestine. The stability of ISDA towards α-chymotrypsin (0.01 mg/ml) was determined at pH 7.4 and 37°C. The hydrolysis followed pseudo first-order kinetics according to the rate parameters presented in *Table 3.13* and was associated with the liberation of a mixture of *in vitro* salicylate metabolites. Although the hydrolysis was more rapid than hydrolysis in aqueous conditions, it was significantly slower than in plasma solution. This result suggests ISDA is a poor substrate for α-chymotrypsin and therefore, may survive the absorption process intact.

The relative susceptibility of ISDA towards hydrolysis in human plasma solution relative to a buffered (pH 7.4) solution containing α-chymotrypsin is depicted in *Fig. 3.25*, showing pseudo first-order rate curves.

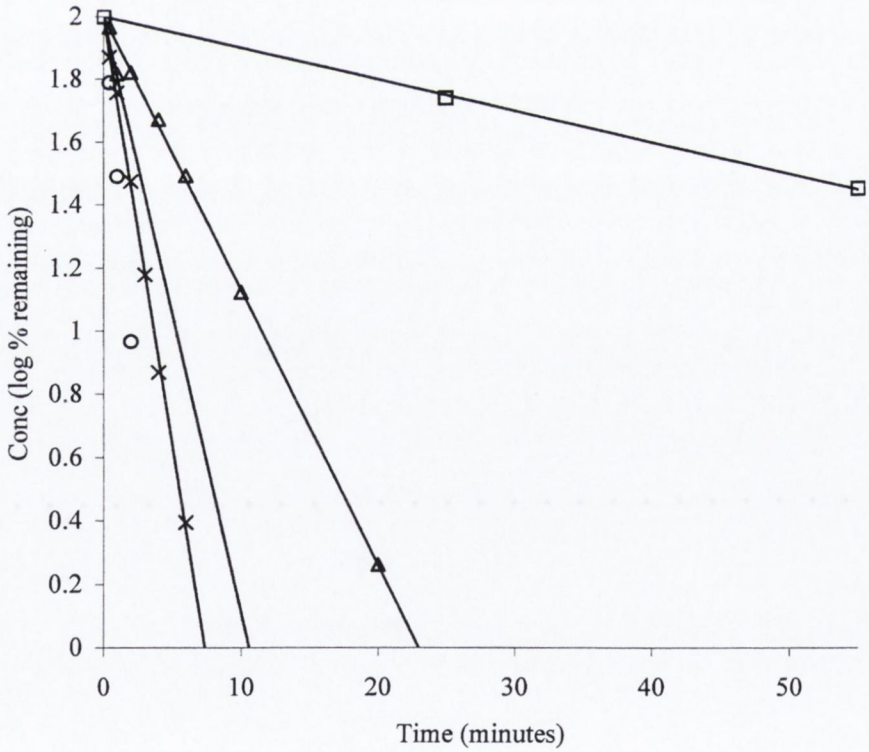



*Figure 3.25 Plot showing the pseudo first-order rate data for the hydrolysis of ISDA at pH 7.4 and 37°C in: 10% human plasma (Δ), 30% human plasma (x), 50% human plasma (o) with α-chymotrypsin (□).*

## 3.6 Investigation of isosorbide-2-aspirinate-5-salicylate as a novel aspirin prodrug

The plasma hydrolysis studies of ISDA (**36**) indicated that isosorbide-2-aspirinate-5-salicylate (**37**), an *in vitro* metabolite of ISDA, might liberate aspirin. This was evident from the degradation pattern of ISDA in 10% human plasma solution, where a shift towards aspirin release corresponded to the hydrolysis of ester **37**. The potential for aspirin release from ester **37** was investigated by hydrolysis studies in human plasma solution. The possible hydrolysis pathways of ester **37** are presented in *Fig. 3.26*. From this work, it transpired that ester **37** is the most successful aspirin prodrug developed thus far, generating up to 91% aspirin in human plasma and 98% aspirin in human serum BuChE. The most favourable result for previously reported aspirin esters was 50% aspirin release [96] (Chapter 1).

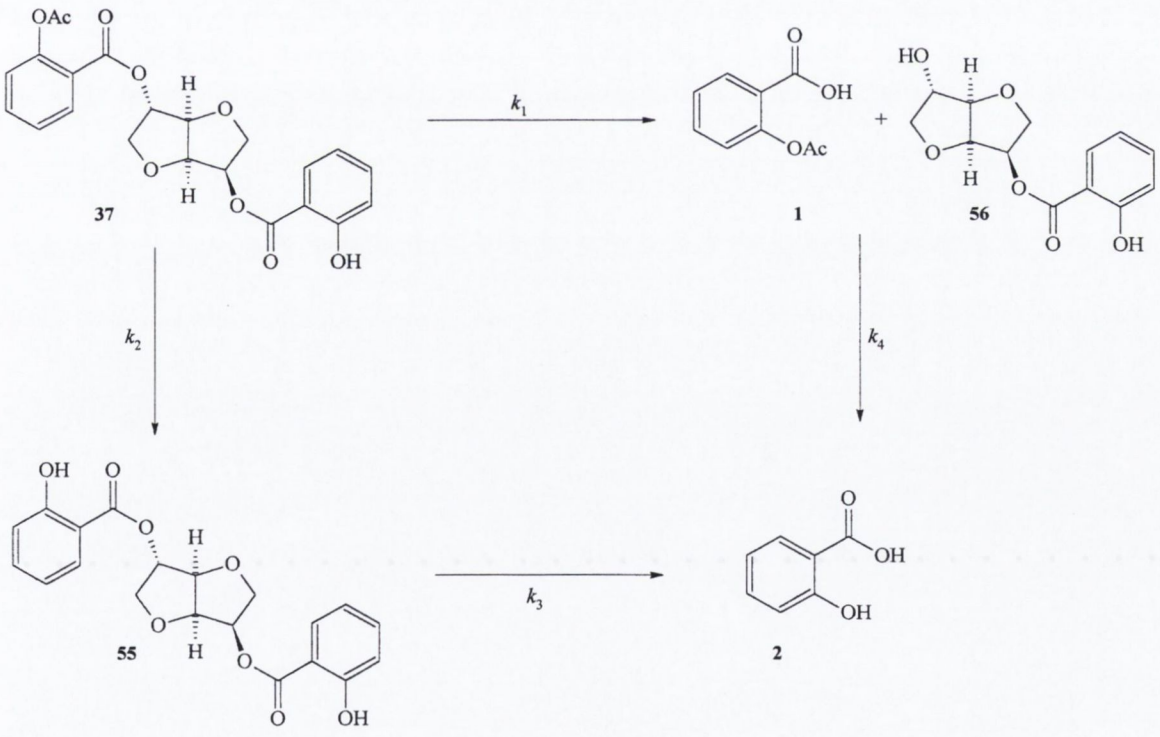



*Figure 3.26 The potential hydrolysis pathways of isosorbide-2-aspirinate-5-salicylate.*

### 3.6.1 Enzyme hydrolysis studies

The hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**) was studied in a number of different enzyme sources to determine its potential as an aspirin prodrug. The hydrolysis studies were carried out as described in Chapter 2. Final samples were analysed according to the gradient HPLC method developed (Section 3.2, *Table 3.3*). The pseudo first-order kinetic data for the hydrolysis studies are presented in *Table 3.14*.

| **Enzyme source** | $K_m$ M ($10^{-4}$) | $V_{max}$ M $min^{-1}$ ($10^{-4}$) | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ mean (s.d., n) | **% Aspirin formed** |
|---|---|---|---|---|---|
| Human plasma 10% | 0.805 | 0.174 | 4.90 | 0.16 (±0.049, n=3) | 73.23 |
| Human plasma 50% | 0.695 | 0.362 | 1.14 | 0.64 (±0.125, n=3) | 80.56 |
| Human serum 50% | 0.927 | 0.851 | 1.14 | 0.61 (±0.29, n=2) | 72.52 |
| Human whole blood | 1.418 | 1.488 | 0.96 | 0.79 (±0.249, n=2) | 46.03 |
| Horse serum BuChE | ___ | ____ | 9.67 | 0.07 (±0.012, n=2) | 43.67 |
| Human serum BuChE | ___ | ____ | 9.49 | 0.07 | 98.0 |
| Rat plasma 10% | 1.314 | 1.750 | 0.47 | 1.49 (±0.566, n=2) | 0.0 |

*Table 3.14 Kinetic data for the hydrolysis of isosorbide-2-aspirinate-5-salicylate in a number of different enzyme sources at pH 7.4 and 37°C.*

The hydrolysis of ester **37** in 10% human plasma solution was associated with the generation of 64.95%-81.5% aspirin. In 50% human serum it generated 67.8%-78.82% aspirin. The hydrolysis of ester **37** in 50% human plasma solution is presented in *Fig. 3.27* and was associated with the liberation of 70.72%-91.5% aspirin along with the release of minor concentrations of isosorbide disalicylate (**55**) and salicylic acid (**2**). In dog and rabbit plasma solution (Section 3.5.1), ester **37** liberated isosorbide disalicylate (**55**) exclusively. These results place ester **37** as the most successful aspirin ester developed to date and indicates that the substituent on the 5-position of the isosorbide ring directs hydrolysis at the carboxylate ester of the aspirinate at the 2-position, in the human BuChE active site. This observation will be investigated further in Chapter 4.

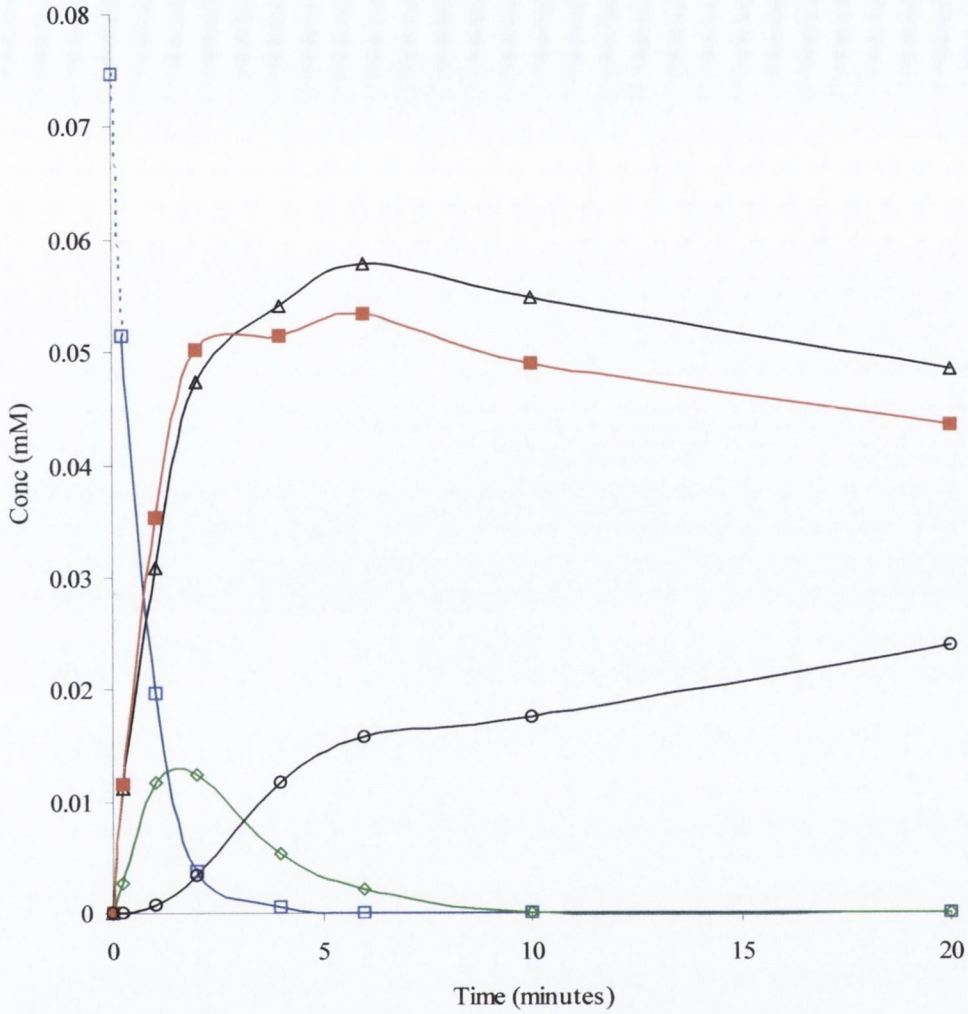



*Figure 3.27 Typical progression curve for the hydrolysis of isosorbide-2-aspirinate-5-salicylate in 50% human plasma (pH 7.4) at 37°C: Isosorbide-2-aspirinate-5-salicylate (□), isosorbide disalicylate (◊), isosorbide-5-salicylate (Δ), aspirin (■) and salicylic acid (o).*

The hydrolysis of ester **37** was studied in human whole blood to mimic conditions *in vivo* since esterases are present in whole blood that are not found in plasma. A typical progression curve for the hydrolysis is presented in *Fig. 3.28.*

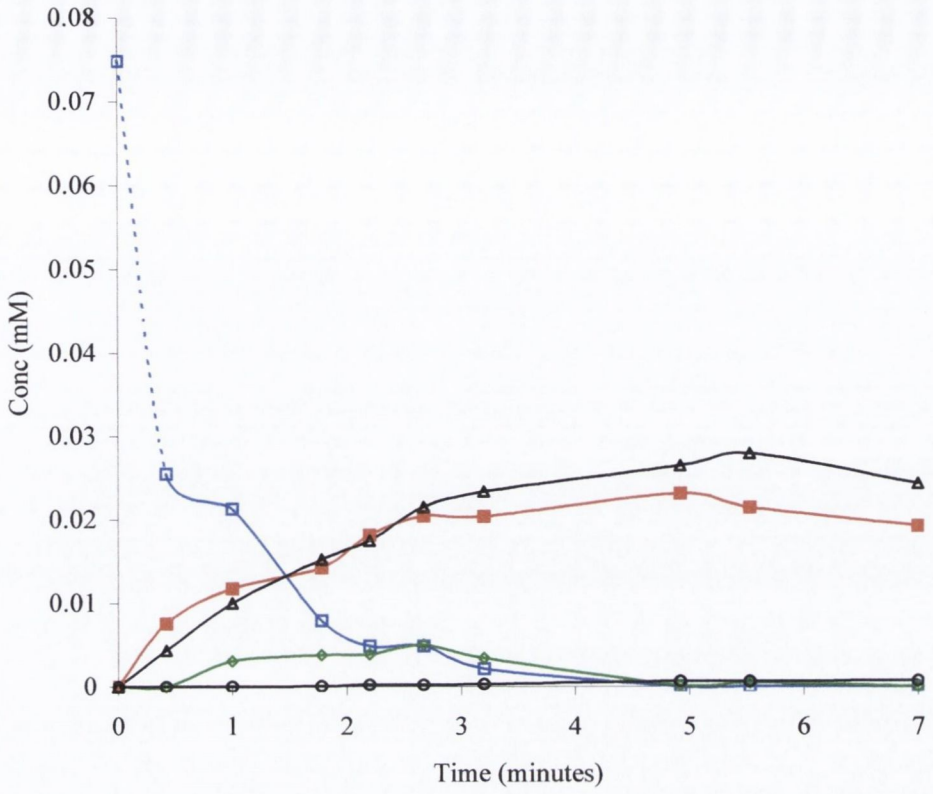



*Figure 3.28 Typical progression curve for the hydrolysis of isosorbide-2-aspirinate-5-salicylate in human whole blood at pH 7.4 and 37°C: Isosorbide-2-aspirinate-5-salicylate (□), isosorbide disalicylate (◊), isosorbide-5-salicylate (Δ), salicylic acid (o) and aspirin (■).*

The hydrolysis of ester **37** in whole blood generated 36.9%-55.16% aspirin, with a mean value of 46% (n = 2). Isosorbide disalicylate (**55**) was the minor product and was ultimately hydrolysed to salicylic acid (**2**). It is evident from the ratio of aspirin (red line) to isosorbide disalicylate (**55**) (green line) generated, that hydrolysis at the carboxylic ester group is the predominant pathway. The enzyme largely responsible for the hydrolysis of ester **37** appears to be butyrylcholinesterase. Its activity in human plasma and whole blood is almost identical [184].

The apparent concentration of aspirin liberated in whole blood was lower than in plasma solution. This may be due to the wider range of proteins present in whole blood, which perhaps possess different specificity towards the substrate and its degradation products. However, it is obvious that some problems exist in the analysis,

which may be related to recovery. Studies involving hydrolysis in whole blood were not validated for the recovery of analytes.

The hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**) was studied in 10% rat plasma solution to establish whether the rat is a suitable experimental model for the analysis of ester **37** *in vivo*. This study should also confirm the presence of a unique BuChE in human plasma, which is responsible for the release of up to 91% aspirin from ester **37**. Ester **37** generated during hydrolysis studies of ISDA (**36**) in dog and rabbit plasma solution does not appear to liberate aspirin in these species.

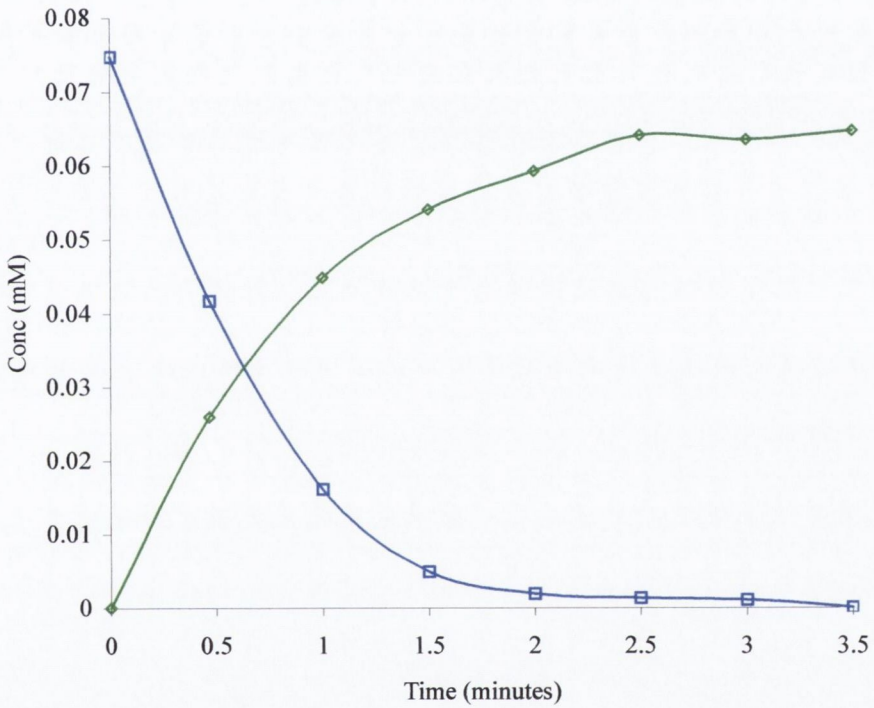



*Figure 3.29 Typical progression curve for the hydrolysis of isosorbide-2-aspirinate-5-salicylate in 10% rat plasma at pH 7.4 and 37°C: Isosorbide-2-aspirinate-5-salicylate (□) and isosorbide disalicylate (◊).*

A typical progression curve for the hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**) in 10% buffered rat plasma (pH 7.4) at 37°C is presented in *Fig. 3.29*. The exclusive product of its hydrolysis was isosorbide disalicylate (**55**), which remained essentially unchanged during the hydrolysis. Similarly, in rabbit and dog plasma solution, ester **37** liberated ester **55** exclusively during the hydrolysis. This study indicates that while BuChE in rat plasma is responsible for the rapid hydrolysis of ester

**37**, aspirin is not released. Therefore, human plasma BuChE is unique among the species investigated, since it promotes the hydrolysis of ester **37** at the carboxylic ester group.

The hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**) was studied in the presence of purified horse serum butyrylcholinesterase (EC 3.1.1.8, 1 mg/10 ml) at 37°C to confirm that this is the enzyme responsible for the observed hydrolysis. The hydrolysis was associated with the release of 40.9%-46.5% aspirin, with a mean value of 43.7%. Isosorbide disalicylate (**55**) was also liberated during the hydrolysis, which ultimately hydrolysed to salicylic acid (**2**). The concentration of aspirin released was somewhat less than the amount generated following incubation in human plasma solution. However, as previously described, horse serum BuChE contains three active site changes in comparison to human serum BuChE [268]. These changes may influence the direction of hydrolysis ester **37** takes in the active site of horse serum BuChE (EC 3.1.1.8).

The hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**) was studied in the presence of purified human serum BuChE (*Table 3.14*) at pH 7.4 and 37°C. Remarkably, the rapid hydrolysis was associated with the generation of 98% aspirin as presented in *Fig. 3.30* and *Fig. 3.31*. This exceptional result places ester **37** as the most successful aspirin prodrug developed to date.

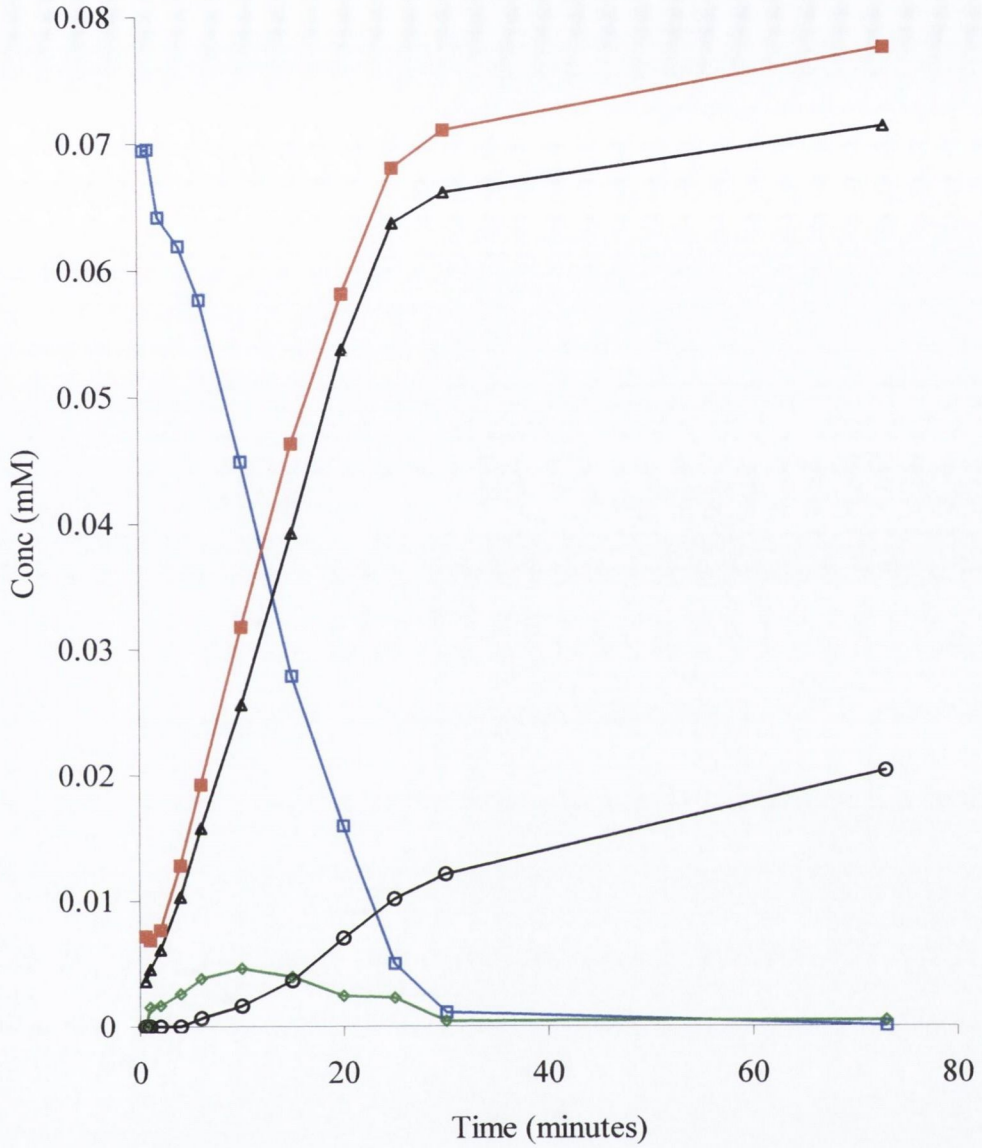



*Figure 3.30 Progression curve for the hydrolysis of isosorbide-2-aspirinate-5-salicylate with purified human serum BuChE at pH 7.4 and 37°C: Isosorbide-2-aspirinate-5-salicylate (□), isosorbide-5-salicylate (Δ), aspirin (■), salicylic acid (o) and isosorbide disalicylate (◊).*

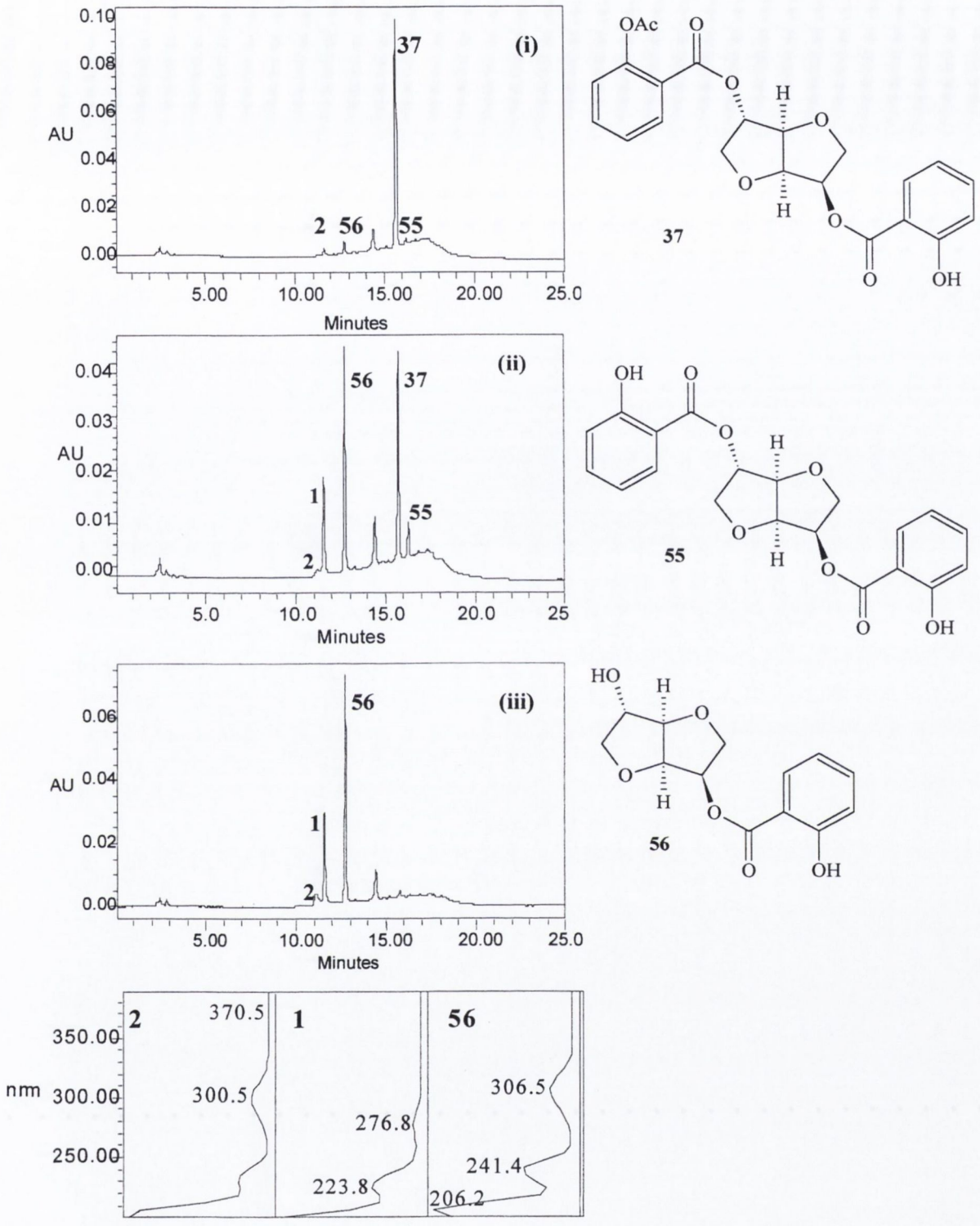



*Figure 3.31 A series of chromatograms (230 nm) obtained following the incubation of isosorbide-2-aspirinate-5-salicylate (**37**) in human serum BuChE at pH 7.4 and 37°C for (i) 0.58 minutes, (ii) 15 minutes and (iii) 30 minutes. Also presented are the PDA spectra of the labelled peaks. Any unlabelled peaks were present in the ester **37** standard. Salicylic acid (**2**), aspirin (**1**), isosorbide-5-salicylate (**56**) and isosorbide disalicylate (**55**).*

The hydrolysis of ester **37** was studied in the presence of α-chymotrypsin (1 mg/ml) at 37°C. The results of this study should give an indication of the stability of this ester towards proteolytic enzymes. Furthermore, information regarding the bioavailability of this ester following oral administration can be obtained, since α-chymotrypsin is known to influence the bioavailability of some esters. The slow hydrolysis proceeded with a rate constant of 0.0018 $min^{-1}$ and a corresponding half-life of 385 minutes. This study indicates the stability of ester **37** towards α-chymotrypsin and perhaps its stability towards other proteolytic enzymes, present in the gastrointestinal tract.

### 3.6.2 Enzyme inhibition studies

The hydrolysis of ester **37** was studied in 50% buffered human plasma (pH 7.4) at 37°C in the presence of specific inhibitors of various classes of esterases to provide further confirmation for the contribution of BuChE to its hydrolysis. The inhibitors employed were EDTA (ethylenediamene tetraacetic acid), BNPP (bis-4-nitrophenylphosphate), eserine (physostigmine), BW254C51 (1:5-bis (4-allyl-dimethyl) ammoniumphenyl-pentan-3-one), dibucaine and iso-OMPA (tetraisopropylpyrophosphoramide). The kinetic data for the inhibition studies are presented in *Table 3.15*.

| **Inhibitor** | **Inhibitor conc.** | **Target enzyme** | $K_{obs}$ $min^{-1}$ mean | (s.d., n) | $t_{1/2}$ min | **% aspirin formed** |
|---|---|---|---|---|---|---|
| EDTA | 2 mM [182] | arylesterase | 0.49 | (±0.075, n=2) | 0.89 | 84.29 |
| BNPP | 10 μM [270] | serine protease | 0.73 | (±0.006, n=2) | 0.95 | 83.96 |
| Eserine | 20 μM [182] | cholinesterase | 0.12 | (±0.003, n=2) | 5.83 | 0.0 |
| BW254c51 | 100 μM | AChE | 0.18 | (±0.003, n=2) | 3.92 | 30.26 |
| | 0.1 μM [271] | | 0.66 | (±0.064, n=2) | 1.06 | 74.21 |
| Dibucaine | 20 μM | BuChE subtype | 0.35 | (±0.07, n=2) | 2.09 | 68.74 |
| | | | 0.15 | (0.013, n=2) | 4.61 | 5.99 |
| iso-OMPA | 10 μM | BuChE | 0.11 | (0.003, n=2) | 6.23 | 0.0 |

*Table 3.15 Kinetic data for the hydrolysis of isosorbide-2-aspirinate-5-salicylate co-incubated with specific esterase inhibitors (pH 7.4, 37°C).*

Inhibition studies in the presence of EDTA and BNPP were performed to confirm that a cholinesterase was responsible for the human plasma hydrolysis of ester **37**, since they do not inhibit cholinesterases, even at concentrations as high as 100 μM. EDTA is an inhibitor of arylesterases, while BNPP inhibits serine proteases [202]. In the presence of BNPP, the hydrolysis was associated with the liberation of 78.47%-90.11% aspirin. In the presence of EDTA, hydrolysis results were comparable, with the liberation of 77.51%-90.41% aspirin. These results confirm that the enzyme responsible for the hydrolysis of ester **37** in plasma is a cholinesterase and not an arylesterase. Supporting evidence for this was obtained from inhibition studies in the presence of eserine. Eserine is a potent inhibitor of cholinesterases (AChE and BuChE) at concentrations as low as $1 \times 10^{-5}$ M [182]. The hydrolysis of ester **37** in 50% human plasma solution, co-incubated with eserine, was significantly slower than in plasma solution alone, with no aspirin being generated. These results confirm that a cholinesterase is responsible for the release of aspirin during the rapid hydrolysis of ester **37** in human plasma solution.

The hydrolysis of ester **37** was studied in 50% buffered human plasma (pH 7.4) at 37°C in the presence of BW254C51. BW254C51 is a specific inhibitor of acetylcholinesterase at concentrations of $10^{-7}$ M. Chen et al. reported that as the concentration of inhibitor is increased, inhibition of BuChE increases and at $10^{-2}$ M, the inhibition of BuChE is essentially complete [271]. As presented in *Table 3.15* at 0.1 μM BW254C51, the hydrolysis of ester **37** was not inhibited (74.13%-74.28% aspirin released). However, at 100 μM, there was a significant change in the rate and pathway of the hydrolysis (21.58%-38.94% aspirin released). This result confirms that acetylcholinesterase does not contribute significantly to the plasma-mediated hydrolysis of ester **37**. It is established that the concentration of acetylcholinesterase (EC 3.1.1.7) is significantly lower than butyrylcholinesterase (EC 3.1.1.8) in plasma [196].

The hydrolysis of ester **37** was also studied in the presence of specific inhibitors of BuChE, including dibuacine and iso-OMPA. Inhibition with dibucaine was studied in two different human plasma sources. From the results of both studies (*Table 3.15*) a significant variation in the extent and pathway of hydrolysis was evident. However, this might be explained by the presence of a dibucaine resistant BuChE subtype present

in one of the plasma sources. The occurrence of an atypical human plasma cholinesterase has been extensively described in the literature [272, 273, 274, 275] and is found in only 3% of the population. This atypical butyrylcholinesterase exhibits a reduced susceptibility to inhibition by dibucaine and several other inhibitors, relative to normal plasma cholinesterase.

Iso-OMPA is a specific potent inhibitor of BuChE [276]. The hydrolysis of ester **37** was studied in 50% buffered human plasma (pH 7.4) at 37°C co-incubated with iso-OMPA (10 μM). As expected, the rate of hydrolysis was significantly slower than hydrolysis in plasma alone. Isosorbide disalicylate (**55**) was the exclusive product of the hydrolysis, with no aspirin being generated. A progression curve for the hydrolysis is presented in *Fig. 3.32*. Inhibition of the plasma hydrolysis of ester **37** by iso-OMPA significantly altered both the rate and pathway of hydrolysis.

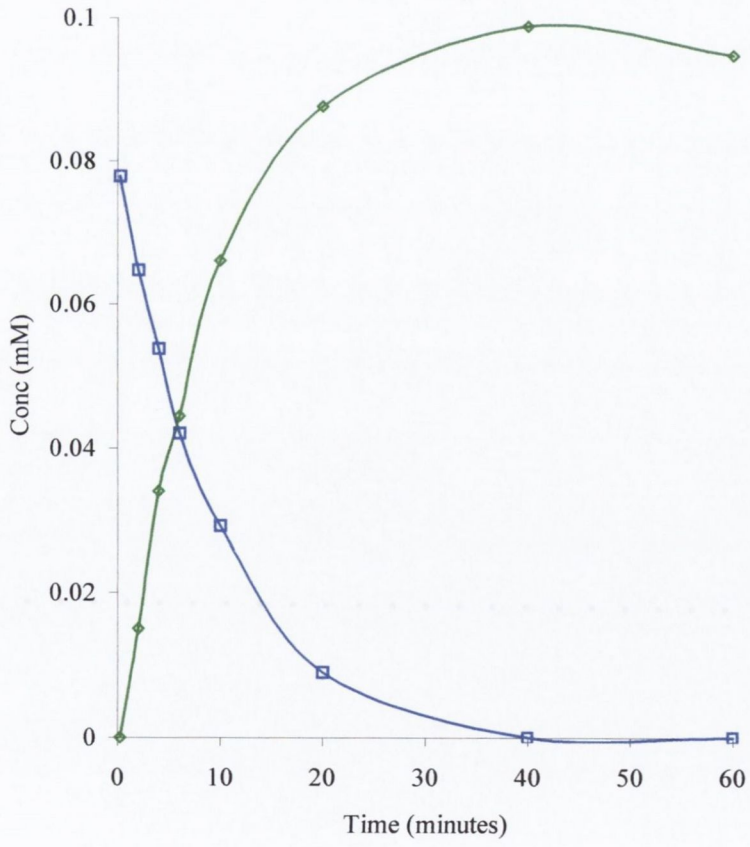



*Figure 3.32 Progression curve for the hydrolysis of isosorbide-2-aspirinate-5-salicylate in 50% human plasma inhibited with iso-OMPA (10 μM): isosorbide-2-aspirinate-5-salicylate (□) and isosorbide disalicylate (◊).*

### 3.6.3 A study of the interaction of isosorbide-2-aspirinate-5-salicylate within the active site of human serum butyrylcholinesterase

We have established that human serum butyrylcholinesterase plays a prominent and highly specific role in the hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**). We were interested in exploring theoretical models of human BuChE that would explain these observations. Surprisingly, while numerous X-ray crystal structures of AchE have been published, no BuChE X-ray model is yet available. However, a well-validated homology model of human BuChE was constructed by Harel et al. (1991) [277] and has been much used in human BuChE studies since. This homology model of human BuChE in PDB (protein database) format was kindly supplied by Harel et al..

Patrick Gardiner performed a docking procedure using ester **37** initially minimised in Macromodel [278] (*Fig. 3.33*). This was then brought into Insight II and backbone aligned within the active site to the prototypical substrate of the enzyme, butyrylcholine, present in Harel's model. Ester **37** and the enzyme were then flexibly minimised using a simulated annealing algorithm from the Affinity module within Insight II. Ester **37** appears to adopt a highly folded conformation and makes a large number of hydrophobic contacts within the enzyme active site. The hydroxy group of the salicylate forms three hydrogen bonding interaction with a Tyr 442 [III] residue, a Gly 80[IV] residue and a Trp 84[V] residue of BuChE. The ester was not ejected from the active site during simulated annealing. In the binding model access to the *O*-acetyl group by the catalytic serine is highly hindered, possibly explaining the unusual preference for the carboxyl ester group.

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[III] Tyrosine
[IV] Glycine
[V] Tryptophan

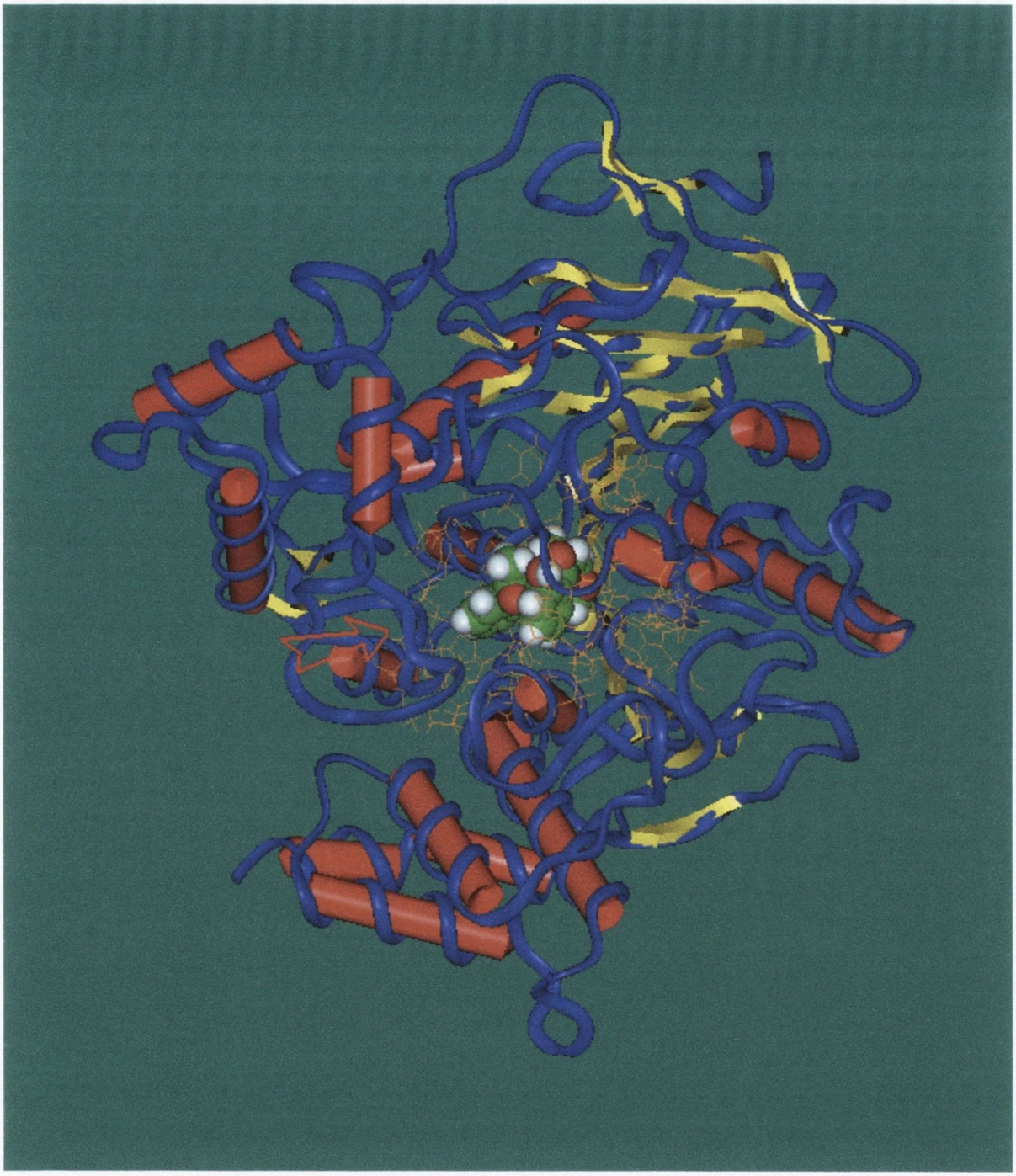


*Figure 3.33 Illustration of isosorbide-2-aspirinate-5-salicylate (CPK representation) docked within the active site of human serum butyrylcholinesterase (ribbon representation). The residues within the 10 Å radius of the active site are shown in orange. The red arrow illustrates the entrance to the active site.*

## 3.7 Conclusions

The previous chapter described the design and *in vitro* analysis of ISMNA (**35**), the mononitrate aspirin ester, which has a known anti-platelet effect [170]. ISMNA liberated up to 78.7% aspirin in 10% rabbit plasma solution. However, the concentration of aspirin liberated in human plasma solution was significantly lower (up to 8.4%). It seemed logical to design an aspirin prodrug around the structure of ISMNA, which could potentially liberate significant concentrations of aspirin in human plasma. It has been shown that the diaspirin ester of isosorbide, ISDA (**36**), is capable of the inhibition of COX-1 *in vitro* in rabbit platelet rich plasma and *ex vivo* in dogs and in human whole blood [168,259].

ISDA (**36**) was synthesised along with all potential metabolites. It was expected that the hydrolysis would be complex, since this molecule is a diaspirin ester and four possible points of hydrolysis exist. ISDA possesses suitable physico-chemical properties for absorption after oral administration. The observed stability of ester **36** to α-chymotrypsin (EC 3.2.41.1) may be useful for the applicability of the ester in decreasing the GI toxicity of aspirin.

The hydrolysis of ISDA (**36**) was studied in the plasma of dog, rabbit and human and the reactions were monitored by a gradient reverse phase LC method. This method was successfully developed for the separation of ISDA and its potential metabolites. In dog plasma solution, the hydrolysis proceeded through the salicylate ($k_2$) route, with no aspirin being generated. In rabbit plasma solution, the hydrolysis proceeded through both the $k_1$ and $k_2$ pathways to liberate a mixture of aspirinate and saliyclate metabolites, including up to 44.33% aspirin. In human plasma, a shift towards aspirin liberation was observed as the *in vitro* metabolite, isosorbide-2-aspirinate-5-salicylate (**37**) was hydrolysed. The hydrolysis of ISDA (**36**) in human plasma solution was associated with the release of up to 60% aspirin. These results explain the anti-platelet effects of ISDA in human and rabbit PRP. However, the results obtained in dog plasma solution do not explain the anti-platelet effect of ISDA in dogs. Experiments are planned to test the possibility that other enzymes in the dog may release aspirin.

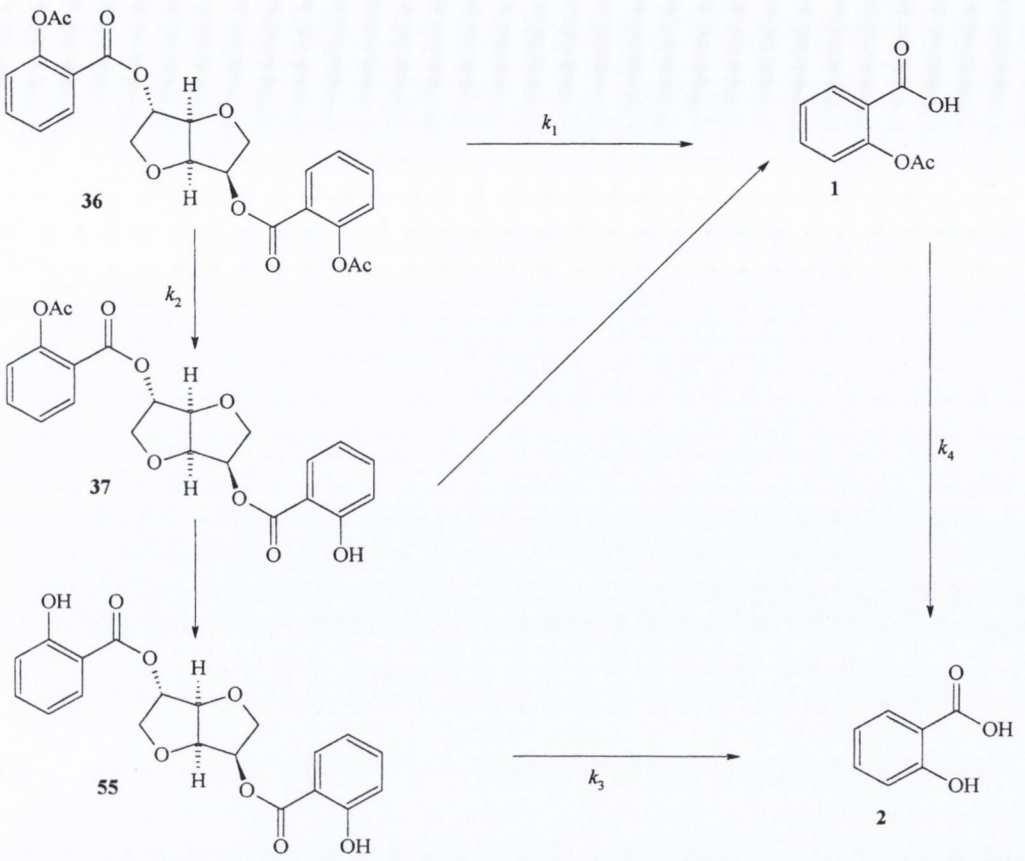



*Figure 3.34 Determined in vitro hydrolysis pathway of ISDA.*

*Fig. 3.34* describes the actual pathway of hydrolysis of ISDA human plasma. The hydrolysis was associated with the liberation of aspirin (**1**) and isosorbide-2-aspirinate-5-salicylate (**37**) initially. Ester **37** was itself hydrolysed to aspirin (**1**) and isosorbide disalicylate (**55**). Salicylic acid (**2**) was the ultimate product of hydrolysis of **37** and **55**.

The enzyme in plasma that is responsible for the hydrolysis of ISDA (**36**) is butyrylcholinesterase (EC 3.1.1.8). Evidence for this was obtained from various studies, such as the total inhibition of plasma catalysed hydrolysis by eserine and hydrolysis in the presence of a purified human serum butyrylcholinesterase preparation (48.5 % aspirin liberated).

Hydrolysis studies of ester **37** in a number of different enzyme sources established that this ester is an excellent carrier for aspirin. Remarkably, in human serum BuChE, the hydrolysis of ester **37** was associated with the liberation of 98%

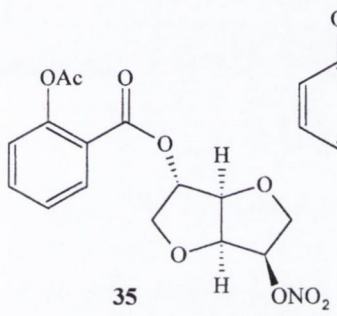
aspirin. It would appear that the salicylate group at the 5-position of isosorbide-2-aspirinate promotes hydrolysis of ester **37** at the carboxylic group, to liberate 98% aspirin. Up to now the best aspirin esters developed liberated 50% aspirin [96]. Therefore, ester **37** is the most successful aspirin prodrug and should form the basis of future investigations of aspirin esters. No other aspirin ester has been shown to liberate aspirin, at similar concentrations, in the presence of human BuChE. The following chapter investigates the effect of various substituents at the 5-position of isosorbide-2-aspirinate-5-substituted esters for their ability to promote aspirin release upon hydrolysis.

# Chapter 4

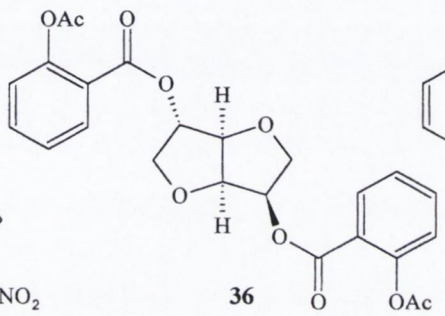
## Identification of structure activity relationship (SAR) between isosorbide-based aspirin esters

## 4.1 Introduction

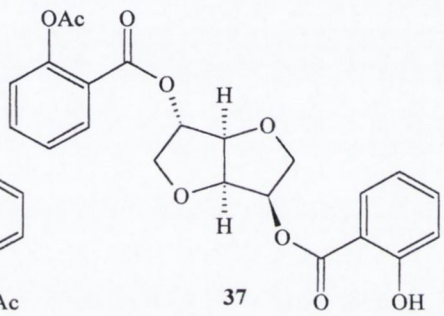
The previous chapters have described the design and evaluation of aspirin esters as potential aspirin prodrugs. These include ISMNA (**35**), ISDA (**36**) and isosorbide-2-aspirinate-5-salicylate (**37**). These esters are similar in structure, possessing an aspirinate substituent at the 2-position of the isosorbide but differing in the substituent at the 5-position. It would appear from enzymatic hydrolysis studies of esters **35**, **36** and **37** that this 5-substituent has a substantial effect on the rate and pathway of hydrolysis at the 2-position (*Table 4.1*). It was confirmed that butyrylcholinesterase (EC 3.1.1.8) was the enzyme responsible for their hydrolysis in plasma (Chapters 2 and 3).



**35**



**36**



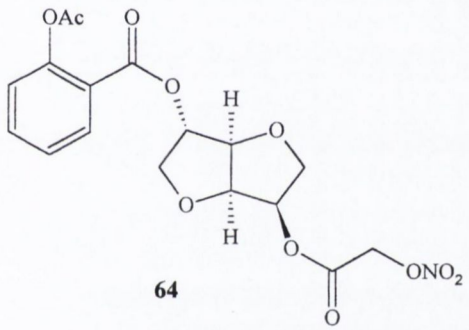
**37**

| Compound No. | Group at 5-position | $k_{obs}$ | $t_{1/2}$ | % Aspirin formed | |
|---|---|---|---|---|---|
| | | $min^{-1}$ | min | mean | (range) |
| **35** | $ONO_2$ | 0.69 | 1.00 | 7.13 | (6.59-8.43) |
| **36** | Aspirin | 0.16 | 4.43 | 51.0 | (40.0-60.0) |
| **37** | Salicylic acid | 0.16 | 4.90 | 73.23 | (64.95-81.5) |

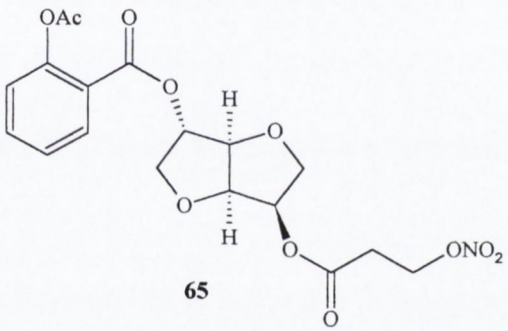
*Table 4.1 Kinetic data for the hydrolysis of ISMNA, ISDA and isosorbide-2-aspirinate-5-salicylate in 10% human plasma at pH 7.4 and 37°C.*

It has been established that in 10% human plasma, ester **35** liberates up to 8.43% aspirin, ester **36** liberates up to 60% aspirin and ester **37** liberates up to 81.5% aspirin. These results indicate that the salicylate group at the 5-position of isosorbide-2-aspirinate promotes hydrolysis at the carboxylic ester group and suppresses hydrolysis at the more labile acetyl ester group.

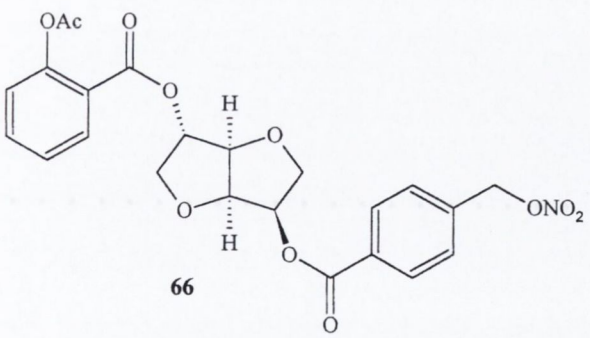
The objective of the current chapter was to introduce a nitric oxide-releasing moiety into the core structure of isosorbide-2-aspirinate-5-salicylate. Novel isosorbide-based aspirin esters having nitrooxy acetate (**64**), nitrooxy propionate (**65**), nitrooxy methyl benzoate (**66**) and bromo acetoxy benzoate (**67**) substituents at the 5-position of isosorbide-2-aspirinate were investigated as potential aspirin prodrugs. Although ester **67** does not possess a nitric oxide-releasing moiety, it might be possible to convert the alkyl bromide to a NO-releasing group. However, attempts to furnish the nitrooxy acetoxy benzoate substituted ester have been unsuccessful to date. The fit of these esters to the active site of butyrylcholinesterase (EC 3.1.1.8) might promote hydrolysis at the carboxylic ester group, liberating aspirin and a nitric oxide donor. These esters should possess superior anti-thrombotic effects to aspirin and perhaps an improved side-effect profile.



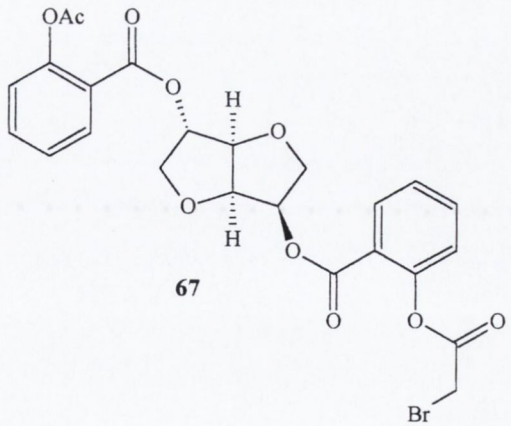

**64**




**65**



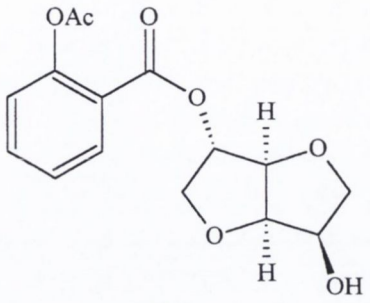

**66**



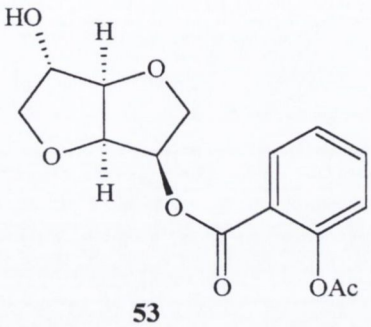

**67**

In order to determine their potential as true aspirin esters, this chapter describes the synthesis and *in vitro* hydrolysis of esters **64**, **65**, **66** and **67**. The hydrolysis of

isosorbide-based aspirin esters, previously synthesised as potential metabolites of ISDA – isosorbide-2-aspirinate (**52**) and isosorbide-5-aspirinate (**53**) – was also studied, to confirm that the position of the substituent on the isosorbide ring has significant effects on the hydrolysis kinetics and products.




**52**




**53**

## 4.1.1 Synthesis of novel isosorbide-based aspirin esters

A series of isosorbide-based aspirin esters, varying the substituents at the 5-position of isosorbide-2-aspirinate, was synthesised according to the general scheme presented in *Fig. 4.1*.

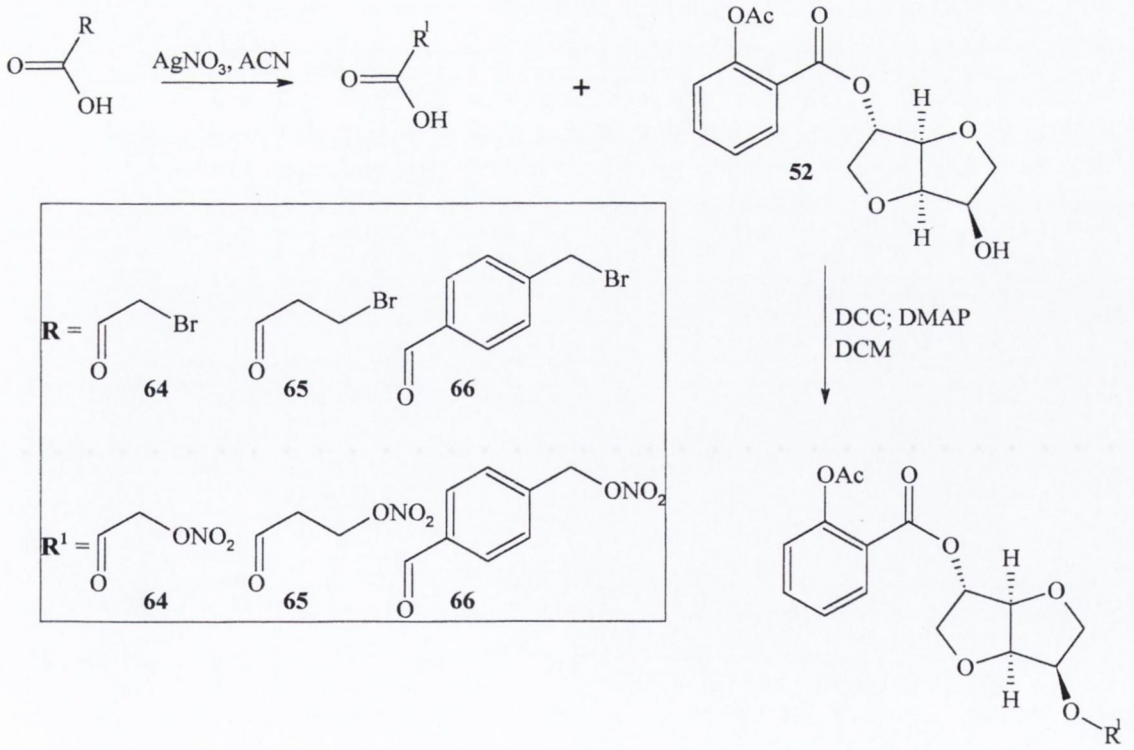



*Figure 4.1 The synthesis of a series of isosorbide-based aspirin esters.*

The first step involved the conversion of the appropriate bromo acid to the corresponding nitrooxy acid: nitrooxy-acetic acid (**68**); 3-nitrooxy-propionic acid (**69**) and 4-nitrooxymethyl-benzoic acid (**70**), which was carried out according to the method of Ferris et al. [279]. The reaction of alkyl halides with silver nitrate is a well-established method of preparing alkyl nitrates. The authors reported the use of acetonitrile due to the high solubility of silver nitrate in this solvent. The progress of the reaction was monitored by the formation of silver bromide, as a grey precipitate. The final step involved the direct esterification of isosorbide-2-aspirinate (**52**) with the appropriate nitrooxy acid - (**68**), (**69**) and (**70**) - in the presence of DCC and DMAP. The target nitroaspirin esters: isosorbide-2-aspirinate-5-(nitrooxy)-acetate (**64**); isosorbide-2-aspirinate-5-(3-nitrooxy)-propionate (**65**) and isosorbide-2-aspirinate-5-(4-nitrooxymethyl)-benzoate (**66**) were afforded in good yield (49.6%-58.52%). NMR, MS and IR confirmed the reaction had been successful. The esters were homogenous by TLC and HPLC.

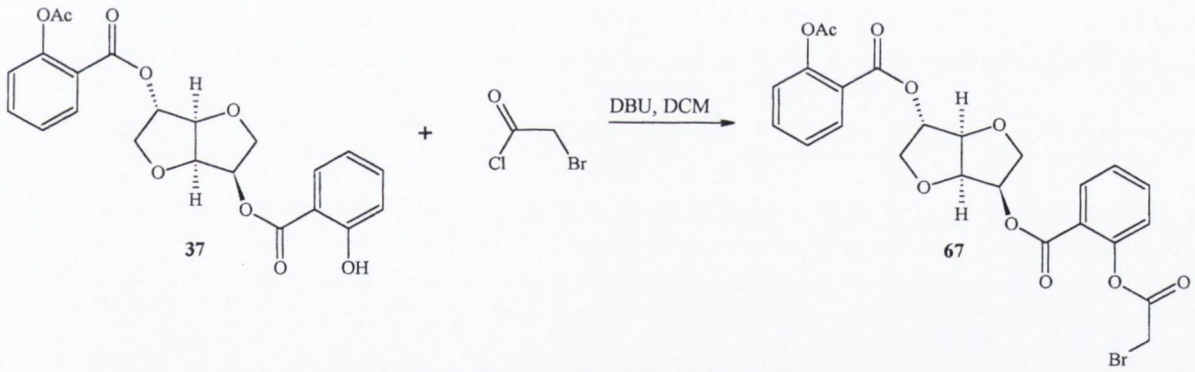



*Figure 4.2 The synthesis of ester **67**.*

We decided to synthesise isosorbide-2-aspirinate-5-(3-(2-bromo-acetoxy))-benzoate (**67**) by a different route, as presented in *Fig. 4.2*. It was anticipated that formation of the nitrooxy ester could be achieved *via* nitration of ester **67**. Isosorbide-2-aspirinate-5-salicylate (**37**) was directly esterified with bromoacetyl chloride in the presence of 1,8-diazabicyclo-undec-7-ene (DBU) and dichloromethane, according to the method of Ono et al. [280]. The reaction proceeded at room temperature overnight and following removal of the DBU-hydrohalides the reaction afforded product (**67**) as an oil in good yield (69.6%). The product was homogenous by TLC and HPLC with additional confirmation from IR, NMR and MS. However, attempts to form the nitrooxy ester of

ester **67** according to the method presented in *Fig. 4.1* were unsuccessful and therefore, *in vitro* studies were carried out on ester **67** itself.

## 4.2 Plasma hydrolysis studies

### 4.2.1 Determination of substituent effect on the plasma hydrolysis of isosorbide-based aspirin esters

Isosorbide-2-aspirinate (**52**) and isosorbide-5-aspirinate (**53**) were synthesised as potential metabolites of ISDA (**36**) according to the method described in Chapter 3. It was of interest to study the hydrolysis of esters **52** and **53** in 10% human plasma (according to the method described in Chapter 2) since any variation in their rate or pathway of hydrolysis is related to the substituents at the 2- and 5-positions of the isosorbide ring. It is already established that the substituent on the isosorbide ring effects its therapeutic profile. The metabolites of ISDN (**39**) (an effective anti-platelet agent) - isosorbide-2-nitrate (IS-2-MN) (**59**) and isosorbide-5-nitrate (IS-5-MN) (**38**) - have themselves been investigated as anti-platelet agents (Chapter 2). However, the 5-nitrate is considered clinically more useful because it releases NO slowly *in vivo* whereas the 2-nitrate is metabolised rapidly, as previously described. Indeed, IS-5-MN (**38**) is the major first-pass metabolite of ISDN (**39**) [165].

| Compound No. | $K_m$ | $V_{max}$ | $t_{1/2}$ | $k_{obs}$ | % Aspirin formed |
|---|---|---|---|---|---|
| | M ($10^{-4}$) | M $min^{-1}$ ($10^{-4}$) | min | $min^{-1}$ | |
| **52** | 0.610 | 0.218 | 4.08 | 0.17 | 2.95 |
| **53** | 1.114 | 0.247 | 4.73 | 0.15 | 0.00 |

*Table 4.2 Kinetic data for the hydrolysis of isosorbide-2-aspirinate and isosorbide-5-aspirinate in 10% human plasma at pH 7.4 and 37°C.*

The rapid hydrolysis of esters **52** and **53** in 10% buffered human plasma (pH 7.4) at 37°C followed pseudo first-order kinetics (*Table 4.2*). The hydrolysis was monitored according to the gradient method described in *Table 3.3*. A typical progression curve for the hydrolysis of ester **52** in 10% human plasma is presented in *Fig. 4.3*. The predominant route of hydrolysis was associated with the liberation of isosorbide-2-

salicylate, which subsequently hydrolysed to salicylic acid (**2**). Only 3% aspirin was generated during the hydrolysis.

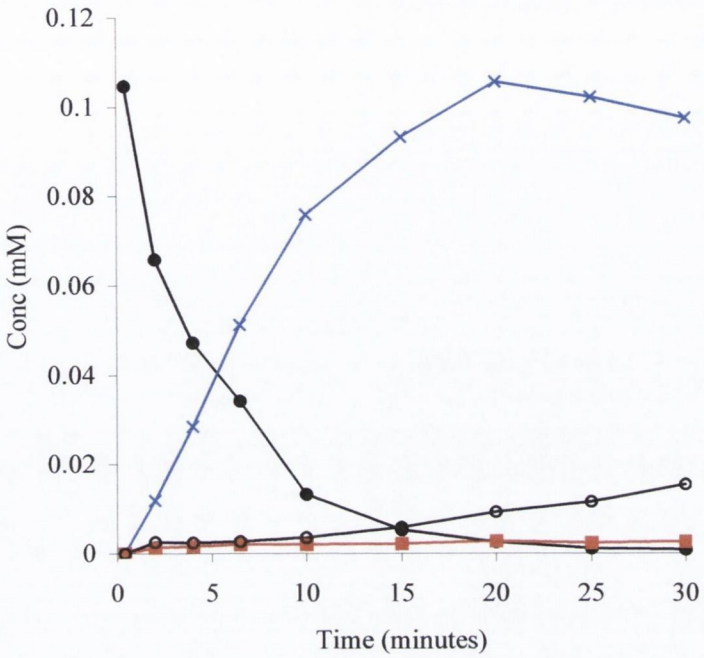



*Figure 4.3 Progression curve for the hydrolysis of isosorbide-2-aspirinate in 10% human plasma at pH 7.4 and 37°C: Isosorbide-2-aspirinate (●), isosorbide-2-salicylate (x), aspirin (■) and salicylic acid (o).*

The hydrolysis of isosorbide-2-aspirinate was associated with the liberation of isosorbide-2-salicylate exclusively. Isosorbide-5-salicylate appears to be more stable towards hydrolysis than isosorbide-2-salicylate, under similar conditions. As described in Chapter 2, the rate of hydrolysis at the 2-position is always expected to be more rapid since the 5-position is more hindered. This study gives an indication of the effect of the substituents on the isosorbide ring for the hydrolysis of isosorbide-based aspirin esters.

Similar results were observed on comparison of the hydrolysis of ISMNA (**35**) and isosorbide-5-aspirinate-2-mononitrate (**51**) (Chapter 2). The hydrolysis of ISMNA in 10% human plasma was associated with the generation of up to 8.43% aspirin whereas similar hydrolysis of isosorbide-5-aspirinate-2-mononitrate was not associated with the generation of aspirin.

### 4.2.2 Plasma hydrolysis studies of isosorbide-based aspirin esters

The hydrolysis of esters **64**, **65**, **66** and **67** was studied in 10% human plasma to determine their potential for aspirin release. It was anticipated that butyrylcholinesterase (EC 3.1.1.8) would be the enzyme responsible for their hydrolysis since isosorbide-2-aspirinate-5-salicylate (**37**) was found to be an excellent substrate for this enzyme. Pooled buffered human plasma (pH 7.4) at 37°C was employed for the study, which was obtained from the blood of a minimum of three healthy human volunteers. The hydrolyses were monitored according to the HPLC gradient method described in *Table 3.3*.

| **Compound No.** | $K_m$ M ($10^{-4}$) | $V_{max}$ M $min^{-1}$ ($10^{-4}$) | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ | **% Aspirin formed** |
|---|---|---|---|---|---|
| **64** | 0.440 | 0.175 | 3.61 | 0.19 | 0.0 |
| **65** | 0.925 | 0.232 | 3.90 | 0.18 | 0.0 |
| **66** | 0.991 | 0.153 | 5.99 | 0.12 | 0.0 |
| **67** | 0.448 | 0.261 | 1.85 | 0.37 | 74.17 |

*Table 4.3 Kinetic data for the hydrolysis of a series of isosorbide-based aspirin esters in 10% human plasma at pH 7.4 and 37°C.*

The 10% human plasma hydrolysis of esters **64**, **65**, **66** and **67** followed pseudo first-order kinetics, as presented in *Table 4.3*. The hydrolysis of each ester proceeded rapidly with the bromo acetoxy benzoate substituted ester (**67**) possessing the shortest half-life. The more hydrophobic nitrooxy methyl benzoate substituted ester (**66**) was the least susceptible to hydrolysis with a half-life of approximately six minutes. Under similar conditions, the half-life of isosorbide-2-aspirinate-5-salicylate (**37**) was 4.9 minutes.

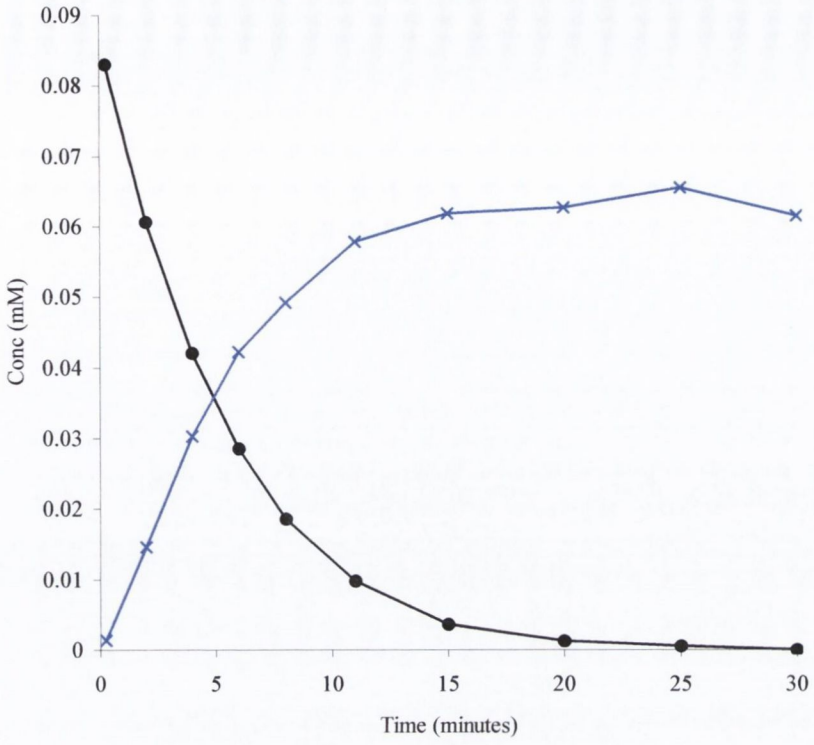



*Figure 4.4 Progression curve for the hydrolysis of ester **64** in 10% human plasma at pH 7.4 and 37°C: ester **64** (●) and salicylate ester (x).*

A progression curve for the hydrolysis of the nitrooxy acetate substituted ester (**64**) in 10% human plasma is presented in *Fig. 4.4*. Its hydrolysis was associated with the exclusive liberation of the salicylate ester with no aspirin being generated. A similar hydrolysis pathway was observed in 10% human plasma solution for esters **65** and **66**. We were disappointed to find that the substituents at the 5-position of esters **64**, **65** and **66** do not promote hydrolysis to liberate aspirin. Significantly, the hydrolysis of ester **67** was associated with the generation of 74.17% aspirin (*Fig. 4.5*), which corresponds with the hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**) – itself an *in vitro* metabolite of ester **67**. It appears that the fit of the hydrophobic substituent at the 5-position of the bromo acetoxy benzoate substituted ester (**67**) in the active site of BuChE promotes hydrolysis at the carboxylic ester group at the 2-position. Since ester **67** bears an alkyl bromide it should be possible to incorporate a nitrooxy group, which is similar in size to the alkyl bromide. However, to date attempts to convert the alkyl bromide to a nitrooxy ester group have proved unsuccessful.

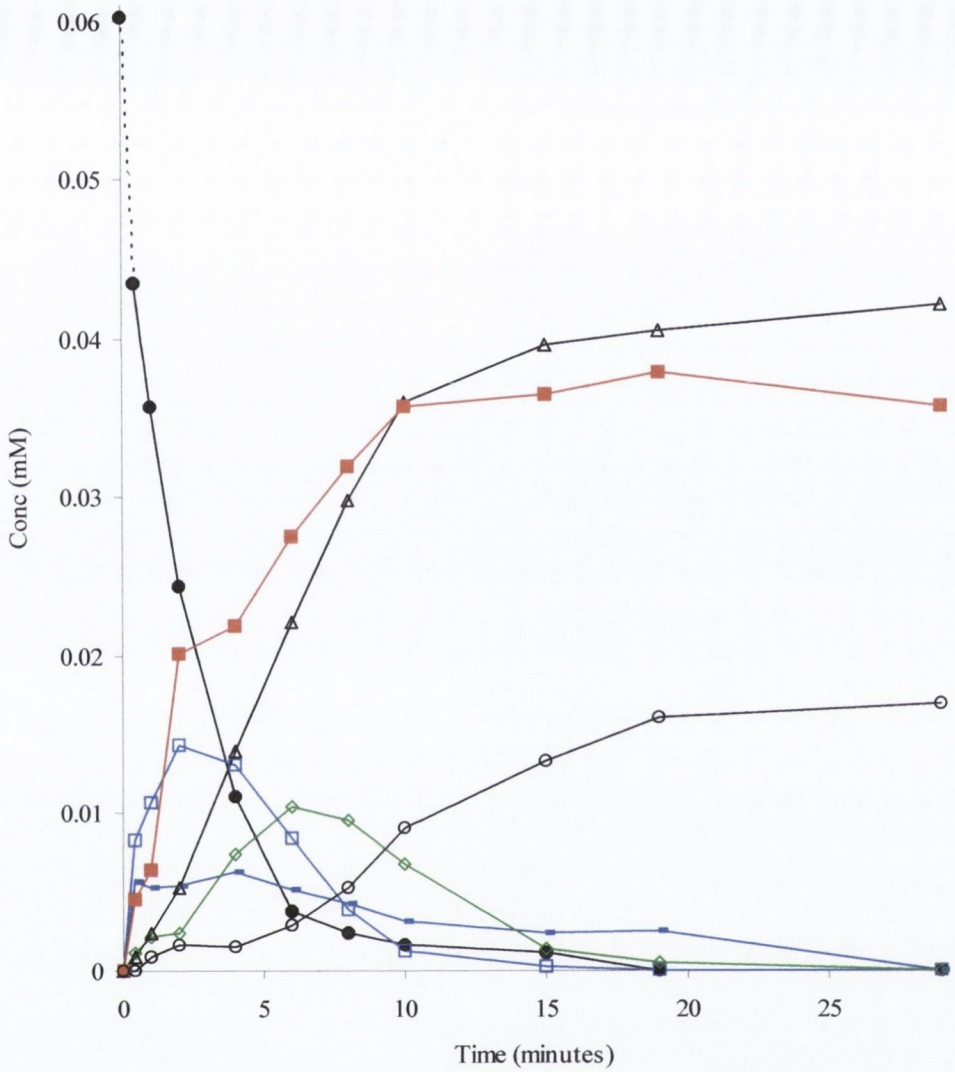



*Figure 4.5 Progression curve for the hydrolysis of ester **67** in 10% human plasma at pH 7.4 and 37°C: Ester **67** (●), salicylate ester (-), isosorbide-2-aspirinate-5-salicylate (□), aspirin (■), salicylic acid (o), isosorbide disalicylate (◊) and isosorbide-5-salicylate (Δ).*

The hydrolysis of ester **67** was also studied in 50% buffered human plasma (pH 7.4) at 37°C. The hydrolysis was associated with the liberation of 71.20% aspirin, which gives a further indication for the potential usefulness of ester **67** as a NO-releasing isosorbide-based aspirin ester.

As previously described, the hydrolysis of isosorbide-2-aspirinate-5-salicylate (**37**) was subject to inter-species variation. In 10% human plasma, up to 81.5% aspirin was released from ester **37**. However, in rat plasma its hydrolysis generated the salicylate ester **55** exclusively. This observation is most likely due to the presence of a unique BuChE type in human plasma (Chapter 3). The hydrolysis of esters **64** and **66** was studied in 10% buffered rat plasma (pH 7.4) at 37°C to determine whether an inter-species variation could be observed in their hydrolysis.

| **Compound No.** | $K_m$ M ($10^{-4}$) | $V_{max}$ M $min^{-1}$ ($10^{-4}$) | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ | **% Aspirin formed** |
|---|---|---|---|---|---|
| **64** | 1.838 | 2.178 | 0.59 | 1.19 | 0.0 |
| **66** | 2.862 | 4.993 | 0.39 | 1.74 | 0.0 |

*Table 4.4 Kinetic data for the hydrolysis of a series of isosorbide-based aspirin esters in 10% rat plasma at pH 7.4 and 37°C.*

The hydrolysis of ester **64** and **66** in 10% rat plasma followed pseudo first-order kinetics as presented in *Table 4.4*. However, the predominant route of hydrolysis was *via* deacetylation, to liberate the corresponding salicylate esters. Interestingly, the rate of their hydrolysis in 10% rat plasma was significantly more rapid than in 10% human plasma (*Table 4.3*). This is surprising since it has been established that the butyrylcholinesterase activity of rat plasma is significantly lower than human plasma [281, 206, 282]. Stedman et al. have reported that rat and rabbit plasma have similar BuChE activity [210]. Perhaps rat plasma possesses a higher content of different esterases, which might be responsible for the observed hydrolysis.

## 4.3 Lipophilicity studies of novel isosorbide-based aspirin esters

The lipophilicites of esters **64**, **65**, **66** and **66** were estimated according to the method described in Chapter 2 and are presented in *Table 4.5*.

| Compound No. | *c*log P | $t_r$ | $k^1$ |
|---|---|---|---|
| **64** | 1.29 | 12.43 | 5.22 |
| **65** | 1.73 | 12.51 | 5.26 |
| **66** | 3.19 | 15.83 | 6.92 |
| **67** | 2.49 | 15.80 | 6.90 |

*Table 4.5 Lipophilicity data for a series of novel isosorbide-based aspirin esters.*

## 4.4 Conclusion

In conclusion, a structure activity relationship can be observed between isosorbide-based aspirin esters. We confirmed that the substituent on the 5-position of isosorbide, having aspirin at the 2-position, significantly affects the extent of aspirin liberation. It seems that the more hydrophobic aryl groups promote hydrolysis at the carboxylic ester group. The bromo acetoxy benzoate substituted ester (**67**) generated 74.17% aspirin in 10% human plasma. This effect appears to be related to the fit of the esters to the active site of human serum butyrylcholinesterase (EC 3.1.1.8). Although this ester bears an alkyl bromide, it should be possible to incorporate a nitrooxy ester group. It is expected that an alkyl bromide group and a nitrooxy group are similar in size and therefore, a similar hydrolysis pattern should be observed.

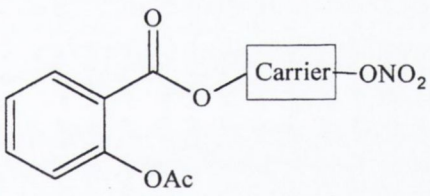
The results of the current work indicate that it should be possible to design a series of isosorbide-based aspirin esters, incorporating a nitric oxide-releasing moiety, as substrates for human butyrylcholinesterase. These esters should bear a hydrophobic group at the 5-position and an aspirinate ester at the 2-position of the isosorbide ring.

# Chapter 5

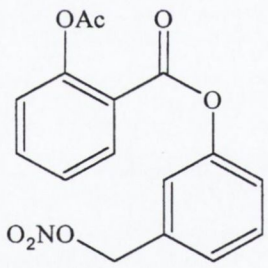
## Design, synthesis and *in vitro* hydrolysis studies of novel nitroaspirin esters

## 5.1 Introduction

The previous chapters have described the synthesis and *in vitro* analysis of isosorbide-based aspirin esters as potential aspirin prodrugs. We have investigated the development of nitric oxide-releasing aspirins in which aspirin (**2**) is linked *via* an ester bond to a carrier molecule bearing a nitrate group. Ideally, these esters cross the gastrointestinal barrier and are then rapidly cleaved by plasma esterases, liberating aspirin and the nitric oxide donor [134]. It is expected that nitro-aspirins (**71**) should exhibit superior anti-thrombotic efficacy to aspirin and possess an improved side-effect profile. As described in Chapter 1, NCX 4016 (**33**) is a nitro-aspirin ester, which possesses anti-platelet properties [148]. The hydrolysis kinetics and pathways of NCX 4016 (**33**) in human plasma have not yet been reported. However, this molecule does not appear to have been appropriately designed for aspirin release in plasma. Similar aryl esters that we have studied liberated no aspirin in human plasma (Chapter 4). It seems likely that the observed anti-platelet properties of ester **33** are due to nitric oxide release [148].

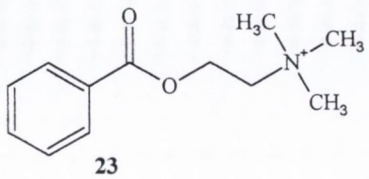



**71**

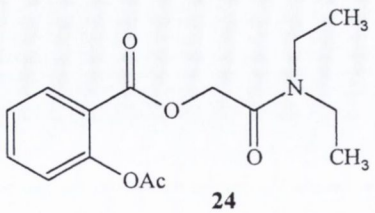



**33**

We have determined that ISMNA (**35**), ISDA (**36**) and isosorbide-2-aspirinate-5-salicylate (**37**) possess a unique fit to plasma butyrylcholinesterase (EC 3.1.1.8) (Chapters 2 and 3). This fit appears to promote hydrolysis at the carboxylic ester group ($k_1$) and suppress hydrolysis at the more labile acetyl group ($k_2$). This work indicated that a true nitro-aspirin might be designed around the correct fit to the human butyrylcholinesterase active site. Ideally, a true nitro-aspirin ester should possess a carrier group that is highly susceptible to butyrylcholinesterase-mediated hydrolysis. Cleavage at this site should be greater than cleavage at the more labile acetyl group ($k_1 > k_2$).

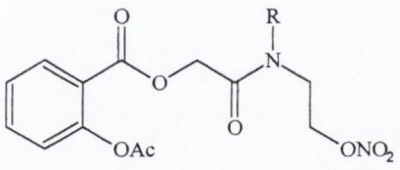


23

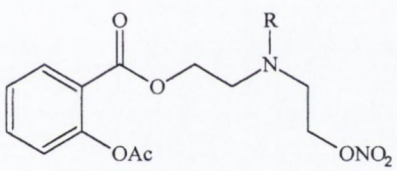


24

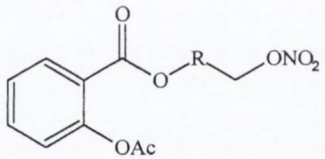
The objective of the current work was to design nitro aspirin esters as substrates for human butyrylcholinesterase (EC 3.1.1.8). Nielsen and Bundgaard reported that a good fit to the trimethyl binding site of BuChE requires an *N,N* disubstituted amine [96]. The authors developed a series of glycolamide esters, such as **24**, with high structural similarity to the choline esters, for example benzoyl choline (**23**). Amongst these esters, which underwent rapid hydrolysis in human plasma, ester **24** liberated 55% aspirin in 10% human plasma [96]. This work presented an opportunity to rationally design a series of nitric oxide-releasing aspirin esters (**72**) by incorporating nitrate ester groups into the side chain of esters of type **24**.



72



73



74

It was also decided to investigate nitrate-bearing choline esters (type **73**), based on the related benzoyl choline (**23**), despite the observation by Bundgaard that choline type aspirin esters failed to liberate aspirin, following rapid hydrolysis in human plasma [96]. A series of esters (type **74**) were also investigated, based on the nitroaspirin ester - NCX 4215 (**34**) (Chapter 1).

The hydrolyses of esters of type **72**, **73** and **74** were studied in human plasma to determine their potential for aspirin release. The elucidation of the hydrolysis pathways of these esters prompted their synthesis.

### 5.1.1 Synthesis of choline aspirin esters

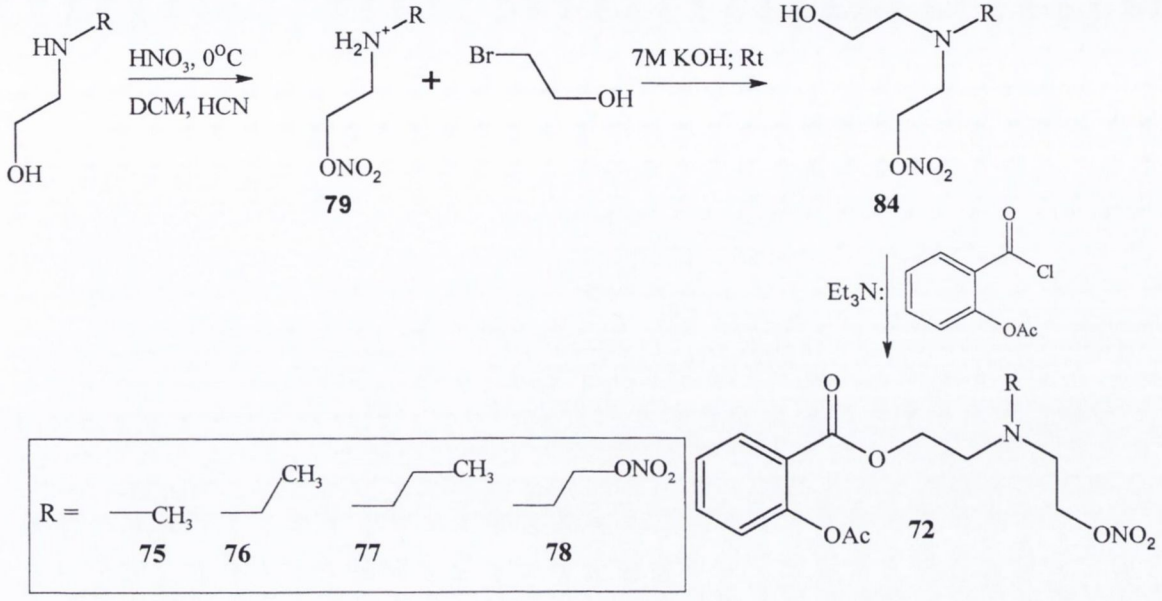



*Fig. 5.1 The synthesis of a series of choline aspirin esters.*

A series of choline aspirin esters in which *N*-methyl nitrate (**75**), *N*-ethyl nitrate (**76**), *N*-propyl nitrate (**77**) and *N,N*-diethyl nitrate (**78**) substituents were introduced, was synthesised according to the scheme presented in *Fig. 5.1*. The first step towards the synthesis of the desired choline aspirin esters involved the conversion of the appropriate ethanolamine to the corresponding nitrooxy amine (type **79**). Various synthetic routes were investigated including the use of a zinc nitrate hexahydrate-dicyclohexyl carbodiimide (DCC) combination, according to the method of Sarma [283]. However, the major drawback of this method was the requirement for formation of the hydrochloride salt of the appropriate ethanolamine; to increase reactivity. The hydrochoride salt was formed from HCl, which was generated from the reaction of methanol and acetyl chloride (20:1). The reaction was continued *in situ* with the addition of the appropriate amino alcohol. However, ultimately the amino alcohol was more reactive towards acetyl chloride than HCl. Nitration of the appropriate amino alcohol was also attempted in a mixture of fuming nitric acid and acetic anhydride [284, 285]. However, this reaction led to the exclusive formation of the *O*-acetylation product of acetic anhydride and the amino alcohol. The most successful method for nitration of the appropriate amino alcohol was that of Romanova et al. [286]. The authors reported

the use of a mixture of fuming nitric acid and dichloromethane, since dichloromethane (87%) dissolves $HNO_3$ with practically no heat evolution. The target products: methyl-(2-nitrooxy-ethyl)-amine (**80**); ethyl-(2-nitrooxy-ethyl)-amine (**81**); propyl-(2-nitrooxy-ethyl)-amine (**82**) and bis-(2-nitrooxy-ethyl)-amine (**83**) separated from the reaction mixture as oils that contained water formed during the reaction. Following the addition of acetic anhydride the target nitrooxyalkyl nitrate products precipitated as crystalline solids in good yield (57.8%-78.55%).

Alkylation of the appropriate secondary amine (**80**, **81**, **82** or **83**) to the corresponding tertiary amine (type **84**) was attempted with the addition of bromo ethanol to a solution containing the appropriate nitrated amino alcohol and triethylamine. However, the nitrated amino alcohol, present as the nitrooxyalkylammonium nitrate salt, proved to be highly insoluble in a range of solvents, including DCM, DMF, ethyl acetate, acetonitrile, THF, acetone and chloroform. Therefore, the appropriate nitrated amino alkyl salt was neutralised and the alkylation was attempted according to the method of Augstein et al. [287]. The method involved reflux of the appropriate amine, bromo ethanol and $K_2CO_3$ in ethanol for 18 hours. However, the desired product was not afforded from this reaction. The most successful method for the alkylation was that of Singh et al. [288]. The authors reported the use of 7M KOH in which the appropriate nitrated amino alcohol was stirred and treated with bromo ethanol. The target tertiary amino nitrooxy alkyl nitrates: 2-[methyl-(nitrooxy-ethyl)-amino)-ethanol] (**85**); 2-[ethyl-(2-nitrooxy-ethyl)-amino]-ethanol (**86**); 2-[propyl-(2-nitrooxy-ethyl)-amino]-ethanol (**87**) and 2-[bis-(2-nitrooxy-ethyl)-amino]-ethanol (**88**) were recovered as oils (26.0-35.12%).

The final step towards the formation of the desired choline aspirin esters, involved esterification of the appropriate nitrooxy alkyl amino ethanol (type **84**) using acetylsalicyloyl chloride. The final products: 2-acetoxy-benzoic acid 2-[methyl-(2-nitrooxy-ethyl)-amino]-ethyl ester (**75**); 2-acetoxy-benzoic acid 2-[ethyl-(2-nitrooxy-ethyl)-amino]-ethyl ester (**76**); 2-acetoxy-benzoic acid 2-[propyl-(2-nitrooxy-ethyl)-amino]-ethyl ester (**77**) and 2-acetoxy-benzoic acid 2-[bis-(2-nitrooxy-ethyl) amino]-ethyl ester (**78**) were obtained as oils (25.4%-37.7%). The products were found to be pure by TLC and HPLC with structural conformation from IR, NMR and MS.

## 5.1.2 Synthesis of glycolamide aspirin esters

Various synthetic routes were investigated for the formation of a series of glycolamide esters in which *N*-methyl nitrate (**96**), *N*-ethyl nitrate (**97**), *N*-propyl nitrate (**98**) and *N,N*-diethyl nitrate (**99**) substituents were introduced. The first route attempted was based on the United States Patent Office patent no. 3,173,900, as presented in *Fig. 5.2* [289].

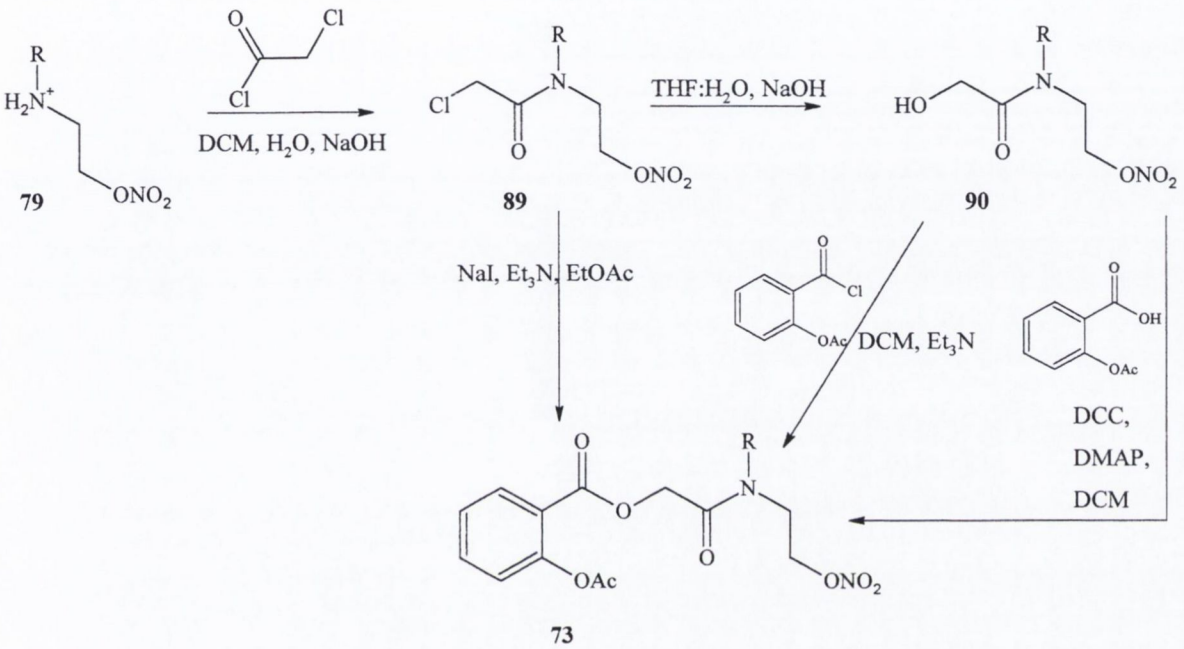
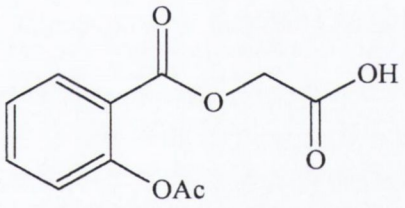



*Fig. 5.2 The attempted synthesis for a series of glycolamide aspirin esters, according to the US patent office patent no. 3,173,900.*

The synthesis of the desired halogenoacetamide (**89**) was attempted in the presence of the appropriate nitrated amine (type **79**), synthesised as described in *Fig. 5.1*. The use of various acid chlorides was investigated including methyl chloroacetate, bromoacetyl chloride and chloroacetyl chloride. When methyl chloroacetate and bromoacetyl chloride were used, the amine (type **79**) was added dropwise, at 0°C, to a stirring solution of the acid chloride in dichloromethane. However, this reaction required initial neutralisation of the nitrated amino alcohol salt, which significantly reduced the yield. The most successful method was that of Chadwick et al. in which the appropriate nitrated amine (type **79**), present as the salt, in dichloromethane was treated with successive portions of chloroacetyl chloride and sodium hydroxide [290,291].

However, this reaction was not reproducible. In some instances, the target compound was present as both the *cis* and *trans* rotamers, evidenced by $^{1}H$ NMR. Conversion to the hydroxy amide (**90**) was achieved by treating the appropriate 2-chloroacetamide (type **89**) with sodium hydroxide (5 M) in a THF and water mixture (1:1). However, this step afforded the target 2-hydroxyacetamide in poor yield. Synthesis of ester type **73** was attempted according to three different methods, as described in *Fig. 5.2*. The first method involved direct esterification of the halagenoacetamide (type **89**) with aspirin (**1**) in the presence of sodium iodide and triethylamine in ethyl acetate, under reflux conditions [96, 292]. On inspection of the NMR and TLC analysis, it was determined that the single spot obtained in the final reaction mixture was the corresponding salicylate ester, formed by hydrolysis at the *O*-acetyl group. Nucleophilic attack on acetylsalicyloyl chloride by the appropriate 2-hydroxyacetamide (**90**) was also attempted but did not afford the target glycolamide ester as a major product. Direct esterification of ester **90** with aspirin, in the presence of DCC and DMAP, afforded the salicylate ester of ester **73**.




**91**

We subsequently investigated the possibility of obtaining the desired glycolamide esters *via* 2-acetoxy-benzoic acid carboxymethyl ester (**91**). Two approaches were considered in the synthesis of acid **91**. Initially, we investigated the formation of the alkene **92** followed by oxidation to the acid **91**, according to the scheme presented in *Fig. 5.3*.

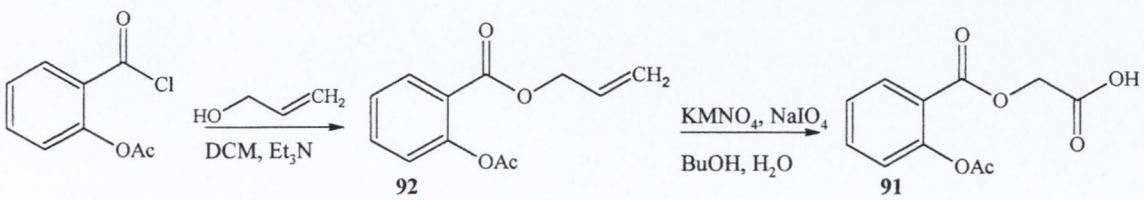



*Fig. 5.3 The synthesis of acid **91** via the alkene **92**.*

The first step involved nucleophilic attack of acetylsalicyloyl chloride by propene-1-ol with the corresponding alkene (**92**) oxidised to the acid (**91**) according to the Von Rudloff oxidation [293]. However, this oxidation was difficult to reproduce.

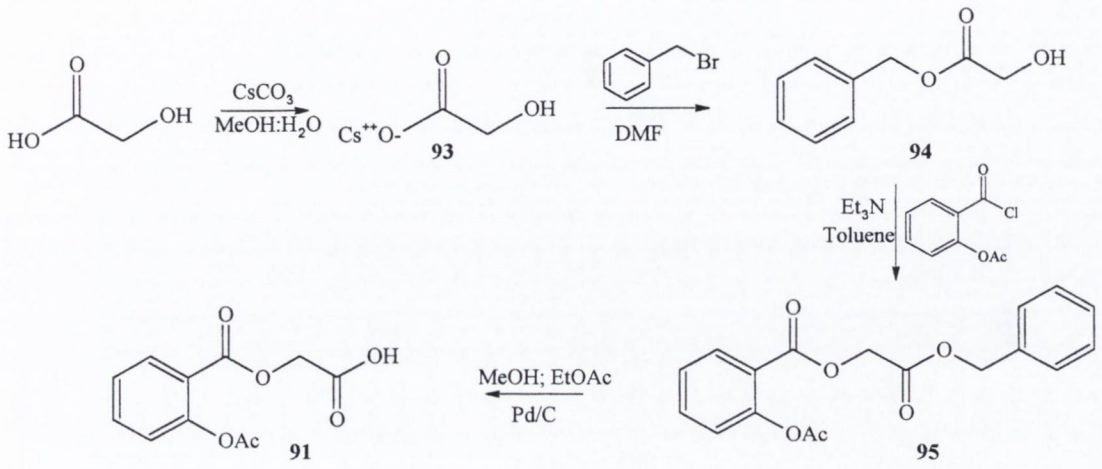



*Fig. 5.4 The synthesis of acid **91** via 1-benzyl-3-hydroxy-propan-2-one (**94**).*

An alternative route towards the synthesis of acid **91** was investigated according to the scheme presented in *Fig. 5.4*. The first step involved the formation of a cesium salt of glycolic acid (**93**) [294]. Cesium salts in a dipolar aprotic solvent like dimethylformamide (DMF) can be readily alkylated with the appropriate alkyl halide [295], which for the purpose of this synthesis was benzyl bromide. The poor solubility of **93** in DMF was overcome by heating the reaction until a slurry was formed, to which benzyl bromide was added. The target alcohol (**94**) was obtained as an oil (54.2%). Nucelophilic attack of acetylsalicyloyl chloride by **94** gave the desired ester **95**, which was deprotected to the acid **91**.

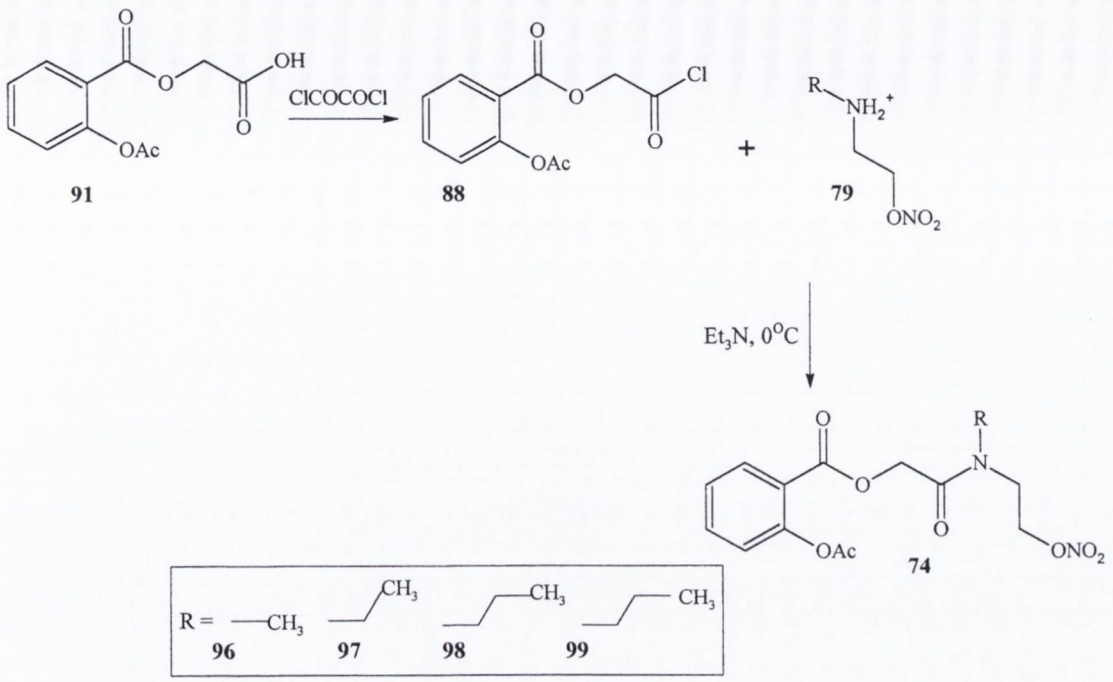



*Fig. 5.5 The synthesis of a series of glycolamide aspirin esters via the acid **91**.*

A series of glycolamide aspirin esters in which *N*-methyl nitrate (**96**), *N*-ethyl nitrate (**97**), *N*-propyl nitrate (**98**) and *N,N*-diethyl nitrate (**99**) substituents were introduced, was synthesised according to the scheme presented in *Fig. 5.5*. Conversion to the acid chloride (**88**) was achieved by treatment of the acid **91** with oxalyl chloride, according to the method of Carroll et al. [296]. The target acid chloride (**88**) could be isolated in good yield (74.4%). The convergent synthesis of the glycolamide aspirin esters involved acylation of the acid chloride **88** with the appropriate nitrooxy amino alcohol – **80**, **81**, **82** and **83** (Section 5.1.1). The target products: 2-acetoxy-benzoic acid-[methyl-(2-nitrooxy-ethyl)-amino]-ethyl ester (**96**); 2-acetoxy-benzoic acid-[ethyl-(2-nitrooxy-ethyl)-amino]-ethyl ester (**97**); 2-acetoxy-benzoic acid-[propyl-(2-nitrooxy-ethyl)-amino]-ethyl ester (**98**) and 2-acetoxy-benzoic acid [bis-(2-nitrooxy-ethyl)-carbamoyl]-methyl ester (**99**) were obtained, following purification, as oils (38.6%-49.6%). IR and NMR confirmed the presence of the major functional groups. The esters were homogenous by TLC and by two different HPLC methods. Bundgaard's *N*-diethyl substituted glycolamide (**24**) was also prepared according to this method.

### 5.1.3 Synthesis of alkyl nitrate aspirin esters

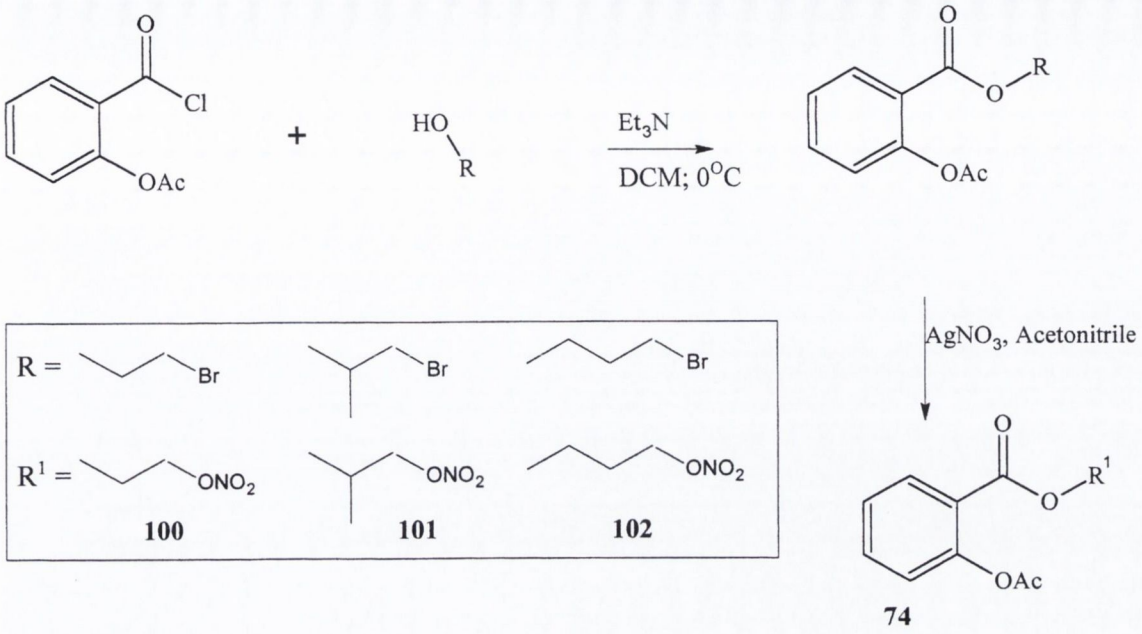



*Fig. 5.6 The synthesis of a series of alkyl nitrate aspirin esters.*

A series of alkyl nitrate aspirin esters in which ethyl nitrate (**100**), iso-propyl nitrate (**101**) and n-propyl nitrate (**102**) substituents were introduced, was synthesised according to the scheme presented in *Fig. 5.6*. Nucleophilic substitution of acetylsalicyloyl chloride on the appropriate bromo alcohol gave the target acetylsalicyoyl alkyl halide: 2-acetoxy-benzoic acid 2-bromo-ethyl ester (**103**); 2-acetoxy-benzoic acid 1-methyl-2-bromo-ethyl ester (**104**) and 2-acetoxy-benzoic acid 3-bromo-propyl ester (**105**). The appropriate acetylsalicyloyl alkyl halide was treated with silver nitrate, in acetonitrile, to give the corresponding nitrate ester: 2-acetoxy-benzoic acid 2-nitrooxy-ethyl ester (**100**); 2-acetoxy-benzoic acid 1-methyl-2-nitrooxy-ethyl ester (**101**) and 2-acetoxy-benzoic acid 3-nitrooxy-propyl ester (**102**) according to the method of Ferris et al. [279]. The target compounds were obtained as oils (50%-71.24%), which were characterised by NMR, MS and IR. The esters were homogenous by TLC and HPLC.

## 5.2 Enzyme hydrolysis studies

The hydrolysis of a series of choline aspirin esters (type **72**), glycolamide aspirin esters (type **73**) and alkyl nitrate aspirin esters (type **74**) was studied in human plasma solution and in the presence of purified enzyme preparations to determine their potential as aspirin prodrugs. Hydrolysis studies were carried out according to the method described in Chapter 2.

### 5.2.1 Enzyme hydrolysis studies of choline aspirin esters

As previously discussed, a series of choline aspirin esters, possessing NO-releasing moieties, were synthesised as substrates for BuChE. Nielsen and Bundgaard have previously shown that aspirin esters of this type undergo rapid hydrolysis, but with no aspirin being liberated [96]. It was anticipated that the incorporation of a nitrate group might alter the pathway and kinetics of hydrolysis towards aspirin release.

The hydrolysis of a series of choline aspirin esters was studied in 10% buffered human plasma (pH 7.4) at 37°C. The hydrolyses were monitored by elution on a Nova-Pak C8 (3.9 x 150 mm) column with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) at 1 ml/minute. This system was capable of the complete separation of the appropriate ester from its potential metabolites. The hydrolysis of each ester followed pseudo first-order kinetics as shown in *Table 5.1*.

| **Compound No.** | $K_m$ | $V_{max}$ | $t_{1/2}$ | $k_{obs}$ | |
|---|---|---|---|---|---|
| | M (x $10^{-4}$) | M $min^{-1}$ (x $10^{-4}$) | min | $min^{-1}$ | |
| | | | | mean | (s.d., n) |
| **75** | 1.235 | 2.637 | 0.36 | 2.62 | (±1.375, n=2) |
| **76** | 3.352 | 2.515 | 1.05 | 0.66 | |
| **77** | 3.073 | 4.619 | 0.65 | 1.33 | (±0.575, n=2) |
| **78** | 12.819 | 7.806 | 1.11 | 0.63 | |

*Table 5.1 Kinetic data for the hydrolysis of a series of choline aspirin esters in 10% human plasma at pH 7.4 and 37°C.*

The hydrolysis of each ester proceeded rapidly with the *N*-methyl nitrate substituted ester (**75**) having the shortest half-life. This was surprising since it was expected that

the *N*-ethyl nitrate substituted ester (**76**) would be most rapidly hydrolysed, since Nielsen and Bundgaard found that among various glycolamide esters of aspirin the *N,N*-diethyl derivative (**24**) showed the highest rate of enzymatic hydrolysis [96]. It was also apparent that the $K_m$ was significantly higher for the *N,N*-diethyl nitrate substituted ester. This may be due to the bulky nature of two nitrooxy groups or to the polarity of the nitrooxy group, which may impart electronic effects in the active site of BuChE.

The hydrolysis of the *N*-methyl nitrate substituted ester (**75**), *N*-ethyl nitrate substituted ester (**76**) and *N,N*-diethyl nitrate substituted ester (**78**) followed the typical pathway of hydrolysis of aspirin esters with exclusive liberation of their corresponding salicylate esters. As previously stated, Nielsen and Bundgaard found that esters of this type are not associated with the generation of aspirin, despite being extremely rapidly hydrolysed [96]. However, a promising result was obtained from the hydrolysis of the *N*-propyl nitrate substituted ester (**77**) in 10% human plasma, which was associated with the generation of 8.4%-10% aspirin, with a mean value of 9.2% (n = 2) (*Fig. 5.7*). It seems that the presence of a NO-releasing moiety in ester **77** promotes hydrolysis at the carboxylic ester moiety. The pathway of hydrolysis of the choline aspirin esters appears to be dependent upon the length of the alkyl chain on the amino nitrogen atom.

While the extent of aspirin release from ester **77** is encouraging, it is not expected to be sufficient to elicit an anti-platelet effect. Perhaps manipulation of its nitrate group would enhance the aspirin release characteristics of ester **77**.

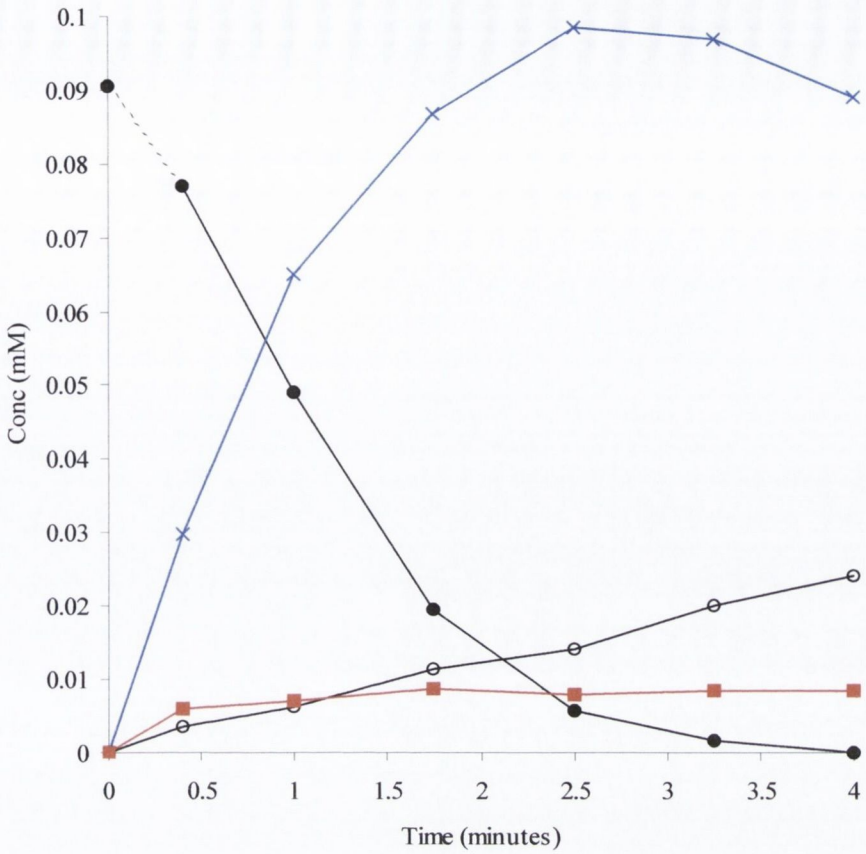



*Figure 5.7 Progression curve for the hydrolysis of ester 77 in 10% buffered human plasma (pH 7.4) at 37°C: Ester 77 (●), salicylate ester (x), aspirin (■) and salicylic acid (o).*

Interestingly, the salicylate esters liberated from the hydrolysis of the choline aspirin esters (blue line in *Fig. 5.7*) possessed high stability, since they were only slowly hydrolysed to salicylic acid. The salicylate esters were expected to undergo rapid enzyme-mediated hydrolysis since these esters were designed as prototypical substrates for butyrylcholinesterase and are a direct analogue of benzoylcholine.

The hydrolysis of the choline aspirin esters **75**, **76**, **77** and **78** was also studied in the presence of purified horse serum butyrylcholinesterase (pH 7.4, 37°C). The hydrolysis followed pseudo first-order kinetics, as presented in *Table 5.2*. The rates of hydrolyses of the *N*-propyl nitrate substituted ester (**77**) and the *N,N*-diethyl nitrate substituted ester (**78**) were comparable, since they were studied at the same enzyme concentration.

| Compound No. | Conc. of BuChE<br>mg/ml | $t_{1/2}$<br>min | $k_{obs}$<br>$min^{-1}$ |
|---|---|---|---|
| **75** | 0.01 | 12.65 | 0.06 |
| **77** | 0.05 | 2.90 | 0.24 |
| **78** | 0.05 | 2.56 | 0.27 |

*Table 5.2 Kinetic data for the hydrolysis of a series of choline aspirin esters with purified horse serum BuChE at pH 7.4 and 37°C.*

The hydrolysis of the *N*-methyl nitrate substituted ester (**75**), *N*-ethyl nitrate substituted ester (**76**) and the *N,N*-diethyl nitrate substituted ester (**78**) was associated with the exclusive liberation of the corresponding salicylate esters. However, the hydrolysis of the *N*-propyl nitrate substituted ester (**77**) was associated with the liberation of 10% aspirin, based on initial ester concentrations (*Fig. 5.8*). The hydrolysis of ester **77** was approximately equal (5% more rapid in plasma) in human plasma solution and in purified horse serum BuChE, based on intrinsic enzyme activities.

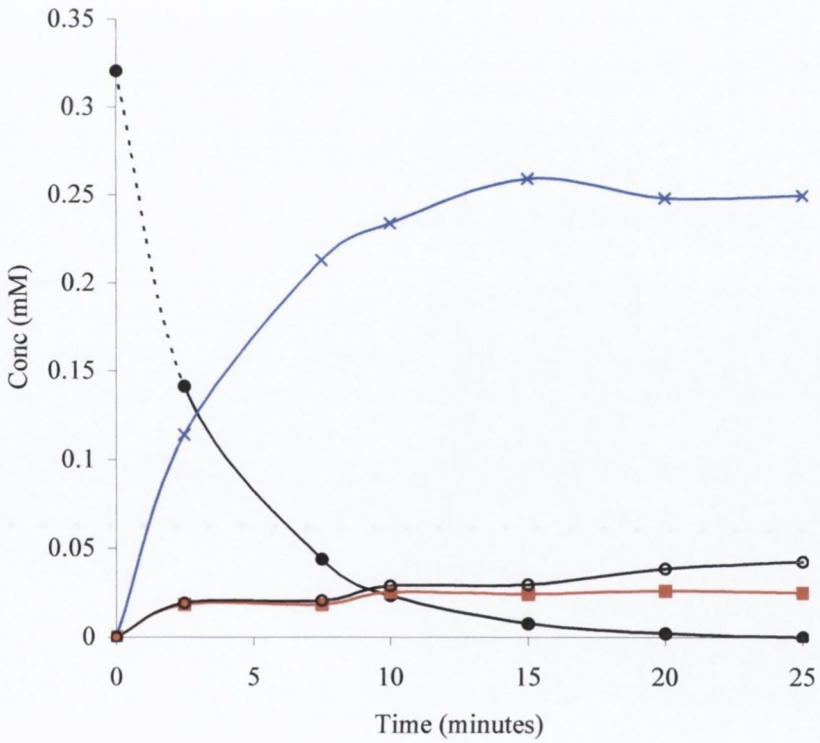



*Figure 5.8 Progression curve for the hydrolysis of ester 77 with purified horse serum BuChE at 37°C: Ester 77 (●), salicylate ester (x), aspirin (■) and salicylic acid (o).*

The hydrolysis pathways of the most promising choline aspirin ester candidate developed to date (ester **77**) are presented in *Fig. 5.9*.

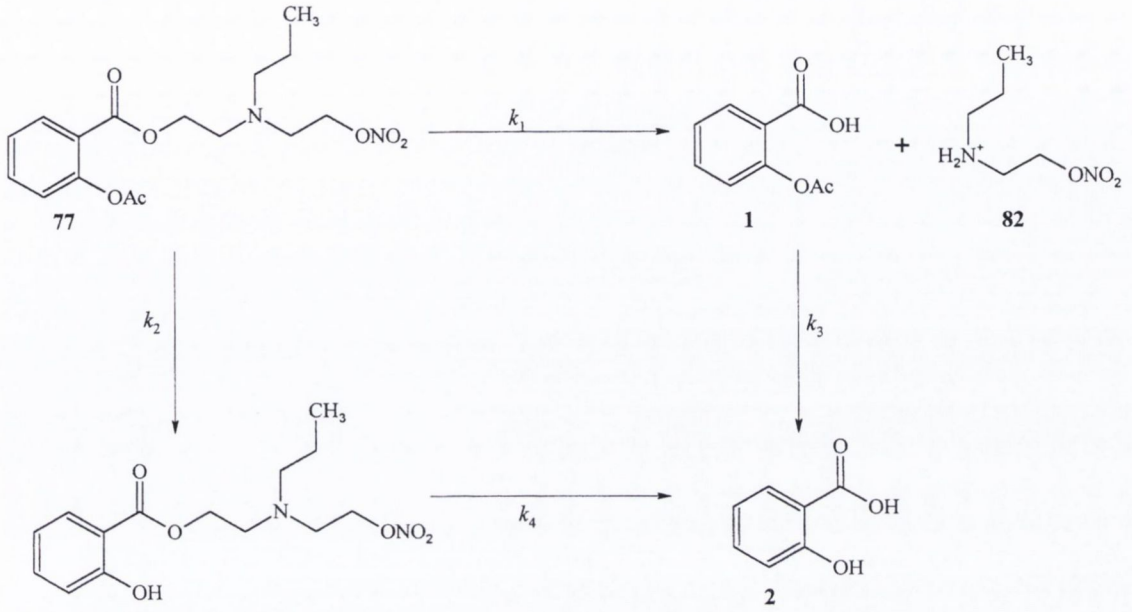



*Figure 5.9 Determined enzyme-mediated hydrolysis pathways of ester 77.*

## 5.2.2 Enzyme hydrolysis studies of glycolamide aspirin esters

As previously discussed, Nielsen and Bundgaard reported a series of glycolamide esters of aspirin that were capable of releasing significant concentrations of aspirin following hydrolysis in plasma. For example, the *N,N*-diethyl substituted ester (**24**) liberated up to 55% in 10% human plasma [96]. We designed a series of glycolamide aspirin esters based on ester **24** with the incorporation of various nitric oxide-releasing moieties into its structure.

The hydrolysis of the *N*-methyl nitrate substituted ester (**96**), *N*-ethyl nitrate substituted ester (**97**), *N*-propyl nitrate substituted ester (**98**) and *N,N*-diethylnitrate substituted ester (**99**) were studied in 10% buffered human plasma (pH 7.4) at 37°C. Final samples were analysed by HPLC under isocratic conditions on a Nova-Pak C8 (3.9 x 150 mm) column eluted with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) at 1 ml/minute. However, the observed pathway of hydrolysis involved the rapid evolution of an extremely complex mixture of degradation products (*Fig. 5.10*). Attempts to identify the components of the mixture

by LC-MS were unsuccessful. Only the PDA UV spectra of each peak were obtained. We also studied the hydrolysis of the *N,N* diethyl ester (**24**), which Bundgaard had reported released 55% aspirin in 10% human plasma. The study was conducted under similar conditions as described above using the same plasma. The hydrolysis was associated with the liberation of up to 65% aspirin (n = 3), which correlates with the previously reported results [96]. These results suggested that the presence of a nitrate group on the *N*-alkyl chain promoted attack of the esters, but not at the typical points of hydrolysis (acetyl or carboxylic ester groups), since neither the salicylate ester or aspirin were generated. Unexpectedly, hydrolysis occured at the amide bond, since the acid **91** and its salicylate ester were identified in the mixture (by PDA and comparison to an external standard of acid **91**). This was surprising, since the stability of amide bonds is well established.

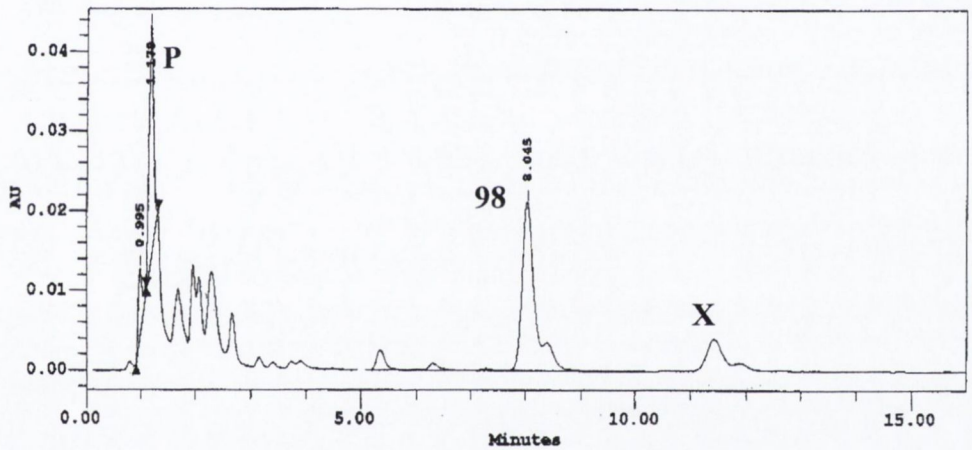



*Figure 5.10 Chromatogram (230 nm) of a sample obtained following incubation of ester **98** in 10% human plasma for 15 minutes. Peaks labelled **P** represent plasma peaks present in the plasma blank. Peaks labelled **X** were present in the standard of ester **98**. All other peaks were liberated during the hydrolysis.*

The hydrolysis of esters **96**, **97**, **98** and **99** was studied in 10% buffered rat plasma (pH 7.4) at 37°C to determine whether the complex hydrolysis described above, in human plasma solution, was species-related. Interestingly, in rat plasma the hydrolysis was associated with the exclusive liberation of the corresponding salicylate ester (*Fig. 5.11*). Although the results in rat plasma were disappointing with respect to aspirin release, the hydrolysis was typical of aspirin esters.

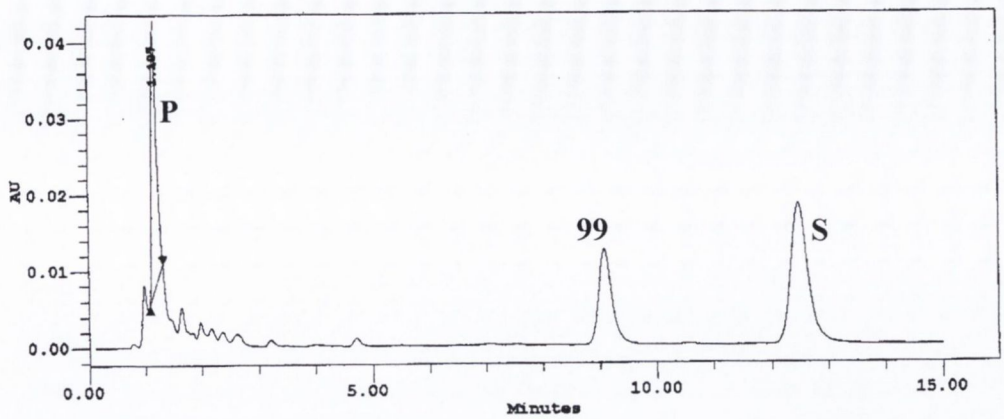



*Figure 5.11 Chromatogram (230 nm) of a sample obtained following incubation of ester **99** in 10% rat plasma for 1 minute. Peaks labelled **P** represent plasma peaks present in the plasma blank. Peak labelled **S** represents the salicylate ester of ester **99**.*

The hydrolysis of the esters was studied in purified horse serum BuChE and occurred through a complex degradation pathway, similar to hydrolysis in human plasma. The predominant point of hydrolysis appeared to be at the amide bond in purified BuChE also.

The hydrolysis of esters **96**, **97**, **98** and **99** was studied in phosphate buffer pH 7.4 (37°C) to determine whether the unusual hydrolysis observed in human plasma was related to their aqueous stability. At pH 7.4, exclusive hydrolysis occurred at the amide group and not at the expected points of hydrolysis (acetyl ester group or carboxylic ester group). Their hydrolysis was also extremely rapid – a characteristic Bundgaard had not reported. It seemed that the presence of a nitrate group activates the amide bond towards rapid hydrolysis under aqueous conditions. Interestingly, Nielsen and Bundgaard have reported that under acidic conditions, the *N*-dimethyl substituted glycolamide ester undergoes cleavage at the amide bond [297]. Perhaps this is an intrinsic characteristic of glycolamide esters, which is enhanced by the nitrate group.

At a later date, we repeated the hydrolysis studies of esters **96**, **97**, **98** and **99** in 10% buffered human plasma (pH 7.4) at 37°C. However, on this occasion the final samples were analysed using a gradient HPLC method on a Nova-Pak C8 (3.9 x 75 mm) column, eluted according to the gradient method detailed in *Table 5.3*.

| **Time** mins | **Flow rate** ml $min^{-1}$ | **% A** | **% B** | **Curve** |
|---|---|---|---|---|
| | 1 | 90 | 10 | |
| 10 | 1 | 10 | 90 | 6 |
| 12 | 1 | 65 | 35 | 6 |
| 17 | 1 | 90 | 10 | 6 |
| 25 | 1 | 90 | 10 | 6 |

*Table 5.3 HPLC gradient method for the separation of a series of glycolamide aspirin esters and their metabolites: A = pH 2.5 phosphate buffer, B = acetonitrile.*

The hydrolyses followed pseudo first-order kinetics, as presented in *Table 5.4*. The rapid hydrolysis of the *N*-ethyl nitrate substituted ester (**97**) is illustrated with a series of chromatograms (*Fig. 5.12*). A similar hydrolysis pathway was observed for the *N*-methyl substituted ester (**96**), the *N*-propyl nitrate substituted ester (**98**) and the *N,N*-diethyl nitrate substituted ester (**99**).

| **Compound No.** | $K_m$ M (x $10^{-4}$) | $V_{max}$ M $min^{-1}$ (x $10^{-4}$) | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ |
|---|---|---|---|---|
| **96** | 5.128 | 3.676 | 1.16 | 0.60 |
| **97** | 3.063 | 2.595 | 1.03 | 0.68 |
| **98** | 4.830 | 3.567 | 1.13 | 0.61 |
| **99** | 5.405 | 2.231 | 6.12 | 0.11 |

*Table 5.4 Kinetic data for the hydrolysis of a series of glycolamide aspirin esters in 10% human plasma at pH 7.4 and 37°C.*

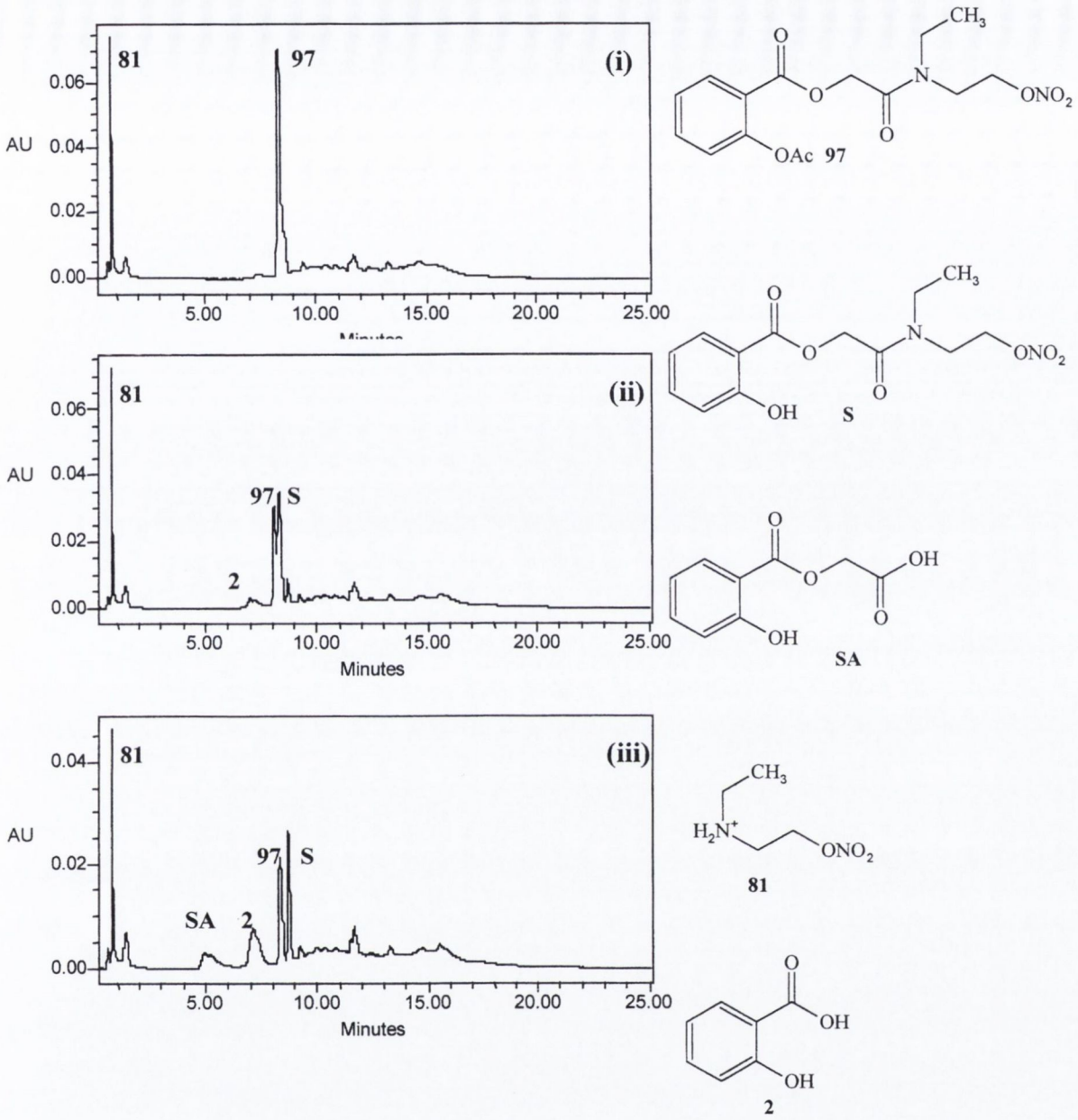



*Figure 5.12 A series of chromatograms (230 nm) obtained following incubation of ester **97** in 10% human plasma for (i) 25 seconds, (ii) 2 minutes and (iii) 6 minutes. Peaks labelled **P** represent plasma peaks present in the plasma blank. Peaks labelled **S** represent the salicylate ester of ester **96**. Peaks labelled **SA** represent the salicylate of the acid **102**.*

The exclusive pathway of hydrolysis for the glycolamide aspirin esters was *via* deacetylation to liberate the corresponding salicylate ester (**S**). While the salicylate ester was more rapidly hydrolysed than the corresponding salicylate choline ester, the hydrolysis was significantly slower than the salicylate ester of **24** [96]. It seems that the presence of a nitrate group suppresses hydrolysis of the salicylate esters. Subsequent products of the hydrolysis included salicylic acid (**2**) and the salicylate of acid **91**, which were identified by PDA detection and by their relative retention times. The rapid evolution of the salicylate of acid **91** confirms the previous observation that the nitrate group assists in hydrolysis at the amide bond. We were disappointed to find that no aspirin was liberated from the hydrolysis of esters **96**, **97**, **98** and **99**, since Bundgaard found that ester **24** liberated 55% aspirin in 10% human plasma [96].

The hydrolysis of the *N*-ethyl nitrate substituted ester (**97**) was subsequently repeated in the presence of horse serum butyrylcholinesterase (0.1 mg/ml) buffered at pH 7.4 (37°C) and final samples were analysed according to the gradient method presented in *Table 3.3*. The rapid hydrolysis followed pseudo first-order kinetics with a $k_{obs}$ of 0.588 $min^{-1}$ and a corresponding half-life of 1.18 minutes. The hydrolysis liberated a mixture of *in vitro* salicylate metabolites, which were identified as the corresponding salicylate ester (**S**), salicylic acid (**2**) and the salicylate of acid **91**. A similar hydrolysis pattern would be expected for esters **96**, **98** and **99**. Once again, in the presence of purified enzyme, hydrolysis of the amide bond was evident.

The hydrolysis of the *N*-propyl nitrate substituted ester (**98**) was repeated in phosphate buffer pH 7.4 and monitored by gradient elution. A series of chromatograms, which illustrate the degradation pattern are presented in *Fig. 5.13*. The rapid degradation was associated with the liberation of acid **91** and the corresponding salicylate ester (**S**). The observed rapid hydrolysis at the amide bond is further confirmation of its surprising instability, even under aqueous conditions. The unexpected stability of the salicylate ester (**S**) observed in human plasma solution, is also evident in *Fig. 5.13*.

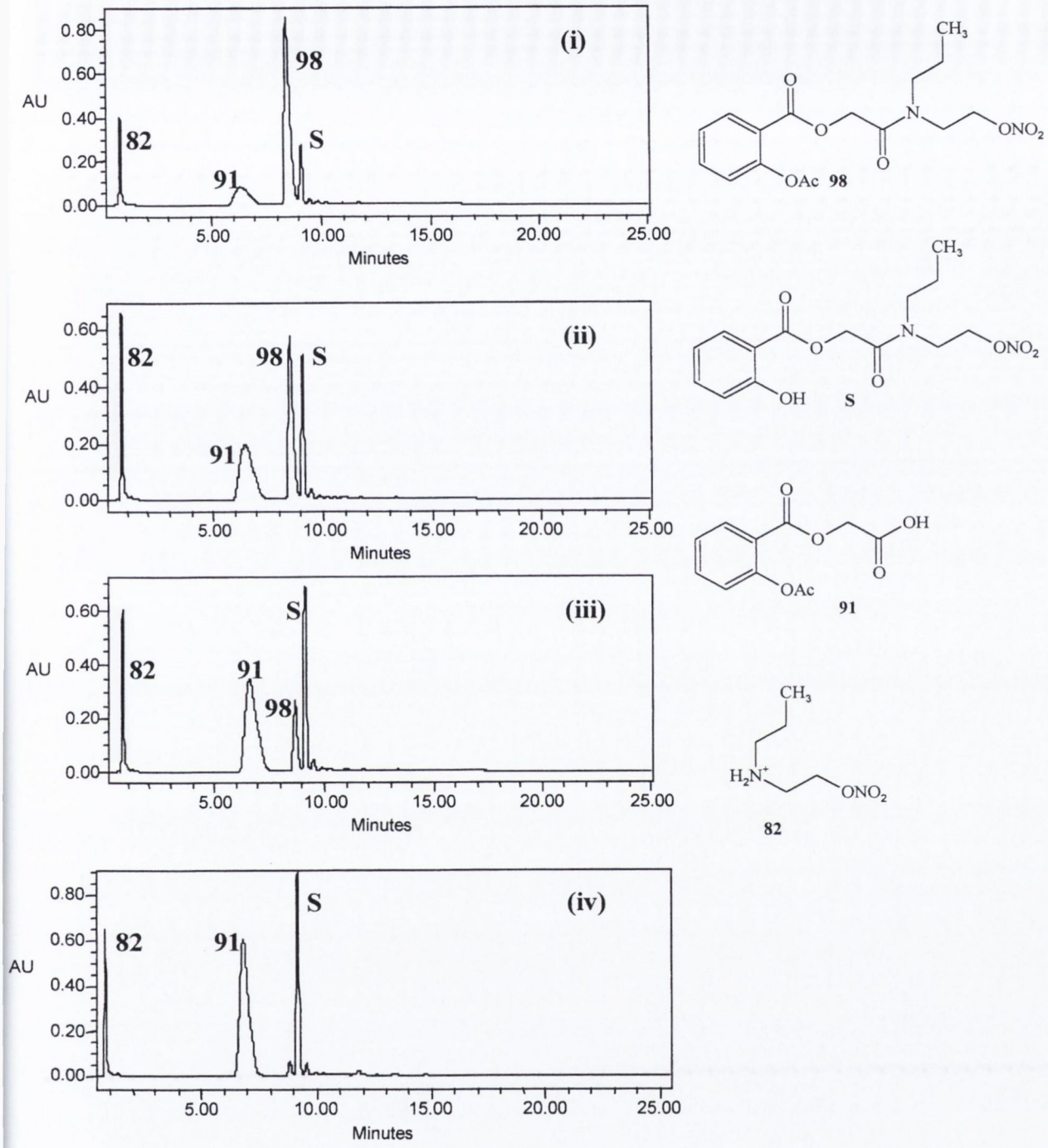



*Figure 5.13 A series of chromatograms (230 nm) obtained following the incubation of the N-propyl nitrate substituted glycolamide ester (**98**) in phosphate buffer pH 7.4 at 37°C for (i) 26 minutes, (ii) 76 minutes, (iii) 2.9 hours and (iv) 9.2 hours. 2-Acetoxy-benzoic acid-[propyl-(2-nitrooxy-ethyl)-amino]-ethyl ester (**98**), salicylate ester of ester **98** (**S**), 2-acetoxy-benzoic acid carboxymethyl ester (**91**), (2-nitrooxy-ethyl)-propyl-amine (**82**).*

To date, we have not identified the complex mixtures of degradation products obtained following initial enzyme-mediated hydrolysis studies of the glycolamide esters. However, it might be possible that the isocratic run time was too short for elution of the salicylate esters (**S**). Therefore, elution of the salicylate ester peak was carried over to the following injection and observed at an earlier retention time then was expected. Another explanation for the unusual hydrolysis observed is that the salicylate esters (**S**) were hydrolysing so rapidly they were not being detected. However, this explanation is less likely since the salicylate esters were present in the chromatograms obtained under gradient conditions. The actual pathways of hydrolysis of the *N*-ethyl nitrate substituted glycolamide ester (**97**) are presented in *Fig. 5.14*. Similar routes of hydrolysis were observed for esters **96**, **98** and **99**.

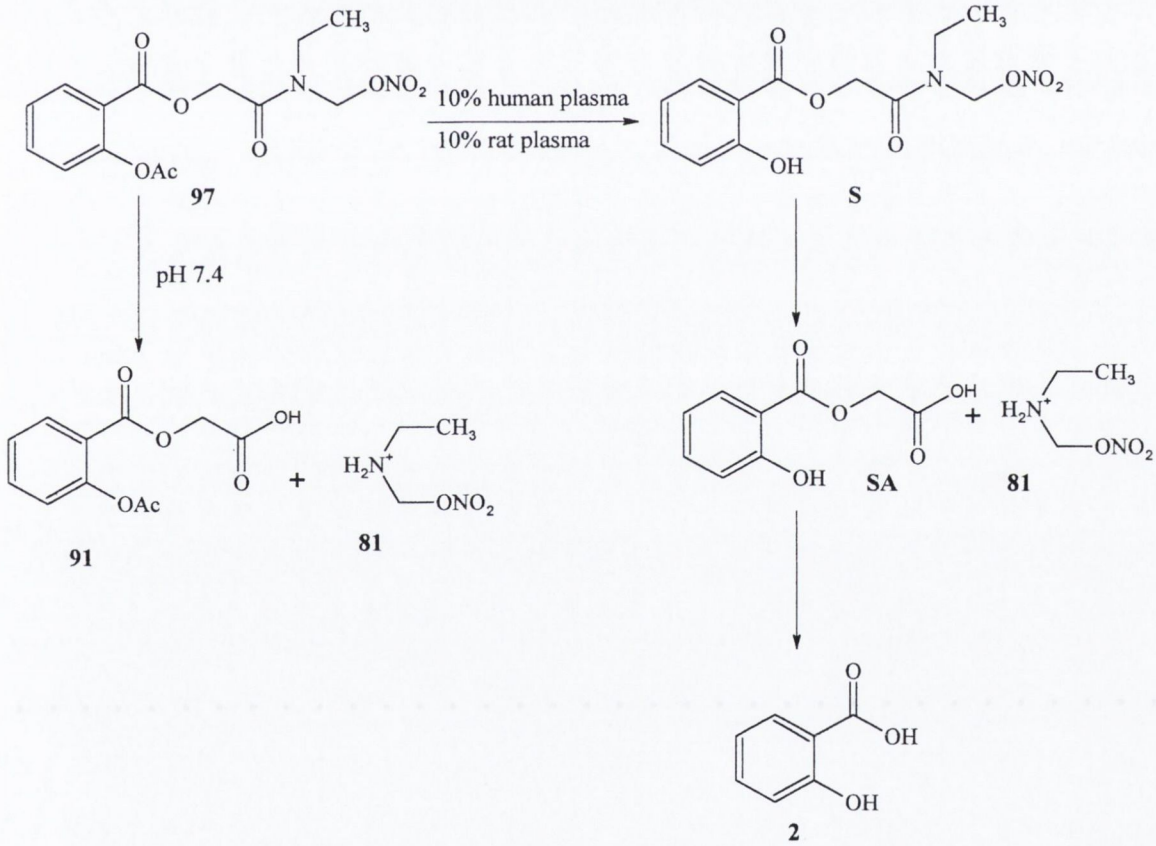



*Figure 5.14 Determined hydrolysis pathways of ester **97**.*

Although no aspirin was released from the present series of glycolamide esters, following plasma hydrolysis, they may potentially release aspirin *in vivo*. It is

established that esterases are present in blood, which are capable of denitration. Therefore, the hydrolysis presented in *Fig. 5.15* for ester **97**, might be envisaged.

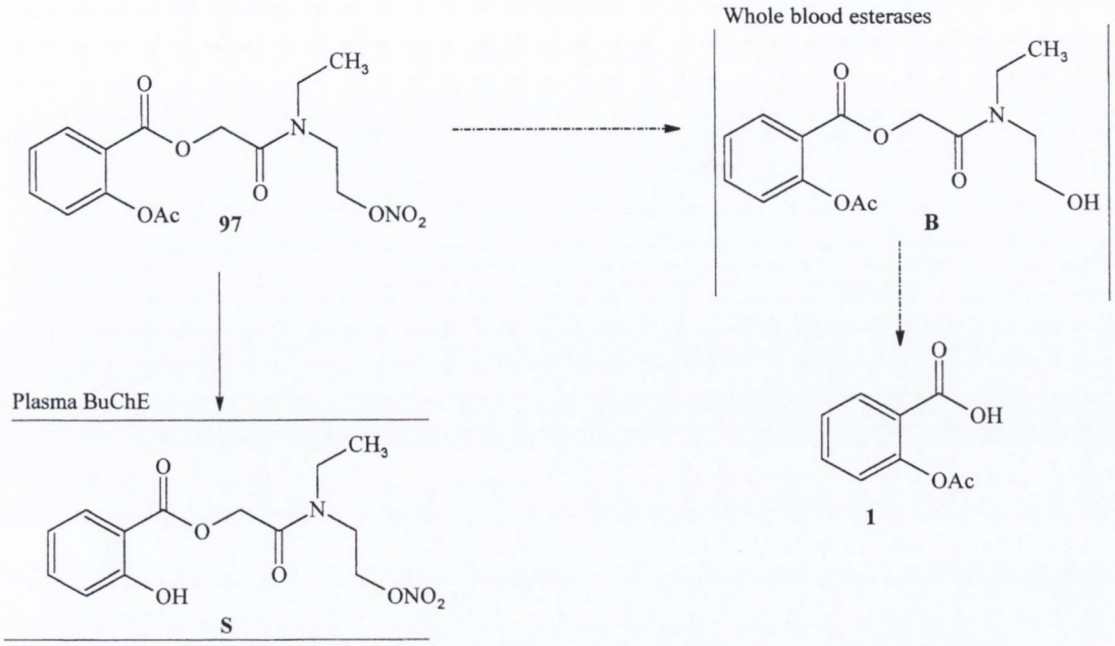
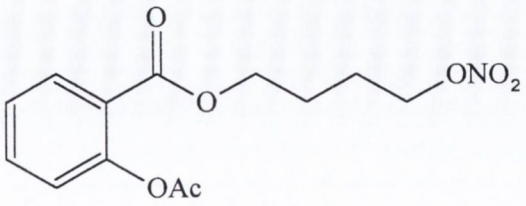



*Figure 5.15 Potential hydrolysis pathway of ester **97** in vivo.*

Since Bungaard established that ester **B** liberates significant concentration of aspirin in plasma, it is not unreasonable to assume that its hydrolysis in whole blood might be associated with the liberation of aspirin.

### 5.2.3 Enzyme hydrolysis studies of alkyl nitrate aspirin esters

A series of alkyl nitrate esters (type **74**) were investigated as potential aspirin prodrugs. As previously described, we investigated a series of glycolamide aspirin esters, which unexpectedly rapidly hydrolysed at the amide bond in human plasma solution and under aqueous conditions (pH 7.4). This pattern might be related to the nitrate group assisting in attack by esterases at the normally highly stable amide bond. A series of alkyl nitrate esters were investigated to determine whether the nitrate group would, in a similar manner, promote hydrolysis at the carboxylic ester group to release aspirin and a NO- releasing moiety. Previously studied aspirin esters of this type, such as ester **34** (NCX 4215), have not been shown to release aspirin *in vitro* (Chapter 1).




**34**

The hydrolysis of a series of alkyl nitrate esters having ethyl nitrate (**100**), iso-propyl nitrate (**101**) and n-propyl nitrate (**102**) substituents was studied in 10% buffered human plasma (pH 7.4) at 37°C. The hydrolysis was monitored on a Spherisorb C18 (4.2 x 250 mm) column eluted under gradient conditions according to the gradient table presented in *Table 5.3*. The hydrolyses followed pseudo first-order kinetics, as shown in the kinetic data presented in *Table 5.5*.

| **Compound No.** | $K_m$ M (x $10^{-4}$) | $V_{max}$ M $min^{-1}$ (x $10^{-4}$) | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ |
|---|---|---|---|---|
| **100** | 4.620 | 2.060 | 1.60 | 0.44 |
| **101** | 7.175 | 4.236 | 1.47 | 0.47 |
| **102** | 2.904 | 1.259 | 2.23 | 0.34 |

*Table 5.5 Kinetic data for the hydrolysis of a series of alkyl nitrate aspirin esters in 10% human plasma at pH 7.4 and 37°C.*

The hydrolysis of the alkyl nitrate aspirin esters proceeded rapidly, with half-lives of less than 2.2 minutes. The observation that the n-propyl nitrate substituted ester (**102**) had the longest half-life may be related to the length of the alkyl chain attached to the carboxylic ester group. In general, esters in which the carbonyl carbon is separated from a nitrate-bearing group by a two-carbon unit are cleaved the most rapidly [96]. Since ester **102** has a three-carbon chain it would be expected to be less susceptible to enzymatic hydrolysis. It also appears that the iso-propyl substituted ester (**101**) has a significantly higher $K_m$ then esters **100** and **102**. This may be due to the introduction of a methylene group into the two-carbon chain, between the carbonyl carbon and the nitrooxy group. It seems this results in a poorer binding of the substrate to butyrylcholinesterase (EC 3.1.1.8), although a high $V_{max}$ is retained.

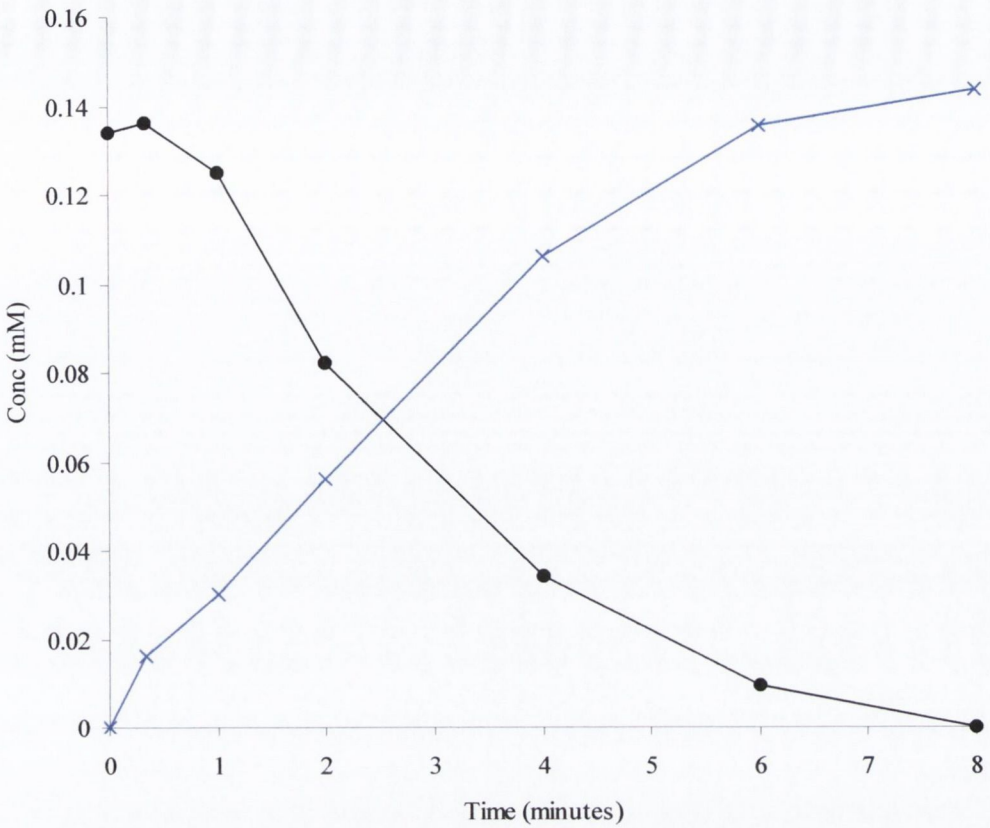



*Figure 5.16 Progression curve for the hydrolysis of ester **100** in 10% human plasma at pH 7.4 and 37°C: Ester **100** (●) and salicylate ester of ester **100** (x).*

A progression curve for the hydrolysis of the ethyl nitrate substituted ester (**100**) in 10% human plasma is presented in *Fig. 5.16*. Its hydrolysis was associated with the exclusive liberation of the salicylate ester. The salicylate ester remained essentially unchanged during the hydrolysis, which is in contrast to the stability of the salicylate esters of our glycolamides esters. However, it is typical of salicylate esters to undergo slow enzymatic hydrolysis. Methyl salicylate has a half-life of 17 hours in 80% human plasma [89]. A similar hydrolysis pattern was observed for the hydrolysis of esters **101** and **102**.

The hydrolysis of esters **100**, **101** and **102** was also studied in purified horse serum butyrylcholinesterase (EC 3.1.1.8) at pH 7.4 and 37°C. The hydrolysis followed pseudo first-order kinetics as presented in *Table 5.6*. The rate of hydrolysis of the ethyl nitrate substituted ester (**100**) and the iso-propyl nitrate substituted ester (**101**) were comparable, at the same enzyme concentration. However, the rate of the hydrolysis of

the n-propyl substituted ester (**102**) was significantly faster, since the enzyme was present at a higher concentration.

| Compound No. | Enzyme conc. mg/ml | $t_{1/2}$ min | $k_{obs}$ $min^{-1}$ |
|---|---|---|---|
| **100** | 0.10 | 5.92 | 0.12 |
| **101** | 0.10 | 9.63 | 0.07 |
| **102** | 0.25 | 2.85 | 0.24 |

*Table 5.6 Kinetic data for the hydrolysis of alkyl nitrate aspirin esters with purified horse serum BuChE at pH 7.4 and 37°C.*

A progression curve for the hydrolysis of the ethyl nitrate substituted ester (**100**) is presented in *Fig. 5.17*. Similar progression curves were obtained for esters **101** and **102**. The hydrolysis proceeded rapidly at the labile acetyl group, liberating the salicylate ester exclusively, which remained essentially unchanged during the study. This was the anticipated pathway of hydrolysis for esters **100**, **101** and **102**, since their hydrolysis in 10% human plasma also occurred *via* deacetylation.

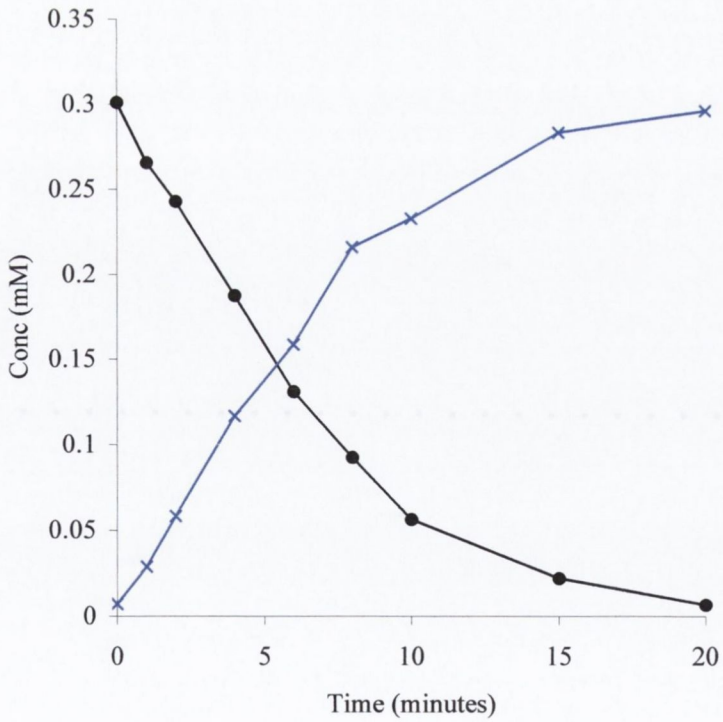



*Figure 5.17 Progression curve for the hydrolysis of ester **100** with purified horse serum BuChE at pH 7.4 and 37°C: Ester **100** (●) and salicylate ester of ester **100** (x).*

The hydrolysis studies of a series of alkyl nitrate aspirin esters confirmed their rapid enzymatic hydrolysis. However, the presence of a nitrate group did not assist in attack at the carboxylic ester group, since no aspirin was liberated during their hydrolysis.

## 5.3 Lipophilicity studies

The lipophilicities of a series of choline aspirin esters (type **72**), glycolamide aspirin esters (type **73**) and alkyl nitrate aspirin esters (type **74**) were estimated according to the method described in Chapter 2. Chromatographic lipophilicity data was also determined by means of the reverse phase HPLC capacity factor ($k^1$), as described in Chapter 2 (*Table 5.7*). The HPLC method involved elution on a Nova Pak C8 (3.9 x 150 mm) column with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) at 1 ml/minute.

| Compound No. | *c*log P | $t_r$ min | $k^1$ | Log $k^1$ |
|---|---|---|---|---|
| **75** | 2.21 | 5.31 | 2.54 | 0.405 |
| **76** | 2.73 | 7.21 | 3.81 | 0.581 |
| **77** | 3.26 | 11.55 | 6.70 | 0.826 |
| **78** | 2.94 | 10.96 | 5.63 | 0.750 |
| **96** | 1.82 | 5.29 | 2.53 | 0.402 |
| **97** | 2.35 | 6.76 | 3.50 | 0.544 |
| **98** | 2.87 | 8.99 | 4.99 | 0.698 |
| **99** | 2.81 | 9.13 | 5.08 | 0.706 |
| **24** | 1.88 | 5.50 | 2.67 | 0.427 |
| **100** | 1.96 | 7.33 | 3.89 | 0.589 |
| **101** | 2.27 | 7.22 | 3.81 | 0.581 |
| **102** | 2.34 | 8.32 | 4.55 | 0.657 |

*Table 5.7 Lipophilic and chromatographic data for a series of choline aspirin esters, glycolamide aspirin esters and alkyl nitrate aspirin esters.*

A plot of the lipophilicity parameters (log $k^1$ and *c*log P) was linear (r = 0.8815), as presented in *Fig. 5.18*. The observed deviation from linearity for the *N*-methyl nitrate substituted aspirin ester (**75**) and the ethyl nitrate substituted alkyl nitrate aspirin ester (**100**) may be related to the retention mechanisms of these esters on the C8 column employed.

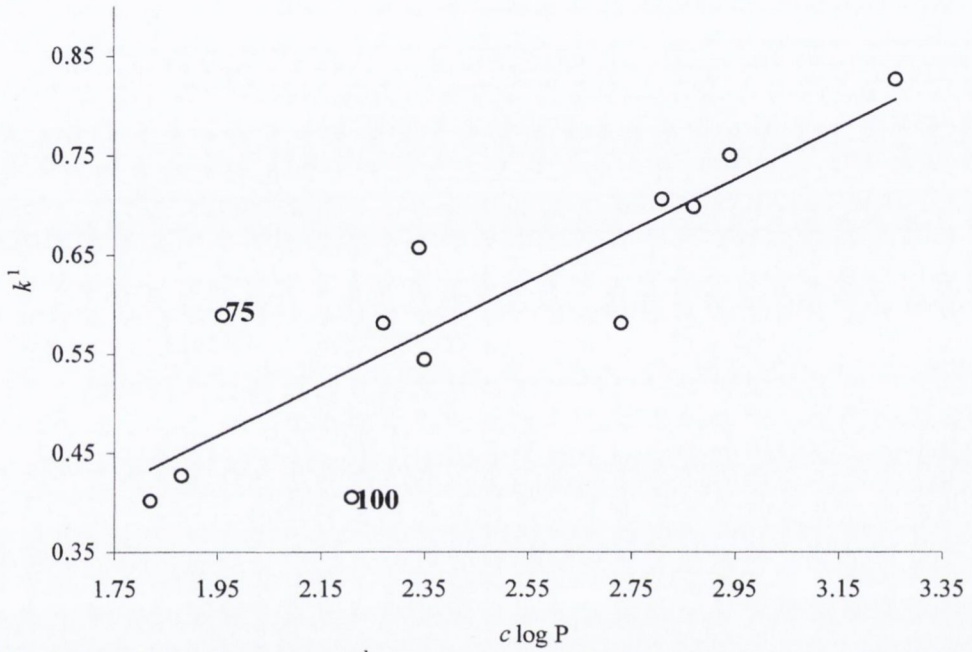



*Figure 5.18 Plot of clog P against $k^1$ for a series of choline aspirin ester, glycolamide aspirin esters and alkyl nitrate aspirin esters.*

The *c*log P data for each ester indicates their suitability for absorption after oral administration. Nielsen and Bundgaard determined the clog P for the *N,N*-diethyl substituted glycolamide ester (**24**) to be 2.06, after partitioning between octanol and water at 22°C [297]. On comparison of the *c*log P data for the choline aspirin esters and the corresponding glycolamide aspirin esters (e.g. ester **75** and **96**), the choline esters were more lipophilic. However, when the amine or amide substituent was an *N,N*-diethyl nitrate group (esters **78** and **99**), the lipophilicities were similar.

## 5.4 Conclusions

It was the aim of the current work to design nitroaspirin esters capable of liberating aspirin and a nitric oxide donor after hydrolysis by plasma esterases. A series of choline aspirin esters (**72**), glycolamide aspirin esters (**73**) and alkyl nitrate aspirin esters (**74**) were investigated.

The esters were synthesised and their observed lipophilicities perhaps indicates their suitability for absorption after oral administration. Their hydrolysis was studied in 10% pooled human plasma to determine their potential as aspirin prodrugs. Esters of type **72**, **73**, and **74** hydrolysed to liberate their corresponding salicylate ester and ultimately salicylic acid. The one exception was the *N*-propyl nitrate choline aspirin ester (**77**), which liberated up to 10% aspirin. The hydrolyses of esters of type **72**, **73** and **74** were also studied in horse serum butyrylcholinesterase (EC 3.1.1.8), to confirm the contribution of this enzyme towards their hydrolyses. Their rapid hydrolysis was accompanied by the liberation of their respective salicylate esters. However, once again it was only the choline aspirin ester **77**, which liberated aspirin (10%).

It may be possible in the future to design nitroaspirin esters around type **72**, **73** and **74**, which would promote hydrolysis at the carboxylic ester group, by manipulation of the NO-releasing moiety. The use of molecular modelling to determine the fit of such esters to the active site of human serum BuChE, which would promote aspirin release, could be useful in this regard (*Fig. 3.30*).

However, given the success of isosorbide-2-aspirinate-5-salicylate (**37**) for aspirin release, in human BuChE, future work should involve the development of a NO-releasing aspirin ester based on ester **37**.

# Chapter 6

## Experimental

## 6.1 Materials

IS-5-MN (**38**) and ISDN (**39**) were obtained from Sifa Ltd., Shannon Industrial Estate, Shannon, Co. Clare, Ireland. Human serum butyrylcholinesterase (EC 3.1.1.8), horse serum butyrylcholinesterase (EC 3.1.1.8), α-chymotrypsin (EC 3.4.21.1) (Bovine pancreas type II), rabbit liver carboxylesterase (EC 3.1.1.1), human serum albumin, eserine, BNPP, iso-OMPA, dibucaine and BW254C51 were all obtained from Sigma-Aldrich Ltd. Citrated rabbit blood was obtained from the marginal ear vein of New Zealand White rabbits held at the Bioresources Unit, Trinity College Dublin. Citrated dog plasma was obtained from the jugular vein of Beagles and Labradors held at the Bioresources Unit, Trinity College Dublin. Citrated rat plasma was obtained by cardiac puncture from rats housed at the Bioresources Unit, Trinity College Dublin. Citrated human plasma was obtained from healthy male and female volunteers from the School of Pharmacy, Trinity College Dublin. HPLC grade solvents were purchased from Riedel-de-Haen and Rathburn Ltd. Non-aqueous solvents were obtained from in-house stills [298]. All other reagents and chemicals were of analytical grade.

## 6.2 Chemistry

### 6.2.1 General procedures

Uncorrected melting points were obtained using a Gallenkamp apparatus. Infra-red (IR) spectra were obtained using a Nicolet 205 FT Infra-red spectrometer. Band positions are given in $cm^{-1}$. Solid samples were obtained by KBr disc: oils were analysed as neat films on NaCl plates. UV spectroscopy was carried out on a Cary 3E UV-VIS spectrophotometer. $^{1}H$ and $^{13}C$ NMR spectra were recorded at 27°C on a Brucker DPX 400 MHz FT NMR spectrometer (400.13 MHz $^{1}H$, 100.61 MHz $^{13}C$), in either $CDCl_3$ (tetramethylsilane as internal standard) or $CD_3OD$. For $CDCl_3$, $^{1}H$ NMR spectra were assigned relative to the TMS peak at 0.00 δ and $^{13}C$ NMR spectra were assigned relative to the middle $CDCl_3$ triplet at 77.00 ppm. For $CD_3OD$, $^{1}H$ and $^{13}C$ NMR spectra were assigned relative to the centre peaks of the $CD_3OD$ multiplets at 3.30 δ and 49.00 ppm respectively. Coupling constants are reported in Hertz. For $^{1}H$ NMR assignments, chemical shifts are reported: shift values (number of protons,

description of absorption, coupling constant(s) where applicable, proton assignment). LRMS were acquired on a Micromass mass spectrophotometer (EI mode) at the Department of Chemistry, Trinity College Dublin. Flash column chromatography was carried out on Merck kieselgel 60 (particle size 0.040–0.063 mm). Thin Layer Chromatography (TLC) was carried out on silica gel Merck F-254 plates.

### 6.2.2 Synthesis

*Isosorbide-5-mononitrate-2-aspirinate* (**35**)

To a solution of IS-5-MN (**38**) (2 g, 10 mmol) in toluene (50 ml) at 0°C, was added triethylamine (2.5 ml, 15 mmol) and acetylsalicyloyl chloride (2.7 g, 12 mmol). The mixture was stirred at room temperature overnight before washing with water (100 ml), HCl (1 M, 50 ml), saturated aqueous $NaHCO_3$ (50 ml) and brine (50 ml). The organic phases were dried over $MgSO_4$ and solvents removed *in vacuo* to afford product as crude oil. This was crystallised from ethanol to yield 1.33 g of product as colourless crystals (37.67%): m.pt. 80-84°C. $IR_{Vmax}$ (KBr): 1769.6 and 1731.8 (C=O), 1651.0 ($NO_2$), 1261.1 (C(O)OR, aromatic), 915.4 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 376.0645 ($M^+$+23), Found: 376.0618 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.36 (3H, s, $OCOCH_3$), 3.93 (1H, dd, J 5.5, 11.0 and 5.5Hz, IS6α-H), 4.10 (3H, m, IS1H[αβ] and IS6H[β]), 4.58 (1H, d, J 5.0Hz, IS3-H), 5.03 (1H, t, J 5.5 and 5.0Hz, IS4-H), 5.38 (1H, m, IS5-H), 5.45 (1H, d, J 3.0Hz, IS2-H), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.32 (1H, t, J 8.0 and 8.5Hz, Ar-H), 7.59 (1H, t, J 8.0 and 8.5Hz, Ar-H), 8.00 (1H, dd, J 1.5 and 2.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.86 ($OCOCH_3$), 69.34 and 73.31 (ISC-1 and ISC-6), 77.97 (ISC-5), 81.29 (ISC-4), 81.53 (ISC-2), 122.66 (ArC-1), 123.87, 126.07, 131.84 and 134.37 (aromatic methine), 150.70 (ArC-2), 163.54 (ArO<u>C</u>O(Me)), 169.62 (ArC(O)OR).

*2-Methyl-2-(isosorbide-5-nitrate-2-)1,3-benzodioxin-4-one* (**41**)

To a solution of IS-5-MN (**38**) (1 g, 5.2 mmol) in chloroform (10 ml) was added acetylsalicyloyl chloride (1.06 g, 5.3 mmol). The mixture was stirred at room temperature for one hour and then brought to reflux for 10 minutes. The mixture was diluted with diethyl ether (50 ml) and washed with saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). The organic phases were dried over $MgSO_4$ and solvents removed *in vacuo* to afford product as crude oil. This was crystallised from diethyl ether and hexane to yield 1.07 g of product as white crystalline material (58.29%): m.pt. 122-125°C. $IR_{Vmax}$ (KBr): 1736.1 (C=O), 1645.2 and 1628.6 ($NO_2$) $cm^{-1}$. LRMS: Requires: 353.0747 ($M^+$), Found: 353.0851 ($M^+$). $^1H$ NMR δ ($CDCl_3$): 1.80 (3H, s, $CH_3$), 3.80 (1H, dd, J 11.2 and 6.6Hz, IS6-H [α]), 3.93 (1H, dd, J 10.3 and 3.3Hz, IS1-H [β]), 3.94 (1H, dd, J 11.2 and 2.9Hz, IS6-H [β]), 4.02 (1H, dd, J 10.2 and 1.1Hz, IS1-H [α]), 4.38 (1H, d, J 4.8Hz, IS3-H), 4.72 (1H, d, J 3.2Hz, IS2-H), 4.87 (1H, t, J 4.8 and 4.8Hz, IS4-H), 5.28 (1H, m, IS5-H), 7.02 (1H, m, Ar-H), 7.17 (1H, m, Ar-H), 7.59 (1H, m, Ar-H), 7.94 (1H, m, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 23.3 ($OCOCH_3$), 69.13 (ISC-6), 75.59 (ISC-1), 75.73 (ISC-4), 81.02 (ISC-3), 81.31 (ISC-5), 8.78 (ISC-2), 112.71, 116.70, 123.54 and 129.62 (aromatic methine), 136.80 (ArC-6), 54.75 (CO), 160.13 (ArC-2).

*Isosorbide mononitrate-2-salicylate* (**42**)

To a solution of IS-5-MN (**38**) (3.88 g, 21.7 mmol) in dichloromethane (50 ml) was added DCC (4.47 g, 21.7 mmol), DMAP (0.27 g, 2.2 mmol) and salicylic acid (**2**) (3 g, 21.7 mmol). The mixture was stirred at room temperature for three hours before filtering and washing the filtrate with HCl (2 x 25 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil, which after recrystallisation from ethyl acetate and hexane (5 ml; 2:1) afforded 2.18 g of product as colourless crystals (32.39%): m.pt. 90-93°C. $IR_{Vmax}$ (KBr): 3228.6 (OH, aromatic), 1676.3 (C=O), 1650.9 ($NO_2$), 1284.4 (C(O)OR, aromatic), 1096.8 (C-O-C), 853.6

($ONO_2$) $cm^{-1}$. LRMS: Requires: 334.0538 ($M^+$+23), Found: 334.0432 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 3.98 (1H, dd, J 5.5 and 6.0Hz, IS6α-H), 4.14 (3H, m, IS1$H_2$[α + β] and IS6$H_2$[β]), 5.04 (1H, d, J 5.0Hz, IS3-H), 5.09 (1H, t, J 5.0 and 5.5Hz, IS4-H), 5.42 (1H, m, IS5-H), 6.04 (1H, m, IS2-H), 6.91 (1H, m, Ar-H), 7.02 (1H, d, J 8.0Hz, Ar-H), 7.50 (1H, m, Ar-H), 7.81 (1H, m, Ar-H), 10.55 (1H, s, Ar-OH). $^{13}C$ NMR ppm ($CDCl_3$): 68.93 and 72.90 (ISC-1 and ISC-6), 77.80 (ISC-5), 80.69 (ISC-2), 81.15 (ISC-4), 86.16 (ISC-3), 108.44 (ArC-1), 117.32, 118.85, 129.43, 135.87 (aromatic methine), 150.59 (ArC-2), 167.42 (ArC(O)OR).

*Isosorbide diaspirinate* (**36**)

To a solution of isosorbide (**40**) (2 g, 13.6 mmol) in toluene (50 ml) at 0°C was added triethylamine (5 ml, 45 mmol) and acetylsalicyloyl chloride (6 g, 30 mmol). The mixture was stirred at room temperature overnight before the addition of ether (20 ml). The solution was then washed with water (100 ml), HCl (1 M, 50 ml), saturated aqueous $NaHCO_3$ (50 ml) and brine (50 ml). The organic phases were combined and dried over $MgSO_4$ and concentrated *in vacuo* to yield product as crude oil, which after crystallisation in ethanol afforded 4.39 g of product as colourless crystals (68.68%): m.pt. 110-112°C. $IR_{Vmax}$ (KBr): 1768.7 and 1727.2 (C=O), 1245.9 (C(O)OR, aromatic), 1194.3 (C-O-C) $cm^{-1}$. LRMS: Requires: 493.1090 ($M^+$+23), Found: 493.4722 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.36 (6H, m, 2 x $OCOCH_3$), 4.05 (4H, m, IS1$H_2$[α + β] and IS6$H_2$[α + β]), 4.63 (1H, d, J 4.5Hz, IS3-H), 4.99 (1H, t, J 5.0 and 5.0Hz, IS4-H), 5.40 (1H, q, J 5.5, 5.0 and 5.5Hz, IS5-H), 5.46 (1H, d, J 3.0Hz, IS2-H), 7.10 (2H, m, Ar-H), 7.37 (2H, m, Ar-H), 7.57 (2H, m, Ar-H), 7.99 (1H, dd, J 1.5 and 1.5Hz, Ar-H), 8.08 (1H, dd, J 1.5 and 1.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.98 ($OCOCH_3$), 70.65 and 73.17 (ISC-1 and ISC-6), 74.51 (ISC-5), 78.51 (ISC-2), 81.03 (ISC-4), 86.11 (ISC-3), 122.70 and 122.75 (2 x ArC-1), 123.83, 123.96, 126.01, 126.06, 131.81, 131.99, 134.16 and 134.24 (aromatic methine), 150.70 and 150.78 (ArC-2), 163.56 and 163.74 (2 x ArO<u>C</u>(O)Me), 169.52 and 169.57 (2 x ArC(O)OR).

*Isosorbide-2-mononitrate* (**59**)

A solution of ISDN (**39**) (5.41 g, 23 mmol) and ferrous sulphate (63.94 g, 23 mmol) in a mixture of methanol and water (140 ml, 4:1) was stirred at room temperature before heating to reflux temperature for 4 hours. The solvents were removed *in vacuo* and the oily residues obtained were reconstituted in dichloromethane and washed with saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (50 ml). After drying over anhydrous $Na_2SO_4$, the solvents were removed *in vacuo* to afford 3.06 g of product as a yellow oil (69.65%). $IR_{\nu max}$ (film): 3444.1 (OH), 1639.9 ($NO_2$), 1274.8 ($NO_2$), 1089.1 (C-O-C), 859.2 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 214.0307 ($M^+$+23), Found: 214.1416 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.79 (1H, d, J 6.5Hz, OH), 3.59 (1H, q, J 5.5, 4.0 and 5.5Hz, IS6α-H), 3.88 (1H, q, J 6.0, 3.5 and 6.0Hz, IS6β-H), 4.12 (2H, d, J 3.0Hz, IS1$H_2$[α+β]), 4.30 (1H, t, J 6.0 and 5.5Hz, IS5-H), 4.55 (1H, d, J 5.0Hz, IS3-H), 4.61 (1H, t, J 5.0 and 5.0Hz, IS4-H), 5.40 (1H, m, IS2-H). $^{13}C$ NMR ppm ($CDCl_3$): 71.51 (ISC-1), 71.99 (ISC-5), 73.27 (ISC-6), 81.97 (ISC-4), 83.88 (ISC-2), 86.24 (ISC-3).

*Isosorbide-5-aspirinate-2-mononitrate* (**51**)

To a solution of isosorbide-2-mononitrate (**59**) (2.6 g, 14 mmol) in dichloromethane (100 ml) at 0°C, was added triethylamine (5.84 ml, 42 mmol) and acetylsalicyloyl chloride (2.78 g, 14 mmol). The mixture was stirred at room temperature overnight before washing with HCl (2 M, 2 x 25 ml), water (2 x 25 ml), saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil, which upon crystallisation in ethanol afforded 1.5 g of product as slightly coloured crystals (30.35%): m.pt. 86-88°C. $IR_{\nu max}$ (KBr): 3401.1 (OH), 1756.0 and 1708.5 (C=O), 627.9 ($NO_2$), 1290.4 ($NO_2$), 1252.3 (C(O)OR, aromatic), 1100.9 (C-O-C), 870.0 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 376.0624 ($M^+$+23), Found: 376.3037 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.38 ($OCOCH_3$), 4.11 (4H, m, IS1$H_2$[α + β] and IS6$H_2$[α + β]), 4.63 (1H, d, J 4.5Hz, IS3-H), 4.96 (1H, t, J 5.5 and 5.0Hz, IS4-H), 5.39 (2H, m, IS2-H and IS5-H), 7.13 (1H, d, J 7.0Hz, Ar-H), 7.35 (1H, t, J 7.0 and 8.3Hz, Ar-H), 7.61 (1H, t, J

7.8 and 7.8Hz, Ar-H), 8.06 (1H, d, J 7.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.51 ($OCOCH_3$), 70.42 and 71.09 (ISC-1 and ISC-6), 73.66 (ISC-5), 80.71 (ISC-4), 84.08 (ISC-2), 85.59 (ISC-3), 122.55 (ArC-1), 123.65, 125.64, 131.43 and 133.83 (aromatic methine), 150.34 (ArC-2), 163.16 (ArO<u>C</u>OMe) and 169.06 (ArC(O)OR).

*Isosorbide-5-aspirinate* (**53**)

A solution of isosorbide-5-aspirinate-2-mononitrate (**51**) (0.32 g, 0.91 mmol) in a mixture of methanol and ethyl acetate (40 ml, 1:1) was stirred for 3 days over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica and the filtrate removed *in vacuo* to yield 0.19 g of product as colourless crystals (67.68%): m.pt. 42-46°C. $IR_{Vmax}$ (KBr): 3210.0 (OH, aromatic), 1756.0 and 1708.5 (C=O), 1627.9 ($NO_2$), 1252.3 (C(O)OR, aromatic), 1100.9 (C-O-C), 870.0 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 331.2710 ($M^+$+23), Found: 331.2963 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.23 (3H, s, $OCOCH_3$), 3.92 (4H, m, $IS1H_2$[α + β] and $IS2H_2$[α + β]), 4.33 (1H, d, J 2.5Hz, IS2-H), 4.42 (1H, d, J 4.5Hz, IS3-H), 4.93 (1H, t, J 5.0 and 5.0Hz, IS4-H), 5.33 (1H, q, J 5.5, 5.0 and 5.5Hz, IS5-H), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.34 (1H, m, Ar-H), 7.55 (1H, m, Ar-H), 8.05 (1H, dd, J 5.0 and 6.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.52 ($OCOCH_3$), 69.96 (ISC-1), 74.25 (ISC-5), 75.05 (ISC-6), 75.57 (ISC-2), 80.78 (ISC-4), 86.41 (ISC-3), 122.64 (ArC-1), 123.46, 125.63, 131.52 and 133.69 (aromatic methine), 150.27 (ArC-2), 163.39 (ArO<u>C</u>OMe) and 169.31 (ArC(O)OR).

*Isosorbide-2-aspirinate* (**52**)

A solution of ISMNA (**36**) (3.5 g, 10 mmol) in a mixture of methanol and ethyl acetate (60 ml, 1:1) was stirred for two days over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica and the filtrate removed *in vacuo* to yield 3 g of crude product. Purification by column chromatography using hexane and ethyl acetate (3:2, 1:1) as the eluant afforded 1.24 g of product as white crystalline material (40.26%): m.pt. 46-48°C. $IR_{Vmax}$ (KBr):

3485.6 (OH), 1767.6 and 1725.2 (C=O), 1251.9 and 1193.9 (C(O)OR, aromatic), 1078.1 (C-O-C) $cm^{-1}$. LRMS: Requires: 331.2710 ($M^+$+23), Found: 331.2961 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.36 (3H, s, $OCOCH_3$), 3.91 (1H, m, IS1H[α]), 4.12 (3H, m, IS1H[β] and $IS6H_2$[β]), 4.33 (1H, m, IS5-H), 4.58 (1H, d, J 4.0Hz, IS3-H), 4.68 (1H, t, J 5.0 and 5.0Hz, IS4-H), 5.44 (1H, d, J 3.5Hz, IS2-H), 7.12 (1H, dd, J 1.0 and 1.0Hz, Ar-H), 7.32 (1H, m, Ar-H), 7.58 (1H, m, Ar-H), 8.03 (1H, dd, J 1.5 and 1.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 71.88 and 72.90 (ISC-1 and ISC-6), 73.13 (ISC-5), 78.49 (ISC-2), 81.56 (ISC-4), 85.13 (ISC-3), 122.29 (ArC-1), 123.39, 125.57, 131.31 and 133.81 (aromatic methine), 150.24 (ArC-2), 163.03 (ArO<u>C</u>(O)Me) and 169.09 (ArC(O)OR).

*Isosorbide dibenzyloxy benzoate* (**60**)

To a solution of isosorbide (**40**) (1 g, 6.8 mmol) in dichloromethane (50 ml) was added DMAP (0.17 g, 1.37 mmol), DCC (2.8 g, 13.6 mmol) and 2-benzyloxy benzoic acid (**61**) (3.12 g, 13.7 mmol). The mixture was stirred at room temperature overnight before filtering and washing the filtrate with HCl (2 x 25 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford 4.4 g of crude product. Purification of 1.6 g by column chromatography over silica gel using petroleum ether and ethyl acetate (3:1) as eluant yielded 1.37 g of product as an oil (35.59%). $IR_{Vmax}$ (film): 1728.0 and 1706.6 (C=O), 1298.2 and 1245.8 (C(O)OR, aromatic), 1079.6 (C-O-C) $cm^{-1}$. LRMS: Requires: 589.5849 ($M^+$+23), Found: 589.6012 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 3.95 (4H, m, $IS1H_2$ [α + β] and $IS6H_2$ [β]), 4.49 (1H, d, J 4.7Hz, IS3-H), 4.73 (1H, t, J 4.7 and 5.2Hz, IS4-H), 5.30 (1H, q, J 4.6, 5.9 and 5.2Hz, IS2-H), 5.40 (1H, d, J 2.4Hz, IS5-H), 7.03 (4H, m, Ar-H), 7.37 (2H, m, Ar-H), 7.43 (5H, m, Ar-H), 7.50 (6H, m, Ar-H), 7.88 (2H, m, 2 x Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 70.21 (ISC-1), 70.58 (2 x BnO<u>$CH_2$</u>Bn), 73.29 (ISC-6), 74.25 (ISC-5), 78.42 (ISC-2), 80.76 (ISC-4), 86.01 (ISC-3), 113.37 and 113.65 (2 x ArC-1), 120.40, 120.47, 127.81, 127.98, 131.86, 132.09, 133.65, 133.71, 136.39 and 136.54 (aromatic methine), 127.11 (2 x aromatic

methine), 127.43 (2 x aromatic methine), 128.45 (2 x aromatic methine), 128.52 (2 x aromatic methine), 158.25 and 158.30 (Ar(OBn)C), 165.45 and 165.53 (ArC(O)OR).

*Isosorbide disalicylate* (**55**)

A solution of isosorbide dibenzyloxy benzoate (**60**) (0.29 g, 0.5 mmol) in a mixture of methanol and ethyl acetate (10 ml, 1:1) was stirred for two days over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica and the filtrate removed *in vacuo* to yield product as crude crystals. Recrystallisation from ethyl acetate and hexane (2 ml, 1:1) afforded 0.08 g of product as a colourless crystalline material (41.45%): m.pt. 115-119°C. $IR_{Vmax}$ (KBr): 3212.2 (OH), 1679.2 and 1674.4 (C=O), 1290.3 and 1250.4 (C(O)OR, aromatic), 1092.7 (C-O-C) $cm^{-1}$. LRMS: Requires: 409.0879 ($M^+$+23), Found: 409.0902 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 4.11 (4H, m, IS1$H_2$[α + β] and IS6$H_2$[β]), 4.70 (1H, d, J 5.0Hz, IS3-H), 5.08 (1H, t, J 5.5 and 5.0Hz, IS4-H), 5.46 (1H, q, J 5.5, 4.5 and 5.5Hz, IS5-H), 5.52 (1H, s, IS2-H), 6.90 (2H, m, Ar-$H_2$), 7.02 (2H, m, Ar-$H_2$), 7.48 (2H, m, Ar-$H_2$), 7.81 (2H, dd, J 1.5 and 1.5Hz, Ar-$H_2$), 10.51 (1H, s, Ar-OH), 10.54 (1H, s, Ar-OH). $^{13}C$ NMR ppm ($CDCl_3$): 70.37 and 72.75 (ISC-1 and ISC-6), 74.26 (ISC-5), 78.25 (ISC-2), 80.73 (ISC-4), 85.67 (ISC-3), 111.28 and 111.41 (2 x ArC-1), 117.26, 117.30, 118.83, 129.44, 135.62 and 135.79 (aromatic methine), 161.36 and 161.44 (ArC-2), 168.74 and 168.84 (ArC(O)OR).

*Isosorbide-2-aspirinate-5-benzyloxy benzoate* (**61**)

To a solution of isosorbide-2-aspirinate (**52**) (1.28 g, 4.2 mmol) in dichloromethane (50 ml) was added benzyloxy benzoic acid (**58**) (0.96 g, 4.2 mmol), DCC (0.87 g, 4.2 mmol) and DMAP (0.05 g, 0.42 mmol). The mixture was stirred at room temperature overnight before filtering and washing the filtrate with HCl (2 x 25 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford 1.81 g of intermediate as crude product. Purification by column chromatography over silica gel

using petroleum ether and ethyl acetate (3:1) as eluant, yielded 1.24 g of product as an oil (57.2%). $IR_{Vmax}$ (film): 1769.9 and 1733.8 (C=O), 1295.8 and 1254.2 (C(O)OR, aromatic), 1072.6 (C-O-C) $cm^{-1}$. LRMS: Requires: 539.1661 ($M^+$+23), Found: 539.0562 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 1.21 (2H, t, J 7.0 and 7.0Hz, BnO$\underline{CH_2}$Bn), 2.30 (1H, s, $OCOCH_3$), 4.00 (4H, m, IS1$H_2$[α +β] and IS6$H_2$[β]), 4.57 (1H, d, J 5.0Hz, IS3-H), 4.97 (1H, t, J 5.0 and 5.5Hz, IS4-H), 5.39 (2H, m, IS2-H and IS5-H), 7.05 (2H, m, 2 x Ar-H), 7.13 (1H, m, Ar-H), 7.44 (8H, m, Ar-H), 7.92 (1H, m, Ar-H), 8.02 (1H, m, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.77 ($OCOCH_3$), 70.51 (BnO$\underline{C}H_2$Bn), 70.57 and 72.94 (ISC-1 and ISC-6), 74.21 (ISC-5), 78.67 (ISC-2), 81.06 (ISC-4), 85.94 (ISC-3), 120.47, 123.73, 125.92, 126.92, 127.83, 131.74, 132.10, 133.69 and 134.11 (aromatic methine), 127.13 and 128.43 (2 x (ArC)), 150.58 (Ar(Asp)C-2), 158.31 (Ar(OBn)$\underline{C}$), 163.50 (ArO$\underline{C}$(O)Me), 165.52 and 169.47 (ArC(O)OR).

*Isosorbide-2-aspirinate-5-salicylate* (**37**)

A solution of isosorbide-2-aspirinate-5-benzyloxy benzoate (**61**) (0.58 g, 1.1 mmol) in a mixture of methanol and ethyl acetate (20 ml, 1:1) was stirred overnight over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica and the filtrate removed *in vacuo* to yield 0.3 g of crude product. Purification by column chromatography using hexane and ethyl acetate (2:1;1:1) as the eluant afforded 0.12 g of product as a white crystalline material (25.49%): m.pt. 82-84°C. $IR_{Vmax}$ (KBr): 3213.8 (OH), 1767.8, 1724.7 and 1683.9 (C=O), 1299.9 and 1249.3 (C(O)OR, aromatic), 1080.5 (C-O-C) $cm^{-1}$. LRMS: Requires: 451.0984 ($M^+$+23), Found: 451.0971 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.37 (1H, s, $OCOCH_3$), 4.10 (4H, m, IS1$H_2$[α + β] and IS6$H_2$[β]), 4.64 (1H, d, J 4.5Hz, IS3-H), 5.04 (1H, t, J 5.0 and 5.5Hz, IS4-H), 5.45 (2H, m, IS2-H and IS5-H), 6.92 (1H, t, J 7.5 and 8.0Hz, Ar-H), 7.02 (1H, d, J 8.5Hz, Ar-H), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.32 (1H, t, J 7.5 and 7.0Hz, Ar-H), 7.49 (1H, m, Ar-H), 7.58 (1H, m, Ar-H), 7.89 (1H, dd, J 1.5 and 1.5Hz, Ar-H), 8.01 (1H, dd, J 1.5 and 1.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.42 ($OCOCH_3$), 70.38 and 72.77 (ISC-1 and ISC-6), 74.31 (ISC-5), 77.98 (ISC-2), 80.65 (ISC-4), 85.71 (ISC-3), 117.48 (Ar(Sal)C-1), 122.27 (Ar(Asp)C-1), 117.29,

118.87, 123.39, 125.59, 129.47, 131.37, 133.85, and 135.59 (aromatic methine), 150.25 (Ar(Asp)C-2), 161.33 (Ar(Sal)C-2), 163.13 (ArO<u>C</u>OMe), 168.84 (ArC(O)OR).

*Isosorbide-5-aspirinate-2-benzyloxy benzoate* (**62**)

To a solution of isosorbide-5-aspirinate (**53**) (0.13 g, 0.4 mmol) in dichloromethane (5 ml) was added benzyloxy benzoic acid (**58**) (0.09 g, 0.4 mmol), DCC (0.08 g, 0.4 mmol) and DMAP (0.01 g, 0.04 mmol). The mixture was stirred at room temperature overnight before filtering and washing the filtrate with HCl (2 x 5 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 5 ml) and water (2 x 5 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford 0.14 g of product as a colourless oil (67.80%). $IR_{Vmax}$ (film): 1759.0 (C=O), 1298.5 and 1245.3 (C(O)OR, aromatic), 1078.4 (C-O-C) $cm^{-1}$. LRMS: Requires: 539.1661 ($M^+$+23), Found: 539.1659 ($M^+$+23). $^1$H NMR δ ($CDCl_3$): 2.36 (1H, s, $OCOCH_3$), 4.01 (4H, m, $IS1H_2$ [α +β] and $IS6H_2$ [β]), 4.48 (1H, d, J 4.5Hz, IS3-H), 4.68 (1H, t, J 5.0 and 5.0Hz, IS4-H), 5.19 (2H, t, J 7.0 and 7.0Hz, BnO<u>$CH_2$</u>Bn), 5.23 (2H, m, IS2-H and IS5-H), 7.15 (2H, m, 2 x Ar-H), 7.24 (1H, m, Ar-H), 7.54 (8H, m, Ar-H), 7.97 (1H, m, Ar-H), 8.12 (1H, m, Ar-H). $^{13}$C NMR ppm ($CDCl_3$) 20.97 ($OCOCH_3$), 70.32 (BnO<u>C</u>$H_2$Bn), 70.68 and 73.45 (ISC-1 and ISC-6), 74.54 (ISC-5), 78.27 (ISC-2), 80.90 (ISC-4), 86.12 (ISC-3), 120.49, 123.92, 127.54, 131.94, 131.97, 132.02, 133.81 and 134.11 (aromatic methine), 126.04 and 128.58 (2 x Ar-C), 150.74 (Ar(Asp)C-2), 158.32 (Ar(OBn)<u>C</u>), 163.75 (ArO<u>C</u>(O)Me), 165.53 and 169.55 (ArC(O)OR).

*Isosorbide-2-salicylate-5-aspirinate* (**54**)

A solution of isosorbide-5-aspirinate-2-benzyloxy benzoate (**62**) (0.13 g, 0.25 mmol) in a mixture of methanol and ethyl acetate (5 ml, 3:1) was stirred overnight over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica and the filtrate removed *in vacuo* to yield 0.05 g of crude product. Purification by column chromatography using hexane and ethyl acetate (2:1) as the eluant afforded 0.02 g of product as a colourless crystalline material

(18.69%): m.pt. 84-86°C. $IR_{Vmax}$ (KBr): 3215.5 (OH), 1765.8, 1728.5 and 1685.6 (C=O), 1309.9 and 1256.3 (C(O)OR, aromatic), 1090.5 (C-O-C) $cm^{-1}$. LRMS: Requires: 451.0984 ($M^+$+23), Found: 451.0952 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.36 (3H, s, $OCOCH_3$), 4.03 (4H, m, $IS1H_2$[α + β] and $IS6H_2$[β]), 4.67 (1H, d, J 4.5Hz, IS3-H), 5.02 (1H, t, J 5.5 and 5.0Hz, IS4-H), 5.38 (1H, m, IS5-H), 5.49 (1H, d, J 3.0Hz, IS2-H), 6.87 (1H, t, J 7.5 and 8.0Hz, Ar-H), 6.98 (1H, d, J 8.5Hz, Ar-H), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.34 (1H, t, J 7.0 and 7.5Hz, Ar-H), 7.47 (1H, m, Ar-H), 7.58 (1H, m, Ar-H), 7.83 (1H, dd, J 1.5 and 1.5Hz, Ar-H), 8.08 (1H, dd, J 2.0 and 1.5Hz, Ar-H), 10.50 (1H, s, Ar-OH). $^{13}C$ NMR ppm ($CDCl_3$): 20.95 ($OCOCH_3$), 70.61 and 73.07 (ISC-1 and ISC-6), 74.78 (ISC-5), 78.75 (ISC-2), 81.16 (ISC-4), 85.98 (ISC-3), 111.67 (Ar(Sal)C-1), 117.55 and 119.25 (aromatic methine), 122.36 (Ar(Asp)C-1), 123.83, 126.10, 129.85, 131.94, 134.98 and 136.19 (aromatic methine), 150.72 (Ar(Asp)AC-2), 161.74(Ar(Sal)C-2), 163.63 (ArOC(O)Me), 169.68 (ArC(O)OR).

*Isosorbide-2-mononitrate-5-salicylate* (**63**)

To a solution of isosorbide-2-mononitrate (**59**) (0.5 g, 2.8 mmol) in dichloromethane (20 ml) was added DCC (0.57 g, 2.79 mmol), DMAP (0.033 g, 0.28 mmol) and salicylic acid (**2**) (0.50 g, 2.8 mmol). The mixture was stirred at room temperature for four hours before filtering and washing the filtrate with HCl (2 x 20 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 20 ml) and water (2 x 20 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product (0.58 g) as an oil (66.60%). $IR_{Vmax}$ (film): 3229.4 (OH, aromatic), 1674.2 (C=O), 1649.8 ($NO_2$), 1281.3 (C(O)OR, aromatic), 1094.5 (C-O-C), 850.2 ($ONO_2$) $cm^{-1}$. HRMS: Requires: 334.0518 ($M^+$+23), Found: 334.0624 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 3.98 (4H, m, IS1-H and IS6-H), 4.60 (1H, d, J 5.0Hz, IS3-H), 4.96 (1H, t, J 5.0 and 5.5Hz, IS4-H), 5.22 (1H, m, IS-5-H), 5.40 (1H, m, IS2-H) 7.02 (1H, m, Ar-H), 7.39 (1H, t, J 7.0 and 7.6Hz, Ar-H), 7.50 (1H, m, Ar-H), 7.91 (1H, d, J 7.5Hz, Ar-H), 10.53 (1H, s, Ar-OH). $^{13}C$ NMR ppm ($CDCl_3$): 70.63 and 70.70 (ISC-1 and ISC-6), 73.79 (ISC-5), 81.01 (ISC-2), 84.49 (ISC-4) 86.31 (ISC-3), 113.62, 120.54, 128.43 and 133.93 (aromatic methine), 158.39 (ArC-2), 165.57 (ArC(O)OR).

*Isosorbide-5-salicylate* (**56**)

A solution of isosorbide-2-mononitrate-5-salicylate (**63**) (0.50 g, 1.25 mmol) in a mixture of methanol and ethyl acetate (20 ml, 1:1) was stirred for three days over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica and the filtrate removed *in vacuo* to yield crude product, which upon crystallisation afforded a white crystalline material (0.23 g, 69.17%). m.pt. 154-156°C. $IR_{Vmax}$ (KBr): 3451.1 (OH), 1686.4 (C=O), 1252.5 (C(O)OR), 1163.7 (C-O-C), 1076.7 (C-O-C) $cm^{-1}$. LRMS: Requires: 289.0688 ($M^+$+23), Found: 289.0702 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 3.93 (2H, d, J 3.0Hz, IS1H[α + β]), 4.02 (2H, d, J 5.0Hz, IS6$H_2$[β]), 4.41 (1H, d, J 3.0Hz, IS2-H), 4.49 (1H, d, J 4.5Hz, IS3-H), 5.01 (1H, t, J 5.5 and 5.0Hz, IS4-H), 5.43 (1H, q, J 5.0, 5.5 and 5.0Hz, IS5-H). $^{13}C$ NMR ppm ($CDCl_3$): 70.72 (ISC-1), 74.86 (ISC-6), 75.63 (ISC-2), 76.19 (ISC-3), 80.67 (ISC-4), 88.46 (ISC-5), 117.73, 119.24, 129.94 and 135.97 (aromatic methine), 150.47 (ArC-2), 167.56 (ArC(O)OR).

*Nitrooxy-acetic acid* (**68**)

A solution of bromoacetic acid (2 g, 14.3 mmol) and silver nitrate (4.89 g, 28.7 mmol) in acetonitrile (40 ml) was allowed to stir at room temperature overnight. The reaction was filtered and the filtrate concentrated *in vacuo*. The oily residues obtained were reconstituted in ethyl acetate and washed with water (2 x 40 ml). After drying over anhydrous $Na_2SO_4$, the ethyl acetate was removed *in vacuo* to afford product (1.5 g) as a green oil (86.60%) [299]. $IR_{Vmax}$ (film): 3381.9 (OH), 1734.9 (C=O), 1224.9 ($NO_2$) $cm^{-1}$. $^1H$ NMR δ ($CDCl_3$): 4.95 (2H, s, $CH_2$), 9.11 (1H, s, OH). $^{13}C$ NMR ppm ($CDCl_3$): 66.64 ($CH_2$), 170.12 (C=O).

*Isosorbide-2-aspirinate-5-(nitrooxy)-acetate* (**64**)

To a solution of isosorbide-2-aspirinate (**52**) (0.49 g, 1.6 mmol) in dichloromethane (10 ml) was added DCC (0.33 g, 1.6 mmol), DMAP (0.02 g, 0.16 mmol) and nitrooxy-acetic acid (**68**) (0.19 g, 1.6 mmol). The mixture was stirred at room temperature

overnight before filtering and washing the filtrate with HCl (2 x 10 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 10 ml) and water (2 x 10 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (5:2, 2:1) as eluant yielded product (0.38 g) as a colourless oil (57.79%). $IR_{Vmax}$ (film): 1759.0 and 1727.5 (C=O), 1643.6 ($NO_2$), 1287.7 ($NO_2$), 1256.3 (C(O)OR, aromatic), 1193.5 (C-O-C) $cm^{-1}$. LRMS: Requires: 411.0802 ($M^+$), Found: 411.3424 ($M^+$). $^1H$ NMR δ ($CDCl_3$): 2.18 (2H, s, $OCH_2O$), 2.36 (3H, s, $OCOCH_3$), 3.61 (2H, q, J 6.0, 3.5 and 6.0Hz, IS1-H), 3.92 (2H, q, J 6.0, 3.5 and 6.0Hz, IS6-H), 4.12 (1H, m, IS4-H), 4.33 (1H, m, IS3-H), 4.58 (1H, d, J 4.0Hz, IS5-H), 4.67 (1H, t, J 5.0 and 5.0Hz, IS2-H), 7.11 (1H, d, J 8.0Hz, Ar-H), 7.33 (1H, t, J 8.0 and 7.5Hz, Ar-H), 7.59 (1H, t, J 7.1 and 8.3Hz, Ar-H), 8.01 (1H, d, J 6.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.91 ($OCOCH_3$), 70.88 ($CH_2$), 72.36 (IS1-C), 73.41 (IS6-C), 73.69 (IS4-C), 78.96 (IS3-C), 82.04 (IS5-C), 85.64 (IS2-C), 122.77 (ArC-1), 123.89, 126.07, 131.81 and 134.31 (aromatic methine), 150.74 (CO), 163.51 (ArO<u>C</u>(O)Me), 169.59 (ArC(O)OR).

*3-Nitrooxy-propionic acid* (**69**)

A solution of 3-bromo-propionic acid (2.0 g, 13 mmol) and silver nitrate (4.44 g, 26 mmol) in acetonitrile (40 ml) was allowed to stir at room temperature overnight. The reaction was filtered and the filtrate concentrated *in vacuo*. The oily residues obtained were reconstituted in ethyl acetate and washed with water (2 x 40 ml). After drying over anhydrous $Na_2SO_4$, the ethyl acetate was removed *in vacuo* to afford product (1.6 g) as a yellow oil (91.17%) [300]. $IR_{Vmax}$ (film): 3044.0 (OH), 1720.3 (C=O), 1637.8 ($NO_2$), 1284.7 ($NO_2$) $cm^{-1}$. $^1H$ NMR δ ($CDCl_3$): 2.76 (2H, t, J 6.0 and 6.0Hz, $CH_2$), 4.66 (2H, t, J 6.0 and 6.0Hz, $CH_2$), 10.19 (1H, s, OH). $^{13}C$ NMR ppm ($CDCl_3$): 31.25 ($CH_2$), 65.59 ($CH_2$), 175.05 (C=O).

*Isosorbide-2-aspirinate-5-(3-nitrooxy)-propionate* (**65**)

To a solution of isosorbide-2-aspirinate (**52**) (0.28 g, 0.9 mmol) in dichloromethane (10 ml) was added DCC (0.19 g, 0.9 mmol), DMAP (0.001 g, 0.09 mmol) and 3-nitrooxy-propionic acid (**69**) (0.14 g, 0.9 mmol). The mixture was stirred at room temperature overnight before filtering and washing the filtrate with HCl (2 x 10 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 10 ml) and water (2 x 10 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed in vacuo to afford product as a crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (5:2, 2:1) as eluant yielded product (0.19 g) as a colourless oil (49.67%). $IR_{Vmax}$ (KBr): 1749.3 and 1722.0 (C=O), 1653.2 ($NO_20$, 1247.8 ($NO_2$), 1198.2 (C-O-C) $cm^{-1}$. LRMS: Requires: 425.0958 ($M^+$), Found: 449.3934 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.36 ($OCOCH_3$), 2.94 (2H, m, $CH_2$), 4.17 (4H, m, IS1-H and IS6-H), 4.53 (2H, m, $CH_2$), 4.67 (1H, d, J 4.5Hz, IS3-H), 5.13 (1H, m, IS4-H), 5.45 (2H, m, IS5-H and IS2-H), 7.18 (1H, d, J 8.0Hz, Ar-H), 7.26 (1H, t, J 7.5 and 8.0Hz, Ar-H), 7.43 (1H, t, J 8.0 and 7.5Hz, Ar-H), 7.96 (1H, d, J 7.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.76 ($OCOCH_3$), 30.24 ($CH_2$), 68.34 (IS5-H), 68.56 (IS1-H), 69.52 ($CH_2$), 74.91 (IS3-H), 78.30 (IS4-H), 81.84 (IS2-H), 85.40 (IS2-H0, 121.54, 125.48, 131.26, 134.30 (aromatic methine), 163.92 (ArOC(O)Me), 166.40 (C=O), 169.34 (ArC(O)OR).

*4-Nitrooxymethyl-benzoic acid* (**70**)

A solution of α-bromo-*p*-toluic acid (5.0 g, 23 mmol) and silver nitrate (7.90 g, 46.5 mmol) in acetonitrile (50 ml) was allowed to stir at room temperature overnight. The reaction was filtered and the filtrate concentrated *in vacuo*. The oily residues obtained were reconstituted in ethyl acetate and washed with water (2 x 50 ml). After drying over anhydrous $Na_2SO_4$, the ethyl acetate was removed *in vacuo* to afford product (4.3 g) as a white crystalline material (94.90%): m.pt. 150-152°C. $IR_{Vmax}$ (KBr): 1701.4 (C=O), 1680.4 ($NO_2$), 1227.3 ($NO_2$) $cm^{-1}$. $^1H$ NMR δ ($CDCl_3$): 5.52 (2H, s, Bn-$CH_2$), 7.53 (2H, d, J 8.0Hz, 2 x Ar-H), 8.17 (2H, d, J 8.0Hz, 2 x Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 73.51 ($CH_2$), 128.49 (2 x aromatic methine), 130.72 (2 x aromatic methine).

*Isosorbide-2-aspirinate-5-(4-nitrooxymethyl)-benzoate* (**66**)

To a solution of isosorbide-2-aspirinate (**52**) (1.2 g, 4 mmol) in dichloromethane (20 ml) was added DCC (0.82 g, 4 mmol), DMAP (0.05 g, 0.4 mmol) and 4-nitrooxymethyl-benzoic acid (**70**) (0.5 g, 4 mmol). The mixture was stirred at room temperature overnight before filtering and washing the filtrate with HCl (2 x 15 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 15 ml) and water (2 x 15 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford a crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (5:2, 1:1) as eluant yielded product (1.14 g) as a green oil (58.52%). $IR_{Vmax}$ (film): 1769.4 and 1725.3 (C=O), 1633.7 ($NO_2$), 1276.8 ($NO_2$), 1195.9 (C-O-C) $cm^{-1}$. LRMS: Requires: 487.1115 ($M^+$), Found: 487.6332 ($M^+$). $^1H$ NMR δ ($CDCl_3$): 2.37 (3H, s, $OCOCH_3$), 4.36 (4H, m, IS1-H and IS6-H), 4.65 (1H, d, J 4.5Hz, IS3-H), 5.05 (1H, t, J 5.0 and 5.5Hz, IS4-H), 5.44 (2H, t, J 5.0 and 5.5Hz, IS5-H and IS2-H), 5.51 (2H, s, $BnCH_2O$), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.33 (1H, t, J 7.5 and 8.0Hz, Ar-H), 7.51 (2H, d, J 7.5Hz, Ar-H), 7.60 (1H, t, J 8.0 and 7.6Hz, Ar-H), 8.01 (1H, d, J 8.0Hz, Ar-H), 8.13 (2H, d, J 8.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.82 ($OCOCH_3$), 70.77 ($CH_2$), 73.16 (IS1-C), 73.55 (IS6-C), 74.63 (IS3-C), 78.47 (IS4-C), 81.09 (IS5-C), 86.07 (IS2-C), 122.68 (ArC-1), 123.79, 125.98, 131.76 and 134.22 (aromatic methine), 128.49 (2 x aromatic methine), 130.37 (2 x aromatic methine), 137.61 (ArC-2), 150.65 (CO), 163.52 (ArOC(O)Me), 165.10 (C=O), 169.54 (ArC(O)OR).

*Isosorbide-2-aspirinate-5-(3-(2-bromo-acetoxy))-benzoate* (**67**)

To a solution of isosorbide-2-aspirinate-5-salicylate (**37**) (0.15 g, 0.35 mmol) and DBU (0.052 ml, 0.35 mmol) in dichloromethane (5 ml) was added bromoacetyl chloride (0.03 ml, 0.35 mmol) and the reaction mixture was allowed to stir overnight. The reaction was washed with water (2 x 5 ml) and the solvents removed *in vacuo* to yield product as a colourless oil (0.13 g, 67.78%). $IR_{Vmax}$ (film): 1765.6 and 1724.3 (C=O), 1608.1 (C=O), 1288.4 and 1251.4 (C(O)OR), 1196.9 and 1135.6 (C-O-C), 732.6 (C-Br) $cm^{-1}$. LRMS: Requires: 548.0318 ($M^+$), Found: 570.4453 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.36 (3H, s, $OCOCH_3$), 4.07 (4H, m, IS1 and 6-H), 4.48 (2H, s, $CH_2$), 4.63

(1H, d, J 5.0Hz, IS4-H), 4.98 (1H, t, J 5.0 and 5.0Hz, IS3-H), 5.40 (2H, m, IS2-H and IS5-H), 7.11 (1H, d, J 8.0Hz, Ar-H), 7.17 (1H, d, J 8.0Hz, Ar-H), 7.32 (1H, t, J 8.0 and 7.5Hz, Ar-H), 7.39 (1H, t, J 8.0 and 7.5Hz, Ar-H), 7.60 (2H, m, 2 x Ar-H), 8.11 (1H, d, J 1.5Hz, Ar-H), 8.12 (1H, d, J 1.5, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.86 ($OCOCH_3$), 40.99 ($CH_2$), 70.47 (IS1-C), 73.24 (IS6-C), 74.69 (IS4-C), 78.40 (IS3-C), 81.01 (IS5-C), 86.01 (IS2-C), 123.61, 123.83, 126.01, 126.65, 131.78, 132.21, 134.26 and 134.42 (aromatic methine), 150.23 and 150.69 (2 x ArC-2), 163.51 (ArO<u>C</u>(O)Me), 166.11 (Ar<u>C</u>(O)$OCH_2Br$), 169.55 (ArC(O)OR).

*Methyl-(2-nitrooxy-ethyl)-amine* (**80**)

To a stirring solution of fuming nitric acid (18.87 ml) and dichloromethane (187.5 ml) cooled to –10°C was added drop wise over 20 minutes, a solution of methylamino ethanol (5 g, 66.6 mmol) in dichloromethane (37.5 ml). After stirring for 15 minutes the solution was treated with acetic anhydride (22.5 ml) and the solution was stirred for a further 15 minutes, after which time product precipitated out as a white crystalline material (4.7 g, 58.80%) [301]: m.pt. 58-60°C. $IR_{Vmax}$ (KBr): 1634.2 ($NO_2$), 1282.0 ($NO_2$), 870.0 ($ONO_2$) $cm^{-1}$. LRMS: Requires 120.0535 ($M^+$), Found: 241.1139 ($2M^+$). $^1H$ NMR δ ($D_2O$): 2.79 (3H, s, $CH_3$), 3.50 (2H, t, J 4.5 and 4.5Hz, $CH_2$), 4.86 (2H, t, J 4.5 and 4.5Hz, $CH_2$). $^{13}C$ NMR ppm ($D_2O$): 35.12 ($CH_3$), 48.17 (N-$CH_2$), 70.12 (ONO-$CH_2$).

*2-[Methyl-(2-nitrooxy-ethyl)-amino]-ethanol* (**85**)

To a solution of methyl-(2-nitrooxy-ethyl)-amine (**80**) (1 g, 8.3 mmol) in KOH (12 ml, 7 M) was added bromoalcohol (2.6 g, 20.8 mmol) and after three days stirring at room temperature the reaction mixture was washed with methanol and the solvent removed *in vacuo*. The residue was columned on silica gel, eluting with ethyl acetate and hexane (1:2) to give the product as a colourless oil (0.37 g, 27.18%). $IR_{Vmax}$ (film): 3420.0 (OH), 1293.4 ($NO_2$), 1124.8 (C-OH), 874.0 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 187.0674 ($M^+$+23), Found: 187.1210 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 3.35 (3H, s, $CH_3$),

3.46 (2H, t, J 4.5 and 4.5Hz, $CH_2$), 3.60 (2H, t, J 5.0 and 4.0Hz, $CH_2$), 3.65 (2H, t, J 5.5 and 5.0Hz, $CH_2$), 3.90 (2H, t, J 5.0 and 5.0Hz, $CH_2$). $^{13}C$ NMR ppm ($CDCl_3$): 39.21 ($CH_3$), 51.86 ($CH_2$), 60.84 ($CH_2$), 66.71 ($CH_2$), 71.78 ($CH_2$).

*2-Acetoxy-benzoic acid 2-[methyl-(2-nitrooxy-ethyl)-amino]-ethyl ester* (**75**)

A solution of 2-[methyl-(2-nitrooxy-ethyl)-amino]-ethanol (**85**) (0.25 g, 1.52 mmol) in dichloromethane (5 ml), cooled to –5°C, was treated with triethylamine (0.23 ml, 2.29 mmol) and acetylsalicyloyl chloride (0.30 g, 1.52 mmol). After stirring overnight the reaction mixture was washed with $Na_2HCO_3$ (2 x 10 ml) and water (2 x 10 ml), dried over anhydrous $MgSO_4$ and the solvents removed *in vacuo*. The resulting yellow oil was purified by column chromatography eluting with ethyl acetate and hexane (1:2) to yield product as a colourless oil (0.20 g, 40.36%). $IR_{Vmax}$ (film): 1759.0 and 1722.3 (C=O), 1607.1 ($NO_2$), 1287.7 ($NO_2$), 1266.8 (C(O)OR, aromatic), 1193.5 (C-O-C), 910.8 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 349.0991 ($M^+$+23), Found: 349.2518 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.35 (3H, s, $OCOCH_3$), 3.44 (3H, s, $CH_3$), 3.77 (4H, t, J 5.5 and 5.0Hz, 2 x $CH_2$), 4.43 (2H, t, J 5.0 and 5.0Hz, $CH_2$), 4.91 (2H, s, $CH_2$), 7.13 (1H, d, J 7.5Hz, Ar-H), 7.34 (1H, t, J 7.5 and 7.6Hz, Ar-H), 7.59 (1H, t, J 8.0 and 7.5Hz, Ar-H), 8.04 (1H, d, J 8.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.99 ($OCOCH_3$), 40.21 ($CH_3$), 52.71 ($CH_2$), 63.73 ($CH_2$), 67.77 ($CH_2$), 69.14 ($CH_2$), 70.91 ($CH_2$), 123.85, 126.05, 131.67 and 134.05 (aromatic methine), 150.80 (CO), 164.21 (ArO<u>C</u>(O)Me), 166.64 (C=O), 169.54 (ArC(O)OR).

*Ethyl-(2-nitrooxy-ethyl)-amine* (**81**)

To a stirring solution of fuming nitric acid (18.87 ml) and dichloromethane (187.5 ml) cooled to –10°C, was added dropwise over 20 minutes, a solution of 2-(ethylamino) ethanol (5 g, 56 mmol) in dichloromethane (37.5 ml). After stirring for 15 minutes the solution was treated with acetic anhydride (22.5 ml) and the solution was stirred for a further 15 minutes, after which time product precipitated out as a white crystalline material (4.5 g, 59.96%) [301]. m.pt. 40-42°C. $IR_{Vmax}$ (KBr) $cm^{-1}$: 1640.2 ($NO_2$), 1281.1

($NO_2$), 874.3 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 134.0691 ($M^+$), Found: 269.2537 ($2M^+$). $^1H$ NMR δ ($D_2O$): 1.27 (3H, t, J 7.5 and 7.0Hz, $CH_3$), 3.14 (2H, t, J 7.3 and 6.0Hz, $CH_2$), 3.45 (2H, m, $CH_2$), 4.80 (2H, s, $CH_2$). $^{13}C$ NMR ppm ($D_2O$): 9.93 ($CH_3$), 42.87 ($CH_2$), 43.70 ($CH_2$), 67.70 ($CH_2$).

*2-[Ethyl-(2-nitrooxy-ethyl)-amino]-ethanol* (**86**)

To a solution of ethyl-(2-nitrooxy-ethyl)-amine (**81**) (1 g, 7.5 mmol) in KOH (12 ml, 7 M) was added bromoalcohol (2.33 g, 18.6 mmol) and after three days stirring at room temperature the reaction mixture was washed with methanol and the solvents removed *in vacuo*. The residue was columned on silica gel, eluting with ethyl acetate and hexane (1:2) to afford the product as an oil (0.45 g, 33.70%). $IR_{Vmax}$ (film): 3426.7 (OH), 1285.1 ($NO_2$), 1126.4 (C-OH) $cm^{-1}$. LRMS: Requires: 201.0824 ($M^+$+23), Found: 201.0849 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 1.08 (3H, m, $CH_3$), 3.42 (2H, t, J 4.5 and 5.0Hz, $CH_2$), 3.56 (2H, t, J 5.0 and 4.5Hz, $CH_2$), 3.61 (2H, t, J 5.0 and 5.0Hz, $CH_2$), 3.71 (3H, q, J 7.0, 7.0 and 7.5Hz, $CH_2$), 3.80 (2H, t, J 5.0 and 5.0Hz, $CH_2$). $^{13}C$ NMR ppm ($CDCl_3$): 10.50 ($CH_3$), 47.06 ($CH_2$), 50.46 ($CH_2$), 60.87 ($CH_2$), 67.04 ($CH_2$), 71.95 ($CH_2$).

*2-Acetoxy-benzoic acid 2-[ethyl-(2-nitrooxy-ethyl)-amino]-ethyl ester* (**76**)

A solution of 2-[ethyl-(2-nitrooxy-ethyl)-amino]-ethanol (**86**) (0.21 g, 1.2 mmol) in dichloromethane (5 ml), cooled to –5°C, was treated with triethylamine (0.18 g, 1.8 mmol) and acetylsalicyloyl chloride (0.23 g, 1.2 mmol). After stirring overnight the reaction mixture was washed with aqueous $Na_2HCO_3$ (2 x 10 ml) and water (2 x 10 ml), dried over anhydrous $MgSO_4$ and the solvents removed *in vacuo*. The resulting yellow oil (0.23 g) was purified by column chromatography eluting with ethyl acetate and hexane (1:2) to yield product as a colourless oil (0.13 g, 31.86%). $IR_{Vmax}$ (film): 1774.1 and 1748.2 (C=O), 1611.4 ($NO_2$), 1256.2 (C(O)OR, aromatic), 1183.2 (C-O-C) $cm^{-1}$. LRMS: Requires: 363.1045 ($M^+$), Found: 363.1980 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 1.26 (3H, m, $CH_3$), 2.37 (3H, s, $OCOCH_3$), 3.77 (4H, m, 2 x $CH_2$), 3.83 (2H,

q, J 7.0, 7.0 and 7.0Hz, $CH_2$), 3.93 (2H, t, J 5.0 and 5.0Hz, $CH_2$), 4.43 (2H, t, J 4.5 and 5.0Hz, $CH_2$), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.34 (1H, t, J 7.5 and 7.0Hz, Ar-H), 7.59 (1H, t, J 8.0 and 7.5Hz, Ar-H), 8.02 (1H, d, J 7.5Hz, Ar-H ). $^{13}C$ NMR ppm ($CDCl_3$): 11.33 ($CH_3$), 20.91 ($OCOCH_3$), 47.95 ($CH_2$), 51.17 ($CH_2$), 63.75 ($CH_2$), 67.84 ($CH_2$), 69.11 ($CH_2$), 123.83, 125.98, 131.66 and 134.01 (aromatic methine), 150.79 (CO), 164.18 (ArOC(O)Me), 169.51 (ArC(O)OR).

*(2-Nitrooxy-ethyl)-propyl-amine* (**82**)

To a stirring solution of fuming nitric acid (12.59 ml) and dichloromethane (125 ml) cooled to –10°C, was added dropwise over 20 minutes, a solution of 2-(propylamino) ethanol (5 g, 48 mmol) in dichloromethane (25 ml). After stirring for 15 minutes the solution was treated with acetic anhydride (15 ml) and the solution was stirred for a further 15 minutes, after which time product was precipitated out as a green oil (5.58 g, 78.55%). $IR_{Vmax}$ (film): 1641.0 ($NO_2$), 1281.2 ($NO_2$) $cm^{-1}$. LRMS: Requires: 148.0848 ($M^+$), Found: 297.3297 ($2M^+$). $^1H$ NMR δ ($D_2O$): 3.48 (3H, t, J 7.0 and 7.6Hz, $CH_3$), 4.23 (2H, s, $CH_2$), 5.57 (2H, d, J 6.0Hz, $CH_2$), 5.99 (2H, s, $CH_2$), 7.35 (2H, s, $CH_2$). $^{13}C$ NMR ppm ($D_2O$): 12.19 ($CH_3$), 21.17 ($CH_2$), 46.64 ($CH_2$), 51.74 ($CH_2$), 70.29 ($CH_2$).

*2-[propyl-(2-Nitrooxy-ethyl)-amino]-ethanol* (**87**)

To a solution of (2-nitrooxy-ethyl)-propyl-amine (**82**) (1 g, 6.1 mmol) in KOH (12 ml, 7 M) was added bromo alcohol (1.9 g, 15 mmol) and after three days stirring at room temperature the reaction mixture was washed with methanol and the solvent removed *in vacuo*. The residue was columned on silica gel, eluting with ethyl acetate and hexane (1:2) to yield product as an oil (0.51 g, 43.55%). $IR_{Vmax}$ (KBr): 3425.6 (OH), 1288.1 ($NO_2$), 1125.4 (C-OH) $cm^{-1}$. LRMS: Requires: 215.0987 ($M^+$+23), Found: 215.0974 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 0.95 (3H, t, J 7.5 and 7.0Hz, $CH_3$), 1.72 (2H, m, $CH_2$), 3.57 (2H, t, J 4.5 and 5.0Hz, $CH_2$), 3.75 (6H, m, 3 x $CH_2$), 3.95 (2H, t, J 5.0

and 5.0Hz, $CH_2$). $^{13}C$ NMR ppm ($CDCl_3$): 10.69 ($CH_3$), 19.50 ($CH_2$), 51.99 ($CH_2$), 54.02 ($CH_2$), 61.34 ($CH_2$), 67.26 ($CH_2$), 72.12 ($CH_2$).

*2-Acetoxy-benzoic acid 2-[(2-nitrooxy-ethyl)-propyl-amino]-ethyl ester* (**77**)

A solution of 2-[propyl-(2-nitrooxy-ethyl)-amino]-ethanol (**87**) (0.39 g, 2 mmol) in dichloromethane (5 ml), cooled to –5°C, was treated with triethylamine (0.31 g, 3.05 mmol) and acetylsalicyloyl chloride (0.40 g, 2 mmol). After stirring overnight the reaction mixture was washed with aqueous $Na_2HCO_3$ (2 x 10 ml) and water (2 x 10 ml), dried over anhydrous $MgSO_4$ and the solvents removed *in vacuo*. The crude oil was purified by column chromatography eluting with ethyl acetate and hexane (1:2) to yield product as a colourless oil (0.18 g, 25.42%). $IR_{Vmax}$ (film): 1763.1 and 1724.8 (C=O), 1632.4 ($NO_2$), 1283.1 ($NO_2$), 1132.1 (C-O-C), 896.2 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 377.1304 ($M^+$+23), Found: 377.2534 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 0.89 (3H, t, J 7.5 and 7.0Hz, $CH_3$), 2.23 (2H, t, J 7.5 and 7.6Hz, $CH_2$), 2.34 (3H, s, $OCOCH_3$), 3.77 (6H, m, 3 x $CH_2$), 3.93 (2H, t, J 5.0 and 5.0Hz, $CH_2$), 4.42 (2H, t, J 4.5 and 5.0Hz, $CH_2$), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.33 (1H, t, J 7.5 and 7.0Hz, Ar-H ), 7.56 (1H, t, J 8.3 and 7.3Hz, Ar-H), 8.01 (1H, d, J 7.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 10.91 ($CH_3$), 19.72 ($CH_2$), 20.87 ($OCOCH_3$), 51.57 ($CH_2$), 54.45 ($CH_2$), 63.71 ($CH_2$), 67.69 ($CH_2$), 69.04 ($CH_2$), 123.77, 125.94, 131.61 and 133.94 (aromatic methine), 150.73 (CO), 164.16 (ArOC(O)Me), 169.47 (ArC(O)OR).

*Bis-(2-nitrooxy-ethyl)-amine* (**83**)

To a stirring solution of fuming nitric acid (2.5 ml) and dichloromethane (25 ml) cooled to –10 °C, was added dropwise over 20 minutes, a solution of diethanolamine (1.05 g, 10 mmol) in dichloromethane (5 ml). After stirring for 15 minutes the solution was treated with acetic anhydride (3 ml) and the solution was stirred for a further 15 minutes, after which time product precipitated out as a white crystalline material (0.78 g, 40.21%) [302]. m.pt. 110-112°C. $IR_{Vmax}$ (KBr): 1648.6 ($NO_2$), 1275.1 ($NO_2$), 925.9 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 194.0413 ($M^+$), Found: 196.0627 ($M^+$). $^1H$ NMR δ

($D_2O$): 3.53 (4H, s, 2 x $CH_2$), 4.82 (4H, s, 2 x $CH_2$). $^{13}C$ NMR ppm ($D_2O$): 44.62 (2 x $CH_2$), 67.46 (2 x $CH_2$).

*2-[Bis-(2-nitrooxy-ethyl)-amino]-ethanol* (**88**)

To a solution of bis-(2-nitrooxy-ethyl)-amine (**83**) (0.85 g, 4.35 mmol) in KOH (5 ml, 7 M) was added bromo alcohol (1.25 g, 10 mmol) and after three days stirring at room temperature the reaction mixture was washed with methanol and the solvents removed *in vacuo*. The residue was chromatographed on silica gel, eluting with ethyl acetate and hexane (1:2) to yield product as a yellow oil (0.27 g, 25.97%). $IR_{Vmax}$ (film): 3409.7 (OH), 1631.9 ($NO_2$), 1288.4 ($NO_2$), 1125.6 (C-O) $cm^{-1}$. LRMS: Requires: 262.0632 ($M^+$+23), Found: 262.0413 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 3.58 (2H, t, J 4.0 and 3.5Hz, $CH_2$), 3.71 (2H, t, J 4.5 and 4.5Hz, $CH_2$), 3.79 (2H, t, J 5.5 and 6.0Hz, $CH_2$), 4.25 (2H, t, J 5.5 and 6.0Hz, $CH_2$), 4.65 (2H, m, $CH_2$), 4.86 (2H, m, $CH_2$). $^{13}C$ NMR ppm ($CDCl_3$): 49.65 ($CH_2$), 52.36 ($CH_2$), 61.30 ($CH_2$), 67.29 ($CH_2$), 68.67 ($CH_2$), 72.17 ($CH_2$).

*2-Acetoxy-benzoic acid 2-[bis-(2-nitrooxy-ethyl) amino]-ethyl ester* (**78**)

A solution of 2-[bis-(2-nitrooxy-ethyl)-amino]-ethanol (**88**) (0.07 g, 0.29 mmol) in dichloromethane (5 ml), cooled to –5°C, was treated with triethylamine (0.04 g, 0.4 mmol) and acetylsalicyloyl chloride (0.05 g, 0.27 mmol). After stirring overnight the reaction mixture was washed with aqueous $Na_2HCO_3$ (2 x 10 ml) and water (2 x 10 ml), dried over anhydrous $MgSO_4$ and the solvents removed *in vacuo*. The crude oil was purified by column chromatography eluting with ethyl acetate and hexane (1:2) to yield product as a colourless oil (0.04 g, 45.8%). $IR_{Vmax}$ (film): 1781.4 and 1734.1 (C=O), 1629.8 ($NO_2$), 1261.4 (C(O)OR, aromatic), 1154.2 (C-O-C), 942.1 ($ONO_2$) $cm^{-1}$. LRMS: Requires: 424.0845 ($M^+$), Found: 424.0713 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.35 (3H, s, $OCOCH_3$), 3.77 (4H, m, 2 x $CH_2$), 4.00 (2H, t, J 5.0 and 5.4Hz, $CH_2$), 4.09 (2H, t, J 5.5 and 5.0Hz, $CH_2$), 4.45 (2H, t, J 4.5 and 4.5Hz, $CH_2$), 4.67 (2H, t, J 5.0 and 5.5Hz, $CH_2$), 7.13 (1H, d, J 8.6Hz, Ar-H), 7.34 (1H, t, J 8.5 and 7.0Hz, Ar-

H), 7.60 (1H, t, J 8.0 and 7.5Hz, Ar-H), 8.02 (1H, d, J 8.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.92 ($OCOCH_3$), 49.64 ($CH_2$), 52.22 ($CH_2$), 63.05 ($CH_2$), 67.27 ($CH_2$) and 68.67 (2 x $CH_2$), 123.82, 126.03, 131.52 and 134.10 (aromatic methine), 164.13 (ArOCO(Me)), 169.45 (ArC(O)OR).

*1-Benzyloxy-3-hydroxy-propan-2-one* (**94**)

A solution of glycolic acid (15.04 g, 0.2 M) and cesium carbonate (31.4 g, 96 mmol) in a methanol and water mixture (1:1, 350 ml) was stirred at room temperature for 30 minutes before removing solvents *in vacuo*. The cesium salt obtained (**93**) as a white crystalline material (35 g, 0.2 M) was stirred in DMF (200 ml) and treated with benzyl bromide (23.5 ml, 0.2 M). The mixture was stirred at room temperature for four hours before diluting in water (200 ml) and extracting into diethyl ether (3 x 200 ml). The solvent was dried over anhydrous $Na_2SO_4$ and removed *in vacuo* to yield product as crude oil, which was purified by column chromatography over silica gel using hexane and ethyl acetate (5:2) as eluant, to afford product (18 g) as a green oil (54.22%) [303]. $IR_{Vmax}$ (film): 3434.2 (OH), 1743.3 (C=O), 1209.2 (C-O), 1094.0 (C-O) $cm^{-1}$. LRMS: Requires: 189.0423 ($M^+$+23), Found: 189.0524 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 4.23 (2H, s, $CH_2$), 5.26 (2H, s, $OCH_2CO$), 7.38 (5H, s, ArH x 5). $^{13}C$ NMR ppm ($CDCl_3$): 60.58 ($CH_2$), 67.03 ($OCH_2O$), 128.31 (2 x Ar-C), 128.48 (Ar-C), 128.55 (2 x Ar-C), 135.05 (CO), 173.10 (C=O).

*2-Acetoxy-benzoic acid benzyloxycarbonyl methyl ester* (**95**)

To a solution of 1-benzyloxy-3-hydroxy-propan-2-one (**94**) (16.72 g, 0.1 M) in toluene (200 ml) was added triethylamine (12.14 g, 0.12 M) and acetylsalicyloyl chloride (20g, 0.1 M). The mixture was allowed to stir at room temperature for 48 hours before washing with saturated aqueous $Na_2HCO_3$ (2 x 100 ml), dilute HCl (2 x 100 ml, 0.1 M) and water (2 x 100 ml). After drying over anhydrous $Na_2SO_4$ the toluene was removed *in vacuo* to afford product (20 g) as white crystalline material (60.98%): m.pt. 68-70$^0$C. $IR_{Vmax}$ (KBr): 1759.8 and 1723.4 (C=O), 1198.4 (C(O)C), 1096.2 (C-O-C) $cm^{-1}$. LRMS: Requires: 351.4085 ($M^+$+23), Found: 350.9534 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$):

2.35 (3H, s, $OCOCH_3$), 4.86 (2H, s, $OCH_2CO$), 5.23 (2H, s, $OCH_2Bn$), 7.15 (1H, d, J 8.2Hz, Ar-H), 7.35 (1H, m, Ar-H), 7.38 (5H, m, Ar-H), 7.61 (1H, t, J 8.2 and 7.6Hz, Ar-H), 8.11 (1H, d, J 7.6Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.86 ($OCOCH_3$), 64.61 ($OCH_2CO$), 70.83 ($OCH_2Bn$), 123.64, 126.17, 128.42, 131.63 and 134.28 (aromatic methine), 128.21 and 128.58 (2 x Ar-C), 151.21 (CO), 168.71 (ArOC(O)Me), 169.32 (ArC(O)OR), 172.48 (C=O).

*2–Acetoxy–benzoic acid carboxymethyl ester* (**91**)

A solution of 2-acetoxy-benzoic acid benzyloxycarbonylmethyl ester (**95**) (20 g, 60.8 mmol) in a methanol and ethyl acetate mixture (1:1, 200 ml) was treated with a catalytic amount of palladium on charcoal and stirred at room temperature, under an atmosphere of hydrogen, for 48 hours. The reaction was filtered through a bed of silica and the filtrate concentrated *in vacuo* to afford product as crude oil, which was purified by column chromatography over silica gel using hexane and ethyl acetate (5:2, 3:2) as eluant to yield product (16 g) as white crystalline material (98.86%) [304]: m.pt. 106-108°C. $IR_{Vmax}$ (KBr): 1752.1 and 1726.1 (C=O), 1246.9 (C(O)OR, aromatic), 1095.7 (C-O-C) $cm^{-1}$. LRMS: Requires: 261.0354 ($M^+$+23), Found: 260.9449 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.35 (3H, s, $OCOCH_3$), 4.84 (2H, s, $CH_2$), 7.13 (1H, d, J 8.0Hz, Ar-H), 7.32 (1H, t, J 7.5 and 7.5Hz, Ar-H), 7.60 (1H, t, J 8.0 and 7.5Hz, Ar-H), 8.09 (1H, d, J 8.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.88 ($OCOCH_3$), 60.64 ($CH_2$), 123.84, 126.07, 131.93 and 134.38 (aromatic methine), 150.85 (CO), 163.58 (ArOC(O)Me), 169.92 (ArC(O)OR), 172.68 (C=O).

*2-Acetoxy-benzoic acid chlorocarbonylmethyl ester* (**88**)

A solution of 2-acetoxy-benzoic acid carboxymethyl ester (**91**) (1 g, 4.2 mmol) at 0°C, under an atmosphere of nitrogen was treated with oxalyl chloride (0.73 ml, 8.4 mmol). The mixture was stirred at 0°C for two hours before removing excess oxalyl chloride and dichloromethane *in vacuo* to afford product (0.8 g) as a white crystalline material (74.40%): m.pt. 110-112°C. $IR_{Vmax}$ (KBr): 1752.0 and 1726.9 (C=O), 1247.0

(C(O)OR, aromatic), 1096.0 (C-O-C), 759.6 (C-Cl) $cm^{-1}$. LRMS: Requires: 279.0016 ($M^+$+23), Found: 279.0126 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.36 (3H, s, $OCOCH_3$), 3.12 (2H, s, $CH_2$), 7.37 (1H, d, J 8.6Hz, Ar-H), 7.65 (1H, t, J 7.5 and 7.5Hz, Ar-H), 8.09 (1H, t, J 7.6 and 8.0Hz, Ar-H), 8.56 (1H, d, J 8.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.91 ($OCOCH_3$), 67.36 ($CH_2$), 124.07, 126.13, 131.90 and 134.82 (aromatic methine), 151.19 (CO), 168.61 (ArOC(O)Me), 169.42 (ArC(O)OR).

*2-Acetoxy-benzoic acid diethylcarbamoyl methyl ester* (**24**)

To a solution of diethylamine (0.057 g, 0.78 mmol) in dry dichloromethane (10 ml) at 0°C was added triethylamine (0.22 ml, 1.56 mmol) and 2-acetoxy-benzoic acid chlorocarbonylmethyl ester (**88**) (0.20 g, 0.78 mmol). The mixture was stirred at room temperature for two hours before washing with NaOH (2 x 10 ml, 10%) and extracting the aqueous phase with chloroform (2 x 10 ml) and dichloromethane (2 x 10 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (4:2) as eluant yielded product (0.11 g) as a colourless oil (48.13%) [96]. LRMS: Requires: 316.1143 ($M^+$+23), Found: 316.1107 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 1.01 (6H, m, 2 x $CH_3$), 2.34 (4H, m, 2 x $CH_2$), 4.90 (2H, s, $OCH_2CO$), 7.11 (1H, d, J 8.5Hz, Ar-H), 7.32 (1H, t, J 7.0 and 8.5Hz, Ar-H), 7.56 (1H, t, J 7.8 and 7.8Hz, Ar-H), 8.15 (1H, d, J 8.0Hz, Ar-H).

*2-Acetoxy-benzoic acid-[methyl-(2-nitrooxy-ethyl)-amino]-ethyl ester* (**96**)

To a solution of methyl-(2-nitrooxy-ethyl)-amine (**80**) (0.29 g, 1.6 mmol) in dry dichloromethane (10 ml) at 0°C was added triethylamine (0.65 ml, 4.75 mmol) and 2-acetoxy-benzoic acid chlorocarbonylmethyl ester (**88**) (0.40 g, 1.6 mmol). The mixture was stirred at room temperature for two hours before washing with NaOH (2 x 10 ml, 10%) and extracting the aqueous phase with chloroform (2 x 10 ml) and dichloromethane (2 x 10 ml). After drying over anhydrous $Na_2SO_4$ the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by

column chromatography over silica gel using hexane and ethyl acetate (4:2) as eluant yielded product (0.21 g) as a yellow oil (38.60%). $IR_{Vmax}$ (film): 1753.1 and 1731.4 (C=O), 1611.3 (CON), 1354.2 and 1298.6 (C-O), 1191.2 (C(O)OR, aromatic) $cm^{-1}$. $^1H$ NMR δ ($CDCl_3$): 2.34 (3H, s, $OCOCH_3$), 3.07 (3H, s, $CH_3$), 3.70 (2H, t, J 5.5 and 5.0Hz, $CH_2$), 4.62 (2H, t, J 5.0 and 5.0Hz, $CH_2$), 4.91 (2H, s, $OCH_2CO$), 7.12 (1H, d, J 7.5Hz, Ar-H), 7.33 (1H, t, J 7.5 and 7.6Hz, Ar-H), 7.58 (1H, t, J 8.0 and 7.5Hz, Ar-H), 8.12 (1H, d, J 8.0Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.88 ($OCOCH_3$), 35.69 ($CH_3$), 46.09 ($CH_2$), 61.61 ($CH_2$), 70.91 ($OCH_2CO$), 123.72, 125.98, 131.92 and 134.17 (aromatic methine), 150.77 (CO), 163.80 (ArOC(O)Me)), 166.64 (CON), 169.57 (ArC(O)OR).

*2-Acetoxy-benzoic acid-[ethyl-(2-nitrooxy-ethyl)-amino]-ethyl ester* (**97**)

To a solution of ethyl-(2-nitrooxy-ethyl)-amine (**81**) (0.15 g, 0.8 mmol) in dry dichloromethane (10 ml) at 0°C was added triethylamine (0.33 ml, 2 mmol) and 2-acetoxy-benzoic acid chlorocarbonylmethyl ester (**88**) (0.20 g, 0.8 mmol). The mixture was stirred at room temperature for two hours before washing with NaOH (2 x 10 ml, 10%) and extracting the aqueous phase with chloroform (2 x 10 ml) and dichloromethane (2 x 10 ml). After drying over anhydrous $Na_2SO_4$ the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (4:1) as eluant yielded product (0.14 g) as a yellow oil (49.44%). $IR_{Vmax}$ (film): 1783.2 and 1761.4 (C=O), 1642.1 (CON), 1396.1 and 1331.2 (C-O), 1204.3 (C(O)OR, aromatic) $cm^{-1}$. LRMS: Requires: 354.1063 ($M^+$), Found: 354.2938 ($M^+$). $^1H$ NMR δ ($CDCl_3$): 1.28 (3H, m, $CH_3$), 2.37 (3H, s, $OCOCH_3$), 3.40 (2H, q, J 7.0, 7.0 and 7.5Hz, $CH_2$), 3.68 (2H, t, J 5.0 and 5.5Hz, $CH_2$), 4.67 (2H, t, J 5.0 and 5.5Hz, $CH_2$), 4.95 (2H, s, $OCH_2CO$), 7.14 (1H, d, J 8.0Hz, Ar-H), 7.34 (1H, t, J 7.6 and 8.0Hz, Ar-H), 7.60 (1H, t, J 8.3 and 7.3Hz, Ar-H), 8.15 (1H, d, J 7.3Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 14.01 ($CH_3$), 20.94 ($OCOCH_3$), 43.26($CH_2$), 43.90 ($CH_2$), 61.46 ($CH_2$), 70.75 ($OCH_2CO$), 123.73, 125.99, 131.98 and 134.18 (aromatic methine), 163.47 (ArOC(O)Me), 166.51 (CON), 169.67 (ArC(O)OR).

*2-Acetoxy-benzoic acid-[propyl-(2-nitrooxy-ethyl)-amino]-ethyl ester* (**98**)

To a solution of (2-nitrooxy-ethyl)-propyl-amine (**82**) (0.33 g, 1.56 mmol) in dry dichloromethane (10 ml) at 0°C was added triethylamine (0.65 ml, 4.68 mmol) and 2-acetoxy-benzoic acid chlorocarbonylmethyl ester (**88**) (0.40 g, 1.56 mmol). The mixture was stirred at room temperature for two hours before washing with NaOH (2 x 10 ml, 10%) and extracting the aqueous phase with chloroform (2 x 10 ml) and dichloromethane (2 x 10 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (4:1) as eluant yielded product (0.27 g) as a yellow oil (47.03%). $IR_{Vmax}$ (film): 1764.2 and 1738.0 (C=O), 1607.1 (CON), 1198.7 (C(O)OR, aromatic) $cm^{-1}$. $^1H$ NMR δ ($CDCl_3$): 0.88 (3H, t, J 7.0 and 7.6Hz, $CH_3$), 1.59 (2H, q, J 7.5, 7.5 and 7.5Hz, $CH_2$), 2.27 (3H, s, $OCOCH_3$), 3.18 (2H, t, J 8.0 and 7.5Hz, $CH_2$), 3.59 (2H, t, J 5.5 and 5.0Hz, $CH_2$), 4.57 (2H, t, J 5.0 and 5.5Hz, $CH_2$), 4.85 (2H, s, $OCH_2CO$), 7.04 (1H, d, J 8.0Hz, Ar-H), 7.26 (1H, t, J 8.5 and 8.0Hz, Ar-H), 7.51 (1H, t, J 8.0 and 7.5Hz, Ar-H), 8.05 (1H, d, J 4.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 11.02 ($CH_3$), 20.98 ($OCOCH_3$), 22.03 ($CH_2$), 44.18 ($CH_2$), 50.20 ($CH_2$), 61.43 ($CH_2$), 70.72 ($OCH_2CO$), 123.73, 126.04, 131.99 and 134.23 (aromatic methine), 150.80 (CO), 163.87 (ArOC(O)Me)), 166.64 (CON), 169.71 (ArC(O)OR).

*2-Acetoxy-benzoic acid [bis-(2-nitrooxy-ethyl)-carbamoyl]-methyl ester* (**99**)

To a solution of bis-(2-nitrooxy-ethyl)-amine (**83**) (0.20 g, 0.8 mmol) in dry dichloromethane (10 ml) at 0°C was added triethylamine (0.33 ml, 2.30 mmol) and 2-acetoxy-benzoic acid chlorocarbonylmethyl ester (**88**) (0.20 g, 0.8 mmol). The mixture was stirred at room temperature for two hours before washing with NaOH (2 x 10 ml, 10%) and extracting the aqueous phase with chloroform (2 x 10 ml) and dichloromethane (2 x 10 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (5:2) as eluant

yielded product (0.12 g) as a colourless oil (36.14%). $IR_{Vmax}$ (film): 1765.4 and 1732.3 (C=O), 1641.2 and 1639.8 (CON), 1211.5 (C(O)OR, aromatic) $cm^{-1}$. $^1H$ NMR δ ($CDCl_3$): 2.35 (3H, s, $OCOCH_3$), 3.74 (4H, t, J 5.5 and 5.0Hz, 2 x $CH_2$), 4.66 (4H, m, 2 x $CH_2$), 4.95 (2H, s, $OCH_2O$), 7.14 (1H, d, J 8.0Hz, Ar-H), 7.36 (1H, t, J 8.0 and 8.0Hz, Ar-H), 7.61 (1H, t, J 7.5 and 7.6Hz, Ar-H), 8.12 (1H, d, J 7.8Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 15.21 ($OCOCH_3$), 45.02 and 45.68 (2 x $CH_2$), 53.37 ($OCH_2O$), 69.69 and 70.61 (2 x $CH_2$), 123.82, 126.09, 131.91 and 134.40 (aromatic methine), 150.87 (CO), 163.87 (ArOC(O)Me), 167.11 (CON), 169.66 (ArC(O)OR).

*2-Acetoxy-benzoic acid 2-bromo-ethyl ester* (**103**)

To a solution of bromoalcohol (1 g, 8 mmol) in dichloromethane (25 ml) at 0°C, was added triethylamine (1.12 ml, 12 mmol) and acetylsalicyloyl chloride (1.59 g, 8 mmol). The mixture was stirred at room temperature overnight before washing with HCl (2 x 25 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (4:1) as eluant yielded product (1.63 g) as a yellow oil (71.49%) [305]. $IR_{Vmax}$ (film): 1766.7 and 1727.0 (C=O), 1195.3 (C(O)O), 1088.3 (C-O-C), 704.5 (C-Br) $cm^{-1}$. LRMS: Requires: 308.9738 ($M^+$+23), Found: 308.9745 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.37 (3H, s, $OCOCH_3$), 3.62 (2H, t, J 6.5 and 6.0Hz, $CH_2$), 4.59 (2H, t, J 6.0 and 6.5Hz, $CH_2$), 7.13 (1H, d, J 8.0Hz, Ar-H), 7.34 (1H, t, J 7.5 and 7.5Hz, Ar-H), 7.59 (1H, t, J 8.8 and 6.7Hz, Ar-H), 8.07 (1H, d, J 7.8Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.95 ($OCOCH_3$), 28.43 ($CH_2$), 64.29 ($CH_2$), 125.98, 129.83, 131.78 and 134.11 (aromatic methine), 158.80 (CO), 163.57 (ArC(O)OR).

*2-Acetoxy-benzoic acid 2-nitrooxy-ethyl ester* (**100**)

A solution of 2-acetoxy-benzoic acid 2-bromo-ethyl ester (**103**) (1.53 g, 6.8 mmol) and silver nitrate (2.31 g, 13.6 mmol) in acetonitrile (25 ml) was allowed to stir at room temperature overnight. The reaction was filtered and the filtrate concentrated *in vacuo*.

The oily residues obtained were reconstituted in ethyl acetate and washed with water (2 x 20 ml). After drying over anhydrous $Na_2SO_4$, the ethyl acetate was removed in vacuo to afford product as crude oil, which was purified by column chromatography over silica gel using hexane and ethyl acetate (5:2) as eluant to yield 1.21 g of product as a yellow oil (66.15%) [306]. $IR_{Vmax}$ (film): 1767.9 and 1728.5 (C=O), 1637.4 ($NO_2$), 1278.4 (ArC(O)OR), 1204.3 ($NO_2$), 1196.3 (C(O)O) $cm^{-1}$. LRMS: Requires: 292.0433 ($M^+$+23), Found: 292.0430 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.37 (3H, s, $OCOCH_3$), 4.58 (2H, t, J 6.0 and 5.3Hz, $CH_2$), 4.78 (2H, t, J 4.5 and 4.5Hz, $CH_2$), 7.14 (1H, d, J 7.5Hz, Ar-H), 7.35 (1H, t, J 7.5 and 7.0Hz, Ar-H), 7.61 (1H, t, J 8.5 and 6.7Hz, Ar-H), 8.03 (1H, d, J 7.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.90 ($OCOCH_3$), 60.77 ($CH_2$), 76.67 ($CH_2$), 123.89, 126.07, 131.80 and 134.34 (aromatic methine), 150.80 (CO), 163.97 (ArOC(O)Me) and 169.54 (ArC(O)OR).

*2-Acetoxy-benzoic acid 1-methyl-2-bromo-ethyl ester* (**104**)

To a solution of 1-bromo-2-propanol (1 g, 7.2 mmol) in dichloromethane (30 ml) at 0°C, was added triethylamine (1.51 ml, 10.8 mmol) and acetylsalicyloyl chloride (1.43 g, 7.2 mmol). The mixture was stirred at room temperature overnight before washing with HCl (2 x 25 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (4:1) as eluant yielded product (1.08 g) as a yellow oil (50.0%). $IR_{Vmax}$ (film): 1768.1 (C=O), 1722.5 (C=O), 1291.5 ($NO_2$), 1255.4 (ArC(O)OR), 1195.7 (C(O)O), 704.5 (C-Br) $cm^{-1}$. LRMS: Requires: 322.9877 ($M^+$+23), Found: 322.9947 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 1.45 (3H, d, J 6.52Hz, $CH_3$), 2.36 (3H, s, $OCOCH_3$), 3.56 (2H, d, J 5.0Hz, $CH_2$), 4.31 (1H, m, CH), 7.12 (1H, m, Ar-H), 7.32 (1H, m, Ar-H), 7.59 (1H, m, Ar-H), 8.04 (1H, m, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 18.49 ($CH_3$), 20.95 ($OCOCH_3$), 69.22 ($CH_2$), 69.73 (CH), 123.45, 125.93, 131.66 and 134.08 (aromatic methine), 150.69 (Ar-C), 163.48 (ArOC(O)Me)), 169.43 (ArC(O)OR).

*2-Acetoxy-benzoic acid 1-methyl-2-nitrooxy-ethyl ester* (**101**)

A solution of 2-acetoxy-benzoic acid 1-methyl-2-bromo-ethyl ester (**104**) (0.56 g, 1.9 mmol) and silver nitrate (0.65 g, 3.8 mmol) in acetonitrile (15 ml) was allowed to stir at room temperature overnight. The reaction was filtered and the filtrate concentrated *in vacuo*. The oily residues obtained were reconstituted in ethyl acetate and washed with water (2 x 15 ml). After drying over anhydrous $Na_2SO_4$, the ethyl acetate was removed in vacuo to afford product as crude oil, which was purified by column chromatography over silica gel using hexane and ethyl acetate (5:2) as eluant to yield 0.41 g of product as a yellow oil (76.25%). $IR_{Vmax}$ (film): 1770.1 (C=O), 1727.0 (C=O), 1634.9 ($NO_2$), 1279.4 (ArC(O)OR), 1195.4 (C(O)O) $cm^{-1}$. LRMS: Requires: 306.0569 ($M^+$+23), Found: 306.0451 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 1.44 (3H, d, J 7.0Hz, $CH_3$), 2.37 (3H, s, $OCOCH_3$), 4.65 (2H, m, $CH_2$), 5.43 (1H, m, CH), 7.13 (1H, d, J 8.0Hz, Ar-H), 7.34 (1H, m, Ar-H), 7.60 (1H, t, J 6.5 and 7.0Hz, Ar-H), 8.00 (1H, d, J 6.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 16.28 ($CH_3$), 20.89 ($OCOCH_3$), 67.37 (CH), 73.76 ($CH_2$), 123.85, 125.97, 131.58 and 134.28 (aromatic methine), 150.85 (CO), 163.59 (ArOC(O)Me), 169.43 (ArC(O)OR).

*2-Acetoxy-benzoic acid 3-bromo-propyl ester* (**105**)

To a solution of 3-bromo-1 propanol (1 g, 7.2 mmol) in dichloromethane (25 ml) at 0°C, was added triethylamine (1.51 ml, 10.8 mmol) and acetylsalicyloyl chloride (1.43 g, 7.2 mmol). The mixture was stirred at room temperature overnight before washing with HCl (2 x 25 ml, 0.1 M), saturated aqueous $NaHCO_3$ (2 x 25 ml) and water (2 x 25 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed *in vacuo* to afford product as crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (3:1) as eluant yielded product (1.63 g) as a yellow oil (75.46%). $IR_{Vmax}$ (film): 1769.7 and 1723.5 (C=O), 1195.2 (C(O)O), 704.3 (C-Br) $cm^{-1}$. LRMS: Requires: 322.9877 ($M^+$+23), Found: 323.0172 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 2.26 (2H, q, J 6.5, 6.5 and 6.0Hz, $CH_2$), 2.34 (3H, s, $OCOCH_3$), 3.49 (2H, t, J 6.5 and 6.5Hz, $CH_2$), 4.41 (2H, t, J 6.0 and 5.5Hz, $CH_2$), 7.10 (1H, d, J 8.0Hz, Ar-H),

7.29 (1H, t, J 7.5 and 8.0Hz, Ar-H), 7.54 (1H, t, J 8.0 and 7.5Hz, Ar-H), 7.99 (1H, d, J 7.5Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.76 ($OCOCH_3$), 29.11 ($CH_2$), 31.43 ($CH_2$), 62.56 ($CH_2$), 123.61, 125.75, 131.37 and 133.71 (aromatic methine), 150.47 (CO), 163.93 (ArOC(O)Me), 169.24 (ArC(O)OR).

*2-Acetoxy-benzoic acid 3-nitrooxy-propyl ester* (**102**)

A solution of 2-acetoxy-benzoic acid 2-bromo-ethyl ester (**105**) (1.08 g, 4.7 mmol) and silver nitrate (1.60 g, 9.4 mmol) in acetonitrile (20 ml) was allowed to stir at room temperature overnight. The reaction was filtered and the filtrate concentrated in vacuo. The oily residues obtained were reconstituted in ethyl acetate and washed with water (2 x 20 ml). After drying over anhydrous $Na_2SO_4$, the ethyl acetate was removed *in vacuo* to afford product as crude oil, which was purified by column chromatography over silica gel using hexane and ethyl acetate (5:2) as eluant to yield 0.81 g of product as a yellow oil (60.89%). $IR_{Vmax}$ (film): 1769.6 and 1723.6 (C=O), 1631.4 ($NO_2$), 1281.3 ($NO_2$), 1259.01 (C(O)OR, aromatic), 1195.5 (C(O)O) $cm^{-1}$. LRMS: Requires: 306.0569 ($M^+$+23), Found: 306.0736 ($M^+$+23). $^1H$ NMR δ ($CDCl_3$): 1.88 (2H, m, $CH_2$), 2.35 (3H, s, $OCOCH_3$), 4.40 (2H, t, J 6.6 and 6.0Hz, $CH_2$), 4.60 (2H, t, J 6.5 and 6.0Hz, $CH_2$), 7.12 (1H, d, J 8.0Hz, Ar-H), 7.33 (1H, t, J 7.5 and 7.6Hz, Ar-H), 7.59 (1H, t, J 8.0 and 7.5Hz, Ar-H), 8.00 (1H, d, J 7.8Hz, Ar-H). $^{13}C$ NMR ppm ($CDCl_3$): 20.87 ($OCOCH_3$), 26.65 ($CH_2$), 60.89 ($CH_2$), 69.70 ($CH_2$), 123.79, 125.97, 131.45 and 134.02 (aromatic methine), 150.74 (CO), 164.05 (ArOC(O)Me), 169.48 (ArC(O)OR).

## 6.3 In vitro studies

### 6.3.1 General procedures

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 Detector controlled by Millennium Chromatography Manager. A second HPLC system was also employed consisting of a Waters 600 pump and controller, Waters 2487 dual λ absorbance detector and a Waters 746 data module. Waters Nova-Pak® C8 (4 μm) columns (3.9 x 75 mm and 3.9 x 150 mm) and Waters Spherisorb® column (4.6 x 250 mm) were employed. HPLC grade mobile phase was obtained using a Millipore Millex LCR 13 0.5 sample filter unit and a HVLP 04700 membrane filter unit. All buffers were prepared according to the Phenomenex computer program. Apparatus required for hydrolysis experiments include IEC Micromax Centrifuge, Fisons Whirlmixer and a Heto Shaker Bath.

### 6.3.2 Solubility Studies

The solubility of the test compounds was studied in a range of solvents. Excess quantities of powdered test compound were added to screw-capped vials and 5 ml of the solvent added. The suspension was vortexed for five minutes and shaken for the required period of time, at 37±0.5°C. The suspensions were sampled at one-hour intervals into a pre-equilibrated syringe 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 in the same concentration range.

### 6.3.3 Aqueous buffer hydrolysis studies

The hydrolysis of the prepared esters was studied in a range of buffers, over a range of pH. A 2 ml aliquot of a 1 mM solution of test compound in acetonitrile was diluted to 10 ml using phosphate, formate, acetate buffers or HCl as appropriate. A constant ionic strength was maintained with the addition of NaCl. 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, and analysed by HPLC.

The concentration of drug remaining in solution was determined by HPLC with external standards run on the same day in the same concentration range.

### 6.3.4 Preparation of biological samples

All blood samples were collected by venipuncture into vials containing citrate solution. For the collection of human blood, 10 ml Sarstedt Monovettes ® containing 1:10 volumes of trisodium citrate solution were used. For the collection of rabbit and dog plasma 25 ml glass vials containing 13 ml sodium citrate were used. For the collection of rat blood 25 ml glass vials containing 2 ml of sodium citrate (3.8% w/v) were used. Plasma samples were obtained by centrifugation of blood at 4,000 rpm for 10 minutes and refrigerated at 4°C, for up to three weeks, until required for testing. Serum samples were obtained by centrifugation of coagulated blood and refrigerated at 4°C for up to three weeks, until required for testing. Whole blood samples were stored at 4°C for 24 hours.

### 6.3.5 Determination of cholinesterase activity

Cholinesterase activity determinations were performed using a modification of the method of Ellman et al [207]. A solution (3.13 ml) comprised of *S*-butyrylthiocholine iodide (100 µl, 10 mmol), DTNB (400 µl, 10 mM) and phosphate buffer pH 8.0 (2.626 ml) was prepared. In order to initiate the reaction, plasma (4 µl) was added. A blank was also prepared without plasma. The reaction was followed spectrophotometrically at room temperature for ten minutes at 412 nm. Enzyme activity was expressed as nmol produced/ml plasma/minute of incubation.

### 6.3.6 Plasma and serum hydrolysis studies

Pooled plasma/serum solutions (4 ml) were prepared by dilution of plasma/serum with pH 7.4 phosphate buffer as appropriate. A 100 µl aliquot of test compound ($1 \times 10^{-4}$ M) in acetonitrile was incubated in the preheated plasma/serum solution (37±0.5°C) and 250 µl aliquots were withdrawn at appropriate time intervals. Samples were transferred to 1.5 ml Eppendorf® tubes containing 500 µl of a 2% w/v $ZnSO_4$-$7H_2O$ solution (acetonitrile and water; 1:1), vortexed and centrifuged for three minutes at

10,000 rpm. The concentration of drug and *in vitro* metabolites remaining in solution were determined by HPLC with external standards run on the same day at the appropriate concentration.

### 6.3.7 Whole blood hydrolysis studies

A 100 µl aliquot of test solution (1 x $10^{-4}$ M) in acetonitrile was incubated in preheated blood (4 ml at 37±0.5°C) and 300 µl aliquots were withdrawn at appropriate intervals. Samples were transferred to 1.5 ml Eppenddorf® tubes containing 1 ml of a 2% w/v $ZnSO_4$-$7H_2O$ solution (acetonitrile and water; 1:1), vortexed and then centrifuged for ten minutes at 5,000 rpm. The samples were then processed as described in Section 6.3.6.

### 6.3.8 Inhibition studies

Plasma hydrolysis studies were performed in the presence of a number of inhibitors to confirm the role of esterases in the hydrolysis of the test compound. The inhibitors were used at appropriate concentrations and incubated (37±0.5°C) in the buffered plasma solution for five minutes before addition of the ester stock solution (Section 6.3.6). The samples were then processed as described in Section 6.3.7.

### 6.3.9 Purified enzyme hydrolysis studies

The test compound (2 x $10^{-4}$M) was incubated at 37±0.5°C in pH 7.4 buffer to which was added the appropriate concentration of enzyme. Aliquots (100 µl) were removed at appropriate time intervals and samples were treated and processed as described in Section 6.3.6. The activity of butyrylcholinesterase and α-chymotrypsin was determined using butyryl thiocholine and *N*-acetyl tyrosine ethyl esters as the respective substrates. The activity of the former was studied according to the method in Section 6.3.5 while the activity of α-chymotrypsin was evaluated according to the method described in Section 6.3.6.

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# Synthesis, hydrolysis kinetics and anti-platelet effects of isosorbide mononitrate derivatives of aspirin

John F. Gilmer[a,*], Louise M. Moriarty[a], Dermot F. McCafferty[b], John M. Clancy[a]

[a]*Department of Pharmaceutical Chemistry, School of Pharmacy, Trinity College, Dublin 2, Ireland*

[b]*Department of Pharmaceutics, School of Pharmacy, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, UK*

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**Abstract**

Two isomeric aspirin derivatives of isosorbide-5-mononitrate (ISMN) were prepared and evaluated as potential mutual prodrugs of aspirin and nitric oxide. The hydrolysis of both compounds was studied in pH 7.4 phosphate buffer solution, buffered α-chymotrypsin solution and 10% buffered rabbit plasma. The benzodioxin-4-one derivative was hydrolysed to salicylic acid and ISMN acetate in buffer solution ($t_{1/2}$ 32.1 h), 10% buffered rabbit plasma ($t_{1/2}$ 25.7 min) and α-chymotrypsin ($t_{1/2}$ 86.6 min). The carboxylic acid ester derivative ISMNA was hydrolysed via the salicylate ester in buffer solution ($t_{1/2}$ 48.5 h) but was rapidly and almost exclusively hydrolysed to aspirin and ISMN in plasma solution ($t_{1/2}$ 2.8 min). The hydrolysis appeared to be enzyme mediated as it was suppressed by co-incubation with eserine. ISMNA was evaluated for its ability to inhibit platelet aggregation in rabbit PRP in response to the following agonists: arachidonic acid (AA) (100 μM), ADP (1.2 μM), phorbol ester (0.5 μM), platelet activating factor (PAF) (5 nM) and the thromboxane mimic U46619 (1.5 μM). ISMNA suppressed platelet response to AA at 1 μM whereas 10 μM aspirin showed no inhibitory effects. © 2001 Published by Elsevier Science B.V.

*Keywords:* Prodrug; Aspirin; ISMN; Nitric oxide; Platelet inhibition

## 1. Introduction

The efficacy of aspirin in the prevention and treatment of cardiovascular and cerebrovascular disorders is now firmly established (Antiplatelet Trialists' Collaboration, 1994). Its antiplatelet activity arises from its ability to inhibit platelet cyclo-oxygenase (COX-1) irreversibly thereby preventing the synthesis of the highly vasopressive and proaggregatory substance thromboxane $A_2$ ($TXA_2$) (Roth et al., 1975). As little as 50–80 mg aspirin daily provides an effective blockade of $TXA_2$ production because the anucleic platelets are unable to synthesise new cyclo-oxygenase (Patrono et al., 1980). However prolonged aspirin use is associated with an increased risk of gastrointestinal bleeding, leading in some instances to haemorrhage and death (Kurata and Abbey, 1990; Cryer et al., 1995). A number of different mechanisms are believed to contribute to this effect. The carboxylic acid group is a topical irritant at the mucosa; low stomach pH promotes ion trapping of the carboxylate in mucosal cells and local inhibition of COX-1 leads to suppression of prostaglandins ($PGE_2$, $PGI_2$), which normally regulate gastric acid secretion and blood flow. There is also evidence for a systemic dimension to aspirin gastropathy, which may be due to COX-1 inhibition but does not require direct contact with the gastric mucosa. This problem has been the focus of intense pharmaceutical attention for many years; however recent endoscopic studies demonstrate that conventional solutions such as enteric coating or buffering are inadequate (Kelly et al., 1996).

One approach that is currently attracting considerable interest is the design of nitric oxide-releasing aspirin derivatives, termed nitro-aspirins or NO-aspirins, in which aspirin is linked to a carrier bearing a nitrate group (Minuz et al., 1998; Napoli et al., 2001). These molecules are designed to cross the gastrointestinal barrier (or skin barrier) and then undergo cleavage by plasma esterases, liberating aspirin and the nitric oxide donor. Nitric oxide release protects the stomach from aspirin-induced gastric erosion by promoting blood flow and reducing leucocyte

*Corresponding author. Tel.: +353-1-608-2795; fax: +353-1-608-2793.

*E-mail address:* gilmerjf@tcd.ie (J.F. Gilmer).

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adhesion while its antithrombotic properties through the cGMP pathway potentiate the antiplatelet effects arising from COX-1 inhibition by aspirin (Del Soldato et al., 1999). Nitro-aspirins may therefore be described as mutual prodrugs of aspirin and nitric oxide. The validity of this strategy has been established in a number of animal models of gastric toxicity including a hemorrhagic shock model (Wallace et al., 1997) an ulcerogenic rat model (Takeuchi et al., 1998) and a diabetic rat model (Tashima et al., 2000). In each study the nitro-aspirin NCX4016 possessed superior gastric tolerability to aspirin. It is notable however that the COX inhibitory potency of NCX4016 is significantly lower than that of aspirin (Wallace et al., 1999). Essential to the success of the nitro-aspirin approach is that the drug liberates aspirin rather than salicylic acid in vivo, since the latter is a weak and reversible COX inhibitor (Danesh et al., 1988). A second and potentially more important design criterion is that nitric oxide release is carefully modulated in vivo to avoid some of the problems associated with nitrate administration such as headache or nitrate tolerance.

We present here an approach that seeks to address these latter design problems by integrating aspirin with isosorbide-5-mononitrate (ISMN). ISMN is a clinically successful long-acting nitrate which is frequently co-prescribed with aspirin, in similar molar quantities, for the prophylaxis of myocardial infarction in patients with unstable angina. Two potentially bioreversible modes of chemically combining aspirin and ISMN were investigated: orthoester or benzodioxin-4-one formation and direct esterification of the aspirin carboxylic acid with ISMN.

## 2. Materials and methods

### 2.1. Materials

Acetylsalicyloyl chloride (95%) was purchased from Fluka Ltd. Aspirin, indomethacin, salicylic acid, arachidonic acid (porcine liver 90%), α-chymotrypsin [EC 3.4.21.1], ADP, heparin, α-D-glucose, U46619, 2-chloroadenosine, PAF-acether, phorbol myristate acetate, eserine and staurosporine were purchased from Sigma-Aldrich Ireland Ltd. ISMN was obtained from SIFA Ltd, Shannon Industrial Estate, Co Clare, Ireland. Citrated rabbit whole blood was obtained from the marginal ear vein of New Zealand White rabbits held at the Bioresources Unit, Trinity College Dublin, Ireland. HPLC grade acetonitrile was purchased from Rathburn Ltd. All other reagents and chemicals were of analytical grade.

### 2.2. Chemistry

#### 2.2.1. 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, Ireland.

#### 2.2.2. Isosorbide-5-mononitrate-2-aspirinate (ismna) 2

A stirred solution of acetylsalicyloyl chloride (5.46 g, 27.5 mmol) in toluene (80 ml) was treated with triethylamine (4.56 ml, 32.75 mmol). The mixture was cooled to 0–5°C when ISMN (**1**) (5 g, 26.2 mmol) was added. Stirring was continued for a further 24 h with exclusion of light while the temperature was allowed to rise to 15°C. The mixture was then poured into 2 M aqueous HCl (20 ml). The organic phase was collected and shaken with 5% aqueous $NaHCO_3$ (20 ml), water (20 ml) and dried over $MgSO_4$. The toluene was removed under vacuum and the resulting oil crystallised twice from ethanol. The resulting solid (7.1 g, 73%) contained below 0.1% of a single impurity by HPLC. M.p. 85–86°C, $\upsilon_{max}$ 1736 (C–O), 1645, 1630 ($NO_2$). $^{1}H$ NMR (300.1 MHz, $CDCl_3$) $\delta$ (ppm) 2.31 (3H, s, OCOMe) 3.88 (1H, dd, $^{1}J$=11.0, $^{2}J$=5.4 Hz, 6α-H), 4.00 (1H, dd, $^{1}J$=11, $^{2}J$=3, Hz, 6β-H), 4.03 (1H, dd, $^{1}J$=11.0, $^{2}J$=3.0 Hz, 1β-H), 4.12 (1H, d, $^{1}J$=10.8 Hz, 1α-H), 4.54 (1H, d, $^{2}J$=4.9 Hz, 3-H), 5.02 (1H, t, $^{2}J$=5.2 Hz, 4-H), 5.35 (1H, dt, $^{2}J$=5.4, $^{2}J$=2.6 Hz, 5-H), 5.4 (1H, d, $^{2}J$=2.5 Hz, 2-H), 7.09 (1H, dd, $^{2}J$=8.0, $^{3}J$=2.2 Hz, Ar3–H), 7.34 (1H, dt, $^{2}J$=7.5, $^{3}J$=1 Hz, Ar5–H), 7.58 (1H, dt, $^{2}J$=9.0, $^{3}J$=2.0 Hz, Ar5–H), 7.85 (1H, dd, $^{2}J$=7.9, $^{3}J$=1.6 Hz, Ar6–H). $^{13}C$ NMR (75.5 MHz,$CDCl_3$) $\delta$ (ppm) 20.7, 69.2, 73.1, 77.8, 81.3, 81.5, 86.3, 122.5, 123.5, 126.0, 131.7, 134.3, 150.5, 163.4, 169.6. MS *m/z* 353, 311. Analysis C51.15:H4.28:N3.80, $C_{15}H_{15}O_9N$ requires C50.99:H4.28:N3.96.

#### 2.2.3. 2-Methyl-2-(isosorbide-5-nitrate-2-)1,3-benzodioxin-4-one 3

Acetylsalicyloyl chloride (1.06 g, 5.3 mmol) and ISMN (**1**) (1 g, 5.2 mmol) were dissolved in chloroform (10 ml). The reaction mixture was stirred at room temperature for 1 h and then brought to reflux for 10 min. The mixture was diluted with diethyl ether (50 ml) and washed successively with sodium bicarbonate (sat. 2×10 ml) and water (2×10 ml), dried over sodium sulfate and filtered. The volatiles were removed under vacuum to afford a pale yellow oil which was crystallised from diethyl ether/hexane to yield **3** (1.07 g, 58%) as a white solid. M.p. 122–125°C, $\upsilon_{max}$ 1736 (C=O), 1645, 1630 ($NO_2$). $^{1}H$ NMR (300.1 MHz,$CDCl_3$) $\delta$ (ppm) 1.80 (3H, s, 2-Me) 3.80 (1H, dd, $^{1}J$=11.2, $^{2}J$=6.6 Hz, 6″α-H), 3.93 (1H, dd, $^{1}J$=10.3, $^{2}J$=3.3 Hz, 1″β-H), 3.94 (1H, dd, $^{1}J$=11.2, $^{2}J$=2.9 Hz, 6″β-H), 4.02 (1H, dd, $^{1}J$=10.2, $^{2}J$=1.1 Hz, 1″α-H), 4.38 (1H, d, $^{2}J$=4.8 Hz., 3″-H), 4.72 (1H, d, $^{2}J$=3.2 Hz, 2″-H), 4.87 (1H, t, $^{2}J$=4.8 Hz, 4″-H), 5.28 (1H, dt, $^{2}J$=5.5, $^{2}J$=2.5 Hz, 5″-H), 7.02 (1H, dd, $^{2}J$=7.0, $^{3}J$=0.5 Hz,

Ar3–H), 7.17 (1H, dt, $^2J$=7, $^3J$=1.0 Hz, Ar5–H), 7.59 (1H, dt, $^2J$=8.3, $^2J$=7.5, $^3J$=2.0 Hz, Ar4–H), 7.94 (1H, dd, $^2J$=7.8, $^3J$=1.0 Hz, Ar6–H). $^{13}$C NMR (75.5 MHz, $CDCl_3$) δ (ppm) 23.3, 68.9, 74.1, 77.6, 81.2, 81.3, 112.7, 116.7, 123.5, 129.6, 136.8, 154.7, 160.1. MS *m/z* 354, 353, 163. Analysis C50.92:H4.20:N3.80, $C_{15}H_{15}O_9N$ requires C50.99:H4.28:N3.96.

### 2.3. Hydrolysis experiments

#### 2.3.1. Aqueous buffer kinetics

The hydrolysis of ISMNA and ISMN benzodioxinone was studied in phosphate buffer at pH 7.4. Buffer solutions containing the esters (0.25 mM) were maintained at 37±0.5°C in sealed glass ampoules in a water bath and samples were withdrawn at appropriate time intervals. Analyses were performed in triplicate using reverse-phase HPLC.

#### 2.3.2. Plasma hydrolysis studies

A 10% plasma solution (4 ml) was prepared by centrifugation of citrated rabbit venous blood and dilution with pH 7.4 phosphate buffer. The compounds ($1\times10^{-4}$ M) were 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 a 2% $ZnSO_4$–$7H_2O$ 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 **2** and **3**. 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.

#### 2.3.3. Enzyme study

Compounds **2** and **3** ($2\times10^{-4}$ M) were incubated at 37°C in pH 7.4 phosphate buffer containing 1 mg/ml α-chymotrypsin. Aliquots were removed at intervals and analysed immediately for remaining compound by direct injection onto the HPLC column. The presence of the enzyme did not have an adverse effect on the chromatography.

#### 2.3.4. 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 detector controlled by Millennium Chromatography Manager. Waters Nova-Pak® C8 (4 μm) columns 3.9×150 mm or 3.9×75 mm were used. The aqueous kinetics study samples were analysed using a gradient mobile phase consisting of phosphate buffer 85% (pH 2.4):acetonitrile 15% grading to acetonitrile 65%:phosphate buffer 35% over 15 min. The capacity factors for each analyte in this system were: ISMN: 0.8, aspirin: 4.6, salicylic acid: 6.2, ISMN-acetate: 7.35, **2**: 14.1, ISMN-Sal: 15.1 and **3**: 14.3. An isocratic mobile phase consisting of phosphate buffer 60% (pH 2.4): acetonitrile 40% was used to analyse the plasma study samples. The capacity factors for each analyte in this system were: aspirin 0.41, salicylic acid 0.58, **2** 4.93, **3** 5.6, ISMN-salicylate 7.31. The flow-rate was 1 ml/min in both methods. The eluent was monitored at 230 nm and peak identity 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 (ICH, 1994). A linear response was observed for each analyte ($r$ >0.998) in the range 1–100 μg/ml. The R.S.D. on multiple injection of each analyte at 10 μg/ml and 100 μg/ml was <2%. The limit of quantitation for the relevant analytes was 1 μg/ml in both methods except for ISMN-sal which had a higher LOQ (4 μg/ml) in the gradient method due to interference from the gradient ramp.

### 2.4. In vitro platelet aggregation studies in rabbit PRP

Venous blood obtained from male New Zealand-derived albino rabbits weighing 2.5–3 kg was mixed with one-tenth volume of trisodium citrate (0.13 M) and centrifuged for 10 min at 220 *g*. The platelet-rich suspension was treated with ISMNA or aspirin at an initial test concentration of 30 μM in aqueous DMSO (0.05%). Aggregation was initiated with AA (100 μM), ADP (1.2 μM), phorbol myristate acetate (PMA) (0.5 μM), platelet activating factor (PAF-acether) (5 nM), or U46619 ($TXA_2$) (1.5 μM). Aggregation was measured over 5 min by optical aggregometry at 37±0.5°C (Born, 1962) using a PAYTON (Model 300BD) from Payton Scientific Inc. Where no antagonist response was observed the measurements were repeated with established antagonists: ADP: 2-chloroadenosine (30 μM), PMA: staurosporine (3 μM), PAF: Web-2086 (0.2 μM), $TXA_2$: BM-13,177 (10 μM), AA: indomethacin (0.3 μM). No inhibition of platelet response was observed when the PRP suspension was incubated with the vehicle. All measurements were performed in duplicate.

## 3. Results and discussion

### 3.1. Chemistry

Isosorbide-5-mononitrate-2-aspirinate (ISMNA) **2** was obtained by treating ISMN **1** with acetylsalicyloyl chloride in toluene in the presence of triethylamine (Fig. 1[i]). The method used to prepare the ISMN-2-benzodioxin-4-one **3**

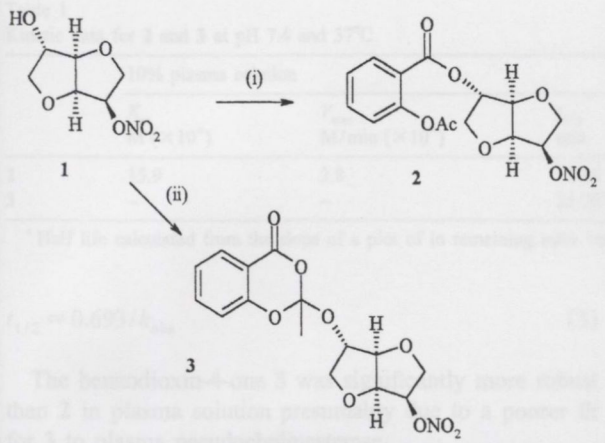



Fig. 1. Scheme showing the synthesis of ISMN-aspirin derivatives: (i) acetylsalicyloyl chloride, $Et_3N$, 24 h, 0–15°C, toluene; (ii) acetylsalicyloyl chloride, $CHCl_3$, 1 h at 25°C then 10 min reflux.

was based on the synthesis of benzodioxinones reported by Hundewadt and Senning (1991) (Fig. 1[ii]). ISMN was stirred with acetylsalicyloyl chloride in anhydrous chloroform for 1 h at RT followed by a brief reflux. Compounds of type **3** were first reported by Rüchardt and Rochlitz (1974) and were extensively investigated as potential aspirin prodrugs by Paris et al. (1980) and later by Senning and colleagues (Ankersen et al., 1989). It is generally believed that benzodioxinones are the kinetic product of nucleophilic attack on acetylsalicoyl chloride whereas esters are the thermodynamic products (Hansen and Senning, 1983). Whether the ester or benzodioxinone is formed appears to be dependent on the presence or absence of base but also to a lesser extent on the solvent, substrate and temperature (Nielsen and Senning, 1990). In the reaction between acetylsalicoyl chloride and ISMN, the role of base is pivotal in determining whether **2** or **3** is formed, whereas the conditions (temperature and solvent) were found by trial and error to be optimal for reaction efficiency (chemical yield and time). We have also prepared **3** by using the aspirin mixed trifluoroacetic anhydride approach reported by Hundewadt and Senning (1991) but the acid chloride reaction was found to be more reproducible and efficient. A key spectroscopic feature distinguishing the isomers **2** and **3** is the upfield position of the 2-methyl group of **3** in the $^1H$ NMR spectrum (1.8 ppm) relative to the acetyl methyl resonance of **2** (2.3 ppm). The shielding of the benzodioxinone methyl group relative to the acetyl methyl signal is an established spectroscopic marker for distinguishing these compounds (Hundewadt and Senning, 1990). In the $^{13}C$ NMR spectrum the $sp^2$ carbonyl carbon of the acetyl group of **2** occurs at 169.6 ppm whereas the corresponding re-hybridized carbon ($sp^3$) of **3** appears at 112 ppm. The two compounds have different HPLC retention times (**2**:15.1 min **3**:15.4 min), melting points (**2**:85–86°C; **3**:122–125°C) and PDA UV spectra (**2**:$\lambda_{max}$ 228, 273 nm; **3**: $\lambda_{max}$ 238, 299 nm). Additionally, the IR spectrum of **2** shows two distinct carbonyl bands whereas a single carbonyl absorbance is observed in the IR spectrum of **3**.

### 3.2. Hydrolysis kinetics

A progression curve for the hydrolysis of **2** in 10% buffered rabbit plasma (pH 7.4) at 37°C appears in Fig. 2. The ester was hydrolysed extremely rapidly ($t_{1/2}$ 2.8 min) in dilute plasma solution presumably due to the presence of plasma pseudocholinesterase. Hydrolysis was suppressed ($t_{1/2}$ 433 min) when **2** was co-incubated with 3 μM eserine (physostigmine), a potent esterase inhibitor. The parameters $K_m$ (Michaelis constant) and $V_{max}$ (maximum rate of substrate consumption) (Table 1) were estimated by fitting depletion data for **2** to the integrated form of the Michaelis–Menten equation (Eq. (1)) as described by Robinson and Characklis (1984).

$$V_{max}\, t = So - S + K_m \ln(So/S) \quad (1)$$

The half-life for **2** presented in Table 1 was calculated from Eq. (2):

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

Depletion of **3** did not appear to obey Michaelis–Menten kinetics in plasma solution. The apparent rate constant for **3** appearing in Table 1 was calculated from the slope of a plot of log remaining compound versus time and the half-life was then obtained from Eq. (3):

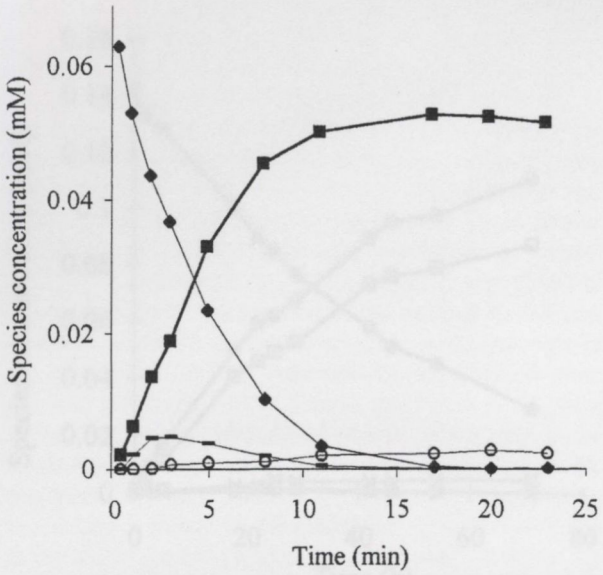



Fig. 2. Plot showing the time course for **2** (♦) in 10% buffered rabbit plasma (pH 7.4) at 37°C and its hydrolysis products: aspirin (■), salicylic acid (○), ISMN-salicylate (–). ISMN was formed too during this process but was not quantitated.

Table 1
Kinetic data for **2** and **3** at pH 7.4 and 37°C

| | 10% plasma solution | | | pH 7.4 buffer | | α-chymotrypsin | |
|---|---|---|---|---|---|---|---|
| | $K_m$ M ($\times 10^4$) | $V_{max}$ M/min ($\times 10^4$) | $t_{1/2}$ min | $k_{obs}$ $h^{-1}$ | $t_{1/2}$ h | $k_{obs}$ $min^{-1}$ | $t_{1/2}$ min |
| **2** | 15.9 | 3.8 | 2.88 | 0.0143 | 48.5 | 0.0041 | 169.02 |
| **3** | – | – | 25.76[a] | 0.0216 | 32.1 | 0.0080 | 86.60 |

[a] Half life calculated from the slope of a plot of ln remaining ester versus time.

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

The benzodioxin-4-one **3** was significantly more robust than **2** in plasma solution presumably due to a poorer fit for **3** to plasma pseudocholinesterase.

Progression curves for the hydrolysis of **2** and **3** in aqueous buffer at pH 7.4 and 37°C appear in Figs. 3 and 4, respectively, while the corresponding half-lives appear in Table 1. In both cases pseudo first-order rate constants ($k_{obs}$) were calculated from linear plots of the log of remaining ester versus time and the half-lives calculated using Eq. (3). Hydrolysis of both **2** and **3** proceeded slowly relative to the buffered plasma experiment. Analysis of depletion data for aspirin incubated in pH 7.4 buffer solution yielded a first order half-life of 18.5 h which is in line with reported values (St Pierre and Jencks, 1968). Aspirin hydrolysis is autocatalysed by the ionised carboxylate group and aspirin esters consequently exhibit higher aqueous stability at pH 7.4.

The hydrolysis of **2** and **3** was also studied in pH 7.4 buffered solution containing α-chymotrypsin, a protease present in the lumen of the gastrointestinal tract which exhibits some esterase activity. Strict pseudo first-order kinetics were observed over several half-lives for the depletion of **2** and **3** with $k_{obs}$ of $4.1\times10^{-3}$ and $8\times10^{-3}$ $min^{-1}$ corresponding to half lives of 169 and 86.6 min, respectively. Thus although hydrolysis in α-chymotrypsin was more rapid than in aqueous buffer it was significantly slower than in dilute plasma. Further experiments are planned to probe the relative stability of **2** and **3** under conditions prevailing in the gastrointestinal tract. However the α-chymotrypsin and aqueous stability results suggest that both compounds may be sufficiently stable to be absorbed intact.

### 3.3. Hydrolysis pathways

Schemes showing hydrolysis pathways for **2** and **3** are presented in Fig. 5. Hydrolysis of aspirin esters may occur simultaneously through two distinct routes; at the carboxylic acid ester bond ($k_1$) liberating aspirin **4** or at the acetyl group leading ultimately to salicylic acid **6** via the salicylate ester **5** ($k_2$) (Fig. 5). The $k_2$ pathway is preferred in plasma, apparently because neutralisation of the car-

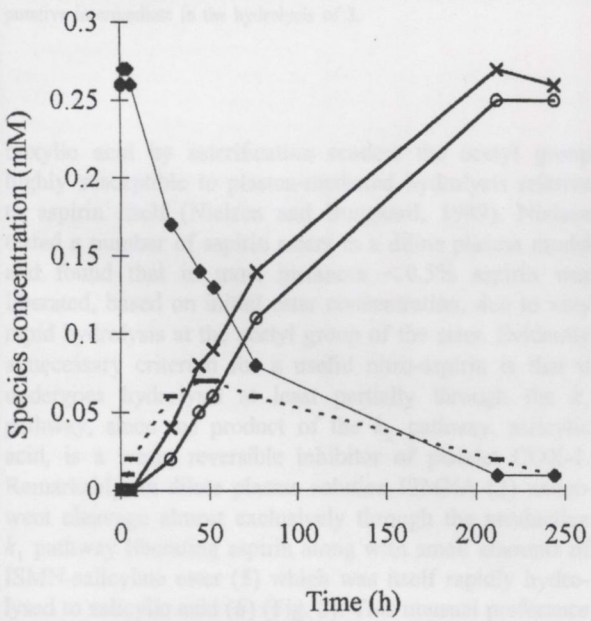



Fig. 3. Plot showing the time-course for **2** (♦) in phosphate buffer (pH 7.4) at 37°C and its hydrolysis products: salicylic acid (○), ISMN-salicylate (–), ISMN (×).

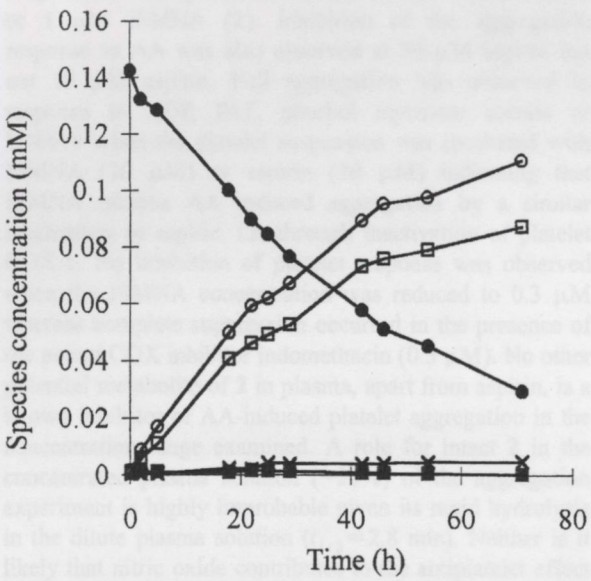



Fig. 4. Plot showing the time-course for **3** (●) in phosphate buffer (pH 7.4) at 37°C and its hydrolysis products: aspirin (■), salicylic acid (○), ISMN (×) and ISMN acetate (□).

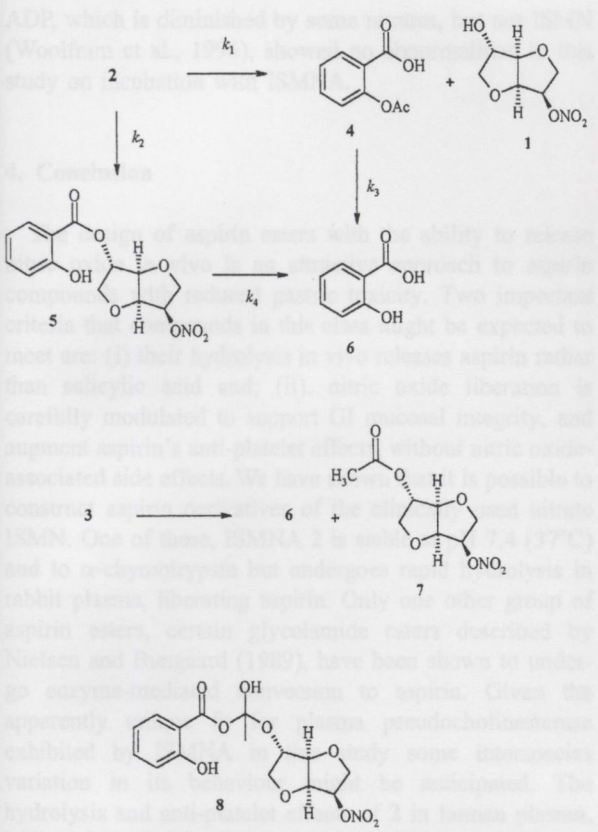


Fig. 5. Scheme showing alternative hydrolysis pathways for ISMNA (**2**) and **3**. In pH 7.4 buffer and α-chymotrypsin solution the rate constant for deacetylation ($k_2$) exceeds the rate constant for hydrolysis to aspirin **4** ($k_1$). In 10% buffered rabbit plasma the $k_1$ pathway to aspirin is dominant. In pH 7.4 buffer, α-chymotrypsin, or dilute rabbit plasma **3** undergoes hydrolysis to salicylic acid (**6**) and ISMN acetate (**7**). Compound **8** is a putative intermediate in the hydrolysis of **3**.

boxylic acid by esterification renders the acetyl group highly susceptible to plasma-mediated hydrolysis relative to aspirin itself (Nielsen and Bungaard, 1989). Nielsen tested a number of aspirin esters in a dilute plasma model and found that in most instances <0.5% aspirin was liberated, based on initial ester concentration, due to very rapid hydrolysis at the acetyl group of the ester. Evidently a necessary criterion for a useful nitro-aspirin is that it undergoes hydrolysis at least partially through the $k_1$ pathway, since the product of the $k_2$ pathway, salicylic acid, is a weak, reversible inhibitor of platelet COX-1. Remarkably, in dilute plasma solution ISMNA (**2**) underwent cleavage almost exclusively through the productive $k_1$ pathway liberating aspirin along with small amounts of ISMN-salicylate ester (**5**) which was itself rapidly hydrolysed to salicylic acid (**6**) (Fig. 2). This unusual preference for the $k_1$ pathway is most likely due to a structural feature of the ISMN 'carrier group' which promotes enzymatic attack at the carboxyl carbon and suppresses attack at the acetyl group. Hydrolysis did however follow the usual $k_2$ pathway when ISMNA was incubated in aqueous buffer with exclusive liberation of ISMN-salicylate **5** (Fig. 3). The benzodioxin-4-one **3** underwent hydrolysis through a different route as illustrated in Fig. 5 with liberation of salicylic acid **6** and ISMN-acetate **7**. The latter was identified by its photodiode array UV spectrum and retention time using an authentic ISMN-acetate standard obtained by acetylation of ISMN. Zahran et al. (1996) have reported that benzodioxin-4-ones of AZT and FLT undergo a similar reaction in growth medium, liberating the corresponding AZT and FLT acetates and salicylic acid. In this instance it might be speculated that hydrolysis proceeds through intermediate **8** which is too labile for detection under reverse-phase HPLC conditions. Hydrolysis of **3** occurred through this pathway in buffer, dilute plasma and in α-chymotrypsin solution. The enzymatic and nonenzymatic hydrolysis of benzodioxin-4-ones has been extensively investigated (Ankersen et al., 1989; Nielsen and Senning, 1990). Hydrolysis of benzodioxin-4-ones in buffer and in dilute plasma generally results in direct liberation of salicylic acid although certain sterically bulky latentiating groups appear to promote hydrolysis with some liberation of aspirin (Hundewadt and Senning, 1990).

### *3.4. In vitro platelet aggregation studies in rabbit PRP*

ISMNA (**2**) was selected for evaluation in an in vitro platelet inhibition model using citrated rabbit platelet rich plasma. Platelet aggregation in response to a variety of agonists was studied using the turbidometric technique (Born, 1962). Complete suppression of the aggregation response to 100 μM AA was observed when platelet suspensions were pre-incubated for 10 min with 30, 10, 3 or 1 μM ISMNA (**2**). Inhibition of the aggregation response to AA was also observed at 30 μM aspirin but not 10 μM aspirin. Full aggregation was observed in response to ADP, PAF, phorbol myristate acetate or U46619 when the platelet suspension was incubated with ISMNA (30 μM) or aspirin (30 μM) indicating that ISMNA inhibits AA induced aggregation by a similar mechanism to aspirin, i.e. through inactivation of platelet COX-1. No inhibition of platelet response was observed when the ISMNA concentration was reduced to 0.3 μM whereas complete suppression occurred in the presence of the potent COX inhibitor indomethacin (0.3 μM). No other potential metabolite of **2** in plasma, apart from aspirin, is a known inhibitor of AA-induced platelet aggregation in the concentration range examined. A role for intact **2** in the concentrated plasma solution (~90%) of the aggregation experiment is highly improbable given its rapid hydrolysis in the dilute plasma solution ($t_{1/2}$=2.8 min). Neither is it likely that nitric oxide contributes to the antiplatelet effect of **2** in this model since significant release from the highly stable ISMN ($t_{1/2}$ 4–6 h in vivo, Wood et al., 1984) is unlikely to have occurred during the 10 min incubation period of the experiment. The aggregation response to

ADP, which is diminished by some nitrates, but not ISMN (Woolfram et al., 1996), showed no abnormalities in this study on incubation with ISMNA.

## 4. Conclusion

The design of aspirin esters with the ability to release nitric oxide in vivo is an attractive approach to aspirin compounds with reduced gastric toxicity. Two important criteria that compounds in this class might be expected to meet are: (i) their hydrolysis in vivo releases aspirin rather than salicylic acid and; (ii). nitric oxide liberation is carefully modulated to support GI mucosal integrity, and augment aspirin's anti-platelet effects, without nitric oxide-associated side effects. We have shown that it is possible to construct aspirin derivatives of the clinically used nitrate ISMN. One of these, ISMNA **2** is stable at pH 7.4 (37°C) and to α-chymotrypsin but undergoes rapid hydrolysis in rabbit plasma, liberating aspirin. Only one other group of aspirin esters, certain glycolamide esters described by Nielsen and Bungaard (1989), have been shown to undergo enzyme-mediated conversion to aspirin. Given the apparently unique fit for plasma pseudocholinesterase exhibited by ISMNA in this study some interspecies variation in its behaviour might be anticipated. The hydrolysis and anti-platelet effects of **2** in human plasma, PRP and whole blood is the subject of current study.

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

John F. Gilmer*, Louise M. Moriarty, Maeve N. Lally, John M. Clancy

*Department of Pharmaceutical Chemistry, School of Pharmacy, Trinity College, Dublin 2, Ireland*

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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

*Corresponding author. Tel.: +353-1-608-2795; fax: +353-1-608-2793.

*E-mail address:* gilmerjf@tcd.ie (J.F. Gilmer).

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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 antiplatelet 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: $\delta_H$ (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). $\delta_C$ (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 ($\mu$) 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 ($1\times10^{-4}$ 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** ($2\times10^{-4}$ 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 ($\mu$) 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

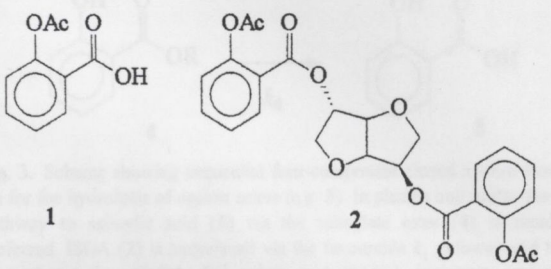



Fig. 1. Chemical structures of aspirin (**1**) and isosorbide diaspirinate (ISDA) (**2**).

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 | $48.5\times10^{-3}$ | 14.29 |
| 1.4 | $20\times10^{-3}$ | 34.65 |
| 1.62 | $11.0\times10^{-3}$ | 62.72 |
| 1.88 | $4.8\times10^{-3}$ | 144.36 |
| 2.24 | $1.6\times10^{-3}$ | 433.13 |
| 2.5 | $2.3\times10^{-3}$ | 300.42 |
| 4.13 | $2.3\times10^{-4}$ | 3013.04 |
| 6.45 | $6.9\times10^{-4}$ | 1004.35 |
| 7.29 | $2.99\times10^{-3}$ | 231.77 |
| 8.1 | $15.9\times10^{-3}$ | 43.33 |
| 9.4 | 0.29 | 2.35 |

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

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

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

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

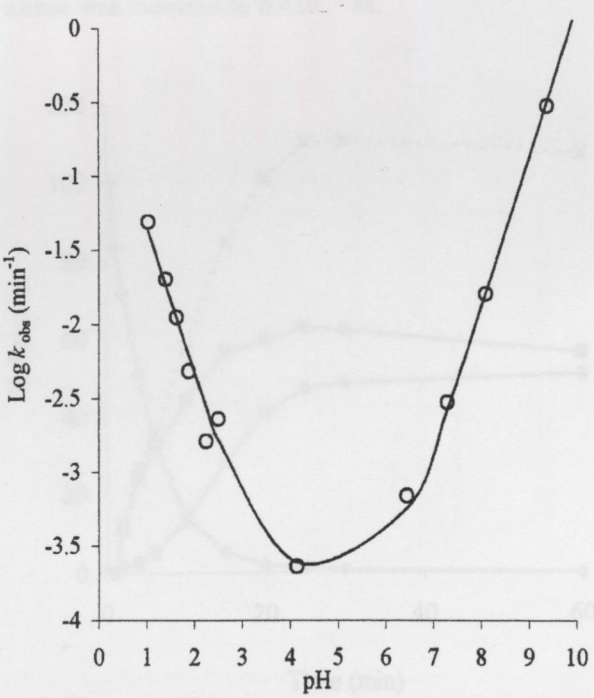



Fig. 2. The pH rate profile for the hydrolysis of **2** in aqueous solution ($\mu = 0.1$) at 37 °C. The points represent log of the pseudo-first-order rate constants determined at each pH from the log of remaining ester versus time, whereas the solid line was constructed using Eq. (1) and the rate constants 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 ($\lambda_{max}$ = 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 $2.33\times10^{-4}$ M and the $V_{max,app}$ value was $4\times10^{-5}$ 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 $6\times10^{-4}$ M.

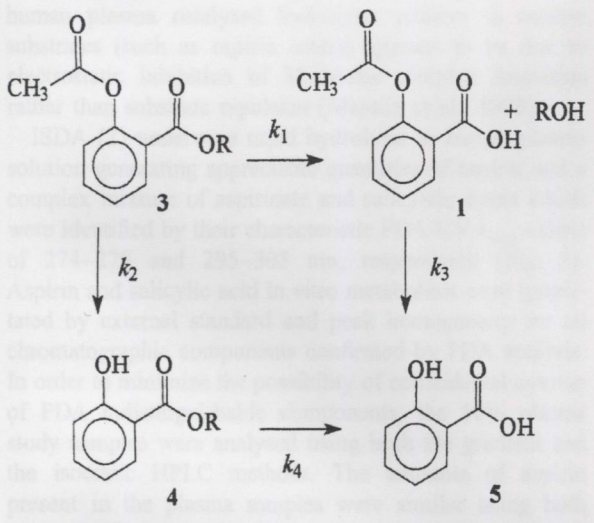



Fig. 3. Scheme showing sequential four-component closed system kinetics for the hydrolysis of aspirin esters (e.g. **3**). In plasma and buffer the $k_2$ pathway to salicylic acid (**5**) via the salicylate ester (**4**) is usually preferred. ISDA (**2**) is hydrolysed via the favourable $k_1$ pathway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

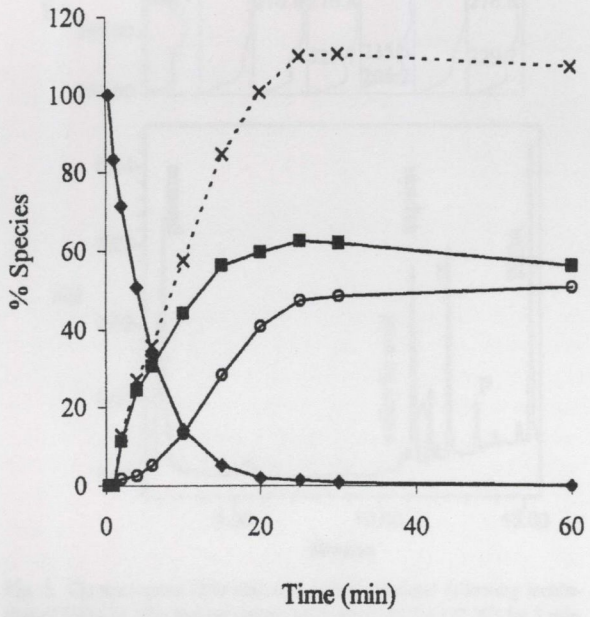



Fig. 4. Plot showing the time course for **2** (♦) in 10% buffered human plasma (pH 7.4) at 37 °C and its hydrolysis products: aspirin (■), salicylic acid (○). The sum of aspirin and salicylic acid is also plotted (×). Other unidentified salicylate metabolites were also formed during this process but 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) | $1.15\times10^{-3}$ | 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 $\lambda_{max}$ 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

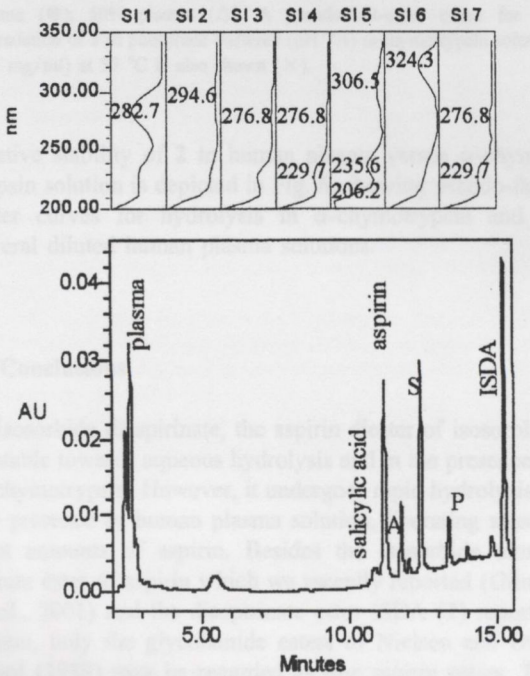



Fig. 5. Chromatogram (230 nm) of a sample obtained following incubation of ISDA in 30% human plasma buffered at pH 7.4 (37 °C) for 3 min. Also shown are PDA spectra of each of the labelled components. Peaks labelled 'plasma' or 'P' were also present in the plasma blank. Peaks labelled 'S' or 'A' have 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.

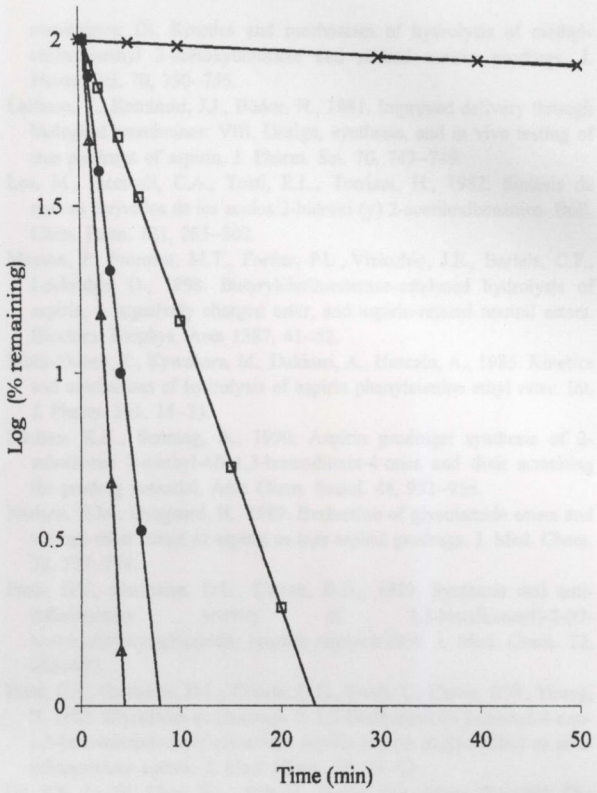



Fig. 6. Plot showing the pseudo-first-order curves for the hydrolysis of **2** in diluted human plasma at pH 7.4 and 37 °C: 10% plasma (□); 30% plasma (●); 50% plasma (△). A pseudo-first-order curve for the degradation of **2** in phosphate buffered (pH 7.4) α-chymotrypsin solution (0.1 mg/ml) at 37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