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Studies into the *in vitro* metabolism and antiplatelet effects of novel aspirin ester prodrugs

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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

2003
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Abstract

Aspirin is the most commonly used antiplatelet agent in the world, and is invaluable in prevention of myocardial infarction, thrombosis and stroke. However prolonged use has been associated with gastrointestinal side-effects such as ulceration and haemorrhage which may be fatal. This thesis describes the design and evaluation of aspirin prodrugs, which may liberate aspirin only on reaching the circulation, thus circumventing the GIT and having a potentially less severe gastrotoxicity profile.

Chapter 1 details the clinical applications of aspirin as an antiplatelet agent and profiles other clinically valuable therapies. Previously reported prodrugs of aspirin are outlined and their potential ability to regenerate aspirin on hydrolysis in the circulation is reviewed. Isosorbide-based aspirin prodrugs developed in our laboratory were found to liberate significant quantities of aspirin on hydrolysis in plasma.

In chapter 2 hydrolysis of isosorbide-2-aspirinate-5-mononitrate (ISMNA) is profiled in plasma from a number of different species. Significant inter-species variation in rate and pathway of prodrug hydrolysis was noted. ISMNA liberated up to 78% aspirin in rabbit plasma, whereas in human plasma only 10% aspirin was detected. Inhibitor studies revealed that butyrylcholinesterase is the enzyme responsible for ISMNA metabolism in human plasma. Studies in Caco-2 cell homogenates suggest that isosorbide-based aspirin prodrugs should be resistant to hydrolysis by gut esterases.

Chapter 3 details an investigation into the potential use of ISMNA, ISDA (isosorbide-diaspirinate) and isosorbide-2-aspirinate-5-salicylate as antiplatelet agents. In platelet and whole blood aggregation experiments ISDA was found to be equipotent with aspirin while Is-2-asp-5-sal was significantly more potent than aspirin at inhibiting platelet aggregation in human whole blood.

Chapter 4 describes a study of the structure-activity relationship (SAR) of isosorbide-2-aspirinate prodrugs with varying substituents at the 5-position of the molecule. Their physicochemical characteristics, ability to liberate aspirin on hydrolysis and their potential antiplatelet effects were investigated. Isosorbide was investigated as a potential carrier for other carboxylic acid drugs such as ibuprofen. A profile of these prodrugs is detailed in Chapter 5.
Acknowledgements

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<td>Arginine</td>
</tr>
<tr>
<td>ASA</td>
<td>Acetylsalicylic acid</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BNPP</td>
<td>bis-4-nitrophenylphosphate</td>
</tr>
<tr>
<td>BSS</td>
<td>Bernard-Soulier Syndrome</td>
</tr>
<tr>
<td>BuChE</td>
<td>Butyrylcholinesterase</td>
</tr>
<tr>
<td>CarbE</td>
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<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
</tr>
<tr>
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<td>Dimethylaminopyridine</td>
</tr>
<tr>
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</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>Dithiobis-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>DTS</td>
<td>Dense tubular system</td>
</tr>
<tr>
<td>EC</td>
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</tr>
<tr>
<td>EDRF</td>
<td>Endothelial-derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanine monophosphate</td>
</tr>
<tr>
<td>GPA</td>
<td>p-guanidino-L-phenylalanine</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectroscopy</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>HTT</td>
<td>Heptadecatrienoic acid</td>
</tr>
<tr>
<td>IEC</td>
<td>International Enzyme Commission</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-red</td>
</tr>
<tr>
<td>ISDA</td>
<td>isosorbide diaspinate</td>
</tr>
<tr>
<td>ISDN</td>
<td>isosorbide dinitrate</td>
</tr>
<tr>
<td>Is₂asp₅sal</td>
<td>isosorbide-2-aspirinate-5-salicylate</td>
</tr>
<tr>
<td>ISMNA</td>
<td>isosorbide mononitrate aspirinate</td>
</tr>
<tr>
<td>ISIS</td>
<td>International Study of Infarct Survival</td>
</tr>
<tr>
<td>iso-OMP</td>
<td>tetraisopropyl.pyrophosphoramide</td>
</tr>
<tr>
<td>k¹</td>
<td>capacity factor</td>
</tr>
<tr>
<td>k₁₀₀</td>
<td>pseudo first-order rate constant</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent Assay</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRMS</td>
<td>Low resolution mass Spectroscopy</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>mg</td>
<td>milligramme</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectroscopy</td>
</tr>
<tr>
<td>MTS</td>
<td>Microtubular system</td>
</tr>
<tr>
<td>NCX</td>
<td>NicOx</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory</td>
</tr>
<tr>
<td>OA</td>
<td>Osteo-arthritis</td>
</tr>
<tr>
<td>OTC</td>
<td>Over-the-counter</td>
</tr>
<tr>
<td>P</td>
<td>Partition Coefficient</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>PD</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode array</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin D&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>Prostaglandin I&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidyl inositol Diphosphate</td>
</tr>
<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl-sulfonylfluoride</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>P-Selectin</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty acid</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Corpuscle</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Sal</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-Activity Relationship</td>
</tr>
<tr>
<td>s.d.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SMILES</td>
<td>Simplified Molecular Input Line Information System</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient Ischaemic Attack</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-Layer Chromatography</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>TXA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Thromboxane A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>half-life</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>µg</td>
<td>microgramme</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Corpuscle</td>
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Chapter 1

Aspirin and other antiplatelet agents
1.1 Introduction

Aspirin (acetylsalicylic acid, ASA) (1) is one of the oldest and most commonly prescribed drugs in the world today and is a valuable anti-inflammatory, anti-pyretic, analgesic and anti-thrombotic agent. Its widespread use is increasing ever further, as long-term therapy is associated with decreased risk of colon cancer and onset of Alzheimer’s disease, both increasingly common with an aging population. However this “wonder-drug” is not the innocuous *panacea* it is commonly perceived to be. Its mechanism of action involves inhibition of the cyclooxygenase enzyme, and this can result in the gastrotoxicity and other adverse effects associated with aspirin. This chapter includes a review of aspirin, its historical background, therapeutic uses and toxicological profile. It focuses on aspirin’s usefulness as an antiplatelet agent, and reviews the other antiplatelet agents in clinical use today. Particular emphasis is given to aspirin prodrugs, designed to be absorbed through the intestinal wall and liberate aspirin *in vivo*, thus retaining the desirable antithrombotic effects of the parent compound, while potentially overcoming the problems of gastrotoxicity and ulceration. A summary of recently developed selective COX-2 inhibitors, designed to achieve maximum anti-inflammatory effects with minimum gastric side-effects, is given.

This chapter also includes a review of aspirin prodrugs developed in our laboratory as prospective antiplatelet agents in an effort to overcome the gastrotoxicity issues associated with aspirin. The objective of this thesis was to evaluate these novel aspirin prodrugs in terms of their potential as aspirin prodrugs and to establish their antiplatelet effect.
1.2 Aspirin – an ancient drug with modern uses

1.2.1 Historical background to aspirin and its pharmacological uses

In 1763, Reverend Stone reported to the Royal Society of London on the antipyretic effects of “fever bark” from English willow \(^1\) (Fig. 1.1), a therapy that had been known and used since the time of Hippocrates (\(\sim 460-377\) BC) \(^2\) and the ancient Egyptians \(^3\). In ancient Egypt the Ebers papyrus recommended the application of dried myrtle leaves to the abdomen to alleviate rheumatic pains from the womb \(^4\). Salicylic acid and the salicylates are constituents of several plants and have long been used in medicinal treatments \(^5\). Salicylic acid is a natural signalling molecule for activation of plant defense. It is synthesised in plant cells in response to environmental injury and serves as a messenger molecule to induce the expression of plant defense-related genes \(^6\). Stone’s advocation of salicylate use was based on the ancient Doctrine of Signatures, whereby the symptoms of the disease give a clue to its treatment. As the willow “delights in a moist or wet soil, where the agues chiefly abound” he dried willow bark, ground it to a powder and successfully treated ague (fever) with 20 grains (about 1g) of the compound \(^7\).

![Fig. 1.1 100-year-old pollarded willow trees](image)

Salicylic acid \(^2\) was first isolated in 1838 by the Italian chemist Raffaele Piria from the glucoside salicin, the active extract in willow bark. It was a potent anti-inflammatory agent but its use in the treatment of rheumatoid arthritis was hindered by its bitter taste \(^8\). In 1893 the German chemist, Felix Hofmann, concerned by his father’s severe arthritis, set about synthesising a more palatable form of salicylic acid. He successfully acetylated the phenol group to produce a pure and stable form of salicylic acid. His employer, the Bayer company, readily
undertook the commercial production of a prodrug of 2, acetylsalicylic acid or aspirin (1) which was registered on Feb 1, 1899. The name Aspirin came from “a” for acetyl and “spir” from the first part of Spirea ulmania, the plant from which salicylic acid was first isolated.

Aspirin has remained the most commonly used drug for relieving pain, inflammation and fever. In the USA today approximately 35 000 kg of aspirin are consumed daily. It has established antithrombotic efficacy and is used in the prevention of myocardial infarction, ischaemia and stroke, while recent research suggests it has a role as a preventative agent in colon and other cancers.
1.2.2 Mechanism of action of aspirin

1.2.2.1 Inhibition of Cyclooxygenase

In 1971, John Vane proposed that the mechanism of action of aspirin and other non-steroidal anti-inflammatory drugs (NSAID’s) was due to their inhibition of prostaglandin (PG) biosynthesis \(^{12}\), based on observations of the effect of 1 on guinea-pig lung \(^{13}\). Since then, there has been intense interest in the enzyme Prostaglandin G/H Synthase [EC 1.14.99.1], known colloquially as cyclooxygenase (COX) \(^{14}\), which is responsible for prostaglandin production \textit{in vivo} \(^{15}\). COX catalyses conversion of arachidonic acid to PGH\(_2\), the first step in the biosynthesis of a class of potent hormones \(^{16}\) including prostaglandins, prostacyclin and thromboxanes \(^{17}\). The prostaglandins are cyclopentane derivatives formed from polyunsaturated fatty acids by most mammalian tissues and by tissues of lower vertebrates and invertebrates \(^{18}\). They have long been known to contribute to pain and inflammation in rheumatic disease, to cytoprotection and maintenance of mucosal integrity in the gastrointestinal tract \(^{19}\), to maintenance of renal blood flow and function, and to haemostasis \(^{20}\). Cyclooxygenase is the primary target of NSAID’s, a collective group of drugs which includes aspirin, the propionic acids such as ibuprofen and naproxen, the acetic acids such as indomethacin and suldinac, and the oxicams typified by piroxicam \(^{11}\).
1.2.2.2 Isoforms of cyclooxygenase

Before the discovery of COX-2, cyclooxygenase was thought to be expressed constitutively with constant levels in individual tissues; prostaglandin synthesis was believed to increase in inflammation due to increased release of precursor. However cyclooxygenase activity increases during inflammation and this increase can be blocked by corticosteroids. This led to the identification of a new inducible isoform (COX-2) in the early 1990s. COX-1 is the constitutive form of the enzyme and appears to be the product of a “house-keeping” gene, supporting the low levels of prostanoid biosynthesis required for maintaining homeostasis; its activation leads to production of prostacyclin, which when released by the endothelium is antithrombogenic and by the gastric mucosa is cytoprotective. The second isoform, COX-2, is induced in a number of cells by pro-inflammatory stimuli and cytokines.

Cyclooxygenase (PGH synthase) in both isoforms is found primarily on the nuclear envelope or endoplasmic reticulum of cells and catalyses the committed step in prostaglandin biosynthesis, a two-step conversion of arachidonic acid (AA) to PGH$_2$ (Fig. 1.2). The initial step involves release and conversion of membrane-bound phospholipid by phospholipase A$_2$ to arachidonic acid. PGG$_2$ and PGH$_2$ are unstable intermediates that react with other enzymes to form a chemically diverse group of prostanoids including PGE$_2$, PGF$_{2\alpha}$, PGI$_2$ and thromboxanes.
A more detailed description of the arachidonic acid cascade and the chemistry of the eicosanoid biosynthetic pathway are presented in Fig 1.3. From cell to cell, specific subsequent enzymes determine the ultimate derivatives of PGH₂ metabolism. For example, in platelets nearly all PGH₂ is converted to the vasoconstricting thromboxane A₂ by thromboxane synthase, while endothelial cells primarily produce the vasodilatory PGI₂. In the gastrointestinal tract, most PG’s are converted to PGE₂, PGF₂α and PGI₂.\(^23\)
Figure 1.3 The arachidonic acid cascade. COX catalyses the oxidation of AA to the hydroperoxy endoperoxide PGG₂ and its subsequent reduction to the hydroxy endoperoxide PGH₂. This is then transformed by a range of enzymes and nonenzymic mechanisms into the primary prostanoids.

The structures of both isoforms of the cyclooxygenase enzyme have been elucidated by X-ray crystallography of their 3D structures. COX-1 and COX-2 are structurally very similar enzymes (see Table 1.1) consisting of a long narrow
channel with a hairpin bend at the end. They are isoenzymes - genetically independent proteins whose genes are located on different chromosomes and show different properties \(^2\). Despite genetic differences, both enzymes exist as homodimers with a molecular mass of approximately 70 kDa per monomer \(^1\), have highly conserved active sites and differ by less than 10% of amino acids within the arachidonic acid binding domain \(^5\).

<table>
<thead>
<tr>
<th></th>
<th>COX-1</th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Homology mRNA</td>
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<td>~60%</td>
</tr>
<tr>
<td>Messenger RNA size</td>
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<td>4.5 kb</td>
</tr>
<tr>
<td>Protein size (^a)</td>
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<td>~70 kDa</td>
</tr>
<tr>
<td>Intracellular location</td>
<td>Endoplasmic reticulum</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td></td>
<td>Nuclear envelope</td>
<td>(some) Nuclear envelope (mostly)</td>
</tr>
<tr>
<td>Regulation</td>
<td>Constitutive</td>
<td>Inducible</td>
</tr>
<tr>
<td>Range of expression</td>
<td>2 to 4 fold</td>
<td>10 to 80 fold</td>
</tr>
<tr>
<td>Tissue expression</td>
<td>Platelets, endothelial cells, stomach, kidney, smooth muscle most tissues</td>
<td>Most tissues especially inflammatory cells, requires stimulation by: growth factors, cytokines and hormones</td>
</tr>
<tr>
<td>Proposed role</td>
<td>&quot;Housekeeping&quot;</td>
<td>Inflammatory response</td>
</tr>
</tbody>
</table>

Table 1.1. Comparison of COX-1 and COX-2 \(^2\). \(^a\) The cyclooxygenase and peroxidase enzyme regions are 90% conserved between the two isoforms

In the C-terminus of the COX-2 polypeptide, the sequence surrounding Ser-516, the aspirin acetylation site residue, is identical to that of COX-1 \(^6\). Although their amino acid homology is only 61% overall, their 3-D X-ray crystal structures are almost superimposable \(^7\). A diagram of the key residues is presented in Fig 1.4. Both isoforms are membrane-associated \(^5\) so when arachidonic acid (AA) is released from damaged membranes, it is ‘sucked’ into the hydrophobic channel, two oxygens are inserted and a free radical extracted, resulting in the five-carbon ring characteristic of prostaglandins \(^2\).
Aspirin irreversibly inhibits COX-1 by acetylation of the serine residue Ser 530, thereby excluding access for AA. It also acetylates an active serine residue in COX-2 (Ser 516) \(^{28}\), but does not block substrate binding \(^{29}\) as the substrate channel is larger and more flexible than that of COX-1 \(^{30}\). Other NSAIDs block COX-1 by hydrogen bonding the polar arginine at position 120. The isoenzymes differ in that COX-1 possesses an isoleucine molecule at position 523 while COX-2 has a smaller valine molecule at the same position. This gives access to a side-pocket in the COX-2 enzyme that is inaccessible in COX-1. Specific selective COX-2 inhibitors utilise this structural difference by possessing a rigid side-extension that can access the COX-2 side pocket. The kinetics of inhibition of the two isoforms by various NSAID’s also varies considerably. COX-1 inhibition is instantaneous and competitively reversible (due to the hydrogen bonding) while COX-2 inhibition is time-dependent with selectivity developing over 15-20 minutes and is essentially irreversible \(^{21}\).
Many of the original NSAIDs were found to have inhibitory activity against both isoforms of the enzyme, and their inhibition of COX-1 is believed to be responsible for their side-effect profile. Indomethacin, which is highly gastrotoxic, is more selective for COX-1, whereas diclofenac is minimally gastrotoxic and much more selective for COX-2. A new generation of selective COX-2 inhibitors have been developed which have good anti-inflammatory activity and are largely free of gastrointestinal side effects. It has been proposed that a third cyclooxygenase isoform exists, COX-3, which is expressed up to 48 hours after an inflammatory event and is more effectively inhibited by paracetamol than either COX-1 or COX-2.

1.2.2.3 Screening for COX-1 and COX-2 selectivity.

Selective cyclooxygenase inhibition is a therapeutically desirable goal, and three different types of \textit{in vitro} assay have been developed to assess COX-1 and COX-2 activity. These are based on

1. Use of nonhuman, purified COX-1 and COX-2 enzymes
2. Use of nonhuman cell lines that inherently express or have been transfected to express only COX-1 or COX-2
3. Components derived from human whole blood that express exclusively either COX-1 or COX-2.

The human whole blood assay is most widely used and is based on the principle that the platelet is a COX-1 specific system while stimulated mononuclear cells are a COX-2 specific system. It has the advantage of using readily available human cells and takes into account the binding of NSAIDs to plasma proteins. Synthesis of thromboxane in platelets during the clotting cascade is used as an indicator of COX-1 activity. Synthesis of PGE$_2$ from monocytes in whole blood exposed to lipopolysaccharide (LPS) is used to quantify COX-2 activity.
The discovery and characterization of COX-2 has led to the search for pharmacologically active agents, which are capable of suppressing inflammation without inducing side effects associated with aspirin and other NSAIDs. These side effects of gastric ulceration and bleeding, renal damage and platelet dysfunction are associated with inhibition of the COX-1 isoform of the enzyme. Selective COX-2 inhibitors offer the potential to inhibit synthesis of inflammatory PGs without affecting PG generation by COX-1 in the GIT, kidney or platelet — in effect an “aspirin without ulcers”\(^{24}\). This would provide a significant improvement over current methods of therapeutic intervention for inflammatory diseases such as arthritis\(^{36}\). Current compounds have been designed to exploit the structural differences between COX-1 and COX-2. As previously discussed, there is a side pocket in the wall of the enzyme’s narrow channel, which is accessible in COX-2 but is blocked in COX-1 by a bulky isoleucine at residue 523 (Fig. 1.4). Many COX-2 inhibitors have structures that exploit this side-pocket by incorporating sulphonyl, sulphone or sulphonamide groups as rigid side-extensions which can access the COX-2 side-pocket but are too bulky to fit within the COX-1 channel\(^{21}\). Structure-activity studies indicate that a cis-stilbene moiety containing a 4-methylsulfonyl or sulphonamide substituent in one of the pendant phenyl rings is required for COX-2 specificity\(^{37}\). In the UK, the National Institute for Clinical Excellence (NICE) has classified four drugs as selective COX-2 inhibitors: celecoxib, etodolac, meloxicam and rofecoxib\(^{38}\) (Fig 1.5).

Nimesulide (3) was launched commercially in 1985. Its molecular structure is capable of accessing the COX-2 side pocket and is 16 times more selective for COX-2 than COX-1. Clinically available as Aulin\(^\circ\) it is a potent analgesic, anti-inflammatory and antipyretic agent in a wide range of conditions\(^{39}\). Nimesulide also has antihistaminic, anticytokinic and anti-oxyradical generating actions, which may help to prevent any exacerbation of inflammation induced by Helicobacter pylori in patients infected with the organism\(^{40}\). In clinical practice however its gastrotoxicity profile has not been shown to be superior to aspirin and other NSAIDs\(^{21}\).

Meloxicam (4) is an NSAID available on prescription in Ireland as Mobic\(^\circ\) (Boehringer Ingelhim). It is a preferential COX-2 inhibitor, which does
not bind to the side pocket of COX-2 but instead exploits a greater degree of flexibility at the apex of the COX-2 channel than the COX-1 apex. Controlled trials show that it has an improved GI toxicity profile relative to other commonly prescribed NSAIDs such as ibuprofen and ketoprofen.

Rofecoxib (5) or Vioxx® is a selective COX-2 inhibitor newly launched on the Irish market by Merck & Co. At doses of 25 – 50 mg daily it is used to relieve symptoms of osteoarthritis. It has been shown to have no effect on gastric mucosal PGE₂ synthesis in vitro compared with the significant inhibition caused by indomethacin, and thus has a large therapeutic window. It is reported to have superior COX-2 selective inhibitory activity compared to celecoxib. However, the VIGOR trial, which compared rofecoxib with naproxen, showed a five-fold increase in myocardial infarction in patients treated with rofecoxib compared with the traditional NSAID group. This is most likely because naproxen protects the cardiovascular system as it inhibits COX-1 thus blocking TXA₂ production. Conversely the selective COX-2 inhibitors primarily block COX-2 in the endothelium, reducing PGI₂ production, which modulates the cardiovascular effects of TXA₂ in vivo.

Celecoxib (6) is a 1,5-diaryl pyrazole-based compound that inhibits recombinant COX-2, and is quoted as being 75-fold more selective for this isoform of cyclooxygenase. Celecoxib’s selectivity appears to result from the phenylsulfonamide moiety, which binds to the side pocket that is restricted in COX-1 but is unoccupied in complexes of COX-2. It has been launched as Celebrex® by Monsanto at doses of 100 mg twice daily for rheumatoid arthritis and 200 mg once daily for osteoarthritis. Preliminary studies suggest that celecoxib is associated with much lower levels of gastric mucosal injury compared with naproxen. The selectivity of this class of highly selective inhibitors is related to their ability to inhibit COX-2 in a time-dependent manner while demonstrating no time-dependent inhibition of COX-1.
Figure 1.5 Selective COX-2 inhibitors

There is considerable interest in these selective COX-2 inhibitors as novel therapeutic agents, which may retard or prevent the development of colon cancer and Alzheimer's disease. However, considerable scrutiny is required to establish the gastric and renal toxicity profile of these compounds before their widespread use is advocated. There are reports which suggest that these drugs may exacerbate symptoms of renal impairment, cause acute renal failure, congestive heart failure, and coronary and cerebrovascular diseases. Because of their selectivity, they have a defined role in inflammatory processes but are incapable of competing with aspirin's cardiovascular protective effects mediated through the inhibition of COX-1.
1.2.3 Therapeutic applications of aspirin

As a nonselective COX inhibitor, aspirin has been widely studied for its anti-inflammatory, antipyretic and antithrombotic traits. Currently its primary use is in prophylaxis of cardiovascular disease, however it has recently been shown to play a role in the reduction of risk of colon cancer and as a protective agent against the onset of Alzheimers disease and other forms of dementia.

1.2.3.1 Anti-inflammatory effects of aspirin

Chronic inflammation is an excellent example of a disease that represents a malfunction of normal host defense systems. Aspirin does not act on a single disease or family of diseases but against a series of events that occur during inflammation arising as the result of injury to cells and tissues. PGE$_2$ is the most important prostaglandin, which mediates the typical symptoms of inflammation: rubor, calor, tumor, dolor and functio laesa. Genetically engineered mice lacking the COX-2 enzyme confirm that it is this inducible isoform of cyclooxygenase which is responsible for hypothalamic PGE$_2$ production during a febrile response. Dilatation of small blood vessels initiates the development of redness and heat; increased vascular permeability results in the characteristic swelling of tissues. Moreover, PGs sensitize peripheral nerve endings and nociceptors to transmit pain signals to the brain and the spinal cord. The major role of aspirin in inflammation involves lowering PGE$_2$ levels by directly inhibiting COX enzyme activity. It also inhibits COX-2 transcription induced by LPS. Interestingly clinically useful actions of aspirin may also be COX-independent as its anti-inflammatory effects are only seen at doses higher than those required for COX inhibition. The salicylates have COX-independent effects, whose physiological relevance is unclear, but whose targets - glucocorticoids receptors, heat shock proteins and nitric oxide synthase - suggest a role in inflammation. Salicylates also suppress tissue inflammation through diminished leukocyte-endothelial cell interactions e.g. aspirin inhibits leukocyte accumulation at sites of tissue injury. It reduces pyrogenic cytokine production and enhances expression of anti-inflammatory molecules, such as adenosine, in areas of inflammation. Evidence
provided by animal models of inflammatory arthritis suggests that increased expression of COX-2 is responsible for increased PG production seen in inflamed joint tissues. Highly selective COX-2 inhibitors are now being developed which have the same anti-inflammatory effects but fewer side effects than the less selective aspirin.

1.2.3.2 Aspirin and colon cancer

Colorectal cancer is a major form of cancer in the western world. In the USA it is the second most common form of cancer after lung cancer. It has been estimated that 50% of the population over 70 years of age have colorectal adenomata and that about 10% of these cases will progress to a cancerous state. Epidemiological studies have shown that long-term consumption of NSAIDs particularly aspirin, greatly reduces the risk of developing colon cancer even at the relatively low doses used as cardioprotectants. This is believed to occur as a result of inhibition of COX-2, which is upregulated in cancer tissue. Elevated levels of PGE₂ have been found in up to 80% of human colon cancer samples compared to normal mucosa. COX-2 activity is also associated with the production of free radicals such as reactive oxygen ions, which are capable of causing cellular mutations. Aspirin has been shown to induce apoptosis during ulcerogenesis and in colonic polyps and carcinomas, leading to a decrease in neoplastic tissue. However aspirin has a short half-life in circulating blood (~ 20 minutes) and is rapidly deacetylated to salicylic acid which has no cyclooxygenase inhibitory activity. Xu et al recently proposed that aspirin and salicylate antineoplastic action is due to the suppression of COX-2 mRNA synthesis at a transcriptional level, thus reducing COX-2 expression. COX-2 has been found to induce angiogenesis in colon cancer cells, its inhibition thus reduces vascular supply delivering nutrients and oxygen to the tumour, which are essential for tumour growth. There may thus be an adjuvant role for selective COX-2 inhibitors in the treatment of tumours as well as a primary role in cancer prevention.
1.2.3.3  Aspirin and Alzheimer's disease

Numerous reports suggest that patient groups who take anti-inflammatory drugs have reduced risk of developing Alzheimer's disease (AD)\textsuperscript{56}. The mechanisms proposed are essentially anti-inflammatory in nature, and reflect the inflammatory events and components in AD lesions\textsuperscript{24}. One theory is that aspirin and other NSAIDs, especially ibuprofen, protect nerve cells from the inflammation associated with amyloid plaques, the protein deposits found in the brain of an AD sufferer\textsuperscript{55}. COX also appears to play a role in postsynaptic signal transduction in the calcium-dependent cascade of pyramidal cells, with excessive stimulation resulting in excitotoxic cell death. These pyramidal cells are found in areas of the cortex and hippocampus, sites of intense neural injury in AD\textsuperscript{57}. COX inhibition may thus alleviate progression of the disease. The new selective COX-2 inhibitors are being evaluated for their efficacy in early treatment of asymptomatic but genetically at risk subjects. If they prove successful their use could result in delaying or even preventing the onset of the disease, with minimum associated gastric side effects\textsuperscript{24}.

1.2.3.4  Aspirin and cardiovascular disease.

The use of aspirin as an antiplatelet agent in cardiovascular and related diseases is further elaborated in \textit{Section 1.3}. In the UK about 25000 people die each year from acute myocardial infarction, other heart disease and stroke, events that occur largely due to platelet activation and thrombosis. A regime of long term, low dose aspirin can reduce the occurrence of, and risk of death from cardiovascular events\textsuperscript{58}. In platelets the only detectable form of cyclooxygenase is COX-1, and loss of AA-induced platelet aggregation is not only a well-established side effect of NSAID treatment, but also the therapeutic aim of the "half an aspirin a day" prophylaxis against thromboembolic disease\textsuperscript{59}. This prophylaxis is achieved through inhibition of COX-1 by aspirin, leading to decreased production of thromboxane A\textsubscript{2} (TXA\textsubscript{2}). Inhibition is irreversible for the lifetime of the platelet in the circulation (8 – 10 days)\textsuperscript{24}. The formation of TXA\textsubscript{2} requires the synthesis of new platelets, which are generated at a daily rate of approximately 10\%\textsuperscript{60}.\textsuperscript{17}
1.2.3.5 Aspirin and diabetes

In patients with diabetes mellitus, coronary artery disease tends to occur with greater severity, with cardiovascular-related deaths being three times more common than in non-diabetic patients. There is evidence to suggest that platelet hyperaggregability plays a role in this phenomenon. Platelets from diabetic patients display increased levels of activation and spontaneous platelet aggregation than in non-diabetic patients. They show higher response to ADP than platelets from healthy controls; this is particularly apparent in diabetes with concomitant microvascular disease. There is enhanced activation of the arachidonate pathway, leading to increased TXA₂ formation. Preventative strategies such as treatment with aspirin have been shown to reduce vascular events in “high risk” patients. In 1999 the American Diabetes Association reported that “98% of all diabetic subjects in the USA are candidates for daily aspirin therapy (80-325 mg of enteric-coated aspirin), while patients with ‘aspirin-allergy’, recent gastrointestinal bleeding or clinically active hepatic disease should be prescribed ticlodipine as an alternative.”

1.2.4 Side-effect profile of aspirin

1.2.4.1 Gastrotoxicity

Aspirin and other NSAID’s are the most commonly used drugs in the world, but their usage is associated with significant adverse effects on the gastrointestinal (GI) tract, with a considerable financial impact on health care systems. Medicaid data from Washington DC for 1981-1983 revealed that 31% of the total cost of care for arthritis patients was for management of GI adverse events. The number of deaths from NSAID-induced bleeding ulcers in the USA alone is comparable to those from AIDS and violent crime. It has been reported that serious gastrotoxicity can arise with even low doses of aspirin, ibuprofen and naproxen such as those available in OTC medications and supermarket products. All COX-
inhibiting NSAIDs currently available on prescription in the USA carry a warning about life-threatening gastrointestinal ulcers. Approximately 65% of all patients regularly consuming aspirin and other NSAIDs will develop small intestinal inflammation, whilst between 10% and 30% will develop peptic ulceration. Even low dose aspirin (75 – 300 mg per day) recommended for the prevention of occlusive vascular disease is potentially toxic to the GIT. Gastric and duodenal petechiae, erosions and endoscopic ulcers are present in more that 60% of patients after 1 month of aspirin therapy. The acidic nature of aspirin contributes to its direct toxic effect on the gastrointestinal mucosa. At low pH values this weak acid is lipid soluble and readily crosses into gastric mucosal cells. Inside the cell, the increased pH causes the aspirin molecule to ionise, effectively trapping it within the cell, and this accumulation disrupts normal cell function. Avoiding contact with the stomach or duodenum does not reduce the risk, because similar odds ratios have been reported for plain, enteric-coated and buffered aspirin. Aspirin has an immediate effect on mucosal integrity, causing a rapid decrease in mucosal potential difference and pH, coincident with a net loss of acid from the stomach lumen. This is consistent with the acid back diffusion and the dyspepsia commonly noted on aspirin administration. The inhibition of COX-1 also decreases production of PGE₂, which maintains the mucous-bicarbonate barrier, regulates gastric submucosal blood flow and promotes recovery after damage to the protective layer. Inhibition of COX-1 by aspirin renders the mucosa more susceptible to damage, and also increases the tendency to bleed because of impairment of platelet aggregation. A Danish study confirmed that even at low doses of aspirin there is an increased risk of upper gastrointestinal bleeding, and the risk is higher still if aspirin is combined with other nonsteroidal anti-inflammatory drugs.

1.2.4.2 Aspirin and asthma

A severe side-effect of aspirin and other NSAIDs is bronchoconstriction with resultant asthmatic episodes. The reduced amount of bronchodilating PGE₂ and a concomitant shift in the metabolic pathway from the cyclooxygenase to the 5-lipoxygenase pathways is thought to be responsible. The 5-lipoxygenase pathway (ref. Fig. 1.3) metabolises "overflow" arachidonic acid, which cannot be
metabolised by the inhibited cyclooxygenase enzyme. The resulting leukotrienes act as potent bronchoconstrictors.

1.2.4.3 Aspirin and renal function

Administration of aspirin and other NSAIDs can lead to renal disorders and promote resultant hypertensive effects. In the kidney prostanoids produced by COX-1 act as vasodilators and help maintain renal plasma flow and glomerular filtration during periods of vasoconstriction. They are important physiological modulators of vascular tone and sodium and water homeostasis in the mammalian kidney, including modulation of glomerular haemodynamics, tubular resorption of sodium and water and regulation of renin secretion. Maintenance of normal kidney function is dependent on PGs in animal models of disease states and in patients with congestive heart failure, liver cirrhosis or renal insufficiency. On inhibition of COX-1 the reduced production of PGs such as PGI₂, PGE₂ and PGG₂ in the renal blood circulation means the rate of glomerular filtration is reduced. In patients with previously reduced renal function, this leads to water retention, hypertension and in some instances renal ischaemia and renal failure.

1.2.4.4 Aspirin and Reye’s syndrome

Reye’s syndrome is a rare, acute and sporadic paediatric liver disorder, which results from an atypical response to viral infection. It is characterized by encephalopathy with pronounced cerebral oedema, and by diffuse fatty infusion of the liver. The illness is biphasic: there is apparent near-recovery from a viral-type prodromal illness followed after a few days by vomiting and altered consciousness. There is a concomitant rise in blood ammonia concentrations and aminase levels. Its occurrence is associated with patients being treated with aspirin during the prodrome stage. As a result of this risk, since 1986, aspirin use is no longer recommended in children under 12 years of age.
1.2.5 Pharmacokinetics and stability of aspirin

All aspects of the pharmacokinetics of aspirin appear to be independent of dose, or duration of dosage. No significant differences were noted between the kinetics of aspirin (375 mg) administered as a single dose or during long-term therapy.

1.2.5.1 Absorption of aspirin

Orally administered aspirin (1) undergoes substantial presystemic hydrolysis in the gut, the gut wall and the liver before entering the presystemic circulation. The absorption half-life of aspirin is less than that of salicylic acid (2) with differences in rate and extent of absorption influenced by pH, pre-absorptive hydrolysis and first-pass effects. Both 1 and 2 are weak acids with a $pK_a$ of 3.6 and 3.0 respectively. They both display a pH dependence on absorption, being most poorly absorbed under alkaline conditions. Passive absorption of aspirin occurs in the stomach and small intestine, with absorption rate being dependent on the rate of stomach emptying.

1.2.5.2 Distribution of aspirin

Both aspirin and salicylic acid are extensively distributed throughout the body following oral administration, as they readily undergo passive diffusion across biological membranes. They are both highly plasma protein bound, with salicylic acid being 80-90% bound at normal therapeutic concentrations. The interaction of aspirin with plasma proteins is more complex, due partly to its ability to acetylate proteins, glycoproteins and lipids in the stomach, liver, kidney and bone marrow.

1.2.5.3 Metabolism of aspirin

Aspirin is rapidly metabolised in vivo to salicylic acid with a half-life in plasma of 15 to 20 minutes. Its oral bioavailability appears to be dose-independent and is approximately 50% of the administered dose. Pre-absorptive hydrolysis occurs in the lumen and is most likely due to enzymatic hydrolysis by brush-border
esterases. Following oral administration, aspirin readily undergoes pre-systemic hydrolysis in the gut and liver, and on reaching the systemic circulation any remaining aspirin is hydrolysed by esterases in plasma and red blood cells. The major metabolic pathway for hydrolysis of aspirin is depicted in Fig. 1.6. The enzyme responsible for hydrolysis of aspirin in plasma and generically known as 'aspirin-esterase' has been identified as butyrylcholinesterase. Salicylic acid remains detectable in plasma for several hours. Unlike aspirin, salicylic acid has no inhibitory effect on platelet COX-1 and so does not contribute to aspirin's antithrombotic effect in vivo.

![Metabolic Pathway Diagram](image)

*Figure 1.6 The metabolic pathway of aspirin and salicylic acid.*
1.2.5.4 Excretion of aspirin

The elimination half-life of aspirin has been determined to be 15-20 minutes, whereas salicylic acid has a much longer elimination half-life of 230-300 minutes. Aspirin metabolism can be determined by monitoring of salicylate and other metabolites in the blood and urine \(^76\).

1.2.5.5 Stability of aspirin

Aspirin undergoes rapid specific base-catalysed hydrolysis in the presence of water \(^81\). This, combined with its poor aqueous solubility makes it unsuitable for formulation as an aqueous suspension \(^82\). It readily undergoes intramolecular nucleophilic catalysis at the carboxylic acid group as shown in Fig. 1.7, hence its poor solubility profile.

![Figure 1.7 The intramolecular nucleophilic catalysis of aspirin](image)

The instability of aspirin in its solid (powder) form in the absence of moisture is insignificant relative to its hydrolysis in the presence of water \(^83\).
1.3 Aspirin as an antiplatelet agent

Although salicylates have been prescribed for symptoms of inflammation for thousands of years, the use of aspirin to prevent arterial thrombosis spans less than four decades. Platelet aggregation plays a fundamental role in the pathophysiology of cardiovascular disorders, and so antiplatelet drugs may be useful in the therapy and prophylaxis of peripheral arterial disease. The clinical indications for aspirin particularly with reference to its use as an antithrombotic agent continue to be redefined. COX-1 is found in all platelets, where the enzyme is responsible for providing precursors for thromboxane synthesis. Teleologically this is important, as platelets are anucleate and so cannot produce an inducible enzyme in response to activating conditions. In the presence of a COX-1 inhibitor such as aspirin, platelets are prevented from generating TXA\(_2\) during aggregation, inhibiting their thrombogenic potential. Aspirin is unique in its irreversible inhibition of COX-1. The other NSAID’s bind reversibly at the active site of the enzyme, depressing platelet thromboxane formation to a small degree, so that platelet function is impaired for only a portion of the dosage interval.

1.3.1 Uses in cardiovascular disease

In the Second International Study of Infarct Survival (ISIS-2), a single aspirin tablet (162.5 mg), administered within 24 hours of onset of a suspected myocardial infarction (MI), and continued daily for five weeks, produced highly significant reductions in the risk of vascular mortality (23%), non-fatal reinfarction (49%) and non-fatal stroke (46%). Aspirin is approximately 50 to 100-fold more selective for COX-1 than COX-2. It is therefore ideally suited to act on anucleated platelets by inducing a permanent defect in thromboxane-dependent platelet function, which cannot be repaired within a 24-hour dosing interval. As it passes through the liver, up to 50% of the aspirin is deacetylated and it becomes further diluted on joining the rest of the venous blood. In humans, aspirin blocks COX activity in platelets within an hour of oral administration. This results in platelet function being inhibited for several days following a single dose of aspirin. Dose regimens
from 25 to 325 mg a day have been suggested for use in antithrombotic prophylaxis or for treatment of acute coronary syndromes, but consensual medical opinion now recommends 75 mg daily as being sufficient. Patients undergoing coronary angioplasty are given aspirin to reduce TXA2 production and platelet clumping, and hence the risk of myocardial infarction.

### 1.3.2 Other antiplatelet agents

Aspirin is the prototype antiplatelet agent – in the ISIS-2 trial aspirin used alone in the early stages of myocardial infarction had an equivalent benefit to thrombolytic therapy, producing significant lasting reductions in mortality rates, recurrent myocardial infarction and stroke with minimal side effects. Impressive benefits were also noted in treatment of patients with stable and unstable angina, symptomatic cerebrovascular disease, peripheral vascular disease following coronary artery bypass grafting and in patients undergoing percutaneous coronary interventions. In many cardiovascular conditions aspirin is the agent of choice as it is inexpensive and generally well tolerated. However, it only inhibits TXA2 production, which is just one of many mediators of platelet activation.

This has led to the search for newer and more potent platelet inhibitors, which are capable of acting on different pathways involved in the complicated process of platelet activation and aggregation.
1.3.2.1 **ADP receptor antagonists**

ADP is a platelet agonist that acts as a local mediator at the site of platelet activation upon its release from dense granules. It can induce full platelet activation and aggregation \(^90\). Clopidrogel (Plavix\(^\text{®}\), 7) and ticlodipine (8) are thienopyridine inhibitors of the platelet ADP receptor \(^91\)\(^92\).

![Chemical structures of Clopidrogel and Ticlodipine]

\[
\begin{align*}
R &= \text{CO}_2\text{CH}_3 \quad \text{Clopidrogel 7} \\
R &= \text{H} \quad \text{Ticlodipine 8}
\end{align*}
\]

These agents do not suppress ADP-induced shape change; rather they act by reducing cAMP levels and suppressing ADP-induced platelet aggregation. They inhibit shear-induced platelet aggregation and platelet adherence to the endothelial matrix \(^92\). Aspirin has no such effect on these parameters. The main indication for ticlodipine use is as an antithrombotic in cardiovascular disease and during cardiovascular interventions associated with the risk of platelet clot formation. However 10-15% of patients treated with ticlodipine experience side-effects including neutropaenia, thrombocytopaenia and aplastic anaemia \(^93\). The CAPRIE trial advocated the use of clopidrogel in prevention of vascular events in patients with a history of myocardial infarction, ischaemic stroke or peripheral vascular disease but its absolute reduction in endpoint over aspirin was only 0.5% \(^94\). It lacks the side-effects associated with ticlodipine and so is favoured as the anti-platelet of choice when aspirin cannot be tolerated for gastrointestinal reasons or in instances of aspirin resistance or aspirin sensitivity.
Agents that increase platelet cAMP or cGMP also have potent antiplatelet effects. Dipyridamole (9) (clinically available as Persantin®) administration results in increased levels of platelet cAMP and cGMP, which in turn suppress platelet activation.

Standard doses do not confer any benefit as monotherapy but a novel, controlled release preparation of 200 mg given twice daily, was as effective as aspirin in patients who had suffered recent transient ischaemic attack (TIA) or stroke. Its main use now is in combination therapy with aspirin, and as an intravenous preparation to precipitate myocardial ischaemia for diagnostic purposes. In combination with aspirin there is a dual effect on the principal mechanisms of platelet function regulation and in some tests of platelet function the final therapeutic effect was greater than the sum of the effects of both drugs used separately.
On activation of platelets the platelet fibrinogen receptor glycoprotein (GP) IIb/IIIa undergoes a conformational change, which increases its affinity for fibrinogen. This can bind adjacent platelets, cross-linking them to initially produce an aggregate, and subsequent thrombosis formation. Therefore, pharmacological blockade of the interaction between fibrinogen and platelet GPIIb/IIIa receptor has emerged as a therapeutic target in prevention of arterial thrombosis, irrespective of the mode of platelet activation. Inhibitors of the receptor were first isolated from the venom of poisonous snakes. Abciximab (a monoclonal antibody inhibitor) was the first GPIIb/IIIa antagonist to reach clinical use. Abciximab (c7E3) is the humanized chimeric Fab fragment of a monoclonal mouse antibody, which once tightly bound in platelets, persists for many days. It is a potent inhibitor of platelet aggregation and its efficacy in prevention of complications during coronary angioplasty and stent placement has been proven in several large scale clinical trials, most significantly the CAPTURE trial of 1997. It is estimated that abciximab is currently used in 40% of coronary interventions in the USA. The potent GPIIb/IIIa receptor antagonists (including newer agents such as tirofiban and eptifibatide) are designed for chronic therapy and monitoring of their use is required to avoid hemorrhagic side-effects. Abciximab, being a chimeric monoclonal antibody has a potential problem of immunogenicity, and the short half-life of tirofiban (1.6 hours) and eptifibatide (0.9 hours) can result in rapid reversal of platelet inhibition upon discontinuation of the drug. For this reason it is unlikely that they will play a significant role in conventional outpatient antiplatelet therapy.
1.4 Pharmacologically active prodrugs of aspirin

Efforts have been made to develop bioreversible prodrugs of aspirin in an attempt to reduce or eliminate gastric irritation and bleeding. Upon administration a successful prodrug should pass intact through the stomach, only being hydrolysed to liberate aspirin in the intestine or following absorption into the circulation.

1.4.1 Glycolamides

The esters of N,N-disubstituted 2-hydroxyacetamides (glycolamides) were shown to be cleaved very rapidly in human plasma by butyrylcholinesterase $^{101}$. Using benzoic acid initially as a model compound, a series of benzoate esters of N-substituted glycolamides were synthesised. These glycolamide esters were found to be suitable prodrug forms, in terms of enzymatic conversion, chemical stability and physical properties $^{102}$. They were rapidly hydrolysed in 50% human plasma to produce benzoic acid. The most prominent structural requirement in order for glycolamide esters to be rapidly hydrolysed was the presence of two substituents on the amide nitrogen atom (i.e. a structural similarity to benzoylcholine, a good substrate for butyrylcholinesterase $^{103}$). The high reactivity of the glycolamide esters of benzoic acid was exploited in the production of glycolamide esters of aspirin $^{104}$, which are susceptible to enzymatic hydrolysis both in vitro and in vivo. The most reactive are the N,N-dimethylglycolamide ester (10) and the N,N-diethylglycolamide ester (11) which liberated 50 and 55% aspirin respectively in a 10% solution of human plasma.
Both esters also had favourable stability, solubility and lipophilicity profiles, (being significantly more stable than aspirin in the pH range 2.0 – 7.0), which enhanced their potential as aspirin prodrugs \(^{105}\). As an extension to these novel aspirin prodrugs, a series of prodrugs of other NSAIDs were studied with respect to enzymatic hydrolysis and aqueous stability \(^{101}\).

### 1.4.2 Nitric-oxide releasing aspirins

An area currently generating much interest is the recent development of nitric oxide-releasing aspirin esters, (NO-aspirins), where aspirin is linked to a carrier molecule containing a NO-releasing moiety \(^{106}\). The nitrate group is linked to the carboxylic acid function of aspirin by an ester bond (R-O-NO\(_2\)) and on enzymatic hydrolysis both aspirin and NO are released into the blood stream \(^{106}\). These molecules are designed to bypass the gastrointestinal system, only releasing the active moieties on ester hydrolysis in the systemic circulation. As discussed in greater detail below, the NO released can counteract aspirin’s inhibition of prostacyclin, and so reduces some of its side-effects of gastrotoxicity \(^{87}\).

#### 1.4.2.1 Physiological and pharmacological effects of nitric oxide

Prostaglandins and nitric oxide (NO) play a major role in maintaining mucosal integrity. NO has cytoprotective effects in the stomach and other organs; in the stomach it is involved in the stimulation of mucous secretion and maintenance of mucosal blood flow \(^{107}\). It is a potent inhibitor of neutrophil adherence *in vivo* and it is hypothesised that NO release may inhibit the leukocyte adherence induced by NSAID’s, thereby preventing NSAID-induced mucosal damage \(^{63}\). Nitric oxide has been identified as endothelial-derived relaxing factor (EDRF), which is involved in the dynamic regulation of vascular smooth muscle tone of blood vessels \(^{108}\). It is synthesised *in vivo* from the guanidino-nitrogen of L-arginine \(^{109}\). NO liberated by inducible NO synthase (iNOS) has been implicated as a mediator of inflammation in rheumatic and autoimmune diseases \(^{110}\). There has been
significant interest in the design of an aspirin prodrug that could liberate NO, thus potentially counteracting the negative effect of PG inhibition at the gastric level. In the healthy cardiovascular system, NO is a potent vasodilator and also protects against thrombosis and atherogenesis, through inhibition of monocytes and platelet adhesion, platelet aggregation and smooth muscle cell proliferation. One disadvantage of nitric oxide donors for anti-thrombotic applications is that drugs such as sodium nitroprusside and glyceryl trinitrate relax vascular smooth muscle, thereby reducing systemic blood pressure. A prodrug that releases NO below the threshold of effects on systemic blood pressure may therefore have a therapeutic as well as a gastroprotective role.

1.4.2.2 NCX 4016 and NCX 4215

In 1997 a patent application was submitted for a series of compounds, described as NO-releasing aspirin prodrugs (Patent no. WO 97/16405). Lechi investigated these compounds for their anti-aggregating activity. Compounds studied included NCX 4215 (12), a nitroxybutyl derivative of ASA and NCX 4016 (13) a 2-acetoxybenzoate 2-[1-nitroxy-methyl]-phenyl ester.

They tested the hypothesis that addition of a nitric oxide-releasing moiety to aspirin would afford a compound that retained its anti-thrombotic activity but had reduced gastrointestinal activity, the rationale being that NO released in the proximity of target tissues, would maintain gastric mucosal flow and prevent leukocyte adherence within the gastric circulation. NCX 4215 was found to
have no inhibitory effect of COX activity yet showed significant inhibition of thrombin-induced aggregation in human platelets, and ADP- and collagen-induced aggregation in rat platelets. In human platelet studies NCX 4215 was significantly more potent than aspirin as an inhibitor of aggregation; this is apparently related to release of NO on metabolism, which causes increased levels of platelet cGMP. However its failure to inhibit thromboxane production suggests that it is not a true aspirin prodrug, and on metabolism no aspirin is liberated. For both compounds enzymatic hydrolysis is required to produce antithrombotic effects; NO release occurs in the presence of plasma or platelets but does not take place spontaneously in buffer alone. Either as a COX-inhibitor or as a nitric oxide donor, NCX 4016 proved to be significantly more potent than NCX 4215, showing significant inhibition of platelet TXA₂ production for up to 24 hours following drug administration. Pharmacokinetic studies revealed that NCX 4016 is metabolised to the salicylate; the weak effects on platelet thromboxane synthesis suggest that little, if any, drug is converted to aspirin. It proved active as an antiaggregating agent against arachidonic-acid induced platelet aggregation but only at high concentrations (1mM) whereas aspirin gave complete inhibition at 2 x 10⁻⁵ M.

NO released from NO donors activates COX, resulting in an increase in prostanoid levels in infarcted heart muscle. They may have a role in conditions where NO and prostanoids are of potential benefit, such as myocardial infarction, hypercoagulable states and disseminated intravascular coagulation. Yamamoto reported that NCX 4016 (65 mg/kg/day) gave significant increases in prostacyclin and TXA₂ production in infarcted heart muscle, with no change in systemic blood pressure. It reduced stenosis following balloon angioplasty in hypercholesterolemic mice, with associated reduction in vascular smooth muscle cell proliferation and macrophage deposition at the site of injury. It has been shown to be cardioprotective in ischaemic conditions, improving postischemic ventricular dysfunction and reducing infarct size. The antiplatelet effect of NCX 4016 differs from that of ASA as it inhibits thrombin-induced platelet adhesion and aggregation. It also reduces cytokine release in human monocytes (which are involved in the initiation of thrombosis) by inhibiting capsase-1.
This inhibition of prothrombotic activities in activated monocytes is believed to be due to both NO release and COX inhibition\textsuperscript{121}.

NCX 4016 was found to induce less gastrototoxicity than aspirin. By inhibiting neutrophil adherence it protects the stomach from shock-induced gastric damage\textsuperscript{122}, whereas aspirin had no such protective effect. Tashima studied the effect of aspirin and related NO-aspirins on the stomachs of diabetic rats. He reported that ASA at 30 mg/kg caused hemorrhagic lesions in STZ-diabetic rats yet NCX 4016 at 190 mg/kg (equivalent to 100 mg/kg ASA) had no associated gastrototoxicity\textsuperscript{123} suggesting its potential use in diabetic patients as an antiplatelet therapy for prevention of microvascular complications.

NCX 4016 has reduced COX inhibitory activity relative to aspirin, whereas NCX 4215 has no effect on the COX enzyme. Pharmacokinetic studies on both compounds shows rapid metabolism in the liver during first-pass, liberating salicylic acid and HBN [3-(nitrooxymethyl)phenol] a NO-liberating molecule\textsuperscript{124}. Crucially, however, no aspirin is detectable during metabolism studies. This suggests that while NCX 4016 in particular is a useful NO-releasing ester with a role to play in cardiovascular diseases, it cannot be considered to be a true aspirin prodrug.
1.4.3 Isosorbide-based aspirin prodrugs

A series of compounds based on the isosorbide sugar molecule have been developed in our laboratory and their ability to liberate aspirin on hydrolysis in plasma solution has been evaluated. ISMNA (Isosorbide-2-aspirinate-5-mononitrate (14)) and ISDA (Isosorbide-Diaspirinate (15)) have been extensively studied as potential aspirin prodrugs. Their hydrolysis in 10% human plasma is associated with liberation of 8.43% and 60.0% aspirin respectively. ISMNA was found to liberate up to 78.9% aspirin in 10% rabbit plasma suggesting that the prodrug has certain structural characteristics that make it a better substrate for esterase in rabbit than in human plasma.

Based on these two compounds the isosorbide-2-aspirinate backbone was used to develop a series of aspirin prodrugs, which varied the substituent at the 5-position of the isosorbide molecule. Inserting a salicylic acid molecule at the 5-position to give isosorbide-2-aspirinate-5-salicylate produced a molecule, which liberated up to 91.5% aspirin in 50% human plasma. This is the most successful aspirin prodrug reported to date and is potentially as potent an antiplatelet agent as aspirin.
1.5 Objectives of this thesis

The objective of this body of work was to evaluate the sugar molecule isosorbide as a potential carrier for aspirin prodrugs. The model compound ISMNA described above (Section 1.4.3) was profiled in a number of different species in an effort to explain the large quantities of aspirin liberated on prodrug hydrolysis in rabbit plasma when compared with humans. This study of interspecies variation in prodrug hydrolysis allows for selection of an animal model for prodrug evaluation, which closely parallels human results.

The isosorbide-aspirin prodrugs were appraised as potential antiplatelet agents and their potency relative to the parent compound, aspirin, was established by means of a variety of in vitro tests.

In an effort to improve the physicochemical properties of the most promising antiplatelet agent, isosorbide-2-aspirinate-5-salicylate, a series of esters were prepared which varied the substituent at the 5-position of isosorbide-2-aspirinate (where R = H, 16).

This was believed to be the key residue in the promotion of hydrolysis at the benzoate ester rather than the more labile acetate group of aspirin ester prodrugs. The ability of these compounds to liberate aspirin on enzymatic hydrolysis in human plasma was also investigated.

Isosorbide was also evaluated as a potential model carrier group in design of prodrugs for other carboxylic acids. Benzoic acid (as a model carboxylic acid) and ibuprofen prodrugs of isosorbide were synthesised and compounds were evaluated for their rate of hydrolysis and ability to regenerate the parent compound on hydrolysis in plasma.
Chapter 2

Species variation in hydrolysis of isosorbide-based aspirin esters
2.1 Introduction

Previous work performed in our laboratory involved the synthesis and evaluation of a series of novel isosorbide-based aspirin prodrugs, which may have potential use as antithrombotic agents. These prodrugs, isosorbide mononitrate 2-aspirinate (ISMNA, 14), isosorbide-diaspirinate (ISDA, 15) and isosorbide-2-aspirinate-5-salicylate (Is-2-asp-5-sal) had very different rates and pathways of hydrolysis when profiled in rabbit, dog, rat and human plasma, as summarized in Table 2.1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Species</th>
<th>Molar % aspirin liberated</th>
<th>$t_{1/2}$ (mins)</th>
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</thead>
<tbody>
<tr>
<td>ISMNA</td>
<td>Rabbit</td>
<td>78.7</td>
<td>3.45</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>8.43</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>&lt;0.5</td>
<td>10.81</td>
</tr>
<tr>
<td>ISDA</td>
<td>Rabbit</td>
<td>36.6</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>60.0</td>
<td>4.43</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>&lt;0.5</td>
<td>24.95</td>
</tr>
<tr>
<td>Is-2-asp-5-sal</td>
<td>Rat</td>
<td>0.0</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>81.65</td>
<td>4.90</td>
</tr>
</tbody>
</table>

Table 2.1. Hydrolysis of isosorbide-aspirin prodrugs in 10% plasma solutions of various species.

This is most likely due to the diversity of enzymes in different species. Variation in enzyme-type or distribution may result in certain of the prodrugs acting as ideal substrates for that particular enzyme. For example is-2-asp-5-sal is metabolised extremely rapidly in human plasma with most hydrolysis directed towards aspirin liberation, whereas in rabbits, ISMNA is the ideal substrate for the plasma esterase responsible for prodrug metabolism. This highlights the difficulties in correlating prodrug behaviour in animal models to those in human studies. Metabolism studies carried out in *in vitro* systems, whether intact cells or purified enzymes, allow for the comparison of intrinsic metabolic potential of drugs in one animal species, or across several species including humans. These studies frequently provide a
rational basis for selection or validation of the animal species to be used in toxicological and pharmacological evaluations. Although sometimes limited to quantitative aspects, predictions of \textit{in vivo} metabolism in humans can be made on the basis of results obtained from certain \textit{in vitro} studies. Consequently in this study ISMNA was profiled in a number of different species to evaluate interspecies variation in the metabolism of aspirin prodrugs and to determine the most suitable model for \textit{in vivo} testing of these compounds.

2.2 Enzymatic hydrolysis of prodrugs

\textit{In vitro} metabolism of each prodrug was tested in plasma solution as this is known to be a rich source of esterases, the enzymes generally responsible for the hydrolysis of ester prodrugs \textit{in vivo}\textsuperscript{129}. The hydrolysis of aspirin esters may occur simultaneously through two distinct and separate routes, according to a competitive sequential four-component closed system (Fig. 2.1)\textsuperscript{130}.

\begin{center}
\includegraphics[width=0.7\textwidth]{hydrolysis_diagram.png}
\end{center}

\textit{Figure 2.1 The hydrolytic pathways of aspirin prodrugs}\textsuperscript{130}.

Hydrolysis can occur via the \(k_1\) pathway at the carboxylic ester bond liberating aspirin, or along the \(k_2\) pathway at the O-acetyl group liberating salicylic acid via
the salicylate ester. In order for a compound to be considered a ‘true’ aspirin prodrug, it is necessary that the hydrolytic rate constant \( k_1 \) is greater than that of deacetylation \( k_2 \). The acetyl group of aspirin is exceptionally labile. Esterification and subsequent neutralisation of the carboxylic acid group during prodrug synthesis renders the acetyl group highly susceptible to plasma-mediated hydrolysis. A successful aspirin prodrug must undergo hydrolysis at the carboxylic ester group at a greater rate than the \( O\)-acetyl group \( (k_1 > k_2) \), otherwise it must be considered to be a prodrug of salicylic acid rather than aspirin\(^{130}\).

There are a large number of different esterases present in living systems, and it was desirable to confirm the enzyme-type responsible for metabolism of our prodrugs. It was also necessary to establish the rate and pathway of metabolism of isosorbide-aspirin prodrugs by esterases of different species.

2.2.1 Esterases

In 1914 Sir Henry Dale suggested that an esterase capable of hydrolysing acetylcholine exists in blood\(^{131}\). Esterases are hydrolases that hydrolyse ester bonds as well as peptides, amides and halides, in the presence of water, and so play an important role in the metabolism of a number of compounds used as drugs in humans\(^{132}\). Some esterases are highly specific for their substrate, while others have wide substrate specificity and are capable of hydrolysing both endogenous and exogenous compounds. They follow the general hydrolysis formula\(^{133}\):

\[
\text{R-} \text{O} \quad + \quad \text{H}_2\text{O} \quad \rightarrow \quad \text{R-} \text{O} \quad + \quad \text{R'}\text{OH}
\]

Classification is difficult, as esterases exhibit overlapping substrate specificities and a single substrate is often hydrolysed by more than one enzyme, albeit at different rates. Esterases can be classified on the basis of their interaction with
organophosphates \(^{134}\). Organophosphates hydrolyse A-esterases including arylesterase, they inhibit B-esterases (acetylcholinesterase, butyrylcholinesterase, carboxylesterase) whereas C-esterases (including acetylesterase) do not interact with them \(^{135}\). While this classification has some merit, the International Union of Biochemistry numerical classification is more widely used today \(^{136} \text{ }^{137}\). Table 2.2 lists the most important esterases and their International Enzyme Commission (EC) classification.

<table>
<thead>
<tr>
<th>EC number</th>
<th>Systematic name</th>
<th>Trivial name</th>
<th>Typical substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.1.1</td>
<td>Carboxylic ester hydrolase</td>
<td>Carboxylesterase</td>
<td>Aliphatic esters</td>
</tr>
<tr>
<td>3.1.1.2</td>
<td>Aryl ester hydrolase</td>
<td>Arylesterase</td>
<td>Aromatic esters</td>
</tr>
<tr>
<td>3.1.1.3</td>
<td>Glycerol-ester hydrolase</td>
<td>Lipase</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>3.1.1.6</td>
<td>Acetic ester</td>
<td>Acetylesterase</td>
<td>Acetic esters</td>
</tr>
<tr>
<td>3.1.1.7</td>
<td>Acetylcholine acetyl hydrolase</td>
<td>Acetylcholinesterase</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>3.1.1.8</td>
<td>Acylcholine acylhydrolase</td>
<td>Cholinesterase</td>
<td>Acylcholine</td>
</tr>
</tbody>
</table>

*Table 2.2 Classification of esterases* \(^{132}\)

A drug may be hydrolysed by more than one esterase at different sites. Aspirin is hydrolysed initially to salicylic acid by liver carboxylesterase during first-pass metabolism. Up to 60% of an oral dose reaches the systemic circulation where it is then further metabolised by plasma cholinesterase, albumin and red blood cell arylesterases \(^{132}\). The three main esterase groups of interest in prodrug hydrolysis are the cholinesterases, arylesterases and carboxylesterases. Cholinesterases are primarily involved in drug metabolism in the plasma; arylesterases, in the plasma and red blood cells, and carboxylesterases, in the liver, gut and other tissues \(^{132}\). The hydrolysis of model substrates by these enzymes is shown in Fig. 2.2.

Serum arylesterase (paraoxonase) is capable of hydrolysing toxic organophosphates, carbamates and aromatic esters \(^{138}\). There is evidence to suggest that it is capable of inactivating toxic by-products, resulting from oxidation of lipid components of the blood low density lipoproteins (LDL) \(^{139}\), thus protecting the mammalian host to some degree against atherosclerosis \(^{140}\). Its activity is reduced
in patients suffering from myocardial infarction, hypercholesterolaemia and diabetes mellitus\textsuperscript{141}.

Aspirin is metabolised to salicylic acid \textit{in vivo}, the initial hydrolysis is due to enzymes known collectively as ‘aspirin esterases’, further hydrolysis is partly due to chemical hydrolysis in aqueous medium \textsuperscript{142}. There is a close relationship between plasma aspirin esterase activity and aspirin disposition and effect. Studies have demonstrated that enzyme activity correlates inversely with the plasma half-life of salicylate. In human plasma, aspirin esterase activity has been identified as plasma cholinesterase. In the gastric mucosa and liver it appears to be associated with carboxylesterases \textsuperscript{132}. Patients with alcoholic liver disease exhibit lower aspirin esterase activity as a result of lowered albumin and cholinesterase levels. Cholinesterases in plasma and serum are also primarily responsible for the hydrolysis of aspirin prodrugs liberating aspirin and other metabolites on metabolism \textit{in vivo}. Our work focuses on the interspecies variation of cholinesterases (both enzyme type and distribution) and how it affects prodrug metabolism \textit{in vitro}. Variations in enzyme activity may result in different hydrolysis kinetics, affecting both rate and pathway of hydrolysis, and thus the amount of aspirin liberated on prodrug metabolism.
Figure 2.2 Hydrolysis of model substrates by esterases.
2.2.2 Cholinesterases

The cholinesterases found in vertebrates are considered to fall into two separate families: true cholinesterase (acetylcholinesterase EC 3.1.1.7, (AChE)) and pseudocholinesterase (butyrylcholinesterase EC 3.1.1.8, (BuChE)). They contain two binding sites at the active centre of the molecule, an anionic and an esteratic site, whereas other esterases lack an anionic site. The anionic site is a negatively charged region of the enzyme surface, which binds the positively charged quaternary nitrogen atom of the substrate or inhibitor. The esteratic site attacks the carboxyl group of the ester linkage and is responsible for the actual hydrolysis and substrate specificity. The participation of a serine residue at the active site has lead to a further classification of AChE and BuChE as serine esterases. Both cholinesterases are widely distributed throughout the body, including brain, serum, liver, intestinal mucosa and other tissues, where they occur in a variety of molecular forms. Although the overall structure of both cholinesterases is similar, the differences in dimensions and the microenvironment of the active site gorge play a significant role in determining the selectivity of substrate and inhibitors for cholinesterases. The volume of the BuChE active-site gorge is ~200 Å³ larger than that of the AChE gorge, which allows for accommodation of larger substrates, in different orientations.

Acetylcholinesterase is found in nervous tissue, striated muscle and red blood cells. It catalyses the rapid hydrolysis of the excitatory neurotransmitter acetylcholine, thereby terminating impulse transmission at cholinergic synapses. However it can also hydrolyse propionylcholine and acetyl-β-methylcholine and their thiocholine analogues. Inhibition studies with AChE have shown that excess acetylcholine substrate inhibits its own rate of hydrolysis. This is believed to be due to the formation of an enzyme-disubstrate complex, which inhibits further hydrolysis of the substrate.

Butyrylcholinesterase found in the circulatory system, has a broader specificity but its specific physiological role is unknown. It is capable of hydrolysing a wide range of choline esters such as acetyl-, propionyl- and butyrylthiocholine and their thio- analogues as well as a number of other esters. Butyrylcholine and butyrylthiocholine are specific substrates for pseudocholinesterase. It can also be distinguished from AChE by its greater...
sensitivity to inhibition by the organophosphate iso-OMPA (tetramonoisopropylpyrophosphate). It is much less sensitive to inhibition by excess substrate than AChE, but it is inhibited by organophosphates. BuChE is synthesised in the liver in parallel with serum albumin and so in hepatic disorders such as liver cirrhosis, serum cholinesterase activity is depressed. Thus measurements of serum cholinesterase levels have been used as markers of many disease states including hepatitis, malaria and liver carcinomas.

The serum cholinesterases are responsible for the hydrolysis of many commonly used therapeutic agents. This hydrolysis may result in the production of a less active metabolite, e.g. aspirin is hydrolysed to salicylic acid, similarly suxamethonium and procaine are metabolised to less active molecules. Conversely hydrolysis may result in the production of a more pharmacologically active agent from the parent prodrug as occurs in heroin, clofibrate, enalapril and benorylate metabolism. The activities of plasma cholinesterase are thus important in the determination of pharmacological action of many agents and measurement of BuChE activity is discussed in greater detail in Section 2.3.4.

### Inhibitors of cholinesterases

Cholinesterases are distinguished from other esterases by a complete inhibition in the presence of 10 μmol/l eserine (physostigmine). Eserine (17) is a potent competitive inhibitor of cholinesterases; it is considered to be a poor substrate of these enzymes and its interaction with cholinesterases was shown to obey classic Michaelis-Menten kinetics. These enzymes are also susceptible to inhibition by specific inhibitors that bind reversibly or irreversibly to the active site. Irreversible inhibitors include organophosphates and carbamates while reversible inhibitors generally bear some structural resemblance to cholinesters.
Dibucaine (18), used clinically as an anaesthetic agent, is a positively charged quaternary ammonium compound which differentially inhibits cholinesterase variants. Certain individuals possess an atypical form of serum cholinesterase which results in an exaggerated response to the muscle relaxant drug, succinylcholine. This atypical esterase is also less susceptible to inhibition by dibucaine, and this anomaly is exploited in diagnostic tests for the condition.

AChE is reversibly inhibited by excess quantities of acetylcholine and is irreversibly inhibited by organophosphate compounds such as DFP (diisopropylfluorophosphate), paraoxon (diethyl p-nitrophenylphosphate) and TEPP (tetrakisethoxy dehydroxyphosphate), which bind the enzyme at the esteratic site. Organophosphates react with the active site serine in AChE forming a stable conjugate, which inactivates the enzyme (Fig 2.1).
BuChE is not as sensitive to inhibition by excess acetylcholine but is inhibited by organophosphates, carbamates, quaternary ammonium salts and eserine. Several inhibitors have been shown to be selective for either AChE or BuChE. Tacrine (9-amino-1,2,3,4-tetrahydroacridine) is found to have a higher affinity for BuChE than AChE; at 2.5 μmol/ml it resulted in complete inhibition of BuChE and 70% of AChE activity. AChE is specifically inhibited by BW284C51 while iso-OMPA is specific for BuChE, which it selectively and irreversibly inhibits.

2.2.4 Species variation in enzyme distribution

As previously outlined in Section 2.2.2, there are two types of cholinesterase present in mammalian blood: acetylcholinesterase (AChE, EC 3.1.1.7), also known as erythrocyte or true cholinesterase and found associated with red blood cells, and butyrylcholinesterase (BuChE, EC 3.1.1.8), also known as pseudocholinesterase, which is found in plasma. Of considerable pharmacological interest is the lack of uniformity in the plasma esterase activities of various mammalian species. Interindividual variation in the activity of esterases influences both the pharmacological and toxicological effects of prodrugs in humans. In 1943 Mendel using methacholine and benzoylcholine (specific substrates for AChE and BuChE respectively) showed that the plasma of six mammalian species and two bird species contained both esterases, with BuChE being predominant in plasma of most species examined, although plasma of sheep had no detectable levels of BuChE. In 1961, Augustsson reported that the cholinesterases of different species induced different rates of hydrolysis for different choline esters. Rat plasma
metabolised propionylcholine most rapidly, guinea-pig and dog preferentially hydrolysed butyrylcholine while rabbit plasma was most efficient at hydrolysing acetyl- and propionylcholine. Inhibition experiments with specific inhibitors have shown that esterases in rabbit serum have properties of both AChE and BuChE, and even some features of carboxylesterase (CarbE).

A variety of xenobiotics are metabolised by carboxyl ester hydrolases, enzymes that exhibit broad substrate specificity and hydrolytic activities and vary among species and individuals. They are present in a wide variety of organs and tissues of many mammalian species, with highest activity occurring in the liver of all species tested. Several isozymes of CarbE are found in the microsomal fraction of various animal species. CarbE activity in rat whole blood was found to be 100 and 400 times higher than that in dog or human whole blood respectively.

Interspecies variation in enzyme distribution is not the only issue to be considered: there is frequently variation in enzymes within the same species, e.g. in addition to the usual form of BuChE, there are approximately nine variants present in human serum, resulting from different gene mutations. Cholinesterase activity is known to vary with sex and age in some species. For example, ritonavir, a potent, orally active HIV-1 protease inhibitor, produced significantly higher plasma levels in female rats than in male rats. This gender specificity is commonly seen in rats but not in dogs or humans.

Diet can also play a major role in esterase activity in different species. Studies by Inoue have shown that esterase activity is elevated in the intestines of rats fed a high-fat diet, and markedly decreases from normal levels in rats fed a fat-free diet or who are fasted. In some in-bred strains of rabbits it was found that variation in the response of plasma cholesterol level to dietary cholesterol was associated with a genetically determined variation in plasma esterases.
2.3 Plasma hydrolysis Studies

2.3.1 Procedures in plasma hydrolysis studies

The hydrolysis of ISMNA (14) has previously been studied in plasma solutions of humans, rabbits and dogs \(^{125,126}\). ISMNA was found to liberate <0.5%, 8.43% and 78.7% aspirin in 10% solutions of dog, human and rabbit plasma respectively. The prodrug was hydrolysed extremely rapidly in all plasma-types (Table 2.1), indicating that 14 is a good substrate for esterases through all species. In dog and human plasma, hydrolysis occurred primarily via the salicylate route \((k_2)\), whereas in rabbit plasma the \(k_1\) pathway leading to aspirin liberation was predominant. This suggests that ISMNA is an excellent substrate for a BuChE variant in rabbit plasma, directing hydrolysis towards optimum aspirin release. To extend this study further, the route of hydrolysis of 14 was examined in a variety of different species to determine if the unusually high liberation of aspirin observed was unique to rabbit plasma.

Whole blood was collected by venipuncture into vessels containing sodium citrate as anticoagulant. It was immediately centrifuged; plasma was collected and stored at 4-8°C until required. Excess plasma was stored at -20°C in quantities suitable for one day's testing. Studies have shown that there is no significant decrease in cholinesterase activity caused by storing samples at -20°C for periods up to 14 months \(^{171}\). The points of sampling and volumes of anticoagulant used are presented in Table 2.3. All blood samples were treated with 10% sodium citrate except some of the rabbit and dog samples, which had a much higher percentage anticoagulant. Data from studies using these samples were used for observation of trends only, and were not incorporated into results presented here.
<table>
<thead>
<tr>
<th>Species</th>
<th>Point of sampling</th>
<th>Ratio of citrate: blood</th>
<th>Total volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Fore-arm</td>
<td>1: 10</td>
<td>10</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Ear</td>
<td>1: 10, 1: 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>Rat</td>
<td>Heart</td>
<td>1: 10</td>
<td>10</td>
</tr>
<tr>
<td>Dog</td>
<td>Jugular</td>
<td>1: 10, 1:1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>Hamster</td>
<td>Heart</td>
<td>1: 10</td>
<td>6</td>
</tr>
<tr>
<td>Guinea-Pig</td>
<td>Ear</td>
<td>1: 10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.3 Preparation of blood samples. <sup>a</sup> Samples of rabbit and dog blood which contained higher levels of anticoagulant were not used in tabulating results.

2.3.2 Species variation in hydrolysis of ISMNA

For each species ISMNA hydrolysis was studied in 10% buffered plasma (pH 7.4) at 37°C according to the experimental procedure in *Chapter 6*. Final samples were analysed by reverse phase HPLC using a mobile phase that afforded good separation of the prodrug and its metabolites. The potential hydrolysis pathway of 14, with its probable metabolites, is presented in *Fig 2.4*. Hydrolysis can occur at the benzoic acid ester bond ($k_1$) liberating aspirin, or at the acetyl ester bond liberating salicylic acid ($k_2$), via ISMN-salicylate (19).
In the development of an aspirin prodrug it is desirable that hydrolysis occur at least partially through the $k_1$ pathway with the liberation of aspirin. The analytical method involved isocratic elution on a Waters Nova-Pak® C8 column (3.9 x 150 mm) with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%) and a flow rate of 1 ml/min. Interferences from sample preparation did not affect peak separation. The concentration of analytes in the plasma samples was calculated using peak areas, with reference to standard samples analysed on the same day under the same chromatographic conditions. The chromatographic method was validated for linearity, precision, sensitivity and specificity for each different type of plasma used. The limit of quantitation for each analyte was 1 µg/ml. The RSD on multiple injection of each analyte at 10
\( \mu g/ml \) and 100 \( \mu g/ml \) was < 1.4%. A linear response was observed for each analyte in the range 1 – 100 \( \mu g/ml \) \((r^2 > 0.998)\).

2.3.2.1 ISMNA in human plasma

The hydrolysis of ISMNA was studied in 10% buffered human plasma (pH 7.4) at 37\(^{\circ}\)C. A typical progression curve for hydrolysis of 14 is presented in Fig. 2.5.

![Figure 2.5 Typical progression curve for the hydrolysis of ISMNA in 10% human plasma at pH 7.4 and 37\(^{\circ}\)C: ISMNA (□), ISMN-salicylate (■), aspirin (○) and salicylic acid (○).](image)

Hydrolysis was associated with the liberation of a mixture of ISMN-salicylate (19), salicylic acid (2) and aspirin (1). Aspirin was the minor product of metabolism with 7.23% detected in plasma samples. This is consistent with results obtained previously \(^{126}\). The small quantities of aspirin liberated by ISMNA may be responsible for the weak anti-platelet effect observed in humans \(^{172}\). However in such low quantities it is unlikely to be useful clinically. The salicylate ester (pink line in Fig. 2.5) showed very rapid hydrolysis to salicylic acid. This was unexpected, as the rate of plasma-mediated hydrolysis of salicylate esters is typically slow, e.g. methyl-salicylate hydrolyses in 80% human plasma with a half-life of 17.6 hours \(^{101}\). It appears that some structural feature of the ISMN group promotes rapid hydrolysis of the salicylate ester in human plasma solution.
Nielsen and Bundgaard reported that for a series of aspirin esters tested in human plasma solution, <0.5% aspirin was liberated, due to rapid hydrolysis at the acetyl group of the ester. This unusual preference for hydrolysis at the carboxyl ester of ISMNA may be due to the ISMN group, which is suppressing attack at the acetyl group. This may be exploited in development of a novel isosorbide-based aspirin prodrug, which promotes hydrolysis exclusively at the carboxyl ester linkage thus optimising aspirin release from the compound.

2.3.2.2 ISMNA in rabbit plasma

The hydrolysis of ISMNA was studied in 10% buffered rabbit plasma (pH 7.4) at 37°C. The pathway for hydrolysis is presented in Fig. 2.6.

Hydrolysis of 14 liberated 58.3% aspirin in 10% rabbit plasma based on initial ester concentrations. While this followed previously observed patterns, release of up to 78.7% aspirin has been noted in some rabbit plasma samples. The slightly lower aspirin release noted in the hydrolysis study presented above may be due to variation in rabbit plasma BuChE between sources. Researchers have reported on a
subtype of BuChE present in rabbit plasma, which is of lower molecular weight, and may be responsible for up to 75% of total cholinesterase activity \(^{173}\). Variation in ester hydrolysis by rabbit sera may be due to variations in distribution of this enzyme subtype \(^{173}\). Hydrolysis of ISMNA is primarily directed towards the carboxylic ester group; the esterase in rabbit plasma differs from the human form in protecting the labile acetyl group from hydrolysis, thus optimising release of aspirin.

2.3.2.3 ISMNA in dog plasma

The hydrolysis of ISMNA was studied in 10% buffered dog plasma (pH 7.4) at 37°C. A typical progression curve for the hydrolysis of ISMNA is presented in Fig 2.7. The results of these hydrolysis studies were consistent with those obtained previously in our laboratory \(^{127}\).

![Figure 2.7 Typical progression curve for the hydrolysis of ISMNA in 10% dog plasma at pH 7.4 and 37°C: ISMNA ( ), ISMN-Salicylate ( ), aspirin ( ), salicylic acid ( ).](image)

The predominant route of hydrolysis in 10% dog plasma proceeds via deacetylation of the prodrug, to liberate ISMN-salicylate (pink line in Fig. 2.7). Once liberated this metabolite is very stable towards further hydrolysis with no observable decrease in concentration noted over the duration of the experiment.
This is in contrast to human plasma (Section 2.3.2.1.) where 19 is rapidly hydrolysed to salicylic acid. This difference may be accounted for by variations in the active site of the esterase between species, whereby ISMN-salicylate is a better substrate for the variant found in human plasma. Aspirin and salicylic acid are minor products of hydrolysis (<5%).

2.3.2.4 ISMNA in hamster plasma

The hydrolysis of ISMNA was studied in 10% buffered hamster plasma (pH 7.4) at 37°C. Analysis was repeated in triplicate and the average progression curve is presented in Fig 2.8.

![Figure 2.8 Typical progression curve for the hydrolysis of ISMNA in 10% hamster plasma at pH 7.4 and 37°C: ISMNA (\(\triangle\)), ISMN-Salicylate (■), aspirin (●), salicylic acid (○).](image)

Similarly to the results obtained in rabbit plasma solution, the hydrolysis of ISMNA in hamster plasma was rapid and associated with the liberation of large quantities of aspirin (range 67.4 – 70.2 %, mean 68.8%). Hydrolysis is promoted at the carboxylic ester group, with the aspirin being a major product of metabolism.
2.3.2.5 ISMNA in rat plasma

The hydrolysis of ISMNA was studied in 10% buffered rat plasma (pH 7.4) at 37°C. Analysis was repeated in triplicate and the average progression curve is presented in Fig 2.9.

The predominant route of hydrolysis of ISMNA in rat plasma is via deacetylation with liberation of ISMN-salicylate as the primary metabolite. As in dog plasma this remains stable to further hydrolysis with only trace amounts of salicylic acid being liberated. This highlights the species variation in ester hydrolysis, whereby 19 is extremely stable in rat and dog plasma, while being rapidly metabolised to salicylic acid in human and rabbit samples. No aspirin was detectable in the plasma samples suggesting that 14 acts as a prodrug for salicylate in rat plasma. Hydrolysis of ISMNA in rat plasma is extremely rapid with a half-life of 2.46 mins.

Figure 2.9 Typical progression curve for the hydrolysis of ISMNA in 10% rat plasma at pH 7.4 and 37°C: ISMNA (□), ISMN-Salicylate (■), salicylic acid (○).
2.3.2.6 ISMNA in guinea-pig plasma

The hydrolysis of ISMNA was studied in 10% buffered guinea-pig plasma (pH 7.4) at 37°C. Analysis was repeated in triplicate and the average progression curve is presented in Fig 2.10.

![Graph](image)

*Figure 2.10 Typical progression curve for the hydrolysis of ISMNA in 10% guinea-pig plasma at pH 7.4 and 37°C: ISMNA (▲), ISMN-Salicylate (■), salicylic acid (○).*

As in rat plasma, ISMNA is hydrolysed very rapidly in guinea-pig plasma, solely *via* the salicylate route generating ISMN-salicylate and salicylic acid as the main metabolites. No aspirin was detectable during the hydrolysis study. Unlike rat and dog plasma however, the ISMN-salicylate is not as stable in the presence of guinea-pig esterases and is further hydrolysed to salicylic acid.
2.3.3 Determination of Michaelis-Menten Parameters

Hydrolysis studies in plasma facilitate the evaluation of such kinetic parameters as the rate constant \( (k_{\text{obs}}) \), half-life \( (t_{1/2}) \) and parameters relating to the fit of data to Michaelis-Menten kinetics. In typical Michaelis-Menten kinetics the rate of hydrolysis initially follows zero-order kinetics, and as the reaction proceeds and substrate concentration decreases the rate becomes first-order. The progression-curve for an enzyme-catalysed reaction commences as linear in the initial-rate phase, but the rate falls off with time. The computer analyses of progression curves for the plasma hydrolysis studies presented above were performed using ‘Scientist’ Micromath Scientific software \(^{174}\). Computer analysis involves a series of mathematical and computational functions, including the derivitisation of an integrated rate equation and its use in a non-linear regression program where \( K_m \) and \( v_{\text{max}} \) are the parameters to be estimated. \( K_m \) is referred to as the ‘apparent affinity constant’, and is the value of \( S \) (substrate concentration) yielding half-maximal rate. It is of importance in establishing the substrate concentration range exhibiting control of the enzymic activity under the given circumstances \(^{175}\). The integrated form of the Michaelis-Menten equation was employed (\textit{Eq. 2.1}), where \( t \) is the independent and \( S \) is the dependent variable.

\[
v_{\text{max}} \cdot t = (S_0 - S) + K_m \cdot \ln \left( \frac{S_0}{S} \right)
\]

(2.1)

The unknown parameters are \( S_0, v_{\text{max}} \) and \( K_m \). \( S_0 \) (initial substrate concentration) is estimated from the intercept of a plot of log of remaining prodrug concentration versus time \(^{176}\). The \( v_{\text{max}} \) and \( K_m \) were given initial estimated values from which the computer fit was generated \(^{174}\).

The half-life and rate constant \( (k_{\text{obs}}) \) data for ISMNA hydrolysis in each species was determined from a plot of the logarithm of remaining ester concentration against time, and are presented in \textit{Table 2.4}. 


57
The rate and pathway of hydrolysis of ISMNA varied greatly between the different species examined. Rabbit and hamster plasma liberated the greatest quantity of aspirin on hydrolysis of the prodrug by plasma esterases, suggesting that ISMNA (14) is a good substrate for a butyrylcholinesterase subtype present in these herbivores. However their rates of hydrolysis varied, with ISMNA being very rapidly metabolised in rabbit plasma ($t_{1/2}$ 2.35 mins) and more slowly in hamster plasma ($t_{1/2}$ 14.0 mins). Dog and human plasma gave similar results with both liberating small amounts of aspirin but most hydrolysis directed via the salicylate route (4.98 and 7.23 % aspirin liberated respectively). 14 was hydrolysed much more rapidly in human plasma ($t_{1/2}$ 1.04 mins) than in dog plasma ($t_{1/2}$ 19.41 mins). Both rat and guinea-pig plasma showed extremely rapid hydrolysis of ISMNA ($t_{1/2}$ 2.46 and 0.11 mins respectively) but in both species hydrolysis was directed solely through the salicylate route with no aspirin detectable in the samples. Rabbit and rat plasma had lowest $K_m$ values (0.108 and 0.110 x $10^{-4}$ M respectively) indicating a stronger affinity of ISMNA for the enzyme-type in these plasma solutions.
Determination of plasma cholinesterase activity

Plasma activity of the esterases can affect the pharmacological activity of commonly used drugs such as aspirin, and the rate of metabolism of ester-type prodrugs. The significantly different rates and pathways of ISMNA hydrolysis of each species examined may be correlated with the BuChE level in each of the different plasma types. The butyrylcholinesterase activity of each of the plasma solutions used in our species variation experiments was monitored to determine if there was a significant interspecies variation in enzyme activity, which may explain this variation in rate and pathway of prodrug metabolism.

The determination of serum BuChE activity is often requested as a test of hepatocellular function, to monitor poisoning and excessive exposure to anticholinesterase organophosphorus insecticides, and to detect patients with atypical forms of the enzyme that could react abnormally to suxamethonium. Blood cholinesterase activity can be measured spectrophotometrically in whole blood, plasma or erythrocytes. Numerous methods have been described to determine the activity of serum cholinesterase including electrometric ΔpH, pH-stat methods and spectrometric methods. The method used in this study was a modification of the Ellman technique and the experimental protocol is described in Section 6.3.4. This method allows for estimation of the activities of cholinesterases in plasma or serum, red blood cells, whole blood, and homogenates of tissues such as lung, liver, kidney, brain and skeletal muscle. Enzyme activity can be estimated by either spectrophotometrically monitoring the rate of thiocholine formation when DTNB (5,5'-dithiobis-2-nitrobenzoic acid) reacts with acetyl, propionyl or butyryl thiocholine as substrate, or the choline formation from benzoylcholine chloride. Acetylthiocholine iodide (ACTI) may be used as a single substrate for determination of both red blood cell and plasma acetylcholinesterase activity. In this experiment the reaction of a thiocholine ester (S-butyrylthiocholine iodide, BTCI, 20) with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 21) as shown in Fig 2.11 was monitored. BTCI is hydrolysed by plasma butyrylcholinesterase at a higher rate than acetyl or propionylcholine, it is the least labile of the thiocholine esters and is relatively specific for BuChE and so is the substrate of choice for this assay. The reaction involves the interaction of thiocholine formed...
from the hydrolysis of butyrylthiocholine by BuChE with the coupling reagent DTNB to release the anion 5-thio-2-nitrobenzoic acid (TNB, 22), which is yellow in colour.\(^{183}\)

![Chemical structures and reactions](attachment:image.png)

**Figure 2.11. Reactions involved in estimating thiocholine ester hydrolysis\(^ {149}\).**

This method involves the measurement of reaction product instead of the remaining intact substrate, and has the advantages of simplicity, high precision, pH constancy, flexibility, short incubation times and continuous increase in colour density as a function of incubation time.\(^ {149}\) It is a rapid, simple, cheap assay and so is the
preferred method for occupational health screening and therapeutic monitoring of pesticide-exposed patients. The assay is performed at pH 7.4 to eliminate non-enzymic hydrolysis of the substrate while retaining a linear response at 37°C.

2.3.4.1 Procedure for determination of plasma esterase activity

Plasma samples from each species – human, rabbit, dog, rat, hamster and guinea-pig were analysed for their butyrylcholinesterase activity on initial sampling and following storage at 4-8°C, according to a modification of the method developed by Ellman. A solution consisting of S-butyrylthiocholine iodide (100 µl, 10 mM), DTNB (400 µl, 10 mM) and phosphate buffer pH 8.0 (2.626 ml) was prepared and the reaction was activated by addition of plasma (4 µl). The liberation of the coloured anion was recorded over a period of 10 mins on a UV spectrophotometer at 412 nm.

2.3.4.2 Results

In plasma samples, differences in the rate of hydrolysis and substrate activity were observed among different species as previously reported. Table 2.5 presents measured activities of butyrylcholinesterase in plasma solutions along with literature values.

<table>
<thead>
<tr>
<th>Species</th>
<th>Measured BuChE activity (nM/ml/min)</th>
<th>Literature BuChE activity (nM/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>2482 (1829-3636)</td>
<td>3300</td>
</tr>
<tr>
<td>Rabbit</td>
<td>775 (305-2128)</td>
<td>750</td>
</tr>
<tr>
<td>Dog</td>
<td>2160 (742-2875)</td>
<td>2300</td>
</tr>
<tr>
<td>Rat</td>
<td>607 (524-644)</td>
<td>720</td>
</tr>
<tr>
<td>Hamster</td>
<td>188 (75-209)</td>
<td>200</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>1200 (400-1600)</td>
<td>2000</td>
</tr>
</tbody>
</table>

Table 2.5 Butyrylcholinesterase activities of plasma from different species.

Multiple isozymic and polymeric forms of pseudocholinesterase are frequently observed in species variation studies, as are the substrate specificity and quantity of enzyme present. Results obtained in this study were consistent with literature values.
findings. Venkataraman reported that rat plasma was four-fold less active than human plasma at hydrolysing butyrylthiocholine iodide, a result noted in our experiments. Dog plasma was found to have similar levels of plasma BuChE to humans, again consistent with literature reports, although most such studies are now performed in canine whole blood. Plasma cholinesterase activity fell in the following order: human > dog > guinea-pig > rabbit > rat > hamster, consistent with previously reported findings. Guinea-pig plasma contained approximately 50% of the activity found in human plasma, while rabbit, rat and hamster plasma showed extremely low BuChE activity. This is consistent with the slower rate of hydrolysis of ISMNA noted in hamster plasma (t_{1/2} 14.0 mins) compared with its rapid hydrolysis in human plasma (t_{1/2} 1.04 mins). In rat plasma it is possible that a variant of BuChE or indeed another enzyme such as carboxylesterase is involved in isosorbide-aspirin prodrug metabolism (t_{1/2} 2.46 mins). This would account for the rapid prodrug hydrolysis in the presence of low BuChE activity and also the exclusive liberation of salicylic acid, with no aspirin being generated.
2.4 Enzyme inhibition studies

2.4.1 Introduction

As discussed in Section 2.2.4, esterases in human plasma are susceptible to inhibition by various specific inhibitors. Butyrylcholinesterase (EC 3.1.1.8) was believed to be the enzyme responsible for ISMNA hydrolysis in plasma. In order to confirm this hypothesis a number of inhibitors were examined for their ability to alter the rate or pathway of hydrolysis of ISMNA.

2.4.2 Procedures in inhibition studies

Enzyme inhibitor studies are considered to be useful analytical tools, since it is well established that the different classes of esterases are inhibited to varying degrees by different inhibitors. The hydrolysis of ISMNA was studied in five different plasma solutions: human, rabbit, rat, hamster and dog in the presence of a variety of inhibitors (PMSF, eserine, dibucaine and iso-OMPA). PMSF (phenylmethylsulfonylfluoride) is an inhibitor of serine proteases (trypsin and chymotrypsin) and mammalian acetylcholinesterase. Dibucaine is specific for a particular butyrylcholinesterase subtype. Eserine is a potent inhibitor of pseudocholinesterase and iso-OMPA is a selective BuChE inhibitor. Rabbit plasma hydrolysis of 14 was also studied in the presence of EDTA, an arylesterase inhibitor. Hydrolysis studies were performed in 50% plasma solutions at pH 7.4 and 37°C according to the experimental procedure described in Section 6.3.7. Plasma was initially incubated with PMSF (100 µM), dibucaine (1 mM), eserine (100 µM), EDTA (1 mM) or iso-OMPA (10 µM) for 60 mins at 37°C. Each plasma solution was then spiked with ISMNA (10 µl of a 4 mg/ml solution in acetonitrile) and sampled in the usual manner. All samples were analysed for concentration of prodrug and its metabolites, using the HPLC method detailed in Section 2.3.2. Control experiments were performed on each plasma solution, on the same day and under the same experimental conditions.
2.4.3 Results of enzyme inhibition studies

Results of inhibitor studies are presented separately for ISMNA hydrolysis in each species tested. All results are compared to a control experiment of plasma hydrolysis of 14 in each species performed on the same day as the inhibitor experiment.

2.4.3.1 Inhibitor studies in human plasma

The hydrolysis of ISMNA was studied in 50% pooled human plasma (pH 7.4, 37°C), co-incubated with a variety of specific inhibitors of a range of different esterases. The aim of this experiment was to confirm the role of butyrylcholinesterase in prodrug hydrolysis. Results are presented in Table 2.6.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Conc.</th>
<th>Target enzyme</th>
<th>( k_{\text{obs}} ) ( \text{min}^{-1} )</th>
<th>( t_{1/2} ) ( \text{min} )</th>
<th>% Aspirin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0.16</td>
<td>4.23</td>
<td>7.63</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>1 mM</td>
<td>BuChE subtype</td>
<td>0.16</td>
<td>4.23</td>
<td>6.75</td>
</tr>
<tr>
<td>PMSF</td>
<td>100 µM</td>
<td>Serine proteases, AChE</td>
<td>0.24</td>
<td>2.86</td>
<td>6.19</td>
</tr>
<tr>
<td>Eserine</td>
<td>100 µM</td>
<td>Cholinesterase</td>
<td>0.03</td>
<td>177.69</td>
<td>0.00</td>
</tr>
<tr>
<td>iso-OMPA</td>
<td>10 µM</td>
<td>BuChE</td>
<td>0.02</td>
<td>38.29</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Table 2.6 Kinetic data for the hydrolysis of ISMNA co-incubated with specific esterase inhibitors in 50% pooled human plasma (pH 7.4, 37°C).

Eserine, which is a non-specific cholinesterase inhibitor, and iso-OMPA, which is a specific BuChE inhibitor in plasma, had the most profound effect on ISMNA hydrolysis in human plasma. Both compounds induced a dramatic alteration in the rate of prodrug hydrolysis. Interestingly no aspirin was liberated during human plasma hydrolysis of ISMNA, on prior incubation with either of these two inhibitors. This suggests that BuChE is the enzyme responsible for ISMNA hydrolysis in human plasma, and inhibition of this enzyme abolishes enzymatic hydrolysis of the prodrug, resulting only in non-enzymatic hydrolysis and subsequent salicylic acid release in vitro.
2.4.3.2 Inhibitor studies in dog plasma

The hydrolysis of ISMNA was studied in 50% solution of dog plasma (pH 7.4, 37°C), co-incubated with a variety of specific inhibitors of a range of different esterases. The aim of this experiment was to confirm the role of butyrylcholinesterase in prodrug hydrolysis. Results are presented in Table 2.7.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Conc.</th>
<th>Target enzyme</th>
<th>$k_{obs}$</th>
<th>$t_{1/2}$</th>
<th>% Aspirin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0.47</td>
<td>1.45</td>
<td>4.92</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>1 mM</td>
<td>BuChE subtype</td>
<td>0.08</td>
<td>8.96</td>
<td>4.12</td>
</tr>
<tr>
<td>PMSF</td>
<td>100 μM</td>
<td>Serine and other proteases, AChE</td>
<td>0.07</td>
<td>9.59</td>
<td>5.73</td>
</tr>
<tr>
<td>Eserine</td>
<td>100 μM</td>
<td>Cholinesterase</td>
<td>0.02</td>
<td>42.52</td>
<td>0.00</td>
</tr>
<tr>
<td>iso-OMPA</td>
<td>10 μM</td>
<td>BuChE</td>
<td>0.01</td>
<td>70.71</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Table 2.7 Kinetic data for the hydrolysis of ISMNA co-incubated with specific esterase inhibitors in 50% dog plasma (pH 7.4, 37°C).

Similarly to human plasma, ISMNA hydrolysis in dog plasma is inhibited by eserine and iso-OMPA. Prodrug hydrolysis in the presence of either of these inhibitors is considerably slower and abolishes the liberation of aspirin noted in uninhibited plasma solutions. This suggests that BuChE is the enzyme responsible for prodrug hydrolysis in dog plasma, and its inhibition by selective inhibitors results only in non-enzymatic prodrug hydrolysis with subsequent liberation of salicylic acid.
2.4.3.3 Inhibitor studies in rabbit plasma

The hydrolysis of ISMNA was studied in 50% solution of rabbit plasma (pH 7.4, 37°C), co-incubated with a variety of specific inhibitors of a range of different esterases. The aim of this experiment was to confirm the role of butyrylcholinesterase in prodrug hydrolysis in rabbits. Results are presented in Table 2.8.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Conc.</th>
<th>Target enzyme</th>
<th>$k_{\text{obs}}$ min$^{-1}$</th>
<th>$t_{1/2}$ min</th>
<th>% Aspirin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0.12</td>
<td>5.78</td>
<td>67.36</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>1 mM</td>
<td>BuChE subtype</td>
<td>0.11</td>
<td>6.13</td>
<td>59.25</td>
</tr>
<tr>
<td>PMSF</td>
<td>100 μM</td>
<td>Serine</td>
<td>0.16</td>
<td>4.39</td>
<td>38.59</td>
</tr>
<tr>
<td>Eserine</td>
<td>100 μM</td>
<td>Cholinesterase</td>
<td>0.07</td>
<td>9.72</td>
<td>0</td>
</tr>
<tr>
<td>iso-OMPA</td>
<td>10 μM</td>
<td>BuChE</td>
<td>0.02</td>
<td>38.29</td>
<td>0.22</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>Arylesterase</td>
<td>0.11</td>
<td>6.25</td>
<td>66.11</td>
</tr>
</tbody>
</table>

Table 2.8 Kinetic data for the hydrolysis of ISMNA co-incubated with specific esterase inhibitors in 50% rabbit plasma (pH 7.4. 37°C).

In accordance with published reports on ISMNA hydrolysis in rabbit plasma, eserine suppressed hydrolysis in rabbit plasma when 14 was co-incubated with the potent esterase inhibitor $^{125}$ That BuChE is the enzyme responsible for prodrug hydrolysis, was confirmed by the similar effect noted when the plasma was exposed to iso-OMPA prior to the hydrolysis experiment. Arylesterases are not involved in the hydrolysis of ISMNA as is evident from the negligible effect of EDTA on rate or pathway of hydrolysis. Rabbit arylesterase has previously been found to be significantly less sensitive to inhibition by EDTA than human esterase, as the rabbit form of the enzyme has a much higher binding affinity for Ca$^{2+}$ and is thus a much more stable form of the enzyme $^{192}$. 

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2.4.3.4 Inhibitor studies in rat plasma

The hydrolysis of ISMNA was studied in 50% solution of rat plasma (pH 7.4, 37°C), co-incubated with a variety of specific inhibitors of a range of different esterases. The aim of this experiment was to confirm the role of butyrylcholinesterase in prodrug hydrolysis. Results are presented in Table 2.9.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Conc.</th>
<th>Target enzyme</th>
<th>$k_{obs}$</th>
<th>$t_{1/2}$</th>
<th>% Aspirin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0.86</td>
<td>0.81</td>
<td>0</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>1 mM</td>
<td>BuChE subtype</td>
<td>0.06</td>
<td>12.27</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>100 μM</td>
<td>Serine proteases, AChE</td>
<td>0.06</td>
<td>11.53</td>
<td>0</td>
</tr>
<tr>
<td>Eserine</td>
<td>100 μM</td>
<td>Cholinesterase</td>
<td>0.40</td>
<td>1.71</td>
<td>0</td>
</tr>
<tr>
<td>iso-OMPA</td>
<td>10 μM</td>
<td>BuChE</td>
<td>0.01</td>
<td>49.86</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.9 Kinetic data for the hydrolysis of ISMNA co-incubated with specific esterase inhibitors in 50% rat plasma (pH 7.4, 37°C).

None of the inhibitors used in this experiment directed the pathway of ISMNA hydrolysis towards the more favourable liberation of aspirin hydrolysis. All inhibitor studies were associated with exclusive release of salicylic acid on prodrug metabolism. ISMNA is very rapidly hydrolysed in rat plasma, and this rate was altered by dibucaine, PMSF and iso-OMPA but not eserine. These findings suggest that an esterase other than BuChE e.g. carboxylesterase, may be involved in prodrug hydrolysis in rat plasma. This is consistent with findings reported in Section 2.3.4.2, where rat plasma was found to have very low levels of BuChE and yet is associated with very rapid prodrug hydrolysis.
### 2.4.3.5 Inhibitor studies in hamster plasma

The hydrolysis of ISMNA was studied in 50% solution of hamster plasma (pH 7.4, 37°C), co-incubated with a variety of specific inhibitors of a range of different esterases. The aim of this experiment was to confirm the role of butyrylcholinesterase in prodrug hydrolysis. Results are presented in Table 2.10.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Conc.</th>
<th>Target enzyme</th>
<th>( k_{\text{obs}} ) min(^{-1} )</th>
<th>( t_{1/2} ) min</th>
<th>% Aspirin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0.199</td>
<td>13.48</td>
<td>71.72</td>
</tr>
<tr>
<td>Eserine</td>
<td>100 µM</td>
<td>Cholinesterase</td>
<td>0.004</td>
<td>187.29</td>
<td>26.39</td>
</tr>
<tr>
<td>iso-OMPA</td>
<td>10 µM</td>
<td>BuChE</td>
<td>0.008</td>
<td>91.19</td>
<td>0.45</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>Arylesterase</td>
<td>0.03</td>
<td>22.94</td>
<td>55.94</td>
</tr>
</tbody>
</table>

*Table 2.10 Kinetic data for the hydrolysis of ISMNA co-incubated with specific esterase inhibitors in 50% hamster plasma (pH 7.4, 37°C).*

In hamster plasma eserine and iso-OMPA were found to significantly lower the rate of ISMNA hydrolysis compared with a control sample. iso-OMPA also abolished aspirin liberation in the plasma samples, suggesting that BuChE is the enzyme responsible for ISMNA prodrug metabolism in hamster plasma. Interestingly EDTA also showed a small reduction in the rate of prodrug metabolism but still gave significant levels of aspirin release on hydrolysis. This may implicate arylesterases in hamster plasma as potentially significant enzymes in prodrug hydrolysis.
Results of all the inhibitor studies performed above confirm that butyrylcholinesterase is the enzyme responsible for ISMNA hydrolysis in plasma solutions. The rate and pathway of hydrolysis inhibition varies between different species, with different concentrations of aspirin being liberated. Rabbit plasma hydrolyses ISMNA (14) almost exclusively through the aspirinate pathway whereas in rat plasma no aspirin is liberated, with only salicylate being detectable in samples. Dog plasma behaves in a manner most similar to human plasma, but for all prodrug evaluations it is desirable to use human plasma if available as significant interspecies variation in enzyme type and distribution occurs.

Sequencing of feline BuChE revealed 70 amino acid differences to the human form of the enzyme, three of which were located in the active site gorge and which may be responsible for kinetic differences in butyrylthiocholine turnover. These included Ala 277 (Leu in cat), Pro 285 (Leu in cat BuChE) and Phe 398 (Ile in cat) \(^{193}\). The structure of the active site of rat BuChE is substantially different to that of humans, most prominently in the acyl-binding pocket where arginine at position 286 in rats is replaced by leucine in humans \(^{194}\). This decreases the size of the binding pocket in rat BuChE, and is reflected in a preference for smaller substrates, which may explain the altered pathway of hydrolysis of ISMNA in rat plasma. Position 390 of the BuChE sequence in human plasma contains glycine, whereas all other completed sequences (mouse \(^{195}\), rabbit \(^{196}\), horse \(^{197}\), cat, tiger \(^{193}\)) contain aspartate. This confers on human BuChE the ability to be inhibited 55% by 5 x 10\(^{-5}\) M sodium fluoride. As guinea-pigs, monkeys and dogs are resistant to this sodium fluoride inhibition, it is postulated that they also possess Asp at position 390. Small changes in amino-acid sequences between species can greatly affect enzymatic drug hydrolysis and inhibition kinetics \(^{195}\), e.g. the carboxylesterases from human and rabbit liver share 81% amino acid homology, but the rabbit enzyme is 100-fold more efficient at converting irinonectan to its potent metabolite SN-38 \(^{199}\).

ISMNA appeared to be the best substrate for the BuChE subtype present in rabbit or hamster plasma, liberating most aspirin on prodrug metabolism. Rabbit and hamsters are exclusively herbivores, while all other species tested are either omnivores (humans, rat, guinea-pig) or carnivores (dog). It is thus postulated that
dietary influences may affect cholinesterase distribution, leading to more favourable ISMNA hydrolysis in some species.

2.5 ISMNA hydrolysis in vegan plasma

As discussed in Section 2.4, ISMNA is hydrolysed via a different pathway in rabbit and hamster plasma than in humans. Hydrolysis is preferentially via the aspirinate route, liberating aspirin as the major product of prodrug hydrolysis. Because both hamster and rabbit are herbivores it was theorized that dietary influences might be responsible for inducing minor changes in the butyrylcholinesterase enzyme-type in these species, which resulted in ISMNA being a better substrate for the enzyme. If this were the case, it may affect the hydrolysis of isosorbide-based aspirin prodrugs and other ester prodrugs in humans who were strict vegetarians or vegans. For this reason, ISMNA hydrolysis in human vegan plasma was examined to detect whether diet plays a major role in prodrug metabolism.

2.5.1 Procedure

Human blood was sampled from a consented female volunteer who follows a strict vegan diet, with no ingestion of animal protein in the form of meat, fish, dairy products or eggs. Blood was centrifuged at 4,000 rpm for 10 mins and plasma was aspirated off and stored at 4-8°C until required for testing. A 10% plasma solution was prepared in phosphate buffer (pH 7.4 0.1 mM) and incubated for 15 mins at 37°C. Plasma was spiked with 100 μl of ISMNA solution (4 mg/ml in acetonitrile) and sampled according to the procedure outlined in Section 6.3.6. A control experiment was performed, using plasma from a female volunteer who was not vegetarian, and all samples were analysed by HPLC under the same experimental conditions.
2.5.2 Results of hydrolysis studies in vegan plasma

Results for ISMNA hydrolysis in vegan plasma are presented in Fig. 2.12 and Table 2.11.

![Graph showing hydrolysis progression](image)

*Figure 2.12 Typical progression curve for ISMNA hydrolysis in 10% vegan plasma (pH 7.4, 37°C): ISMNA □, ISMN-2-salicylate ■, salicylic acid●, aspirin ●.*

On comparison with control experiments (ref. Fig. 2.5) it is evident that there is no significant difference between pathways hydrolysis of ISMNA in ordinary and vegan human plasma. Both plasma types show hydrolysis mainly through the salicylate pathway with ~10% aspirin being generated.

<table>
<thead>
<tr>
<th>Plasma type</th>
<th>( k_{\text{obs}} ) (mins)</th>
<th>( t_{1/2} ) (mins)</th>
<th>% aspirin liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordinary</td>
<td>0.5808</td>
<td>1.19</td>
<td>10.11</td>
</tr>
<tr>
<td>Vegan</td>
<td>0.4818</td>
<td>1.44</td>
<td>10.17</td>
</tr>
</tbody>
</table>

*Table 2.11 Comparison of ISMNA hydrolysis in 10% human plasma solutions from ordinary and vegan sources.*
There was no significant change in rate of hydrolysis observed either, with both plasma samples showing a comparable $t_{1/2}$ of approximately 1.1-1.4 mins, again comparable to results obtained in hydrolysis experiments reported in Section 2.2.3. This suggests that there are no dietary implications for ISMNA hydrolysis in humans. Differences in the pathway of hydrolysis of ISMNA noted in rabbit and hamster plasma, which direct hydrolysis towards the more stable benzoate ester group, thus optimising liberation of aspirin, are most likely due to genetic differences in BuChE, consistent with interspecies variation.

2.6 Caco-2 cell studies

2.6.1 Introduction to Caco-2 cells

Isosorbide-based aspirin prodrugs showed promising levels of aspirin release during plasma hydrolysis studies, especially Is-2-asp-5-sal which was associated with production of ~80% aspirin in human plasma. The gastrointestinal tract (GIT) represents the first barrier encountered by orally delivered drugs which, in order to be absorbed, have first to pass through the intestinal epithelium. In prodrug development it is important to investigate the mechanisms of the parent compound’s intestinal transport and biotransformation, in order to develop compounds which efficiently cross the intestinal barrier while retaining their therapeutic properties. Experiments were performed in Caco-2 cell homogenates as a further in vitro study to determine their potential as true aspirin prodrugs. ISMNA, ISDA and Is-2-asp-5-sal were examined to establish their respective pathways and rates of hydrolysis in intestinal cell homogenates.

Caco-2 cells originated from a human colon adenoma cell culture of a 72 year-old caucasian male. When cultured as a monolayer, they provide an excellent screening model for investigations into drug transport and drug permeation processes in the GIT. In culture, the cells spontaneously differentiate into polarized cell monolayers with microvilli on their apical surfaces. They form tight junctions after attaining confluency, exhibiting transporting epithelia. The carrier-mediated transport systems found normally in the small intestine are
expressed in Caco-2 cells, as are receptors and many enzyme activities such as alkaline phosphatase, aminopeptidase-N, sucrose-isomaltase and lactase. Studies have also reported on the metabolic capabilities of Caco-2 cells, including metabolism of fatty acids and testosterone, the glucuronide conjugation of 4-nitrophenol and fatty acid esterification. Thus Caco-2 cells not only possess suitable properties for studying drug transport, they also reflect drug metabolism in the intestinal epithelium. Studies in Caco-2 cells are used as an in vitro model to assess the metabolic stability of therapeutic agents in the GIT at a cellular level. Caco-2 cells have been shown to have BuChE activity, which was sensitive to inhibition by iso-OMPA. This was the enzyme believed to be responsible for hydrolysis of isosorbide-aspirin prodrugs in plasma (Section 2.4.3.1). For such prodrugs to be clinically useful, it is essential that they pass intact through the GIT, releasing their active aspirin molecule only on enzymatic cleavage in plasma. If the prodrug is poorly resistant to esterase activity, rapid intracellular conversion by esterases inside the mucosal cells may result in the entrapment of the acidic acetylsalicylic acid in the mucosa, exposing the sensitive tissue to the topical irritant effect of the weak acid and its potential ulcerogenic effects.

2.6.2 Procedure in Caco-2 cell hydrolysis studies

2.6.2.1 Preparation of cell homogenates

Caco-2 cell homogenates were grown in 75 cm³ culture flasks at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity. When cells had achieved confluence cells were homogenized and the homogenate was centrifuged at 12,000 rpm for 15 mins at 4 °C. The supernatant was harvested and stored in quantities sufficient for a days testing, at -20 °C, until required. Protein content of all solutions was determined prior to assay, using a BCA Protein Assay kit, by means of a modification of the method first described by Lowry. Prior to use in enzyme assays, protein concentration of all preparations was adjusted to 0.33 mg/ml.
2.6.2.2 Prodrug hydrolysis experiments

Experimental protocol is described in more detail in Section 6.5.3. A 50% solution of cell homogenate was prepared and allowed to equilibrate at 37 °C for 20 mins. The cell suspension was then spiked with drug solution (10^{-4} M in acetonitrile). At regular intervals aliquots were removed and adding to a 2% zinc sulfate solution quenched enzyme activity. Eppendorfs were centrifuged at 10,000 rpm for 3 mins, and supernatant was analysed by HPLC using the appropriate conditions and mobile phase for each prodrug.

2.6.2.3 HPLC methods for isosorbide-based aspirin prodrugs

As described in Section 2.3.2, ISMNA plasma hydrolysis experiments were performed using an isocratic HPLC method of analysis. This gave optimum separation of aspirin and salicylic acid in the plasma samples. The same method (Table 2.12) was used for Caco-2 homogenate samples and again was found to give good separation of all analytes.

<table>
<thead>
<tr>
<th>Column</th>
<th>Waters Nova-Pak® C₈ 3.9 x 150 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>Waters 996 PDA (λ 235 nm)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>phosphate buffer pH 7.4: acetonitrile 60: 40</td>
</tr>
</tbody>
</table>

*Table 2.12 HPLC conditions for analysis of ISMNA in Caco-2 cell homogenate*

ISDA and Is-2-asp-5-salicylate required a gradient method of analysis to ensure that all metabolites were adequately separated. Samples were run on a Waters Nova-Pak® C₈ 3.9 x 150 mm column, with a flow rate of 1 ml/min using a mixture of phosphate buffer pH 3.0 (A) and acetonitrile (B). Details of the gradient method used are given in Table 2.13.
Both analytical methods were validated for linearity, precision, sensitivity and specificity. A linear response was observed for each analyte ($r > 0.999$) in the range 1-100 μg/ml. The limit of quantitation for the relevant analytes was 1.0 μg/ml and the RSD on multiple injection of each analyte at 10 μg/ml and 100 μg/ml was <1.98%.
2.6.3 Results of hydrolysis studies

2.6.3.1 Hydrolysis of ISMNA in Caco-2 cell homogenate

ISMNA was hydrolysed very slowly in Caco-2 cells as shown in Figure 2.13. The prodrug has a half-life of 82.5 minutes in cell solutions compared with a half-life of ~1.2 mins in human plasma. Most hydrolysis is via the salicylate route with only 2.26% aspirin being liberated.

![Figure 2.13 ISMNA hydrolysis in Caco-2 cell homogenate (pH 7.4, 37°C): ISMNA □, ISMN-2-salicylate ■, salicylic acid ○, aspirin ●.](image)

The slow hydrolysis by cell esterases suggests that ISMNA as an aspirin prodrug would not be significantly metabolised on passage across the gastric mucosa. The majority of the prodrug should pass intact into the plasma, where it can then be hydrolysed more favourably by plasma butyrylcholinesterase to liberate larger amounts of aspirin (up to 10%).
2.6.3.2 Hydrolysis of ISDA in Caco-2 cell homogenate

Similarly to ISMNA, the diaspirinate prodrug ISDA displayed very slow metabolism by Caco-2 cell esterases, as shown in Fig. 2.14.

![Graph](image)

Figure 2.14 ISDA hydrolysis in Caco-2 cell homogenate (pH 7.4, 37°C): Isosorbide-diaspirinate ◦, Isosorbide-disalicylate ■, Isosorbide-2-aspirinate/-5-aspirinate ▲, Isosorbide-2-aspirinate-5-salicylate ▲, Isosorbide-2-salicylate/-5-salicylate □, aspirin ●, salicylic acid ○

The prodrug had a half-life of 52.9 minutes and liberated 9.22% aspirin. This is significantly slower than previously reported prodrug hydrolysis results in human plasma, where ISDA has a $t_{1/2}$ of 1.1 mins and generates up to 60% aspirin. From these results it can be deduced that 15 is markedly stable in gut cell homogenate and is resistant to hydrolysis by gut enzymes. This implies that the prodrug might pass across the GIT relatively intact, resisting hydrolysis until it reaches the esterases in the circulation.
2.6.3.3 Hydrolysis of is-2-asp-5-sal in Caco-2 cell homogenate

Previous work on isosorbide-2-aspirinate-5-salicylate suggests that this is one of the most successful aspirin prodrugs reported to date. It has been associated with release of up to 80% aspirin in human plasma hydrolysis experiments. Its hydrolysis was examined in 50% Caco-2 cell homogenate and the results are presented in Fig. 2.15.

Is-2-asp-5-sal was hydrolysed slowly in Caco-2 cell homogenate (t\textsubscript{1/2} 58.6 ± 7.33 mins), and was associated with release of 0.84% aspirin. Again hydrolysis was considerably slower than in plasma hydrolysis experiments (t\textsubscript{1/2} 4.90 mins). This indicates that the aspirin prodrug is particularly stable in gut homogenate, and so is expected to resist hydrolysis by gut enzymes, ensuring that aspirin is not liberated \textit{in vivo} until it has reached the circulation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.15.png}
\caption{Is-2-asp-5-sal hydrolysis in Caco-2 cell homogenate (pH 7.4, 37°C): Isosorbide-2-aspirinate-5-salicylate, Isosorbide-disalicylate, Isosorbide-2-aspirinate/-5-aspirinate, Isosorbide-2-salicylate/-5-salicylate, aspirin, salicylic acid.}
\end{figure}
2.7 Plasma hydrolysis of other aspirin prodrugs.

2.7.1 Validation of plasma hydrolysis experiments

Plasma hydrolysis experiments form the basis of much of our analytical work on aspirin prodrugs. They are vital in determining the rate and pathway of prodrug analysis in humans and other species. Liberation of aspirin rather than salicylic acid on metabolism by plasma esterases is the ultimate goal of aspirin prodrug development. To establish that plasma hydrolysis studies in our laboratory were a validated method of determining the usefulness or otherwise of our prodrugs, a series of prodrugs, which had been reported previously in the literature, were evaluated. These included phenyl aspirinate (23)\textsuperscript{105}, guaiacol-aspirinate (24) and benorylate (25).

\[
\begin{align*}
&\text{23} \\
&\text{24} \\
&\text{25}
\end{align*}
\]
Compounds were tested in 10% human plasma solutions and kinetics of hydrolysis were compared with those reported in the literature to assess the usefulness of this hydrolysis model as a screening tool for aspirin prodrugs. Prodrugs were also examined in 10% rabbit plasma to determine whether ISMNA is unique in directing most hydrolysis through the aspirinate route on metabolism by rabbit plasma esterases (Section 2.3.2.2).

2.7.2 Synthesis of aspirin prodrugs

Compound 23 was prepared by treating acetylsalicyloyl chloride with phenol in the presence of DCM and triethylamine to yield product as white crystals. Compound 24 was synthesised by esterification of acetylsalicylic acid with o-methylphenol (guaiacol) under basic conditions. Compound 25 was formed by reacting paracetamol with acetylsalicyloyl chloride in the presence of DCM and triethylamine, at room temperature.

2.7.3 Plasma hydrolysis studies

2.7.3.1 Human plasma hydrolysis experimental protocol

All compounds were tested in human plasma to determine their rate of hydrolysis and to investigate whether aspirin was liberated during metabolism by plasma esterases. The hydrolysis experiment is described in greater detail in Section 6.3.6. Pooled plasma solutions (4 ml) were prepared by centrifugation of citrated human venous blood and dilution of the resultant plasma supernatant with phosphate buffer (pH 7.4). The plasma was spiked with 100 µl of test compound (as a 1 x 10⁻⁴ M solution in acetonitrile) and incubated at 37°C. Aliquots of 250 µl were removed at appropriate time intervals, quenched, centrifuged and supernatant analysed by HPLC.
2.7.3.2 Rabbit plasma hydrolysis experimental protocol

The three esters were examined in rabbit plasma to determine their rate of hydrolysis and also to investigate whether hydrolysis was directed more favourably to liberation of aspirin. The hydrolysis experiment is outlined in greater detail in Section 6.3.6. Pooled plasma solutions (4 ml) were prepared by centrifugation of citrated rabbit blood (sampled from the ear-vein), spiked with 100 μl of test compound (as a 1 x 10^{-4} M solution in acetonitrile) and incubated at 37°C. 250 μl aliquots were removed at appropriate time intervals, quenched, centrifuged and supernatant analysed by HPLC.

2.7.3.3 Chromatographic conditions

HPLC was performed using a Waters Nova-Pak ® C_{8} (4 μm) column, 3.9 x 150 mm. All three esters were analysed using the same mobile phase: phosphate buffer pH 7.4: acetonitrile, 60:40 and a flow rate of 1 ml/min, as this was found to give optimum separation of the aspirin and salicylic acid peaks.
2.7.4 Results of prodrug hydrolysis experiments in human plasma

2.7.4.1 Hydrolysis of phenyl aspirinate in human plasma

Phenyl aspirinate was tested for hydrolysis in 10% human plasma. The results of the experiment are presented in Fig. 2.16.

Hydrolysis of phenyl aspirinate was extremely rapid in human plasma with a half-life of 1.51 mins. Hydrolysis was directed primarily via the salicylate route with only 3.35% aspirin liberated. This correlates well with reported literature values for phenyl aspirinate hydrolysis ($t_{1/2}$ 1.3 mins and 0% aspirin liberated)\textsuperscript{105}. This indicates that esterification of aspirin with an aromatic group is unsuitable as a model for development of a prodrug which would regenerate aspirin in the circulation. The correlation of our results with literature findings validate the plasma hydrolysis model as a method of screening potential aspirin prodrugs.
2.7.4.2 Hydrolysis of guaiacol-aspirinate in human plasma

Guaiacol-aspirinate (O-methyl phenyl acetylsalicylic acid) was observed for its hydrolysis kinetics in 10% human plasma. The results are presented in Fig 2.17.

![Graph showing hydrolysis of guaiacol-aspirinate](image)

Figure 2.17 Hydrolysis of guaiacol-aspirinate in 10% human plasma (pH 7.4, 37°C): guaiacol-aspirinate (○), guaiacol-saliclylate (■), aspirin (●), salicylic acid (○).

Again prodrug metabolism was extremely rapid with a \( t_{1/2} \) of 54 s. Hydrolysis occurred almost exclusively along the salicylate pathway with formation of negligible quantities of aspirin (0.03% based on initial ester concentration). This confirms that esterification of aspirin with an aromatic group alone is not sufficient to suppress hydrolysis of the labile acetyl group.
2.7.4.3 Hydrolysis of benorylate in human plasma

As a further validation of the plasma hydrolysis model for evaluation of aspirin prodrugs, benorylate (4-acetamidophenyl acetylsalicylate or paracetamol aspirinate) was examined in 10% human plasma. The results of this analysis are depicted in Fig. 2.18.

Figure 2.18 Hydrolysis of paracetamol-aspirinate in 10% human plasma (pH 7.4, 37°C): paracetamol-aspirinate (%), paracetamol-salicylate (◻), paracetamol (◆), salicylic acid (○).

Hydrolysis in human plasma was exceptionally fast with a $t_{1/2}$ of 40.5 s and was directed exclusively through the salicylate pathway with no aspirin detectable throughout the experiment. The hydrolysis kinetics of benorylate have been reported previously and results here correlate well in terms of pathway and rate

All three esters tested in this experiment are associated with rapid hydrolysis and low levels of aspirin liberation. In contrast the isosorbide-aspirinate esters have comparatively slower rates of hydrolysis in the same human plasma model, with correspondingly higher levels of aspirin being liberated. This suggests that isosorbide esters are preferable at promoting aspirin release by suppressing aspirin hydrolysis at the more labile acetyl group, in addition to accelerating hydrolysis at the carrier ester group.
2.7.5 Results of prodrug hydrolysis experiments in rabbit plasma

2.7.5.1 Phenyl aspirinate in rabbit plasma

Phenyl aspirinate was tested in 10% rabbit plasma and the results are depicted graphically in Fig. 2.19.

![Graph showing hydrolysis of phenyl aspirinate in 10% rabbit plasma](image)

*Figure 2.19 Hydrolysis of phenyl aspirinate in 10% rabbit plasma (pH 7.4, 37°C): phenyl aspirinate (○), phenyl salicylate (■), aspirin (●), salicylic acid (○).*

Hydrolysis was fast ($t_{1/2}$ 1.79 mins) and was associated with liberation of 11.6% aspirin. Compound 23 does not display the dramatic liberation of aspirin observed on hydrolysis of ISMNA in rabbit plasma. This suggests that the structural characteristics of 23 direct hydrolysis towards the more labile acetyl group of the aspirin prodrug, with subsequent liberation of salicylate as the primary product of esterase metabolism.
2.7.5.2 Guaiacol-aspirinate in rabbit plasma

Guaiacol-aspirinate was examined in 10% rabbit plasma and rate and route of hydrolysis were compared with results in human plasma. The hydrolysis is depicted graphically in Fig. 2.20.

![Figure 2.20 Hydrolysis of guaiacol-aspirinate in 10% rabbit plasma (pH 7.4, 37°C): guaiacol-aspirinate (○), guaiacol-salicylate (■), aspirin (●), salicylic acid (○).](image)

Hydrolysis was slower in rabbit than in human plasma ($t_\text{v}$ 4.62 mins) and was associated with liberation of up to 10.5% aspirin. This compared with hydrolysis kinetics of 24 in human plasma where 0.03% aspirin was detectable in plasma samples. While the result is not as striking as ISMNA in rabbit plasma (70% aspirin generated), it does suggest that aspirin prodrug hydrolysis is promoted at the carboxylic ester group to a greater extent in rabbit plasma than occurs in human plasma.
2.7.5.3 Benorylate in rabbit plasma

To confirm the hypothesis above, that aspirin prodrugs are hydrolysed more favourably by rabbit plasma esterases to liberate aspirin *in vitro*, paracetamol-aspirinate was examined for its hydrolysis kinetics in 10% rabbit plasma. The results are presented in Fig. 2.21.

![Hydrolysis of paracetamol-aspirinate in 10% rabbit plasma (pH 7.4, 37°C): paracetamol-aspirinate (○), paracetamol-salicylate (■), paracetamol (△), salicylic acid (○), aspirin (●)](image)

Benorylate was rapidly hydrolysed in rabbit plasma ($t_{1/2}$ 0.76 mins) and up to 20.8% aspirin was liberated. Once more rabbit plasma generated significantly more aspirin on ester hydrolysis of 25 than human plasma (no aspirin detectable in samples).

These results suggest that an esterase variant in rabbit plasma hydrolyses aspirin esters by directing hydrolysis towards the benzoic acid and away from the more labile acetyl group, relative to human plasma. The result is most striking with ISMNA suggesting that this is an excellent substrate for the enzyme, directing hydrolysis towards maximum aspirin liberation. It is also possible that there are other enzymes in human plasma that hydrolyse the acetate ester linkage more rapidly than in rabbit plasma.
2.8 Conclusions

The present chapter examines species variation in hydrolysis of isosorbide-based aspirin prodrugs. The hydrolysis of ISMNA was tested in human, rabbit, dog, rat, hamster and guinea-pig plasma. Significant differences in rate and pathway of hydrolysis were noted, with rabbit and hamster plasma being associated with greatest release of aspirin (58 and 69% respectively). The observed aspirin release was not related to BuChE activity of their plasma, as human and dog plasma possesses significantly higher enzyme activities but do not generate comparable concentrations of aspirin (7 and 5 % aspirin respectively).

Inhibitor studies confirmed that BuChE is the enzyme responsible for metabolism of ISMNA in all species, with exposure to eserine and iso-OMPA (butyrylcholinesterase inhibitors) resulting in abolition of enzymatic prodrug hydrolysis in all species. A genetic variant of BuChE, perhaps specific to herbivores, was suspected to be responsible for the predominance of aspirin generation on hydrolysis of ISMNA in both rabbit and hamster. However, hydrolysis characteristics of the prodrug in plasma of humans who follow a strict vegetarian diet did not differ significantly from that of normal human plasma samples. This indicated that the species variation in enzyme distribution is genetically determined, and is not likely to result from dietary changes within one generation.

ISMNA, ISDA and is-2-asp-5-sal were tested in Caco-2 cell homogenates to determine their resistance to gut esterases. All displayed very slow metabolism in Caco-2 cells (t ½ 82.5, 52.9 and 58.6 mins respectively) and were not associated with release of large quantities of aspirin. This suggests that these prodrugs may be absorbed intact through the gut mucosal layer on passage through the GIT with no significant prodrug hydrolysis and aspirin release occurring only on hydrolysis by plasma esterases in the circulation.

To confirm the validity of the plasma hydrolysis model as a method of screening aspirin prodrugs, and also to display the superior aspirin release of our isosorbide-aspirin prodrugs as compared with other aspirin esters, a series of compounds were synthesised in-house. Phenyl aspirinate, guaiacol-aspirinate and
benorylate were all rapidly hydrolysed in human plasma with no significant aspirin concentration being detectable in plasma samples. The results obtained correlated well with literature values, thus validating our experimental protocol. The three prodrugs were also examined in 10% rabbit plasma to see if the liberation of up to 78.7% aspirin from ISMNA in rabbits was an anomalous result. The three prodrugs gave significantly increased aspirin release (11.57, 10.5 and 22.79% respectively) compared with human plasma. However most hydrolysis was still directed through the salicylate pathway. This suggests that ISMNA is an excellent substrate for a BuChE variant found in rabbits and hamsters. It may be possible to exploit its basic structure to give prodrugs, which are a better fit for the active site of the enzyme in humans, thus optimising aspirin generation \textit{in vivo}.

Work performed in this section cautions against use of results from just one species in the development of prodrugs for another. This is especially true for aspirin esters where there is significant variation in rate and pathway of hydrolysis between human and other plasma sources.
Chapter 3

Antiplatelet effects of isosorbide-based aspirin prodrugs
3.1 Introduction to platelet aggregation

As discussed in Chapter 1, aspirin is one of the most useful and commonly prescribed antiplatelet agents in clinical practice today. Its popularity as an antiplatelet therapy is inherently linked to its mechanism of action, irreversible inhibition of COX-1 in platelets. It was not until the landmark thrombolytic and unstable angina trials of the 1980s and 1990s that its full potential in the treatment of cardiovascular disease was appreciated. These clinical trials have validated use of an antiplatelet agent in several cardiovascular conditions. Aspirin is usually the agent of choice as it is inexpensive and generally well tolerated; however side-effects, particularly gastrointestinal, can limit its use. Acute coronary syndromes including myocardial infarction and unstable angina are frequently characterized by thrombosis associated with rupture of atherosclerotic plaques. Exposure of blood to procoagulant surfaces can trigger platelet activation and aggregation. Because aspirin is regarded as a fairly weak antiplatelet agent, both antiplatelet and antithrombotic agents are thus critical in the treatment of patients with acute coronary symptoms. In this chapter, the isosorbide-based aspirin prodrugs ISMNA, ISDA and isosorbide-2-aspirinate-5-salicylate are evaluated for their efficacy as novel antiplatelet agents. As discussed in Chapter 2, their ability to liberate aspirin on hydrolysis in plasma has previously been established. Their potency, relative to aspirin, as inhibitors of platelet aggregation is established by means of in vitro testing, and their potential as alternative antiplatelet therapies to aspirin is discussed.

3.1.1 Physiology of blood

Blood accounts for one quarter of the extracellular fluid in the body, total blood volume being 7% of total body weight. It carries out various transport functions to maintain the internal environment, such as uninterrupted delivery of oxygen to support aerobic metabolism, supply of nutrients such as amino acids, fatty acids, sugars, ions and trace materials, maintenance of correct concentrations of water,
electrolytes and hydrogen ions in the interstitial fluid and removal of waste products of cellular metabolism. Blood also plays a vital role in thermal regulation. Plasma is the fluid portion of the blood, within which the cellular components are suspended. Water accounts for 92% of plasma weight, proteins for 7% and the remaining 1% consists of dissolved organic molecules such as amino acids, glucose, vitamins and dissolved oxygen and carbon dioxide. Plasma proteins (albumin, globulins and fibrinogen) are synthesized in the liver and maintain higher osmotic pressure in plasma than in surrounding interstitial fluid. They participate in many in vivo functions including blood clotting and defensive action against foreign bodies, and act as carriers for hormones, cholesterol and ions such as iron (Fe$^{2+}$) and drug molecules (Table 3.1).

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>Liver (multiple types)</td>
<td>Major contributor to osmotic pressure of plasma, carrier for various substances</td>
</tr>
<tr>
<td>Globulins</td>
<td>Liver and lymphoid tissue (multiple types)</td>
<td>Clotting factors, enzymes, antibodies, carriers for various substances</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Liver</td>
<td>Forms fibrin threads essential to clotting</td>
</tr>
</tbody>
</table>

Table 3.1 Functions of plasma proteins

Three main cellular components are present in blood: red blood cells (erythrocytes, RBCs), white blood cells (leukocytes, WBCs) and platelets. Leukocytes are the only fully functional cells in the circulation; red blood cells lose their nucleus before they enter the bloodstream and platelets are annucleate cell fragments.

RBCs play a key role in transport of oxygen and carbon dioxide between the lungs and tissues. They are the most abundant cell type in the blood. One microlitre of blood contains about 5 million RBCs compared with only 4-11,000 WBCs and 2-5 x 10$^5$ platelets. They are disk-shaped cells which survive in the circulation for 120 ± 20 days before rupturing and allowing their intracellular
components to be recycled into the circulation. Erythrocyte deformability is one of the most important factors affecting blood flow and is achieved by means of the RBCs biconcave shape (i.e. high surface/volume ratio). The oxygen-carrying pigment of RBCs is haemoglobin; a complex quaternary protein consisting of four globular protein chains, each of which is wrapped around an iron-containing haem group. Approximately 70% of all iron in the body is situated in the haem groups of RBCs.

White blood cells play a key role in defence against parasites, bacteria and viruses. There are five types of mature white blood cells: lymphocytes, monocytes, neutrophils, eosinophils and basophils. Monocytes that leave the circulation and enter tissues develop into macrophages. Neutrophils, monocytes and macrophages are collectively known as phagocytes, as they can engulf and ingest foreign particles such as bacteria (phagocytosis). Lymphocytes are also called immunocytes, as they are responsible for specific immune responses. Basophils, eosinophils and neutrophils are termed granulocytes as they contain cytoplasmic inclusions that give them a granular appearance. Basophils leave the circulatory system and enter tissues in order to carry out their secretory function as mast cells, releasing histamine, heparin and other mediators of inflammation on activation.

3.1.2 Role of platelets

When a blood vessel is damaged, under normal physiological conditions the role of platelets in the circulation is to arrest the loss of blood. This process involves the rapid adhesion of platelets to the exposed sub-endothelium, followed by platelet-to-platelet adherence. This culminates in the formation of a platelet plug, which temporarily seals off the damaged vessel wall. As such, platelets play a fundamental, life-saving role in haemostasis and repair at sites of vascular injury. In contrast, in pathophysiological conditions such as atherosclerosis, arterial thrombus formation may limit the blood supply to nearby tissues, resulting in ischaemia and/or the progression of atherosclerotic lesions and resultant complications in coronary and cerebrovascular systems. Factors that initiate thrombosis include vascular damage, stimulation of platelets and activation of the coagulation cascade.
3.1.3 Platelet Physiology

Platelets are smooth-surfaced, discoid, amnucleate cells, originating from megakaryocytes in the bone marrow and are found circulating in the bloodstream. They are the smallest corpuscular components, with a diameter of 2-4 μm. Platelets have a life span of approximately 8-10 days and their removal from the circulation occurs as a result of changes in mitochondrial function during platelet aging. Internally there is a cytoskeleton (microtubules and microfilaments), two membrane systems (the endoplasmic reticulum or dense tubular system and the plasma membrane-derived surface connected canalicular system), mitochondria and four types of granules (α-granules, dense granules, lysosomes and peroxisomes).

Each platelet is surrounded by a plasma membrane, essentially a phospholipid bilayer extending into the internal canalicular system in order to enhance surface area. Glycoproteins protrude through the membrane, acting as platelet receptors for both activating and inhibiting agents. The platelet membrane also contains a large number of receptors which specifically bind agonists that stimulate platelet response e.g. ADP, adrenaline, collagen, thrombin, serotonin and platelet-activating factor (PAF).

Internally, the dense tubular system is where calcium is sequestered and enzymes involved in prostanoid synthesis are located. Granules act as storage systems for the platelet; α-granules contain mainly proteins (including fibrinogen, platelet-derived growth factor and von Willebrand factor), while dense granules are a rich source of ADP, serotonin and calcium. In platelets, calcium and cyclic AMP serve as second messengers, controlling platelet activation and inhibition of activation respectively.

3.1.4 Platelet Activation

Platelet activation and subsequent aggregation is a complex process and many of the underlying intracellular signals have yet to be defined. Activation of platelets can occur for a variety of reasons – contact with artificial surfaces, immunological processes, mechanical alteration or pharmacological intervention. Platelets are activated by several physiological (thrombin, collagen, ADP, adrenaline,
vasopressin, serotonin) and non-physiological (cyclic endoperoxides) substances

Upon activation all platelets react with the same series of responses

I. Shape change
II. Aggregation
III. Three secretory processes
IV. Liberation of arachidonic acid, which is rapidly converted to prostaglandins and lipoxygenase products.

On binding of an activating agent to its receptor on the platelet surface a signalling molecule such as calcium, diacylglycerol (DAG) or inositol 1,4,5-triphosphate (IP$_3$) is released internally, which ultimately result in release of AA, secretion of many factors and the expression of several surface receptors, e.g. fibrinogen receptor GPIIb/IIIa. Fibrinogen binding to its specific platelet receptors associated with GPIIb/IIIa appears to be a prerequisite for platelet aggregation. This process is depicted in Fig. 3.1.

![Figure 3.1 Activation of platelets in vivo. PLC = phospholipase C, PLA$_2$ = phospholipase A$_2$, PIP = phosphatidyl inositol diphosphate, PS = P-Selectin, PKC = protein kinase C, CM = calmodulin, IP$_3$ = inositol triphosphate, DAG = diacylglycerol, AC = actin, PD = phosphodiesterase, DTS = dense tubular system, MTS = microtubular system](image-url)
Agonist binding to specific membrane receptors results in the activation of phospholipase C, which releases inositol triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ generates increased levels of intracellular Ca$^{2+}$. This increased Ca$^{2+}$ activates phospholipase A$_2$ and results in granule release. PLA$_2$ also liberates AA from the plasma membrane and this is converted to thromboxane A$_2$ (TXA$_2$) by cyclooxygenase (COX). DAG activates protein kinase C (PKC), leading to phosphorylation of various intracellular proteins and activation of GPIIb/IIIa. Histamine is synthesised and liberated from platelets following stimulation and is believed to be another intracellular messenger for platelet aggregation. Platelet activation prompts the secretion of contents from the three types of platelet storage vessels: α-granules, lysosomes and dense bodies. The α-granules fuse with the canalicular membrane system, enabling extrusion of their contents to the exterior.

Upon activation, platelets lose their discoid shape, become spherical, and extend long spiky pseudopods while the internal organelles expel their contents (Fig. 3.2). This is a rapid process, with shape change occurring within 50 msec of stimulation. These pseudopods allow platelets to become enmeshed, forming a thrombus or clot. The growth of aggregates begins as an apparently random attachment of individual platelets that act as nucleation sites for thrombi.

![Figure 3.2 Resting and activated platelets](image)

The aggregation of platelets is correlated with the time sequence of conversion from spheres to spiny spheres. On stimulation with adrenaline however, platelets
can aggregate without undergoing shape change, indicating that fibrinogen receptors can also be expressed exclusive of shape change\textsuperscript{226}.

### 3.1.5 Inducers of platelet aggregation

#### 3.1.5.1 Thrombin

Thrombin is a serine protease which catalyses the cleavage of fibrinogen by the thrombin receptor. It thus activates both platelets and the coagulation system and so is considered to play a central role in haemostasis and thrombosis\textsuperscript{227}. Thrombin-induced platelet aggregation is mediated through the stimulation of prostaglandin synthesis, with phospholipase A\textsubscript{2} activation as the triggering step. This in turn caused arachidonic acid liberation and thromboxane formation\textsuperscript{228}.

#### 3.1.5.2 ADP

ADP has been recognised as an inducer of platelet aggregation since the early 1960's. It plays a key role in platelet activation because it amplifies the platelet responses induced by other agonists on its release from platelet dense granules. ADP acts through binding to both low- and high-affinity membrane receptors\textsuperscript{90}, and is liberated on metabolism of adenosine triphosphate (ATP) in platelets on commencement of the clotting cascade\textsuperscript{229}. Although not as powerful as collagen and thrombin it can still produce full platelet activation and aggregation. The addition of exogenous ADP to PRP stimulates platelet shape change and aggregation\textsuperscript{226}. It significantly reduces agonist-induced platelet aggregation at low concentrations (1-10 nM) and stimulates aggregation at high concentrations (1-10 \textmu M)\textsuperscript{230}.

#### 3.1.5.3 Arachidonic acid

An alternative pathway of platelet stimulation is via liberation of arachidonic acid from membrane phospholipids by the direct action of phospholipase A\textsubscript{2}, or by the indirect action of phospholipase C. Platelets contain virtually no free arachidonic acid. It is released from platelet membrane phospholipids as a result of activation of phospholipases and then further metabolised via the cyclooxygenase pathway to prostaglandin endoperoxides and thromboxane A\textsubscript{2} (see Fig 3.3) which induce
platelet responses \textsuperscript{231}. TXA\textsubscript{2}, which is the predominant arachidonate metabolite, invokes platelet aggregation and vascular smooth muscle constriction and plays an important role in arterial thrombosis. It is thus considered to be a pathological factor in cardiovascular disease \textsuperscript{232}. TXA\textsubscript{2} has a very short half-life (approx 30 s) and is rapidly hydrolysed to its inactive metabolite TXB\textsubscript{2} \textsuperscript{90}. Several investigators have observed that addition of exogenous AA (30-50 \textmu M) to platelet suspension results in platelet responses independent of TXA\textsubscript{2} production \textsuperscript{231}. As discussed in \textit{Chapter 1}, aspirin inactivates cyclooxygenase by acetylating a serine residue at position 529 within the active site of the enzyme, preventing TXA\textsubscript{2} and PGI\textsubscript{2} production. The inhibition of platelet cyclooxygenase is non-competitive and irreversible \textsuperscript{233} and lasts for the life span of the platelet (8-10 days) \textsuperscript{90}. However it also inhibits synthesis of PGI\textsubscript{2} in vascular endothelial cells potentially inducing stomach ulcers and other gastrointestinal toxic effects, restricting its clinical use \textsuperscript{234}.
Platelet activation results in activation of phospholipase A$_2$ and release of AA from platelet membranes. COX converts this to PGG$_2$ and PGH$_2$, from where thromboxane synthase and prostacyclin synthase convert them to TXA$_2$ and PGI$_2$ respectively. TXA$_2$ is released from the platelet and binds to specific membrane receptors resulting in further platelet activation.

3.1.6 Platelet Inhibitors

Platelet inhibition can involve one or more aspects of platelet behaviour – adhesion, activation, aggregation or secretion.

Platelet adhesion to the site of vessel wall injury is mediated by von Willebrand factor that binds to GPIIb/IIIa receptors. Inhibition of platelet adhesion can occur by altering the platelet membrane, plasma cofactors of platelet adhesion, or the adhesive glycoproteins in the subendothelium of the atherosclerotic plaque.
Inhibitors of platelet adhesion include von Willebrand factor monoclonal antibodies, aurintricarboxylic acid, and GPIIb/IIIa receptor antagonists such as abciximab (Section 1.3.2.3).

Inhibition of platelet activation is mediated by several agents such as prostacyclin (PGI$_2$) \(^{{226}}\), PGE$_2$, thromboxane antagonists and prostanoid analogs. PGI$_2$, an AA metabolite of endothelial cells is the most potent inhibitor of platelet aggregation \(^{{222}}\). However the clinical use of PGI$_2$ and PGE$_2$ is limited by their extremely rapid metabolism \textit{in vivo} \((t_{1/2} \sim 10 \text{ sec})\), their effect on vascular tone (causing systemic hypotension) and their rapid first-pass effect (up to 70% clearance in the lungs) \(^{90}\). TXA$_2$ synthase inhibitors such as picotamide are limited by accumulation of PGH$_2$, itself a potent platelet stimulus, following enzyme inhibition \(^{{237}}\).

Agents that inhibit platelet aggregation include aspirin and the NSAIDs, nitric oxide, clopidogrel and dipyridamole, omega-3 fatty acids and vitamin E. The intravascular release of PGI$_2$ and NO under normal arterial flow conditions inhibit platelet adhesion and aggregation by increasing cAMP and cGMP levels. The actions of platelet-derived pro-aggregatory and vasoconstrictor substances such as 5-hydroxytryptamine (5-HT) and TXA$_2$ are opposed by the release from endothelial cells of vasodilators such as prostacyclin and NO which also inhibit platelet aggregation \(^{{238}}\).

3.1.7 Platelet and bleeding disorders

Bernard-Soulier Syndrome (BSS) is a rare congenital bleeding disorder manifested by prolonged bleeding time, large but fragile platelets and thrombocytopenia. The biochemical defect involves a deficiency of the glycoprotein Ib/IX complex and glycoprotein V proteins, which form a noncovalent complex on the platelet surface membrane \(^{220}\). Definitive diagnosis requires a platelet aggregation study; BSS is characterized by an isolated defect in ristocetin-induced agglutination \(^{90}\).

Glanzmann’s thrombasthenia is a rare autosomal recessive platelet disorder defined by an abnormality in the GPIIb/IIIa integrin. Consequently platelets fail to bind fibrinogen or to aggregate upon activation \(^{90}\). It is characterised by prolonged
bleeding time, absence of platelet aggregation, defective clot retraction and decreased fibrinogen binding to platelets\textsuperscript{220}.

3.1.8 Anticoagulants

When measuring platelet aggregation responses, it is necessary to prevent blood coagulation on sampling by means of an anticoagulant. This creates a non-physiological milieu for the platelets and entails the risk of artificial modification of platelet responses\textsuperscript{239}. The nature of the anticoagulant used can greatly affect the platelets response to a surface. Calcium-chelating agents such as EDTA inhibit thrombus formation at low concentrations, and decrease platelet adhesion at high concentrations. Platelet activation begins immediately after blood withdrawal and is most pronounced in EDTA-anticoagulated samples\textsuperscript{240}. Heparin has been used as an anticoagulant in studies \textit{ex vivo}, and does not appear to alter platelet accumulation, however studies \textit{in vitro} using heparin are difficult as in some donors spontaneous platelet clumping may occur. At present, citrate appears to be a superior anticoagulant to EDTA or heparin. Sodium citrate chelates $\text{Ca}^{2+}$ in blood and thus suppresses blood coagulation\textsuperscript{239}. However some reports suggest that the low $\text{Ca}^{2+}$ concentration and pH reduction induced by citrate\textsuperscript{241} may influence the outcome of platelet aggregation under certain conditions e.g. adrenaline induces the formation of platelet aggregates in blood anticoagulated with citrate but not at physiological $\text{Ca}^{2+}$ concentration\textsuperscript{242}. 
3.2 Platelet Aggregation studies

3.2.1 Introduction to tests for platelet function

Because platelets play such an important role in the pathophysiology of cardiovascular disease and its associated risk factors, many techniques have been developed to quantify the extent of platelet activation \(^{243}\). Morphological studies have used electron microscopy to evaluate the change in shape of platelets and flow-cytometric analyses reveal the status of the platelet cell surface. However these methods cannot investigate platelet function, as complex cell-fixing procedures are required \(^{244}\). The luminescence method monitors the release of ATP from dense granules by means of a firefly luciferin-luciferase assay in whole blood or PRP \(^{245}\). The two most popular methods in clinical use today are the optical (turbidometric) and impedance (electrical aggregation) techniques. These same techniques are also used to investigate new antiplatelet agents and evaluate their potency relative to established therapies. In both assays blood is used within 8 hours of sampling, as on storage platelets rapidly lose their viability and their functions of aggregation, adhesion and secretion \(^{246}\). The life-span of platelets stored at 22°C is higher than that of refrigerated platelets \(^{219}\) so samples are retained at room temperature until required for testing. Cooling human platelets below −20°C results in marked shape change, increased intracellular calcium and actin polymerisation and α-granule release \(^{247}\), thus samples cannot be frozen for later use. Platelet function must be examined in a physiological milieu, be it plasma or blood, as it has been shown that aspirin is 1500 times less potent at inhibiting arachidonate-induced aggregation in platelets suspended in buffer than in plasma \(^{248}\).

3.2.1.1 Photometric aggregation

Optical (turbidometric) platelet aggregometry was one of the first techniques developed to assess platelet function. It was designed by Born in 1962 and was the first effective method of quantifying platelet aggregation \(^{249}\). It involves measurement of the changes in light transmittance through a platelet sample
following induction of platelet aggregation. Platelet-rich plasma samples are stirred in a cuvette at 37°C between a light source and a photo multiplier tube. Upon addition of a platelet agonist platelets aggregate, decreasing the volume occupied by the platelets and giving a concomitant increase in transmitted light, which is proportional to the aggregation response. (Fig. 3.4) Stirring at 1,000 rpm is employed to ensure even distribution of aggregating agent throughout the sample, although experiments have shown that in an unstirred sample optical density decreased during the same time period as in a stirred sample ²⁴⁹.

![OPTICAL METHOD](image)

Figure 3.4. Analysis of platelet aggregation in stirred PRP. On adding a trigger (agonist) platelets begin to aggregate resulting in increased light transmission through the sample ²⁵⁰.

Turbidometric platelet aggregation facilitates distinguishing between the primary and secondary phases of aggregation, and acts as a measure of platelet function. It allows for detection of the shape change, rate of aggregation and maximum aggregation of platelets ²⁵¹. There are however many disadvantages to this method: platelet function in vitro does not necessarily reflect platelet function in vivo, sample aging occurs due to the time taken to prepare the PRP, and the presence of lipids in PRP or PPP can alter the wavelength of observation. Most importantly, centrifugation of whole blood to obtain PRP modulates platelet behaviour. Platelets are heterogeneous in size, density and metabolic activities, and it is likely that subpopulations of platelets are lost during centrifugation that may be an
important determinant of platelet function *in vivo*. Leukocytes can generate the potent antiaggregating agent prostacyclin, and their removal may result in platelets behaving differently in blood than in PRP. Erythrocytes release ADP, which can induce the aggregation of platelets, thus isolation of platelets from blood removes many extraneous factors which may influence their behaviour. Centrifugation may also induce platelet activation and affect sensitivity to aggregatory stimulus. Impedance aggregometry, which is described in further detail below, has been reported to be a more sensitive method than the turbidometric technique in detecting the loss of platelet function.

3.2.1.2 Electrical impedance

Because of disadvantages of the optical method of aggregometry listed above, an aggregometer that quantifies platelet function within a whole blood sample was developed, which uses electrical impedance as a means of assessing aggregation. This method was first reported by Cardinal and Flower in 1980. A sample of whole blood is stirred at 37°C between two platinum electrodes set at a fixed distance. When exposed to an agonist, platelets aggregate around the electrode effectively increasing electrical impedance (see Fig. 3.5). Electrical microscopy indicates that during initial contact with blood the electrodes become coated with a monolayer of platelets. In the absence of an aggregating agent no further interactions occur between the platelets and the electrodes, and conductance between the two electrodes is constant. On addition of an aggregating agent such as arachidonic acid however, platelets adhere to the platelet monolayer coated on the electrodes and there is a gradual accretion of platelets at these sites. This coats the electrodes and impairs conductance between them. The change in resistance is related to the mass of the platelet aggregate on the electrode tip and increased impedance can be monitored on a chart recorder. The Chrono-Log impedance electrode consists of two fine palladium wires, bent into evenly spaced loops, with both ends secured to a printed circuit board. A small voltage difference is applied across the wires to measure the impedance of the sample. The electrode is designed to be used in a 0.40-inch inside diameter cuvette with a sample volume of 1 ml. The position of the wires is critical, as they must be located in an area where they are completely covered by the sample, where there is
sufficient stirring with an even flow pattern and where they do not interfere with the stir bar.\textsuperscript{245}

Figure 3.5. Analysis of platelet aggregation in stirred whole blood. On adding a trigger (agonist) platelets adhere to the electrode impeding the flow of charge through it.\textsuperscript{250}

The main advantage of this method is that whole blood is a more accurate reflection of the \textit{in vivo} condition of platelets and no modification of the sample is required (apart from the use of a small quantity of anti-coagulant). It requires only small volumes of blood and the assay can be performed quickly and conveniently. It is especially useful when the blood to be tested has an abnormally low or high haematocrit, low platelet count or abnormally large platelets, which may be lost on centrifugation (as occurs in Bernard-Soulier Syndrome).\textsuperscript{258} It is also beneficial in the testing of hyperlipidemic plasma samples where the turbidometric method is not applicable.\textsuperscript{259} Its only disadvantage relative to the turbidometric method is its increased variability of results and low reproducibility.\textsuperscript{254} With the impedance method, shape change, disaggregation and biphasic aggregation response are not detectable and the response to adrenaline is poor.\textsuperscript{251}
3.2.2 Procedures in human platelet aggregation studies using aspirin and isosorbide-based aspirin prodrugs.

Human platelet rich plasma (PRP) was prepared by centrifugation of anticoagulated blood at 800 rpm for 10 minutes at room temperature. The PRP was aspirated off and remaining cells were centrifuged at 3,200 rpm for 10 minutes to give platelet poor plasma (PPP) for the blank. Platelet counts were obtained for all PRP samples using a Sysmex XE-2100 Automated Haematology Analyser (Sysmex Corp. Kobe, Japan). All samples used in experiments had a platelet count of 250 – 300 x 10^9/L.

Experimental protocol is given in greater detail in Section 6.4.2. 250 μl of PRP was equilibrated at 37°C for 3 minutes while stirring at 1000 rpm following incubation with an inhibitor at known concentration in DMSO. Each channel was blanked with 250 μl of PPP, then samples were activated with agonist and aggregation patterns were monitored for 10 minutes.

3.2.3 Controls and solvent checks

Dimethyl sulfoxide (DMSO) is one of the most commonly used solvents in platelet aggregation experiments. In high concentrations (above 0.25%) it may induce a concentration dependent change in platelet cytoplasmic ionised calcium. Before each experiment a control was run using PRP to establish normal aggregation response to each agonist. A sample of PRP was also incubated with 10 μl of DMSO for 10 minutes at 37°C before challenging with the same agonists to ensure that the solvent was not having any inherent inhibitory effect on the aggregation response.
3.2.4 Comparison of aspirin and isosorbide-based aspirin prodrugs.

Aspirin was evaluated for its antiplatelet effect in human PRP. An IC_{50} for aspirin was established by examining a range of concentrations of aspirin from 1-100 μM (all in DMSO). This was compared with the inhibition obtained for isosorbide-diaspirinate (ISDA) and isosorbide-2-aspirinate-5-mononitrate (ISMNA) over the same concentration range. Solubility was an issue for the aspirin prodrugs; at higher concentrations (50-100 μM) they tended to crash out of DMSO at 0.5% so all samples were prepared in 1% DMSO. This was checked at the start of each experiment to ensure that at these higher concentrations there was no inhibitory effect attributable to the solvent phase.

Because aspirin has known COX-1 inhibitory effects, preventing arachidonic-acid induced aggregation in platelets, all compounds were analysed for their inhibitory effect on aggregation initiated by AA (0.5 mM).

3.2.5 Results of platelet aggregation experiments

On repeated testing of the three compounds, ISDA (IC_{50} = 4.34 μM) was found to be equipotent with aspirin (IC_{50} = 5.23 μM). ISDA liberates up to 60% aspirin on metabolism in human plasma \textsuperscript{127}, suggesting that there is another factor contributing to its antiplatelet effects besides aspirin release. It is possible that one or more metabolites of ISDA, apart from aspirin, have inherent COX-1 inhibitory activity.

ISMNA was a considerably less potent platelet inhibitor, almost 20-fold less effective than aspirin or ISDA (IC_{50} 85 μM). Again this is consistent with plasma hydrolysis studies, which show liberation of up to 8.43% aspirin on hydrolysis of ISMNA by plasma esterases in 10% plasma solution \textsuperscript{127}.

Sample platelet aggregation profiles for PRP control and in the presence of 1% DMSO are shown in Fig. 3.6

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On addition of aggregating agent (AA 0.5 mM) (visible on the curve as a small spike) platelets begin to aggregate, giving increased light transmission through the sample. The aggregometer records this as a percentage; uninhibited PRP normally gives 90-100% transmission. Sample aggregation profiles of ISDA over a range of concentrations are presented in Fig 3.7.

Figure 3.6 Sample platelet aggregation profiles showing normal PRP aggregation patterns (1) and in the presence of 1% DMSO (2)

Figure 3.7. Inhibition of platelet aggregation induced by ISDA.
Similarly the platelet aggregation profile of aspirin in the same concentration range and under the same experimental conditions is presented in Fig. 3.8.

Percentage platelet inhibition was plotted against concentration for each compound and their IC\textsubscript{50} values calculated using the Prism computer program. Thus ISDA can be compared directly with aspirin to evaluate its effectiveness as an antiplatelet agent. These results are depicted in Fig 3.9.
From the graph it is evident that aspirin and ISDA are equipotent, with regard to their ability to inhibit arachidonic acid induced platelet aggregation. Both compounds gave complete inhibition of aggregation at 7 μM while below 2 μM neither had any effect on platelet response to AA. Results were reproducible over a number of days testing, as is evident from the narrow standard deviation of results.
3.3 Whole blood aggregation studies

3.3.1 Procedures in whole blood aggregation studies

Human whole blood was sampled by venipuncture from consented adult volunteers and used within two hours of sampling. Experimental protocol is given in greater detail in Section 6.4.2. A 500 μl aliquot of whole blood was added to 500 μl of physiological saline and allowed to incubate at 37°C for 10 minutes in the incubation chamber of a Chrono-Log® Whole Blood Aggregometer [model 591/592]. The sample was then transferred to the assay well of the aggregometer, baseline was established and the appropriate volume of reagent was added. Aggregation was measured over 6 mins with impedance output recorded on a chart recorder. When testing inhibitors, whole blood was incubated with the appropriate concentration of drug in 10 μl of DMSO at 37°C for a specified length of time before adding the stimulant.

3.3.2 Controls and solvent checks

Control experiments were performed throughout the course of each experiment to establish normal aggregation responses. Prior to each experiment, a sample of whole blood was incubated with 10 μl of DMSO for 10 mins at 37°C with stirring to ensure the solvent was having no inhibitory effect on the aggregation response (as depicted in Fig. 3.10).

3.3.3 Comparison of aspirin with other aspirin prodrugs.

Aspirin was evaluated for its antiplatelet effect in human whole blood. An IC₅₀ for aspirin inhibition of AA-induced platelet aggregation was established by examining a range of drug concentrations from 1-100 μM (all in DMSO). This was compared with the inhibition obtained for isosorbide-diaspirinate (ISDA) and isosorbide-2-aspirinate-5-mononitrate (ISMNA) over the same concentration range. A metabolite of ISDA, isosorbide-2-aspirinate-5-salicylate was also examined in whole blood to determine its potency relative to the parent compound
and to aspirin. It has been established that in human serum BuChE, the hydrolysis of this ester is associated with the liberation of up to 98% aspirin, significantly more than previously reported aspirin prodrugs. To establish its superiority as a prodrug, a further series of aspirin prodrugs including guaiacol-aspirinate, phenyl aspirinate and benorylate were also evaluated for their antiplatelet effects. To overcome solubility issues all samples were made up in DMSO, such that on addition of 10 μl of drug solution to the blood sample the final concentration of solvent was no greater than 1%.

3.3.4 Results of whole blood aggregation experiments

Whole blood aggregometry allows for the testing of platelet aggregation with little sample preparation and consequently is considered to be a closer approximation of the physiological milieu. Isotonic saline is recommended as the diluent of choice, as it increases the extent of platelet aggregation. Also, use of a diluting procedure means less blood is required for routine platelet tests in whole-blood aggregometry (10 tests per 5 ml blood) compared with turbidimetry (6-8 tests per 5 ml blood). Results obtained by the impedance and turbidometric methods may not be exactly comparable, but trends are expected to be similar. The time to reach maximal aggregation in electrical aggregometry is longer than for the turbidometric method. This is possibly due to the flow forces in the electrical aggregometer: adherent platelets must resist the shear forces at the surface of the electrode in order to remain attached. Flow forces have been shown to delay the growth of thrombi as a shear stress of 50 dynes/cm² can result in low levels of ADP release from platelets. In whole blood the presence of erythrocytes has also been shown to interfere with thrombus formation due to mechanical collision of the red blood cells with platelet aggregates. This explains the apparent lower potency of antiplatelet agents in the impedance as opposed to the turbidometric method of evaluating platelet aggregation.
In the whole blood assay aspirin and ISDA were shown to be almost equipotent with aspirin having a slightly lower IC$_{50}$ (35 μM) than ISDA (40 μM). Sample traces from the chart recorder are shown in Fig 3.10 and Fig 3.11. In each assay a baseline was established before addition of the aggregating agent to the chamber. The initial upward inflection corresponds to platelet change on activation and prior to platelet aggregation.

![Figure 3.10. Whole blood aggregation profiles following addition of 0.5 mM arachidonic acid. 1. Control (whole blood + 1% DMSO), 2. Aspirin 10 μM, 3. Aspirin 30 μM.](image)
Percentage platelet inhibition was plotted against concentration for each compound and their IC\textsubscript{50} values were calculated using the Prism computer program. This allowed for a direct comparison of ISDA with aspirin to evaluate its effectiveness as an antiplatelet agent in human whole blood. The results of this analysis are depicted graphically in Fig 3.12.
The antiplatelet effect of aspirin is reported to be greater in whole blood than in platelet-rich plasma and the effect is potentiated in the presence of leukocytes. In all the whole blood studies there was considerable variation in results. Platelet aggregation in whole blood is also sex-related with greater *in vitro* aggregability observed in females than in males. This is to be expected in biological samples, as there are many factors that influence platelet aggregation in whole blood including ingested medication, diet, alcohol and nicotine intake. Alcohol has been shown to inhibit arachidonic acid release and thromboxane biosynthesis during platelet activation. Females respond to alcohol ingestion with greater platelet inhibition than men and whole-blood aggregation is a more sensitive indicator of platelet reactivity. All blood donors had not taken aspirin or other NSAIDs for two weeks prior to sample collection, but no dietary or other restrictions were imposed, which may explain the considerable variation noted in some of the graphs presented.
ISMNA was evaluated for its efficacy as an anti-platelet agent against AA-induced aggregation of human whole blood according to the method described in Section 3.3.1. As is evident from Fig. 3.13, ISMNA is significantly less potent an antiplatelet agent than either aspirin or ISDA under the same experimental conditions. In pooled human whole blood, ISMNA has an IC$_{50}$ of 205 µM. As in PRP experiments, this correlates with a lower percentage of aspirin being liberated on hydrolysis of this prodrug by plasma esterases.

![Figure 3.13 Plot of ISMNA inhibition of AA-induced platelet aggregation in human whole blood.](image)

While ISMNA does exhibit some antiplatelet activity, its lower potency relative to aspirin means it is unlikely to be as effective a clinical agent.

### 3.3.4.3 Isosorbide-2-aspirinate-5-saliclylate

One of the main metabolites of ISDA is isosorbide-2-aspirinate-5-saliclylate, which has been shown to liberate up to 91.5% aspirin in 50% human plasma solution during hydrolysis studies. This is the most effective aspirin prodrug reported to date at liberating the parent compound on hydrolysis by plasma enzymes, favouring the aspirinate rather than the salicylate route of hydrolysis. Its inhibition of platelet aggregation is profiled in Fig. 3.14.
Figure 2.14 Plot of Is-2-asp-5-sal inhibition of AA-induced platelet aggregation in human whole blood.

Isosorbide-2-aspirinate-5-salicylate was found to be significantly more potent than aspirin or ISDA at inhibiting AA-induced platelet aggregation. The calculated IC$_{50}$ for the isosorbide prodrug is 12 $\mu$M, almost three-fold more potent than aspirin under the same experimental conditions (Section 3.4.4.1). Of the three isosorbide-based aspirin prodrugs evaluated, this is the most potent antiplatelet agent, showing significantly more platelet inhibition than aspirin.

3.3.4.4 Other aspirin prodrugs

In order to confirm that it is is-2-asp-5-salicylate which is the active metabolite of ISDA, a series of experiments were performed on another metabolite of ISDA namely isosorbide-2-aspirinate. A series of aspirin prodrugs synthesised in house were also examined: phenyl aspirinate, benorylate and guaiacol-aspirinate. Each compound was examined at a single concentration of 100 $\mu$M in human whole blood and results of their effect on AA-induced platelet aggregation are presented in Fig. 3.15.
As is evident in the histogram, only isosorbide-2-aspirinate-5-salicylate has a significant effect on platelet aggregation, giving 88.9% inhibition of aggregation at 100 μM. The ISDA metabolite isosorbide-2-aspirinate was not as an effective antiplatelet agent, inducing just 40.55% inhibition of platelet aggregation at 100 μM. Benorylate (25) is the most potent of the aspirin prodrugs resulting in 31.15% inhibition, while phenyl aspirinate (23) and guaiacol-aspirinate (24) are relatively weak inhibitors of AA-induced platelet aggregation (19.45 and 5.85% inhibition respectively) at 100 μM.
3.4 ADP – induced platelet aggregation

3.4.1 Introduction

ISDA was evaluated for its inhibitory effects on ADP-induced platelet aggregation in male and female whole blood. It has been shown that response to ADP, as an aggregating agent is sex-related, with female platelets having greater sensitivity to ADP. It was thus desirable to consider both responses separately.

3.4.2 Procedure

Aspirin and ISDA in a range of different millimolar concentrations were prepared in DMSO. As per Section 3.3.1, 500 μl of whole blood was incubated with 500 μl physiological saline in the presence of 10 μl of drug solution. After the appropriate incubation time (10 mins for aspirin, 20 mins for ISDA, (see Section 3.5)) samples were challenged with 10 μl of 10 μM ADP solution and aggregation was quantified relative to control samples tested under the same experimental conditions.

3.4.3 Results

The response of female whole blood to ADP in the presence of aspirin and ISDA is shown in Figure 3.16. ISDA is significantly more potent than aspirin at inhibiting ADP-induced platelet aggregation, with an IC₅₀ of 375 μM compared with aspirin, IC₅₀ 775 μM (Ref. Table 3.2).
Similarly in male whole blood, 15 appears to be two-fold more potent than aspirin at inhibiting ADP-induced platelet aggregation (Fig. 3.17). ISDA has an estimated IC$_{50}$ value of 380 $\mu$M compared to that of aspirin (800 $\mu$M).
On direct comparison of results (Table 3.2), it is evident that in both males and females, ISDA is at least twice as potent as aspirin at inhibiting ADP-induced aggregation. The IC$_{50}$ values obtained for aspirin in males compare well with literature values, however those in female blood are lower than quoted.

<table>
<thead>
<tr>
<th></th>
<th>ISDA (IC$_{50}$ μM)</th>
<th>Aspirin (IC$_{50}$ μM)</th>
<th>Literature values for Aspirin (IC$_{50}$ μM)$^{268}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>375</td>
<td>775</td>
<td>1154</td>
</tr>
<tr>
<td>Male</td>
<td>380</td>
<td>800</td>
<td>694</td>
</tr>
</tbody>
</table>

*Table 3.2 IC$_{50}$ values for inhibition of ADP-induced aggregation by aspirin and ISDA in male and female whole blood.*

A marked difference between the inhibitory effects of aspirin in the different sexes was not noted. However, clinical trials have raised the possibility that the effects of antiplatelet drugs such as aspirin may be much less apparent in females than males $^{269}$. The literature values quoted involve use of a lower concentration of ADP as aggregatory stimulus (2.5 μM as compared with 10 μM used in this experiment). A higher concentration was used in our experiment based on manufacturer’s recommendations, to ensure all responses could be accurately recorded. While it would be desirable to perform the experiment under strict dietary controls, it is also possible that marked differences in sensitivity to ADP in males and females is not as evident at higher concentrations of the aggregating agent. ISDA’s superior potency to aspirin in inhibition of ADP-induced aggregation is however undisputable, and suggests its value as a prophylactic agent against undesirable thrombo-embolic events.
3.5 Effect of incubation time on antiplatelet effect

3.5.1 Introduction

To establish whether ISDA itself has any inherent antiplatelet activity, or if it requires hydrolysis to aspirin or other active metabolites, a series of time-course experiments were performed, in the Chrono-Log® Whole Blood Aggregometer. This experiment was performed as part of a $2^3$ factorial design experiment (detailed in Appendix 1) and all statistical analysis was performed using Minitab®, a statistical package for PCs.

3.5.2 Procedures

Whole blood aggregation experiments were performed as detailed in Section 3.3.1. 50 μM ISDA in DMSO was incubated in each blood sample for varying lengths of time over a period of 20 mins, before challenging samples with 0.5 mM AA and analysing results on a chart recorder.
3.5.3 Results

There was a direct correlation between incubation time and efficacy of 15 as an antiplatelet agent (Fig 3.18). With no incubation, ISDA (50 μM) had no inhibitory effect on platelet aggregation, while at 20 mins incubation time 100% aggregation was observed.

![Graph showing effect of ISDA (50 μM) on human whole blood aggregation at varying incubation times.](image)

\[ r^2 = 0.9505 \]

Statistical analysis using Minitab demonstrates that the incubation time of ISDA is a significant main effect \( p = 0.0215 \), i.e. ISDA’s antiplatelet effect is not immediate, rather it requires incubation for up to 20 minutes to achieve its maximum potency. This suggests that ISDA itself is an inactive prodrug, which requires metabolism by plasma enzymes and liberation of other more active metabolites, including aspirin, to establish its antiplatelet efficacy. The large error bars are commonly observed in experiments performed using the electrical impedance method of platelet aggregometry.²⁵⁴
3.6 Eserine inhibition of ISDA’s antiplatelet effect

3.6.1 Introduction

Enzyme and enzyme inhibitor studies performed in plasma infer that butyrylcholinesterase is the enzyme responsible for isosorbide prodrug metabolism in vivo, liberating aspirin, which is then capable of cyclooxygenase inhibition (Section 2.4). To establish that prodrug metabolism is essential for antiplatelet effect, eserine was chosen as a butyrylcholinesterase inhibitor for ISDA hydrolysis in whole blood.

3.6.2 Procedures in inhibitor studies

Procedures for inhibitor studies were as described in Section 3.3.1. Whole blood was incubated in the presence of 0.5 mg/ml of eserine in a Chrono-Log® Whole Blood Aggregometer and 100 μl of aspirin or ISDA, before inducing platelet aggregation using arachidonic acid.

3.6.3 Results of inhibitor studies

Eserine at a concentration of 0.5 mg/ml was shown to completely inhibit the antiplatelet effect of ISDA. (Table 3.3) Experiments in the absence of an inhibitor show that ISDA has an IC₅₀ of 40 μM in whole blood stimulated with 0.5 mM AA. However in the presence of eserine this antiplatelet effect is abolished. Aspirin at the same concentration retains its ability to inhibit platelet aggregation.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Ohm Response (± s.d.)</th>
<th>% Inhibition (± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISDA (100 μM)</td>
<td>18.5 ± 0.81</td>
<td>2.63 ± 3.71</td>
</tr>
<tr>
<td>Aspirin (100 μM)</td>
<td>0 ± 0.00</td>
<td>100 ± 0.00</td>
</tr>
</tbody>
</table>

*Table 3.3  Effect of eserine on the antiplatelet effect of aspirin and ISDA, n=3*

These results correlate with plasma hydrolysis experiments, which show that eserine inhibits metabolism of ISDA by butyrylcholinesterase. No aspirin is liberated from the prodrug and so no antiplatelet effect is observed. This confirms that 15 is an inactive prodrug, which requires metabolism in vivo to liberate the parent compound aspirin or other active metabolites in the circulation.
3.7 Malondialdehyde assay

Malondialdehyde (MDA) is a by-product of the arachidonic acid pathway in platelets, and as such can be used as a marker of cyclooxygenase activity \textit{in vitro}. The following section details the pharmacological significance of MDA and its use in an assay to establish COX-inhibitory activity of aspirin prodrugs.

3.7.1 Introduction to Malondialdehyde

Malondialdehyde is a volatile, low molecular weight, short-chain 1, 3-dicarbonyl compound and a moderately weak acid (pK\textsubscript{a} = 4.46)\textsuperscript{270}.

In solution and in the gas phase MDA is rapidly enolized between two intramolecularly hydrogen-bonded asymmetric forms with a low barrier to interconversion via a symmetrically hydrogen-bonded resonance structure (Fig. 3.19).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_19.png}
\caption{Structural chemistry of MDA\textsuperscript{270}.}
\end{figure}
3.7.2 Formation of Malondialdehyde

MDA is one of several low-molecular weight end products formed on decomposition of primary and secondary lipid peroxidation products and also on cyclooxygenase-catalysed oxygenation of arachidonic acid in the synthesis of prostanoids. Inhibition of MDA synthesis is thus considered to be consistent with COX inhibition activity. It is a reactive entity \textit{in vivo} and can form stable derivatives with some biomolecules. However it is not just the intrinsic activity of MDA that is particularly significant, rather the pathway from which it arises. Platelet arachidonate metabolism via the thromboxane pathway can be monitored in suspensions of activated human platelets by measurement of MDA.

3.7.2.1 MDA formation from non-lipid biomolecules

It has been shown that exposure to ionising radiation or oxidative stress yields quantitative amounts of MDA. Purported non-lipid sources include proteins, bile pigments, nucleic acids and carbohydrates. It has been used as a marker of oxidative DNA damage by free radicals, but no evidence exists that MDA represents a primary oxidative-injury product from a non-lipid biomolecule.

3.7.2.2 MDA formation from lipid molecules

MDA is an end product of nonenzymatic, metabolically uncoupled polyunsaturated fatty acids containing at least two conjugated double bonds. The metabolically uncoupled oxygenation of polyunsaturated fatty acids (PUFA), leading to lipid-derived MDA is a process synonymous with degradation of oils, foodstuffs and lipid-rich biomolecular assemblies (membranes, lipoproteins etc). Thus MDA formation has long been used as a marker of rancidity in the food industry. Polymerisation of the cell membrane of erythrocytes on ageing liberates MDA. This alters the deformability and fluidity necessary for RBCs survival in the circulatory system, thus leading to removal of aging cells. In living systems, prostaglandin production by platelets is essential to the process of platelet aggregation. These transient intermediates are then rapidly metabolised to nonprostanate substances including MDA. This production pathway is illustrated in Figure 3.20. Thromboxane synthase generates MDA via the conversion of PGH$_2$ to thromboxane A$_2$ (TXA$_2$), which rapidly hydrolyses to...
TXB$_2$ $^{276}$, with the concomitant formation of 12 (L)-hydroxy-5,8,10-heptadecatrienoic acid (HTT). Since thromboxane synthase generates equimolar amount of these three products, the efficacy of enzymatic MDA formation is $\sim 30\%$ $^{270}$. As the prostanoid intermediates are unstable in aqueous media, any direct attempt to quantify their production is difficult. The indirect measurements of the more stable metabolites, MDA, TXB$_2$ or HHT are thus valuable indicators of enzyme activity in the arachidonic acid pathway.
Figure 3.20 Malondialdehyde formation during eicosanoid biosynthesis.
3.7.3 Malondialdehyde in disease states

Although MDA is a metabolite of the pathway described in Fig. 3.2, it also has biological significance in its own right. It has been shown to possess carcinogenic/mutagenic properties and is a useful marker for various disease states.

Inflammation associated with infectious and degenerative diseases, such as hepatitis C, lead to activation of cells such as neutrophils and endothelial cells, which may produce oxidative stress. Lipid-peroxidation is induced and MDA is among the many aldehydes produced on degradation of lipid hydroperoxides. As oxidative stress increases in hepatitis C and other liver disorders, there is a corresponding increase in levels of MDA. Thus specific assays of MDA have potential use in the clinical management of chronic hepatitis C patients as the affectivity of interferon treatment could be tested by serial MDA determinations during the course of the treatment.

Many abnormalities of platelet function are associated with diabetes mellitus, leading to an increased risk of thrombosis and premature arteriosclerosis. Decreased levels of MDA are found to be significant in patients with Type II diabetes, especially those with poor metabolic control of their condition, most likely due to late vascular complications associated with the disease.

Lipid peroxidation is also considered to be important in the development of arteriosclerosis and to be involved with the aging process and clinically significant disorders such as cancer and cardiovascular disease. As MDA is one of the major secondary oxidation products of peroxidized polyunsaturated fatty acids, and is known to be elevated in these disease states, it is widely used as an index of lipoperoxidation.
3.7.4 Quantification of MDA levels

MDA levels are routinely measured as a means of quantifying levels of lipid peroxidation. Derivitisation allows indirect evaluation of MDA production as a fluorescent, pigmented, UV-absorbing, or volatile reaction product. The most commonly used derivatisation procedure involves the use of 2-thiobarbituric acid (TBA). Like all barbituric acids it acts as a nucleophile in acid-catalysed reactions, precipitating aromatic aldehydes and ketones as stable coloured derivatives. MDA readily undergoes a nucleophilic addition reaction with TBA in an acid medium to generate a red/pink fluorescent MDA: TBA adduct. This derivatisation assay is a useful way of estimating COX-1/ thromboxane synthase inhibitory activity of compounds. As MDA is a direct stable by-product of arachidonic acid metabolism, cyclooxygenase inhibition will result in decreased levels of MDA production relative to controls. Thus determination of MDA production can estimate arachidonic acid and prostaglandin metabolism at a specific platelet level. As MDA, HHT and TXB2 are formed in a 1:1:1 ratio, inhibition of MDA production can also be correlated with decreased liberation of TXA2 in vivo. This facilitates the measurement of COX-1 inhibition and prostaglandin biosynthesis and thus the antiplatelet effect of drugs, without recourse to the more expensive and time-consuming TXB2 ELISA assay.
In this experiment we examined the effect of aspirin and ISDA on MDA production in human whole blood. Inhibition of MDA formation was understood to be related to inhibition of the cyclooxygenase enzyme, and thus was used as an indicator of the potency of each agent as an antiplatelet drug.

### 3.7.5 MDA assay

The experimental protocol used in this assay is described in more detail in Chapter 6. Blood was sampled from healthy human volunteers who had not taken any NSAIDs in the previous seven days. There is no significant statistical difference between basal levels of MDA in male and female blood. All blood was tested within 4 hours of sampling using the Chrono-Log® Whole Blood Aggregometer. Blood was incubated with the test compound before challenging with arachidonic acid. Following aggregation, plasma was acidified with trichloroacetic acid (TCA) and then centrifuged. Sample acidification can induce turbidity or precipitate nonlipid sample constituents (e.g. proteins) and so centrifugation is a
necessary step to clarify the sample prior to analysis. Supernatant was mixed with equal volumes of thiobarbituric acid (TBA), (0.53% in phosphate buffer pH 7.4) and heated. Samples were analysed by HPLC using a mobile phase consisting of 50:50 methanol: phosphate buffer pH 7.4 and a detection wavelength of 532 nm. MDA peaks were quantified relative to controls run under the same experimental conditions. Malondialdehyde \textit{bis}(dimethyl acetyl) is the recommended standard, as it is readily hydrolysed to MDA in an acidic environment.

3.7.6 Results of MDA assay

Both aspirin and ISDA were examined for their inhibitory effect on MDA production in the concentration range 1-100 \( \mu M \). Experiments were carried out in triplicate and adjusted with reference to a blank sample to account for basal levels of MDA. Percentage inhibition was then determined relative to control samples, which were incubated with 10 \( \mu l \) of DMSO rather than drug solution. A cumulative plot of experimental results is presented in Fig. 3.22.

![Figure 3.22 Aspirin versus ISDA inhibition of MDA production in human whole blood: Aspirin ( ), ISDA ( )](image)

Figure 3.22 Aspirin versus ISDA inhibition of MDA production in human whole blood: Aspirin ( ), ISDA ( )
ISDA showed marked inhibition of MDA production in platelets stimulated with arachidonic acid. ISDA is almost equipotent with aspirin as an inhibitor of MDA synthesis (IC$_{50}$ of 24 μM compared with aspirin IC$_{50}$ of 17 μM). The slightly lower potency of ISDA relative to the parent compound may be due to incomplete hydrolysis of the prodrug within the time constraints of this experiment. Results obtained correlate closely with those for the whole blood aggregometer where aspirin was found to be slightly more potent than ISDA at inhibiting platelet aggregation with an IC$_{50}$ of 35 μM compared with ISDA IC$_{50}$ 40 μM. (Section 3.3.4.1). 100% inhibition of MDA formation is not observed as MDA is produced from PGH$_2$ not only enzymatically by thromboxane synthase but also non-enzymatically.

The determination of MDA production has been proposed as a useful assay method in the evaluation of drugs impairing platelet function. There is precedence in the literature for use of an aggregometer to induce MDA production. Rajtar examined the effects of diazepam on rat platelets by challenging rat PRP with AA in an aggregometer, stopping the reaction with trichloroacetic acid and processing samples further for MDA determination. The correlation between the reduction in platelet aggregation and the synthesis of MDA suggested that the observed effect of diazepam is due to the inhibition of the cyclooxygenase pathway of AA metabolism in the platelet.

HPLC provides a sensitive, rapid means of determining MDA levels in plasma solutions. Aspirin inhibits the production of MDA after platelet stimulation with both thrombin (10-25 units/ml) or sodium arachidonate (0.5-2.25 mM) and this effect is both time and concentration dependent. It has been shown to inhibit the synthesis of prostaglandin endoperoxides by platelets as well as the formation of their products PGE$_2$, PGF$_{2α}$, HTT and MDA, and these results are replicable in our study. In platelets, prostaglandin synthesis involves enzymatic transformation of arachidonic acid into unstable intermediates, which in turn are broken down to form MDA. In this study it is evident that in stimulated platelets both aspirin and ISDA inhibit formation of MDA to a comparable extent, thus validating the proposal that ISDA is a potent aspirin prodrug, liberating aspirin on hydrolysis by plasma esterases. It inhibits cyclooxygenase activity in the arachidonic acid cascade in a concentration-dependent fashion, as is evident from its ability to reduce MDA production.
3.8 ELISA assay for Thromboxane B\textsubscript{2}

3.8.1 Introduction to the ELISA assay

As explained in previous sections aspirin irreversibly acetylates the amino terminal serine in the active site of cyclooxygenase, the initial enzyme in the prostaglandin and thromboxane biosynthetic pathway \textsuperscript{28}. This results in reduced formation of the cyclic endoperoxide intermediates and the end products of arachidonic acid metabolism (PG's and TXA\textsubscript{2}) \textsuperscript{294}. Because of the short biological half-life of the physiologically active TXA\textsubscript{2} its first metabolite TXB\textsubscript{2} has traditionally been accepted as an indicator of TXA\textsubscript{2} production \textsuperscript{295}. This indirect method is widely used for measurement of TXA\textsubscript{2} concentrations in biological samples \textsuperscript{296}. The immunoassay of eicosanoids is known to be a very sensitive and specific method suitable for detecting minute amounts of those substances in biological material. Measurement of the major platelet cyclooxygenase product, TXB\textsubscript{2}, provides a direct method for evaluation of the antiplatelet effect of aspirin and related aspirin prodrugs. Serum TXB\textsubscript{2} measurements are used as a reflection of platelet cyclooxygenase activity for \textit{in vivo} and \textit{ex vivo} drug evaluation. The assay is based on the generation of endogenous thrombin from prothrombin, occurring within minutes of contact of whole blood to glass tubes at 37°C \textsuperscript{297}. Whole blood TXB\textsubscript{2} production measured by ELISA is thus a reflection of the cyclooxygenase activity of the platelet COX-1 \textsuperscript{298}.

3.8.2 ELISA assay

An \textit{in vitro} COX-1 assay was performed using human whole blood containing no anticoagulant \textsuperscript{299}. Aliquots of 500 μl were immediately transferred to siliconized microcentrifuge tubes preloaded with 2 μl of either DMSO or a test compound at final concentration of 0.1 – 100 μM. The tubes were vortexed and incubated at 37°C for 1 hour, to allow the blood to clot. At the end of the incubation period serum was obtained by centrifugation (12,000 rpm for 10 mins). The supernatant was aspirated off and assayed for TXB\textsubscript{2} using an enzyme immunoassay kit (R&D Systems, Oxon, UK) according to the manufacturers instructions.
3.8.3 Results of ELISA assay

The compounds tested for their ability to inhibit COX-1 were aspirin, ISDA, is-2-asp-5-sal and is-2-asp-5-(4-nitrobenzoate). Quantitation of TXB\(_2\) levels was achieved by means of a standard calibration curve (13.7 - 10,000 pg/ml), performed on the same day as the prodrug assays. The calibration curve is presented in Fig. 3.23. Each concentration was measured in duplicate and corrected to account for non-specific binding in each assay well.

Figure 3.23. Calibration curve of log TXB\(_2\) conc. (pg/ml) versus optical density \(\lambda\) (\(r^2 = 0.988\))

Inhibition curves for the experimental compounds are presented in Fig. 3.24. Each compound was tested in triplicate at each different concentration. The amount of TXB\(_2\) produced was calculated with reference to the calibration curve presented above. Basal levels of TXB\(_2\) in human whole blood in the absence of any inhibitor were determined to be 63.2 ng/ml TXB\(_2\) (range 60.4 - 64.7 ng/ml). Literature values for TXB\(_2\) levels vary considerably depending on the treatment of platelets prior to the assay. Human serum has been reported previously to contain 224.4 ± 81.3 ng/ml \(^{294}\) while washed platelets were quoted as containing from 24 ± 6 pg/ml \(^{300}\) to 46.3 ng/ml \(^{296}\). Human whole blood as used in this experiment has previously been quoted as containing > 1 ng/ml as it was below the limits of quantitation of the particular assay \(^{298}\).
The production of TXB₂ following blood coagulation was used as an indication of COX-1 activity in the blood. This reaction has been shown to be sensitive to the action of aspirin and is a well-characterized method with which to measure platelet COX-1 activity in human whole blood. In this particular assay, aspirin was found to be the most potent inhibitory agent for COX-1. Aspirin at 100 μM allowed for liberation of only 0.91% TXB₂ due to inhibition of COX-1, whereas at 0.01 μM liberation of 86.59% TXB₂ was observed. ISDA and is-2-asp-5-sal inhibited COX-1 with approximately equal potency. ISDA at 100 μM was associated with 28.52% TXB₂ production while is-2-asp-5-sal gave 31.62% production of TXB₂ at 100 μM. In this assay the aspirin prodrugs thus appeared less effective than aspirin at inhibiting COX-1. This may be due to the limitations of the assay rather than reduced potency of the prodrugs. Because it is necessary to isolate serum, whole blood is sampled into tubes containing no anticoagulant. The blood tends to clot within 15 minutes, which gives very little time for prodrug metabolism by plasma esterases. Isosorbide-2-aspirinate-5-[4-nitrobenzoate] showed no appreciable inhibition of TXB₂ production, which correlates with lack of release of aspirin in plasma hydrolysis studies (Section 4.3.2). Thus the results of this assay, in conjunction with those of the MDA assay, confirm that the isosorbide-based aspirin prodrugs achieve their antiplatelet effect by inhibition of the COX-1 enzyme, but conclusions about their potency are drawn only from
coagulation experiments where the incubation step so vital in liberating parent aspirin in vivo can be completed.
There are methodological difficulties associated with the measurement of TXB₂, due to the artificial production of TXB₂ by platelets during blood collection. This explains why, for is-2-asp-5-sal, more than 100% production of TXB₂ is noted.

3.9 Studies in canine blood

Previous work on ISDA in dog blood revealed that it was a potent inhibitor of TXB₂ production in serum, although little aspirin was liberated on hydrolysis by plasma esterases. It gave profound sustained suppression of serum TXB₂ levels after oral administration to dogs. These studies suggest that either ISDA or a metabolite directly inhibits COX-1 or another pathway in platelet activation such as lipid release or thromboxane synthesis. This section describes aggregation experiments in canine blood, which attempted to evaluate the potency of ISDA as an antiplatelet agent.

3.9.1 ISDA in canine PRP

Platelet aggregation in canine PRP was examined, using arachidonic acid to induce maximal platelet aggregation. ISDA and aspirin were evaluated for their ability to inhibit AA-induced aggregation.

3.9.1.1 Experimental protocol

20 ml of canine blood was sampled from the jugular of beagle dogs and an FBC was carried out on all samples prior to centrifugation to ensure that platelet numbers were between 200-300 x 10⁹/cm³. Blood was centrifuged to isolate PRP then further centrifuged to yield PPP as a blank. Samples were incubated with 100 μM aspirin or ISDA, before challenging with arachidonic acid (0.1-10 mM).
3.9.1.2 Results of PRP experiments
The optical method for evaluation of platelet aggregation was unsuitable for use with canine blood. There was great variability in response to arachidonic acid with some samples showing 98% aggregation response to 8 mM AA, while other samples failed to respond at all. At lower and higher concentrations of agonist there was no response observed.

3.9.2 ISDA in canine whole blood

Because platelet aggregation experiments using beagle blood failed to show any aggregation in response to arachidonic acid using the turbidometric method, it was decided to repeat the analysis using the impedance method and whole blood from a labrador dog. Blood was tested for its response to arachidonic acid and the effect of aspirin and ISDA on inhibiting AA-induced aggregation.

3.9.2.1 Experimental protocol for canine whole blood.
Whole blood (20 ml) was sampled from the cephalic vein of a labrador, using sodium citrate as anticoagulant. As per other whole blood aggregation experiments, dog blood was incubated in a Chrono-Log® Whole blood Aggregometer. Blood was challenged with 0.5 mM arachidonic acid and the aggregation response was monitored on a chart recorder. ISDA and aspirin were examined for their inhibitory effect in canine whole blood by incubation of each at 40 μM, prior to challenging the blood with AA.

3.9.2.2 Results of canine whole blood aggregation experiments
Maximal aggregation in labrador whole blood was observed at 0.5 mM arachidonic acid. On increasing the concentration to 1.0mM the aggregation response was abolished. Both aspirin and ISDA gave full inhibition of canine whole blood at 40 μM as shown in Fig 3.25. Aggregation patterns were similar to those observed in human whole blood. Dog platelets have many functional characteristics which are similar to human platelets, and their response to platelet inhibitory drugs such as aspirin is nearly equal to that of human platelets, as was noted during the course of this experiment.
Figure 3.25 Inhibition of AA-induced platelet aggregation by ISDA and aspirin: 1 = control with 10 μl DMSO, 2 = ISDA 40 μM, 3 = Aspirin 40 μM.

Use of AA at high concentrations (0.5-1 mM) may not induce platelet activation response in canine platelets. They do respond at lower AA concentrations, which implies that at higher agonist concentrations it may induce the formation of AA-inhibitory metabolites (prostacyclin), thus impairing platelet activation responses. It is known that there is marked inter-species variation in whole blood aggregometry, suggesting that modulations in aggregation by other blood elements probably differ in each species and with stimulation agonists. Differences in canine platelet aggregation exist in response to ADP and collagen, arachidonic acid and thromboxane A₂. When using canine platelets individual variation needs to be taken into consideration. Chignard observed that in many instances dog PRP fails to aggregate on exposure to arachidonic acid, which explains the negative results obtained in the turbidometric analysis detailed above. The arachidonate pathway is intact but dog platelets uniformly fail to aggregate in response to the generation of PGH₂ and TXA₂.
Results of platelet activation responses to AA vary among different breeds of dogs with beagle results being quite variable (as evident in our experiments). German Shepherd dogs do not respond to AA while Irish Setters display both platelet aggregation and secretion when stimulated with the same AA concentrations. This suggests that in dogs AA-induced platelet activation is not only dose- but also breed-dependent, and that individual differences within the same breed may also exist\textsuperscript{305}. 
This chapter examined the potential use of ISMNA, ISDA and isosorbide-2-aspirinate-5-salicylate as antiplatelet agents. The compounds were synthesised in-house and their potency, relative to the parent compound aspirin, was ascertained. All three compounds had previously been evaluated in plasma hydrolysis studies in human blood: ISMNA liberated up to 8.4% aspirin in 10% human plasma while ISDA was associated with the generation of up to 60% aspirin. Is-2-asp-5-sal confirms the importance of the 5-position of the isosorbide-2-aspirinate backbone liberating up to 91.5% aspirin in in vitro hydrolysis studies.

ISMNA and ISDA were evaluated for their antiplatelet effect in PRP using the optical turbidometric assay. ISDA was found to be equipotent with aspirin (IC$_{50}$ 4.3 and 5.2 µM respectively), while ISMNA was almost 10-fold less potent (IC$_{50}$ 85 µM) consistent with its liberation of less than 10% aspirin on hydrolysis by plasma esterases.

In whole blood assays using the impedance method of aggregometry, ISDA was again found to be equipotent with aspirin (IC$_{50}$ 40 and 35 µM respectively). Once more ISMNA was found to be considerably less potent than aspirin (IC$_{50}$ 250 µM). Isosorbide-2-aspirinate-5salicylate was approximately 3-fold more potent than aspirin at inhibiting arachidonic-acid-induced platelet aggregation (IC$_{50}$ 12 µM). Other potential aspirin prodrugs such as benorylate and guaiacol-aspirinate had little antiplatelet effect even at the relatively high doses of 100 µM. These isosorbide-based aspirin prodrugs are the first reported instance of prodrugs which are known to liberate aspirin on hydrolysis by plasma esterases and which parallel aspirin’s antiplatelet effects in vitro.

Results for inhibition of ADP-induced aggregation experiments in human whole blood revealed that ISDA is a significantly more potent inhibitor of platelet aggregation than aspirin. Factorial design experiments demonstrated that the optimum incubation time for isosorbide-based aspirin prodrugs prior to aggregation experiments is 20 minutes, presumably to allow for hydrolysis of the parent compound by plasma esterases to liberate aspirin and other active metabolites. Experiments with eserine confirmed that butyrylcholinesterase is the enzyme required for this essential in vivo prodrug hydrolysis. ISDA behaves as a
model prodrug, lacking any significant pharmacological effect until its hydrolysis by plasma enzymes, whereby it liberates the parent compound.

That these novel prodrugs achieve their antiplatelet effect by inhibition of cyclooxygenase-1 in platelets was established by means of the MDA assay, which quantifies production of an end-product of the arachidonic-acid cascade following platelet activation. Again ISDA was shown to be equipotent to aspirin at inhibiting production of MDA. These results were confirmed by means of an ELISA assay, which detects production of TXB$_2$, another end product of the arachidonic acid cascade. ISDA and is-2-asp-5-sal were shown to have an inhibitory effect on COX-1, which while not as significant as that of aspirin, can be explained by constraints of the experiment which do not permit sufficient incubation time to ensure hydrolysis of the parent compound.

Studies of ISDA's potency relative to aspirin in canine whole blood were also performed. This confirmed that ISDA does have an antiaggregatory effect equivalent to aspirin in labrador blood, although its hydrolysis is not associated with any liberation of aspirin in dog plasma studies.

This chapter confirms the potential usefulness of the isosorbide-based aspirin prodrugs as antiplatelet agents. They are selective for COX-1 in platelets, and is-2-asp-5-sal in particular has been shown to be the first reported prodrug to release solely aspirin on hydrolysis in plasma. If toxicity studies in the GIT establish their safety profile, they will be contenders to replace aspirin as the antiplatelet of choice in patients who cannot tolerate aspirin.
Chapter 4

An investigation into the structure-activity relationship of a series of aspirin prodrugs based on isosorbide.
4.1 Introduction

4.1.1 Prodrugs of aspirin and other carboxylic acids

A prodrug has been defined as a pharmacologically inactive derivative of a parent drug molecule, which requires spontaneous or enzymatic transformation in the body to release the active drug. Prodrugs are designed to increase metabolic stability, aqueous solubility, eliminate secondary effects or improve delivery characteristics, taste or smell. Of major advantage in the design of prodrugs is that if the prodrug and the inert moiety are both inactive then the pharmacology and/or toxicology are limited to that of the original drug. Esters are among the most prevalent prodrug types due to the predominance of carboxylic and hydroxyl substituents in drug molecules along with the availability of enzymes in the living systems capable of hydrolysing them. As described in Chapter 1, the pharmacological usefulness of aspirin as an antithrombotic agent is limited by its gastrotoxicity profile. It has been shown that up to 70% of people taking aspirin experience occult gastric bleeding. It is thus desirable to develop an ester prodrug of aspirin, which would be hydrolysed by plasma esterases to release the active aspirin in the blood after absorption from the gastrointestinal tract.

4.1.2 Typical hydrolysis pathway of aspirin prodrugs

There are two main categories of aspirin prodrug - derivatives that are cleaved by enzymes in vivo to regenerate the parent drug and those that are non-enzymatically hydrolysed to give the same. One of the major limiting factors in the development of aspirin prodrugs is the lability of the acetyl ester group in aspirin. Esterification of the carboxyl group renders the acetyl group very susceptible to enzymatic cleavage, thus liberating salicylic acid as the major metabolite. While salicylic acid is itself an anti-inflammatory agent, aspirin is a far more potent analgesic and antithrombotic agent and so delivery to the bloodstream in the intact form is desirable. The typical hydrolysis pathway for aspirin prodrugs...
is shown in Fig. 4.1. As detailed in Chapter 2, in order for a compound to be considered a true aspirin prodrug it must undergo hydrolysis preferentially at the carboxylic ester group rather than the O-acetyl group i.e. it is necessary that $k_1 > k_2$. If $k_2 > k_1$ the compound is instead considered to be a prodrug of the salicylate ester $^{307}$.

There have been numerous attempts to produce esters of acetylsalicylic acid, which will protect the labile acetyl group during hydrolysis to regenerate aspirin \textit{in vivo} on enzymatic hydrolysis.
4.1.3. Specific aspirin prodrugs designed to date.

4.1.3.1 Acylals of aspirin

Aspirin was converted to its 1-ethoxyethyl ester (26) in an attempt to reversibly mask the acidic carboxylic group in order to reduce local irritation. It was found to liberate aspirin on aqueous hydrolysis and this hydrolysis was first-order with respect to solvent and pH independent.

\[
\begin{align*}
\text{H}_3\text{C} & - \text{O} \\
\text{O} & - \text{CH}_3 \\
\text{O} & - \text{CH}_3 \\
\text{O} & - \text{O} \\
\text{C} & - \text{O} \\
\end{align*}
\]

There are no reported studies on the pathway or rate of hydrolysis of this prodrug in vitro or in vivo, and so it cannot be assumed to be a true aspirin prodrug.

4.1.3.2 Benorylate

In 1963 a novel ester called Benorylate (4-acetamidophenyl 2-acetoxybenzoate, 25), a potential prodrug of aspirin and paracetamol, was synthesised by Robertson. When administered at relatively high doses (8 g/day) for prolonged periods of time it has been used clinically in the treatment of rheumatoid arthritis and other chronic rheumatic diseases. The potential pathways of hydrolysis of the prodrug are presented in Fig 4.2.
Benorylate (25) is rapidly hydrolysed by esterases in gut mucosal cells and in the blood stream, liberating principally paracetamol and salicylic acid. Hydrolysis via the aspirin route was minor with only trace amounts of aspirin detected in the plasma, which may be a result of impurities in the analgesic formulation. In liver microsomal fractions the rate of removal of the acetyl group from benorylate was about ten times faster than for aspirin. Clinically, it was found to be as effective an analgesic and anti-inflammatory agent as soluble aspirin in treatment of rheumatic conditions, with appreciably lower incidence of
side-effects. Significantly lower levels of gastrointestinal blood loss were noted in patients on benorylate as opposed to aspirin regimens.

Benorylate cannot be considered a true aspirin prodrug, as its hydrolysis does not liberate the parent compound. Its therapeutic activity as an anti-inflammatory agent is due to the production of salicylic acid and paracetamol on metabolism. The slower absorption of benorylate may be due to lower absorption rate across the gastric mucosa, or its low aqueous solubility.

4.1.3.3 Methylsulfinylmethyl ester

Methylthiomethyl and β-ethylthiomethyl esters are used as protective groups for carboxylic esters as they are readily cleaved in alkaline conditions to liberate the free acid. A series of related aspirin esters were synthesised including the methylthiomethyl (27), methylsulfinylmethyl (28) and methylsulfonylmethyl (29) derivatives.

In human plasma, all three esters had shorter half-lives than aspirin (~ 2 hrs). Hydrolysis rate constants were first order for all three prodrugs. In vivo studies in dogs revealed that the methylsulfinyl derivative (28) is a true aspirin prodrug, giving detectable levels of aspirin in the blood following oral administration. Metabolic studies in 10% human plasma by Bundgaard revealed that compounds 28 and 29 liberated 30% and 20% aspirin respectively on hydrolysis by human plasma esterases. Studies on percutaneous absorption through excised mouse skin revealed that the prodrug has a penetration rate twice that of the parent aspirin but cleavage occurs primarily via the salicylate route. These prodrugs may
therefore be unsuitable as true aspirin prodrugs for transdermal formulations. There have been no studies reported which examine the antiplatelet effects of these thioesters of aspirin. Similarly, their susceptibility to aqueous hydrolysis has not been studied.

4.1.3.4 Phenylalanine esters

Amino acids are desirable prodrug moieties as they are non-toxic and have a broad range of properties (they are usually classified as nonpolar, polar, acidic and basic), enabling them to dramatically alter the physical properties of the parent carboxylic acid. Phenylalanines esters of aspirin were synthesised, which were expected to undergo α-chymotrypsin mediated hydrolysis of the ester or amide group followed by carboxypeptidase cleavage of the amide-releasing aspirin in the intestinal membrane wall. This would reduce the local irritation effects of aspirin on the gastric mucosa. Prodrugs synthesised included aspirin phenylalanine ethyl ester (30), aspirin phenylalanine amide (31) and aspirin phenylacetic ethyl ester (32).

Where \( R = \)

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Enzymatic kinetic studies revealed that compound **30** was the best substrate for \( \alpha \)-chymotrypsin \( (K_m \ 1 \times 10^{-6} \ \text{mol/l}) \) \(^{327}\), while compound **32** was the best substrate for carboxypeptidase \( (K_m \ 1.3 \times 10^{-4} \ \text{mol/l}) \) \(^{328}\). Hydrolysis studies showed that compound **30** was the most promising true aspirin prodrug as *in vitro* studies in enzyme solution confirmed the regeneration of aspirin on enzymatic hydrolysis of the prodrug. However *in vivo* studies have not been reported and low aqueous solubilities of all compounds may present formulation issues \(^{326}\). A subsequent study by Muchi-EIdeen determined that the aspirin generation reported in initial studies was in fact produced on hydrolysis of aspirin anhydride, present as an impurity in the formulation \(^{329}\).

**4.1.3.5 Amino acid ethyl esters**

Consequent to work on aspirin prodrugs of phenylalanine the ethyl esters of aspirin \( \text{L-arginine (33)} \) and aspirin \( \text{\( p \)-guanidino-L-phenylalanine (34)} \) were synthesised \(^{330}\) in an effort to produce water-soluble prodrugs, which would liberate aspirin on enzymatic cleavage by trypsin and carboxypeptidase B.

![Diagram](image-url)
The ester bonds of both prodrugs were good substrates for the trypsin enzyme but generated salicylic acid on hydrolysis. They were poor substrates for cholinesterases and as such did not function as aspirin prodrugs.  

4.1.3.6 Benzodioxinones

In the benzodioxinone series of aspirin esters the carboxyl and acetyl groups are incorporated into a common ortho ester function (35). The R substituent is chosen to ensure an appropriate rate of hydrolysis as well as an acceptable hydrophilic/lipophilic ratio.  

In hydrolysis studies in water-dioxane mixtures only the tert-butyl derivative released aspirin in detectable amounts. Hundewand and Senning synthesised compound 36 (2-Methyl-2-[2-(methoxy)phenoxy]-4H-1,3-benzodioxin-4-one) which on hydrolysis in 10% human plasma liberated ~70% aspirin with a half-life of 80 minutes. This is one of the most promising compounds reported to date. However stability studies in the pH range 3.0 to 7.4 suggest that compound 36 is too unstable to be useful as an aspirin prodrug.

Nielsen and Senning investigated a series on 15 esters where the tert-butyl moiety of 35 was substituted for other tertiary aliphatic alkyl substituents (e.g. R = 3-methyl-2-hexyl). Nine of the prodrugs were sufficiently stable to be considered as potential aspirin prodrugs and only four of these were found to liberate aspirin.
in plasma solution. All the prodrugs studied were hydrolysed too rapidly however to be considered as prospective aspirin prodrugs.

4.1.3.7 Triglycerides

Triglycerides, whereby aspirin has replaced one or more of the fatty acids in the structure, have been synthesised (37). The incorporation of the water-soluble aspirin into a lipid structure renders the prodrug fat-soluble, thereby enhancing absorption. Hydrolysis by pancreatic lipases would theoretically cleave the fatty acids leaving aspirin as a monoglyceride, which could be cleaved in the blood or mucosa to release aspirin.

\[
\text{CH}_2\text{OR}_1 \\
\text{CHOR}_2 \\
\text{CH}_2\text{OR}_3
\]

Where R₁, R₂ and R₃ are H or fatty acyl and at least one is OR₁.

Hydrolysis studies focused only on quantification of plasma salicylate levels following ingestion by rats; there is no evidence to suggest that these are true aspirin prodrugs, which liberate ASA on metabolism in vivo. In rats the prodrugs appeared to induce a low degree of gastric ulceration. However this may be due to the lack of aspirin generation on prodrug hydrolysis.
4.1.3.8 Formylphenyl esters

A series of 2-, 3- and 4-formylphenyl (R = CHO) (38) and 4-substituted 2-formylphenyl (39) aspirins were synthesised by Bowden in an attempt to determine whether the benzoic acid ester function of aspirin undergoes alkaline hydrolysis more readily than the acetyl group.

There was quantitative formation of aspirin and the respective formylphenol under alkaline conditions due to intramolecular catalysis. In the carrageenan-induced rat paw oedema model for anti-inflammatory activity the 2-formylphenyl aspirin showed potent anti-inflammatory activity, whereas compound 39 lacked any significant activity. This test was repeated for similar prodrugs of indomethacin and other NSAIDs and in all cases the 2-formylphenyl esters were more potent than the parent compound. However no data exists on whether these aspirin esters liberate aspirin on enzymatic hydrolysis in plasma solution. It is not clear that the intramolecularly catalysed reaction leading to aspirin would successfully compete with the rapid deacetylation reaction likely to occur at more physiological pH values on contact with plasma- or other esterases.
4.1.3.9 2-Nitroimidazoles

2-Nitroimidazoles are designed for targeting of hypoxic tissues and as such are of interest in the delivery of aspirin to areas of rheumatoid joint inflammation (known to be associated with hypoxic conditions) and other damaged tissues where poor oxygenation prevails. The nitroimidazole prodrug of aspirin undergoes reductive elimination (Fig. 4.3) to liberate aspirin \[338\].

![Figure 4.3 Mechanism for the reductive elimination of aspirin from 2-nitroimidazole-aspirinate.](image)

It has enhanced aqueous solubility but the ester linkage is susceptible to cleavage by non-specific esterases. It is therefore unlikely to be suitable as a potential aspirin prodrug.

4.1.3.10 Glycolamides

In 1987, Bundgaard reported the synthesis of a series of benzoic acid esters of 2-hydroxyacetamides (glycolamides) \[40\], which were rapidly hydrolysed in 50% human plasma \[339\]. The optimum structure for a rapid rate of enzymatic hydrolysis was the glycolamide structure with two substituents on the amide nitrogen atom. The high reactivity of the N,N-disubstituted glycolamide esters towards cholinesterase can be explained by the structural similarity of the esters to benzoylcholine \[41\].
The glycolamide esters synthesised were very susceptible to hydrolysis by plasma cholinesterase and also had good stability in aqueous solution. This was subsequently exploited in the synthesis of glycolamide esters of aspirin. The N,N-dimethyl and N,N-diethylglycolamide esters liberated the largest quantity of aspirin in 10% human plasma (50 and 55% respectively). These esters are chemically stable, with water solubilities and lipophilicities favourable for delivery. The same basic structure was also incorporated into a series of prodrugs of other NSAIDs such as ibuprofen, ketoprofen, naproxen and mefenamic acid in an effort to improve their delivery characteristics.

4.1.4 Isosorbide-based aspirin prodrugs

Previous work in our laboratory has determined that of the three main isosorbide-based aspirin prodrugs under investigation (ISMNA, ISDA and Is-2-asp-5-sal), it is isosorbide-2-aspirinate-5-salicylate (42) which possesses a unique fit for plasma butyrylcholinesterase (EC 3.1.1.8). This optimal fit promotes hydrolysis at the carboxylic ester group and suppresses hydrolysis at the more labile acetyl group, giving primarily aspirin liberation on metabolism in human plasma. (Table 4.1, Reproduced with permission).
<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Group at 5-position</th>
<th>$k_{obs}$ min$^{-1}$</th>
<th>$t_{1/2}$ min</th>
<th>% Aspirin formed mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISMNA</td>
<td>ONO$_2$</td>
<td>0.69</td>
<td>1.00</td>
<td>7.13 (6.59-8.43)</td>
</tr>
<tr>
<td>ISDA</td>
<td>Aspirin</td>
<td>0.16</td>
<td>4.43</td>
<td>51.0 (40.0-60.0)</td>
</tr>
<tr>
<td>Is-2-asp-5-sal</td>
<td>Salicylic acid</td>
<td>0.16</td>
<td>4.90</td>
<td>73.23 (64.95-81.5)</td>
</tr>
</tbody>
</table>

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.

The substituent at the 5-position on the isosorbide ring appears to be crucial for determining the route of hydrolysis of the prodrug. A salicylate molecule in the 5-position has been found to liberate optimal levels of aspirin by directing hydrolysis through the 2-carboxylate ester linkage as opposed to the more labile acetate group. Is-2-asp-5-salicylate has been associated with liberation of up to 81.5% aspirin in human plasma hydrolysis studies. This is one of the most significant aspirin esters reported to date, the salicylic acid group in the 5-position directing the major pathway of hydrolysis towards the liberation of aspirin from the 2-position. It might be possible to modify the 5-position in an effort to produce a compound with more desirable physico-chemical properties, while retaining or even improving the aspirin release characteristics of the prodrug.

In order to further explore the dependence of aspirin release characteristics on the nature of the substituent at the 5-position, a series of compounds was synthesised using the isosorbide-2-aspirinate structure with varying substituents at the 5-position. It was also desirable to determine if the physico-chemical properties of the prodrug could be manipulated to optimise characteristics such as aqueous solubility and lipophilicity, while maintaining their ability to release aspirin.
4.2 Synthesis of esters of isosorbide-2-aspirinate

4.2.1 Synthesis of alkyl derivatives of isosorbide-2-aspirinate

Instead of an aromatic group at the 5-position of the isosorbide molecule an alkyl group was substituted. A series of alkyl esters, whereby methyl (43) and ethyl (44) substituents were introduced, were synthesised according to the scheme presented in Fig. 4.4.

![Figure 4.4](image)

Figure 4.4 The synthesis of a series of alkyl derivatives of isosorbide-2-aspirinate

Isosorbide-2-aspirinate was stirred in dichloromethane at 0°C in the presence of triethylamine and one molar equivalent of the appropriate alkyl anhydride. Compounds were obtained as crude oils which were purified by flash chromatography over silica gel to yield products as white crystals whose purity was confirmed by TLC and HPLC, and structural confirmation by IR, NMR and HRMS.
4.2.2 Synthesis of aromatic derivatives of isosorbide-2-aspirinate.

The 5-position of the isosorbide molecule was also examined for the effect of substitution of an aromatic ester on the rate and pathway of hydrolysis. Once more, isosorbide-2-aspirinate was used as the starting material and a range of esters were produced according to the scheme presented in Fig. 4.5. The synthesis involved the coupling of isosorbide-2-aspirinate and the relevant acid in the presence of dicyclohexylcarbodiimide (DCC), catalysed by dimethylaminopyridine (DMAP). In the case where the acid bore an acylatable functional group, it was added at 0°C to suppress self-acylation. The acids used included benzoic acid (45), nicotinic acid (46), iso-nicotinic acid (47), benzyloxybenzoic acid (48), anthranillic acid (49), 3-toluic acid (50), 2-anisic acid (51), 3-anisic acid (52) and 4-anisic acid (53). All products were purified by column chromatography to yield esters as crystalline materials whose purity was confirmed by TLC and HPLC and structural characteristics characterised by NMR, IR and HRMS.
Where R =

![Chemical structure](image)

**Figure 4.5 The synthesis of a series of aromatic esters of isosorbide-2-aspirinate**

A further series of isosorbide-2-aspirinate esters were prepared from the appropriate acid chlorides according to the scheme presented in Fig. 4.6. Nucleophilic substitution of the acid chloride by the alcohol moiety of the isosorbide molecule gave the appropriate aromatic ester.
Where R =

![Chemical structures](image)

Figure 4.6 The synthesis of a series of aromatic esters of isosorbide-2-aspirinate from their corresponding acid chlorides.

The acid chlorides 2-toluoyl chloride (54), 4-toluoyl chloride (55) and 4-nitrobenzoyl chloride (56) were reacted with isosorbide-2-aspirinate in the presence of triethylamine in toluene at 0°C. All compounds were purified by column chromatography over silica gel; purity was verified by TLC and HPLC, while structure was confirmed by NMR, IR and HRMS.
4.3 Plasma hydrolysis studies

The hydrolysis patterns of the fifteen different prodrugs of isosorbide-2-aspirinate, whose synthesis is described above, were studied in buffered human plasma solution to determine their potential as novel aspirin prodrugs. Any variation in rate or pathway of hydrolysis compared to the parent isosorbide-2-aspirinate-5-salicylate is most probably related to the change in substituent at the 5-position. Hydrolysis studies were performed as detailed in Chapter 6.

4.3.1 Development of HPLC methods for analysis of aspirin prodrugs.

The rate and pathway of hydrolysis for each compound was examined in 10% and 50% solutions of buffered pooled human plasma (pH 7.4, 37°C). All analysis of samples was performed using either isocratic or gradient HPLC methods as described in Table 4.2. All samples were monitored initially using an isocratic reverse phase HPLC method on a Nova-Pak® C8 column (3.9 x 150 mm), using a mobile phase comprising of phosphate buffer pH 2.5 (60%) and acetonitrile (40%). For several compounds this method gave good separation of the parent compound peak from that of its metabolites especially the salicylates. The isocratic method was validated for linearity, precision, sensitivity and specificity. A linear response was observed for each prodrug (r > 0.990) 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%. The limit of quantitation for prodrugs, aspirin and salicylic acid was 5 μg/ml.
### Table 4.2 Methods of HPLC analysis for aspirin prodrugs

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Ester type</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>acetate</td>
<td>isocratic</td>
</tr>
<tr>
<td>44</td>
<td>proprionate</td>
<td>isocratic</td>
</tr>
<tr>
<td>45</td>
<td>benzoate</td>
<td>gradient (method 1)</td>
</tr>
<tr>
<td>46</td>
<td>nicotinate</td>
<td>isocratic</td>
</tr>
<tr>
<td>47</td>
<td>iso-nicotinate</td>
<td>isocratic</td>
</tr>
<tr>
<td>48</td>
<td>benzoylexybenzoate</td>
<td>gradient (method 2)</td>
</tr>
<tr>
<td>49</td>
<td>2-aminobenzoate</td>
<td>isocratic</td>
</tr>
<tr>
<td></td>
<td>(Anthranilate)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3-methylbenzoate</td>
<td>gradient (method 3)</td>
</tr>
<tr>
<td>52</td>
<td>2-methoxybenzoate</td>
<td>gradient (method 3)</td>
</tr>
<tr>
<td>52</td>
<td>3-methoxybenzoate</td>
<td>gradient (method 3)</td>
</tr>
<tr>
<td>53</td>
<td>4-methoxybenzoate</td>
<td>gradient (method 3)</td>
</tr>
<tr>
<td>54</td>
<td>2-methylbenzoate</td>
<td>isocratic</td>
</tr>
<tr>
<td>55</td>
<td>4-methylbenzoate</td>
<td>isocratic</td>
</tr>
<tr>
<td>56</td>
<td>4-nitrobenzoate</td>
<td>gradient (method 2)</td>
</tr>
</tbody>
</table>

For some prodrugs the isocratic method detailed above did not give sufficient separation of the prodrug and metabolite peaks and co-elution of peaks was observed. In these instances the analytical method was changed to a gradient one to alter the retention of desired peaks. The column selected was also used for isocratic elution – Waters Nova-Pak® C8 3.9 x 150 mm column. The pH of phosphate buffer in the mobile phase was increased to 3.19, and ratios of buffer to acetonitrile were varied over time. The different gradient tables found to give optimum peak separation for the different compounds are presented in Table 4.3.
<table>
<thead>
<tr>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (mins)</td>
<td>%A</td>
<td>%B</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>18</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>28</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

*Table 4.3 Reverse phase HPLC gradient methods for isosorbide-2-aspirinate prodrugs. A = phosphate buffer pH 3.19 and B = acetonitrile, on a Waters Nova-Pak® C8 3.9 x 150 mm column.*

Each gradient method was validated for linearity, precision, sensitivity and specificity. A linear response was observed for each prodrug in the range 1-100 μg/ml (r > 0.991). The RSD on multiple injection of each compound at 10 μg/ml and 100 μg/ml was < 1.8%. The limit of quantitation for the relevant analytes was 1 μg/ml. The gradient methods developed were successfully employed in the assay of plasma samples of all synthesised prodrugs.

### 4.3.2 Hydrolysis studies in human plasma

Each compound was examined for rate and pathway of hydrolysis in both 10% and 50% solutions of pooled human plasma sampled from healthy volunteers (n = 4) who had not taken any NSAID’s in the two weeks prior to blood collection. For any of the esters to possess clinical efficacy, a considerable portion of each molecule must be hydrolysed *in vivo* to liberate aspirin. Rate data and % aspirin liberated for each ester are presented in *Table 4.4.*
<table>
<thead>
<tr>
<th>compound no.</th>
<th>group at 5-position</th>
<th>10%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>Plasma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t_{\text{1/2}}$ min</td>
<td>% aspirin</td>
</tr>
<tr>
<td>42</td>
<td>salicylic acid$^1$</td>
<td>4.90</td>
<td>72.23</td>
</tr>
<tr>
<td>43</td>
<td>acetate</td>
<td>3.63</td>
<td>1.17</td>
</tr>
<tr>
<td>44</td>
<td>propionate</td>
<td>3.76</td>
<td>6.82</td>
</tr>
<tr>
<td>45</td>
<td>benzoate</td>
<td>3.32</td>
<td>18.93</td>
</tr>
<tr>
<td>46</td>
<td>nicotinate</td>
<td>1.29</td>
<td>27.58</td>
</tr>
<tr>
<td>47</td>
<td>iso-nicotinate</td>
<td>3.68</td>
<td>18.76</td>
</tr>
<tr>
<td>48</td>
<td>benzoyloxybenzoate</td>
<td>20.63</td>
<td>12.87</td>
</tr>
<tr>
<td>49</td>
<td>2-aminobenzoate</td>
<td>50.22</td>
<td>2.82</td>
</tr>
<tr>
<td>50</td>
<td>3-methyl benzoate</td>
<td>9.72</td>
<td>38.54</td>
</tr>
<tr>
<td>51</td>
<td>2-methoxy benzoate</td>
<td>3.59</td>
<td>5.16</td>
</tr>
<tr>
<td>52</td>
<td>3-methoxybenzoate</td>
<td>3.28</td>
<td>18.91</td>
</tr>
<tr>
<td>53</td>
<td>4-methoxy benzoate</td>
<td>3.17</td>
<td>2.53</td>
</tr>
<tr>
<td>54</td>
<td>2-methyl benzoate</td>
<td>2.17</td>
<td>46.02</td>
</tr>
<tr>
<td>55</td>
<td>4-methyl benzoate</td>
<td>6.23</td>
<td>15.85</td>
</tr>
<tr>
<td>56</td>
<td>4-nitrobenzoate</td>
<td>2.86</td>
<td>18.46</td>
</tr>
<tr>
<td>15</td>
<td>Aspirin$^1$</td>
<td>4.43</td>
<td>51.0</td>
</tr>
<tr>
<td>14</td>
<td>ONO$_2$$^1$</td>
<td>0.90</td>
<td>7.13</td>
</tr>
<tr>
<td>16</td>
<td>OH$^1$</td>
<td>4.08</td>
<td>2.95</td>
</tr>
</tbody>
</table>

Table 4.4. Kinetic data for the hydrolysis of isosorbide-2-aspirinate prodrugs in 10% and 50% human plasma solutions at pH 7.4 and 37°C; performed previously by Moriarty et al$^{127}$.

For all compounds, similar quantities of liberated aspirin were noted in 10 and 50% plasma solutions, while a notable increase in rate of hydrolysis was observed for all compounds on increasing the concentration of plasma from 10 – 50%. It has been established that in 10% human plasma, isosorbide-2-aspirinate-5-salicylate liberates up to 81.5% aspirin with a half-life of 4.4 mins and in 50% plasma
aspirin release of up to 91.5% has been observed. While substitution at the 5-
position of isosorbide was capable of giving marked increases in the rate of
metabolism (e.g. compound 46, $t_{1/2} = 0.37$ mins, and compound 54, $t_{1/2} = 2.17$
mins) in 10% plasma, there was no significant aspirin release noted. Compound 54
(isosorbide-2-aspirinate-5- [2-methyl] benzoate) was the most promising prodrug
based on initial test results, having a relatively fast metabolism combined with
reasonable aspirin release (46.02%) in 10% plasma and 58.5% aspirin in 50%
plasma. Its hydrolysis pathway is shown in Fig. 4.7. It differs from the parent
compound by having a methyl (-CH$_3$) group on the 2-position of the benzene ring
as opposed to the hydroxyl (-OH) group of isosorbide-2-aspirinate-5-salicylate. By
retaining many of its structural characteristics of the model prodrug, it appears to
be a good substrate for the BuChE enzyme active site, directing hydrolysis away
from the labile acetate group of the aspirin substituent, and thus increasing the
concentration of aspirin liberated on hydrolysis.

![Figure 4.7 Progression curve for the hydrolysis of ester 54 (isosorbide-2-
aspirinate-5-2’ methylbenzoate) in 10% human plasma at pH 7.4 and 37°C: ester
54 (○), salicylate ester (■), aspirin (●) and salicylic acid (○).]

In general, the alkyl substituents (compounds 43 and 44) gave a marked reduction
in percentage aspirin liberated, (1.17 and 6.86 % respectively, ester 43 progression
curve is presented in Fig 4.8), while aromatic substituents varied from very long half-life (e.g. compound 49) to very rapid metabolism *in vitro* (compound 46).

![Fig. 4.8 Progression curve for the hydrolysis of ester 43 (isosorbide-2-aspirinate-5-acetate) in 10% human plasma at pH 7.4 and 37°C: ester 43 (○), salicylate ester (■), aspirin (●) and salicylic acid (○).](image)

This plasma hydrolysis study confirms that salicylic acid ester is the optimum substituent on isosorbide-2-aspirinate, which directs hydrolysis to the carboxyl group rather than the acetate group, thus liberating aspirin and acting as a true aspirin prodrug. Varying the substituent gives a marked reduction in aspirin release, suggesting that these compounds are not as good a fit for the active site of serum butyrylcholinesterase. Results of this SAR study also indicate that 2-substitution on an aryl ring at the 5-position of isosorbide gives the optimum configuration for directing hydrolysis towards maximum aspirin release.
4.4 Whole blood aggregation studies

All the compounds analysed in plasma hydrolysis experiments (ref. Table 4.4) were examined for their antiplatelet effect in human whole blood. Blood was freshly sampled from healthy human volunteers who had not ingested any NSAIDs for two weeks prior to blood donation. All experiments were performed in the Chrono-Log® Whole Blood Aggregometer according to the experimental protocol outlined in Section 6.4.3. All compounds were prepared to a final concentration of 100 μM in DMSO and allowed to incubate in the blood for 20 mins prior to challenging samples with 0.5 mM arachidonic acid. Results of this study are presented in Fig. 4.9.

As is evident from the plot presented above, four of the prodrugs tested, isosorbide-2-aspirinate-5-salicylate (42), isosorbide-2-aspirinate-5-nicotinate (46), isosorbide-2-aspirinate-5-iso-nicotinate (47) and isosorbide-2-aspirinate-5-[4-
nitrobenzoate] (56) showed almost complete inhibition of AA-induced platelet aggregation at 100 μM. It has previously been reported (Section 3.3.4.3) that compound 42 is a very potent antiplatelet agent with an IC$_{50}$ value of 12 μM in human whole blood, significantly more potent than aspirin (IC$_{50}$ 35 μM) under the same experimental conditions.

Compounds 46, 47 and 56 were further investigated over a range of different concentrations and it was determined that they have IC$_{50}$ values of 65, 53 μM and 50 μM respectively. These compounds are less potent than 42 at inhibiting AA-induced platelet aggregation in whole blood. They contain relatively polar aromatic groups at the 5-position of the isosorbide molecule, confirming that such a configuration is optimum for in vivo antiplatelet potency. Significantly, compounds associated with greater release of aspirin in plasma hydrolysis studies e.g. 3-methylbenzoate (50) and 2-methylbenzoate (54) showed less potency at inhibition of platelet aggregation. These compounds were very poorly soluble in DMSO, which is the only solvent recommended for in vitro studies involving blood samples. This may preclude sufficient enzymatic hydrolysis of these prodrugs by plasma esterases, even following incubation for 20 mins in whole blood. This would result in incomplete aspirin liberation and a possible underestimation of the potency of their antiplatelet effects.
4.5 Physico-chemical studies

The lipophilicity and aqueous solubility of all synthesised prodrugs were determined, as these are regarded as useful indicators of the extent of absorption of a drug following oral administration. The rate of absorption of acidic NSAIDs and their effect on the gastric mucosa is related to their lipid solubility.\(^{341}\)

4.5.1 Lipophilicity

The partition coefficient of a prodrug is a key parameter in its design. In order to possess pharmacological activity, the prodrug must be absorbed, distributed and metabolised in the body, all of which requires its efficient passage across phospholipid cell membranes.\(^{342}\) Lipophilicity has been shown to be an important physico-chemical parameter for the efficacy of NSAIDs.\(^{22}\) It is also one of the major determinants for the transfer of drugs into the central nervous system across the blood-brain barrier.\(^{343}\) The lipophilicity of a compound is determined by its partition coefficient between a bilayer lipid membrane (represented by \(n\)-octanol) and water.\(^{310}\) Good absorption of orally administered drugs is attainable when the clog P value > 2.\(^{344}\)

4.5.1.1 Lipophilicity studies

The lipophilicities of all compounds were estimated using SMILES, a computer-based chemical notation system designed to calculate the clog P values for each fragment of the molecule. The estimated log P values for each of the aspirin prodrugs synthesised are presented in Table 4.5. It has previously been shown that on increasing the lipophilicity of aspirin esters, the rate of deacetylation increased correspondingly.\(^{105}\) This indicates that an aspirin prodrug should not be too lipophilic as otherwise it risks becoming a prodrug for salicylic acid rather than releasing aspirin on hydrolysis \textit{in vivo}. However, in this series of isosorbide-aspirin esters there was no correlation between lipophilicity and \% aspirin released.
<table>
<thead>
<tr>
<th>Compound no.</th>
<th>$c$ log P</th>
<th>$t_r$ (min)</th>
<th>$k^1$</th>
<th>log $k^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>3.04</td>
<td>24.767</td>
<td>13.15</td>
<td>1.12</td>
</tr>
<tr>
<td>43</td>
<td>1.19</td>
<td>6.925</td>
<td>2.96</td>
<td>0.47</td>
</tr>
<tr>
<td>44</td>
<td>1.71</td>
<td>12.166</td>
<td>5.95</td>
<td>0.77</td>
</tr>
<tr>
<td>45</td>
<td>2.71</td>
<td>14.459</td>
<td>7.26</td>
<td>0.86</td>
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<td>46</td>
<td>1.36</td>
<td>5.265</td>
<td>2.01</td>
<td>0.30</td>
</tr>
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<td>47</td>
<td>1.36</td>
<td>4.125</td>
<td>1.36</td>
<td>0.13</td>
</tr>
<tr>
<td>48</td>
<td>4.37</td>
<td>49.228</td>
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<td>1.43</td>
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<td>8.913</td>
<td>4.09</td>
<td>0.61</td>
</tr>
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<td>50</td>
<td>3.20</td>
<td>19.667</td>
<td>10.24</td>
<td>1.01</td>
</tr>
<tr>
<td>51</td>
<td>2.81</td>
<td>9.994</td>
<td>4.71</td>
<td>0.67</td>
</tr>
<tr>
<td>52</td>
<td>2.81</td>
<td>15.978</td>
<td>8.13</td>
<td>0.91</td>
</tr>
<tr>
<td>53</td>
<td>2.81</td>
<td>16.787</td>
<td>8.59</td>
<td>0.93</td>
</tr>
<tr>
<td>54</td>
<td>3.20</td>
<td>28.781</td>
<td>15.44</td>
<td>1.19</td>
</tr>
<tr>
<td>55</td>
<td>3.20</td>
<td>22.303</td>
<td>11.74</td>
<td>1.07</td>
</tr>
<tr>
<td>56</td>
<td>2.45</td>
<td>14.215</td>
<td>7.12</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 4.5. Lipophilic and chromatographic data for potential isosorbide-based aspirin prodrugs.

The results suggest that esters 42, 44, 45, 49 and 56 possess optimum lipophilicity for good oral absorption. Much work has been done on establishing a relationship between partition coefficient data and reverse phase HPLC retention times. The chromatographic lipophilicity parameter was determined from the reverse phase
HPLC capacity factor ($k^l$). The capacity factor is calculated according to Eq. 4.1, where $t_r$ is the retention time of the solute and $t_o$ is the elution time of the system.

$$k^l = \frac{(t_r - t_o)}{t_o} \quad (4.1)$$

The retention time of each prodrug was determined using an isocratic reverse phase HPLC method; compounds were eluted on a Waters Nova-Pak® C8 column (3.9 x 150 mm) using a mobile phase consisting of phosphate buffer pH 7.4 (60%) and acetonitrile (40%) and a flow rate of 1 ml/min. The retention times and corresponding capacity factors are presented in Table 4.5. A linear relationship is expected between lipophilicity parameters log $k^l$ and clog P. A plot of this relationship is shown in Fig. 4.10 ($r^2 = 0.8491$).

![Figure 4.10 Plot of clog P versus log $k^l$ for isosorbide-based aspirin prodrugs.](image)

4.5.2 Aqueous solubility studies

The solubility of all prodrugs was determined over a period of 6 hours in water, according to the experimental protocol described in Section 6.3.2. Briefly, 0.01 g of each ester was shaken in 10 mls of HPLC grade water at 37°C and filtered samples were analysed by HPLC using an isocratic elution method. Quantitation
was achieved by analysis of external standards dissolved in acetonitrile, under the same HPLC conditions and in a similar concentration range. *Table 4.6* presents the solubility of all prodrugs in aqueous solution (pH 7.0) as measured in µg/ml.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Water (pH 7.0) µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>n/a</td>
</tr>
<tr>
<td>43</td>
<td>18.82</td>
</tr>
<tr>
<td>44</td>
<td>57.71</td>
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<tr>
<td>45</td>
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<td>46</td>
<td>87.94</td>
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<td>47</td>
<td>207.26</td>
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<td>48</td>
<td>0.51</td>
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<tr>
<td>49</td>
<td>9.55</td>
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<tr>
<td>50</td>
<td>0.58</td>
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<tr>
<td>51</td>
<td>9.99</td>
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<tr>
<td>52</td>
<td>15.65</td>
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<tr>
<td>53</td>
<td>0.31</td>
</tr>
<tr>
<td>54</td>
<td>5.34</td>
</tr>
<tr>
<td>55</td>
<td>8.03</td>
</tr>
<tr>
<td>56</td>
<td>1.72</td>
</tr>
</tbody>
</table>

*Table 4.6 Aqueous solubility of a series of isosorbide-based aspirin esters*

Esters 46 (isosorbide-2-aspirinate-5-nicotinate) and 47 (isosorbide-2-aspirinate-5-iso-nicotinate) were significantly more soluble than the other esters with an aqueous solubility of 87.94 and 207.26 µg/ml respectively.
4.6 Conclusions

Previous work in our laboratory has identified isosorbide-2-aspirinate-5-salicylate as one of the most promising aspirin prodrugs developed to date. In 50% human plasma it is found to liberate up to 91.5% aspirin, more than any other prodrug reported in literature to date. It is an excellent substrate for human serum butyrylcholinesterase (EC 3.1.1.8) where its enzymatic hydrolysis is associated with liberation of 98.0% aspirin. However it has a poor aqueous solubility and so may present problems for formulation and bioavailability on oral dosage.

In an effort to improve the physico-chemical characteristics while retaining the optimum structural fit for the enzyme a series of aspirin prodrugs were developed based on the isosorbide-2-aspirinate structure, but varying the substituent at the 5-position. A number of aliphatic and aromatic groups were esterified at the 5-position of the molecule and their lipophilicity, aqueous solubility and kinetics and route of hydrolysis in human plasma was established.

A structure-activity relationship can be detected for the isosorbide-based aspirin prodrugs. Varying the substituent at the 5-position of isosorbide significantly affects the rate and extent of aspirin liberation from the 2-position. The 2-methylbenzoate ester (54) was the optimum prodrug synthesised, liberating 46.02% aspirin in a 10% solution of pooled human plasma. None of the other prodrugs produced significant levels of aspirin, their substituents directing hydrolysis towards the more labile acetate group and thus acting ultimately as prodrugs for salicylic acid. Aqueous solubility was greatly improved by changing the salicylate group at the 5-position to the corresponding iso-nicotinate acid. However there was a corresponding significant reduction in the levels of aspirin generated (18.76%).

In conclusion, the work presented here indicates that it is possible to alter the physico-chemical properties of an isosorbide-2-aspirinate prodrug to improve its aqueous solubility. While some of the esters synthesised in this study had better solubility, they all displayed decreased aspirin generation in human plasma when compared to the parent compound isosorbide-2-aspirinate-5-salicylate. This is still the most promising aspirin prodrug reported in the literature to date. However
results indicate the importance of the 5-position on the isosorbide ring in determining physicochemical properties as well as directing the route of hydrolysis. Further work at this 5-position with more water-soluble groups (perhaps containing a phosphate moiety) may identify a substituent which liberates as much aspirin as the salicylate derivative but with improved delivery characteristics.
Chapter 5

Design, synthesis and \textit{in vitro} hydrolysis studies of novel ester prodrugs of benzoic acid and ibuprofen.
5.1 Introduction

5.1.1 Isosorbide-based aspirin prodrugs

Previous chapters have described the design and evaluation of prospective aspirin prodrugs, based on the isosorbide sugar moiety, which may have potential as antithrombotic agents. These esters include ISMNA (14), ISDA (15) and isosorbide-2-aspirinate-5-salicylate (42). Enzymatic hydrolysis studies in 10% human plasma suggest that the 5-substituent has a pivotal role to play in governing the rate and pathway of hydrolysis at the 2-position.

![Chemical structures 14, 15, and 42](image)

Previous work in this area has illustrated that in 10% human plasma, isosorbide-2-aspirinate-5-salicylate had a half-life of 4.9 mins and generates up to 81.5% aspirin on hydrolysis \textit{in vitro} \textsuperscript{347}. The salicylate moiety at the 5-position of the isosorbide molecule appears to be the most favourable substituent for optimising aspirin release \textit{in vitro} on hydrolysis of the prodrug. The objective of this work was to
evaluate the potential of isosorbide as a carrier group for other acid drugs such as ibuprofen, having previously established its efficacy in aspirin prodrugs. The model compounds selected for this study were benzoic acid (57) and ibuprofen (2-(4-isobutylphenyl) propionic acid) (58).

Benzoic acid was chosen for its structural similarity to salicylic acid and other clinically used agents such as the uricosuric agent probenecid. It is a good prototype for carboxylic acid drugs and is commonly used as a model compound in prodrug hydrolysis studies. Ibuprofen (a commonly prescribed NSAID) similarly to aspirin has inherent anti-inflammatory activity due to its inhibition of the cyclo-oxygenase enzyme in both its constitutive (COX-1) and inducible (COX-2) forms. However its potential usefulness as an anti-inflammatory agent is hindered by its side-effect profile. Long-term use of ibuprofen can lead to gastrointestinal side effects such as ulceration and bleeding.

5.1.2 Prodrug esters of carboxylic acids

There have been many attempts to modify the side effect profile of commonly used NSAID’s such as ibuprofen and indomethacin by synthesising prodrugs which are themselves devoid of irritant effects but release the parent compound on metabolism following absorption. Ester prodrugs of many such drug molecules have been synthesised, mainly because of the predominance of hydroxyl and
carboxylic groups in most NSAIDs, as well as the proliferation of esterases in vivo capable of hydrolysing the prodrug. Temporary masking of the carboxylic acid function in NSAIDs has been proposed as a promising means of reducing or abolishing gastrointestinal toxicity due to direct mucosal contact. Ester prodrugs of anti-inflammatory agents should be readily hydrolysed following absorption to release the parent active acid in the blood, and require physicochemical properties (i.e. aqueous solubility and lipophilicity), which are favourable for peroral absorption. On enzymatic hydrolysis in plasma or blood, it is desirable that a sizeable proportion of the parent compound is liberated unchanged. For example the much-reduced anti-inflammatory activity observed for the methyl and ethyl esters of naproxen and fenbrufen relative to the free acids may be ascribed to the resistance of these esters to hydrolysis in vivo.

Topical application of NSAIDs is an attractive alternative to oral delivery especially in the treatment of local inflammation. Topically applied NSAIDs are effective in pain therapy of acute and chronic inflammatory skin diseases such as acute and post-herpetic neuralgia. However most NSAIDs have limited skin permeability as they tend to be ionised over the normal physiological pH range of the dermal tissues (pH 4 - 7.4) with a resulting decrease in permeability. Ibuprofen is a very lipophilic drug (clog $P = 3.6$) and has a depot effect in the stratum corneum resulting in low systemic absorption and bioavailability. For this reason the prodrug approach has been suggested as an attractive method of enhancing transdermal delivery of compounds such as ibuprofen, naproxen and ketoprofen.
5.1.3 Esters of benzoic acid

5.1.3.1 Glycolic acid derivatives

A series of esters of benzoic acid including glycolic acid derivatives were synthesised, and their hydrolysis was studied in alkaline solution and human plasma in order to obtain information on the chemical and enzymatic lability of a broad spectrum of esters of the same acid. All esters hydrolysed to give benzoic acid except the benzoylglycolic acid esters (59), which predominantly hydrolysed to give benzoylglycolic acid.

\[
\text{R} = \begin{cases} 
\text{CH}_3 \\
\text{CH}_2\text{CH}_3 \\
\text{H}_2\text{C} - \text{C}_6\text{H}_4
\end{cases}
\]

These esters have been characterised as having high chemical reactivity and an incomplete conversion to the parent compound (benzoic acid) and so their usefulness as potential esters of aspirin or other carboxylic acid drugs is limited.

5.1.3.2 Glycolamide esters

A large series of glycolamide esters of benzoic acid have been synthesised (60). Benzoic acid is typically used in such studies as a model of a carboxylic acid drug. It was found that some of the N, N-disubstituted glycolamides were very rapidly hydrolysed (t½ < 5 s in 50% plasma).
As discussed in Chapter 4 the N, N-diethylglycolamide ester of benzoic acid bears a structural resemblance to benzylocholine, a prototypical substrate for butyrylcholinesterase. The benzoic acid model demonstrates that ester derivatives can be obtained with almost any desired water solubility or lipophilicity while retaining high lability to enzymatic hydrolysis.

5.1.4 Esters of ibuprofen and other NSAID's

5.1.4.1 Benzoic acid ester

There have been several attempts to design prodrugs of ibuprofen, one of the most promising to date being a phenyl ester prodrug of ibuprofen (61)\(^{355}\). This ester showed significantly reduced gastric ulcerogenicity compared to the parent ibuprofen, while retaining the anti-inflammatory and analgesic activity in carrigeenan-induced rat paw oedema studies.

![Image of ibuprofen ester](attachment:ibuprofenester.png)

There was no hydrolysis of the prodrug in simulated gastric fluid \(^{355}\), which indicates that the ester is not hydrolysed prior to absorption from the GIT, thus eliminating the local contact effect associated with mucosal damage.
5.1.4.2 Glycolamide esters

Studies on glycolamide esters of benzoic acid showed that optimum structural requirement for rapid rate of hydrolysis is the glycolamide ester combined with two substituents on the amide nitrogen atom. A number of such esters of commonly prescribed NSAIDs including naproxen, ketoprofen, indomethacin and ibuprofen were synthesised. Where

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>62a</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>62b</td>
<td>C₂H₅</td>
<td>C₂H₅</td>
</tr>
<tr>
<td>62c</td>
<td>CH₃</td>
<td>CH₂CONH₂</td>
</tr>
</tbody>
</table>

In the ibuprofen series of glycolamide esters (62a-c, shown above) it was found that substituting two ethyl groups on the amide (62b) gave the most significant increase in rate of metabolism and correspondingly the fastest half-life (4.0 mins) in an 80% solution of human plasma. However the compound was an oil, which can be difficult to incorporate into pharmaceutical preparations. Reducing molecule size to two methyl substituents (62a) gave an increased half-life (8.6 mins) with a preferable crystalline structure while changing R₂ to a carbamoyl group (62c) gave a further increase in half-life (9.6 mins). All three prodrugs are metabolised to the parent ibuprofen in plasma with promising hydrolysis kinetics. Gastric toxicity studies have not been reported but it is possible that this series of esters may provide a useful means of protecting the carboxylic acid function of ibuprofen and other NSAIDs on passage through the GIT, while retaining a high capacity to release the parent active drugs on metabolism by plasma enzymes.
Furoxan prodrugs of ibuprofen.

Furoxans (1,2,5-oxadiazole 2-oxides) generate nitric oxide in the presence of thiol cofactors, and this liberated NO is known to have a protective effect on gastric mucosa by promoting mucous secretion and increasing mucosal blood flow. Altering substituents on the furoxan ring can modify the amount and rate of NO generation. A series of prodrugs were designed with ibuprofen joined by an ester linkage to one of two furoxan rings, which retain the ability to release nitric oxide.

\[
\begin{align*}
\text{63a} & \quad n = 1, R = \text{PhSO}_2 \\
\text{63b} & \quad n = 1, R = \text{PhS}
\end{align*}
\]

The benzenesulfonyl derivative (63a) generated 46.8% ibuprofen and 34.7% NO in human plasma after 6 hours incubation, while the phenylthiofuroxan (63b) liberated 35.6% ibuprofen and 9.5% NO under the same conditions. The compounds had similar anti-inflammatory activity to ibuprofen in the carrageenan-induced rat paw oedema assay but displayed significantly reduced gastric ulcerogenicity. They also had superior antiaggregatory activity to ibuprofen. This suggests their ability to liberate NO could possibly confer on these furoxan prodrugs a more clinically acceptable toxicity profile. However no human in vitro studies have been reported to date on this series of prodrugs.
5.1.4.4 2-Formylphenyl esters

Studies on 4-substituted 2-formylphenyl aspirins (Chapter 4) revealed that these esters act as true aspirin prodrugs by an intramolecular catalytic route. This series was extended to include prodrugs of ibuprofen (64), indomethacin and ketoprofen.

![Chemical Structure](image)

Formylphenol and ibuprofen were produced in quantitative yield on alkaline and neutral hydrolysis studies of prodrug 64. The ibuprofen prodrug was not tested for anti-inflammatory activity but the 2-formyl esters of aspirin and indomethacin showed increased anti-inflammatory activity compared with the parent NSAID in the carrageenan-induced paw oedema test.
5.1.4.5 Cyclic amide derivatives of ibuprofen

N-Hydroxymethylsuccinamide (HMSI, 65) and N-hydroxymethyl isatin (HMIS, 66) prodrugs of ibuprofen, indomethacin, aspirin and naproxen were synthesised as potential prodrugs with an improved therapeutic index. The promoieties are themselves derivatives of endogenous substances.

On hydrolysis studies in 80% human plasma, and 10% rat liver homogenate all prodrugs were found to be converted quantitatively to the parent NSAIDs with aspirin undergoing further hydrolysis to salicylic acid. The HMSI prodrug of ibuprofen has a half-life of 19.2 mins in 80% human plasma, while the HMIS half-life is 17.05 minutes. The 65 series of esters had higher chemical stability than the corresponding 66 analogues, which may result from their higher lipophilicities. All compounds were found to be acid stable, which should enable such prodrugs to avoid the direct contact effects with the stomach mucosa as well as the local inhibition of prostaglandins. The ulcerogenicity of the ibuprofen prodrugs was not reported, however the indomethacin prodrugs appeared to induce considerably less ulceration than parent compound or aspirin.
5.2 Synthesis of novel ester prodrugs of benzoic acid and ibuprofen.

A series of esters of benzoic acid and ibuprofen were synthesised in-house using the isosorbide sugar molecule as carrier, and varying the substituent at the 5-position. These were essentially modifications of the basic prodrug structure of ISMNA (14). The objective of this study was to establish if the exceptionally rapid hydrolysis characteristics of ISMNA and is-2-asp-5-sal could potentially be exploited in the design of other NSAID ester prodrugs.

5.2.1 Synthesis of benzoic acid ester prodrugs

A series of three prodrugs of benzoic acid were synthesised: isosorbide-2-benzoate (67), isosorbide-2-benzoate-5-mono nitrate (68) and isosorbide-dibenzoate (69). Compounds 67 and 68 were synthesised according to the scheme presented in Fig. 5.1.

5.2.1.1 Synthesis of isosorbide-2-benzoate-5-mono nitrate (68)

Isosorbide mononitrate (ISMN) was esterified using benzoyl chloride in the presence of triethylamine, to give isosorbide-2-benzoate-5 mono nitrate (68) as colourless oil, which recrystallised in ethanol to yield product as white crystals. Ester 68 was characterised by NMR, HRMS and IR and was homogeneous by TLC and HPLC.
5.2.1.2 Synthesis of isosorbide-2-benzoate (67)

Ester 68 was denitrated by catalytic hydrogenation at the 5-nitroxy position using palladium on charcoal under an atmosphere of hydrogen to give isosorbide-2-benzoate (67) as white crystals whose purity was ascertained by NMR, IR and HRMS.

\[ \text{HO} \quad \text{Cl} \quad \text{O} \quad \text{H} \quad \text{O} \quad \text{DCM} \quad \text{Et}_3\text{N} \]

\[ \text{O} \quad \text{MeOH, EtOAc} \quad \text{H}_2, \text{Pd/C} \]

**Figure 5.1 The synthesis of a series of isosorbide-based benzoate esters**
5.2.1.3 Synthesis of isosorbide-dibenzoate (69)

![Chemical structure of isosorbide-dibenzoate (69)]

Isosorbide-dibenzoate (69) was synthesised in accordance with the scheme presented in Fig. 5.2. The reaction was found to go to completion on addition of isosorbide to 2 molar equivalents of benzoyl chloride at 0°C in toluene, to which was added triethylamine. The dibenzoate was recrystallised in a mixture of hexane and ethyl acetate to yield product as white crystalline material, which was characterised, by NMR, IR and HRMS. The ester was homogeneous by TLC and HPLC.
5.2.2 Synthesis of isosorbide-based ibuprofen prodrugs

A series of ibuprofen prodrugs incorporating the isosorbide backbone and varying the substituent at the 5-position were synthesised in-house. These included isosorbide-2-ibuprofenate (70), isosorbide-5-mononitrate-2-ibuprofenate (71) and isosorbide-diibuprofenate (72).

5.2.2.1 Synthesis of isosorbide-5-mononitrate-2-ibuprofenate

Isosorbide-5-mononitrate-2-ibuprofenate (71) was synthesised in accordance with the reaction scheme shown in Fig. 5.3. The synthesis involved the coupling of IS-5-MN and S-ibuprofen in the presence of dicyclohexylcarbodiimide (DCC) and a catalytic amount of dimethylaminopyridine (DMAP).

Figure 5.3. Synthesis of isosorbide-2-ibuprofenate-5-mononitrate (71) and isosorbide-2-ibuprofenate (70).
To prevent formation of the anhydride of ibuprofen it was added dropwise to the reaction vessel. The reaction yielded product as white crystalline material whose structure was confirmed by NMR, IR and HRMS and which proved homogeneous by TLC and HPLC.

5.2.2.2 Synthesis of isosorbide-2-ibuprofenate (70)

Compound 71 (prepared above, Section 5.2.2.1) was dissolved in glacial acetic acid to which was added zinc powder (see Fig. 5.3). Following denitration at the 5-position, product was recovered as white crystals, which were characterised by NMR, IR and HRMS.
Isosorbide-diibuprofenate was synthesised according to the scheme presented in Fig 5.4.

Isosorbide was reacted with S-ibuprofen, in the presence of dicyclohexylcarbodiimide (DCC) and a catalytic amount of dimethylaminopyridine (DMAP). Product was isolated by flash chromatography to yield a waxy white solid whose structure was confirmed by IR, NMR and HRMS. The sample was homogeneous by TLC and HPLC.
5.3 Physico-chemical properties

The lipophilicity and aqueous solubility of all six compounds were determined. These can be useful tools in the prediction of the extent of absorption of a drug following oral administration.\textsuperscript{358}

5.3.1 Lipophilicity studies

The lipophilicities of the benzoate and ibuprofen esters were estimated using the SMILES computer program - a computer-based chemical notation system designed for modern chemical information processing (Table 5.1). The estimation centres on the calculation of clog P values for each fragment in the molecule. Optimum absorption of orally administered drugs generally correlates with a clogP value greater than or equal to 2.0.\textsuperscript{344} The estimated log P values for each ester are presented in Table 5.1. Results suggest that esters 69 and 72 have too high a lipophilicity, which would result in most of the drug partitioning into the lipid phase, rendering them poorly bioavailable. The ibuprofen esters 70 and 71 have optimum lipophilicity required for oral absorption while the benzoic esters 67 and 68 have very low lipophilicities potentially resulting in low rates of passive diffusion.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>cLog P</th>
<th>$t_r$</th>
<th>$k^1$</th>
<th>Log $k^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
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<td>5.091</td>
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<tr>
<td>58</td>
<td>3.68</td>
<td>13.819</td>
<td>6.896</td>
<td>0.84</td>
</tr>
<tr>
<td>67</td>
<td>0.79</td>
<td>2.617</td>
<td>0.49</td>
<td>-0.31</td>
</tr>
<tr>
<td>68</td>
<td>0.15</td>
<td>2.664</td>
<td>0.52</td>
<td>-0.28</td>
</tr>
<tr>
<td>69</td>
<td>3.17</td>
<td>19.028</td>
<td>9.87</td>
<td>0.99</td>
</tr>
<tr>
<td>70</td>
<td>2.75</td>
<td>12.979</td>
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<td>0.81</td>
</tr>
<tr>
<td>71</td>
<td>3.62</td>
<td>17.378</td>
<td>8.23</td>
<td>0.92</td>
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<tr>
<td>72</td>
<td>7.08</td>
<td>60.010</td>
<td>33.28</td>
<td>1.52</td>
</tr>
</tbody>
</table>

Table 5.1 Lipophilic and chromatographic data for isosorbide-based prodrugs of benzoic acid and ibuprofen
Chromatographic lipophilicity data was also determined by utilising the reverse phase HPLC capacity factor ($k'$), as described in Chapter 4. There should be good correlation between the capacity factor and the lipophilicity of the compounds as reflected in their clogP data. HPLC analysis involved isocratic elution of all compounds on a Waters Nova-Pak® C8 (3.9 x 150 mm) column with a mobile phase consisting of phosphate buffer pH 2.5 (60%) and acetonitrile (40%); flow rate of 1 ml/min. Gradient elution is not used in this determination as it is not possible to obtain a real capacity factor for a system under gradient conditions.

For the benzoate esters a plot of lipophilicity parameters (log $k'$ and clogP) was linear ($r^2 = 0.9622$) as shown in Fig. 5.5.

![Figure 5.5](image)

**Figure 5.5** Plot of clogP versus log $k'$ for benzoic acid, isosorbide-2-benzoate, ISMN-benzoate and isosorbide dibenzoate.

Similarly for the ibuprofen ester series a linear relationship was evident on plotting the lipophilicity parameters (log $k'$ and clogP, $r^2 = 0.9726$) as presented in Fig. 5.6.
Figure 5.6 Plot of $c\log P$ versus $\log k'$ for ibuprofen, isosorbide-2-ibuprofenate, ISMN-2-ibuprofenate and isosorbide-diibuprofenate.

This correlation of capacity factors with HPLC retention times validates the estimated lipophilicity data, and the SMILES program used to estimate them.
5.3.2 Aqueous solubility studies

The solubility of all compounds was determined over a six-hour period in water. Saturated solutions of the esters were shaken in 10 mls of deionised water at 37°C and at regular hourly intervals 1ml aliquots were removed, filtered and analysed by isocratic elution on a reverse phase Nova-Pak® C8 column (3.9 x 150 mm) using a variety of different mobile phases as shown in Table 5.2, to give optimum separation of metabolites.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Mobile phase</th>
<th>Ratio of constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>Phosphate buffer pH 2.5: acetonitrile</td>
<td>60 : 40</td>
</tr>
<tr>
<td>67</td>
<td>Acetate buffer pH 5.0 : acetonitrile</td>
<td>60 : 40</td>
</tr>
<tr>
<td>69</td>
<td>Acetate buffer pH 5.0 : acetonitrile</td>
<td>60 : 40</td>
</tr>
<tr>
<td>70</td>
<td>Phosphate buffer pH 2.5 : acetonitrile</td>
<td>65 : 35</td>
</tr>
<tr>
<td>71</td>
<td>Acetate buffer pH 5.0 : acetonitrile</td>
<td>50 : 50</td>
</tr>
<tr>
<td>72</td>
<td>Phosphate buffer pH 3.0 : acetonitrile</td>
<td>30 : 70</td>
</tr>
</tbody>
</table>

Table 5.2. Mobile phases employed in HPLC analysis of isosorbide-based prodrugs.

The aqueous solubilities of the tested esters varied significantly over the course of the experiment. The concentrations of all compounds were measured in µg/ml with reference to calibration curves in the same concentration range, under the same analytical conditions. The results of all solubility studies are presented in Table 5.3. The solubility of 58 was also determined in order to validate the method. Ibuprofen was found to have a solubility of 10.85 µg/ml in water after 6 hours incubation time. Previous solubility studies determined the aqueous solubility of
ibuprofen to be $7.0137 \times 10^{-2}$ mg/ml after 24 hours incubation at 37°C. This result is reasonably similar to that obtained in our experiments; variation in results can be accounted for by subtle methodological differences. At pH 7.4, (in PBS buffer), ibuprofen has a reported solubility of 6.02 mg/ml. From the data presented below, ester 67 has the highest solubility whereas ester 72 has the lowest aqueous solubility (0.32 μg/ml after 6 hours). This corresponds to the clog P data generated for the compounds with ester 72 being a large hydrophobic molecule with poor aqueous solubility.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Solubility (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td>861.23</td>
</tr>
<tr>
<td>68</td>
<td>11.16</td>
</tr>
<tr>
<td>69</td>
<td>44.68</td>
</tr>
<tr>
<td>70</td>
<td>26.83</td>
</tr>
<tr>
<td>71</td>
<td>129.02</td>
</tr>
<tr>
<td>72</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Table 5.3. Solubility of prodrugs in water at 37°C*
5.4 Plasma hydrolysis studies

5.4.1 Plasma hydrolysis studies of isosorbide-based benzoic acid esters

The hydrolysis of esters 67, 68 and 69 was studied in a range of plasma solutions to determine the effect of substitution at the 2- and 5-positions of the isosorbide molecule on the rate of hydrolysis of the prodrug. It was anticipated that the 5-position would play the most important role in influencing the kinetic properties of the prodrug. Each compound was analysed in varying concentrations of pooled buffered human plasma (pH 7.4) at 37°C, with all samples being monitored under the relevant HPLC conditions as described in Table 5.2. The results of all hydrolysis studies are presented in Table 5.4. Sample progression curves for the hydrolysis of ISMN-benzoate in 10% plasma and isosorbide-dibenzoate in 20% human plasma are presented in Fig. 5.7 and 5.8 respectively.

<table>
<thead>
<tr>
<th>ISMN-benzoate</th>
<th>Isosorbide-2-benzoate</th>
<th>Isosorbide-dibenzoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>% plasma</td>
<td>t_{1/2} (mins)</td>
<td>% plasma</td>
</tr>
<tr>
<td>10</td>
<td>3.5 ± 0.35^i</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>1.78</td>
<td>20</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>50</td>
<td>0.55</td>
<td>50</td>
</tr>
</tbody>
</table>

*Table 5.4 Kinetic data for the hydrolysis of a series of isosorbide-benzoate esters in different concentrations of human plasma. (^i n=2, ^ii n=3).*
Figure 5.7. ISMN-2-benzoate hydrolysis in 10% human plasma, pH 7.4, 37°C: ISMN-benzoate (○), benzoic acid (▲).

Figure 5.8. Isosorbide-dibenzoate hydrolysis in 20% human plasma, pH 7.4, 37°C: ISDB (○), Is-2-benzoate (■), benzoic acid (▲).
In 10% human plasma isosorbide dibenzoate (69) is most rapidly hydrolysed prodrug with a half-life of 1.21 ± 0.49 min. The next fastest hydrolysis is of ISMN-2-benzoate (68) with a $t_{1/2}$ of 3.5 ± 0.35 min and isosorbide-2-benzoate (67) being the most slowly hydrolysed ($t_{1/2}$ 20.58 ± 4.81 min). There was thus a marked increase in rate of hydrolysis with increasing size of the substituent at the 5-position. Ester 69 is the largest of the benzoic acid esters and the most lipophilic, with a $clogP$ of 3.17 (ref. Table 5.1) yet the benzoate substituent at the 5-position confers on it more rapid hydrolysis characteristics, possibly by enhancing the substrate's fit for the active site of the esterase. The more polar ester 67, which has a hydroxy group at the 5-position, appears to be a poorer substrate for the enzyme, and this is reflected in its slower rate of hydrolysis. On increasing the concentration of plasma there is a corresponding increase in the rate of hydrolysis of all esters with the same pattern evident at 50% plasma, whereby ester 69 is the most rapidly hydrolysed and ester 67 is the slowest.

5.4.2 Plasma hydrolysis studies of other benzoate prodrugs

It must be noted that the hydrolysis times of all the isosorbide-based benzoate ester prodrugs are extremely rapid with respect to other commonly used benzoate prodrug standards. In order to confirm the potential usefulness of isosorbide as a carrier molecule for carboxylic acids, plasma hydrolysis studies were performed for two commonly used benzoic acid standards, phenyl benzoate (73) and ethyl benzoate (74).

![Chemical Structures]

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Compound 73 was synthesised in-house (Ref. Chapter 6), while 74 was purchased from Sigma Chemicals. The hydrolysis of both benzoate esters was examined in a solution of 80% pooled human plasma, according to the experimental protocol detailed in Section 6.3.6 and samples were analysed for remaining ester concentration by HPLC. Results for the hydrolysis studies are presented in Table 5.5.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>$t_{1/2}$ (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>23.89</td>
</tr>
<tr>
<td>74</td>
<td>163.4</td>
</tr>
</tbody>
</table>

*Table 5.5. Rates of hydrolysis of a series of benzoate prodrugs in 80% human plasma.*

Hydrolysis of both esters was considerably slower than for the isosorbide-benzoate esters. ISMN-benzoate has a $t_{1/2}$ of 0.55 mins in 50% plasma, whereas phenyl benzoate has a $t_{1/2}$ of 24 mins in 80% plasma. The results obtained in this study closely parallel previously reported literature values for the rate of hydrolysis of 74 in 80% plasma ($t_{1/2}$ 210 mins). Hydrolysis of 73 has not been reported in the literature, but the structurally similar phenyl ester of 2-methylbenzoic acid has a $t_{1/2}$ of 24 mins in 80% plasma. This study confirms that isosorbide is one of the most promising compounds for use in the design of carboxylic acid prodrugs. A structural characteristic of the compound enables it to serve as a good substrate for esterase in human plasma, resulting in rapid rates of hydrolysis, with quantitative liberation of the parent compound.
5.4.3 Plasma hydrolysis studies of isosorbide-based ibuprofen esters

The hydrolysis of esters 70, 71 and 72 was examined in pooled buffered human plasma (pH 7.4) to determine if the effect of substituent size at the 5-position affected rate of hydrolysis of the ester in a similar way to that observed with the benzoate esters. Each compound was examined in 50% human plasma at 37°C with all samples analysed by HPLC under the relevant conditions as reported in Table 5.2. The results of the hydrolysis study are presented in Table 5.6. All studies were performed in duplicate. Sample progression curves for the hydrolysis of isosorbide-diibuprofenate and isosorbide-2-ibuprofenate are presented in Fig. 5.9 and 5.10 respectively.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>$t_{1/2}$ (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>56.70 ± 11.27</td>
</tr>
<tr>
<td></td>
<td>11.27</td>
</tr>
<tr>
<td>71</td>
<td>33.75 ± 2.20</td>
</tr>
<tr>
<td>72</td>
<td>38.72 ± 2.14</td>
</tr>
</tbody>
</table>

Table 5.6. Rates of hydrolysis of a series of isosorbide esters of ibuprofen in 50% human plasma.

Figure 5.9. Isosorbide-diibuprofenate hydrolysis in 50% human plasma, pH 7.4 and 37°C. ISDI (○), Ibuprofen (■).
From Table 5.6 it is evident once more that the presence of a small hydroxy group at the 5-position of the isosorbide molecule (ester 70) results in a marked decrease in the rate of hydrolysis of the prodrug. On increasing the size of the substituent to a nitroxy group (ester 71) or the larger ibuprofen molecule (ester 72) there is a marked decrease in the half-life of the compounds (72, \( t_{1/2} = 38.72 \) mins). This is the same pattern as was observed with the benzoate ester prodrugs.

\[ \text{Figure 5.10. Isosorbide-2-ibuprofenate hydrolysis in 50\% human plasma, pH 7.4 and 37ºC. IS-2-ibuprofen (\%), ibuprofen (\%).} \]

The hydrolysis of 72 is associated with the liberation of up to 68.4\% ibuprofen (Fig. 5.9), while metabolism of 70 by plasma esterases generates 69.19\% of the parent compound (Fig. 5.10). This compares extremely favourably with the furoxan esters of ibuprofen which only liberated 46.8\% ibuprofen after 6 hours incubation in human plasma \( ^{348} \).

The significantly longer half-lives observed in 50\% plasma, on comparing the two series of isosorbide-based compounds indicates that the isosorbide-ibuprofen prodrugs are not as good a substrate for the plasma esterase responsible for prodrug hydrolysis as benzoic or salicylic acid prodrugs. The ibuprofen group at the 2-position of the series of prodrugs is considerably larger than that of the benzoates. This suggests that a benzoate or other small aromatic group such as
aspirin may be optimum at the 2-position. However the hydrolysis of 72 is still considered relatively rapid, and its high rates of ibuprofen regeneration on hydrolysis suggest that it has a potential clinical role in the sustained release of ibuprofen on metabolism in the circulation.

5.4.4 Enzyme studies of benzoate esters

It was thought that the enzyme responsible for the hydrolysis of the isosorbide-benzoate esters in plasma was butyrylcholinesterase (E.C. 3.1.1.8). Their hydrolysis was studied using purified horse serum butyrylcholinesterase, as it is known to have high homology with human serum butyrylcholinesterase and similar substrate specificity. Each ester was tested in a solution of enzyme (0.01 mg/ml) in phosphate buffer pH 7.4 at 37°C. Samples were taken at regular time intervals and analysed by HPLC under the conditions described in Table 5.2. Kinetic data is presented in Table 5.7.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>$k_{obs}$</th>
<th>$t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\min^{-1}$</td>
<td>$\min$</td>
</tr>
<tr>
<td>67</td>
<td>0.012</td>
<td>82.5</td>
</tr>
<tr>
<td>68</td>
<td>0.023</td>
<td>43.04</td>
</tr>
<tr>
<td>69</td>
<td>0.39</td>
<td>2.51</td>
</tr>
</tbody>
</table>

*Table 5.7 Kinetic data for the hydrolysis of benzoic acid esters in butyrylcholinesterase preparations (pH 7.4, 37°C)*

Paralleling the results of the plasma hydrolysis studies it can be seen that ester 69 (isosorbide-dibenzoate) is a good substrate for the butyrylcholinesterase enzyme. Its structural similarity to isosorbide-2-aspirinate-5-salicylate confirms that compared to other substituents at the 5-position, an aromatic ring confers optimal rates of enzymatic hydrolysis. This is possibly because this molecular configuration fits more accurately into the active site of the butyrylcholinesterase enzyme.
5.5 Conclusion

This study confirms that esterification of NSAIDs containing a carboxylic acid function, such as ibuprofen, with the isosorbide sugar molecule is a useful method of developing prodrug derivatives of these compounds. Isosorbide esters described above have a high capacity to liberate the parent active molecule under conditions similar to those encountered in vivo. It is also possible to modify the physicochemical aspects of the prodrug to obtain one with more favourable properties such as increased aqueous solubility.

The results of this chapter further confirm the importance of the substituent at the 5-position of isosorbide. Increasing the size of the substituent at the 5-position markedly increases the rate of hydrolysis of the prodrug at the 2-position. A small substituent such as a hydroxy group at position 5 tends to lead to a long half-life in plasma, whereas increasing the size of the substituent to an aromatic group will increase the rate of hydrolysis to more pharmaceutically attractive levels.

Hydrolysis of ibuprofen prodrugs is somewhat slower than the benzoate series of isosorbide prodrugs. This suggests that the larger ibuprofen molecule at the 2-position of the isosorbide group is too bulky to act as a good substrate for plasma esterase. A smaller aromatic group, such as aspirin or benzoate, at the 2-position appears to be optimum for rapid prodrug hydrolysis in vivo. The rapid rate of hydrolysis of isosorbide-2-aspirinate-5-salicylate, as discussed in Chapter 4 may not extend to larger acids such as ibuprofen. The high lipophilicity of the isosorbide esters, in particular isosorbide-diibuprofenate, combined with their poor aqueous solubility, may discourage their development as prodrugs. However the rapid hydrolysis rates of the benzoates compared with the literature standards phenol-benzoate and ethyl-benzoate indicate that isosorbide as a carrier for carboxylic acid prodrugs has certain structural characteristics which enable it to act as an excellent substrate for plasma esterase (most probably butyrylcholinesterase).
6.1 Materials

IS-5-MN and ISDN were obtained from Sifa Ltd., Shannon Industrial Estate, Shannon, Co. Clare, Ireland. Salicylic acid, acetylsalicyloyl chloride and acetylsalicylic acid were acquired from Riedel-de-Haën and isosorbide from Aldrich-Chemie, Steinheim. HPLC grade solvents were purchased from Rathburn Ltd. and Riedel-de-Haen. Non-aqueous solvents were obtained from in-house stills. All other reagents and chemicals were of analytical grade. 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, EDTA (ethylenediamine tetraacetic acid), adenosine-5'-diphosphate, arachidonic acid (as sodium salt), BNPP, iso-OMPA, PMSF and dibucaine were all obtained from Sigma-Aldrich Ltd. Arachidonic acid (as oil) for use in platelet aggregation studies was purchased from Bio Data Corporation. For the MDA assay, 1,1,3,3-tetramethoxypropane (malondialdehyde bis (dimethyl acetal)) was purchased from Aldrich while 4,6-dihydroxypyrimidine-2-thiol (TBA) was obtained from Sigma. 0.9% NaCl solution was purchased from Braun Medical and trichloroacetic acid from BDH Chemicals. Citrated blood collection tubes (Sarstedt Monovettes®, 10 mls) were obtained from Sarstedt Ltd. Citrated human plasma was sampled from healthy human volunteers in the Student Health Centre, Trinity College Dublin. Citrated rabbit blood was sampled from the marginal ear vein of New Zealand White rabbits housed in the Bioresources Unit, Trinity College Dublin. Citrated dog blood was sampled from the jugular vein of labradors and beagles held at the Bioresources Unit, Trinity College Dublin. Citrated rat blood was obtained by cardiac puncture of Wister rats held at the Bioresources Unit, Trinity College Dublin. Citrated hamster blood was sampled from the heart of hamsters held at the Bioresources Unit, Trinity College Dublin. Citrated guinea-pig blood was obtained by cardiac puncture of a male guinea-pig held at the Bioresources Unit, Trinity College Dublin.
6.2 Chemistry

6.2.1 General Experimental Procedures

6.2.1.1 Melting Points
Uncorrected melting points were obtained using a Gallenkamp melting point apparatus.

6.2.1.2 Infra-red Spectra
Infra-red (IR) spectra were obtained using a Perkin Elmer 205 FT Infrared Paragon 1000 spectrometer. Band positions are given in cm$^{-1}$. Solid samples were obtained by KBr disc: oils were analysed as neat films on NaCl plates.

6.2.1.3 Ultra-violet spectra
UV spectroscopy was performed on a Cary 3E UV-VIS spectrophotometer.

6.2.1.4 Nuclear Magnetic Resonance (NMR) spectra
$^1$H and $^{13}$C NMR spectra were recorded at 27$^\circ$C on a Brucker DPX 400 MHz FT NMR spectrometer (400.13 MHz $^1$H, 100.61 MHz $^{13}$C), in either CDCl$_3$ or CD$_3$OD, (tetramethylsilane as internal standard). For CDCl$_3$, $^1$H NMR spectra were assigned relative to the TMS peak at 0.00 $\delta$ and $^{13}$C NMR spectra were assigned relative to the middle CDCl$_3$ triplet at 77.00 ppm. For CD$_3$OD, $^1$H and $^{13}$C NMR spectra were assigned relative to the centre peaks of the CD$_3$OD multiplets at 3.30 $\delta$ and 49.00 ppm respectively. Coupling constants were reported in hertz (Hz). For $^1$H NMR assignments, chemical shifts are reported: shift values (number of protons, description of absorption (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s) where applicable, proton assignment).

For all NMR assignments of isosorbide-aspirin prodrugs, the aromatic substituent at the 5-position of isosorbide was labelled Ar$_1$, while the aromatic substituent at the 2-position was referred to as Ar$_2$. Ar$_3$ refers to any further aromatic substituents on isosorbide.
6.2.1.5 Mass Spectra

HRMS was performed on a Micromass mass spectrophotometer (EI mode) at the Department of Chemistry, Trinity College Dublin.

6.2.1.6 Chromatography

Flash chromatography was performed on Merck Kieselgel 60 particle size 0.040-0.063 mm). Thin layer Chromatography (TLC), for which Rf values are quoted, was performed on silica gel Merck F-254 plates. Compounds were visually detected by UV absorbance at 254 nm +/- or iodine staining.

6.2.2 Synthesis

Isosorbide-2-aspirinate-5-mononitrate (14)

To a solution of IS-5-MN (5 g, 26.65 mmol) in 100 ml toluene at 0°C was added triethylamine (5.52 ml, 3.96 mmol) and acetylsalicyloyl chloride (6.31 g, 31.74 mmol). The reaction was returned to room temperature and allowed to stir for 6 hours before washing with water (2 x 50 ml), HCl (1 M, 2 x 50 ml), saturated aqueous NaHCO₃ (2 x 50 ml) and brine (100 ml). The organic phase was dried with Na₂SO₄ and solvent removed in vacuo to yield product as oil. This was crystallised from ethanol to yield 5.42 g of product as white crystals. (58.05%): m.pt. 82-84°C. IRνmax (KBr): 1757.6 and 1733.4 (C=O), 1651.8 (NO₂), 1261.4 (C(O)OR, aromatic), 915.5 (ONO₂) cm⁻¹. HRMS: Requires: 376.0645 (M⁺+23), Found: 376.0640 (M⁺+23). ¹H NMR δ (CDCl₃): 2.37 (3H, s, OCOCH₃), 3.93 (1H, dd, J 6.0, 11.5 and 6.0 Hz, IS6α-H), 4.09 (3H, m, IS1H [αβ] and IS6H [β]), 4.58 (1H, d, J 4.5Hz, IS3-H), 5.03 (1H, t, J 5.0 and 5.5 Hz, IS4-H), 5.38 (1H, m, IS5-H), 5.45 (1H, d, J 3.0 Hz, IS2-H), 7.12 (1H, d, J 8.0 Hz, Ar-H), 7.33 (1H, t, J 7.5 and 8.0 Hz, Ar-H), 7.60, (1H, t, J 7.5 and 8.0 Hz, Ar-H), 8.01 (1H, d, J 7.5 Hz, Ar-H). ¹³C NMR ppm (CDCl₃): 20.40 (OCOCH₃), 68.88 and 72.84 (ISC-1 and ISC-6), 77.50 (ISC-5), 80.83 (ISC-4), 81.08 (ISC-2), 122.19 (ArC-1), 123.41, 125.61, 131.37, 133.92 (aromatic methine), 150.24 (ArC-2), 163.09 (ArOCO(Me)), 169.17 (ArC(O)OR).
A solution of ISMNA (8 g, 22.66 mmol) was stirred in 50 ml glacial acetic acid in the presence of zinc powder (5.97 g, 90.64 mmol). The reaction was allowed to stir for 48 hours until product was completely denitrated. The reaction mixture was diluted with 100 ml water, and then extracted into 50 ml dichloromethane. The organic phase was washed with three 100 ml aliquots of water, saturated aqueous NaHCO₃ (100 ml) and brine (50 ml). The organic phase was dried with MgSO₄ and solvent removed in vacuo to yield product as yellow oil. This was crystallised in a mixture of hexane: ethanol (1:3) to produce 4.51 g of product as white crystalline material (65.56%): m.pt. 58-60°C. IRmax (KBr): 3391.9 (OH), 1767.1 and 1725.2 (C=O), 1253.1 and 1191.8 (C(OR), aromatic), 1092.8 (C-O-C) cm⁻¹. HRMS: Requires: 331.0794 (M⁺+23), Found: 331.0808 (M⁺+23). ¹H NMR δ (CDCl₃): 2.36 (3H, s, OCOCH₃), 3.94 (1H, m, ISIH [α]), 4.09 (1H, d, ISIH [β]), 4.12 (1H, d, IS6H [β]), 4.36 (1H, q, IS5-H), 4.59 (1H, d, J 4.52 Hz, IS3-H), 4.70 (1H, t, J 4.52, 5.04 Hz, IS4-H), 5.47 (1H, d, J 3 Hz, IS2-H), 7.14 (1H, dd, J 8.0 and 8.0 Hz, ArH-3), 7.33 (1H, m, ArH-5), 7.61 (1H, t, J 1.5 and 1.5, ArH-4), 8.02 (1H, dd, J 2.0 and 1.5 Hz, ArH-6). ¹³C NMR ppm (CDCl₃): 20.43 (OCOCH₃), 71.88 (ISC-1), 72.94 (ISC-6), 73.23 (ISC-5), 78.48 (ISC-2), 81.59 (ISC-4), 85.15 (ISC-3), 122.29 (ArC-1), 123.41, 125.59, 131.34 and 133.85 (aromatic methine), 150.24 (ArC-2), 163.03 (ArOCOMe), 169.25 (ArC(OR)).

Isosorbide diaspirinate (15)

To a solution of isosorbide (2 g, 13.68 mmol) in toluene (50 ml) at 0°C was added triethylamine (2.5 ml, 35.0 mmol) and acetylsalicyloyl chloride (6 g, 30.28 mmol). The reaction was stirred at room temperature for 24 hours, then washed with water (90 ml), saturated aqueous NaHCO₃ (30 ml), HCl (1 M, 30 ml), and further water (2 x 30 ml). The organic phase was dried over Na₂SO₄ and dried in vacuo to give product as brown crystals. On re-crystallisation in ethanol, product as colourless crystals was obtained (5.88 g, 90.07%): m. pt. 108-110°C. IRmax (KBr): 1768.0 and 1725.9 (C=O), 1253.8 (C(OR), aromatic), 1191.8 (C-O-C) cm⁻¹. HRMS: Requires: 471.1291 (M⁺+23), Found: 471.1285 (M⁺+23). ¹H NMR δ (CDCl₃): 2.38 (6H, m, 2 x OCOCH₃), 4.07 (4H, m, IS1H [α + β] and IS6H₂[α + β]), 4.63
(1H, d, J 5.0 Hz, IS3-H), 5.00 (1H, t, J 5.0 and 5.5 Hz, IS4-H), 5.40 (1H, q, J 5.5, 5.0 and 5.5 Hz, IS5-H), 5.47 (1H, d, J 3.0, IS2-H), 7.13 (2H, m, Ar-H), 7.37 (2H, m, Ar-H), 7.57 (2H, m, Ar-H), 8.00 (1H, dd, J 1.5 and 1.5 Hz, Ar-H), 8.09 (1H, dd, J 1.5 and 1.5 Hz, Ar-H). $^{13}$C NMR ppm (CDCl$_3$): 20.51 and 20.61 (2 x OCOCH$_3$), 70.28 and 72.26 (ISC-1 and ISC-6), 74.05 (ISC-5), 78.00 (ISC-2), 80.61 (ISC-4), 85.65 (ISC-3), 122.16 and 122.21 (2 x ArC-1), 123.41, 123.54, 125.65, 125.70, 131.42, 131.61, 133.84 and 133.91 (aromatic methine), 150.24 and 150.33 (ArC-2), 163.13 and 163.27 (2 x ArOC(O)Me), 169.24 and 169.29 (2 x ArC(O)OR).

Isosorbide-2-aspirinate-5-benzyloxy benzoate (48)

To a solution of isosorbide-2-aspirinate (0.27 g, 0.87 mmol) in dichloromethane (20 ml) was added benzyloxy benzoic acid (0.20 g, 0.87 mmol), DCC (0.18 g, 0.87 mmol) and DMAP (0.01 g, 0.09 mmol). The reaction vessel was stirred at room temperature for 24 hours before filtering and washing the filtrate with HCl (30 ml, 0.1 M), saturated aqueous NaHCO$_3$ (30 ml) and water (2 x 30 ml). After drying over anhydrous Na$_2$SO$_4$, the dichloromethane was removed in vacuo to give 0.7 g of crude product as colourless oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (3:1) as eluant yielded 0.19 g of product as white crystals (41.5%): m.pt. 76-78°C. IR$_{\text{vmax}}$ (KBr): 1772.7 and 1726.2 (C=O), 1276.6 (C(O)OR aromatic), 1078.1 (C-O-C) cm$^{-1}$. HRMS: Requires: 541.1475 (M$^+$+23), Found: 541.1460 (M$^+$+23). $^1$H NMR δ (CDCl$_3$): 2.05 (2H, s, ArOCH$_2$Ar), 2.36 (3H, s, OCOCH$_3$), 3.92 (1H, q, J 5.0, 5.04 and 5.0 Hz, IS$_6$a-H), 4.02 (3H, m, IS$_1$H[α+β]), 4.13 (1H, q, J 7.04, 7.0 and 7.56 Hz, IS$_6$H-β), 4.62 (1H, d, J 5.0 Hz, ISH-3), 5.02 (1H, t, J 5.04 and 5.0 Hz, ISH-4), 5.19 (2H, s, ISH-5), 5.39 (2H, m, ISH-5), 7.03 (2H, m, 2 x Ar-H) 7.11 (1H, d, J 7.56 Hz, Ar-H) 7.33 (2H, m, Ar-H), 7.41 (2H, t, J 6.04 and 7.04 Hz, Ar-H), 7.48 (3H, m, Ar-H) 7.58 (1H, m, Ar-H), 7.92 (1H, dd, J 1.52 and 2.0 Hz, Ar-H) 8.01 (1H, dd, J 1.48 and 2.0 Hz, Ar-H). $^{13}$C NMR ppm (CDCl$_3$): 20.49 (OCOCH$_3$), 70.12 (ISC-6), 70.19 (ISC-2), 72.59 (ISC-5), 73.84 (ISC-3), 78.28 (ArOCH$_3$), 80.48 (ISC-1), 85.59 (ISC-4), 113.18 (Ar$_2$C-5), 119.29 (Ar$_2$C-1). 120.10 (Ar$_2$C-3), 122.30 (Ar$_1$C-5), 123.41 (Ar$_1$C-1), 125.64 (Ar$_1$C-3), 126.78 (Ar$_3$C-2 and Ar$_3$C-6), 127.51 (Ar$_3$C-4), 128.08 (Ar$_3$C-3 and Ar$_3$C-6), 128.13 (Ar$_1$C-2), 131.44 (Ar$_2$C-2), 131.81 (Ar$_1$C-4) 133.44
(Ar₂C-4), 136.09 (Ar₃C-1), 150.21 (Ar₁C-6), 157.95 (Ar₂C-6), 163.16 (ArOC(O)Me), 165.20 (ArC(O)OR), 169.28 (ArC(O)OR).

*Isosorbide-2-aspirinate-5-salicylate (42)*

A solution of isosorbide-2-aspirinate-5-benzyloxybenzoate (0.42 g, 0.8 mmol) in methanol: ethyl acetate (15 ml, 1:1) was stirred for 24 hours with palladium on charcoal under an atmosphere of hydrogen. The reaction mixture as filtered and solvent removed *in vacuo* to yield 0.33 g of crude product. Purification by column chromatography using hexane and ethyl acetate (2:1) as eluant yielded 0.16 g of product as white crystalline material (47.0 %): m.pt. 81-84°C. IR *v*max (KBr): 3215.8 (OH), 1765.4 and 1724.2 (C=O), 1298.7 and 1250.2 (C(O)OR, aromatic), 1076.3 (C-O-C) cm⁻¹. HRMS: Requires: 451.0984 (M⁺+23), Found: 451.0972 (M⁺+23). ¹H NMR δ (CDCl₃): 2.36 (1H, s, OCOCH₃), 4.11 (4H, m, IS₁H[α+β] and IS₆H [β]), 4.60 (1H, d, J 4.5 Hz, ISH-3), 5.05 (1H, t, J 5.0 and 5.5 Hz, ISH-4), 5.44 (2H, m, ISH-2 and ISH-5), 6.90 (1H, t, J 7.5 and 8.0 Hz, Ar-H), 7.05 (1H, d, J 8.5 Hz, Ar-H), 7.12 (1H, d, J 8.0 Hz, Ar-H), 7.34 (1H, t, J 7.0 and 7.5 Hz, Ar-H), 7.50 (1H, m, Ar-H), 7.61 (1H, m, Ar-H), 7.90 (1H, dd, J 1.5 and 1.5 Hz, Ar-H), 8.02 (1H, dd, J 1.5 and 1.5 Hz, Ar-H). ¹³C NMR ppm (CDCl₃): 20.41 (OCOCH₃), 70.32 (ISC-1), 72.76 (ISC-6), 74.32 (ISC-5), 77.20 (ISC-2), 80.64 (ISC-4), 85.70 (ISC-3), 17.42 (Ar₂C-1), 122.26 (Ar₁C-1). 117.29 118.87 123.39 125.59 129.47 131.37 133.86 135.60 150.25(Ar₁C-2), 161.30 (Ar₂C-2), 163.20 (ArOC(O)CH₃), 168.87(ArC(O)OR).

*Benorylate (4-acetamidophenyl 2-acetoxybenzoate) (25)*

To a solution of paracetamol (0.5 g, 3.31 mmol) in dichloromethane (50 ml) at 0°C was added triethylamine (0.40 ml, 3.96 mmol) and vessel was allowed equilibrate for 30 mins. The reaction vessel was brought to room temperature and acetylsalicyloyl chloride (0.72 g, 3.63 mmol) was added, then allowed to stir for 6 hours. The solution was washed in water (2 x 20 ml), HCl (2 x 20 ml), and saturated aqueous NaHCO₃ (30 ml). The organic layer was dried over MgSO₄ and concentrated *in vacuo* to give product as white amorphous powder (0.49 g, 47.4%).
m.pt. 176-178°C. IR v_{\text{max}} \ (\text{KBr}): 3307.8 \ (\text{N-H stretching}), \ 1772.4 \ \text{and} \ 1741.1 \ (\text{C=O}), \ 1665.1 \ (\text{C=O amide stretch}), \ 1607.2 \ (\text{C=C, aromatic}), \ 1559.9 \ (\text{N-H bending}) \ \text{cm}^{-1}. \ \text{HRMS: Requires: 336.0848 (M}^+23), \ \text{Found: 336.0832 (M}^+23). \ ^1\text{H NMR} \ \delta \ (\text{CDCl}_3): \ 2.21 \ (3\text{H, s, COCH}_3), \ 2.33 \ (3\text{H, s, OCOCH}_3), \ 7.18 \ (1\text{H, d, } J \ 7.5 \ \text{and} \ 7.5 \ \text{Hz, Ar-H}), \ 7.57 \ (2\text{H, d, } J \ 9.0 \ \text{Hz, Ar-H}), \ 7.66 \ (1\text{H, m, Ar-H}), \ 8.23 \ (1\text{H, dd, } J \ 1.5 \ \text{and} \ 1.5 \ \text{Hz, Ar-H}). \ ^{13}\text{C NMR ppm (CDCl}_3): 20.59 \ (\text{ArOCOCH}_3), \ 24.06 \ (\text{NHCOCH}_3), \ 120.48 \ (\text{Ar}_2\text{C-2 and Ar}_2\text{C-4}), \ 121.66 \ (\text{Ar}_1\text{C-5}), \ 121.99 \ (\text{Ar}_2\text{C-1 and Ar}_2\text{C-5}), \ 123.57 \ (\text{Ar}_1\text{C-1}), \ 125.79 \ (\text{Ar}_1\text{C-3}), \ 131.79 \ (\text{Ar}_1\text{C-2}), \ 134.21 \ (\text{Ar}_1\text{C-4}), \ 136.2044 \ (\text{Ar}_2\text{C-3}), \ 146.14 \ (\text{Ar}_2\text{C-6}), \ 154.43 \ (\text{Ar}_1\text{C-6}), \ 167.89 \ \text{ArCOOA}r), \ 169.39 \ (2 \times \text{COCH}_3).

**Phenyl aspirinate (23)**

Phenol (0.215 g, 2.28 mmol) was dissolved in dichloromethane (50 ml), and triethylamine added (0.38 ml, 2.75 mmol). Reaction was equilibrated for one hour then acetylsalicyloyl chloride (0.50 g, 2.52 mmol) was added and reaction was stirred at room temperature for 4 hours. Reaction was washed with water (2 x 25 ml), HCl (1M, 25 ml) and saturated aqueous NaHCO$_3$ (25 ml). Organic phase was dried over MgSO$_4$ and solvent evaporated in vacuo to give product as white powder (0.54 g, 84.3%): m.pt. 86-88°C. IR v_{\text{max}} \ (\text{KBr}): 3061.0 \ (\text{OH}), \ 1762.0 \ \text{and} \ 1736.5 \ (\text{C=O}), \ 1606.0 \ (\text{C=C, aromatic}), \ 1255.9 \ \text{and} \ 1184.8 \ (\text{C(O)OR, aromatic}), \ 1054.3 \ (\text{C-O-C}) \ \text{cm}^{-1}. \ \text{HRMS: Requires: 279.0633 (M}^+23), \ \text{Found: 279.0632 (M}^+23). \ ^1\text{H NMR} \ \delta \ (\text{CDCl}_3): 2.44 \ (3\text{H, s, OCOCH}_3), \ 7.20 \ (3\text{H, t, } J \ 5.04 \ \text{and} \ 2.0 \ \text{Hz, Ar}_2\text{H-2, Ar}_2\text{H-4 and Ar}_2\text{H-6}), \ 7.30 \ (2\text{H, t, } J \ 8.0 \ \text{and} \ 7.56 \ \text{Hz, Ar}_1\text{H-3 and Ar}_1\text{H-5}), \ 7.44 \ (3\text{H, m, Ar}_2\text{H-3 and Ar}_2\text{H-5}), \ 7.67 \ (1\text{H, dd, } J \ 7.52 \ \text{and} \ 6.0 \ \text{Hz, Ar}_1\text{H-4}), \ 8.26 \ (1\text{H, dd, } J \ 1.76 \ \text{and} \ 1.52 \ \text{Hz, Ar}_1\text{H-2}). \ ^{13}\text{C NMR ppm (CDCl}_3): 20.59 \ (\text{OCOCH}_3), \ 121.25 \ (\text{Ar}_1\text{C-5}), \ 122.13 \ (\text{Ar}_2\text{C-2 and Ar}_2\text{C-6}), \ 123.59 \ (\text{Ar}_1\text{C-1}), \ 125.67 \ (\text{Ar}_1\text{C-3}), \ 125.76 \ (\text{Ar}_2\text{C-4}), \ 129.15 \ (\text{Ar}_2\text{C-3 and Ar}_2\text{C-5}), \ 131.79 \ (\text{Ar}_1\text{C-2}), \ 134.15 \ (\text{Ar}_1\text{C-4}), \ 150.09 \ (\text{Ar}_2\text{C-1}), \ 150.73 \ (\text{Ar}_1\text{C-6}), \ 162.54 \ (\text{ArOCO Ar}), \ 169.34 \ (\text{OCOCH}_3).
Guaiacol-aspirinate (2-methoxyphenyl 2-acetoxybenzoate) (24)

To a solution of guaiacol (2-methoxyphenol, 0.27 ml, 2.28 mmol) in dichloromethane (50 ml) was added triethylamine (0.38 ml, 2.75 mmol) and the reaction was allowed to stir at room temperature for one hour. Acetylsalicyloyl chloride (0.50 g, 2.52 mmol) was added and reaction vessel was stirred for 24 hours. Organic phase was washed with water (2 x 25 ml), HCl (0.1 M, 25 ml), saturated aqueous NaHCO₃ (25 ml) and dried over MgSO₄. Solvent was removed in vacuo to give yellow oil, which on recrystallisation in hexane yielded product as yellow crystals (0.66 g, 92.22%): m.pt. 71-73°C. IRvmax (KBr): 2037.9 (C-H stretching), 1749.7 (C=O), 1608.0 (C=C, aromatic), 1255.4 and 1198.1 (C(O)OR, aromatic), 1051.8 (C-O-C) cm⁻¹. HRMS: Requires: 309.0739 (M⁺+23), Found: 309.0747 (M⁺+23).¹H NMR δ (CDCl₃): 2.32 (3H, s, OCOCH₃), 3.86 (3H, s, ArCOCH₃), 7.01 (2H, m, Ar₂H-3 and Ar₂H-5), 7.17 (2H, m, Ar₂H-2 and Ar₂H-4), 7.26 (1H, m, Ar₁H-5), 7.40 (1H, t, J 7.52 and 7.52 Hz, Ar₁H-3), 7.65 (1H, m, Ar₁H-4), 8.28 (1H, dd, J 1.52 and 2.04 Hz, Ar₁H-2).¹³C NMR ppm (CDCl₃): 20.59 (OCOCH₃), 30.51 (ArOCH₃), 55.41 (ArOCOAr), 112.08 (Ar₂C-5), 120.37 (Ar₂C-3), 122.49 (Ar₁C-5), 123.55 (Ar₂C-2), 125.70 (Ar₁C-1), 126.65 (Ar₁C-3), 132.00 (Ar₂C-4), 133.93 (Ar₁C-2), 139.13 (Ar₁C-4), 150.72 (Ar₂C-1), 150.77 (Ar₁C-6), 162.01 (Ar₂C-6), 169.26 (OCOCH₃)

Isosorbide-5-mononitrate-2-benzoate (68)

ISMN (1g, 5.58 mmol) was stirred in dichloromethane (50 ml) to which was added triethylamine (0.94 ml, 6.70 mmol). After equilibration at room temperature benzoyl chloride (0.59 g, 5.58 mmol) was added and the reaction vessel was stirred at room temperature for 24 hours. Reaction was washed with water (2 x 40 ml), HCl (40 ml) and saturated aqueous NaHCO₃ (40 ml), and dried over MgSO₄. Organic solvent was removed by rotary evaporation to give yellow oil, which crystallised in ethanol to yield colourless crystals as product (1.29 g, 78.2%): m.pt. 66-68°C. IRvmax (KBr): 2983.6 and 2910.3 (C-H, stretching), 1720.1 (C=O), 1633.9 (C=C, aromatic), 1287.6 and 1265.9 (C(O)OR, aromatic), 1098.3 (C-O-C) cm⁻¹. HRMS: Requires: 318.0587 (M⁺+23), Found: 318.0577 (M⁺+23).¹H NMR δ (CDCl₃): 3.97 (1H, q, J 5.52, 6.0 and 5.52 Hz, ISH-4), 4.08 (1H, dd, J 2.52 and 3.0
Hz, ISH-1, ISH-2), 4.17 (2H, m, ISH-5 and ISH-3), 4.66 (1H, d, J 5.04 Hz, ISH-6), 7.46 (2H, t, J 7.56 and 7.52 Hz, ArH-3 and ArH-5), 7.59 (1H, t, J 7.52 and 7.5 Hz, ArH-4), 8.04 (2H, d, J 7.56 Hz, ArH-2 and ArH-6). $^{13}$C NMR ppm (CDCl$_3$): 62.41 (ISC-1), 64.62 (ISC-5), 70.16 (ISC-4), 75.22 (ISC-6), 79.99 (ISC-3), 81.70 (ISC-2), 128.46 (ArC-3 and ArC-5), 129.78 (ArC-2 and ArC-6), 130.54 (ArC-1), 132.84 (ArC-4), 167.01 (OCOAr)

**Isosorbide-2-benzoate (67)**

ISMN-benzoate (1.2g, 4.06 mmol) was dissolved in a mixture of methanol and ethyl acetate (1:1, 60 ml) and stirred for 48 hours over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica and solvent removed in vacuo to give yellow oil. Recrystallisation in a minimum amount of an ethanol: hexane mixture afforded product as colourless crystals (0.68 g, 66.9%): m.pt. 68-70°C. IR $\nu_{max}$ (KBr): 1725.4 (C=O), 1654.3 (C=C, stretching), 1265.9 and 1113.0 (C(O)OR, aromatic), 1044.6 (C-O-C) cm$^{-1}$. HRMS: Requires 273.0739 (M$^+$+23), Found: 273.0722 (M$^+$+23). $^1$H NMR $\delta$ (CDCl$_3$): 2.71 (1H, d, J 7.0 Hz, OH), 3.63 (1H, t, J 6.52 and 6.0 Hz, ISH-4), 3.95 (2H, t, J 6.04 and 6.0 Hz, ISH-1 and ISH-2), 4.16 (1H, m, ISH-3), 4.36 (1H, t, J 6.0 and 6.04 Hz, ISH-6), 4.65 (1H, d, J 4.52 Hz, ISH-5), 7.47 (2H, t, J 8.0 and 7.52 Hz, ArH-3 and ArH-5), 7.59 (1H, t, J 7.56 and 7.52 Hz, ArH-4), 80.4 (2H, d, J 7.52 Hz, ArH-2 and ArH-6). $^{13}$C NMR ppm (CDCl$_3$): 72.37 (ISC-6), 73.51 (ISC-1), 73.63 (ISC-2), 78.86 (ISC-5), 82.06 (ISC-4), 85.71 (ISC-3), 128.43 (ArC-3 and ArC-5), 129.43 (ArC-2 and ArC-6), 129.70 (ArC-1), 133.37 (ArC-4), 165.47 (ArOCO-).

**Isosorbide-2,5-dibenzoate (69)**

Isosorbide (2.0 g, 13.68 mmol) was dissolved in toluene (50 ml) at 0°C to which was added triethylamine (5 ml, 04.5 mmol), and benzoyl chloride (5.18 ml, 27.37 mmol). The reaction vessel was stirred at room temperature for 48 hours before washing with HCl (25 ml, 1M), water (2 x 25 ml), saturated aqueous NaHCO$_3$ (5 ml) and brine (25 ml). Solution was dried over MgSO$_4$ and solvent was removed in
**Phenyl benzoate (73)**

To a solution of phenol (0.42 g, 4.46 mmol) in dichloromethane (50 ml) was added triethylamine (0.75 ml, 5.34 mmol) and benzoyl chloride (0.52 g, 4.95 mmol). The reaction mixture was stirred at room temperature for 12 hours before washing with water (2 x 25 ml), HCl (25 ml) and saturated aqueous NaHCO₃ (25 ml). Solvent was removed *in vacuo* to give product as yellow oil, which was recrystallised in ethanol to afford white crystals (0.42 g, 47.5%) m.pt. 70-72°C. IR<sub>max</sub> (KBr): 3058.6 (C-H stretching), 1730.3 (C=O), 1596.7 (C=C, stretching), 1262.9 and 1198.7 (C(O)OR, aromatic), 1063.0 (C-O-C) cm⁻¹. HRMS: Requires: 221.0578 (M⁺+23), Found: 221.0580 (M⁺+23). ¹H NMR δ (CDCl₃): 7.18 (2H, d, J 6.52 Hz, Ar₂H-2 and Ar₂H-6), 7.24 (1H, q, J 7.52, 9.56 and 7.52 Hz, Ar₂H-4), 7.36 (2H, m, Ar₂H-3 and Ar₂H-5), 7.48 (2H, t, J 7.56 and 6.0 Hz, Ar₁H-3 and Ar₁H-5), 7.61 (1H, m, Ar₁H-4), 8.17 (2H, dd, J 1.0 and 2.0 Hz, Ar₁H-2 and Ar₁H-4). ¹³C NMR ppm (CDCl₃): 121.21 (Ar₂C-2 and Ar₂C-6), 125.39 (Ar₂C-4), 128.08 (Ar₂C-3 and Ar₂C-5), 129.05 (Ar₁C-3 and Ar₁C-6), 129.64 (Ar₁C-1), 133.11 (Ar₁C-4), 150.44 (Ar₂C-1), 164.67 (ArOCO).

**Isosorbide-2-mononitrate**

ISDN (0.75 g, 3.17 mmol) was stirred in a mixture of methanol: water (50 ml, 4:1) in the presence of ferrous sulphate (4.12 g, 14.8 mmol), before refluxing in an oil
bath at 98°C for 3 hours. Solvent was removed in vacuo and residue was dissolved in dichloromethane (75 ml) before washing in water (2 x 25 ml), saturated aqueous NaHCO₃ (25 ml) and brine (25 ml). The organic phase was dried over MgSO₄ and solvent removed in vacuo to give product as yellow oil (0.41 g, 67.5%). IR

\[ \text{IR } \text{vmax} \text{ (film): 3442.9 (OH), 1639.8 (NO}_2\text{), 1275.1 (NO}_2\text{), 1089.3 (C-O-C), 859.3 (ONO}_2\text{)} \text{ cm}^{-1}. \]

HRMS: Requires: 214.1407 (M⁺+23), Found: 214.1413 (M⁺+23). \(^1\)H NMR \( \delta \) (CDCl₃): 2.76 (1H, d, J 6.5 Hz, OH), 3.58 (1H, q, J 5.5, 4.0 and 5.5 Hz, IS6α-H), 3.88 (1H, q, J 6.0, 3.5 and 6.0 Hz, IS6β-H), 4.14 (2H, d, J 3.0Hz, IS1H₂ [α+β]), 4.32 (1H, t, J 6.0 and 5.5 Hz, ISH-5), 4.53 (1H, d, J 5.0 Hz, ISH-3), 4.60 (1H, t, J 5.0 and 5.1 Hz, ISH-4), 5.43 (1H, m, ISH-2). \(^1\)C NMR ppm (CDCl₃): 71.50 (ISC-1), 72.02 (ISC-5), 73.29 (ISC-6), 82.04 (ISC-2), 86.24 (ISC-3).

**Isosorbide-2-aspirinate-5-acetate (43)**

To a solution of isosorbide-2-aspirinate (0.2 g, 0.65 mmol) in dichloromethane (20 ml) was added triethylamine (0.09 ml, 0.65 mmol) and acetic anhydride (0.06 ml, 0.65 mmol). The reaction vessel was stirred at room temperature for 24 hours before washing with water (2 x 20 ml), HCl (1M, 30 ml), saturated aqueous NaHCO₃ (30 ml) and drying over MgSO₄. Solvent was removed by rotary evaporation to yield 0.52 g of crude product. Purification by column chromatography using hexane and ethyl acetate (3:2) as eluant afforded product as white crystalline material (0.1 g, 43.8%). m.pt. 96 – 98°C. IR

\[ \text{IR } \text{vmax} \text{ (KBr): 2966.9 and 2928.6 (C-H stretching), 1751.6 and 1734.0 (C=O), 1607.8 (C=C stretching), 1262.0 and 1193.9 (C(O)OR aromatic), 1082.5 (C-O-C) cm}^{-1}. \]

HRMS: Requires: 373.0899 (M⁺+23), Found: 373.0877 (M⁺+23), \(^1\)H NMR \( \delta \) (CDCl₃): 2.13 (3H, s, IS-OCOCH₃), 2.37 (3H, s, Ar-OCOCH₃), 3.85 (1H, q, J 5.52, 4.52 and 4.96 Hz, IS6α-H), 3.99 (1H, q, J 6.0, 3.52 and 6.04 Hz, IS6β-H), 4.10 (2H, t, J 3.52 and 2.0 Hz, IS1H₂[α+β]), 4.59 (1H, d, J 4.52 Hz, ISH-3), 4.90 (1H, t, J 5.0 and 5.04 Hz, ISH-4), 5.19 (1H, d, J 5.52 Hz, ISH-5), 5.44 (1H, d, J 5.52 Hz, ISH-2), 7.12 (1H, d, J 8.04 Hz, ArH-4), 7.33 (1H, t, J 7.52 and 7.56 Hz, ArH-2), 7.59 (1H, m, ArH-3), 8.01 (1H, dd, J 6.04 Hz, ArH-1). \(^1\)C NMR ppm (CDCl₃): 20.43 (ArOCOCH₃), 20.18 (IS-OCOCH₃), 69.91 (ISC-2), 72.78 (ISC-6), 73.52 (ISC-5), 78345 (ISC-3), 80.32 (ISC-1), 122.29 (Ar-C-5), 123.39 (Ar-C-3), 125.58 (Ar-C-3), 131.36 (Ar-C-2), 216
133.81 (ArC-4), 154.32 (ArC-6), 167.15 (OCOAr), 168.48 (ArOCOCH₃), 171.27 (OCOCH₃).

Isosorbide-2-aspirinate-5-[2-methylbenzoate] (54)

To a solution of isosorbide-2-aspirinate (0.2 g, 0.65 mmol) in dichloromethane (15 ml) was added triethylamine (0.11 ml, 0.98 mmol) and 2-toluoyl chloride (0.09 ml, 0.72 mmol). The reaction mixture was stirred at room temperature for 24 hours and then was washed with water (2 x 25 ml), HCl (1 m, 25 ml) and saturated aqueous NaHCO₃ before drying over anhydrous MgSO₄. Solvent was removed in vacuo to give 0.41 g of crude product as brown oil. Purification by column chromatography using hexane and ethyl acetate (2:1) as eluant gave product as yellow oil. This was recrystallised in ethanol to yield product as a white solid (0.11 g, 39.6%) m pt. 104–106°C. IRmax (KBr): 2987.1 and 2922.8 (C-H stretching), 1762.0 and 1718.1 (C=O), 1259.5 and 1199.8 (C(O)OR aromatic), 1072.4 (C-O-C) cm⁻¹. HRMS: Requires: 449.1212 (M^+23), Found: 449.1238 (M^+23), ¹H NMR δ (CDCl₃): 2.38 (3H, s, OCOCH₃), 2.65 (3H, s, ArCH₃), 4.01 (1H, dd, J 5.52 and 5.52 Hz, IS₆-H[α]), 4.12 (3H, m, IS₁H[αβ] and IS₆H[β]), 4.66 (1H, d, J 4.52 Hz, ISH-3), 5.04 (1H, t, J 5.04 and 5.0 Hz, ISH-4), 5.41 (1H, q, J 5.52, 5.52 and 5.52 Hz, ISH-5), 5.47 (1H, d, J 2.0 Hz, ISH-2), 7.13 (1H, dd, J 1.0 and 1.0 Hz, ArH-4), 7.33 (1H, t, J 7.0 and 6.52 Hz, ArH-2), 7.59 (1H, t, J 6.52 and 6.52 Hz, ArH-3), 8.02 (1H, dd, J 1.52 and 2.0 Hz, ArH-1). ¹³C NMR ppm (CDCl₃): 20.43 (Ar-CH₃), 21.12 (OCOCH₃), 70.29 (ISC-1), 72.72 (ISC-6), 73.81 (OC(O)Ar), 78.22 (ISC-4), 80.52 (ISC-2), 85.63 (ISC-3), 122.32 (Ar₁C-1), 123.38 (Ar₁C-4), 125.58 (Ar₁C-2 and Ar₂C-4), 128.46 (Ar₂C-2 and Ar₂C-5), 130.19 (Ar₂C-3), 133.78 (Ar₁C-3), 140.08 (Ar₂C-1), 150.26 (Ar₁C-5), 163.12 (ArOCOME), 169.15 (ArCOOR)

Isosorbide-2-ibuprofenate-5-mononitrate (71)

S-Ibuprofen (5g, 24.2 mmol) was stirred at 0°C in dichloromethane (50 ml) to which was added DMAP (2.95 g, 24.1 mmol) and DCC (5 g, 24.2 mmol). The reaction vessel was brought to room temperature; ISMN (4.35 g, 24.2 mmol) was added and reaction was stirred for 4 hours. The organic phase was washed with

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HCl (1M, 50 ml), water (50 ml), saturated aqueous NaHCO₃ (50 ml) and brine (50 ml) after filtration of DCU precipitate. Solvent was removed in vacuo to give crude product as yellow oil, which was recrystallised in ethanol to yield product as white crystals (3.98 g, 43.3%) m.pt 59-60°C. \( \text{IR}_{\text{vmax}} \) (KBr): 2954.1 (C-H stretching), 1731.7 (C=O), 1642.7 (C=C), 1281.1 and 1197.7 (C(O)OR, aromatic), 1092.0 (C-O-C) cm⁻¹. HRMS: Requires: 402.1529 (M⁺+23), Found: 402.1526 (M⁺+23). \(^1\)H NMR \( \delta \) (CDCl₃): 0.92 (6H, d, J 6.52 Hz, 2 x CH₃), 1.49 (3H, d, J 7.0 Hz, CHCH₃), 2.19 (1H, s, CH(Me)₂), 2.47 (2H, d, J 7.04 Hz, CH₂Ar), 3.72 (IH, m, ArCHCH₃), 3.86 (1H, q, J 5.52, 5.52 and 6.0 Hz, ISH-4), 4.01 (1H, m, ISH-1 and ISH-2), 4.26 (1H, d, J 4.52 Hz, ISH-6), 4.52 (1H, t, J 5.0 and 5.04 Hz, ISH-3), 5.23 (1H, d, J 2.52 Hz, ISH-5), 7.15 (4H, dd, J 8.0 and 8.04 Hz, 4 x Ar-H). \(^1^C\) NMR ppm (CDCl₃): 17.68 (COCHCH₃), 21.87 (2 x CH₃), 29.68 (CH(Me)₂), 44.42 (COCHCH₃), 44.54 (CHCH₂Ar), 68.69 (ISC-1), 73.02 (ISC-6), 76.23 (ISC-5), 80.77 (ISC-4), 80.94 (ISC-3), 85.99 (ISC-1), 126.59 (ArC-2 and ArC-6), 128.95 (ArC-3 and ArC-5), 136.23 (ArC-1), 140.39 (ArC-4), 173.8 (OOCOCH).

Isosorbide-2-ibuprofenate (70)

Isosorbide-2-aspirinate-5-mononitrate (3.9 g, 9.39 mmol) was stirred in glacial acetic acid (50 ml) to which was added zinc powder (2.45 g, 9.39 mmol). The reaction vessel was stirred at room temperature for 48 hours before washing with water (3 x 50 ml) and saturated aqueous KHCO₃ (3 x 50 ml). Solvent was removed in vacuo to yield product as crude oil, which was crystallised in ethanol to yield product as white crystals (0.67 g, 19.5%) m.pt. 60-61°C. \( \text{IR}_{\text{vmax}} \) (KBr): 3447.8 (OH), 2954.1 (C-H stretching), 1726.5 (C=O), 1267.9 and 1178.9 (C(O)OR aromatic), 1091.6 (C-O-C) cm⁻¹. HRMS: Requires: 357.4071 (M⁺+23), Found: 357.4082 (M⁺+23). \(^1\)H NMR \( \delta \) (CDCl₃): 0.89 (6H, d, J 6.52 Hz, 2 x CH₃), 1.48 (3H, d, J 7.04 Hz, COCHCH₃), 1.84 (1H, m, CH (Me)₂), 2.17 (1H, s, -OH), 2.44 (2H, d, J 7.04 Hz, ArCH₂), 3.52 (1H, q, J 6.04, 3.0 and 6.04 Hz, COCH-), 3.69 (1H, q, J 7.0, 7.2 and 7.04 Hz, ISH-4), 3.83 (1H, q, J 6.0, 3.52 and 6.04 Hz, ISH-1 and ISH-2), 3.99 (2H, m, ISH-6), 4.24 (2H, d, J 4.0 Hz, ISH-3), 4.44 (1H, t, J 5.0 and 4.52 Hz, ISH-5), 7.09 (4H, dd, J 8.04 and 8.04 Hz, 4 x Ar-H). \(^1^C\) NMR ppm (CDCl₃): 17.73 (COCHCH₃), 21.91 (2 x CH₃), 29.73 (CH(Me)₂), 44.43 (COCHMe), 44.54 (CH₂CH(Me)₂), 71.82 (ISC-6), 73.03 (ISC-1), 76.28 (ISC-2), 218
Isosorbide-2,5-diibuprofenate (72)

S-ibuprofen (4.13g, 20 mmol) was dissolved in dichloromethane (50 ml) to which was added DMAP (1.22 g, 20 mmol) and DCC (2.063 g, 20 mmol) at 0°C. The reaction vessel was brought to room temperature and isosorbide (1.46 g, 10 mmol) was added. The reaction was stirred for 48 hours before washing with water (2 x 50 ml), HCl (50 ml), saturated aqueous NaHCO₃ (50 ml) and brine (50 ml). Solvent was removed *in vacuo* to yield a white waxy solid as product (3.2 g, 86.5%). m.pt. 57-59°C. IR νmax (KBr): 3439.8 (OH), 2961.3 (C-H stretching), 1725.6 and 1714.6 (C=O), 1269.6 (C(O)OR aromatic), 1108.3 (C-O-C) cm⁻¹. HRMS: Requires: 545.2879 (M⁺+23), Found: 545.2873 (M⁺+23). ¹H NMR δ (CDCl₃): 0.91 (4H, d, J 6.52, 4 X methyl), 1.49 (3H, d, J 7.0 Hz, COCHCH₃), 1.54 (3H, d, J 7.0 Hz, COCH₃), 1.85 (2H, m, CH(CH₃)₂), 2.19 (2H, s, CH(CH₃)₂), 2.46 (4H, d, J 7.04 Hz, 2 x Ar-CH₂), 3.76 (6H, m, ISIH₂ [α+β]) and IS₆H₂ [α+β]), 4.23 (1H, d, J 4.52 Hz, ISH-3), 4.73 (1H, t, J 5.52 and 5.0, ISH-4), 5.07 (1H, dd, J 5.52 and 5.52, ISH-5), 5.15 (1H, d, J 3.0 Hz, ISH-2), 7.09 (4H, d, J 8.04 Hz, Ar₁H-3, Ar₁H-5, Ar₂H-3 and Ar₂H-5), 7.21 (4H, q, J 8.04, 7.52 and 8.04 Hz, Ar₁H-2, Ar₁H-6, Ar₂H-2 and Ar₂H-6). ¹³C NMR ppm (CDCl₃): 17.73 (2 x ArCHCH₃), 21.93 (4 x CH₃), 30.51 (2 x CH(CH₃)₂), 38.73 (2 x ArCHCH₃), 44.56 (2 x ArCH₂), 70.16 (ISC-2 and ISC-6), 76.27 (ISC-3 and ISC-5), 80.27 (ISC-1 and ISC-4), 128.86 (ArC-2 and ArC-6), 128.95 (ArC-5 and ArC-3), 136.73 (ArC-1), 140.21 (ArC-4), 173.76 (2 x OCO).

Isosorbide-2-aspirinate-5-benzoate (45)

To a solution of isosorbide-2-aspirinate (1.0 g, 3.25 mmol) in dichloromethane (20 ml) was added benzoic acid (0.59 g, 4.88 mmol), DCC (1.34g, 6.49 mmol) and DMAP (0.38 g, 3.11 mmol). The reaction mixture was allowed to stir at room temperature for three hours before filtering off precipitate and washing the filtrate with HCl (30 ml, 1M), saturated aqueous Na₂HCO₃ (30 ml) and water (3 x 30 ml).
It was dried over anhydrous Na$_2$SO$_4$ and solvent was removed in vacuo to give colourless oil, which was recrystallised in ethanol to afford product as white crystals (1.13 g, 84.3%): m.pt. 80-82°C. IR$_{\text{vmax}}$ (KBr): 2991.1 and 2932.9 (C-H, stretching), 1762.9 and 1720.6 (C=O), 1275.5 and 1199.1 (C(O)OR aromatic), 1078.4 (C-O-C) cm$^{-1}$. HRMS: Requires: 435.1056 (M$^+$+23), Found: 435.1043 (M$^+$+23). $^1$H NMR $\delta$ (CDCl$_3$): 2.37 (3H, s, OCOCH$_3$), 4.07 (1H, m, IS1\text{a-H}), 4.11 (3H, m, IS6H[\alpha+\beta] and IS1\text{b-H}), 4.65 (1H, d, J 5.0 Hz, ISH-5), 5.05 (1H, t, J 5.5 and 5.0 Hz, ISH-3), 5.56 (1H, m, ISH-4), 7.13 (1H, d, J 8.04 Hz, Ar$_1$H-5 and Ar$_1$H-3), 7.27 (1H, t, J 8.04 and 6.52 Hz, Ar$_2$H- and Ar$_2$H-5), 7.33 (1H, d, J 7.56 Hz, Ar$_1$H-4), 7.49 (2H, t, J 7.52 and 7.56 Hz, Ar$_2$H-5), 8.01 (1H, d, J 7.56 Hz, Ar$_1$H-2), 8.12 (2H, d, J 7.52 Hz, Ar$_2$H-2 and Ar$_2$H-6). $^{13}$C NMR ppm (CDCl$_3$): 20.44 (OCOCH$_3$), 70.42 (ISC-1), 72.79 (ISC-6), 73.97 (ISC-5) 78.16 ISC-2), 80.67 (ISC-4), 85.67 (ISC-3), 123.39 (Ar$_1$C-5), 125.58 (Ar$_1$C-1), 128.00 (Ar$_1$C-3), 129.31 (Ar$_2$C-3 and Ar$_2$C-5), 131.39 (Ar$_1$C-2 and Ar$_2$C-6), 132.82 (Ar$_2$C-2 and Ar$_2$C-1), 133.79 (Ar$_2$C-4), 134.81 (Ar$_1$C-4), 154.32 (Ar$_1$C-6), 167.10 (OCOCH$_3$), 168.2 (OCOA$_1$ and OCOA$_2$).

**Isosorbide-2-aspirinate-5-[4-methylbenzoate] (55)**

A solution of isosorbide-2-aspirinate (0.2 g, 0.65 mmol) was dissolved in toluene at 0°C to which was added triethylamine (0.13 mls, 0.98 mmol) and 4-toluoyl chloride (0.93 ml, 0.78 mmol). The reaction vessel was returned to room temperature and allowed to stir for 10 hours, then washed with HCl (30 ml, 1M), saturated aqueous NaHCO$_3$ (30 ml), water (3 x 30 ml) and saturated NaCl solution (30 ml). The reaction was dried with anhydrous Na$_2$SO$_4$ and solvent was removed in vacuo using ethyl acetate as co-solvent to give crude product. Purification by column chromatography using hexane and ethyl acetate (9:1) as eluant gave product as white crystals (0.1 g, 35.99%): m.pt. 102-104°C. IR$_{\text{vmax}}$ (KBr): 2982.7 and 2923.6 (C-H stretching), 1763.9 and 1717.8 (C=O), 1608.5 (C=C), 1275.4 and 1202.0 (C(O)OR), 1100.3 (C-O-C) cm$^{-1}$. HRMS: Requires: 449.1212 (M$^+$+23), Found: 449.1229 (M$^+$+23), $^1$H NMR $\delta$ (CDCl$_3$): 2.19 (3H, s, OCOCH$_3$), 2.43 (3H, s, Ar-CH$_3$), 4.05 (2H, d, J 5.0 Hz, IS1H$_2$[\alpha+\beta] and IS6H$_2$[\alpha+\beta]), 4.09 (2H, t, J 4.04 and 3.52 Hz, ISH-6), 4.14 (1H, t, J 7.04 and 7.52 Hz, ISH-5), 4.63 (1H, d, J 5.0 Hz, ISH-3), 5.03 (1H, t, J 4.8 and 5.0, ISH-4), 5.44 (2H, m, ISH-2), 7.11 (1H, d, J
8.04 Hz, Ar-H), 7.27 (2H, d, J 8.56 Hz, Ar-H), 7.33 (1H, t, J 7.52 and 7.52 Hz, Ar-H), 7.55 (1H, t, J 1.52 and 6.04 Hz, Ar-H), 8.00 (3H, m, Ar-H). $^{13}$C NMR ppm (CDCl$_3$): 13.71 (ArCH$_3$), 20.54 (OCOCH$_3$), 70.49 (ISC-1), 72.75 (ISC-6), 73.78 (ISC-5), 78.13 (ISC-4), 80.72 (ISC-2), 85.62 (ISC-3), 122.25 (Ar$_1$C-1), 123.38 (Ar$_1$C-4), 125.64 (Ar$_1$C-6), 126.26 (Ar$_1$C-2 and Ar$_2$C-4), 128.74 (Ar$_2$C-2 and Ar$_2$C-5), 129.36 (Ar$_2$C-6), 131.42 (Ar$_2$C-3), 133.87 (Ar$_1$C-3), 143.62 (Ar$_2$C-1), 150.22 (Ar$_1$C-5), 163.15 (ArOCOCH$_3$), 165.48 (IS-OCOAr), 169.27 (ArCOO).

Isosorbide-2-aspirinate-5-propionate (44)

Isosorbide-2-aspirinate (0.3 g, 0.98 mmol) was dissolved in dichloromethane (20 ml) to which was added proprionic anhydride (0.14 ml, 1.07 mmol) and triethylamine (0.09 ml, 1.07 mmol). This was allowed to stir at room temperature for 24 hours before washing with HCl (30 ml, 1M), saturated aqueous NaHCO$_3$ (30 ml) and water (2 x 30 ml). Reaction was dried over anhydrous Na$_2$SO$_4$ and solvent was removed in vacuo to yield crude product as a yellow oil (0.19 g). Purification by column chromatography using hexane and ethyl acetate (5:2) as eluant yielded product as white crystals (0.3 g, 84.3%): m.pt. 54-56°C. IR$_{\text{vmax}}$ (KBr): 2989.0 and 2933.0 (C-H stretching), 1764.0 and 1734.5 (C=O), 1606.3 (C=C stretching), 1254.3 and 1193.6 (C(O)OR, aromatic), 1080.6 (C-O-C) cm$^{-1}$. HRMS: Requires: 387.1056 (M$^{+}$+23), Found: 387.1069 (M$^{+}$+23). $^1$H NMR $\delta$ (CDCl$_3$): 1.19 (3H, t, J 8.04 and 7.52 Hz, CH$_3$), 2.37 (3H, s, OCOCH$_3$), 2.44 (2H, q, J 7.52, 8.04 and 7.52 Hz, OCH$_2$), 3.86 (1H, q, J 5.52, 4.52 and 5.04 Hz, IS$_6\alpha$-H), 3.98 (1H, q, J 5.52, 4.04 and 6.0 Hz, IS$_6\beta$-H), 4.08 (2H, m, IS$_1$H$_2[\alpha+\beta]$), 4.59 (1H, d, J 4.52 Hz, ISH-3), 4.91 (1H, t, J 5.0 and 5.04, ISH-4), 5.20 (1H, q, J 5.04, 6.0 and 5.52 Hz, ISH-5), 5.43 (1H, d, J 3.0 Hz, ISH-2), 7.12 (1H, dd, J 1.0 and 1.0 Hz, ArH-4), 7.33 (1H, t, J 1.0, 6.56 and 8.0 Hz, ArH-2), 7.59 (1H, t, J 6.0 and 6.52 Hz, ArH-3), 8.01 (1H, dd, J 1.48 and 2.0 Hz, ArH-1). $^{13}$C NMR ppm (CDCl$_3$): 8.62 (CH$_2$CH$_3$), 20.49 (OCOCH$_3$), 26.84 (OCOCH$_2$), 70.08 (ISC-2) 72.72 (ISC-6), 73.32 (ISC-5), 78.12 (ISC-3), 80.37 (ISC-1), 85.45 (ISC-4), 122.22 (ArC-5), 123.41 (ArC-1), 125.64 (ArC-3), 131.41 (ArC-2), 133.89 (ArC-4), 150.24 (ArC-6), 163.09 (OCOAr), 169.28 (OCOCH$_3$), 173.40 (OCOCH$_2$).
Isosorbide-2-aspirinate (0.2 g, 0.65 mmol) was dissolved in toluene (15 ml) at 0°C, to which was added DCC (0.13 g, 0.65 mmol) and DMAP (0.08 g, 0.07 mmol). After 10 mins the reaction vessel was returned to room temperature and 3-toluic acid (0.09 g) was added and allowed to stir for 24 hours. After washing with HCl (30 ml, 1M), saturated aqueous NaHCO₃ (30 ml), saturated brine solution (30 ml) and water (3 x 30 ml) the reaction mixture was dried over anhydrous Na₂SO₄ and solvent removed in vacuo to yield crude product as a clear oil. Purification by column chromatography using hexane and ethyl acetate (3:2) as eluant yielded product as white crystals (0.12 g, 43.2%): m.pt. 96-98°C. IR ν max (KBr): 2987.1 and 2922.8 (C-H stretching), 1762.0 and 1718.1 (C=O), 1259.5 and 1199.8 (C(O)OR, aromatic), 1072.4 (C-O-C) cm⁻¹. HRMS: Requires: 449.1212 (M⁺+23), Found: 449.1234 (M⁺+23), ¹H NMR δ (CDCl₃): 2.36 (3H, s, OCOCH₃), 2.43 (3H, s, ArCH₃), 4.09 (4H, m, IS₁-H₂[α+β] and IS₆-H₂[α+β]), 4.65 (1H, d, J 5.0 Hz, ISH-3), 5.04 (1H, t, J 5.04 and 5.0 Hz, ISH-4), 5.43 (2H, m, ISH-5 and ISH-2), 7.12 (1H, d, J 8.0 Hz, ArH-4), 7.35 (3H, m, ArH-2), 7.58 (1H, q, J 1.0, 6.56 and 1.48 Hz, ArH-3), 8.01 (1H, dd, J 1.0 and 1.52 Hz, ArH-1). ¹³C NMR ppm (CDCl₃): 20.42 (ArCH₃), 20.79 (OCOCH₃), 70.39 (ISC-1), 72.77 (ISC-6), 73.92 (ISC-5), 78.19 (ISC-4), 80.66 (ISC-2), 85.66 (ISC-3), 122.35 (Ar₁C-1), 123.39 (Ar₁C-4), 125.57 (Ar₁C-6), 126.45 (Ar₁C-2), 12.89 (Ar₂C-4), 129.80 (Ar₂C-5), 131.37 (Ar₂C-3), 133.77 (Ar₂C-1), 150.25 (Ar₂C-5), 163.15 (ArOCOCH₃), 165.58 (ISOCOAr), 169.12 (ArOCO).

Isosorbide-2-aspirinate-5-nicotinate (46)

Isosorbide-2-aspirinate (0.3 g, 0.98 mmol), in dichloromethane (20 ml) at 0°C was stirred for 10 mins in the presence of DCC (0.2 g, 0.98 mmol) and DMAP (0.12 g, 0.98 mmol). The reaction vessel was returned to room temperature, nicotinic acid (0.12 g, 0.98 mmol) was added and allowed to stir for 24 hours. The reaction mixture was washed with HCl (20 ml, 1M), saturated aqueous NaHCO₃ (20 ml), water (3 x 20 ml), dried over anhydrous Na₂SO₄ and solvent removed in vacuo to give product as a crude oil (0.95 g). Purification by column chromatography over silica gel using dichloromethane and ethyl acetate (95:5) as eluant yielded product.
as white crystals (0.12g, 29.7%): m.pt. 94-96°C. IR\text{\textsubscript{\text{vmax}} (KBr)}: 3327.6 (N=C), 2929.6 (C-H stretching), 1731.7 and 1718.7 (C=O), 1654.4 (C=C stretching), 180.7 and 1195.9 (C(O)OR aromatic), 1090.4 (C-O-C) cm\textsuperscript{-1}. HRMS: Requires: 436.1008 (M\textsuperscript{+}+23), Found: 436.1011 (M\textsuperscript{+}+23). \textsuperscript{1}\text{H NMR} \delta (CDCl\textsubscript{3}): 2.36 (3H, s, OCOCH\textsubscript{3}), 4.11 (9H, m, IS1-H\textsubscript{2}[\alpha+\beta] and IS6-H\textsubscript{2}[\alpha+\beta]), 6.64 (1H, d, J 4.52 Hz, ISH-3), 5.05 (1H, t, J 5.04 and 5.52 Hz, ISH-4), 5.46 (2H, dd, J 2.0 and 2.52 Hz, ISH-5 and ISH-2), 7.11 (1H, d, J 8.52 Hz, Ar\textsubscript{1}H-2), 7.32 (1H, q, J 6.52, 8.04 and 8.52 Hz, Ar\textsubscript{1}H-3), 7.43 (1H, q, J 6.53, 8.04 and 8.52 Hz, Ar\textsubscript{1}H-5), 7.59 (1H, t, J 6.04 and 6.52 Hz, Ar\textsubscript{1}H-4), 8.00 (1H, dd, J 1.52 and 2.0 Hz, Ar\textsubscript{2}H-5), 8.34 (1H, m, Ar\textsubscript{2}H-6), 8.82 (1H, dd, J 2.0 and 1.48 Hz, Ar\textsubscript{2}H-4), 9.28 (1H, d, J 2.0 Hz, Ar\textsubscript{2}H-2). \textsuperscript{13}\text{C NMR} ppm (CDCl\textsubscript{3}): 20.52 (OCOC\textsubscript{3}), 79.32 (ISC-1), 72.75 (ISC-6), 74.38 (ISO\textsubscript{C(O)}Ar), 74.43 (ISC-5), 78.27 (ISC-4), 80.60 (ISC-2), 85.60 (ISC-3), 122.26 (Ar\textsubscript{1}C-1), 122.88 (Ar\textsubscript{1}C-4), 123.38 (Ar\textsubscript{2}C-4), 125.57 (Ar\textsubscript{1}C-6), 131.35 (Ar\textsubscript{2}C-6), 133.83 (Ar\textsubscript{1}C-2), 136.68 (Ar\textsubscript{1}C-3), 150.55 (Ar\textsubscript{2}C-5), 153.30 (Ar\textsubscript{2}C-1), 164.13 (Ar\textsubscript{1}C-5), 164.13 (Ar\textsubscript{2}C-3), 170.59 (Ar\textsuperscript{\text{COOR}}).

\textit{Isosorbide-2-aspirinate-5-\{iso-nicotinate\} (47)}

Isosorbide-2-aspirinate (0.2 g, 0.65 mmol) was dissolved in dichloromethane (20 ml) at 0°C to which was added DCC (0.13 g, 0.65 mmol) and DMAP (0.08 g, 0.65 mmol). After 10 mins the reaction vessel was returned to room temperature and iso-nicotinic acid (0.08 g, 0.65 mmol) was added and stirred for 24 hours. The reaction was washed with HCl (20 ml, 1M), saturated aqueous NaHCO\textsubscript{3} (20 ml), water (3 x 20 ml), dried over anhydrous MgSO\textsubscript{4} and solvent removed \textit{in vacuo} to yield product as white powder (0.17 g, 63.1%): m.pt. 86-88°C. IR\text{\textsubscript{\text{vmax}} (KBr)}: 3327.8 (N=C), 2929.3 (C-H stretching), 1751.8 and 1710.7 (C=O), 1628.0 (C=C stretching), 1249.0 and 1194.1 (C(O)OR aromatic), 1082.8 (C-O-C) cm\textsuperscript{-1}. HRMS: Requires: 436.1008 (M\textsuperscript{+}+23), Found: 436.1004 (M\textsuperscript{+}+23). \textsuperscript{1}\text{H NMR} \delta (CDCl\textsubscript{3}): 2.37 (3H, s, OCOCH\textsubscript{3}), 4.09 (5H, m, IS1-H\textsubscript{2}[\alpha+\beta] and IS6-H\textsubscript{2}[\alpha+\beta]), 4.65 (1H, d, J 4.52 Hz, ISH-3), 5.05 (1H, t, J 5.52 and 5.04 Hz, ISH-4), 5.46 (2H, dd, J 5.52 and 5.04 Hz, ISH-5 and ISH-2), 7.12 (1H, d, J 7.04 Hz, Ar\textsubscript{1}H-2), 7.33 (1H, m, Ar\textsubscript{1}H-3), 7.59 (2H, t, J 6.04 and 6.04 Hz, Ar\textsubscript{1}H-5 and Ar\textsubscript{1}H-4), 7.90 (1H, d, J 5.04 Hz, Ar\textsubscript{2}H-6), 8.01 (1H, dd, J 2.0 and 1.52 Hz, Ar\textsubscript{2}H-2), 8.84 (1H, s, Ar\textsubscript{2}H-5), 8.98 (1H, s, Ar\textsubscript{2}H-3).
**Isosorbide-2-aspirinate-5-[4-methoxy]-benzote (53)**

Isosorbide-2-aspirinate (0.2 g, 0.65 mmol) was dissolved in toluene (15 ml) at 0°C to which was added DMAP (0.08 g, 0.65 mmol) and DCC (0.13 g, 0.65 mmol). After 10 mins the reaction vessel was returned to room temperature, 4-anisic acid (4-methoxybenzoic acid) (0.10 g, 0.65 mmol was added and allowed to stir for 12 hours. The reaction mixture was washed with HCl (20 ml, 1M), saturated aqueous NaHCO₃ (20 ml), saturated brine solution (20 ml) and water (3 x 20 ml), dried over anhydrous Na₂SO₄ and solvent removed in vacuo to yield product as a crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (2:1) as eluant yielded product as white crystals (0.17 g, 58.9%): m.pt. 141-144°C. IR: νmax (KBr): 2994.1 and 2936.7 (C-H stretching), 1764. and 724.9 (C=O), 1605.8 (C=C stretching), 1260.5 (C(O)OR, aromatic), 1078.6 (C-O-C) cm⁻¹. HRMS: Requires: 465.1162 (M⁺+23), Found: 465.1157 (M⁺+23).¹H NMR δ (CDCl₃): 2.32 (3H, s, OCOCH₃), 3.84 (3H, s, ArOCH₃), 3.99 (1H, m, IS6-Hα), 4.07 (6H, m, IS1-H₂[α+β] and IS6-Hβ), 4.59 (1H, d, J 4.52 Hz, ISH-3), 4.98 (1H, t, J 5.52 and 5.0 Hz, ISH-4), 5.38 (1H, t, J 5.0 and 5.52 Hz, ISH-5), 5.43 (1H, d, J 2.0 Hz, ISH-2), 6.91 (2H, d, J 8.52 Hz, Ar₂H-3 and Ar₂H-5), 7.08 (1H, d, J 8.0 Hz, Ar₁H-4), 7.28 (1H, t, J 7.56 and 9.52 Hz, Ar₁H-2), 7.54 (1H, t, J 8.0 and 7.52 Hz, Ar₁H-3), 7.99 (3H, q, J 9.0, 7.04 and 8.04 Hz, Ar₁H-1, Ar₂H-2 and Ar₂H-6). ¹³C NMR ppm (CDCl₃): 20.48 (ArOCOCH₃), 59.83 (ArOCH₃), 70.41 (ISC-1), 72.82 (ISC-6), 73.58 (ISO₂COAr), 76.58 (ISC-5). 78.26 (ISC-4), 80.47 (ISC-2), 85.46 (ISC-3), 113.33 (Ar₂C-3 and Ar₂C-5), 121.46 (Ar₁C-5), 122.35 (Ar₂C-1), 123.36 (Ar₁C-1), 125.54 (Ar₁C-3), 131.35 (Ar₁C-2), 133.73 (Ar₂C-2 and Ar₂C-6), 133.76 (Ar₁C-4), 150.23 (Ar₁C-6), 163.12 (ArOCH₃ and Ar₂C-4), 165.09 (ArCOOR), 169.08 (ArCOOR), 170.52 (ArOCOCH₃).

**Isosorbide-2-aspirinate-5-[2'-methoxy]-benzote (51)**

Isosorbide-2-aspirinate (0.2 g, 0.65 mmol) was dissolved in toluene (15 ml) at 0°C to which was added DMAP (0.08 g, 0.65 mmol) and DCC (0.13 g, 0.65 mmol). After 10 mins the reaction vessel was returned to room temperature, 2-anisic acid
(2-methoxybenzoic acid, 0.10 g, 0.65 mmol) was added and allowed to stir for 12 hours. The reaction mixture was washed with HCl (20 ml, 1M), saturated aqueous NaHCO₃ (20 ml), saturated brine solution (20 ml) and water (3 x 20 ml), dried over anhydrous Na₂SO₄ and solvent removed in vacuo to yield product as a crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (3:1) as eluant yielded product as white crystals (0.23 g, 79.8%): m.pt. 132-134°C. IR \( \nu_{\text{max}} \) (KBr): 2920.5 (C-H stretching), 1764.9 and 1720.4 (C=O), 1253.2 (C(O)OR, aromatic), 1075.2 (C-O-C) cm⁻¹. HRMS: Requires: 465.1162 (M⁺+23), Found: 465.1131 (M⁺+23), \(^1\)H NMR δ (CDCl₃): 2.89 (3H, s, OCOCH₃) 3.95 (3H, m, ArOCH₃), 4.06 (1H, m, IS6-Hα), 4.14 (3H, m, IS1-H₂[α+β] and IS6-Hβ), 4.64 (1H, d, J 5.0 Hz, ISH-3), 5.03 (1H, t, J 5.04 and 5.52 Hz, ISH-4), 5.40 (1H, t, J 5.0 and 5.52 Hz, ISH-5), 5.45 (1H, d, J 2.0 Hz, ISH-2), 7.01 (2H, q, J 4.52, 2.52 and 6.0 Hz, Ar₃H-3 and Ar₅H-5), 7.11 (1H, d, J 8.04 Hz, Ar₇H-2), 7.31 (1H, m, Ar₁H-3), 7.50 (1H, m, Ar₁H-4), 7.58 (1H, m, Ar₁H-5), 7.88 (1H, dd, J 2.04 and 1.52 Hz, Ar₂H-4), 8.01 (1H, dd, J 1.52 and 1.48 Hz, Ar₂H-6). \(^{13}\)C NMR ppm (CDCl₃): 20.42 (ArOCOCH₃), 55.52 (ArOCH₃), 70.41 (ISC-1), 72.66 (ISC-6), 73.69 (ISO.COAr), 76.58 (ISC-5), 78.25 (ISC-4), 80.56 (ISC-2), 85.64 (ISC-3), 111.74 (Ar₁C-2), 118.83 (Ar₂C-1), 122.39 (Ar₂C-5), 122.91 (Ar₁C-5), 123.38 (Ar₁C-1), 125.42 (Ar₁C-3), 125.56 (Ar₁C-2), 131.38 (Ar₂C-6), 133.45 (Ar₁C-4), 133.75 (Ar₂C-4), 150.24 (Ar₁C-6), 159.09 (Ar₂C-6), 164.79 (ArCOOR), 169.13 (ArO(OC)(CH₃)).

**Isosorbide-2-aspirinate-5-[3'-methoxy]-benzoate (52)**

Isosorbide-2-aspirinate (0.2 g, 0.65 mmol) was dissolved in toluene (15 ml) at 0°C to which was added DMAP (0.08 g, 0.65 mmol) and DCC (0.13 g, 0.65 mmol). After 10 mins the reaction vessel was returned to room temperature, 3-anisic acid (3-methoxybenzoic acid) (0.10 g, 0.65 mmol) was added and allowed to stir for 12 hours. The reaction mixture was washed with HCl (20 ml, 1M), saturated aqueous NaHCO₃ (20 ml), saturated brine solution (20 ml) and water (3 x 20 ml), dried over anhydrous Na₂SO₄ and solvent removed in vacuo to yield product as a crude oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (3:1) as eluant yielded product as white crystals (0.23 g, 79.8%): m.pt. 132-134°C. IR \( \nu_{\text{max}} \) (KBr): 2920.5 (C-H stretching), 1764.9 and 1720.4 (C=O), 1253.2 (C(O)OR, aromatic), 1075.2 (C-O-C) cm⁻¹. HRMS: Requires: 465.1162 (M⁺+23), Found: 465.1131 (M⁺+23), \(^1\)H NMR δ (CDCl₃): 2.89 (3H, s, OCOCH₃) 3.95 (3H, m, ArOCH₃), 4.06 (1H, m, IS6-Hα), 4.14 (3H, m, IS1-H₂[α+β] and IS6-Hβ), 4.64 (1H, d, J 5.0 Hz, ISH-3), 5.03 (1H, t, J 5.04 and 5.52 Hz, ISH-4), 5.40 (1H, t, J 5.0 and 5.52 Hz, ISH-5), 5.45 (1H, d, J 2.0 Hz, ISH-2), 7.01 (2H, q, J 4.52, 2.52 and 6.0 Hz, Ar₃H-3 and Ar₅H-5), 7.11 (1H, d, J 8.04 Hz, Ar₇H-2), 7.31 (1H, m, Ar₁H-3), 7.50 (1H, m, Ar₁H-4), 7.58 (1H, m, Ar₁H-5), 7.88 (1H, dd, J 2.04 and 1.52 Hz, Ar₂H-4), 8.01 (1H, dd, J 1.52 and 1.48 Hz, Ar₂H-6). \(^{13}\)C NMR ppm (CDCl₃): 20.42 (ArOCOCH₃), 55.52 (ArOCH₃), 70.41 (ISC-1), 72.66 (ISC-6), 73.69 (ISO.COAr), 76.58 (ISC-5), 78.25 (ISC-4), 80.56 (ISC-2), 85.64 (ISC-3), 111.74 (Ar₁C-2), 118.83 (Ar₂C-1), 122.39 (Ar₂C-5), 122.91 (Ar₁C-5), 123.38 (Ar₁C-1), 125.42 (Ar₁C-3), 125.56 (Ar₁C-2), 131.38 (Ar₂C-6), 133.45 (Ar₁C-4), 133.75 (Ar₂C-4), 150.24 (Ar₁C-6), 159.09 (Ar₂C-6), 164.79 (ArCOOR), 169.13 (ArO(OC)(CH₃)).
acetate (3:1) as eluant yielded product as white crystals (0.23 g, 79.8%): m.pt. 125-128°C. IR$_{\text{vmax}}$ (KBr): 2980.9 (C-H stretching), 1768.3 and 1723.8 (C=O), 1298.5 and 1253.5 (C(O)OR, aromatic), 1075.9 (C-O-C) cm$^{-1}$. HRMS: Requires: 465.1162 (M$^+$+23), Found: 465.1168 (M$^+$+23), $^1$H NMR $\delta$ (CDCl$_3$): 2.36 (3H, s, OCOCH$_3$), 3.87 (3H, s, ArOCH$_3$), 4.05 (1H, d, J 5.0 Hz, IS6-Ha), 4.09 (2H, t, J 3.0 and 2.52 Hz, IS1-H$_2$[$\alpha$+\$]), 4.14 (2H, d, J 7.52 Hz, IS6-H$\beta$), 4.64 (1H, d, J 5.04 Hz, ISH-3), 5.03 (1H, t, J 5.04 and 5.52 Hz, ISH-4), 5.43 (1H, q, J 5.0, 5.52 and 5.52 Hz, ISH-5), 5.47 (1H, s, ISH-6), 7.13 (2H, q, J 4.52, 2.52 and 6.0 Hz, Ar$_2$H-3 and Ar$_2$H-5), 7.33 (2H, m, Ar$_1$H-2 and Ar$_1$H-3), 7.58 (2H, m, Ar$_1$H-4 and Ar$_1$H-5), 7.69 (1H, d, J 7.52 Hz, Ar$_2$H-4), 8.01 (1H, dd, J 1.52 and 1.52 Hz, Ar$_2$H-6). $^{13}$C NMR ppm (CDCl$_3$): 20.41 (ArOCOCH$_3$), 54.99 (ArOCH$_3$), 70.43 (ISC-1), 72.74 (ISC-6), 74.05 (ISO$\text{CO}$Ar), 76.58 (ISC-5), 78.17 (ISC-4), 80.49 (ISC-2), 85.67 (ISC-3), 113.96 (Ar$_2$C-2), 119.21 (Ar$_2$C-4), 121.67 (Ar$_2$C-5), 122.34 (Ar$_2$C-6), 123.38 (Ar$_1$C-1), 125.56 (Ar$_1$C-3), 129.03 (Ar$_2$C-5), 130.39 (Ar$_1$C-2), 131.36 (Ar$_2$C-1), 133.77 (Ar$_1$C-4), 150.24 (Ar$_1$C-6), 159.20 (Ar$_2$C-3), 163.14 (Ar$\text{COOR}$), 169.11 (ArOCOCH$_3$).

*Isosorbide-2-aspirinate-5-(4-nitrobenzoate) (56)*

Isosorbide-2-aspirinate (0.2g, 0.65 mmol) was dissolved in DCM (10 mls) at room temperature. To the reaction vessel was added 4-nitrobenzoylchloride (0.15g, 0.78 mmol) and triethylamine (1.12 ml, 0.78 mmol). The reaction was allowed to stir at room temperature for 48 hours before washing with HCl (20 ml, 1M), saturated aqueous NaHCO$_3$ (25 ml), saturated brine solution (20 ml) and water (2 x 20 ml), dried over anhydrous Na$_2$SO$_4$ and solvent removed *in vacuo* to yield product as a crude yellow oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (3:2) as eluant yielded product colourless oil which when recrystallised in ethanol afforded product as white crystals (0.15g, 50.5%). m.pt. 66-68°C. IR$_{\text{vmax}}$ (KBr): 1772.7 and 1726.2 (C=O), 1276.6 (C(O)OR, aromatic), 1078.1 (C-O-C) cm$^{-1}$. HRMS: Requires: 480.0907 (M$^+$+23), Found: 480.0922 (M$^+$+23), $^1$H NMR $\delta$ (CDCl$_3$): 2.34 (3H, s, OCOCH$_3$), 4.07 (4H, m, ISH-3), 4.64 (1H, d, J 4.52 Hz, ISH-1 and ISH-4), 5.04 (1H, t, J 5.04 and 5.0 Hz, ISH-5), 5.45 (2H, m, ISH-2 and ISH-6), 7.10 (1H, dd, J 1.0 and 1.0 Hz, Ar$_1$H-2), 7.31 (1H, m,
Isosorbide-2-aspirinate-5-(2-aminobenzoate) (49)

Isosorbide-2-aspirinate (0.69g, 2.2 mmol) was dissolved in DCM (20 mls) to which was added DCC (0.44g, 2.2 mmol) and DMAP (0.05 g, 0.22 mmol) and the reaction vessel was stirred at 0°C for 10 minutes. After returning to room temperature, anthranilic acid (0.29g, 2.2 mmol) was added allowed to stir for 3 hours. The reaction mixture was washed with HCl (20 ml, 1M), saturated aqueous NaHCO₃ (20 ml), saturated brine solution (20 ml) and water (2 x 20 ml), dried over anhydrous Na₂SO₄ and solvent removed in vacuo to yield product as crude yellow oil. Purification by column chromatography over silica gel using hexane and ethyl acetate (4:1) as eluant yielded product as a yellow solid (0.39g, 41.5%). Product was stored at 0-4°C until required for testing. m.p.t. 150-152°C. IR νmax (KBr): 3434.4 (N-H stretching), 2920.5 (C-H stretching), 1742.7 (C=O), 1548.0 (N-H bending), 1220.9 and 1158.6 (C(O)OR, aromatic), 1047.4 (C-O-C) cm⁻¹. ¹H NMR δ (CDCl₃): 2.07 (3H, s, OCOCH₃), 4.04 (2H, m, NH₂), 3.88 (1H, q, J 5.52, 4.52 and 5.0 Hz, ISC-1), 4.16 (2H, m, ISC-2 and ISC-5), 4.69 (2H, dd, J 4.52 and 4.52, ISH-1 and ISH-3), 4.95 (1H, t, J 5.04 and 5.0 Hz, ISH-4), 6.69 (1H, t, J 57.52 and 7.52 Hz, Ar₂H-5), 6.91 (1H, t, J 8.0 and 7.04, Ar₂H-3), 7.01 (2H, d, J 8.52, Ar₁H-3 and Ar₁H-5), 7.31 (1H, m, Ar₂H-4), 7.51 (1H, m, Ar₁H-4), 7.82 (1H, dd, J 2.0 and 2.0 Hz, Ar₂H-2), 7.92 (1H, d, J 7.04, Ar₁H-2). ¹³C NMR ppm (CDCl₃): 20.13 (ArOCOCH₃), 69.82 (ISC-1), 70.02 (ISC-5), 75.01 (ISC-2), 75.03 (ISC-6), 79.29 (ISC-3), 81.86 (ISC-4), 115.02 (Ar₂C-5), 117.49 (Ar₂C-1), 119.32 (Ar₂C-3), 121.63 (Ar₁C-5), 123.59 (Ar₁C-1), 125.42 (Ar₁C-3), 130.23 (Ar₁C-2), 130.59 (Ar₂C-2), 133.27 (Ar₁C-4), 133.36 (Ar₂C-4), 147.99 (Ar₂C-6), 154.32 (Ar₁C-6), 167.02 (OCOAr), 167.06 (OCOAr), 168.92 (OCOCH₃).
6.3 In vitro studies

6.3.1 General procedures and Equipment

High performance liquid chromatography was performed on a system comprising a Waters 600 pump and controller, Waters 717 Autosampler and a Waters 996 Photodiode Array Detector controlled by Millennium Chromatography Manager. HPLC columns used included Waters Nova-Pak® C8 (4μm) columns (3.9 x 75 mm and 3.9 x 150 mm) and a Waters Spherisorb® column (4.6 x 250 mm). HPLC grade water, used in preparation of all mobile phases, was obtained from a Millipore Millex LCR 13 0.5 sample filter unit and a HVLP 04700 membrane filter unit. All buffers were prepared to the required ionic strength using the calculations of the Phenomenex computer prediction program. Further apparatus required for hydrolysis experiments included the IEC Micromax centrifuge (for centrifugation of Eppendorf 1.5 ml tubes), a Fisons Whirlmixer and a Heto Shaker Bath for incubation of all samples. Plasma samples were centrifuged in a Harrier centrifuge.

6.3.2 Solubility Studies

The aqueous solubility of all compounds was examined by weighing powdered sample (0.01 g) into a screw-capped glass vial to which 10 mls of HPLC grade water was added. The suspension was vortex-mixed and then allowed to shake at 37°C ± 0.5°C for the duration of the experiment. At hourly intervals, samples were withdrawn using a pre-equilibrated syringe and filtered through a Whatman® 0.45μM membrane filter. Samples were analysed by HPLC using the appropriate mobile phase, and concentration of drug was determined with reference to calibration curves run on that day in the same concentration range and under the same experimental conditions.

6.3.3 Aqueous buffer hydrolysis studies

The dependence of rate of hydrolysis on pH was examined for a number of ester prodrugs over a range of pHs. For each study a 2 ml aliquot of a 1 mM solution of
test compound in acetonitrile was diluted to 10 ml in a screw-capped vial using phosphate, acetate, formate buffers or HCl as required. NaCl was added to maintain a constant ionic strength. Each buffer solution was shaken at 37±0.5°C for the required time interval with sampling at appropriate intervals. Samples were analysed by HPLC, using the appropriate mobile phase, and concentration of drug was determined with reference to calibration curves run on that day in the same concentration range and under the same experimental conditions.

6.3.4 Preparation of biological samples

All blood samples were collected by venipuncture into vials containing sodium citrate solution as anticoagulant. Human blood and dog blood were sampled into 10 ml Sarstedt Monovettes containing 1:10 vol of trisodium citrate solution (3.8 % w/v). Rabbit, rat, hamster and guinea pig bloods were sampled into screw-capped glass vials containing 1: 10 vol of sodium citrate (3.8% w/v). Whole blood used in aggregation experiments was stored for no longer than 36 hours under refrigerated conditions (2-4°C). After 36 hours, blood samples were found to have a diminished aggregatory response. Plasma samples were obtained by centrifugation of whole blood at 4000 rpm for 10 minutes. Plasma was aspirated off and stored in siliconized tubes at 2-4°C for no more than three weeks. Serum samples were obtained by allowing uncitrated blood samples to coagulate at room temperature and centrifuging the coagulated blood at 4,000 rpm for 10 minutes. Serum was aspirated off and stored in siliconized tubes at 2-4°C for no longer than three weeks.

6.3.5 Determination of plasma cholinesterase activity

Cholinesterase activity in plasma solution was determined using a modification of the technique described by Ellman. S-butryrylthiocholine iodide solution was prepared (10 mM in deionised water) and 100 μl was added to a cuvette containing DTNB (400 μl, 10 mM in phosphate buffer pH 7.0) and phosphate buffer pH 8.0 (2.626 ml). The reaction was initiated by addition of 4μl of plasma, and the reaction was followed spectrophotometrically at 22°C for 10 mins at 412 nm. The
thiocholine formed during hydrolysis of the BTCI rapidly reacts with the DTNB to release a yellow anion. A blank that includes reagents but no plasma was also tested under the same experimental conditions to control for non-enzymic hydrolysis of BTCI. Enzyme activity was expressed as nmol/ml of plasma/min.

6.3.6 Plasma and serum hydrolysis experiments

Pooled plasma/serum solutions (4 ml) were prepared to the correct strength by dilution of the sample with phosphate buffer pH 7.4 (e.g. for a 10% solution 0.4 ml of plasma/serum was added to 3.6 ml of phosphate buffer pH 7.4). Following equilibration of the plasma/serum sample at 37±0.5°C 100 μl of a stock solution of test compound in acetonitrile (1 x 10⁻⁴ M) was added and 250 μl aliquots were removed at specified time intervals. Samples were transferred to 1.5 ml Eppendorf® tubes containing 500 μl of a 2% w/v solution of ZnSO₄.7H₂O (water: acetonitrile, 1:1). Tubes were vortexed for 2 minutes, then centrifuged at 10,000 rpm for 3 minutes at room temperature. Supernatant was aspirated off and analysed by HPLC. The concentration of test compound and metabolites were determined with reference to calibration curves run on that day in the same concentration range and under the same experimental conditions.

6.3.7 Inhibition studies

In order to confirm the role of esterases in the metabolism of the various prodrugs in plasma/serum it was necessary to perform hydrolysis studies as described in Section 6.3.5 in the presence of inhibitors specific for various plasma enzymes. Inhibitors were used in appropriate concentrations and were incubated with plasma solutions at 37±0.5°C for 20-60 minutes prior to spiking with 100 μl of stock solution of the prodrug in acetonitrile. 250 μl aliquots were removed at specified time intervals. Samples were transferred to 1.5 ml Eppendorf® tubes containing 500 μl of a 2% w/v solution of ZnSO₄.7H₂O (water: acetonitrile, 1:1). Tubes were vortexed for 2 minutes, then centrifuged at 10,000 rpm for 3 minutes at room temperature. Supernatant was aspirated off and analysed by HPLC. The concentration of test compound and metabolites were determined with reference to
calibration curves run on that day in the same concentration range and under the same experimental conditions.

6.4 Platelet and whole blood aggregation experiments

6.4.1 Blood collection

For platelet aggregation studies, blood was sampled from a single male volunteer in the Haematology Department, St. James’ Hospital, Dublin, who had not taken any medication for three weeks prior to blood collection. All samples were collected by venipuncture into 10ml Sarstedt Monovettes® containing 1:10 vol of 3.2% trisodium citrate solution as anticoagulant. Samples were kept at room temperature 20-22°C and were used within 4 hours of sampling.

For whole blood aggregation studies male and female healthy human volunteers were consented as blood donors, none of whom had taken any medication for three weeks prior to blood sampling. All blood samples were taken in the Student Health Centre, Trinity College Dublin. Samples were collected by venipuncture into 10ml Sarstedt Monovettes® containing 1:10 vol of 3.2% trisodium citrate solution. Samples were stored at room temperature and used within two hours of sampling.

6.4.2 Platelet aggregation studies

All platelet aggregation studies were performed in the Haematology Department of St. James’ Hospital, Dublin. Platelet-Rich Plasma (PRP) was prepared by centrifuging anticoagulated blood samples at 800 rpm for 10 minutes at room temperature. The PRP was aspirated off and remaining blood was centrifuged at 3200 rpm for 10 minutes. The supernatant as platelet poor plasma (PPP) was aspirated off and stored separately until required. Platelet counts were obtained for PRP samples on a Sysmex XE-2100 Automated Haematology Analyser, Sysmex Corp, Kobe, Japan. All PRP samples used had a final platelet count of 250-300 x10^9 cells/litre.
250 μl of PRP was dispensed into each of four test tubes [7.25 x 55 mm] and placed in the aggregometer heating block at 37°C for three minutes to allow the sample to equilibrate with stirring at 1,000 rpm. If inhibitors were to be examined samples were pre-incubated with the required molarity of the test compound in dimethyl sulfoxide (DMSO). Each aggregation channel was blanked with 250 μl of PPP before analysis of samples. The PRP sample was transferred to the pre-blanked channel and activated by addition of a suitable quantity of agonist (AA 0.5 mM, ADP 10 μM, collagen 5 μg/ml). Aggregation patterns were monitored for 10 minutes. An IC_{50} value for each COX inhibitor was determined by examining inhibition of aggregation over a range of concentrations.

### 6.4.3 Whole Blood aggregation studies

500 μl of whole blood was mixed with 500 μl of physiological saline (0.9%) in a Chrono-Log® 2 ml disposable sample vial, and allowed to incubate at 37°C for three minutes in the incubation chamber of a Chrono-Log® Whole Blood Aggregometer [model 591/592]. The blood sample was then transferred to the assay well of the aggregometer, the electrode was inserted and once the baseline was established the appropriate volume of reagent was added (see Section 6.4.2). Aggregation was monitored over a period of 6 minutes using a chart recorder. When testing inhibitors of platelet aggregation whole blood was pre-incubated with appropriate concentrations of the inhibitor in DMSO at 37°C for a specified length of time (10 or 20 minutes with stirring) before adding the stimulant.

### 6.4.4 Malondialdehyde assay

Blood was used within 4 hours of sampling. 500 μl of blood was diluted with 500 μl of physiological saline and incubated in the incubation chamber of a Chrono-Log® Whole Blood Aggregometer with 10 μl of drug solution in DMSO or solvent alone for 10 minutes with stirring at 1000 rpm at 37°C. 10 μl of a 0.5 mM solution of arachidonic acid was added to activate platelets and aggregation response was measured over 6 minutes. The sample was then centrifuged at 10,000 rpm for 10
mins and the plasma aspirated off. 400 μl of trichloroacetic acid (5% in distilled water) was mixed with 600 μl of plasma, vortexed and samples re-centrifuged at 10,000 rpm for 10 mins. 750 μl of acidified supernatant was mixed with 750 μl of thiobarbituric acid solution (0.53% in phosphate buffer pH 7.4) and heated for 30 mins at 80°C. Samples were allowed to cool and the pink adduct was analysed by HPLC. HPLC conditions were as follows: mobile phase – 50:50 methanol: phosphate buffer pH 7.4, injection volume 20 μl, flow rate 1 ml/min, detector wavelength 532 nm. The MDA peak had a retention time of 3.5-3.9 mins under these conditions.

MDA was quantified with reference to a calibration curve run on the same day under the same experimental conditions. The calibration curve was performed as follows. Potassium phosphate buffer (pH 7.4, 700 μl) was mixed with 400 μl of trichloroacetic acid (5% in distilled H₂O) and 260 μl of 1,1,3,3-tetramethoxypropane (0.1, 0.2, 0.5 and 1.0μM). 500 μl of each solution was mixed with 500 μl of TBA (0.53% in phosphate buffer pH 7.4) and heated for 30 minutes at 80°C, cooled to room temperature and analysed by HPLC.

6.4.5 ELISA assay for TXB₂

Blood was sampled from a healthy human volunteer into Sarstedt® Monovettes containing no anticoagulant. 500 μl of blood was immediately transferred into siliconized microcentrifuge tubes preloaded with either 2 μl of DMSO or test compound in DMSO. Tubes were vortexed for 30 seconds and then incubated at 37°C for one hour. Serum was obtained by centrifugation of the clotted samples at 12,000 rpm for 10 minutes. The supernatant was aspirated off and stored at −20°C until required for testing. Serum was assayed for TXB₂ levels using an enzyme immunoassay kit (R&D Systems, Oxon, UK). Prior to testing, all serum samples were diluted 1:10 using the assay buffer provided. The ELISA assay was then performed according to the manufacturers instructions and the optical density of each well was measured using a microplate reader set to 405 nm with wavelength correction set between 570 and 590 nm.
6.5 Caco-2 cell studies

6.5.1 Preparation of cell homogenates

Cells were kindly donated by the Department of Pharmaceutics, Trinity College Dublin. Cells were grown in 75cm² culture flasks at 37°C, in an atmosphere of 5% CO₂ and 90% relative humidity. They were maintained in Dulbecco’s minimum essential medium (DMEM) containing 100 U/ml penicillin, 100 mg/ml of streptomycin, 1% non-essential amino acids and 10% fetal bovine serum (FBS). When required, cells were freshly scraped in 5 ml of phosphate buffered saline (PBS) at 0°C and transferred to a non-siliconized test-tube. The cell suspension was homogenized by passage through a 20G needle (repeated 20 times). The homogenate was then centrifuged at 12,000 rpm for 15 mins at 4°C. The supernatant was harvested and stored at –20°C until required.

6.5.2 Protein assay

Prior to use, the protein content of all samples was determined using a BCA Protein Assay Kit (Pierce Diagnostics, Rockford, Ill.). This is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. It is based on the reduction of Cu²⁺ to Cu⁺ by protein in an alkaline medium, which can be detected using the chelating agent BCA. The water-soluble complex formed exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a broad working range of 20 µg/ml to 2,000 µg/ml. Protein concentration in the tested sample was determined by comparison with a calibration curve run of standards in the range 0.025 – 1.0 mg/ml. Each sample was tested in triplicate. The average protein concentration in homogenate samples was 0.33 mg/ml.
6.5.3 Hydrolysis experiments

Caco-2 homogenate samples (stored in 1.5 ml Eppendorfs at -20°C) were brought to room temperature when required for testing. Homogenate (1 ml) was diluted with phosphate buffer (pH 7.4, 0.016M) to give 2 ml of a 50% solution. This was allowed to incubate with shaking in a screw-capped vial for 20 mins at 37°C. When the sample had equilibrated, 50 μl of a stock solution of prodrug (1 x 10^{-4} M in acetonitrile) was added and vortexed. 200 μl aliquots were removed at regular intervals and enzyme activity was quenched by adding the samples to Eppendorf tubes containing 500 μl of zinc sulfate solution (2% w/v in 50:50 water: acetonitrile). These were centrifuged at 10,000 rpm for 3 minutes, supernatant was aspirated off and analysed by HPLC using the appropriate HPLC conditions for each prodrug being analysed.
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aminocarboxyloxymethyl esters as prodrugs of carboxylic acid agents. *Bioorganic and Medicinal Chemistry* **10** 809-816


Appendix 1

Use of factorial design to determine the variables affecting inhibition of whole blood aggregation by isosorbide-diaspirinate *.

* Submitted as part of the Diploma in statistics and Quality Improvement, 2001, in the Department of Statistics, Trinity College Dublin.
A.1 Theory of Experimental Design

The traditional approach to designing experiments is to investigate one parameter at a time. Factorial design experiments are a much more efficient way of identifying factors involved in a process. It is effectively a statistical method for process optimisation \(^1\). A large volume of information can be gained from a relatively small-scale study, which allows one to decide which parameters are significant and which are passive as it allows for the investigation of multiple parameters at various levels. As well as being much more efficient than the traditional approach, factorial experiments offer the added advantage of being able to identify whether factors are interdependent i.e. interactions between factors. An interaction exists if the effect of one factor, Factor A, changes with a change in a second factor, Factor B. In the absence of an interaction factor A will not be affected by any change in factor B. It is of particular benefit where there are multiple factors, which might influence the results of a process, but it is unlikely that all factors have a significant effect.

A.2 Objectives of this study

This study investigated the potential factors affecting whole blood aggregation and its inhibition by the aspirin prodrug isosorbide-diaspirinate (ISDA). Citrated human blood was used according to the experimental protocol described in Section 6.4.3. in a Chrono-Log\textsuperscript{®} Whole Blood Aggregometer. Clotting responses were induced using arachidonic acid and thrombin as initiators of clotting cascade. The design used in this study was a 2\(^3\) factorial and involved eight design points corresponding to each factor at two levels. The factors being investigated are as listed above. The low and high levels used for each of these factors are set out in Table 1.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Low Level (-)</th>
<th>High Level (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Incubation time at 37°C</td>
<td>10 mins</td>
</tr>
<tr>
<td>B</td>
<td>Stirring of incubated sample</td>
<td>No</td>
</tr>
<tr>
<td>C</td>
<td>Aggregating reagent used</td>
<td>Thrombin</td>
</tr>
</tbody>
</table>

Table 1. Definition of high and low levels of each factor.

When there are three factors at each of two levels there are eight combinations and so eight different design points. This can be represented by the corners of a cube. There are three replicates at each design point; the mean is calculated and represents the result as shown in Figure 1.

Fig. 1 Cube diagram representing eight design points of the factorial experiment
This design plan can be summarised in a design matrix as shown in Table 2.

<table>
<thead>
<tr>
<th>Design Points</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
<th>ABC</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>( Y_1 )</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>( Y_2 )</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>( Y_3 )</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>( Y_4 )</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>( Y_5 )</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>( Y_6 )</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>( Y_7 )</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>( Y_8 )</td>
</tr>
</tbody>
</table>

Table 2. The Expanded Design Matrix

Each row in the design matrix describes one experimental run that is to be carried out. Thus row 3 indicates a run with A low, B high and C low. From Table 1 this corresponds to incubation time of 10 mins with stirring and thrombin as aggregating agent.

Using the average \( \bar{Y} \) values as determined from triplicate runs, the effects could be calculated in the following manner.

The main effect of A:

\[
\text{Average release at high A} - \text{Average release at low A} = \frac{\bar{Y}_2 + \bar{Y}_4 + \bar{Y}_6 + \bar{Y}_8 - \bar{Y}_1 + \bar{Y}_3 + \bar{Y}_5 + \bar{Y}_7}{4}
\]

Alternatively the sum of the products of the A column in the design matrix and the results column, divided by half the number of design points (i.e. 4), will produce the same result.

Main effect of A = \( \frac{1}{4}(\bar{Y}_1 + \bar{Y}_2 - \bar{Y}_3 + \bar{Y}_4 - \bar{Y}_5 + \bar{Y}_6 - \bar{Y}_7 + \bar{Y}_8) \)
Similarly this can be done for factors B (Stirring)

Main effect of B = \( \frac{1}{4}(-Y_1 - Y_2 + Y_3 + Y_4 - Y_5 - Y_6 + Y_7 + Y_8) \)

And for C (aggregating agent):

Main effect of C = \( \frac{1}{4}(-Y_1 - Y_2 - Y_3 - Y_4 + Y_5 + Y_6 + Y_7 + Y_8) \)

A and B can be said to interact if the effect of changing A at low B is different from the effect of changing A at the high level of B. The AB interaction can be defined as half this difference:

\[ \frac{1}{2}(\text{effect A at high B} - \text{effect A at low B}) \]

Since C is not involved it is convenient to collapse the cube over C to get the square:

\[
\begin{array}{ccc}
\frac{\bar{Y}_1 + \bar{Y}_5}{2} & & \frac{\bar{Y}_4 + \bar{Y}_8}{2} \\
\frac{\bar{Y}_3 + \bar{Y}_7}{2} & B & \frac{\bar{Y}_2 + \bar{Y}_6}{2}
\end{array}
\]

Effect of A at high B = \( \frac{\bar{Y}_4 + \bar{Y}_8}{2} - \frac{\bar{Y}_1 + \bar{Y}_7}{2} \)

Effect of A at low B = \( \frac{\bar{Y}_2 + \bar{Y}_6}{2} - \frac{\bar{Y}_1 + \bar{Y}_5}{2} \)

This gives the AB interaction:
\[
\begin{align*}
= \frac{1}{2} \left( \frac{1}{2} (\overline{Y_4} + \overline{Y_8} - \overline{Y}_3 - \overline{Y}_7) - \frac{1}{2} (\overline{Y}_2 + \overline{Y}_6 - \overline{Y}_1 - \overline{Y}_5) \right) \\
= \frac{1}{4} \{ \overline{Y}_1 - \overline{Y}_2 - \overline{Y}_3 + \overline{Y}_4 + \overline{Y}_5 - \overline{Y}_6 - \overline{Y}_7 + \overline{Y}_8 \}
\end{align*}
\]

Similarly the BC and AC interactions are obtained in a similar way.

\[
\begin{align*}
\text{AC interaction} &= \frac{1}{4} \{ \overline{Y}_1 - \overline{Y}_2 + \overline{Y}_3 - \overline{Y}_4 - \overline{Y}_5 + \overline{Y}_6 - \overline{Y}_7 + \overline{Y}_8 \}
\end{align*}
\]

\[
\begin{align*}
\text{BC interaction} &= \frac{1}{4} \{ \overline{Y}_1 + \overline{Y}_2 - \overline{Y}_3 - \overline{Y}_4 + \overline{Y}_5 - \overline{Y}_6 - \overline{Y}_7 + \overline{Y}_8 \}
\end{align*}
\]

These calculations are done automatically using Minitab, a statistical package which also tests for statistical significance.

A.3 Materials and Methods

Healthy human volunteers were consented as blood donors. Males and females were used, none of whom had taken any NSAID’s in the previous seven days.

A.3.1 Blood Collection

All blood samples were collected by venipuncture into 10ml Sarstedt monovettes® containing 1:10 vol of 3.2 % trisodium citrate solution. Samples were kept at room temperature 20-22°C until required for testing.

A.3.2 Whole Blood Aggregation using Electrical Impedance method

Whole blood was used within 2 hours of sampling. 500 µl of blood was mixed with 500 µl of physiological saline and allowed to incubate at 37°C for 10 mins in the incubation well of a Chrono-Log® whole blood aggregometer model 591/592. The sample was then transferred to the assay well, baseline was established and appropriate volume of reagent was added. Aggregation was
monitored over 6 mins. When testing inhibitors whole blood was pre-incubated with appropriate concentrations of inhibitor in DMSO at 37°C for a specified length of time before adding the stimulant.

ISDA was the prodrug tested for its inhibitory effect on platelet aggregation at concentrations of 50 μM in DMSO. Aggregation was initiated using 0.5 mM Arachidonic acid (as the sodium salt from porcine liver, obtained from Sigma) or 1 μg/ml of Thrombin (as a lyophilised powder from Bovine plasma, obtained from Sigma).

All replicates were carried out on a single days testing in a randomised fashion as generated by Minitab.
Results on the Whole Blood aggregometer are quantified as a digital reading of the Ohm response. A trace was also generated on a pen chart recorder. Full aggregation is illustrated as a curve while inhibition of the platelet aggregation cascade is illustrated as a straight line, i.e. there is no increase in electrical resistance as there are no platelets aggregating on the electrode. A typical aggregometer trace for uninhibited whole blood is as seen in Fig A.2.

These responses are quantified as percentage inhibition in relation to a control. The significance of effects that may enhance or adversely affect the response is easily visualised but difficult to analyse without a statistical model. Analysing the data using Minitab gives a far more detailed picture of the responses. Printouts in the form of that shown in Appendix 1 give direct information on the factors that are significant in the system including interaction effects and their associated significance. Interaction plots give visual illustrations of the interactions profile.
Fig. A.3 Interaction plot between incubation time and effect of stirring.

A two-way interaction is observed between incubation time and presence or absence of stirring (Fig. A.3). The greatest inhibition effect is seen at long incubation times with stirring of the sample during the incubation period.

Fig. A.4 Interaction plot between incubation time and reagent used.

The arachidonic acid samples are more sensitive to incubation times (Fig. A.4) as a greater inhibitory response is seen with arachidonic acid 0.5 mM when incubated
for 20 mins. Thrombin-challenged samples are less affected by incubation time, showing poor inhibition at both low and high levels. (p=0.105)

Fig. A.5 Interaction plot of reagent used and stirring

The reagent used is affected by the presence of stirring. Arachidonic acid requires stirring to give enhanced inhibition response while thrombin is unaffected by stirring, giving poor inhibition at both low and high levels.

The main effects plot (Fig.A.6) indicates that the most important parameter is the presence or absence of stirring (p=0.000). The reagent used also significantly affects the inhibitory response obtained (p=0.025) but incubation time is not a relevant factor (p=0.497)
Fig. A.6 Main effects plot for three parameters being investigated, incubation time, stirring and reagent used.

There are no significant three-way interactions observed (p=0.273)

A.5 Discussion

From the results obtained, and as illustrated in the main effects plots, it is clear that the best inhibition of platelet aggregation using ISDA 50 μM is obtained using arachidonic acid 0.5 mM and stirring the blood sample during the incubation process. The length of incubation time is not as relevant as the other two factors. The most significant interaction is between stirring and incubation time. This can be explained as the difference between reagents. In the presence of the cyclooxygenase enzyme AA is converted to Thromboxane A\(_2\), which is a potent platelet agonist. This gives the characteristic curve seen in fig. 3. However Thrombin is considerably more potent than AA and has one major complication in whole blood aggregation testing: cleavage of fibrinogen and formation of a clot. It is therefore much more difficult to inhibit the aggregatory response to thrombin, in fact previous work done by this author suggests that it cannot be inhibited with Aspirin 100 μM which can be inhibited by all other platelet agonists, including ADP, AA and ristocetin. For AA incubation time of 20 minutes with stirring is optimum to allow the ISDA to permeate the sample and penetrate platelet membranes.
A.6 Conclusions

A real platelet inhibition effect is seen with ISDA 50 μM when platelets are incubated for 20 mins at 37°C with stirring of the sample. This can be used as the optimum conditions for examining different concentrations of this and other aspirin ester prodrugs, and also to compare their potency to aspirin. Thrombin is not as suitable a reagent as it is too potent an agonist, causing clot formation on the electrode and showing no inhibition of the sample. Incubation times of 10 minutes or not stirring the sample also gives a less effective inhibition as the prodrug is not sufficiently able to permeate cell membranes and achieve its pharmacological effect.
## Appendix 1a.

### Fractional Factorial Fit: Aggregation response versus time, stirring, reagent

#### Estimated Effects and Coefficients for Platelet aggregation (coded units)

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>Coef</th>
<th>SE Coef</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>43.131</td>
<td>3.681</td>
<td>11.72</td>
<td>0</td>
<td>.000</td>
</tr>
<tr>
<td>time</td>
<td>5.111</td>
<td>2.555</td>
<td>3.681</td>
<td>0.69</td>
<td>0.497</td>
</tr>
<tr>
<td>stirring</td>
<td>41.818</td>
<td>20.909</td>
<td>3.681</td>
<td>5.68</td>
<td>0.000</td>
</tr>
<tr>
<td>reagent</td>
<td>18.208</td>
<td>9.104</td>
<td>3.681</td>
<td>2.47</td>
<td>0.025</td>
</tr>
<tr>
<td>time*stirring</td>
<td>18.999</td>
<td>9.500</td>
<td>3.681</td>
<td>2.58</td>
<td>0.020</td>
</tr>
<tr>
<td>time*reagent</td>
<td>5.569</td>
<td>2.785</td>
<td>3.681</td>
<td>0.76</td>
<td>0.460</td>
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<tr>
<td>stirring*reagent</td>
<td>12.649</td>
<td>6.325</td>
<td>3.681</td>
<td>1.72</td>
<td>0.105</td>
</tr>
<tr>
<td>time<em>stirring</em>reagent</td>
<td>8.351</td>
<td>4.175</td>
<td>3.681</td>
<td>1.13</td>
<td>0.273</td>
</tr>
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</table>

#### Analysis of Variance for Aggregation response (coded units)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects</td>
<td>3</td>
<td>12638.0</td>
<td>12638.0</td>
<td>4212.7</td>
<td>12.96</td>
<td>0.000</td>
</tr>
<tr>
<td>2-Way Interactions</td>
<td>3</td>
<td>3311.9</td>
<td>3311.9</td>
<td>1104.0</td>
<td>3.40</td>
<td>0.044</td>
</tr>
<tr>
<td>3-Way Interactions</td>
<td>1</td>
<td>418.4</td>
<td>418.4</td>
<td>418.4</td>
<td>1.29</td>
<td>0.273</td>
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<tr>
<td>Residual Error</td>
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<td>5202.8</td>
<td>5202.8</td>
<td>325.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure Error</td>
<td>16</td>
<td>5202.8</td>
<td>5202.8</td>
<td>325.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>21571.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Unusual Observations for Aggregation response

<table>
<thead>
<tr>
<th>Obs</th>
<th>C8</th>
<th>Fit</th>
<th>SE Fit</th>
<th>Residual</th>
<th>St Resid</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>63.640</td>
<td>33.337</td>
<td>10.411</td>
<td>30.303</td>
<td>2.06R</td>
</tr>
<tr>
<td>16</td>
<td>90.450</td>
<td>60.453</td>
<td>10.411</td>
<td>29.997</td>
<td>2.04R</td>
</tr>
<tr>
<td>23</td>
<td>27.270</td>
<td>60.453</td>
<td>10.411</td>
<td>-33.183</td>
<td>-2.25R</td>
</tr>
</tbody>
</table>

R denotes an observation with a large standardized residual

#### Least Squares Means for Aggregation response

<table>
<thead>
<tr>
<th>Term</th>
<th>Mean</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>time -1</td>
<td>40.58</td>
<td>5.206</td>
</tr>
<tr>
<td>time 1</td>
<td>45.69</td>
<td>5.206</td>
</tr>
<tr>
<td>stirring</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
-1 22.22 5.206
1 64.04 5.206
**reagent**
-1 34.03 5.206
1 52.24 5.206
**time**\***stirring**
-1 -1 29.17 7.362
1 -1 15.28 7.362
-1 1 51.99 7.362
1 1 76.10 7.362
**time**\***reagent**
-1 -1 34.26 7.362
1 -1 33.80 7.362
-1 1 46.90 7.362
1 1 57.58 7.362
**stirring**\***reagent**
-1 -1 19.44 7.362
1 -1 48.61 7.362
-1 1 25.00 7.362
1 1 79.47 7.362
**time**\***stirring**\***reagent**
-1 -1 -1 25.00 10.411
1 -1 -1 13.89 10.411
-1 1 -1 43.52 10.411
1 1 -1 53.71 10.411
-1 -1 1 33.34 10.411
1 -1 1 16.67 10.411
-1 1 1 60.45 10.411
1 1 1 98.48 10.411

**Alias Structure**

Isosorbide-based aspirin prodrugs
II. Hydrolysis kinetics of isosorbide diaspirinate
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Abstract
Aspirin prodrugs have been intensively investigated in an effort to produce compounds with lower gastric toxicity, greater stability or enhanced percutaneous absorption, relative to aspirin. This report describes the hydrolysis kinetics and aspirin release characteristics of isosorbide diaspirinate (ISDA), the aspirin diester of isosorbide. ISDA underwent rapid hydrolysis when incubated in phosphate buffered human plasma solutions (pH 7.4) at 37 °C, producing appreciable quantities of aspirin. In 30% human plasma solution the half-life was 1.1 min and 61% aspirin was liberated relative to the initial ester concentration. The hydrolysis kinetics of ISDA were monitored in aqueous solution at 37 °C over the pH range 1.0-3.9. 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. Loftsson 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 Banggaard, 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 Galigiardi, 1963), benzodioxinone derivatives (Ankersen et al., 1989; Nielsen and Senning, 1990), acyl 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 allyl and aryl esters (Rainsford and Whitehouse, 1976, 1980), triglycerides (Kumar and Billimoria, 1978; Paris et al., 1979, 1980), acyloxyalkyl esters (Los et al., 1982), sulfanyl or sulfonyl esters (Loftsson and Bodor, 1981a,b; Loftsson et al., 1981), phenylalanine derivatives (Banerjee and Amidon, 1981a,b,c; Muhi-Eideen et al., 1985), amino acid derivatives (Tsunematsu et al., 1991), glycolamide esters (Nielsen and Banggaard, 1989), and...
indoleiones 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 (Gilmor 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 (ISDIA), 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, Benorilate (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 ISDIA.

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 butyrycholinesterase [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. 1H and 13C 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 1H and 13C NMR.

2.3.2. Synthesis of 2,5-diacetyloxysalicyloxy-1,4:3,6-dianhydro-o-glucitol (isosorbide-2,5-diaspirinate) or ISDIA (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 acetylsalicyloyl 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: δH (300 MHz, CDCl3) 2.34 (3H, s, OCOMe), 2.36 (3H, s, OCOMe), 3.94–4.13 (4H, m, ArH-5), 7.07–7.14 (2H, m, ArH-3), 7.27–7.37 (2H, m, ArH-4), 4.98 (1H, m, IS-4-H), 5.35–5.46 (2H, m, IS-2-H, IS-3-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-5), 131.77, 131.96 (ArC-4), 134.18, 134.26 (ArC-3), 122.53, 122.65 (ArC(O)OR). C, H requires 61.28%, 4.71%; found 61.46%, 4.72%. FAB^+ (m/z) 471.12 (MH^+).
2.4. Solubility

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

2.5. Hydrolysis experiments

2.5.1. Aqueous buffer kinetics

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

2.5.2. Plasma hydrolysis studies

Pooled plasma solutions (4 ml) were prepared by centrifugation of citrated human venous blood and dilution of the resultant plasma supernatant with pH 7.4 phosphate buffer as appropriate. A 100 μl aliquot of the test compound (1×10⁻⁴ M) in acetonitrile was incubated in the preheated solution (37±0.5 °C) and 250 μl aliquots were withdrawn at appropriate intervals. Samples were transferred to 1.5 ml Eppendorf tubes containing 500 μl of 2% ZnSO₄·7H₂O in MeCN–H₂O (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 butyrylthiocholine as substrate (Chatonnet and Lockridge, 1989). Values were typically between 2200 and 4000 nmol/ml plasma/min.

2.5.3. Enzyme study

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

2.6. Chromatography

High-performance liquid chromatography was performed using a system consisting of a Waters 600 pump and controller, Waters 717 autosampler and a Waters 996 photodiode-array (PDA) detector controlled by Millennium Chromatography Manager. A Waters Nova-Pak® C₈ (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 (pH 3.2)–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 acetylsalicyloyl 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 acetylsalicyloyl chloride and triethylamine in toluene. The identity of these compounds was confirmed by 1H and 13C NMR spectroscopy.

3.2. Hydrolysis kinetics

The hydrolysis of ISDA (2) was monitored in aqueous solution at 37 °C over the pH range 1.03–9.4. The hydrolysis was observed to follow pseudo-first-order kinetics over several half-lives. The rate of hydrolysis was found to be independent of ionic strength or buffer concentration at pH 2.8, nevertheless a constant ionic strength (μ) of 0.1 was maintained in all solutions. Pseudo-first-order plots for the decomposition of 2 were constructed from the logarithm of remaining ester versus time. The pseudo-first-order rate constants (kobs) 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 (kΗ) and water catalysed (k0) or specific base-catalysed (kOH) processes and the overall profile can consequently be accounted for by the expression

\[ k_{obs} = k_0 + k_\text{H}_2\text{O} + k_{\text{OH}}a_{\text{OH}} \]  

(1)

where \( a_\text{H} \) and \( a_{\text{OH}} \) represent the hydrogen ion and hydroxide ion activity. The hydroxide ion activity at 37 °C was calculated from the expression (Harned and Hamer, 1933)

\[ \log a_{\text{OH}} = \text{pH} - 13.6 \]  

(2)

Second-order rate constants for the specific base (kOH) and specific acid (kΗ) catalysed reactions were determined from data at high and low pH, respectively. The apparent first-order rate constant for spontaneous decomposition (k0) 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

\[ k_0 = 0.452 \text{ M}^{-1} \text{ h}^{-1} \]

![Fig. 1. Chemical structures of aspirin (1) and isosorbide disaspirinate (ISDA) (2).](image)

![Table 1](image)

<table>
<thead>
<tr>
<th>pH</th>
<th>kobs (h⁻¹)</th>
<th>t1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.03</td>
<td>4.85×10⁻³</td>
<td>14.29</td>
</tr>
<tr>
<td>1.4</td>
<td>2×10⁻³</td>
<td>34.65</td>
</tr>
<tr>
<td>1.62</td>
<td>1.1×10⁻³</td>
<td>62.72</td>
</tr>
<tr>
<td>1.88</td>
<td>4.8×10⁻³</td>
<td>144.36</td>
</tr>
<tr>
<td>2.24</td>
<td>1.6×10⁻³</td>
<td>433.13</td>
</tr>
<tr>
<td>2.5</td>
<td>2.3×10⁻³</td>
<td>300.42</td>
</tr>
<tr>
<td>4.13</td>
<td>2.3×10⁻⁴</td>
<td>3013.64</td>
</tr>
<tr>
<td>6.45</td>
<td>6.9×10⁻⁴</td>
<td>1004.35</td>
</tr>
<tr>
<td>7.29</td>
<td>2.99×10⁻³</td>
<td>231.77</td>
</tr>
<tr>
<td>8.1</td>
<td>15.9×10⁻⁴</td>
<td>43.33</td>
</tr>
<tr>
<td>9.4</td>
<td>0.29</td>
<td>2.35</td>
</tr>
</tbody>
</table>

![Fig. 2. The pH rate profile for the hydrolysis of 2 in aqueous solution (μ = 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.](image)
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

\[ t_{1/2} = \frac{0.693}{k_{\text{obs}}} \]  

(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 \( k_1 \) 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 (\( \lambda_{\text{max}} = 295-306 \text{ nm} \)).

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 \( K_{m,\text{app}} \) (Michaelis constant) and \( V_{\text{max,app}} \) (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):

\[ V_{\text{max}}t = S_0 - S + K_m \ln(S_0/S) \]  

(4)

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

\[ t_{1/2} = \frac{0.693}{V_{\text{max}}/K_m} \]  

(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 M 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 \( 6 \times 10^{-4} \text{ M} \).
Table 2
Kinetic data for 2, benorylate, guaiacol aspirinate in the presence of various enzyme preparations at pH 7.4 and 37 °C

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

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: $k_3 \gg k_1$). Aspirin esters do not in general undergo hydrolysis in aspirin in human plasma and most if not all aspirin esters reported thus far should therefore be more appropriately termed salicylic acid prodrugs (Nielsen and Bungaard, 1989). Aspirin is a poor substrate for human plasma butyrylcholinesterase and its half-life in human plasma is about 120 min (Cham et al., 1979), considerably longer than the half-life of related aspirin esters (Nielsen and Bungaard, 1989). The high stability of aspirin towards human plasma catalysed hydrolysis relative to neutral substrates (such as aspirin esters) appears to be due to electrostatic inhibition of Michaelis complex formation rather than substrate repulsion (Masson et al., 1998).

ISDA (2) underwent rapid hydrolysis in human plasma solution generating appreciable quantities of aspirin and a complex mixture of aspirinate and salicylate esters which were identified by their characteristic PDA-UV λ$_{max}$ values of 274–278 and 295–305 nm, respectively (Fig. 5). Aspirin and salicylic acid in vitro metabolites were quantitated by external standard and peak homogeneity for all chromatographic components confirmed by PDA analysis. In order to minimize the possibility of coincidental overlap of PDA indistinguishable components, the 10% plasma study samples were analysed using both the gradient and the isocratic HPLC methods. The amounts of aspirin present in the plasma samples were similar using both methods. Hydrolysis in pH 7.4 phosphate-buffered human plasma solutions (10–50%) was associated with the generation of 40–60% aspirin based on the initial molar quantity of 2, with a mean value of 51% in 10% human plasma (n=6). This places the diester 2 among the most successful aspirin prodrugs reported to date and confirms that a feature of the isosorbide group confers extremely rapid hydrolysis on its 2-esters in mammalian plasma. In order to validate this experiment, two putative aspirin prodrugs, Benorylate (paracetamol aspirinate), and Guaiacol aspirinate, were incubated in pH 7.4 buffered human plasma (10%) at 37 °C. Both esters were rapidly hydrolysed with half-lives of 40 and 54 s, respectively. Hydrolysis of these two esters occurred almost exclusively along the $k_3$ pathway, as depicted in Fig. 3, with the
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$_2$O, 10 µg/ml; pH 6.8, 10.9 µg/ml), however its stability towards hydrolysis by α-chymotrypsin was high ($t_{1/2} \approx 203$ 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.

**4. Conclusions**

Isosorbide diastipininate, 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 mononitrile ester of aspirin which we recently reported (Gilmer et al., 2001) and the diastipinate 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.
References


