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MEASUREMENT OF SALICYLIC ACID, 2,3-DIHYDROXYBENZOIC ACID, 2,5-DIHYDROXYBENZOIC ACID IN HUMAN SERUM BY HPLC WITH ELECTROCHEMICAL DETECTION : METHOD DEVELOPMENT AND VALIDATION

by

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A thesis submitted to the Department of Pathological Biochemistry (research carried out at Crichton Royal Laboratory, Dumfries), University of Glasgow, in partial fulfilment of the requirements for the degree of Medical Science.

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ABSTRACT

Free radicals, the highly reactive, unstable molecular species implicated in the development of many diseases, are introduced and the analytical methods available for their estimation reviewed. One approach, the measurement of the aspirin metabolites salicylic acid (SA) and 2,3- and 2,5-dihydroxybenzoic acid (DHBA) is fully examined.

The improvement and validation of a method for the routine measurement of SA in-patients receiving prophylactic doses of aspirin resulted in an assay with the following characteristics:-

0.5ml serum, acidified to pH 2.5, and applied to a HAX™ (Jones Chromatography) mixed phase SPE column preparation in succession with methanol and pH 2.5 acetate buffer. After further buffer washes, the SA was eluted with methanol containing 3%NH_3 (v/v). The dried extract was reconstituted in 500µl HCl (0.05M).

HPLC separation, free from interference, was achieved on a 250 x 4.6 mm C_{18} column of 5µ particle size, at a temperature of 30°C, using a mobile phase of 30mM citrate buffer pH 4.75 containing 28.6% (v/v) methanol flowing at 1.0ml/min. The electrochemical detector (Antec Ltd) oxidising potential was set at 1.10V.

Mean SA extraction efficiencies were 85% over a 0.1 - 50µM linear range. The internal standard, 4- methylsalicylic acid (4-MeSA), was selected from a number of candidates, based on chromatographic, voltammetric and extraction characteristics. Intra-assay precision was 7.1, 6.2 and 4.6% (CV, n = 9), at 1.5, 15 and 35µM respectively; inter-assay precision was 16.9, 8.5 and 6.6% (CV, n = 9) at 1.5, 15 and 35µM respectively. SA was stable for 13 days in water, hydrochloric acid and serum at pH 7.3 - 7.5 and pH 2.5 at -30°C, 4°C and room temperature.

Development of an assay for 2,3-DHBA and 2,5-DHBA was only partially completed as described below:-

2,3-DHBA and 2,5-DHBA were also extracted by SPE as described above. Chromatography took place on a similar C_{18} column at 30°C at a flow rate of 1.0ml/min. Successful resolution of analytes, IS and unknown extracted peaks was obtained using 30mM citrate buffer containing no methanol, by modification of the pH to 5.3. However, a gradient of increasing methanol concentration was added to elute the polar compounds and shorten run times. An optimum oxidation potential of 0.75V was required for the detection of 2,3- and 2,5-DHBA making the selection of a new IS, 3,4-DHBA, with a similar voltammogram, necessary.

Preliminary extraction efficiencies for 2,3- and 2,5-DHBA at a concentration of 100nM were found to be 79% and 73% respectively. 2,3- and 2,5-DHBA were found to be stable for up to 2.5 hours in aqueous solution at room temperature. Both analytes are therefore less stable than SA. Precision data are required to complete the validation of this assay.

Clinical application of the SA method was carried out using blood from 8 diabetic patients known to be taking low doses of aspirin. SA was detected in all specimens, with levels of SA ranging from 0.23 - 10.51μM after administration of low-doses of aspirin from 75 - 300mg/day.

Blank serum samples from subjects known not to have taken aspirin were analysed for SA (n = 6) and 2,3 and 2,5-DHBA (n = 11) by the methods described above. Unexpectedly, SA was detected in all samples in the range 15 - 75pmol/ml. Similarly, peaks with the same retention time as 2,3- and 2,5-DHBA were found in all samples. The presence of all three analytes was confirmed by voltammetric comparison with standard solutions. The origin of these analytes in samples from

control subjects is known, but since SA, 2,3- and 2,5-DHBA have been detected in a number of different foodstuffs, [62, 65] a dietary source should be considered.

With little further development, the methods described above are sufficiently sensitive, robust and reliable for use in measuring the extent of free radical involvement in various disease states by assaying relative levels of these analytes following low-dose aspirin administration.

CONTENTS

EXPERIMENTAL

RESULTS

DISCUSSION

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADP - adenosine diphosphate

ASA - acetylsalicylic acid

AUX - auxiliary electrode

BHA - butylated hydroxyanisole

BHT - butylated hydroxytoluene

CV - coefficient of variation

C_{18} - octadecylsilane

2,3-DHBA - 2,3-dihydroxybenzoic acid

2,5-DHBA - 2,5-dihydroxybenzoic acid

3,4-DHBA - 3,4-dihydroxybenzoic acid

3,4-DHPAA - 3,4-dihydroxyphenylacetic acid

2,5-DHPAAGL - 2,5-dihydroxyphenylacetic acid gamma lactone

4-MeSA - 4-methylsalicylic acid

DNA - deoxyribonucleic acid

EC - electrochemical

ECD - electrochemical detection

E_{ox} - oxidation potential

ESR - electron spin resonance

Exp - experiment

GC - gas chromatography

GC/MS - gas chromatography mass spectrometry

GSH - glutathione

HCl - hydrochloric acid

HPLC - high performance liquid chromatography

IS - internal standard

LOD - limit of detection

MDA - malondialdehyde

MI - myocardial infarction

MP - mobile phase

ND - none detected

NSAID - non-steroidal anti-inflammatory drug

REF - reference electrode

ROS - reactive oxygen species

SA - salicylic acid

SOD - superoxide dismutase

SPE - solid phase extraction

TBA - thiobarbituric acid

t_r - retention time

UV - ultraviolet

V - volt

WE - working electrode

INTRODUCTION

1. ACETYLSALICYLIC ACID (ASA)

1.1 Background

In a letter to the Right Honourable George, Earl of Macclesfield, president of the Royal Society, April 25th, 1763 the Rev Mr. Edmund Stone wrote,

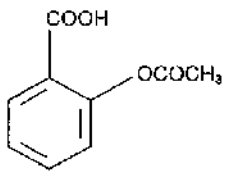
"There is a bark of an English tree, which I have found by experience to be a powerful astringent, and very efficacious in curing anguish and intermitting disorders."

What Stone had actually discovered was a group of compounds known as the salicylates. The bark of the willow tree (*Salix alba*) is astringent because it contains high levels of salicin, the glycoside of salicylic acid. Half a century later French and German chemists competed to find the active ingredient of the willow bark. Not until 1838 did Raffaele Piria of Pisa, working in Paris, give the compound the name by which it is known today: salicylic acid (SA).

Acetylsalicylic acid commonly known as 'Aspirin' (Fig 1a, p.1) was not discovered until 1898 at the Bayer division of I.G. Farben. The new drug substance was named aspirin, the "a" from acetyl and the "spirin" from the German Spirsaure (the French root would have yielded asalicylin).[1]

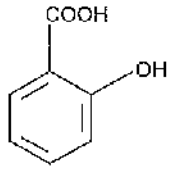
Fig. 1

(a)



Acetylsalicylic acid
(ASA)

(b)



Salicylic acid
(SA)

SA (Fig 1b, p1) is the primary metabolite of aspirin and great interest has been shown in this drug and its metabolite for almost 100 years. Aspirin is widely used as an over the counter and prescription drug for treating many conditions. Its mechanism of action, to some extent, still remains a subject of dispute, particularly because it has a multiplicity of actions both central and peripheral. Its convenience as a research tool is unquestioned in that it is a representative organic acid that can be isolated easily and detected with great sensitivity by chromatographic means, is of low toxicity suitable for human consumption and exhibits a variety of different metabolic pathways.

1.2 Chemistry and stability

Aspirin is a non-steroidal anti-inflammatory drug (NSAID). It is the salicylate ester of acetic acid and has a chemical formula $C_9H_8O_4$ with a molecular weight of 180.2. *In vivo*, the drug rapidly hydrolyses to salicylate and acetate. Aspirin occurs as colourless or white crystals that are usually tubular or needle-like, or as a white crystalline powder and may have a faint odour. Its solubility in different solutions is: 1 in 300 of water; 1 in 6 of alcohol; 1 in 17 of chloroform and 1 in 15 of ether. It is soluble in solutions of acetate and citrates and decomposes in solutions of alkali hydroxides and carbonates.

Aspirin is stable in dry air, but in moist air or in aqueous or alcoholic solutions the drug gradually hydrolyses to salicylate and acetate and emits a strong vinegar like odour. The rate of hydrolysis is increased by heat and is pH dependent.

In aqueous solutions aspirin is most stable at pH of 2-3, less stable at a pH of 4-8 and least stable at a pH less than 2 or greater than 8. In a saturated aqueous solution at a pH of 5-7, aspirin is almost completely hydrolysed within one week at 25°C. [2]

1.3 Pharmacology

Aspirin is used for its antithrombotic, antipyretic and antirheumatic effects. Although aspirin hydrolyses to salicylate and acetate, the drug does not require hydrolysis to produce its effects and appears to have some pharmacological effects that are distinct from those of the salicylates. The ability of aspirin to acetylate proteins *e.g.* platelet proteins, results in some inhibition of platelet aggregation, which other currently available salicylates do not exhibit.

In terms of haematological effects, aspirin inhibits platelet aggregation induced by epinephrine or low concentrations of collagen but not that induced by thrombin or high concentrations of collagen.[3] Aspirin inhibits the second phase of platelet aggregation by preventing release of adenosine diphosphate (ADP).

Like the analgesic and anti-inflammatory effects, the effects of aspirin on platelets appear to be mainly associated with inhibition of prostaglandin synthesis.[3] Aspirin irreversibly acetylates and inactivates cyclo-oxygenase in circulating platelets and has been extensively investigated for potential therapeutic effects in the prevention of thrombosis, particularly arterial thrombosis.[4] This inactivation prevents platelet synthesis of prostaglandin endoperoxides and thromboxane A_2, compounds which induce platelet aggregation and constrict arterial smooth muscle. Since cyclo-oxygenase in platelets is not resynthesized, this effect of aspirin on platelet function persists for the lifespan of platelets (4-7 days).

1.4 Pharmacokinetics

Approximately 80-100% of an oral dose of aspirin is absorbed from the gastro-intestinal tract. However, the actual bioavailability as unhydrolysed aspirin is lower since aspirin is partially hydrolysed in the gastro-intestinal mucosa to SA during absorption and on first passing through the liver.[5]

The rate of absorption of aspirin depends on various factors, including the dosage form, with aqueous solutions being most rapidly absorbed. Following oral administration of single doses of rapidly absorbed aspirin dosage forms, salicylate is detected in serum within 5-30 minutes and peak serum salicylate concentrations are attained within 0.25-2 hours. *In vitro* dissolution of film coated tablets does not differ from that of uncoated plain tablets, but the film coated tablets do not undergo dissolution in the mouth during administration.[4,5]

1.5 Disposition in the body

Aspirin is rapidly and widely distributed into most body tissues and fluids.[6] The volume of distribution of aspirin and SA is about 0.15litres/kg of body weight. Aspirin is poorly bound to plasma proteins with only one third of the unhydrolysed form of the drug bound in serum at a salicylate concentration of 120ug/ml.

1.6 Uses of ASA

Aspirin is used acutely for its analgesic, antipyretic and anti-inflammatory effects as well as in prevention of myocardial infarction (MI) and stroke. The principal adverse effects associated with aspirin are gastro-intestinal, but other side effects do exist. Aspirin is used to a very limited extent in children because of the risk of Reye's syndrome.

Aspirin has been used extensively in the treatment of coronary artery disease where it has been found to reduce the risk of death or non-fatal recurrent MI in patients surviving an MI, although the precise value and optimal dosage and timing of prophylactic aspirin therapy in these patients remains to be fully elucidated.[7-10]

The report of the 'Physicians Health Study'[11] suggests that aspirin therapy of 325mg once every other day can reduce the overall risk of MI, both non-fatal and fatal, by 50% in men.

2. FREE RADICAL THEORY

2.1 Free radicals. What are they?

A free radical is any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital.[12] Free radicals are extremely reactive species with rate constants of the order of 10^5-10^{10} $M^{-1}s^{-1}$, their reactivity due to the unpaired electron in an outer orbital. Since these radical reactions proceed so rapidly, special techniques are required to measure their rates.

Pulse radiolysis is one of these techniques and involves placing a solution of the compound in a reaction cell and pulsing the solution with ionising radiation from a linear accelerator. Specific radicals can be generated and their reactions followed by change in light absorption of the cell components on an oscilloscope.

The most important free radical reactions take place *in vivo* in the form of univalent biochemical redox reactions with oxygen being the main component of most free radical species.

The term "reactive oxygen species" (ROS) is a collective one that not only includes species such as hydroxyl and superoxide radicals, but also some non-radical derivatives which are nonetheless very important in free radical activity such as hydrogen peroxide and hypochlorous acid.

2.2 Hydroxyl free radical production

Hydroxyl free radicals are produced in two main ways: reaction of transition metal ions with hydrogen peroxide; and homolytic fission of water caused by background exposure to ionising radiation.

2.2.1 Fenton chemistry

Transition metal ions in the first row of the d-block in the periodic table have unpaired electrons with the exception of zinc. However, copper does not readily fit the definition of a transition metal since the d orbital is full but it can readily lose two electrons to form Cu^{2+}, one from the 4s and one from the 3d orbital. An important feature of transition metals is their variable valence states which allows changes in oxidation state involving one electron. This is crucial in their role in free radical production.

In 1894 Fenton observed that a mixture of hydrogen peroxide and Fe(II) salts reacted with many biological molecules and that the reactivity was probably due to the reactivity of the hydroxyl radical. This reaction is known as the Fenton reaction (See eq^{n} 1)

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + \underline{HO^{\cdot}} \quad (eq^{n}\ 1)$$

Oxygen itself is a diradical (2 unpaired electrons in π^{*} 2p antibonding molecular orbital) and univalent reduction of this molecule produces the superoxide radical (1 paired and 1 unpaired electron in π^{*} 2p antibonding molecular orbital). In an aqueous environment this superoxide radical can react to form hydrogen peroxide (both π^{*} 2p antibonding molecular orbitals are filled). Hydrogen peroxide itself is not a free radical but in the presence of Fe(II), Fenton reactions occur. A Fenton reaction occurs when hydrogen peroxide reacts not only with ferrous iron [Fe(II)] but also with other transition metals such as copper. The resulting ferric iron (Fe^{3+}) can then be reduced, by reducing agents such as ascorbate, in the presence of superoxide to recycle ferrous iron (Fe^{2+}) and a chain reaction is set up. (See eq^{n} 2)

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2 \qquad (eq^{n}\ 2)$$

Iron and copper are available *in vivo* and are capable of catalysing radical reactions but organisms take great care in the handling of iron using both transport (transferrin) and storage (ferritin and haemosiderin) proteins therefore minimising the amount of free iron within cells and extracellular fluids. Iron is released from proteins (haemoglobin, myoglobin) at low pH after damage by peroxides by reductive mobilisation involving a superoxide radical.[13]

2.2.2 Ionising radiation

The other main way in which hydroxyl radicals are produced is by ionising radiation. The main component of all living cells is water and exposure of cells to ionising radiation such as X-rays or γ-rays ultimately leads to the production of hydroxyl free radicals. In contrast, ultraviolet (UV) radiation does not have sufficient energy to ionise water molecules but has sufficient energy to generate hydroxyl radicals by the homolytic fission of hydrogen peroxide.

One of the most susceptible targets for hydroxyl free radical attack is cellular deoxyribonucleic acid (DNA). This causes the formation of double strand breaks which cannot be repaired by the cell. Oxygen enhances the damage caused by ionising radiation through the generation of superoxide radicals thus leading to the production of more hydroxyl radicals.[14] However, the knowledge of the mechanisms of damage to DNA by free radicals may be of therapeutic benefit in the killing of tumour cells.

2.3 Free radical measurement

The very high reactivity of radical species makes direct measurement of free radicals *in vivo* extremely difficult. Many of the methods that have been developed are based on measuring the extent of damage a free radical causes to endogenous macromolecules such as lipids, proteins, carbohydrates and DNA.

2.3.1 Electron spin resonance (ESR)

ESR is considered to be the 'gold standard' technique in the identification of free radicals but unfortunately this technique has a very limited place in the analysis of samples from patients. The toxic nature of the spin traps does not permit administration to humans for studies *in vivo*.[15]

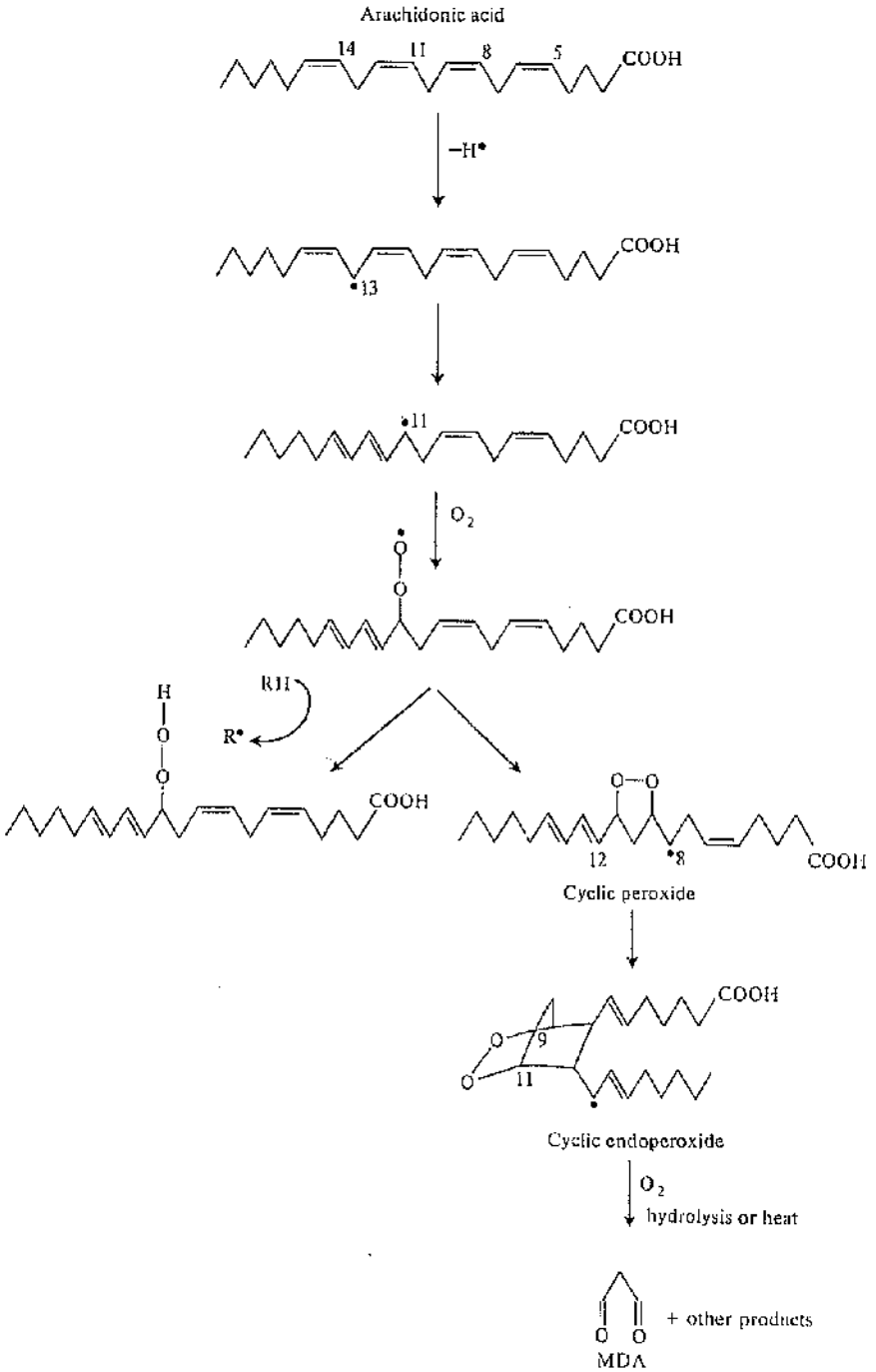
2.3.2 Lipid peroxidation

Polyunsaturated fatty acids are especially susceptible to attack by free radicals. Those incorporated into lipids are readily attacked by free radicals becoming oxidised into lipid peroxides.[16]

Lipid peroxidation is initiated by attack on a fatty acid or a fatty acyl side chain by any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. The greater the number of double bonds present the more susceptible and therefore the easier it is to abstract the hydrogen from the methylene carbon. Polyunsaturated fats are particularly susceptible to attack due to the high number of double bonds present. The resulting carbon centred radical can have several fates, but in the presence of oxygen it forms a peroxyl radical. Peroxyl radicals can attack fatty acid side chains, removing hydrogen and so propagating the chain reaction known as lipid peroxidation (Fig 2, p.9).

Lipid peroxide measurement is the most widely used measurement of the rate of peroxidation of membrane lipids or fatty acids. At each intermediate stage in the lipid peroxidation process different compounds are formed. Individual techniques have been used to detect these compounds but no one method by itself can be said to be an accurate measure of lipid peroxidation.

Fig. 2 Proposed mechanism for the formation of lipid hydroperoxides and cyclic peroxides from arachidonic acid. Initial abstraction of H at C-13 is shown. H can also be abstracted at C-10 or C-7, giving several other peroxide end products.



2.3.3 Thiobarbituric acid (TBA) test

The TBA test is by far the most commonly used assay because of its simplicity and cheapness. The sample under investigation is heated at acid pH with TBA to produce a pink chromagen ($[TBA]_2$-malondialdehyde). This absorbs at a wavelength of about 532nm and fluoresces at 553nm. This test measures the amount of malondialdehyde (MDA) present at the end of the peroxidation process.

The first biological use of the TBA reaction was in 1944 where it was observed that brain tissue incubated in the presence of oxygen gave a colour with 2-thiobarbituric acid.[17]

Colorimetric assays have been shown to have low specificity and more recently fluorimetric assays have become more popular due to better specificity and sensitivity obtained. Extraction of the coloured product into n-butanol using spectrofluorimetric detection (Excitation wavelength 532nm, emission wavelength 553nm) has provided better results.[18]

Various high performance liquid chromatography (HPLC) methods have been introduced to improve specificity, but the problem with HPLC methods still lies in the variation in reference ranges from laboratory to laboratory depending on which TBA test is employed.

The TBA test works well when applied to specific biological matrices but its application to body fluids and tissue extracts has produced a number of problems.[19-21]

2.3.4 Peroxide measurement

Measurement of peroxides can be demonstrated using a variety of different techniques.

Iodometric measurement can be used for measuring lipid peroxides. The lipid peroxides produced are capable of oxidising iodide (I^-) ions to iodine (I_2) which can be estimated by titration with sodium thiosulphate.[22] Glutathione peroxidase reacts with hydrogen peroxide and fatty acid hydroperoxides simultaneously oxidising glutathione (GSH) to its oxidised form.[23] Gas chromatography mass spectrometry (GC/MS) has been used to measure both lipid peroxide products and aldehydes.[20]

2.3.5 **Conjugated dienes**

Conjugated dienes are formed by the peroxidation of unsaturated fatty acids and absorb UV light in the range 215-250nm. Conjugated diene measurement is a useful measure of the damage caused in the early stages of the peroxidation process. HPLC has been used to separate conjugated dienes and it was reported that one intermediate product formed was an isomer of linoleic acid, octadeca-9(cis),11(trans)-dienoic acid.[24] However, this isomer was later identified as resulting from bacterial fatty acid metabolism.[25] Conjugated dienes can be found in the diet of animals. This results in background levels and provides a problem when quantifying the degree of tissue damage incurred.[26] The main difficulty with diene conjugate measurement in biological materials is the high background absorption at 230nm to 235nm due to polyunsaturated fatty acids and breakdown products of lipid peroxides as well as haem proteins, purines and pyrimidines. Improved resolution of the conjugated diene peak using second derivative spectroscopy has been achieved.[27]

2.3.6 **Hydrocarbon gas measurement**

The hydrocarbon gases pentane and ethane can be measured during the decomposition of lipid peroxides. These gases can be measured using GC/MS, but only small amounts are produced.[28]

Diene conjugation and direct measurement of lipid peroxides tell us about damage caused in the early stages of the lipid peroxidation process, whereas measurement of aldehydes and hydrocarbon gases highlight the damage caused towards the end of lipid peroxidation. The method to use will depend at which stage in the peroxidation process is required to be looked at. In many cases it will be advisable to use more than one method due to the non-specific nature and potential problems associated with these methods.

Free radicals not only cause damage to lipids but can also attack proteins, carbohydrates and DNA.

2.3.7 **Protein oxidation**

Protein oxidation involves the free radical attack of aromatic amino acids, cysteine and disulphide bonds and to a lesser extent proline residues. The characteristic feature of free radical reactions involving proteins is the induction of autofluorescence with excitation and emission maxima in the region of 360nm and 460nm respectively.[29,30]

2.3.8 **Carbohydrate oxidation**

It is well documented that simple monosaccharides autoxidise under physiological conditions generating superoxide[31]. The formation of this superoxide on the protein backbone, in close association with aromatic amino acids enhances the oxidation and crosslinking of such proteins.

2.3.9 **DNA oxidation**

DNA is an important cellular target for free radical attack *in vivo*. Attack of the hydroxyl radical on DNA produces a large number of different chemical species. Oxygen free radicals can induce damage to DNA yielding strand breaks and include conversion of thymine into thymine glycol and 5-hydroxymethyluracil, and of guanine residues into 8-hydroxyguanine. DNA repair systems involved in repairing attack by oxygen/hydroxyl radicals act by an excision repair system. In its simplest form a damaged part of the DNA strand is removed, new DNA synthesis fills the gap and finally a DNA ligase enzyme joins the newly synthesised DNA to the rest of the strand.

2.4 Aromatic hydroxylation

A knowledge of the oxidation of aromatic compounds by metal ion/hydrogen peroxide complexes has existed for over 80 years.

In order that *in vivo* studies can be carried out it is necessary that the aromatic compounds involved have a sufficiently high concentration to compete with other scavenger molecules and the products of hydroxylation do not react further or form enzyme mediated products.[32]

At low pH, in the absence of oxidising metal ions, benzoic acids favour decarboxylation reactions. In the case of SA, catechol is formed. Under physiological conditions (pH 7.4, metal ions and oxygen present), hydroxylation is the predominant reaction observed.

The hydroxylation of aromatic compounds takes place in a systematic way with the attachment of the polar hydroxyl radical to each ring position dependent on the electron donating/withdrawing properties of the substituent attached to the ring. Raghaven and Steenken demonstrated that the fractions of hydroxyl attack at the *ipso*, *ortho*, *meta* & *para* positions of phenol are 0.08, 0.48, 0.08 and 0.36 respectively.[33] Electrophilic reaction of the hydroxyl radical with phenol shows a strong preference for attack at the ring positions activated by the phenolic hydroxyl group *i.e. ortho* & *para* positions.[34]

Aromatic hydroxylation assays were first introduced into biological systems by Halliwell[35] using the xanthine/xanthine oxidase system as the source of hydroxyl radicals and salicylate as the aromatic detector molecule.

Hydroxylation of benzene occurs[36] but it is the hydroxylation of aromatic derivatives of benzene which have been studied more fully. Three aromatic compounds that have been used are phenol, phenylalanine and SA, with anisole and aniline used to a lesser extent.[37] These have been shown to hydroxylate in a similar manner to that of phenol.

2.4.1 **Phenol hydroxylation**

Raghaven and Steenken investigated the mechanism of attack of hydroxyl radicals on phenol (As above, p.13). From this *in vitro* study they found that 4 intermediate dihydroxycyclohexadienyl radicals were formed giving four possible hydroxyl products after oxidation. In this instance catechol and hydroquinone (1,2 & 1,4-dihydroxybenzene respectively) were preferentially formed. An *in vitro* study by Floyd *et al* produced similar findings for phenol with undetectable amounts of resorcinol (1,3-dihydroxybenzene) detected. Raghaven and Steenken used pulse radiolysis with optical detection, whereas Floyd *et al* used three different hydroxyl-generating systems: ADP/Fe(II)/H_2O_2,; hypoxanthine/xanthine oxidase; and UV photolysis of water. HPLC with electrochemical detection (ECD) was used to detect the hydroxylated products in the Floyd method.[38]

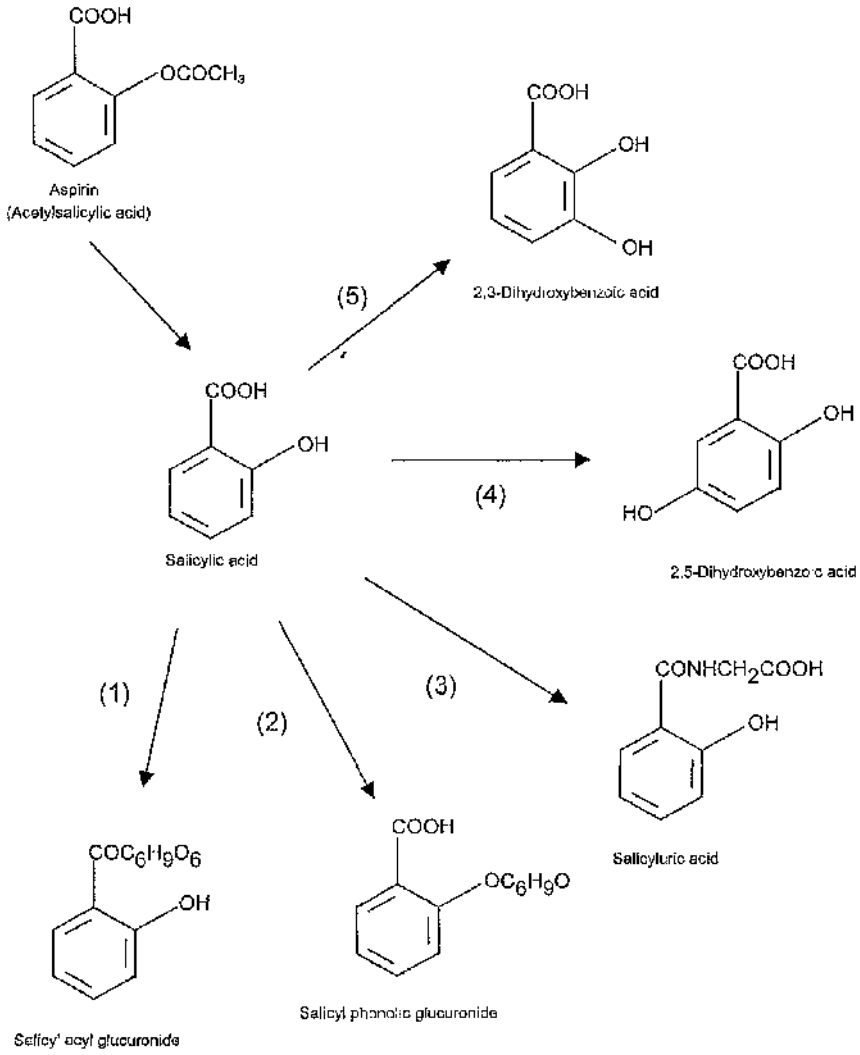
2.4.2 **Phenylalanine hydroxylation**

When the essential amino acid phenylalanine is administered to humans the enzyme L-phenylalanine hydroxylase converts it into L-*p*-tyrosine. However, if a hydroxyl radical attacks the D- or L- phenylalanine then three isomers *ortho*-tyrosine, *meta*-tyrosine and *para*-tyrosine are produced. This was shown *in vitro* (ozonised aqueous solutions) and *in vivo* by showing that blood from premature babies appears to be capable of catalysing hydroxyl radical formation via Fenton reactions involving iron.[39]

2.4.3 **Salicylate hydroxylation**

A lot of interest has been shown in salicylate hydroxylation since aspirin is a widely used medication and is rapidly hydrolysed to SA with further metabolites being produced (Fig 3, p.15).

Fig. 3 Main enzymatic and oxidative by-products of human aspirin metabolism



(1), (2) & (3) - enzyme mediated salicylate by-products.

(4) - hydroxylation and hydroxyl free radical mediated salicylate by-product.

(5) - hydroxyl free radical mediated salicylate by-product.

The first work to be carried out using aspirin and hence SA for hydroxyl radical detection *in vivo* was by Halliwell *et al*.[36] Floyd *et al* used the same three hydroxyl generating systems *in vitro* as used when investigating the hydroxylation of phenol to see if SA would behave in a similar manner, and confirmed that 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) were predominantly formed.[38]

Ingelman-Sundberg *et al* [32] later demonstrated that although 2,5-DHBA is formed by hydroxyl free radical attack on SA, microsomal fractions from mammals treated with inducers of cytochrome P-450 also produce 2,5-DHBA. However, 2,3-DHBA formed under the same conditions occurred only by the primary reaction of hydroxyl free radicals with SA.

2.5 Antioxidants

An antioxidant is any compound that, when present in low concentrations compared to those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate.[12]

Antioxidants can be divided into two main classes; chain-breaking antioxidants and preventive antioxidants.

Chain-breaking antioxidants react with radicals to produce a relatively stable antioxidant radical compound causing chain reaction breakdown. Preventive antioxidants rapidly scavenge active species before they can interact with susceptible targets. These are simplified definitions as many antioxidants have several mechanisms of action.

Many of the non-enzyme antioxidants exist in extracellular fluids. α-tocopherol, a chain breaking antioxidant, delays lipid peroxidation by scavenging intermediate products. However, chain breaking antioxidants such as α-tocopherol, can have a pro-oxidant effect towards non-lipids under certain conditions often because they can bind Fe(III) ions and reduce them to Fe(II), hence a catalytic Fenton reaction can take place. In most extracellular fluids the major defence against oxidants may be to prevent metal ions from participating in radical reactions.

Uric acid, albumin, haptoglobin, and hemopexin have been shown to inhibit various free radical reactions by acting in this manner.[40,41] Ceruloplasmin, an important extracellular antioxidant,[42] oxidises Fe^{2+} to Fe^{3+}, which swiftly binds to transferrin, as will any iron mobilised from serum ferritin.

The food industry relies heavily on chain-breaking antioxidants including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). These protect food lipids in a similar manner to the way α-tocopherols do in lipid peroxidation.

Many plant phenolics have been described as antioxidants since they inhibit lipid peroxidation but they can also accelerate oxidative damage to non-lipid biomolecules such as DNA. This occurs by the reduction of Fe^{3+} to Fe^{2+} or by oxidation to produce superoxide and hydrogen peroxide.

Enzyme antioxidants that exist *in vivo* occur intracellularly as preventive antioxidants. Superoxide dismutase (SOD) is a preventive antioxidant that rapidly converts superoxides to hydrogen peroxide. The hydrogen peroxide produced can then interact with catalase, another preventive antioxidant. Haemoglobin and myoglobin can convert hydrogen peroxide to water and oxygen and work most effectively in the presence of reducing agents such as ascorbate. In the absence of a reducing agent free iron may eventually be released.

3. ANALYTICAL METHOD REVIEW

3.1 Review of analytical methods available for measurement of Salicylic acid (SA) in serum plasma and urine.

SA is commonly administered in its acetylated form as aspirin and can be measured after the administration of low doses of aspirin (typically <300mg). This requires highly sensitive and specific methods. Many different techniques have been employed for the measurement of SA in serum, plasma and urine, however, most of these methods lack the required sensitivity to measure SA in patients administered with low doses of aspirin. A review of different analytical techniques used for measuring SA is provided in a paper by Stewart and Watson where UV, fluorimetric, colorimetric, chromatographic, immunoassay and enzymatic methods are discussed.[43]

Gas chromatography (GC) techniques have been used for detecting SA but in many cases are not the method of choice as they require complex derivatisation procedures which are time consuming, impractical and unattractive for routine use in many laboratories. Pederson and Fitzgerald developed a GC/MS method using a stable isotope dilution assay which detects both labelled and unlabelled aspirin and SA in plasma following co-administration experiments.[44] This produced sensitive and specific results, with a limit of detection of 72pmol/ml (10ng/ml), but involves complex deuterium labelling experimental preparation.

HPLC has proved the most convenient chromatographic tool for routine analysis of SA. HPLC with UV detection has been widely used and is suitable for toxicological purposes where low sensitivity is not required. In the paper by Shen *et al* [45] they use an ion-pair HPLC method with UV detection at 229nm. Extraction recoveries of 92-100% and coefficient of variation (CV) of less than 10% at 72, 720 and 7200nmol/mL (n=6) were achieved, however, the limit of detection (LOD) was 720pmol/ml, indicating that this was a relatively insensitive method.

Fung and Luk reported a method for the determination of SA in various pharmaceutical formulations and foods by differential-pulse voltammetry using a glassy carbon electrode.[46]

Hassan and Hamada have also reported a selective determination of salicylate in pharmaceutical formulations using a liquid membrane electrode.[47]

To date, there are few reports on the use of amperometric detection of SA from biological matrices. Selinger and Purdy reported the determination of SA and its metabolites in blood plasma by HPLC with amperometric detection and back-extraction, giving limits of detection for SA in the submicromolar range.[48] Evans *et al* investigated the oxidation of SA at a planar glassy carbon electrode and showed that the electrode reaction was found to be dependent on the pH and the ionic strength of the acetate buffer, which contained 35% methanol. The average extraction recovery from serum was about 60% with an CV of 5.8%.[49]

Of the methods described above for measuring SA, many use a liquid-liquid extraction process using HPLC with UV or fluorescence detection which are relatively insensitive. Very few papers describe methods involving ECD. Liquid chromatography-electrochemistry has proven to be a viable tool for solving a wide variety of analytical problems, primarily in biomedical research and one of the main aims of this thesis was to develop a sensitive and robust method for the determination of SA in serum at levels equivalent to those of patients taking low doses of aspirin.

3.2 Review of analytical methods available for the estimation of 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) in serum plasma and urine.

Measurement of free radicals *in vivo* is difficult due to their high reactivity. In order to estimate the damage caused by free radicals, secondary products such as aromatic hydroxylation products can be quantified directly by chromatographic techniques. Two such compounds that are formed as a result of the hydroxyl radical attack of SA are 2,3-DHBA and 2,5-DHBA.

Aromatic hydroxylation assays were first used in 1978 using colorimetric techniques[35] but HPLC methods using both UV and EC detection are now preferred due to their enhanced specificity and sensitivity.

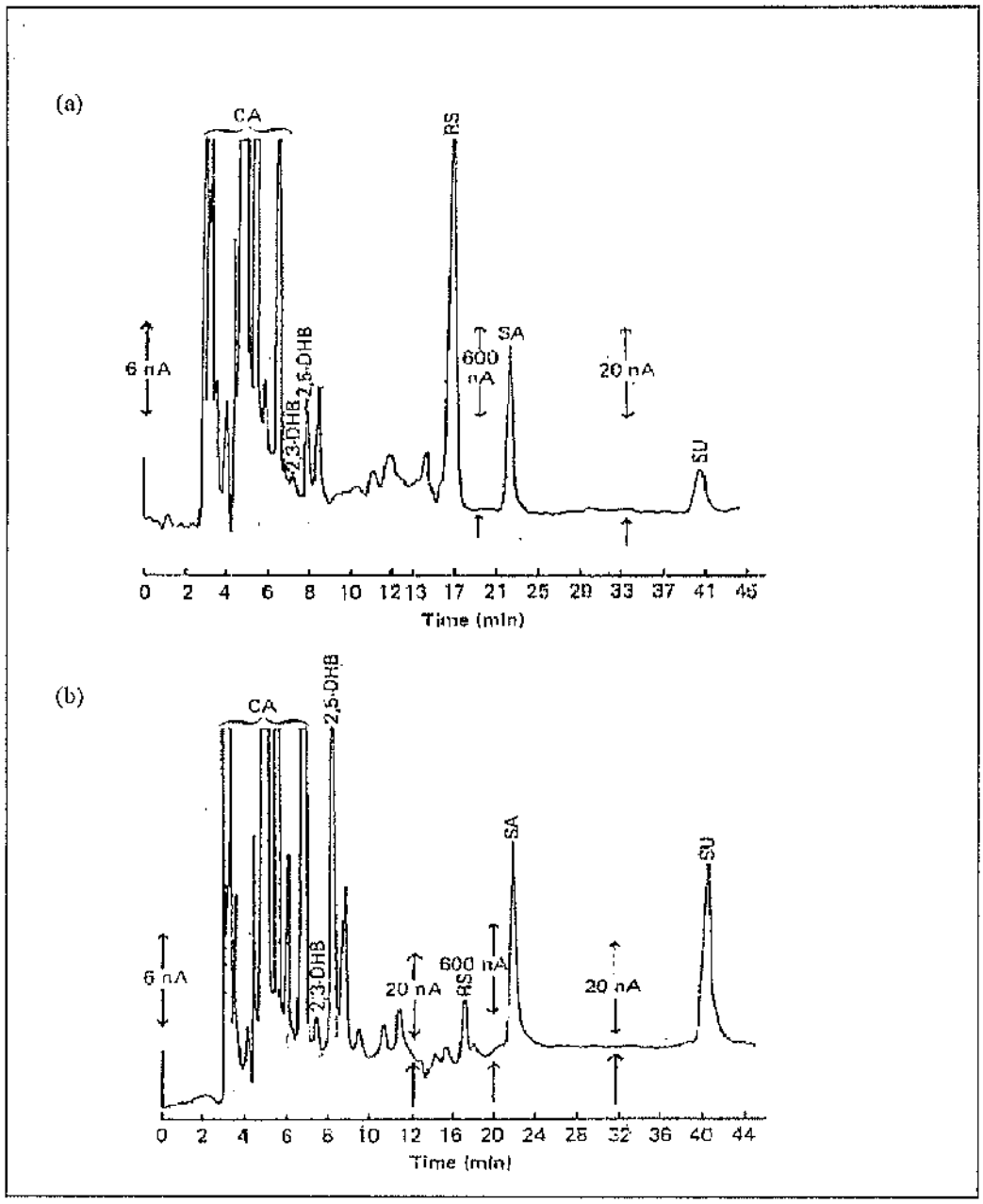
Grootveld and Halliwell investigated aromatic hydroxylation as a potential measure of hydroxyl radical attack using SA as the aromatic scavenger molecule. They proposed that conversion of SA or other aromatic compounds into specifically hydroxylated products would be a useful marker of hydroxyl free radical attack.[50] Grootveld and Halliwell later showed that 2,3-DHBA was a product of human aspirin metabolism using HPLC with ECD and mass spectrometry (MS) to identify the 2,3-DHBA. They found it to be present in low concentrations in comparison with other metabolites that were produced.[51]

Work carried out by Ingelman-Sundberg *et al* confirmed that 2,3-DHBA was not formed by the catalytic hydroxylation of microsomal P-450 but that its conformational isomer 2,5-DHBA was produced.[32] Hence, measurement of 2,3-DHBA and not 2,5-DHBA could provide a means of monitoring hydroxyl radical attack. Following on from the work carried out by Ingelman - Sundberg, Halliwell *et al* highlighted the above findings in a paper entitled 'A short cautionary note'[52]

Floyd *et al*[38] suggested that formation of both 2,3-DHBA and 2,5-DHBA maybe a way of determining hydroxyl radical activity in biological systems. Detection and quantitation of the hydroxylation products obtained and formed during hydroxyl radical generation had been demonstrated in "biologically meaningful systems". The three systems utilised were ADP/Fe(II)/H_2O_2, hypoxanthine/xanthine oxidase plus chelated iron and UV photolysis of hydrogen peroxide. ECD was carried out at 0.80V for 2,3-DHBA and 2,5-DHBA but baseline resolution was not achieved in standard solutions. No extraction from biological matrices was carried out to demonstrate the absence of interference from other extracting compounds.

While the above method did not involve extraction from a biological matrix such as serum, plasma or urine, Grootveld and Halliwell discussed the potential measure of aromatic hydroxylation products *in vivo*.[50] They found that attack by hydroxyl radicals, generated by a Fenton system, upon SA produced 2,3-DHBA and 2,5-DHBA as the major products. HPLC with

Fig. 4 Chromatograms showing separation of an extract from (a) plasma and (b) rheumatoid plasma from healthy volunteers taking aspirin (from Grootveld paper - see Ref 50).



ECD was used to identify and quantify 2,3-DHBA and 2,5-DHBA in human plasma and synovial fluid. Although *in vivo* measurement was carried out using a liquid-liquid extraction process,baseline resolution was not achieved. Extracts from both plasma and synovial fluid show very busy chromatograms at an oxidation potential (E_{ox}) of 0.96V (Fig 4a&b, p.21).

In 1992 an Italian group carried out a study utilising salicylate hydroxylation as an early marker of *in vivo* oxidative stress in diabetic patients.[53] As well as using salicylate hydroxylation, they used the TBA reaction in parallel and found that in diabetic patients higher levels of 2,3-DHBA were found, but that the TBA values between the two groups were very similar. Their results suggested that it might be useful to reveal *in vivo* oxidative stress independently from the peroxidation of lipids.

From a review of the of the work carried out on the detection of 2,3-DHBA and 2,5-DHBA from biological matrices, there has been a number of key validation parameters omitted including poor resolution of the analytes of interest and internal standard (IS) and no precision or stability work carried out on the analytes of interest or the IS. Of all the HPLC methods considered none have met the required sensitivity and specificity which are essential to permit determination of the metabolites of aspirin following the administration of low doses <300mg/day.

4. ELECTROCHEMISTRY

4.1 Introduction to electrochemistry

Since 1974, when the first commercially available HPLC ECD was introduced, a considerable number of articles on design, performance and application have appeared in the scientific literature and today HPLC-ECD is widely accepted as a sensitive and selective technique for analysis of electroactive substances.

4.2 Theory of ECD

4.2.1 Detection principle

ECD of an analyte in the effluent of an HPLC-column is based on electrochemical conversion (electrolysis) of the analyte at a measuring electrode, known as the working electrode (WE), placed in the effluent stream. Electrolysis is activated by applying a voltage (potential) difference between the electrode and the mobile phase containing the analyte and results in electron transfer between electrode and analyte. The resulting "electron flow" (electrical current) is amplified and converted into a signal that we recognise as a peak in the chromatogram. Extremely small currents can be measured representing analyte quantities in the picogram region or less.

Electron transfer from the analyte to the WE is called oxidation (WE is the anode). Electron transfer from the WE to the analyte is called reduction (WE is cathode).

Oxidation of an analyte is favoured when the potential of the WE is made more positive; reduction is favoured when the potential is made more negative.

The WE potential is measured relative to a reference electrode, which has a fixed potential difference measured against the mobile phase.

4.2.2 The three-electrode detection system

Detector response is strongly influenced by voltage changes between the electrode and the mobile phase. A constant and known potential difference between the WE and the mobile phase is therefore a vital requirement to obtain stable, reproducible and predictable detector response.

A counter electrode, also known as the auxiliary electrode (AUX), is used to apply the potential difference between the mobile phase and the WE. This facilitates electrolysis of the analyte.

In principle, this two-electrode arrangement would be sufficient for electrolysis, however, due to "polarisation" processes in the electrode/mobile phase boundary layer and potential "drop" caused by electrical resistance of the mobile phase, the potential applied on the AUX versus WE may differ substantially from the potential of the mobile phase versus WE.

A stable and predictable potential difference between mobile phase and WE is therefore impossible with a two-electrode system. A third electrode known as the reference electrode (REF) is therefore required to monitor the potential of the mobile phase. Its potential versus mobile phase is constant and known, and is invariant with respect to mobile phase composition and current between the AUX and the WE.

4.2.3 Selecting a detector potential

To determine optimum WE-potential for a certain analysis we need to know how detector response (electrolysis current) is related to the WE-potential (voltage). Such a relationship (known diagrammatically as a voltammogram) provides all the information necessary to determine the optimum detection potential for a certain analyte under given chromatographic conditions.

4.2.4 Selectivity

Selective detection is based on the fact that different electroactive substances have maximum detector response at different potentials. Since the number of electroactive substances that can be electrolysed increases with increasing WE potential, optimum selectivity is obtained at minimum WE potential. The final choice for a detection potential usually is a compromise between sensitivity, stability of response and selectivity.

4.3 Electrode materials

4.3.1 WE material

The ideal WE should be electrochemically inert, able to be polished to minimise "internal pore surface", since internal pore surface contributes to noise (analyte molecules have no time to diffuse into the pores during passage along the WE surface), and is chemically inert.
Materials that match the above description are gold, platinum, gold/mercury amalgam, silver and carbon.
In the case of oxidative detection, carbon is the best choice in nearly all cases, therefore, glassy carbon (an amorphous glass-like carbon) is the standard WE material in most cells for HPLC-EC. Vitreous (glassy carbon) has been widely applied in electroanalytical work with, again, a good grade of glassy carbon being first choice for most oxidative applications. However, solid electrodes such as these are susceptible to adsorption[54] or surface fouling and surface oxidation. Fortunately a surface current can be identified since the peak height is directly proportional to the applied potential. The major practical problem of working solid electrodes is the maintenance of a uniform working electrode surface.

4.3.2 **Auxiliary electrode**

The auxiliary electrode must be electrochemically and chemically inert and therefore carbon is the electrode material chosen. It has to be electrochemically inert in order to have a low signal/noise ratio and a constant baseline and chemically inert to prevent reaction with analytes which may be oxidised.

A certain knowledge of the principles of electrochemistry is therefore essential to enable optimum HPLC-ECD conditions to be created.

5. SOLID PHASE EXTRACTION (SPE)

5.1 Background to SPE

SPE is a physical extraction process involving a liquid and a solid phase. Efficient extraction depends on the competing interactions between the isolate and the solvent the isolate is dissolved in, with the solid phase component of the column.

Over the past 20 years, SPE has become an important process in the preparation of biological specimens for routine analysis using different analytical techniques.

It wasn't until the early 1970s that significant advances were made with the introduction of silica columns as the stationary phase. Small disposable columns containing silica, bonded silica and other bonded phases were later introduced.

5.2 Bonded silicas

The reaction of organosilanes with activated silica results in the formation of bonded silicas with the functional group of the organosilane attached to the silica substrate through a silyl ether linkage. In the case of large functional groups e.g. octadecylsilane (C_{18}) being attached there is steric hindrance and as a result there are a large number of unreacted silanol groups (Si-OH) present which are free to undergo polar interactions. If these unreacted sites are not required a further reaction called endcapping can take place and this results in the formation of Si-CH_3, thus deactivating the silanol groups. The intent is to create a surface whose principal properties are due to the functional group allowing minimal interactions with the silica substrate. However, the process of endcapping only deactivates a maximum of 50% of the active silanol groups (Phase Separations seminar).

In practice, bonded silicas are stable within a pH range of 1-14 since degradation of the sorbent is a finite process and sorbents are exposed to solvents for only short periods of time.

5.3 Selection of a suitable column

There are two different approaches for choosing a suitable sorbent. The sorbent can either retain the analyte of interest and release the unwanted components by the use of a suitable eluent. Otherwise, the sorbent can have a low affinity for the analyte and retain the unwanted components.

Both the nature of the sorbent and the sample therefore play important roles in the extraction process.

5.4 Interactions

The most common sorbent interactions can be grouped into one of four categories: non-polar, polar, ion-exchange, and covalent.

5.4.1 Non-polar interactions

Non-polar interactions occur between the carbon-hydrogen bonds of the sorbent functional group and the carbon-hydrogen bonds of the isolate. These forces are sometimes known as van der Waals or dispersion forces.

The most widely used sorbent for non-polar interactions is C_{18}. Since most organic molecules have some degree of non-polar character then non-polar interactions are not very selective.

5.4.2 Polar interactions

Polar interactions include hydrogen bonding, dipole-dipole and π-π in which distribution of electrons between individual atoms in the functional groups is unequal causing polarity. Some of the functional groups that display these type of interactions are hydroxyl, amines, carbonyls, aromatic rings and hetero atoms such as oxygen, nitrogen, sulphur and phosphorus.

Due to the polar nature of the silica substrate (silanol groups), polar interactions are characteristic of all bonded silicas. The interaction of these silanol groups are known as secondary interactions and are most significant in non-polar solvents.

5.4.3 Ionic interactions

Ionic interactions occur between the isolate molecules carrying a charge and a sorbent carrying an opposite charge to that of the isolate. Cationic (positively charged) examples include amines and inorganic cations such as calcium. Anionic (negatively charged) examples include carboxylic acids and phosphates.

For ion-exchange interactions to take place two criteria must be met:

1/ Both the isolate and sorbent have to be in a suitable pH environment to be charged.

2/ The solvent must not contain high concentrations of competing species.

It is, therefore, essential that pKa values of isolates are known.

5.4.4 Covalent interactions

Covalent interactions are those resulting in formation of covalent bonds between the sorbent and the isolate molecule. These interactions are designed to be reversible with a change to the solvent environment. These sorbents are less commonly used but are highly selective.

Almost all available sorbents are capable of more than one type of interaction and this has to be taken into consideration when choosing a suitable column.

5.5 **Advantages of SPE over conventional extraction techniques**

There are a number of advantages of using SPE over other more conventional types of extraction. High recovery of an analyte make an assay procedure more sensitive enabling a smaller sample volume to be used and quantified. The benefit of this is seen in studies where small sample volumes are required *e.g.* young children, neonates.

SPE manifolds are commercially available and can be used to reduce the time involved in carrying out an extraction procedure. This allows sampling of highly volatile or photosensitive analytes.

In addition to a reduction in time and labour, much less solvent is required for SPE extraction procedures in comparison to conventional techniques such as liquid-liquid extraction. This results in less waste disposal, fire hazard and generally benefits a safer working environment.

6. Aims and objectives of the current investigation

The main study aims at the outset of the work for this thesis were:-

1. To develop and improve on published methods the measurement of 2,3-DHBA, 2,5-DHBA and SA in human serum by utilising HPLC with ECD. The process of SPE would produce a "cleaner" extract thereby reducing chromatographic interference and allowing the three analytes of interest to be separated.

2. To employ an appropriate IS to account for extraction losses.

3. To introduce autosampling and computerised data handling to facilitate a greater throughput of samples.

4. To confirm the identity of putative 2,3-DHBA, 2,5-DHBA and SA peaks through the use of alternative techniques such as GC/MS.

5. To validate the improved methods for measuring concentrations of 2,3-DHBA, 2,5-DHBA and SA by carrying out precision, accuracy, linearity, stability and recovery studies.

EXPERIMENTAL

1. MATERIALS

1.1 Chemicals

β-resorcylic acid (2,4-dihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), α-resorcylic acid (3,5-dihydroxybenzoic acid), 3,4-dihydroxbenzoic acid, *p*-hydroxybenzoic acid (4-hydroxybenzoic acid), isovanillic acid (3-hydroxy-4-methoxybenzoic acid), 4-methylsalicylic acid, 3-methylsalicylic acid (Sigma Chem.Co.); *m*-anisic acid (3-methoxybenzoic acid), *m*-hydroxybenzoic acid (3- hydroxybenzoic acid), 2,3-dimethoxybenzoic acid, 3-methoxysalicylic acid, 2- bromobenzoic acid, 2-iodobenzoic acid, 2-chlorobenzoic acid, 3-fluorobenzoic acid, 2,5-dihyroxyphenylacetic acid gamma-lactone, 2,5-dihdroxyphenylacetic acid, 3-hydroxy-4-methylbenzoic acid, 2-hydroxy-3-isopropylbenzoic acid, salicyluric acid, salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, (Aldrich Chem.Co.); HPLC grade sodium acetate trihydrate, (Fisons); HPLC grade water and methanol (Rathburn Chem.Ltd.); benzoic acid, tri-sodium citrate, acetic acid, 35% ammonia solution, hydrochloric acid sp.gr. 1.18 (BDH Lab.Supplies).

1.2 Serum specimens for standard curves

Blood samples were taken from volunteers from the Biochemistry Department, Dumfries and Galloway Royal Infirmary. These volunteers were not consuming aspirin. The samples were left to coagulate for 30 minutes before being spun in a centrifuge at 3000g for 10 minutes. The serum was then removed and stored at -30°C.

2. APPARATUS

2.1 HPLC apparatus

The HPLC system consisted of a Jasco 851-AS Intelligent Sampler, a Jasco PU-980 Intelligent HPLC Pump, a Jasco LG-980-02 Ternary Gradient Unit, a Jour Research X-Act 4 Channel Degasser and an Antec Decade Electrochemical Detector (Presearch). Data was acquired using Minichrom, a chromatography data capture system which processes raw data, via an ETHERNET network using a VG Chromatography Server (Fisons). The analytical column was a 25cm × 4.6mm Apex Octadecyl 5µ Column (Jones Chromatography) and the column was held within the main cavity of the electrochemical detector at a constant temperature of 30°C. A combination pH electrode (Russell Electrodes) was used to measure pH. All solutions were filtered through 0.22 micron filters (MSI), an injection loop of 100µl was used and the flow rate was 1.0ml/min. Identification of peaks was by comparison of retention times (t_r) with standard material. Straight line calibration curves were calculated by peak height ratios of extracted material with IS.

2.2 SPE apparatus

The SPE system consisted of a Vac-Elut Extraction System and SPE Columns - HAX 200mg/10ml XL Column Reservoir (Jones Chromatography) and a Techne Dri-Block DB-3 & SC3 Sample Concentrator (Techne (Cambridge) Ltd.)

2.2.1 SPE procedure

Solid phase columns were placed on the Vac-Elut system and to each individual column was added 2mls of methanol (column solvation) followed by 2mls of acetate buffer (9.6mM) adjusted to pH 2.5 using glacial acetic acid (column pre-equilibration). The specimen was added to the column reservoir and allowed to flow through the column (no vacuum necessary at this stage). 1ml of pH 2.5 acetate buffer was used to "wash" the column and then allowed to dry under

vacuum for 5 minutes. A rack of screw-capped glass tubes were placed on the Vac-Elut system and the tip of the delivery needle wiped dry. 5mls of a 3%(v/v) ammonia in methanol was added to the column and the eluent collected. The eluent was evaporated at 55°C under a stream of nitrogen, reconstituted in 0.5mls of 0.05M HCl and 50µl was injected onto the analytical column.

2.3 Calculating extraction efficiencies

Detection of a substance by the ECD was represented on the chromatogram by a peak. In order to determine the efficiency of extraction for SA (or 2,3-DHBA and 2,5-DHBA) using peak heights, a known amount of SA is added to serum, extracted and injected and peak heights compared against a known amount of standard SA injected. The following relationship is then applied:

$$\text{Extraction efficiency of SA} = \frac{\text{Peak height of SA from extracted sample}}{\text{Peak height of SA from standard solution}} \times 100$$

3. METHOD DEVELOPMENT AND VALIDATION OF SA ASSAY

3.1 Mobile phase

When the method was set up at first there was no methanol present in the mobile phase (tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid). When a standard SA solution was injected the t_r of the corresponding peak was 28 minutes. In order to try and elute the SA peak more quickly, 4 mobile phases were prepared, each consisting of a different methanolic concentration. 50μM standards of SA were prepared and analysed to see where the analyte peak eluted.

3.2 Blank sera specimen analysis

Serum that had been stored at -30°C was thawed and 1.0M hydrochloric acid (HCl) added to 0.5ml aliquots to bring them to a pH of about 2.5. Serum was extracted and analysed in order to look at the quality of the chromatography produced.

3.3 Voltammetric behaviour of SA

3.3.1 Voltammetric behaviour of SA at a glassy carbon electrode

A 1mM stock solution of SA in water was prepared and made up to a final concentration of 10μM. 50μl of this stock solution was then injected onto the column (69ng of SA) and the E_{ox} of the ECD was varied between 0.90 and 1.30V. It was believed that the E_{ox} for SA would be higher than that for 2,3-DHBA since there is one less hydroxyl group.

3.3.2 Voltammetric comparison of standard SA and blank sera peaks having the same retention time (t_r) using SPE.

0.5mls of human serum from subjects known not to have taken aspirin was adjusted to pH 2.5 using 1.0M HCl and extracted using SPE. A 1mM stock solution of SA in water was prepared and diluted to produce a solution containing 500nM of SA. The E_{ox} of the ECD was varied between 1.10-1.25V and the blank extract and the standard SA were injected onto the analytical column to provide peak height response at the different E_{ox} voltages. Peak height ratios for both the serum and standard injections were calculated using the acquired data.

3.4 Extraction efficiency of SA

3.4.1 Extraction efficiency of SA from human serum

7mls of serum was thawed, adjusted to pH 2.5 using 1.0M HCl and divided into 7 equal aliquots. One of these aliquots was kept as a blank and the remaining 6 were spiked with different amounts of SA (0.625, 1.25, 2.5, 5.0, 10 and 20nmol/ml). Extraction efficiencies for SA were calculated as described in section 2.3, p.34.

3.4.2 Effect on extraction efficiencies of SA by varying the serum pH before extraction

An experiment was carried out to determine the effect of varying the serum pH from the proposed pH of 2.5 during sample preparation, prior to SPE. As part of method robustness it was essential to ensure that the extraction efficiencies would not vary significantly with small changes in pH of the serum. Five separate 1.0ml aliquots of serum were spiked with 50μM SA and prepared by adding different amounts of 1.0M HCl. This provided a range of serum pH values around pH 2.5, the pH at which it was thought maximum extraction efficiencies would be attained. The pH of the five serum samples were 2.07, 2.33, 2.42, 2.53, and 2.71. These samples were extracted as described in section 2.2.1, p.33 and extraction efficiencies calculated.

3.5 Internal standard (IS) for SA assay

Various compounds with structural similarities to SA were investigated as possible IS. Four important factors had to be taken into consideration in the search for a suitable IS and these were: the voltammetric behaviour of the compound; the SPE behaviour; the t_r and resolution of peaks in the chromatogram; and the compound chosen should not be produced by metabolic pathways.

3.5.1 Compounds examined for suitability as an IS

Table 4, p.56 shows the compounds that were tested to discover whether or not they would be suitable to be used as an IS. The mobile phase used on days 1, 2 and 3 contained 20%(v/v) methanol in tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid. On day 4 the mobile phase consisted of a methanolic concentration of 16.7%(v/v). A 10μM standard solution of SA and 50μM of each potential IS were prepared and injected onto the analytical column to determine the t_r of each with respect to each other. In each experiment, extraction of human blank serum was carried out to indicate what regions of a blank chromatogram were available for "insertion" of trial ISs.

3.5.2 Voltammetric behaviour of 2,5-dihydroxyphenylacetic acid γ lactone (2,5-DHPAAGL) at a glassy carbon electrode

A 1mM stock solution of 2,5-DHPAAGL was prepared and diluted to a final concentration of 10μM. 50μl of the 10μM 2,5-DHPAAGL solution was injected onto the column with the E_{ox} of the ECD varied between 0.70 and 1.15V. A mobile phase consisting of 20%(v/v) methanol in tri-sodium citrate (30mM) was adjusted to pH 4.75 using glacial acetic acid.

3.5.3 SPE of 2,5-DHPAAGL

Baseline resolution of the 2,5-DHPAAGL and SA was achieved and both peaks were free from interference from blank serum, therefore SPE was carried out to ensure that the 2,5-DHPAAGL was extracting efficiently.

1mM stock solutions of 2,5-DHPAAGL and SA were prepared, diluted and 50µl of 10µM 2,5-DHPAAGL and SA standards were injected onto the analytical column. Blank serum was thawed and the pH adjusted to 2.5 using 1.0M HCl. The serum was spiked with 50µl of 100µM 2,5-DHPAAGL and 50µl of 100µM SA. Spiked and unspiked sera were extracted in duplicate and prepared for analysis. The extracts were injected onto the analytical column. The t_r of the extracted peaks were compared with the t_r of standard 2,5-DHPAAGL and SA peaks. The mobile phase consisted of 20%(v/v) methanol in tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid.

3.5.4 SPE of 4-methylsalicylic acid (4-MeSA)

4-MeSA and 3-methylsalicylic acid (3-MeSA) were both well resolved from SA and were free from interference from blank serum. 4-MeSA was considered first since the t_r was shorter than that of 3-MeSA and a standard solution of 3-MeSA was found to have an impurity present which produced another peak in the chromatography. 1mM stock solutions of both SA and 4-MeSA were prepared, diluted and 50µl of each 10µM standard solution injected onto the column. 3mls of sera was thawed and adjusted to pH 2.5 using 1.0M HCl and divided into 3 equal aliquots of 1ml. One aliquot of serum was spiked with 50µl of 100µM 4MeSA and 50µl of 100µM SA, another with 50µl of 100µM SA and the final aliquot was used as a blank. The aliquots were extracted in duplicate (2 x 0.5ml) and the t_r of standard solutions and extracted samples for 4-MeSA and SA were compared after injection onto the column. The mobile phase consisted of 28.6%(v/v) methanol in tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid. It

was hoped that the increased methanol content in the mobile phase would allow faster elution of the 4-MeSA peak.

3.5.5 Voltammetric behaviour of 4-MeSA at a glassy carbon electrode

In order to optimise the chromatographic conditions a voltammogram was carried out. A 1mM stock solution of 4-MeSA was prepared and diluted. 50µl of a 10µM 4-MeSA standard was injected onto the column with the E_{ox} on the ECD varied between 0.90 and 1.25V.

3.5.6 Extraction efficiency of 4-MeSA

6mls of serum was thawed, adjusted to pH 2.5 using 1.0M HCl and divided into 6 equal aliquots of 1ml. One aliquot was used as a blank and the remaining 5 were spiked with different concentrations of 4-MeSA. The extraction efficiency of 4-MeSA was determined. The mobile phase consisted of 28.6%(v/v) methanol in citrate/acetate with the pH adjusted to 4.75 using glacial acetic acid.

3.5.7 Linearity of 4-MeSA

A 1mM stock solution of 4-MeSA was prepared and diluted. 16 different concentrations of 4-MeSA were injected onto the column and the peak height plotted against the concentration of 4-MeSA injected.

3.6 Range and linearity

3.6.1 Range and linearity of SA

The linearity of SA after extraction was determined over the concentration range 0.1-50μM. This was the range anticipated in the serum of patients receiving low dose aspirin. 50μl volumes of different concentrations of standard SA solutions were added to 0.5ml aliquots of pooled human control serum and acidified to pH 2.5 using 1.0M HCl.

3.6.2 Level of IS required for calibration curves

The median serum level of SA expected during low dose aspirin therapy was 10μmol/l. In order to arrive at an appropriate concentration of IS (4-MeSA) to spike the serum with, it was estimated that a 25μmol/l solution of 4-MeSA would give a peak height ratio of SA/IS of 1 at the 10μmol/ml SA concentration. 50μl injections of aqueous SA (1.5, 15 & 45μmol/l) and 4-MeSA (25μmol/l) were made onto the HPLC system at an E_{ox} of 1.10V.

The detector response of both compounds were compared after extraction, and a 4-MeSA concentration selected based on the extraction recovery of 4-MeSA relative to that of SA.

3.6.3 Pooled serum from patients

Serum from patients was collected and then assayed to ensure that no subjects had taken any aspirin. Serum samples were pooled from patients and aliquotted into 6 equal groups. The pooled sera in each group was thoroughly mixed and the pH of each pool was adjusted to pH 2.5 using 1.0M HCl and extracted in duplicate. This serum was to be used for preparation of standards for calibration curves.

3.6.4 Extracted SA calibration curve from human serum

A range of SA concentrations in pooled human serum were prepared between 0.1-50μM. 4-MeSA was added to give a final concentration in extracted serum of 25μM at each level. These samples were extracted and analysed by HPLC with ECD at an E_{ox} of 1.10V.

3.7 Inter and intra-assay precision and accuracy of SA analysis

Concentrations of SA in pooled human serum were prepared within the concentration range of the calibration curve at concentrations of 1.5, 15 and 35μM. Aliquots of these solutions were stored at -30°C during the timescale of the precision analysis. Sufficient serum standards were prepared to allow 10 determinations at each concentration on a single day and for duplicate measurement to be made at each concentration on 10 separate days to provide data for both intra and inter-assay precision respectively. Aliquots were thawed and analysed by reference to calibration standards (1, 5, 10, 20 & 50μM) prepared using pooled human serum on the day of measurement of the stored specimens. The data generated was compared with expected concentrations of freshly prepared standards as an estimate of accuracy for the SA analysis.

3.8 Stability of SA

3.8.1 Stability of aqueous SA stored at 3 different temperatures

1mM stock solutions of standard SA and IS were prepared and diluted to give aliquots consisting of concentrations of 1.5, 15 & 35μM. These were stored in glass tubes with teflon lined screw caps at room temperature, 4°C and -30°C over a period of 20 days. Aliquots were removed and analysed by direct injection onto the column with the E_{ox} of the ECD set at 1.10V.

3.8.2 Stability of SA made up in 0.05M HCl and water

1mM stock solutions of SA made up in both 0.05M HCl and water were prepared to investigate the effect of pH on the stability of SA. Aliquots were stored at -30°C and investigated as described above covering a period of 11 days.

3.8.3 Stability of SA in serum stored at serum pH and pH 2.5

1.5, 15 and 35μM concentrations of SA were prepared in serum with the pH adjusted to 2.5 with 1M HCl or in serum where no HCl was added (pH 7.5-7.8). Aliquots of serum were stored in glass tubes at -30°C. These were removed at intervals covering a period of 13 days, extracted and analysed.

3.9 Clinical application

3.9.1 Measurement of SA levels in 8 diabetic patients taking low dose aspirin

Blood samples collected from diabetic outpatients at Dumfries and Galloway Acute & Maternity National Health Service Trust, who were known to be taking aspirin at low dose levels (75-300mg/day), were allowed to clot and the serum was separated after centrifugation and stored at -30°C until analysis. The 8 samples were thawed, mixed and 0.5mls extracted and analysed in duplicate. The aspirin dose was recorded for each subject but not the time after dosing that the blood sample was taken.

4. METHOD DEVELOPMENT OF 2,3-DHBA AND 2,5-DHBA ASSAY

4.1 SPE procedure

The SPE procedure was the same as used for the SA assay described previously in section 2.2.1, p.33.

4.2 Voltammetric behaviour of 2,3-DHBA and 2,5-DHBA at a glassy carbon electrode

1mM stock solutions of 2,3-DHBA and 2,5-DHBA were prepared and diluted to give a concentration of 10nM. 50µl of each standard solution was injected onto the analytical column and the E_{ox} of the ECD varied between 0.35 and 0.80V.

4.3 Confirmation of the presence of 2,3-DHBA and 2,5-DHBA in human blank serum

4.3.1 Patient samples (n=11) analysed for the presence of 2,3-DHBA and 2,5-DHBA.

Blood samples were taken from 11 subjects known not to be taking aspirin. Serum was removed and the pH adjusted to 2.5 using 1.0M HCl. Following extraction, chromatograms of extracted serum and standard 2,3-DHBA and 2,5-DHBA were compared. The E_{ox} was 0.75V.

4.3.2 Comparison of peak height ratios for blank serum unknown peaks, 2,3-DHBA and 2,5-DHBA by varying the E_{ox}.

Blank serum was adjusted to pH 2.5 using 1.0M HCl and extracted. 1mM stock solutions of 2,3-DHBA and 2,5-DHBA were prepared and diluted to a concentration of 10nM. Three different E_{ox} values were applied (0.45, 0.55 and 0.65V) to compare peak height ratios of 2,3-DHBA and 2,5-DHBA at the different E_{ox} values against those of the unknown peaks with identical t_r to 2,3-DHBA and 2,5-DHBA in extracted blank serum.

4.4 Extraction efficiencies of 2,3-DHBA and 2,5-DHBA

6mls of human blank serum were thawed, the pH adjusted to 2.5 using 1.0M HCl and divided into 6 equal aliquots of 1ml. Two of these were used as blanks, 2 were spiked with 100nM of 2,3-DHBA and the final 2 spiked with 100nM of 2,5-DHBA.

4.5 Stability of 2,3-DHBA and 2,5-DHBA while using the HPLC autosampler

1mM stock solutions of 2,3-DHBA and 2,5-DHBA were prepared in water, diluted to a concentration of 100nM and 50µl injected onto the column using the autosampler. Nine injections of a mixture of 2,3-DHBA and 2,5-DHBA were run over a period of 4 hours. The E_{ox} was 0.75V.

4.6 IS for 2,3-DHBA and 2,5-DHBA assay

4.6.1 3,4-dihydroxyphenylacetic acid (3,4-DHPAA)

3,4-DHPAA had been used in previous work carried out by a group at the Royal Infirmary in Glasgow. This compound was known to be well resolved from 2,3-DHBA and 2,5-DHBA and it was thought it could be used as the IS. No other method development work had been carried out using 3,4-DHPAA.

4.6.2 Voltammetric behaviour of 3,4-DHPAA at a glassy carbon electrode

A 1mM stock solution of 3,4-DHPAA was prepared and diluted to a concentration of 10nM. 50µl of the 10nM solution was injected onto the column and the E_{ox} of the ECD was varied between 0.35 and 0.80V.

4.6.3 Stability of 3,4-DHPAA while using the HPLC autosampler

1mM stock solutions of 2,3-DHBA, 2,5-DHBA and 3,4-DHPAA were prepared in water and diluted to give a 100nM mixture of 2,3-DHBA, 2,5-DHBA and 3,4-DHPAA. A series of 50µl injections of the mixture were injected onto the analytical column and data recorded. The E_{ox} was 0.75V.

4.6.4 Continued search for a suitable IS for 2,3-DHBA and 2,5-DHBA assay

Using the basis for choosing an IS described previously in section 3.5, p.37, six potential candidates were investigated further:

1/ 3,4-dihydroxybenzoic acid
2/ 2,4-dihydroxybenzoic acid
3/ 3,5-dihydroxybenzoic acid
4/ 3-hydroxybenzoic acid
5/ 4-hydroxybenzoic acid
6/ 3,4,5-trihydroxybenzoic acid

A 1mM stock solution of each of the above compounds was prepared in 0.05M HCl and diluted to a concentration of 100nM. 50µl of each compound was manually injected onto the column. The E_{ox} was 0.75V.

4.6.5 Stability of 3,4-dihydroxybenzoic acid (3,4-DHBA)

A 1mM stock solution of 3,4-DHBA was prepared, diluted and assayed to ensure stability in 3 different aqueous environments including water, 0.05M HCl and mobile phase. The stability was tested over a period of 10 hours at room temperature. The E_{ox} was 0.75V.

4.7 Optimisation of chromatographic and extraction conditions.

Mobile phase consisting of tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid, resulted in slight overlap of the 2,3-DHBA and 2,5-DHBA peaks. In order to produce better resolution of these two analytes, the chromatographic conditions were altered.

4.7.1 Tri-sodium citrate (30mM) mobile phase at pH 4.75 examining the effect of overlap of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA peaks with increasing analyte concentration

A mobile phase was prepared consisting of tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid. 3 mixtures containing different concentrations of each were prepared in water (30, 100, 170nM each of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA) and 50µl injected onto the column to see what effect increased concentration would have on the resolution of the peaks. These concentrations were chosen because they were thought to be in the approximate range of levels in subjects taking low-dose aspirin.

4.8 Search for a more suitable mobile phase

4.8.1 Addition of 4.75% acetonitrile to the mobile phase

A mobile phase was prepared containing 4.75% acetonitrile(v/v) in tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid. 100nM solutions of 2,3-DHBA and 2,5-DHBA were prepared from 1mM stock solutions and 50µl of each was manually injected to ascertain whether better resolution had been achieved.

4.8.2 Addition of 1.5% methanol to the mobile phase

A mobile phase was prepared containing 1.5% methanol in tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid. The same solutions as in Exp 4.8.1 were used and the same amount manually injected.

4.8.3 Variation in pH of the mobile phase

The pH of the tri-sodium citrate (30mM) mobile phase was varied with pH values of 4.75, 5.0, 5.2, 5.3 and 5.4 being investigated. A 100nM aqueous mixture of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA was prepared and 50µl injected onto the column. Chromatographic data, including the t_r of the analytes, using the different mobile phases were collected. The effect of the mobile phase pH on detector response of the compounds of interest was investigated also.

4.9 Optimisation of SPE for 2,3-DHBA and 2,5-DHBA assay.

4.9.1 Analysis of SPE eluent fractions for 2,3-DHBA and 2,5-DHBA

During SPE, 5mls of eluent (3%(v/v) ammonia in methanol) were added to the solid phase column to elute the 2,3-DHBA and 2,5-DHBA. In order to examine this elution process, 0.5ml fractions were collected, dried under nitrogen at 55°C, reconstituted in 0.5mls of 0.05M HCl and 50µl of each extract injected onto the analytical column. From each 0.5ml increment collected, recoveries were calculated to see how much of each analyte was present in each 0.5ml fraction.

4.9.2 SPE using new mobile phase

A mobile phase of tri-sodium citrate (30mM) with the pH adjusted to 5.3 using glacial acetic acid was prepared. A serum sample from a subject not consuming aspirin was extracted. 50µl of the extract was injected onto the column to determine changes in t_r of unknown peaks from blank

serum using the new mobile phase. These were compared to standard mixtures of the 3 analytes of interest.

4.9.3 Setting up a gradient to elute unwanted peaks

The use of gradient elution was investigated in order to reduce the amount of time between injections, since it had been observed that some peaks in the serum extracts had a t_r of 120 minutes. Two mobile phases were used in order to set up the gradient: A - tri-sodium citrate (30mM) with a pH of 5.3; B -28.6%(v/v) methanol in tri-sodium citrate (30mM) with a pH of 5.3. Four different gradient programmes were set up on the gradient unit and are shown below.

Program 1

Time (mins)	Step time (mins)	%A	%B
0.0	0.0	100	0
9.5	9.5	100	0
14.5	5.0	0	100
20.0	5.5	0	100
22.0	2.0	100	0

Program 2

Time (mins)	Step time (mins)	%A	%B
0.0	0.0	100	0
7.0	7.0	100	0
8.0	1.0	0	100
15.0	7.0	0	100
16.0	1.0	100	0

Program 3

Time (mins)	Step time (mins)	%A	%B
0.0	0.0	100	0
6.0	6.0	100	0
6.1	0.1	0	100
15.0	8.9	0	100
15.1	0.1	100	0

Program 4

Time (mins)	Step time (mins)	%A	%B
0.0	0.0	100	0
6.0	6.0	100	0
6.1	0.1	0	100
17.5	11.4	0	100
17.6	0.1	100	0

4.10 Carryover of peaks

4.10.1 Checking for carryover of peaks

Gradient program 3 was found to give the quickest throughput of samples using the mobile phases A and B as described in section 4.9.3, p.48. A serum sample was extracted and the gradient program used to inject the extracted serum sample followed by mobile phase to check for carryover of the 2,3-DHBA, 2,5-DHBA and 3,4-DHBA from serum.

4.10.2 **The effect of the number of washes of the autosampler needle on carryover**

Gradient program 3 was used and an aqueous mixture of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA was prepared (170nM of each). The washing feature on the autosampler allows a maximum of 5 washes between injections. Five samples were injected, and the number of washes varied between 1 and 5, to see what effect the number of washes would have on carryover. The wash solution was 50% methanol(v/v) in 0.05M HCl.

4.10.3 **Investigation of different wash solutions**

Four wash fluids were examined, including: 50% methanol(v/v) in 0.05M HCl (5 washes) with the outside of the autosampler needle wiped thoroughly; 0.05M HCl on its own (5 washes); 0.1M HCl to see if a stronger acid could prevent the carryover from occurring (5 washes); 0.2M HCl (5 washes). Stock solutions of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA were prepared to a concentration of 170nM each and the post wash solution was 0.05M HCl.

RESULTS

1. METHOD DEVELOPMENT AND VALIDATION OF SA ASSAY

1.1 Mobile Phase

The t_r of the SA with different methanolic concentrations in the mobile phase are shown in Table 1, p.52. From this it was shown that for a mobile phase consisting of 28.6%(v/v) methanol, the SA peak eluted with a t_r of about 6 minutes. A peak in the chromatogram of the serum blank shown in Fig. 5a, p.54 at a t_r of approximately 5 minutes did not allow further optimisation of the mobile phase conditions. It was believed that a further increase in the organic content of the mobile phase would reduce the resolution between the peak in the blank sera and the SA peak.

1.2 Blank sera specimen analysis

A typical chromatogram showing an extracted blank serum sample from a subject not consuming aspirin is shown in Fig. 5a, p.54. The mobile phase used consisted of 28.6%(v/v) methanol.

1.3 Voltammetric behaviour of SA

1.3.1 Voltammetric behaviour of SA at a glassy carbon electrode

A voltammogram was drawn for SA (Fig.6a, p.58). The optimum potential for the determination of SA was found from the position of the plateau on the hydrodynamic wave, in this case around 1.10V.

1.3.2 Voltammetric comparison of standard SA and blank sera peaks having the same t_r using SPE

At maximum sensitivity, a peak occurred at the same t_r as SA in the "blank" sera. Voltammograms were drawn indicating that the blank peak was probably due to SA. Carryover from previous

injections was carefully excluded (Table 2, p.52). All blank sera were found to contain SA at concentrations less than 75pmol/ml.

Table 1 The retention times (t_r) of SA using different concentrations of methanol in the mobile phase (30mM sodium citrate/acetate with the pH adjusted to pH 4.75 using glacial acetic acid).

Methanol concentration % (v/v)	**t_r of SA (mins)**
9.0	11.94
16.7	8.01
20.0	6.83
28.6	5.82

Table 2 A voltammetric comparison of SA and a compound present in all blank sera having the same t_r.

E_{ox} (V)	**Standard peak height in mm (% change from 1.10V)**	**Blank sera peak height (%change from1.10V)**
1.10	18.3	1.5
1.15	46.1 (252)	3.3 (220)
1.20	127.5 (697)	9.6 (640)
1.25	55.1 (301)	3.6 (240)

1.4 Extraction efficiency of SA

1.4.1 Extraction efficiency of SA from human serum

The mean extraction efficiency of SA was found to be 85% (CV = 12.6%, n=6).

1.4.2 Effect on extraction efficiencies of SA by varying the serum pH before extraction

The effect of varying the pH of serum samples before application to the solid phase columns is shown in Table 3, p.55. From the table we can see that the extraction efficiencies were >96% across the pH range 2.07-2.71.

1.5 IS for SA assay

1.5.1 Compounds examined for suitability as an IS

The t_r of various compounds which were investigated to see whether they could be used as IS are shown in Table 4, p.56. Of the compounds analysed, very few were considered as potential IS. A number of the compounds did not oxidise at the E_{ox} of SA (1.10V), therefore no peak on the chromatogram was detected, some interfered with endogenous peaks present in blank serum and others co-eluted with the SA peak. 2,5 DIIPAAGL had a t_r of 10.42 minutes, compared to 6.90 minutes for SA and was the first compound to be investigated as a potential IS. It did not interfere with any of the peaks extracted from blank serum. 4-MeSA and 3-MeSA oxidised at the same E_{ox} as SA and were well resolved from interfering peaks, but had longer elution times.

Fig. 5 Chromatograms of (a) extracted blank serum from a healthy volunteer not consuming aspirin, (b) blank serum spiked with SA and 4-MeSA and extracted and (c) human serum from a volunteer taking low dose aspirin spiked with IS and extracted.

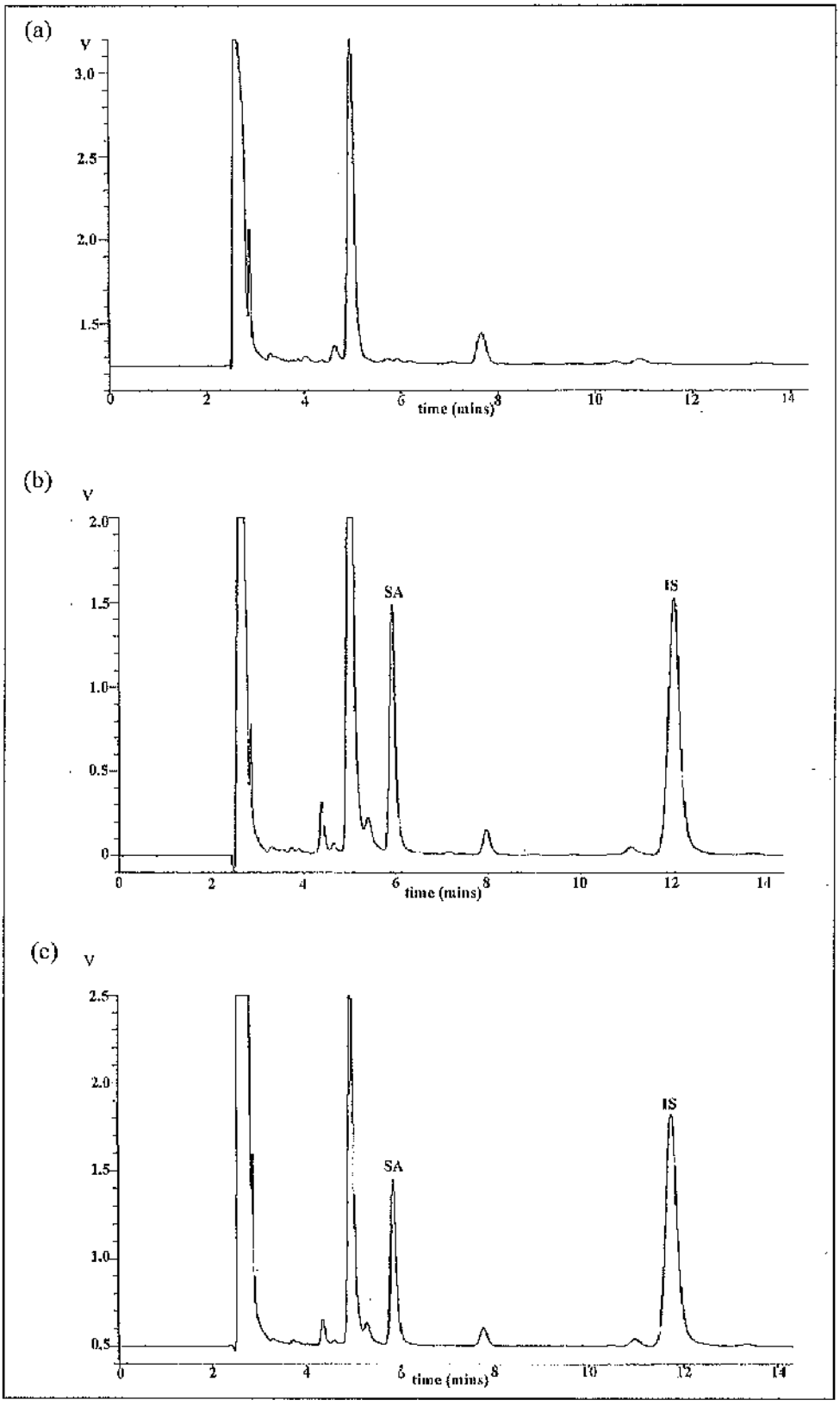


Table 3 The effect of pH on extraction efficiencies of SA in human serum.

Volume of 1.0M HCl added (µl)	Serum pH	Extraction efficiency of SA (%)
130	2.07	96
128	2.33	98
126	2.42	98
125	2.53	101
120	2.71	102

1.5.2 Voltammetric behaviour of 2,5-DHPAAGL at a glassy carbon electrode

A voltammogram was drawn for 2,5-DHPAAGL by plotting the recorded peak height, which corresponds to peak current, versus the applied E_{ox} between the range 0.70-1.15V. The optimum potential for the determination of 2,5-DHPAAGL was found from the position of the plateau on the hydrodynamic wave, in this case around 1.10V, very similar to that of SA.

1.5.3 SPE of 2,5-DHPAAGL

The t_r of standard solutions of SA and 2,5-DHPAAGL were found to be 6.72 and 10.57 minutes respectively. After SPE, an unspiked extract of serum was injected onto the analytical column and there was found to be no interfering peak present at the t_r of the standard 2,5-DHPAAGL. Thereafter, the spiked extract was injected and no peak was observed at the t_r of 2,5-DHPAAGL. This extraction process was repeated with the same result indicating that 2,5-DHPAAGL was either not extracted or that it was unstable.

Table 4 List of compounds investigated for possible use as the IS for SA

NAME OF COMPOUND	RT (mins)	RT of SA (mins)
3-methoxysalicylic acid (1)	6.58	6.87
benzoic acid (1)	ND	6.87
3,4,5-trihydroxybenzoic acid (1)	2.96	6.87
4-hydroxybenzoic acid (1)	5.88	6.87
3-hydroxybenzoic acid (1)	5.52	6.87
2,4-dihydroxybenzoic acid (1)	3.44	6.87
3,4-dihydroxybenzoic acid (1)	3.96	6.87
3,5-dihydroxybenzoic acid (1)	3.15	6.87
2,3-dimethoxybenzoic acid (2)	ND	6.90
3-methoxybenzoic acid (2)	ND	6.90
3-hydroxy-4-methoxybenzoic acid (2)	7.48	6.90
3-hydroxy-4-methylbenzoic acid (2)	18.15	6.90
2-hydroxy-3-isopropylbenzoic acid (2)	ND	6.90
2,5-dihydroxyphenylacetic acid (2)	3.43	6.90
2,5-dihdroxyphenylacetic acid gamma-lactone (2)	10.42	6.90
2-bromobenzoic acid (3)	ND	6.79
2-chlorobenzoic acid (3)	ND	6.79
2-iodobenzoic acid (3)	6.78	6.79
3-fluorobenzoic acid (3)	ND	6.79
3-methylsalicylic acid (4)	24.13	8.15
4-methylsalicylic acid (4)	19.72	8.15

ND - no peak detected.
(1), (2), (3) & (4) - corresponds to the 4 days on which analysis took place.

1.5.4 **SPE of 4-MeSA**

The t_r of SA and 4-MeSA were found to be 5.82 and 11.78 minutes respectively. After SPE, the blank serum sample was injected and there was found to be no interfering peak present at the t_r of 4-MeSA (11.78 minutes). Following injections of the extract spiked with 4-MeSA and SA, two peaks were observed which eluted at the same t_r as standard 4-MeSA and SA (Fig.5b, p.54).

1.5.5 **Voltammetric behaviour of 4-MeSA at a glassy carbon electrode**

A voltammogram was drawn for 4-MeSA (Fig.6b, p.58), with the optimum applied E_{ox} for the determination of 4-MeSA found from the position of the plateau on the hydrodynamic wave, in this case sufficiently close to that of SA (1.10V) to allow the latter to be used for the detection of both compounds with acceptable sensitivity.

1.5.6 **Extraction efficiency of 4-MeSA assay**

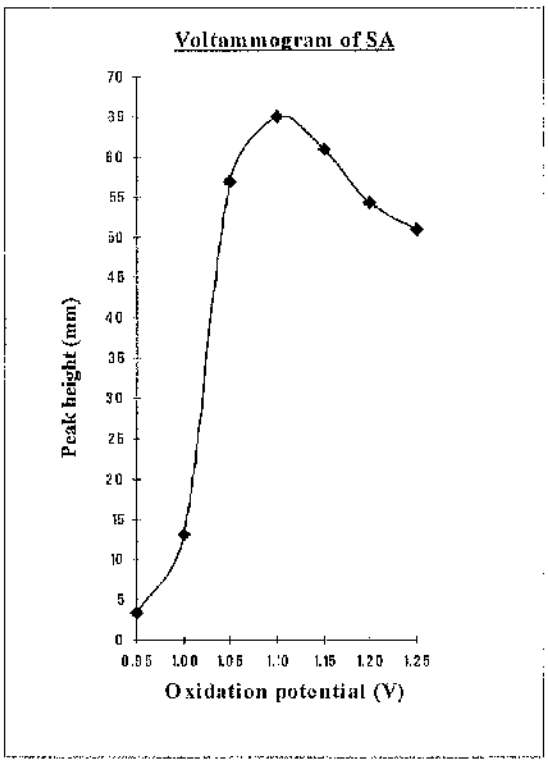
The mean extraction efficiency of 4-MeSA from serum was found to be 91% (CV = 4.1%, n=5). Results were calculated by comparing peak areas of standard solutions of 4-MeSA with equivalent concentrations to those of the extracts.

1.5.7 **Linearity of 4-MeSA assay**

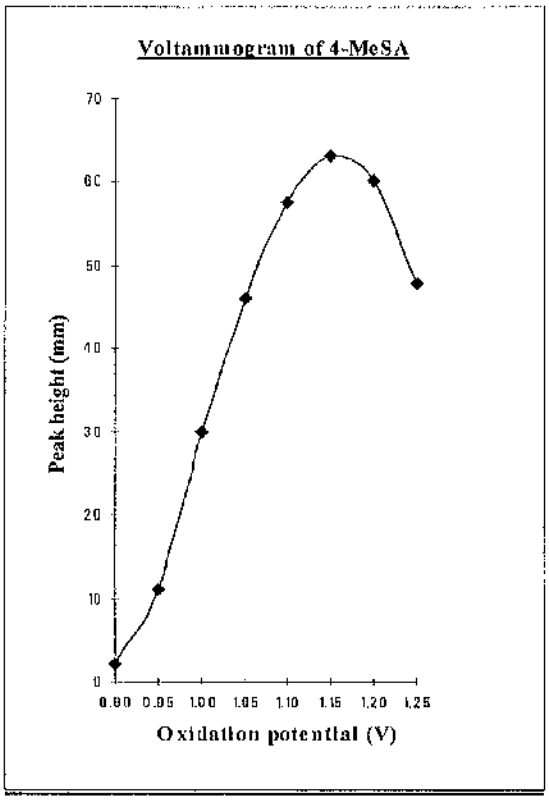
The 4-MeSA peak response was found to be linear over a concentration range of 0.1-50μM.

Fig. 6 Voltammogram of (a) SA and (b) 4-MeSA

(a)



(b)



1.6 Range and linearity

1.6.1 Range and linearity of SA analysis

The SA peak response was linear over a concentration range of 0.1-50μM with a linear regression equation y = 0.244286x(+/-0.003007) -2.429(+/-1.518) where n=8 and the standard error s = 1.949. The limit of detection defined as that amount of solute producing a signal three times the standard deviation of the noise was 15pmol/ml (103pg) of SA.

1.6.2 Level of IS required for calibration curves

The level of IS required for calibration curves to allow accurate peak quantitation across the 0.1-50μM SA standards range was found to be 25μM. This gave a peak/height ratio of around 1 at the median serum level of SA expected during low dose aspirin therapy.

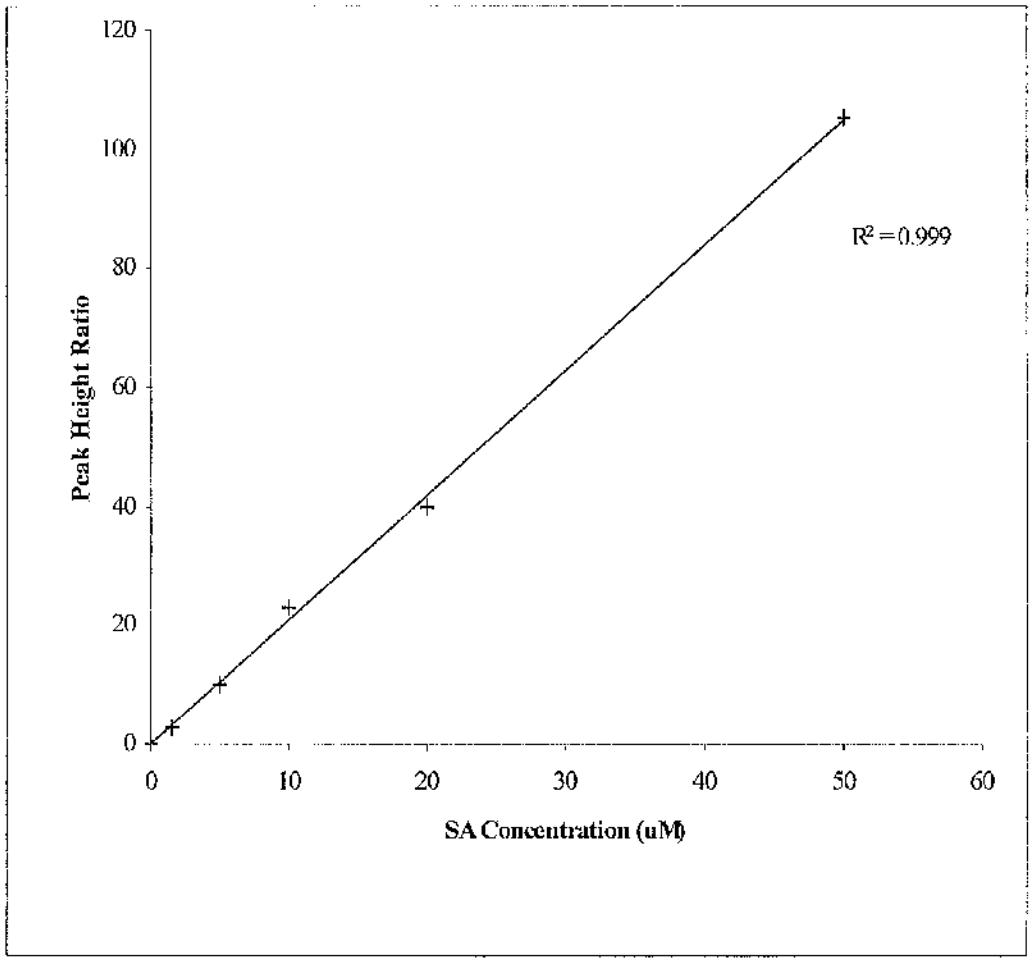
1.6.3 Pooled serum from patients

6 pools of serum from 5 patients were analysed and it was found that 2 out of 6 of the groups had interfering peaks from patients who had taken aspirin. The resulting 4 group's sera were pooled together, and aliquots of 3mls stored in the freezer at -30°C for use as blank sera in the preparation of standard curves.

1.6.4 Extracted SA calibration curve from human serum

A typical standard curve covering the concentration range expected from subjects consuming low dose aspirin is shown in Fig.7, p.60 with correlation coefficients greater than 0.997 in all cases. Correlation coefficients were calculated by the Minichrom data handling system.

Fig.7 A typical standard curve of peak height ratio versus SA concentration (μM).



1.7 Inter and intra-assay precision of SA analysis

The results of the inter and intra assay precision of 3 levels prepared and stored at -30°C are shown below in Tables 5-8, p.62 & 63. Intra-assay precision was better than that of inter-assay, as expected, and in both cases improved with increasing concentration.

1.8 Inter and intra-assay accuracy of SA analysis

Inter-assay accuracy of SA at 1.5μM, 15μM and 35μM was found to be 87.1%, 87.0% and 94.0% respectively, where n = 9 at each concentration. Intra-assay accuracy of SA at 1.5μM, 15μM and 35μM was found to be 84.1%, 94.7% and 93.4% respectively, where n = 10 at 1.5μM and 35μM and n = 9 at 15μM.

1.9 Stability of SA

After 13 days of storage there were no significant changes (outwith the inter assay precision) in the 1.5, 15 and 35μM solutions of SA in water and HCl kept at room temperature, or in serum at pH 7.3-7.5 and pH 2.5 kept at -30°C. However, when aqueous solutions of SA were examined after 20 days at room temperature, 4°C and -30°C the 1.5μM solution alone showed a significant decline of 18.2%.

Table 5 Inter-assay results at 3 concentrations of SA after extraction from human serum.

DAY NO		SA CONCENTRATION (μM)	
1	1.32	11.35	33.05
2	1.79	14.15	38.88
3	1.31	11.98	35.66
4	1.69	13.56	39.90
5	1.37	11.73	34.50
6	1.47	12.85	35.05
7	1.54	12.96	35.99
8	1.63	14.10	39.61
9	2.14	14.27	36.36

Table 6 Inter-assay variation at 3 concentrations of SA after extraction from human serum.

	MEAN (μM)	CV (No.of samples)
LOW (1.5μM)	**1.58**	**16.9% (n=9)**
MED (15μM)	**12.99**	**8.5% (n=9)**
HIGH (35μM)	**36.56**	**6.6% (n=9)**

Table 7 Intra-assay results at 3 concentrations of SA after extraction from human serum.

SAMPLE NO.		SA CONCENTRATION (μM)	
1	1.55	15.33	38.05
2	1.80	14.55	34.09
3	1.62	13.42	38.53
4	1.82	14.71	37.89
5	1.89	13.88	35.91
6	1.78	15.80	38.62
7	1.85	15.77	35.07
8	1.55	13.43	36.61
9	1.73	14.76	35.59
10	1.80	-----	38.90

Table 8 Intra-assay variation at 3 concentrations of SA after extraction from human serum.

	MEAN(μM)	CV (No. of samples)
LOW (1.5μM)	**1.74**	**7.1% (n=10)**
MED (15μM)	**14.63**	**6.2% (n=9)**
HIGH (35μM)	**36.93**	**4.6% (n=10)**

Table 9 Levels of SA in diabetic patients taking low dose aspirin with corresponding doses.

PATIENT NO.	DOSE (mg/day)	SA (μM)
1	75	0.62
2	75	3.44
3	75	5.26
4	75	10.51
5	150	0.82
6	150	1.51
7	150	8.93
8	300	0.23

1.10 Clinical application

1.10.1 Measurement of SA levels in 8 diabetic patients taking low dose aspirin

The levels of SA in patients taking low dose aspirin are shown in Table 9, p.64 along with respective doses. The range of levels to be expected from patients taking low dose aspirin is from 0.23-10.51μM, albeit from a limited number of analyses. The time of the last dose relative to the blood sampling time was not recorded, and variations in this interval may add to the variability in levels from different administered doses.

2. METHOD DEVELOPMENT OF 2,3-DHBA & 2,5-DHBA ASSAY

2.1 Voltammetric behaviour of 2,3-DHBA and 2,5-DHBA at a glassy carbon electrode.

Voltammograms were drawn for 2,3-DHBA and 2,5-DHBA (Fig.9a & b, p.67). The optimum potentials for the determination of 2,3-DHBA and 2,5-DHBA were found from the position of the plateaus on the hydrodynamic waves, in this case between 0.70-0.75V for both analytes.

2.2 Confirmation of the presence of 2,3-DHBA and 2,5-DHBA in human blank serum in subjects not taking aspirin.

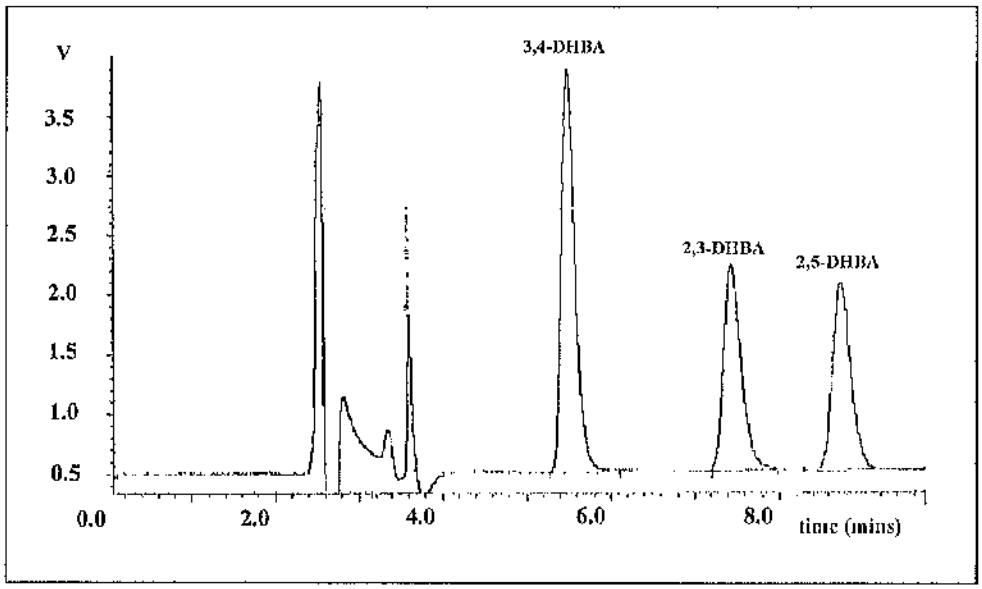
2.2.1 Patient samples (n=11) analysed for the presence of 2,3-DHBA and 2,5-DHBA

Chromatograms of all of the patient samples assayed showed the presence of unknown peaks with t_r identical to 2,3-DHBA and 2,5-DHBA (Fig. 8b, p.66).

2.2.2 Comparison of peak height ratios for blank serum unknown peaks, 2,3-DHBA and 2,5-DHBA by varying the E_{ox}

In order to obtain evidence of identity beyond similarity of retention time, blank serum extracts and standard solutions of 2,3-DHBA and 2,5-DHBA were subjected to HPLC at E_{ox} 0.45V, 0.55V and 0.65V. Heights of serum derived peaks with retention times similar to these standards, obtained at different E_{ox}'s, were expressed as ratios (0.55/0.45 ; 0.65/0.55, 0.65/0.45) and compared with those of the standards (Table 10, p.68). Voltammetrically, the bank serum peak of t_r 6.2 minutes was similar to 2,3-DHBA, while the serum peak of t_r 6.6 minute was similar to 2,5-DHBA.

Fig. 8 Chromatograms showing (a) a standard containing 3,4-DHBA, 2,3-DHBA and 2,5-DHBA (75nM of each) and (b) an extract from a subject known not to have consumed aspirin.



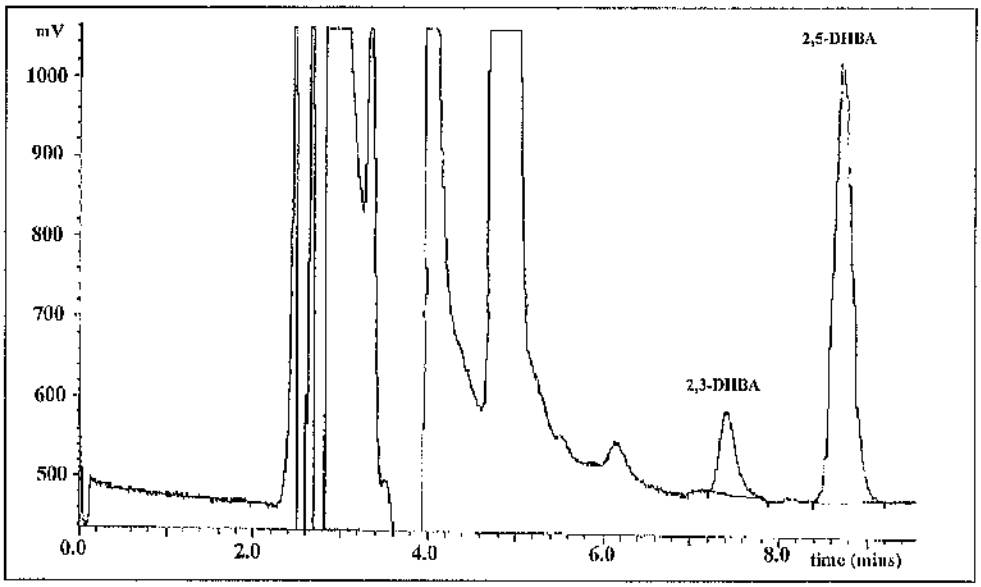
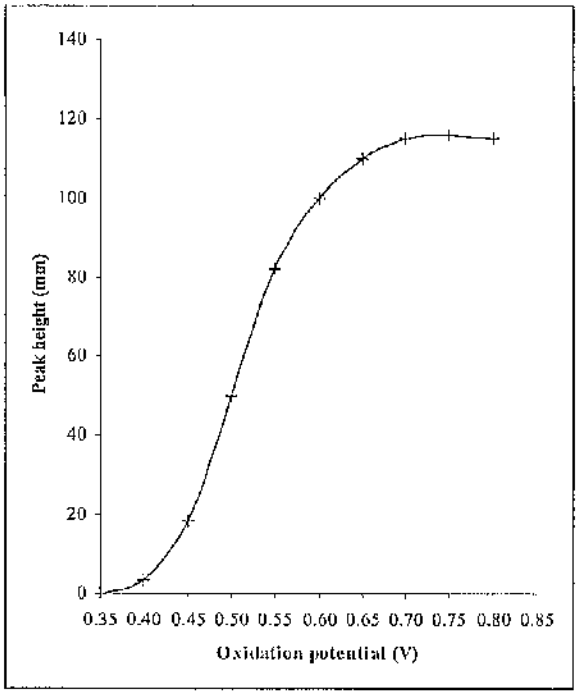


Fig.9 Voltammogram of (a) 2,3-DHBA and (b) 2,5-DHBA.

(a)



(b)

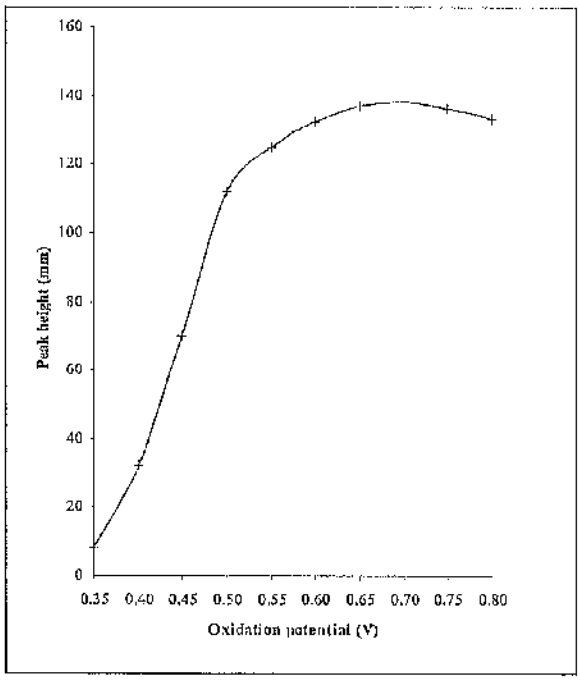


Table 10 Verification of peaks from control serum and aspirin takers identified by t_r comparison of peak height ratios obtained at different E_{ox}.

SAMPLES	PEAK HEIGHT RATIOS		
DW blank serum	0.55V/0.45V	0.65V/0.45V	0.65V/0.55V
t_r of first unknown in blank serum: 6.25min	4.00	1.45	5.80
t_r of second unknown in blank serum: 6.68min	1.50	1.21	1.82
t_r of 2,3-DHBA*: 6.21min	4.49	1.28	5.73
t_r of 2,5-DHBA*: 6.64min	1.90	1.07	1.97

* - The 2,3-DHBA and 2,5-DHBA standard solutions were both prepared to a concentration of 10nM.

Table 11 Stability of 2,3-DHBA and 2,5-DHBA over a period of 4 hours using the autosampler.

Time of injection (hrs)	2,3-DHBA Pk.ht.(mV)	%Degradation of 2,3-DHBA	2,5-DHBA Pk.ht.(mV)	%Degradation, of 2,5-DHBA
0	425.7	0.0	479.0	0.0
0.5	420.9	1.2	474.2	2.0
1.0	413.0	3.0	441.3	7.9
1.5	403.9	5.2	438.5	8.5
2.0	416.5	2.2	433.3	9.5
2.5	391.5	8.2	438.7	8.4
3.0	399.4	6.2	367.2	23.7
3.5	348.3	18.3	430.9	10.0
4.0	407.7	4.3	389.0	18.8

2.3 Extraction efficiencies of 2,3-DHBA and 2,5-DHBA

The extraction efficiencies of 2,3-DHBA and 2,5-DHBA were calculated in duplicate. Since 2,3-DHBA and 2,5-DHBA were thought to be present at low concentrations in the blank sera, the extraction efficiencies were calculated by subtracting the peak height of the blank levels from the final peak height of the spiked sample. Extraction efficiencies of 79% and 73% for 2,3-DHBA and 2,5-DHBA were calculated respectively.

2.4 Stability of 2,3-DHBA and 2,5-DHBA while using the HPLC autosampler

Results indicated (Table 11, p.68) that 2,3-DHBA and 2,5-DHBA were only stable in aqueous solution for a period of 2.5 hours. This was based on setting an acceptance criteria of less than 10% degradation of the analyte compared to the peak height of injection at time zero. The results show that the two analytes are relatively unstable in aqueous solution.

2.5 IS for 2,3-DHBA and 2,5-DHBA assay

2.5.1 Voltammetric behaviour of 3,4-DHPAA at a glassy carbon electrode

A voltammogram was drawn for 3,4-DHPAA. The optimum potential for the determination of 3,4-DHPAA was found from the position of the plateau on the hydrodynamic wave, in this case around 0.70V, similar to the position of the plateau for 2,3-DHBA and 2,5-DHBA.

2.5.2 **Stability of 3,4-DHPAA while using the HPLC autosampler**

Repeat injections of 3,4-DHPAA were shown to be stable for only 1.5hrs, but when mixtures of 2,3-DHBA, 2,5-DHBA and 3,4-DHPAA were injected, the 3,4-DHPAA degraded significantly faster compared to when 3,4-DHPAA was injected alone (Table 12, p.70). Injections where mixtures of 3,4-DHPAA, 2,3-DHBA and 2,5-DHBA were injected are highlighted in the table by the use of bold type print.

Table 12 Stability of 3,4-DHPAA over a period of 6 hours using the autosampler.

Injection time (hrs)	3,4-DHPAA Pk ht(mV)	% Degradation of 3,4-DHPAA
0.0	253.4	0.0
0.5	238.9	5.7
1.0	**158.6**	**37.4**
1.5	230.2	9.2
2.0	198.5	21.7
2.5	**111.6**	**56.0**
3.0	195.1	23.0
3.5	222.8	12.1
4.0	**62.7**	**75.3**
4.5	202.0	20.3
5.0	168.6	33.5
5.5	**52.7**	**79.2**
6.0	192.6	24.0

2.5.3 Continued search for a suitable IS for 2,3-DHBA and 2,5-DHBA assay

The t_r of the 2,3-DHBA and 2,5-DHBA were 8.36 and 8.96 minutes respectively. Of the six compounds injected, only two oxidised with the E_{ox} at 0.70V. 3,4-DHBA had a t_r of 11.35 minutes and 3,4,5-trihydroxybenzoic acid a t_r of 5.65 minutes.

2.5.4 Stability of 3,4-DHBA

The stability of 3,4-DHBA in different aqueous solutions was carried out over a period of 9 hours. The results are shown in Tables 13a, b & c, p.80.

2.6 Optimisation of chromatographic and extraction conditions

2.6.1 Tri-sodium citrate (30mM) mobile phase at pH 4.75; examining the effect of overlap of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA peaks with increasing analyte concentration.

As the concentration of the 3 analytes at the 3 different analyte concentrations increased the more poorly resolved they became. However at concentrations thought to approximate the levels in patients taking low dose aspirin, the resolution of the peaks was satisfactory.

2.7 Search for a more suitable mobile phase

The mobile phase consisting of tri-sodium citrate (30mM) with the pH adjusted to 4.75 using glacial acetic acid resulted in the retention times of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA being 8.34, 8.93 and 11.34 minutes respectively. In order to baseline resolve the 2,3-DHBA and 2,5-DHBA peaks, some alterations to the mobile phase content were made.

2.7.1 **Addition of 4.75% acetonitrile to the mobile phase**

Addition of the 4.75% acetonitrile to the mobile phase. In comparison with the original mobile phase, the t_r of the 2,3-DHBA, 2,5-DHBA and 3,4-DHBA using 4.75% acetonitrile were 5.00, 5.08 and 5.72 minutes respectively. From these results we can see that the acetonitrile causes elution of all 3 analytes more quickly but the 2,5-DHBA peak co-elutes with the 2,3-DHBA peak giving poorer resolution (Fig.10a, p.73).

2.7.2 **Addition of 1.5% methanol to the mobile phase**

The t_r for 2,3-DHBA, 2,5-DHBA and 3,4-DHBA when 1.5% methanol was incorporated into the mobile phase were 7.18, 7.68 and 8.99 minutes respectively. The addition of methanol made each analyte elute more quickly but resolution was not improved over the original mobile phase due to "saddling" of the 2,3-DHBA and 2,5-DHBA peaks (Fig.10b, p.74).

2.7.3 **The effect of varying the pH of the mobile phase on resolution**

5 separate mobile phases were prepared with pH values of 4.75, 5.0, 5.2, 5.3 and 5.4 and the t_r and the detector response of each analyte noted under the different pH conditions (Table 14, p.81). Chromatograms at each pH are shown in Fig.11a-e, p.75-79) which demonstrate the significant effect the pH of the mobile phase has on the 3 analytes. As the pH increases from 4.75 - 5.4 the 2,3-DHBA and 2,5-DHBA peaks move very slowly apart *i.e.* the 2,3-DHBA elutes more quickly and the 2,5-DHBA more slowly. As the pH is increased, the 3,4-DHBA peak elutes much more rapidly and passes across the 2,3-DHBA and the 2,5-DHBA at pH 5.0 and elutes before them at pH 5.2. For every increase in pH of 0.1, the 3,4-DHBA elutes more quickly by around 0.85 minutes.

Fig. 10a Chromatogram showing the effect of adding 4.75% acetonitrile to the mobile phase on the resolution of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA.

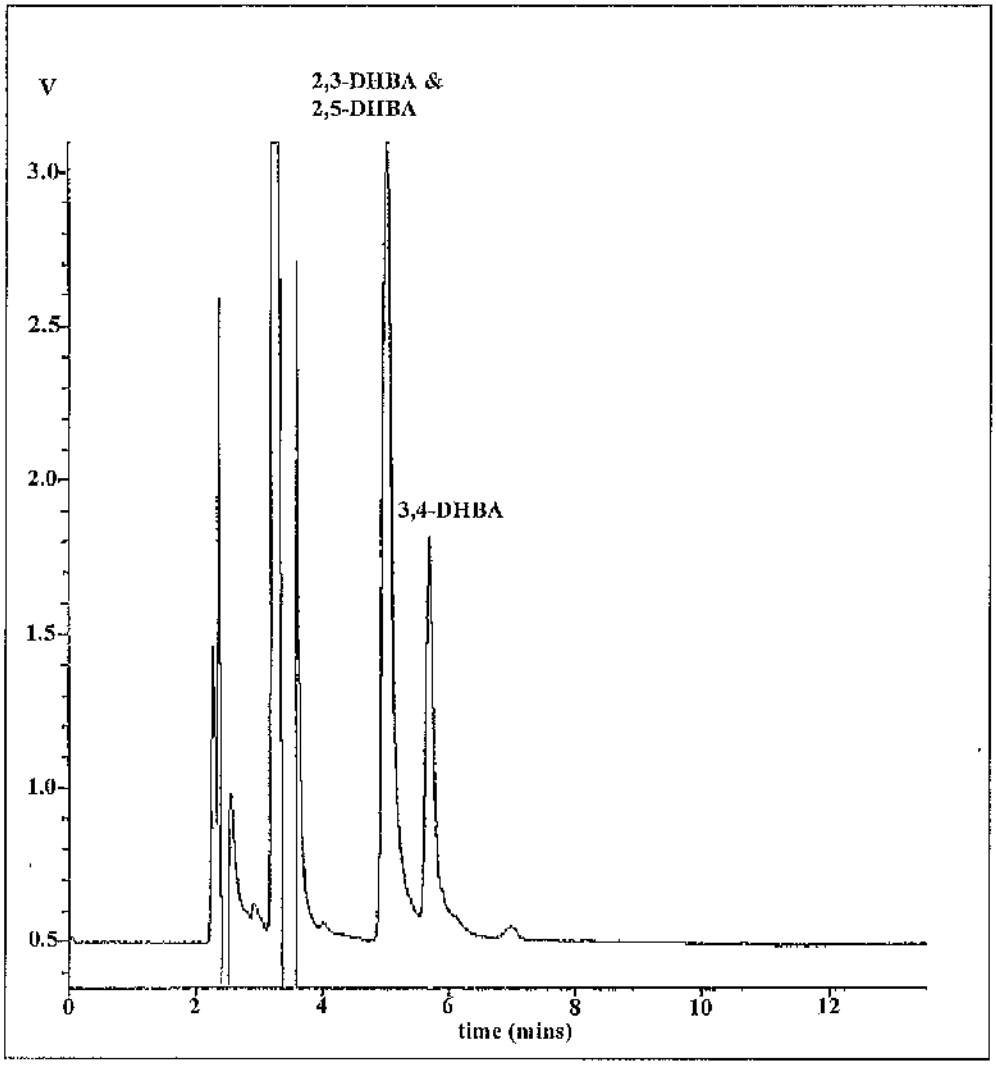


Fig.10b Chromatogram showing the effect of adding 1.5% methanol to the mobile phase on the resolution of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA.

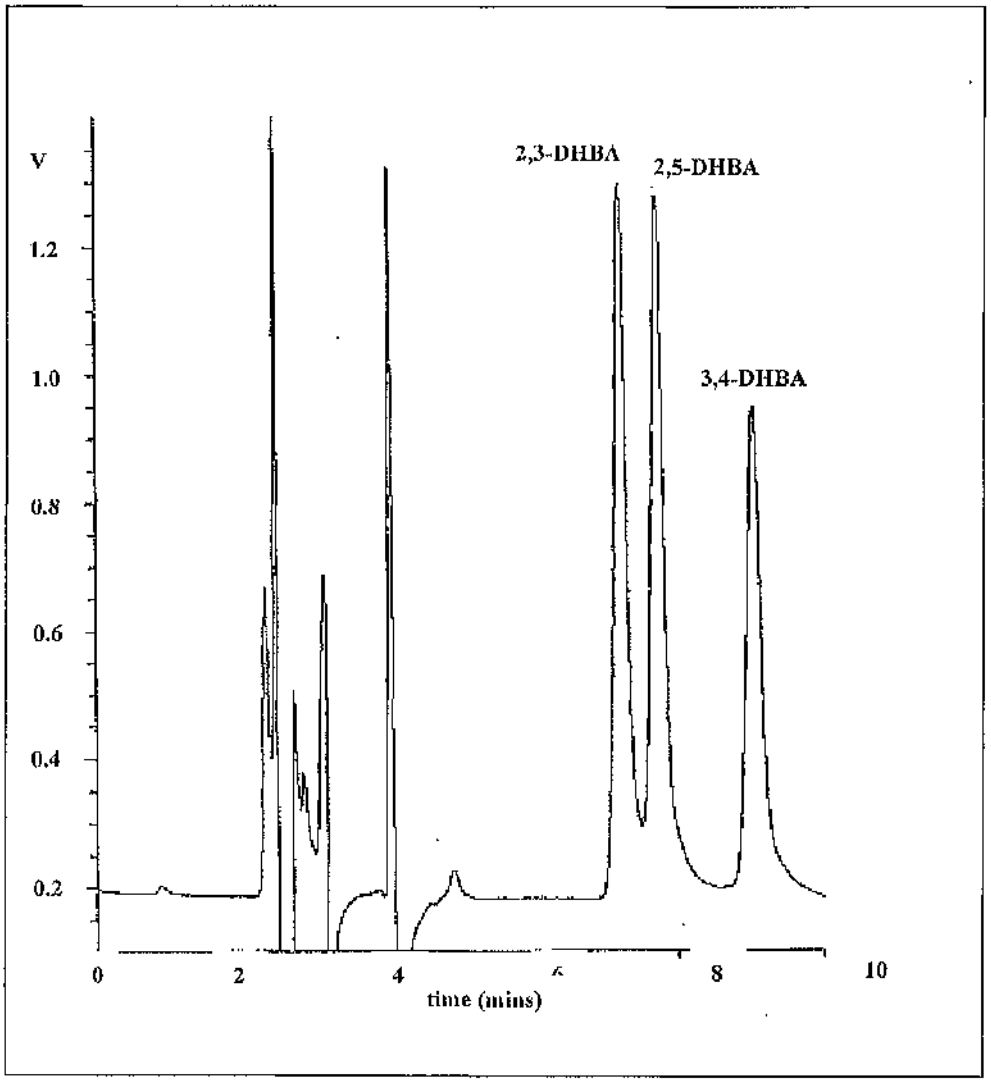


Fig. 11a Chromatogram showing the effect of pH on the resolution of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA at pH 4.75.

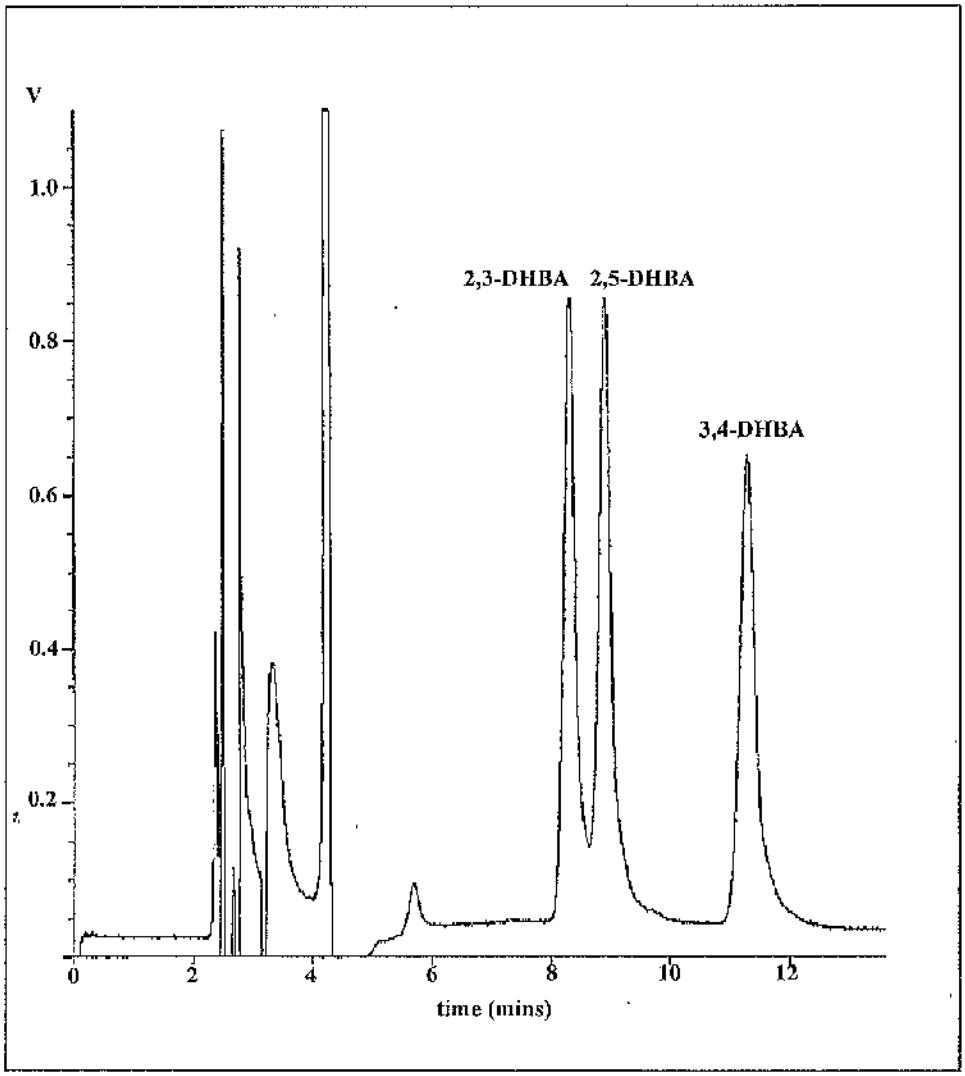


Fig. 11b Chromatogram showing the effect of pH on the resolution of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA at pH 5.0.

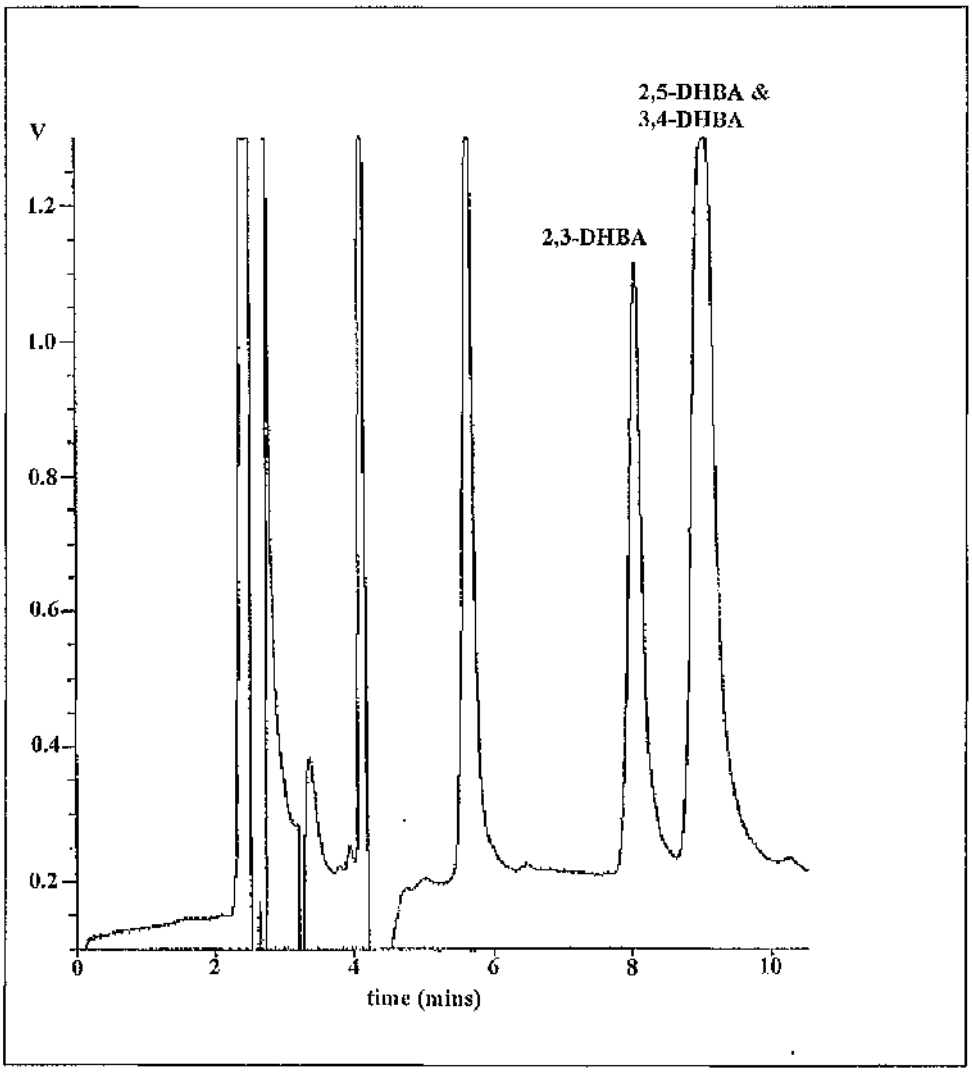


Fig. 11c Chromatogram showing the effect of pH on the resolution of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA at pH 5.2.

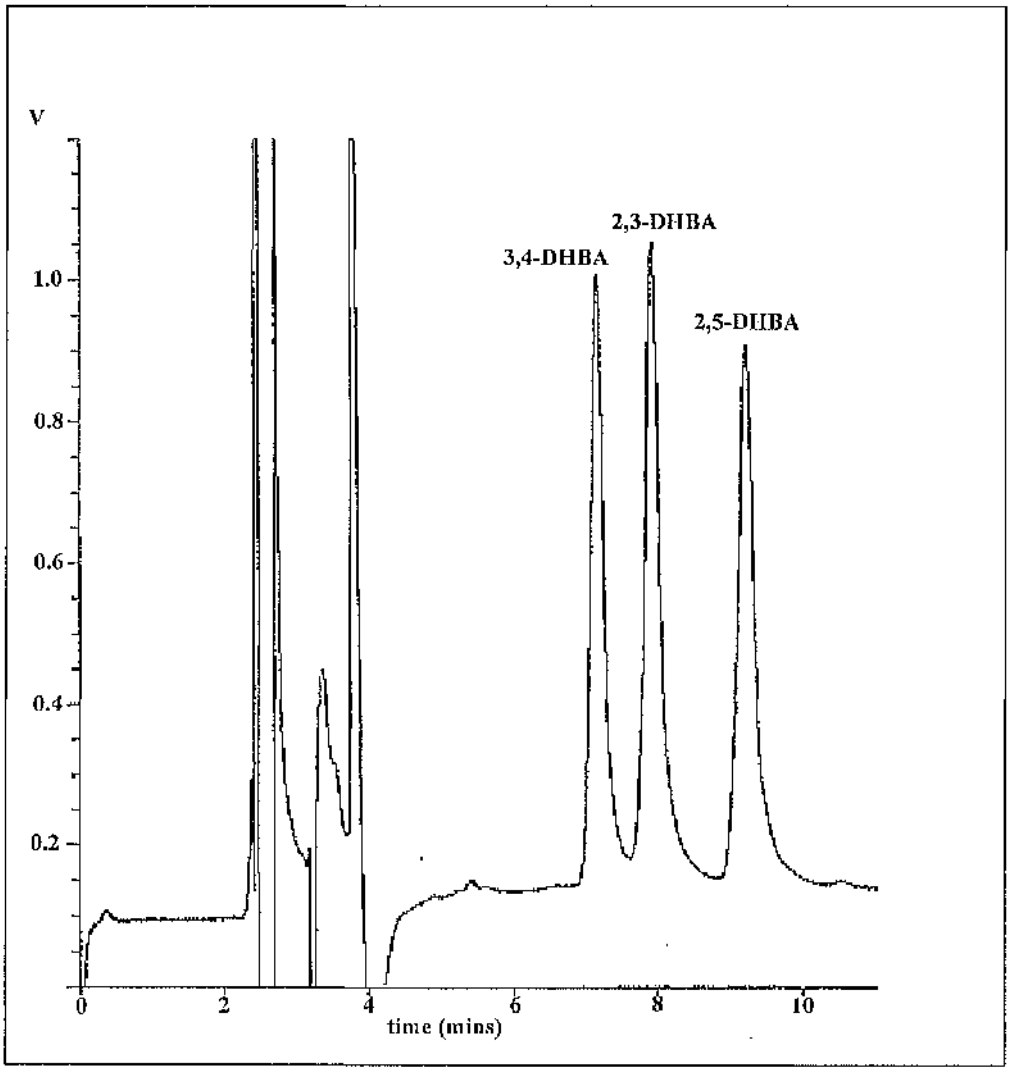


Fig. 11d Chromatogram showing the effect of pH on the resolution of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA at pH 5.3.

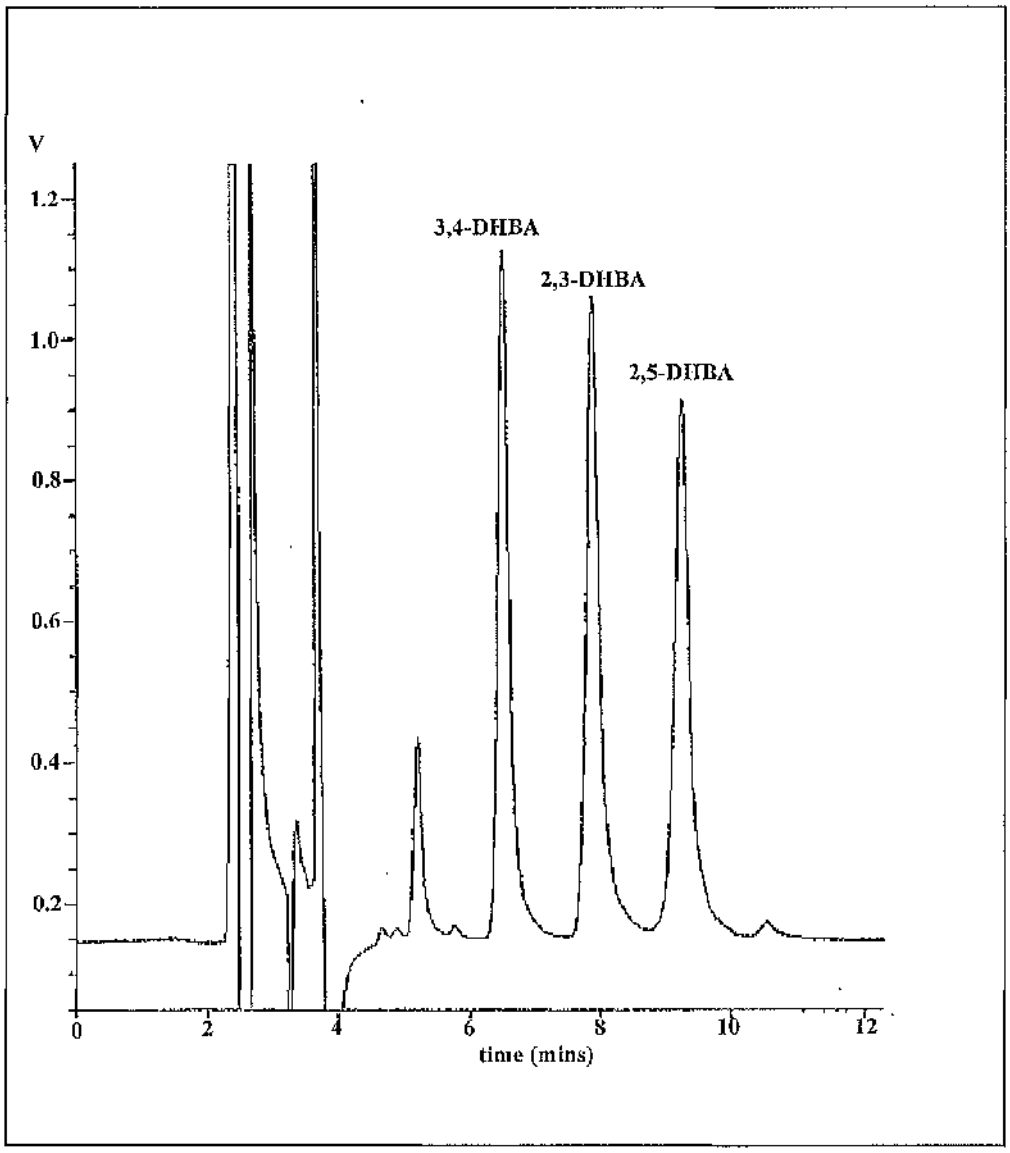


Fig. 11e Chromatogram showing the effect of pH on the resolution of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA at pH 5.4.

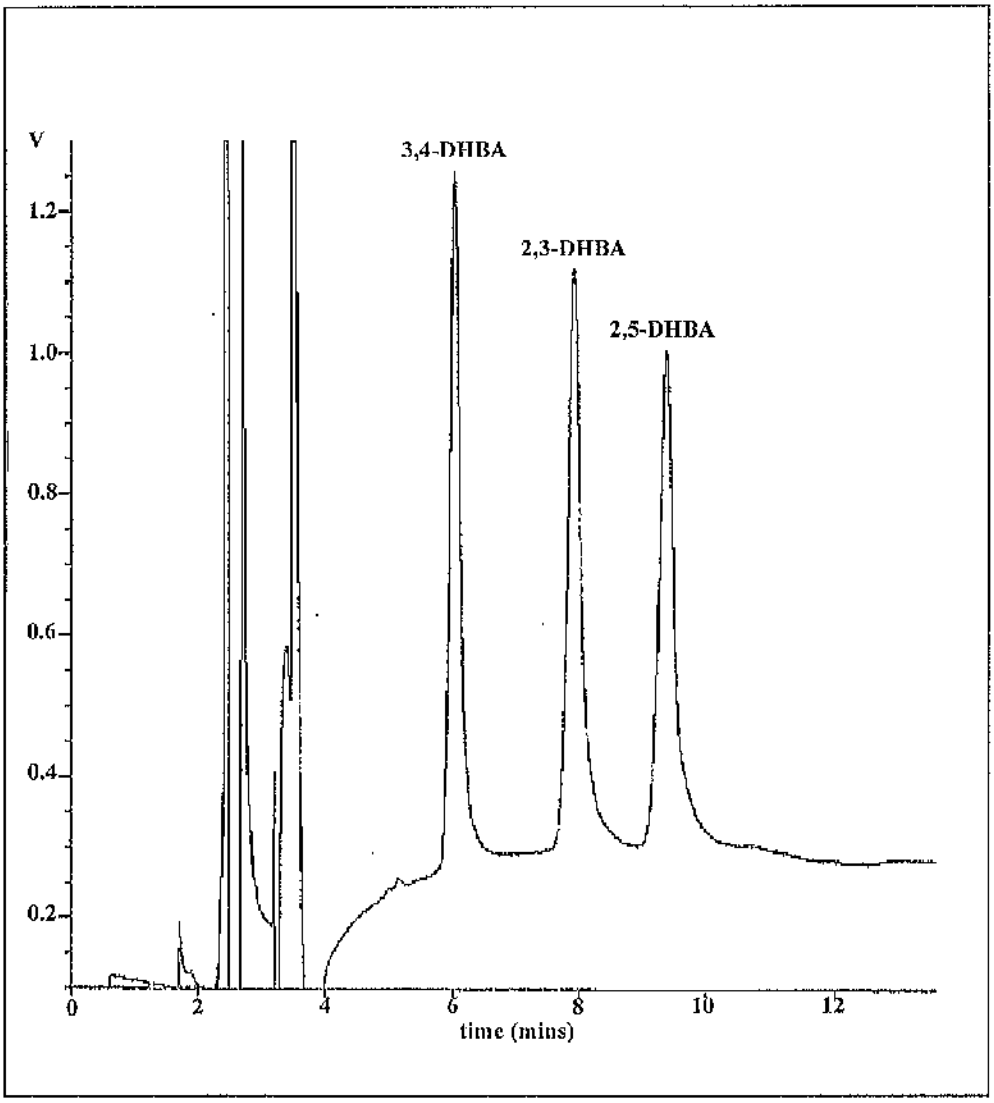


Table 13 Stability of 3,4-DHBA over a period of 9 hours in (a) H_2O, (b) 0.05M HCl and (c) mobile phase (MP)

(a) 3,4 DHBA in H_2O

Time (hrs)	PkHt (mVs)
0	1972.4
4.00	1968.2
4.67	1966.5
5.33	1958.8
6.00	1936.4
6.67	1922.5
7.33	1937.7
8.00	1939.7
8.67	1922.5

(b) 3,4 DHBA in 0.05M HCl

Time (hrs)	PkHt (mVs)
0	2863.8
4.00	2861.5
4.67	2864.1
5.33	2864.7
6.00	2870.2
6.67	2865.0
7.33	2863.6
8.00	2858.0
8.67	2842.0

(c) 3,4 DHBA in MP

Time (hrs)	PkHt (mVs)
0	2582.7
4.00	2580.2
4.67	2591.5
5.33	2593.8
6.00	2585.2
6.67	2590.4
7.33	2596.7
8.00	2590.7
8.67	2571.9

Table 14 The t_r and detector response of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA under differing mobile phase pH conditions.

	Pk Ht (mVs)			t_r (mins)		
pH	2,3-DHBA	2,5-DHBA	3,4-DHBA	2,3-DHBA	2,5-DHBA	3,4-DHBA
4.75	2636.3	2493.1	1879.4	8.34	8.93	11.34
5.0	2687.7	**Overlap**	**Overlap**	8.05	9.07	9.07
5.2	2640.7	2164.3	2633.7	7.93	9.25	7.20
5.3	2853.9	2559.2	2712.6	7.88	9.25	6.52
5.4	2322.5	1984.3	2858.0	7.92	9.38	5.92

Table 15 Percentage carryover of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA after different numbers of washes with 50% methanol/water.

	% Carryover		
No of washes	**3,4-DHBA**	**2,3-DHBA**	**2,5-DHBA**
1	2.4	14.4	11.2
2	1.8	17.2	8.5
3	1.2	11.5	4.5
4	1.6	8.9	5.4
5	1.5	5.5	2.9

2.8 Optimisation of SPE for 2,3-DHBA and 2,5-DHBA assay

2.8.1 Analysis of SPE eluent fractions for 2,3-DHBA and 2,5-DHBA

In the first 1.0ml of eluent collected, 85% and 88% of 2,3-DHBA and 2,5-DHBA were recovered respectively (% of total recovery), with very little collected in the final 4mls as shown in Fig.12, p.83. To obtain a good recovery of 2,3-DHBA and 2,5-DHBA from the SPE process, less eluent could be used.

2.8.2 SPE of blank serum using the new mobile phase at pH 5.3.

Serum was extracted from a subject known not to be taking aspirin and the t_r of the unknown peaks in blank serum, thought to be 2,3-DHBA and 2,5-DHBA, had identical t_r to standard solutions of 2,3-DHBA and 2,5-DHBA. Although the pH of the mobile phase had changed, the two unknown peaks co-eluted with the 2,3-DHBA and 2,5-DHBA standards suggesting the presence of these compounds in non-aspirin takers.

2.8.3 Setting up a gradient to elute unwanted peaks.

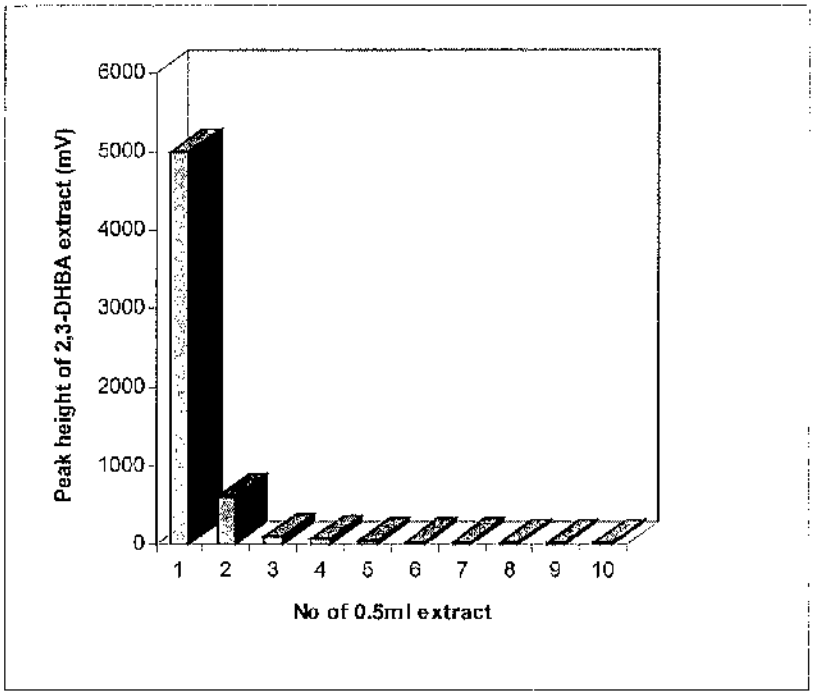
4 gradient programmes were set up in order to reduce the time at which the final peak eluted. Chromatograms were obtained showing the elution of late eluting peaks at around 30 minutes. The program that allowed subsequent injections to be analysed with the shortest time between injections was program 3.

2.9 The effect of carryover of peaks

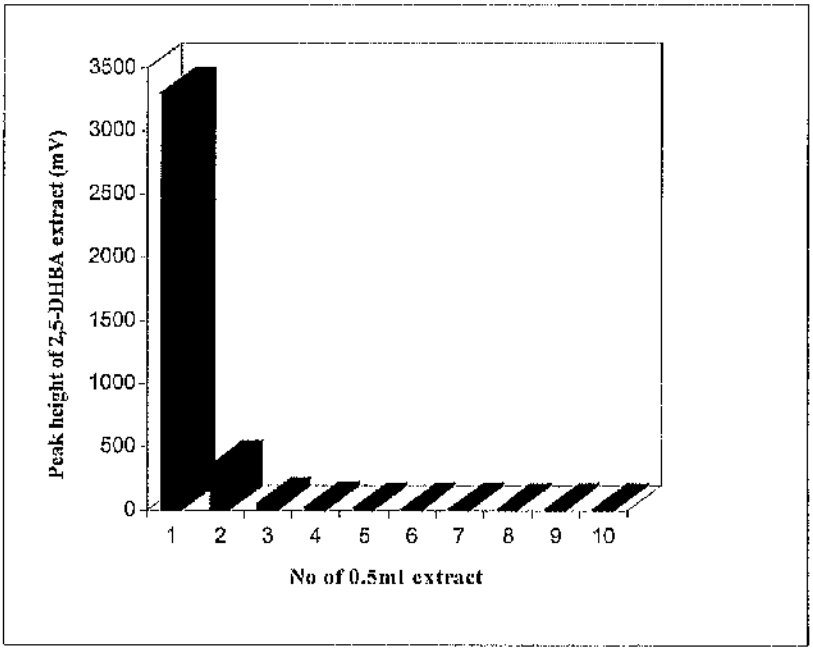
After injection of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA with an injection of 0.05M HCl between each standard, carryover existed from one sample to the next. In order to overcome this problem, two experiments were carried out.

Fig. 12 Analysis of SPE eluent fractions for 2,3-DHBA and 2,5-DHBA showing peak heights obtained from each 0.5ml extracted.

(a)



(b)



2.9.1 **The effect of the number of washes of the autosampler needle on carryover.**

The results of the effect of carryover after injections of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA followed by varying number of washes (1-5) with 50% methanol/water are shown in Table 15, p.81. We can see that although results do improve with the number of washes, there is still some carryover of each analyte after the maximum number of washes.

2.9.2 **The investigation of different wash solutions.**

The effect of four different wash solutions on carryover of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA were investigated with the results shown in Table 16, p.84. We can see that 0.2M HCl eliminates carryover of 2,3-DHBA and 2,5-DHBA from standard solutions but that a small amount of 3,4-DHBA still remains.

Table 16 Percentage carryover of 2,3-DHBA, 2,5-DHBA and 3,4-DHBA using different wash solutions.

Wash solution (5 washes between inj.)	% Carryover		
	3,4-DHBA	2,3-DHBA	2,5-DHBA
50%MeOH:0.05M HCl	1.0	6.6	3.8
0.05mM HCl	0.7	4.5	2.5
0.10mM HCl	0.8	2.5	0.0
0.20mM HCl	1.0	0.0	0.0

DISCUSSION

1.1 Optimisation of chromatographic conditions for the SA assay.

The optimisation of the chromatographic conditions in the development of an HPLC method is essential in order to be able to produce a robust and reliable method. Some of the factors that were taken into account when developing the SA method are discussed below.

The first step in the development of the method for SA was to find a suitable mobile phase. An ideal mobile phase could be defined as one that allows good resolution of the analytes of interest from any interference after extraction and which results in a short elution time leading to a fast throughput of samples. The mobile phase used for the identification of SA in our developed method was similar to that used by Grootveld and Halliwell for the detection of 2,3-DHBA and 2,5-DHBA, structurally similar compounds to SA (See Fig.3, p.15 for 2,3-DHBA and 2,5-DHBA structures).[50]

One way in which to elute peaks more quickly under reversed phase conditions is to change the polarity of the mobile phase. In this case the methanol content of the mobile phase was increased and we can see from Table 1, p.52 that as you increase the organic content of the mobile phase then the peaks elute more quickly. The mobile phase comprising of a methanolic concentration of 28.6%(v/v) results in a t_r of 5.82 minutes for SA and good resolution from any possible interference after extraction from serum (Fig 5b, p.54). Evans *et al* investigated the oxidation of SA at a glassy carbon planar electrode and showed that the electrode reaction was found to be dependent on the pH and the ionic strength of the buffer content of the mobile phase.[49] Further work showed that a maximal electrochemical signal was obtained with a supporting electrolyte concentration of 0.06mol dm^{-3} acetate buffer in 35% methanol (pH 5.0). The magnitude of the response was observed to increase by about 10% when the methanol concentration was decreased from 35% to 8%. However, this was thought to be due to solubility of SA or possibly changes in conductivity of the electrolyte.

Chromatographic run times are dependent on the mobile phase content and determined by the elution time of analytes, IS and of any background peaks which might interfere with subsequent injections, these latter being a particular problem at low analyte concentrations. At low SA concentrations (0.1-50μM), the cleaner extracts obtained using SPE allowed a run time of 35 minutes to be achieved. Evans *et al* found it necessary to reduce the methanol content of the mobile phase from 35% to 8% to separate SA from interfering peaks.[49] As a result of reducing the organic content of the mobile phase, they obtained a t_r of 11 minutes for SA suggesting a longer run time for their method, however, no data was provided on run time in their paper. Vree *et al*, however, reported a t_r of 32 minutes for SA in their non-extraction assay but used a gradient method to elute unwanted peaks, thereby allowing a quicker throughput of samples.[55] A further 5 minutes was required to elute late interfering peaks, and in addition, time would be required for equilibration of the HPLC system to the initial mobile phase conditions. The time between each injection would then be approximately 50 minutes, almost twice as long as our current method. Once a suitable mobile phase had been chosen which provided good resolution of the SA peak and satisfactory run times, a suitable extraction technique for extracting SA from serum had to be developed. In the literature either direct injection or liquid-liquid extraction techniques are described but it was thought that SPE may be a better option in order to obtain "cleaner" chromatograms thereby permitting shorter elution times and a greater potential choice of IS. Initially HAX columns (anion exchange columns) were used since it was believed that SA could undergo ionic interactions under suitable pH conditions with an ionic exchange column. The SPE procedure that was followed suggested adjusting the pH of the serum to 2.5 using 1.0M HCl. The pKa value of the carboxylic acid group of SA is 2.98. According to the acidic dissociation constant, less than half of the carboxylic acid groups of SA will be negatively charged at a pH of 2.5, therefore it would be expected that the extraction recoveries achieved at this pH would be less than 50% if an anion exchange column was used. Interestingly, the extraction recoveries using these conditions were of the order of 85%, not a theoretically achievable value based on anion exchange alone.

The manufacturer of the columns (Jones chromatography) was contacted and they explained that the packing material contained not only anion exchange material but also some non-polar material. Other interactions between the sorbent packing material and the analyte were thought to be taking place. The structure of SA (Fig 1b, p.1) would suggest that non-polar interactions could take place between the SPE column and the four ring carbons of the benzene ring which have no substituents attached.

Stewart *et al* reported that C_{18} columns (Bond-Elut octadecylsilane) produced recoveries of 91.8% for SA in acidified plasma but that other columns, including anion exchange columns, produced recoveries consistently below 15% from acidified plasma.[56] Moore and Tebbett, looking at the rapid extraction of anti-inflammatory drugs in whole blood for HPLC analysis, reported that C_8 columns produced the best recoveries (85% for SA at 15ug/ml, 100μM).[57]

The two references suggest that acidified plasma will undergo non-polar interactions with high extraction recoveries. Another plausible explanation is that carboxylic acid and hydroxyl functional groups may interact with free silanol groups on the surface of the sorbent via hydrogen bonds. It is likely that a combination of these interactions is responsible for the high extraction recoveries obtained during this work.

Before it was discovered that the SPE columns contained non-polar packing material, optimisation of the extraction procedure was investigated by varying serum pH between 2.0 and 2.7, extracting each sample and monitoring extraction recoveries. During routine experiments involving extraction, serum pH varied from experiment to experiment and sample to sample since additions of small amounts of HCl caused variable pH values. On assessing what effect this had on extraction efficiencies, it was found to have no significant effect on the SA recoveries (Table 3, p.55). This finding supports the hypothesis that non-polar interactions are taking place since pH does not seem to have any significant effect on the extraction recoveries.

When validating methods which involve extraction procedures, it is essential that a suitable IS can be found. One of the advantages of using SPE when looking for an IS is that cleaner extracts

were produced, resulting in fewer interfering peaks therefore more baseline where there was no interference present for an IS to elute. This enabled an IS to be chosen more easily.

Interfering peaks were a problem for Grootveld *et al*, including those due to catecholamine peaks (Fig.4a, p.21),[50] whilst Vree *et al* published a chromatogram containing many peaks in the first 20 minutes where it would not be possible for an IS to elute due to interference.[55] Evans *et al* published chromatograms which were crowded with peaks and they were unable to find a suitable IS.[49] Shen *et al* also published chromatograms which were very crowded and although an IS was chosen, complete resolution of the IS and interfering peaks was not achieved.[45]

After looking at a number of compounds (Table 4, p.56) a suitable IS was found; 4-MeSA. 2,5-DHPAAGL was investigated as a possible IS since it was well resolved from any interference and had similar voltammetric behaviour to SA. However, when blank serum was spiked with 2,5-DHPAAGL and extracted, no peak corresponding to 2,5-DHPAAGL was found to be present in the chromatogram of the extracted sample. It was likely that an intermediate reaction had taken place causing breakdown of the lactone during the extraction process. It is known that acyclic esters and cyclic esters (lactones) undergo hydrolysis at acid pH to yield carboxylic acids and alcohols and this may have been the fate of the 2,5-DHPAAGL.

4-MeSA was found to elute free from any interference from extracted serum with a t_r of 12 minutes (Fig.5b, p.54). 4-MeSA had a similar voltammetric profile to that of SA (Fig.6a&b, p.58), extracted with good efficiency and standard curves produced correlation coefficients of typically greater than 0.997 using 4-MeSA in peak height ratio calculations (Fig.7, p.60). 4-MeSA was found to be linear over a concentration range (0.1-50μM) equivalent to that of the SA standard curve range. The difficulty in finding a suitable IS is clearly shown in published methods where no IS was used by Evans *et al*[49] and Reidl [58], and although a large number of IS (35) were examined by Siebert and Bochner, none were found to be suitable.[59] Shen *et al* claimed to have found a suitable IS in *m*-hydroxybenzoic acid, but chromatograms in their paper are crowded, with the SA and IS peaks on a steep decline and not completely resolved.[15] Mays *et al*[60] and

Eliott Cham *et al*[61] used mephenytoin and *o*-methoxybenzoic acid respectively as their IS with good resolution of the SA from the IS, but these methods lack the required sensitivity to detect low levels of SA produced after oral administration of low-dose aspirin.

Very few methods, with the exception of Evans *et al*[49], use ECD. The method described in this thesis permits concentrations of SA standards equivalent to those of patients taking low-dose aspirin (0.1-50μM) to be detected.

Precision was thought to be an important factor since very low analyte concentrations were to be quantified. Air bubbles were a common source of problems even when the mobile phase was continually sparged with helium. An on-line degassing unit was acquired in order to overcome this difficulty, thereby substantially reducing the amount of dissolved air. Air bubbles frequently congregated in the reference electrode (part of the main cell component), causing poor electrical conductance in the salt bridge (3.0M KCl). This resulted in noisy baselines and poor detector response. The setting up of the reference electrode was a process of trial and error but with experience using this equipment, these problems with the air bubbles became less of an issue. After setting up the main cell of the detector and allowing equilibration time, analysis took place with further problems arising from fouling of the glassy carbon electrode. This caused increased background noise and reduced sensitivity. HPLC grade water and smaller filter pore size were introduced for removing dissolved gases. The pore size of the filters were changed from 0.45 micron to 0.22 micron to minimise the amount of dissolved air. The supplier of the ECD informed us that the cartridges used for filtering the water release tertiary amines and these amines have the capability of fouling the carbon electrode when oxidised. To clean the glassy carbon surface after light fouling, a piece of tissue saturated with ethanol/methanol was used to wipe the surface clean. If there were deposits engrained on the surface and the surface was not clear after light cleaning, then it was necessary for diamond slurry to be applied to a glass-backed polishing mat and the surface polished in this manner. Thus, as a result of the careful set up of the ECD, greater sensitivity was achieved compared with previously published work (Table 17, p.90).

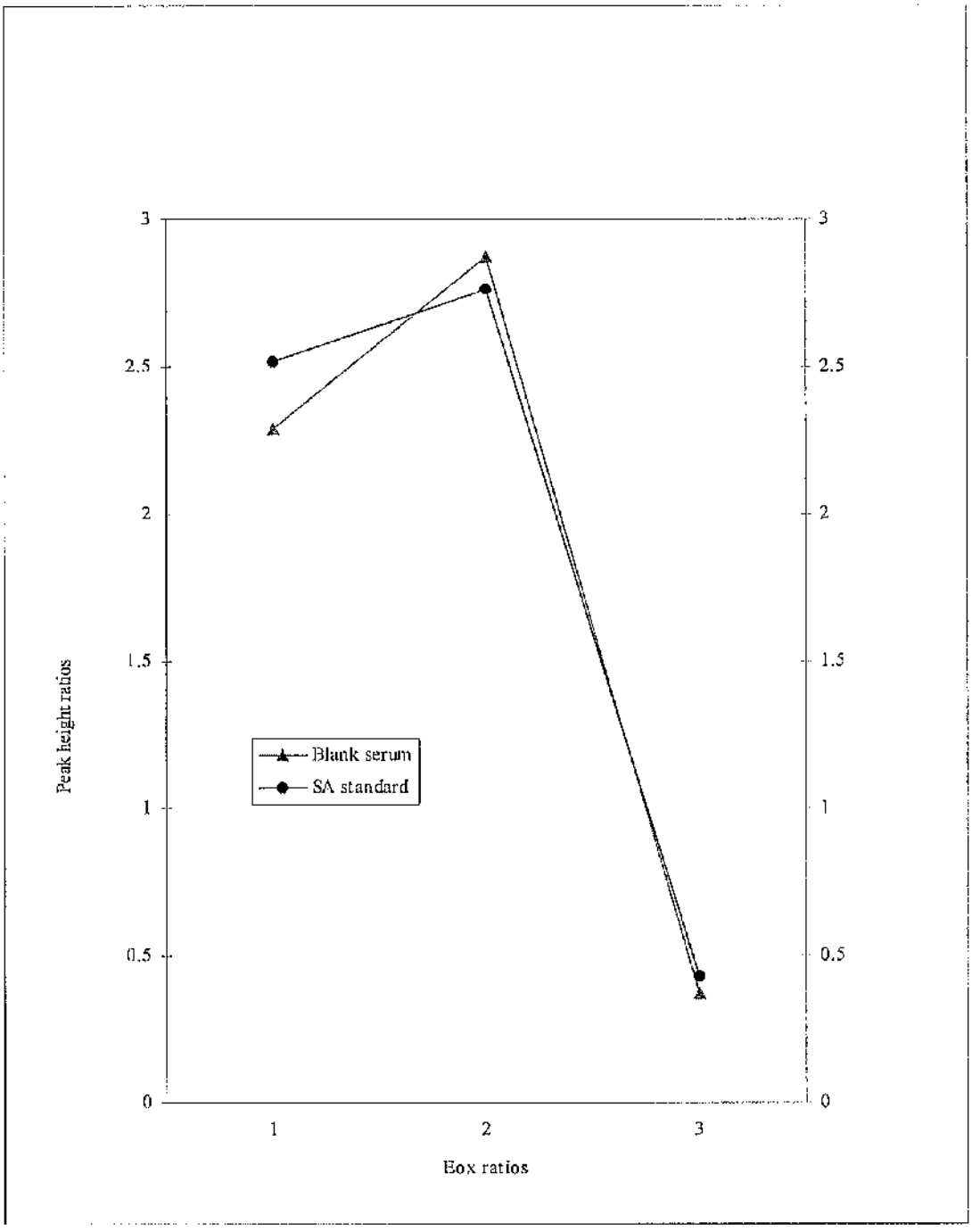
Table 17 List of limit of detection (LOD) values from a selection of recently published work.

Author	Biological medium	Limit of detection (LOD)
Current method	**serum**	**15pmol/ml**
Eliott Cham *et al*[61]	plasma	22pmol/ml
Siebert and Bochner[59]	plasma	111pmol/ml
Shen *et al*[45]	plasma	720pmol/ml
Evans *et al*[49]	serum	1.5nmol/ml
Vree *et al*[55]	plasma	1.5nmol/ml
Reidl[58]	plasma	1.5nmol/ml
	urine	750pmol/ml

Table 18 List of extraction efficiencies from a selection of recently published work.

Author	Biological medium	Extraction efficiency (%)
Elliot Cham *et al*[61]	plasma	99-102
Shen *et al*[45]	plasma	92-100
Mays *et al*[60]	urine	98
	plasma	85
Current method	**serum**	**85**
Reidl[58]	plasma	89
Siebert and Bochner[59]	plasma	70
Evans *et al*[49]	serum	60

Fig. 13 Comparison of voltammograms of extracted blank serum and standard SA



The combination of SPE (cleaner extracts) and sensitive detection (ECD) allowed much lower concentrations of SA to be detected. At maximum sensitivity a peak occurred at the same t_r as SA in the "blank" sera. This could have been due to several factors, including interference, or that salicylate was already present in human serum at very low concentrations. Voltammograms of extracted serum and standard SA were plotted from the data, which showed that they had a similar voltammetric profile (Fig 13, p.91). Results indicated (Table 2, p.52) that the peaks in human serum were probably due to SA (carryover from previous injections was carefully excluded). It was found that all "blank" sera obtained from normal subjects not consuming aspirin or other salicylate drugs contained SA at concentrations less than 75pmol/ml.

Swain *et al* reported information on food products which are known to contain SA, and this may be a source of the very low levels found in blank sera.[62] Hence, with very low dose aspirin treatment, the serum SA concentration which is measured may not originate solely from the aspirin. This is now an area of salicylate research that is being followed up in the labs at the Crichton Hospital. Published methods that have sufficient sensitivity to detect low SA levels have also shown small interfering peaks at the same t_r as SA in blank plasma or serum. Siebert and Bochner published a paper that showed a chromatogram that had such an interference, although they did not discuss the origin of this in their paper.[59]

1.2 Method validation of SA assay

After optimisation of the chromatographic conditions had been successfully completed, work had to be carried out to validate the method. A method can only be considered to be validated after certain criteria have been met.

Comparison of extraction efficiencies with other published methods indicates that the method described in this thesis provides satisfactory extraction efficiency (Table 18, p.90).

Great care had to be taken when adding methanol in the initial preparation of the SPE column to prevent disturbance of the sorbent materials. If the sorbent bed was disturbed poorer extraction efficiencies resulted. Methanol was added dropwise to the columns and allowed to seep through under gravity in to prevent this from happening.

The SA peak response was linear over a concentration range of 0.1-50μM extracted from 0.5ml of sera (Fig.7, p.60). Since sera was to be used in the preparation of calibration curves, it was decided that it would be of benefit if a source of sera other than human sera could be found. New born calf sera was extracted but unknown interfering peaks were present which co-eluted with the SA and IS peak. Sera from patients was pooled and extracted to ensure that there was no significant interfering peaks present with the same t_r as SA or IS. These non-interfering pooled samples were stored in 3ml aliquots at -30°C until required for use in the preparation of calibration curves to provide linearity data.

Experiments were set up involving both intra- and inter-assay precision. Precision is the closeness of agreement between independent test results obtained under prescribed conditions. Each day an assay was carried out, a blank serum sample was extracted to ensure no interfering peaks were present at the t_r of the SA or the IS (selectivity). Precision is an essential element in validating an analytical method to show that the method is not susceptible to change both from day to day and within day variations. Results from our work indicated that both inter and intra-assay precision were adequate with the greatest variation being shown, not surprisingly, at the lowest level concentration (Table 6, p.62). The level of accuracy of the method was calculated using the data

obtained from the precision work and the results obtained showed the method to be satisfactorily accurate. In addition, stability experiments were carried out over a period of 13 days under different conditions. Instability was not found to be a problem.

The sensitivity, reproducibility, precision and accuracy as demonstrated in the results section indicates that this assay of SA could be applied to research studies involving patients with different pathologies taking low dose aspirin. The analysis of serum specimens obtained from diabetic patients confirmed this.

2.1 Optimisation of chromatographic and extraction conditions for 2,3-DHBA and 2,5-DHBA assay.

Optimisation of the chromatographic and extraction conditions for 2,3-DHBA and 2,5-DHBA was carried out in a similar manner to that for SA.

A suitable mobile phase had to be chosen that would provide good resolution of 2,3-DHBA, 2,5-DHBA and the chosen IS. As a starting point in finding a suitable mobile phase, the literature was reviewed and the chromatographic conditions of other research groups considered. Floyd *et al* used a mobile phase consisting of 30mM acetic acid and 30mM citric acid. The mobile phase was then titrated to a pH of 3.0 with solid sodium hydroxide followed by sodium acetate to a final pH of 3.6 and this was continually sparged using nitrogen gas.[38] The resulting published chromatogram shows insufficient resolution of the 2,3-DHBA and 2,5-DHBA peaks, therefore, this mobile phase was not considered. Moreover this research employed standard solutions only, such that interferences in serum from an extraction process were not taken into account.

Of work that has been carried out on serum, Grootveld *et al* used a mobile phase consisting of 30mM sodium citrate/27mM acetate buffer at a pH of 4.75.[50] They stated that 2,5-DHBA was found to elute very close to their IS, 3,4-DHBA. However, the chromatography in general was unsatisfactory and contained significant interference.

Udassin *et al*[63] and Das *et al*[64] employed the same mobile phase used by Floyd *et al*[38] but also added 0.2g/l sodium azide. However, the chromatography produced from extracted body fluids was very poor. Ghiselli *et al*[53] used a similar mobile phase to Grootveld and Halliwell[50] with the only difference being the methanolic concentration. Poor resolution of the 2,3-DHBA and 2,5-DHBA peaks was obtained.

The mobile phase that was considered in this work was 100% 30mM tri-sodium citrate buffer, with the pH adjusted to 4.75 using glacial acetic acid. The pH of the mobile phase was later changed to pH 5.3 because of relatively poor resolution of the 2,3-DHBA from the 2,5-DHBA when a new, "identical" analytical column was purchased. This was probably due to column to column variation which occurs as a result of slight differences in the behaviour of different batches of the same packing material resulting in differences in the column chemistry.

The pH was altered to eliminate any future problems with resolution and provide a more robust method. However, some development work was carried out using the initial mobile phase conditions at a pH of 4.75 and is described below.

Voltammograms (Fig.9a & b, p.67) of 2,3-DHBA and 2,5-DHBA were drawn and it was shown that the applied potential across the glassy carbon electrode which produced the maximum current response was around 0.70V. This applied potential was similar to that found in published work. Floyd *et al* applied a potential of 0.80V,[38] Grootveld *et al* a potential of between 0.60-0.68V,[50] Das *et al* a potential of 0.60V [64] and Ghiselli *et al* a potential of 0.76V.[53] Hence, the published values of applied potential varies from 0.60-0.80V, agree with the value of 0.70V used in our current method. The variability in E_{ox} applied by the various research groups could be caused by a number of factors. As shown by Evans *et al*, mobile phase content can affect the voltammetric behaviour of SA and the geometry of the electrode and the electrode material may also have an effect.[49]

Extracts of blank sera from subjects not consuming aspirin showed the presence of small interfering peaks at the same t_r as 2,3-DHBA and 2,5-DHBA. It was thought that 2,5-DHBA

could be present since work carried out by Ingelman-Sundberg *et al* on microsomal fractions from mammals treated with inducers of cytochrome P-450 indicated that salicylate was metabolised to 2,5-DHBA.[32] It was also shown that under the same conditions, although 2,5-DHBA was found no 2,3-DHBA was formed. To help characterise the unknown peaks with t_r values similar to 2,3-DHBA and 2,5-DHBA, the ratios of blank unknown peaks and standard solutions of 2,3-DHBA and 2,5-DHBA were calculated at different E_{ox} values and the values compared. The measured ratios were similar (Table 10, p.68), indicating that it was likely that 2,3-DHBA and 2,5-DHBA were present in blank sera. Further work carried out in this laboratory supports this view (C. Blacklock, personal communication), with the unknown peaks having similar t_r values to 2,3-DHBA and 2,5-DHBA using three different mobile phase conditions.

When carrying out the development of the SA method it was discovered that small amounts of SA were present in extracted blank sera raising the possibility that 2,3-DHBA is generated by hydroxyl free radical attack on any SA present. SA is known to occur in many different foodstuffs and wines.[62]

A letter in the Lancet by Muller and Fugelsang indicated that 2,3-DHBA, 2,5-DHBA and SA were present in a selection of Californian wines, both red and white.[65] They stated that more SA, 2,3-DHBA and 2,5-DHBA are present in red wines than in white but that levels in white wine of SA, 2,3-DHBA and 2,5-DHBA were still significant.

Extraction conditions for 2,3-DHBA and 2,5-DHBA were identical to those described for the SA method but the selection of a suitable IS again proved difficult. 3,4-DHPAA seemed a suitable candidate since it had similar voltammetric behaviour to 2,3-DHBA and 2,5-DHBA. However, when 3,4-DHPAA was prepared in solution as an aqueous mixture with 2,3-DHBA and 2,5-DHBA, the 3,4-DHPAA peak heights dropped markedly when compared to when it was injected individually (Table 12, p.70). A further 6 compounds were examined with only two oxidising at the same applied E_{ox} as 2,3-DHBA and 2,5-DHBA. These were 3,4-DHBA and gallic acid (3,4,5-trihydroxybenzoic acid). 3,4-DHBA was chosen because of its structural similarity to 2,3-DHBA

and 2,5-DHBA and it was thought that it would have similar physicochemical behaviour for extraction purposes.

It was at this stage of the development of the method that resolution of the 2,3-DHBA and 2,5-DHBA deteriorated due to the introduction of a new column even though chromatographic conditions were unchanged. The resolution of the 2,3-DHBA, 2,5-DHBA and 3,4-DHBA analytes at a concentration thought to approximate the levels of those taking low dose aspirin was poor with saddling of the peaks introduced. In order to try and improve resolution, two components of the mobile phase were looked at which were believed might help to resolve this problem.

Firstly, small amounts of organic solvent were added to the mobile phase to try and separate the peaks of interest. 4.75% v/v of acetonitrile was added to the mobile phase buffer, but the three analyte peaks eluted more quickly with the 2,3-DHBA and 2,5-DHBA peaks virtually co-eluting, and the 3,4-DHBA peak poorly resolved from these co-eluting peaks (Fig.10a, p.73). 1.5% v/v of methanol was added to the buffer, but increased saddling of the 2,3-DHBA and 2,5-DHBA peaks (Fig.10b, p.74) was observed. Addition of organic solvents clearly did not improve the resolution and so the pH of the mobile phase was investigated as a means to improve resolution.

A range of mobile phases with different pH values were prepared with the pH ranging from 4.75 - 5.4. Standard solutions of 2,3-DHBA, 2,5-DHBA and IS were chromatographed. As the pH of the mobile phase increased, the t_r gap of the 2,3-DHBA and 2,5-DHBA peaks increased, and simultaneously, the 3,4-DHBA peak eluted more quickly at a rate of approximately 0.80 minutes per 0.1pH unit change with respect to the 2,3-DHBA and 2,5-DHBA peaks (Table 14, p.81 & Fig.11a-e, p.75-79). When the pH of the mobile phase was 5.3, resolution of the 3 analytes was improved with detector response for 2,3-DHBA and 2,5-DHBA also improved. Poor resolution of the 2,3-DHBA and 2,5-DHBA is a problem observed in previously published methods. None of the published methods to date show satisfactory resolution of these two analytes, whether a standard solution or extracted plasma or sera are used. Floyd *et al* state that the 2,3-DHBA and 2,5-DHBA compounds have similar t_r, but are separated enough from each other to differentiate

between them.[38] This suggests that baseline resolution has not been achieved. They examined different mobile phases and found the most suitable to contain 30mM acetic acid, 30mM citric acid, with the pH altered to 3.0 with solid sodium hydroxide and then with sodium acetate to a final pH of 3.6. Method development carried out by this group was done using aqueous solutions, thereby avoiding the need to consider potential interfering analytes present in body fluids.

Grootveld *et al* used three separate methods employing different chromatographic conditions in each.[50] In "method A" (specific for 2,3-DHBA and 2,5-DHBA), diethylether was used as the extracting solvent and the mobile phase consisted of 100% 30mM sodium citrate/27.7mM acetate buffer pH 4.75. Chromatograms show that 2,3-DHBA and their IS, 3,4-DHBA, are well resolved but the paper states that 2,5-DHBA has a similar t_r to 3,4-DHBA. This suggests that co-elution is a problem with the IS but no chromatogram is provided to show this. A chromatogram of an extract from a healthy control not consuming aspirin is provided with a large peak, which may be equivalent to 2,5-DHBA, shown to elute at a similar t_r to 3,4-DHBA. Extracts from plasma and knee joint synovial fluid showed 2,3-DHBA poorly resolved from unknown peaks, thought to be catecholamines (Fig.4, p.21). Das *et al*[64] used the same method as Floyd[38] but the 2,3-DHBA and 2,5-DHBA peaks look as if there are other peaks co-eluting with them. Ghiselli[53] used a similar method to Grootveld and Halliwell[50] but resolution of the 2,3-DHBA and 2,5-DHBA was poor. The chromatography and resolution achieved in our current method is a significant improvement over those methods previously published. SPE of blank sera using the new mobile phase conditions still produced two peaks at the same t_r as 2,3-DHBA and 2,5-DHBA.

Now that the chromatographic conditions were optimised as far as the resolution of the 2,3-DHBA, 2,5-DHBA and IS were concerned, a further problem arose. Data from an injection from an extracted sample was acquired overnight. The chromatography was looked at in the morning and it was observed that a broad peak eluted from the column with a t_r of 120 minutes (2hrs). This resulted in carryover of this late eluting peak into subsequent injections. In order to prevent this

carryover and to prevent such potentially long run times, a gradient method was introduced. The two solutions acting as mobile phases in the gradient set up were the normal mobile phase (pH adjusted to 5.3) (A) and 28.6%(v/v) 30mM tri-sodium citrate with the pH adjusted to 5.3 (B). Methanol had been used for mobile phase (B) but it was discovered that methanol on its own caused uncontrollable baseline drift, probably due to leakage of small amounts of methanol into the salt bridge. Four different programs were set up and extracts injected onto the column. Optimum gradient conditions were attained using Program 3.

Further optimisation of the extraction procedure took place to see if the amount of eluent in the final stage of the SPE procedure could be reduced from 5mls thus providing a more concentrated extract. Fractions of eluent were collected and individually injected to show that 85% and 88% of 2,3-DHBA and 2,5-DHBA were recovered respectively from the first 1ml collected (% of total recovery), with very little collected in the final 4mls.

Finally, before the method could be validated, carryover of analytes between injections had to be resolved. Since such low concentrations of analytes were required to be detected, any degree of carryover could contribute significantly to the signal of a subsequent injection. To investigate this problem, two experiments were carried out, the first being to increase the number of flushes between injections from 1 to 5. A small percentage of carryover still existed even after five washes (Table 15, p.81), with the 3,4-DHBA carryover being the most significant (5.5%). Secondly, different flush solvents were prepared and showed that 0.2M HCl removed all the 2,3-DHBA and 2,5-DHBA and only left 1.0% of 3,4-DHBA (Table 16, p.84).

The measurement of 2,3-DHBA and 2,5-DHBA has been investigated by few research workers, but those methods published use ECD. Chromatographic and extraction conditions were developed, employing similar conditions to those used for the determination of SA. The applied E_{ox} and the mobile phase pH was changed to 0.70V and pH 5.3 respectively. In addition, the isocratic conditions were replaced by a gradient method to elute a late unwanted peak.

The development work carried out in optimising both the chromatographic and extraction conditions has significantly improved this method over anything that is currently available in other published methods for measuring levels of 2,3-DHBA and 2,5-DHBA in subjects consuming low doses of aspirin.

FUTURE WORK

1. To complete "validation" of the assay for serum SA.

2. To carry out pharmacokinetic studies to determine the rate of formation of SA in serum after administration of different doses of ASA (low to high dose). This information would determine the time at which blood samples could be taken to give maximum concentrations of SA. By carrying out studies at different doses, this would also allow monitoring of 2,3-DHBA, ensuring that complete quenching had taken place. The concentration of hydroxyl free radicals in subjects could then be monitored. In order to get a meaningful measure of hydroxyl radical production it would also be necessary to carry out analysis on "blank" sera to assess baseline concentrations of 2,3-DHBA.

3. To carry out studies on groups of patients with different pathologies where hydroxyl free radical damage is suspected e.g. schizophrenics, diabetics and patients who suffer from coronary heart disease. By measuring concentrations of SA and 2,3-DHBA in these subjects it would allow estimation of the relative concentrations of free hydroxyl radicals being quenched by the SA to form 2,3-DHBA.

4. To compare results from differing assays estimating free radical activity.

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